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Characterisation of environmental stress biomarkers in *Prodiamesa olivacea* (Diptera) and their analysis and comparative evaluation in two chironomid species for ecotoxicity studies in natural scenarios

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Toda cadena es tan fuerte como el eslabón más débil

The show must go on

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TABLE OF CONTENTS

(Click on any page number to return here)

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

LIST OF FIGURES

LIST OF FIGURES

LIST OF FIGURES

ABBREVIATIONS

20E 20-hydroxyecdysone

AA Amino Acid

AMP Antimicrobial Peptide

ASTM American Society for Testing Material

BBP Benzyl Butyl Phthalate

BCA Bicinchoninic Acid

BP Base Pair

BPA Bisphenol A

BP3 Benzophenone 3

BSA Bovine Serum Albumin

CA *Corpora Allata* gland

CAT Catalase

cDNA Complementary DNA

CECs Contaminants of Emerging Concern

CoRAP Community Rolling Action Plan

DTNB 5,5-dithiobis-(2-nitrobenzoic Acid

DTPA Diethylenetriaminepenta-acetic Acid

EC European Commission

EcR Ecdysone Receptor

ECHA European Chemicals Agency

EHEC Hydrobiological Field Station "Encoro do Con"

EDCs Endocrine Disrupting Chemicals/compounds

EDTA Ethylenediaminetetra-acetic Acid

EEA European Economic Area

EFSA European Food Safety Authority

EP Emerging Pollutant

ER Estrogen Receptor

ERA Ecological Risk Assessment

ABBREVIATIONS

ERR Estrogen-related Receptor

EtOH Ethanol

EU European Union

FDA Food and Drug Administration

GAPDH Glyceraldehyde-3-phosphate dehydrogenase

G3P Glyceraldehyde-3-Phosphate

GC-MS Gas Chromatography-Mass Spectrometry

GO Gene Ontology

GR Glutathione Reductase

GSH Glutathione

GST Glutathione S-transferases

GPx Glutathione Peroxidase

HPLC-MS High-Performance Liquid Chromatography-Mass Spectrometry

HSE Heat-shock Element

HSF Heat-shock Factor

HSP Heat-shock Protein

INT 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride

IMD Immune Deficiency Pathway

JAK/STAT Janus Kinase/signal Transducer and Activator of Transcription pathway

JH Juvenile Hormone

JHEH Juvenile Hormone Epoxide Hydrolase

JNK JUN N-terminal Kinase pathway

kDa Kilodaltons

mRNA messenger RNA

MRP Multidrug Resistance-associated Proteins

NADPH Nicotinamide Adenine Dinucleotide Phosphate-oxidase

NCBI National Centre for Biotechnology Information

NIH National Institute of Health

NS Not Significant

OECD Organisation for Economic Co-operation and Development

ORFs Open Reading Frames

PAH Polycyclic Aromatic Hydrocarbons

PCPs Personal Care Products

PEC Predicted Environmental Concentration

PG Prothoracic Gland

PGRP Peptidoglycan Recognition Protein

PMS Post-mitochondrial Supernatant

PNEC Predicted No-Effect Concentration

PPAR Peroxisome Proliferator-Activated Receptor

PPCP Pharmaceutical and Personal Care Product

PRP Pattern Recognition Protein/receptor

PTTH Prothoracicotropic Hormone

qPCR quantitative Polymerase Chain Reaction

REACH Registration, Evaluation, Authorisation and Restriction of Chemicals

RNA Ribonucleic Acid

RT Room Temperature

ABBREVIATIONS

RT qPCR Real Time quantitative PCR

ROS Reactive Oxygen Species

ROS-RNS Reactive Oxygen and Nitrogen Species

SOD Superoxidase Dismutase

SVHC Substances of Very High Concern

TCA Trichloroacetic Acid

TDI Tolerable Daily Intake

TDS Total Dissolved Solids

TNB 5-thio-2-nitrobenzoic Acid

USP Ultraspiracle

US-EPA The United States Environmental Protection Agency

UV Ultraviolet

UVR Ultraviolet Radiation

WFD Water Framework Directive

WHO World Health Organization

WWTP Wastewater Treatments Plant

ABSTRACT

1. ABSTRACT

Along with traditional ecotoxicological research using model organisms, toxicological studies in non-model organisms are being considered a complement to achieve more robust methodologies. This integrative strategy allows us to figure out the complexity of exposures in natural ecosystems. The main objective of this work has been to characterise the molecular effects that three common environmental pollutants (BBP, butyl benzyl phthalate; BPA, bisphenol A; and BP3, benzophenone 3), as well as heat stress provoke in natural populations of two aquatic invertebrates: the non-model species *Prodiamesa olivacea* (Chironomidae, Diptera) and model organism in ecotoxicology *Chironomus riparius* (Chironomidae, Diptera).

The genus *Chironomus* has four standardised OECD tests for evaluating water and sediment toxicity, and different species can be used to assess classical toxicity parameters. In contrast, *Prodiamesa* is rarely used for toxicity studies, although it is an interesting toxicological species because it shares habitats with *Chironomus* while requiring less extreme conditions and higher oxygen levels. These different requirements are particularly interesting to assess the different responses of both species to stress conditions.

In the present research, 4th instar larvae of both chironomids were collected in a contaminated river (Sar) from Galicia (Spain) and independently exposed to 1 µg/L BBP, BPA or BP3 for 4 h and 24 h. Additionally, to evaluate heat-shock impacts, larvae were exposed to 35 °C and 39 °C for 30', 60', 120' and 120' followed by 2 h at RT. The effects were measured by analysing transcriptional and enzymatic alterations of different biomarkers of interest.

The results have allowed us to identify and characterise for the first time in *P. olivacea* new early biomarkers of effect involved in the cells stress response (*Hsp27*, *Hsp60*, *Hsp70*, *Hsc70*, *Cdc37*), the endocrine system (*EcR*, *Kr-h1*, *JHEH*), biotransformation and oxidative responses

(*MnSOD*, *CAT*, *PHGPx*, *Cyp4g15*, *Cyp6a-14-like*, *Cyp6a2-like*), the immune system (*PGRP*, *C-type lectin*, *TOLL*, *JAK/hopscotch*) and the energy metabolism (*GAPDH*). Differential species-specific toxic responses have been described following exposure to xenobiotics and heat stress, with *P. olivacea* being the dipteran that showed the highest sensitivity in most of the studied biomarker genes.

BBP was the most toxic to *P. olivacea* larvae among the three chemical tested, leading to quick and robust inhibition of genes related to critical metabolic pathways. All three compounds interfered with the endocrine system, triggering an ecdysteroid-antagonist effect. The comparative transcriptional analysis between *C. riparius* and *P. olivacea* revealed that exposure to xenobiotics resulted in strong early transcriptional activation in *C. riparius*, leading to downregulation in *P. olivacea*. Heat-shock drastically activated the cell stress response and inhibited the immune system in *P.* olivacea, while both responses were activated in *C. riparius*. These effects could compromise the adaptive capacity and survival of *P. olivacea* and highlights the high tolerance of *C. riparius* to extreme environments.

The differential responses detected between *P. olivacea* and *C. riparius* provide novel information on the harmful effects of BBP, BPA, BP3 and heat-shock on these aquatic midges and highlight the potential of *P. olivacea* to be considered as a suitable sentinel organism for ecotoxicity studies in natural scenarios.

RESUMEN

1. RESUMEN

Junto con los métodos tradicionales de investigaciones ecotoxicológicas utilizando organismos modelo, los estudios toxicológicos en organismos no modelo se están considerando un complemento perfecto para conseguir metodologías más robustas. Esta estrategia integradora nos permite descifrar la complejidad de las exposiciones en los ecosistemas naturales. El objetivo principal de este trabajo ha sido caracterizar los efectos moleculares que tres contaminantes ambientales comunes (BBP, butil bencil ftalato; BPA, bisfenol A; y BP3, benzofenona 3), así como el estrés por choque térmico provocan en poblaciones naturales de dos invertebrados acuáticos: la especie no modelo *Prodiamesa olivacea* (Chironomidae, Diptera) y el organismo modelo en ecotoxicología *Chironomus riparius* (Chironomidae, Diptera).

El género de los quironómidos, un género de mosquitos no picadores, cuenta con cuatro ensayos estandarizados y aceptados internacionalmente con fines regulatorios para evaluar la toxicidad del agua y los sedimentos. En todos ellos, se utilizan especies de quironómidos y se analizan parámetros clásicos de toxicidad. Por el contrario, *Prodiamesa* rara vez se usa para estudios de toxicidad, pese a ser una especie interesante desde el punto de vista toxicológico ya que comparte hábitats con *Chironomus* pero requiere condiciones menos extremas y niveles de oxígeno más altos. Esta divergencia de requerimientos es particularmente interesante para evaluar las diferentes respuestas de ambas especies a condiciones de estrés.

En esta investigación de tesis doctoral, se recolectaron larvas de cuarto estadío de ambos quironómidos en un río contaminado (Sar) de Galicia (España) y se expusieron de forma independiente a 1 µg/L BBP, BPA o BP3 durante 4 h y 24 h. Además, para evaluar el impacto del choque térmico, las larvas se expusieron a 35 °C y 39 °C durante 30', 60', 120' and 120' seguido de 2 h a temperatura ambiente. Los efectos se midieron

analizando alteraciones transcripcionales y enzimáticas de diferentes biomarcadores de interés.

Los resultados han permitido identificar y caracterizar por primera vez en *P. olivacea* nuevos biomarcadores tempranos de efecto implicados en la respuesta de estrés celular (*Hsp27*, *Hsp60*, *Hsp70*, *Hsc70*, *Cdc37*), el sistema endocrino (*EcR*, *Kr-h1*, *JHEH*), respuestas de biotransformación y estrés oxidativo (*MnSOD*, *CAT*, *PHGPx*, *Cyp4g15*, *Cyp6a-14-like*, *Cyp6a2-like*), el sistema inmune (*PGRP*, *C-type lectin*, *TOLL*, *JAK/hopscotch*) y el metabolismo energético (*GAPDH*). Se han descrito respuestas tóxicas diferenciales específicas de especie tras la exposición a xenobióticos y choque térmico, *siendo P. olivacea* el díptero que mostró una mayor sensibilidad en la mayoría de los biomarcadores moleculares estudiados.

De entre los tres contaminantes de estudio, BBP fue el compuesto más tóxico para las larvas de *P. olivacea* ya que produjo una clara y rápida inhibición de genes relacionados con vías metabólicas esenciales. Los tres compuestos interfirieron con el sistema endocrino desencadenando un efecto antagonista de las hormonas esteroideas. El análisis transcripcional comparativo entre *C. riparius* y *P. olivacea* reveló que la exposición a xenobióticos desencadenó una temprana y aguda activación transcripcional en *C. riparius*, en contraposición al descenso de la actividad transcripcional observado en *P. olivacea*. El choque térmico activó drásticamente la respuesta de estrés celular e inhibió el sistema inmune en *P. olivacea*, mientras que ambas respuestas se activaron en *C. riparius*. Estos efectos podrían comprometer la capacidad de adaptación y supervivencia de *P. olivacea* y destaca la alta tolerancia de *C. riparius* a ambientes extremos.

Las respuestas diferenciales detectadas entre *P. olivacea* y *C. riparius* proporcionan información novedosa sobre los efectos nocivos del BBP, BPA y BP3, así como del choque térmico en estos mosquitos acuáticos y destacan el potencial de *P. olivacea* para ser considerado un organismo centinela adecuado para estudios de ecotoxicidad en escenarios naturales.

INTRODUCTION

2. INTRODUCTION

2.1. Environmental contamination

Nowadays, industrial and economic development is a double-edged sword. On the one hand, it is a fact that represents an improvement in the quality of the population's life. On the other hand, it is one of the largest anthropogenic threats to the planet. The exponential growth of the world's population in recent decades is translated into a high demand to produce new chemicals of anthropogenic origin. This leads to their indiscriminate consumption and, as an endpoint, the dumping of their waste products in an uncontrolled way. Pesticides, industrial additives, mineral wastes, surfactants, drugs, illicit drugs, steroid hormones, personal care products, or compounds called "lifestyle" such as caffeine or nicotine are just a few examples of synthetic organics products found in water (Carrasco *et al*., 2017).

Although the aquatic environment is the main target of pollution due to the bioaccumulative capacity of these substances and the water cycle, the truth is that land, sea, and air are affected by the emission of a wide variety of pollutants of human origin, leading to an increasingly accelerated environmental deterioration, which is a major concern. The fact that contaminants reach the aquatic environment implies their integration into sediments, which involves their adsorption and their persistence along the time and into organisms which may have direct or indirect toxic effects and/or bioaccumulate through the trophic chain from benthic organisms to higher predators, including humans. In addition, since contaminants can influence animal welfare and may alter trait variance of individuals, chemical contamination can act as a potent evolutionary force resulting in ecosystem changes via effects on wildlife (Saaristo *et al*., 2018).

2.2. Development of ecotoxicological assays

As a need to unify water management actions in the European Union (EU), in the early 21st century, the Water Framework Directive (WFD) (Directive 2000/60/EC), was developed and approved by the European Parliament and Council. The WFD main aim is protecting inland surface waters, transitional waters, coastal waters and groundwater in qualitative terms and thus ensuring their sustainability. To do that, WFD establishes a water status classification depending on three elements that are mandatory to study in all water bodies: biological, hydromorphological, and physical-chemical elements (WFD, 2000). By doing that, the ecological status of any water body can be defined as high, good, moderate, poor or bad. According to this Directive, all the Member States are expected to have at least good quality in all their water bodies (WFD, 2000).

The study of the biological element is based on the presence, absence or abundance of several organisms. These organisms are selected by their ecology relevance or due to their suitable characteristics as bioindicators by analysing the response of different parameters considered as biomarkers.

According to the Food and Drug Administration and the National Institute of Health (FDA-NIH, 2016), the basic definition of biomarkers is "*a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention*". Bioindicators are organisms or communities of organisms, which reactions are observed representatively to evaluate a situation, giving clues for the status of the whole ecosystem (Gerhardt, 2002). In toxicology, bioindicators are used as pollution markers to assess stress responses, possible effects and bioaccumulation as an early warning on biomonitoring.

In general terms, three different organisms are representing key taxonomic groups that are routinely used in water ecotoxicology studies: algae, aquatic invertebrates, and fish.

Algae are one of the most important primary producers in aquatic ecosystems since they are situated at the bottom of the trophic chain and are essential for ecosystem functioning (Silva *et al*., 2009). Growth rate, population density or species diversity are water quality parameters commonly used. Several guidelines establish their use as test organisms in the monitoring of water quality (DIN, 1991; ISO, 2012; OECD, 2011a).

Invertebrates play essential and diverse ecological roles in many aquatic ecosystems (Gillespie *et al*., 1998). Among them, species such as *Daphnia magna* (Crustacea, Cladocera), *Lumbriculus variegatus* (Annelida, Oligochaeta), *Potamopyrgus antipodarum* (Gastropoda, Littorinimorpha), *Chironomus riparius* or *Chironomus tentans* (Insecta, Diptera) are sediment-associated organisms that have become increasingly important in toxicity test (OECD, 2004a,b,c 2007, 2010, 2011b, 2016). These model organisms have some characteristics in common that facilitates investigation such as short life cycle, which minimise experimentation time, or the minimum space and laboratory equipment necessary to maintain the cultures.

Lastly, fish represent the largest and most diverse group of vertebrates (Yancheva *et al*., 2015). Different characteristics make them excellent as an experimental model in water toxicity assessments such as their greater sensitivity to changes in the aquatic environment compared to invertebrates, their tendency to accumulate contaminants several times higher than in the ambient media, their presence at different trophic levels, or their well-developed osmoregulatory, endocrine, nervous and immune system (Yancheva *et al*., 2015). Moreover, over the last years, the number of works related to endocrine disruption on fish are gaining

relevance (Cao *et al*., 2019; Fraser *et al*., 2017; Han *et al*., 2010; Jiang, J. *et al*., 2015; Viganò *et al*., 2020). *Danio rerio*, *Oryzias latipes*, *Pimephales promelas* or *Gasterosteus aculeatus* are key fish species proposed by OECD for ecotoxicology tests. Among others, mortality, growth, reproduction, fecundity or metabolism are classical parameters of evaluation (OECD 2009, 2012, 2013, 2015).

To improve human and environmental health protection from chemical risks, on June 1st, 2007, the European Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) entered into force in the EU. The REACH Regulation (EC 1907/2006) is based on a standardised identification and characterisation of toxic effects of chemical substances, and it places responsibility on industry to manage risks and to provide safety information. To date, 2022, the information related to more than 23445 chemical substances from all the European Economic Area (EEA) countries is stored in a central database in the European Chemicals Agency (ECHA), of which more than 15000 substances are available to download. In terms of chemical safety, this allows to a better hazard knowledge and as of last, safer and more committed handling of chemicals.

Chemical risk assessment allows a better characterisation of the possible toxic effects of certain substances. This is useful for the control of exposures and management of chemical products. A good risk characterisation consists of a quantitative and qualitative estimation of the harmful effects associated with them, taking as the main step their toxicological assessment. The application of REACH regulation implies an increase in the number of toxicity tests for these compounds, which is associated with an increase in the quantity of animal tests carried out. However, REACH is balanced between increasing the knowledge related to possible chemical hazards and avoiding unnecessary animal testing. Therefore, registrants of new substances are called to carry out further

tests only when they have exhausted all other relevant available data sources. Essential resources provided by REACH to minimise animal tests are data sharing of companies producing or importing the same substances, and also the use of alternative methods. On May 30th, 2008, the Council Regulation (EC) No 440/2008 was implemented modifying the Commission Regulation No 260/2014 of January 24th, 2014. The (EC) No 440/2008 establishes the test procedures following REACH Regulation, developing methods such as substitution, reduction and refinement of animal experimentation.

The term ecotoxicology has evolved since the first definition by Professor Jean-Michel Jouany in 1971: "*the study of nuisance effects on the relationships between organisms and their environment*". Nowadays, ecotoxicology is an integrative science that considers physical, chemical, biological, and social factors (e.g. human health) from the molecular level to the biosphere (Férard, 2013). Insects are largely used as model species in these multi-disciplinary studies for several reasons that make them perfect over other species for toxicity assessment. As stated before, their short life cycle permits minimise experimentation time, and they are easy to maintain and breed on the laboratory. Moreover, in comparison to other animals, insects expose a far greater surface area relative to its volume (Matsumura, 1985). Finally, as the endocrine system of insects is the best described among all the invertebrates, they are a powerful tool in the study of potential endocrine-related effects. *Drosophila melanogaster*, *Apis mellifera*, *Bombyx mori*, *Locusta migratoria* or *C. riparius* are just a few examples of insect model organisms routinely used as bioindicators in life science research laboratories to assess possible toxic effects of compounds of interest by analysing responses of a wide variety of parameters considered as biomarkers.

In this sense, a biomarker is defined as "*a change in biological response, ranging from molecular through cellular and physiological*

responses to behavioural changes, which can be related to exposure or toxic effects of environmental chemicals" (Peakall, 1994). Although a wide range of toxicology studies carried out in freshwater has focused on the use of fish or macroinvertebrates (Clarke, 1994), in practice, macroinvertebrates constitute, by far, the most used group to evaluate environmental stress (Hellawell, 1986). Furthermore, among macroinvertebrates, the use of chironomids as environmental bioindicators have highly increased in the last decades (Rosenberg, 1992), apart from their extensive use in different disciplines such as forensic Medicine, Ecology, Paleogeography, Paleoclimatology or Paleoecology (Brooks and Birks, 2001; González Medina *et al*., 2015; Massaferro *et al*., 2014; Walker, 1987).

2.3. Chironomids as ecotoxicology model organisms: *Chironomus riparius* systematic, biology and ecology

Chironomids (Diptera, Chironomidae) are a large group of more than 15000 known species of non-biting midges that represent one of the most abundant benthic invertebrate group in freshwater ecosystems (Armitage *et al*., 1995). Besides, it is the most abundant and diversified insect group in inland water ecosystems. These macroinvertebrates are of particular interest since they constitute a crucial element in fish and aquatic bird diet as well as in detritivorous and organic matter recycling processes (Rieradevall *et al*., 1995). They present important advantages, some of them shared with all the macroinvertebrates, although in some cases these pros are more pronounced and in others are less favourable (Rosenberg, 1992).

Some of the advantages of using chironomids in biomonitoring are (Gómez-Sande, 2018):

- **Ubiquitous occurrence**. Composition and structure of benthic communities are similar among the same type of freshwater ecosystems situated at different zoogeographic regions, and also among different types of aquatic ecosystems located in the same area. Due to this fact, the same environmental water assessment methodology can be applied to a wide variety of ecosystems just by making small changes.

- **High species richness** of *Chironomidae* compared to other benthic macroinvertebrates. This group confers versatility and sensitivity to any methodology based on them upon a broad spectrum of responses to environmental stressors.

- **Sedentary nature.** Without considering the aerial phases and drifting, benthic organisms have reduced vital territory, which allows effective spatial analyses of pollutant or disturbance effects.

- **Relatively long-life cycles** compared to other biotic groups. This permits the detection of past changes, and it allows to have a temporal record of water perturbations, which may hamper the elucidation of temporal changes that took place time ago.

- **Simple qualitative sampling.** Required equipment for sampling is basic and inexpensive, which facilitates their use in various sites and situations.

- **Data analysis.** There are many methods developed and widely used in community-level biomonitoring which can be

used as a starting point for the optimisation of new data analysis methods.

Nevertheless, there are few limitations of using chironomids as freshwater bioindicators. As it happens with the advantages, most of these cons are shared with the use of benthic community (Gómez-Sande, 2018):

- **Variability in responses**. *Chironomidae* family is composed of a wide variety of several species. Therefore, different responses between species can occur in the account of the same environmental stressor. For this reason, it is essential to know all the local fauna and to optimise monitoring programs.

- **Response to factors other than water quality**. Due to natural conditions such as current velocity or nature of the substrate, distribution and abundance of the individuals can be affected. It is essential to have a good ecological knowledge of involved species and to perform an accurate experimental design.

- **Seasonality in abundance and distribution**. Chironomid abundance and distribution is affected by seasonality, which can generate sampling problems during specific periods in specific habitats or in comparing samples taken in different seasons. This problem can be avoided by having a good knowledge of life histories.

- **Drift behaviour**. Drift may carry invertebrates into habitats in which they do not normally occur, thereby giving false information. It is essential to have knowledge of habitat preferences and drift behaviour of species.

- **Taxonomic difficulties** with larvae. Some of chironomids groups are well known, and several dichotomic keys allow their identification in the larval stage. However, there are some knowledge gaps in the identification of their instar larvae or even species with indistinguishable instar larvae phases.

- **Time-consuming** quantitative sample processing and identification. Quantitative sampling requires a high number of individuals to achieve reasonable precision, identification and classification. Alternative sampling design and the use of rapid assessment techniques could help to get around this problem.

Taking all of this into account and given the fact that chironomids are exposed to multiple contaminants during their most critical and long-life cycle stage (larva), chironomids are perfect environmental indicator organisms. They are used in the evaluation of the aquatic toxicology of chemical substances to assess the impact of contaminants and any kind of hydrological disturbance. Since *Chironomus* spp. are considered as model species, they are largely used at organism, population, cellular and molecular level in standard freshwater and whole sediment toxicity tests for ecological risk assessment (ERA). Different institutions such as the United States Environmental Protection Agency (US-EPA), the Organisation for Economic Co-operation and Development (OECD) or the American Society for Testing Material (ASTM), among others, have published different standardised ecotoxicology tests based on the use of a wide spectrum of *Chironomus* spp. such as *C. riparius*, *C. tentans*, *C. dilutus* or *C. plumosus* (US-EPA, 1996, 2000; OECD, 2004b, c, 2010, 2011b; ASTM, 2010).

2.3.1. Systematic, identification and morphology

C. riparius is an aquatic dipteran that belongs to *Chironomidae* family; a very diversified monophyletic group of nematocera dipterans, and *Chironominae* subfamily. The *Chironomidae* family comprises at least 10000 species in more than 400 genera (Armitage *et al*., 1995; Saether, 2000).

Chironomid larvae are eucephal, they present a head capsule completely sclerotised and the size of fourth instar larvae varies between 2 and 30 mm (Lindegaard, 1997). Their body is divided into three main parts: head, thorax and abdomen. Thorax consists of three segments called prothorax, mesothorax and metathorax. At the end of the third or beginning of the fourth instar larvae, takes place the formation of structures called imaginal discs in the prothorax that will form the wings and imago legs. These structures play an important role in the characterisation of chironomid individuals and have been largely described for *C. riparius* (Goddeeris *et al*., 2001). Larvae of *C. riparius* contain widely separated triangular ventromental plates on the ventral side of the head capsule and lacks long hairs on them (Sundermann *et al*., 2007).

2.3.2. Distribution, microhabitat and water type

C. riparius is widely distributed in the Northern hemisphere at temperate latitudes, usually in organic enriched water. There is practically no water habitat in which these larvae cannot live. Despite they are frequently abundant in organic polluted conditions, they may also be dominant in temporary or newly created water bodies, or in water subject to other forms of pollution where few other species are present (Pinder, 1986). *C. riparius* larvae are considered as opportunistic species with a

remarkable range of ecological tolerance which allows them to exploit environments with little insect competition (Pinder, 1986).

2.3.3. Biology and physiology

Chironomid's life cycle is composed of four stages of different duration: egg, larva (with four different instars), pupa and imago (Figure 1). The three first immature stages take place in, or closely associated with, any kind of aquatic environment including special habitats such as axillary water of plants, water-filled tree holes (dendrotelmata) or thermal springs (Cobo, 1988; Lindegaard, 1997). Contrary to the first three stages, the imago is aerial, which allows the mosquitos to disperse and colonise new zones easily as well as to recover the distance lost due to drift.

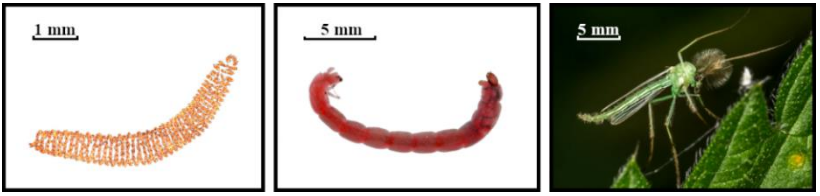


Figure 1. *Chironomus riparius* life cycle. From left to right: egg mass, fourth instar larva and imago male (courtesy of Steve Lanigan www.flickr.com/photos/148652836@N08/).

Depending on the species, the time of the year or the availability of the food, the whole life cycle can take place in few days, or it can last more than a year (Lindegaard, 1997). As guidance, Servia (2001) reported that *C. riparius* larvae maintained in laboratory-controlled conditions (20 °C - 24 °C), complete the whole life cycle in around twenty-one days. Larvae live associated to sediments. Upon completion of the larval life stage, the larva constructs a tube-like shelter and attaches itself with silken

secretions, secreted by their salivary glands, to the surrounding substrate and pupation occurs. After pupation, pupae actively swim to the surface of the water, and adults emerge from the pupal exuviae (Kranzfelder *et al*., 2015). Given that chironomids lack stigma openings of the tracheal system to the environment, they are apneustic insects (Roskosch *et al*., 2011). Their survival is subject to the presence of relatively high oxygen levels to deep into sediments composed of high concentration of organic matter where they might experience a drastic decline in oxygen levels in a few millimetres of depth (Watling, 1991). As an evolutionary adaptation, many larvae, among them *C. riparius*, present haemoglobin in their haemolymph in a concentration proportional to the local oxygen deficit (Weber *et al*., 1985).

Chironomid's larvae present high tolerance to a wide range of pH, oxygen levels, salinity and sediment particle size (Al-Shami *et al*., 2010; Bervoets *et al*., 1996; Orendt, 1999; Suedel and Rodgers, 1994). They are capable of surviving in the organic matter of highly contaminated aquatic environments and can reach population densities of up to 100000 *Chironomus* larvae per m2 (McLachlan, 1977).

A perfect description of the mode of life of these organisms was made by Lindegaard (1997). He described them as opportunist species that grow fast while the environmental conditions are favourable (e.g. temperature, food availability) but are capable of slowing down their development and growth when conditions are not the most suitable.

2.3.4. Use in ecotoxicology

In last decades, there has been an increasing interest in the use of *Chironomus riparius* Meigen, 1804 as a model organism in aquatic toxicological studies (Janssens de Bisthoven *et al*., 1998; Muñiz-González, 2021; Muñiz-González and Martínez-Guitarte, 2020; Planelló *et al*., 2011,

2008; Servia *et al*., 2004; Warwick, 1985). As stated above, *C. riparius* is widely distributed in the northern hemisphere at temperate latitudes, and it can be usually found in organically enriched water (Péry and Garric, 2006). Larvae of this aquatic dipteran are submitted to acute and/or chronic chemical exposures, and classical life cycle endpoints such as survival, growth or reproduction are assessed as toxicological biomarkers. Moreover, in the last years, mouthpart deformities and fluctuating asymmetry have been reported as sublethal effects of environmental pollutants during larval development (Planelló *et al*., 2013; Servia *et al*., 2004). However, before the onset of sublethal effects on life cycle endpoints, toxicant-exposed organisms are already impacted at the cellular level. In this sense, polytene chromosomes of salivary glands make it possible to visualise the genotoxic effects of a wide spectrum of chemicals (Ilkova *et al*., 2017; Michailova *et al*., 2006; Morcillo *et al.*, 1997, 1993). Complementary to these traditional approaches, in recent years molecular biomarkers including gene transcription and enzyme activity have been demonstrated to be effective for the early detection of exposure to toxic compounds (Aquilino *et al*., 2016; Herrero *et al*., 2018; Planelló *et al*., 2010, 2008).

The fact that *Chironomus* species can survive under extreme environmental conditions of temperature, oxygen concentration, pH or salinity, with no presence of deformities, diseases or stress signals makes them very tolerant organisms. In terms of water quality monitoring, this capacity of resistance and resilience makes difficult the classification of water quality based only on the health status of natural populations of species of this mosquito.

Concerning this problem, one possible solution is the use of more sensitive species as a complement of tolerant species to obtain missing information. Ideally, the best organisms to choose are those that share the same ecological niche, as both can be found in the same places and

circumstances. In this sense, *Prodiamesa olivacea* (Meigen, 1818) is an aquatic dipteran that belongs to the *Chironomidae* family and shares the same ecological niche than *C. riparius*, sharing also physiological and ecological characteristics. Moreover, Servia and collaborators (1998) showed that while *P. olivacea* is more abundant during January-April, populations of *C. riparius* are more abundant during June-August and November-December. This seasonal variation confers a great advantage given that it makes possible the study of water and sediment toxicity during any season of the year.

2.4. *Prodiamesa olivacea* systematics, biology and ecology

2.4.1. Systematic, identification and morphology

As well as *C. riparius*, *P. olivacea* is an aquatic dipteran that belongs to *Chironomidae* family and instead of *Chironominae* subfamily, it belongs to *Prodiamesinae* subfamily (Gómez-Sande, 2018). As seen in Figure 2, both species of study used in this thesis are phylogenetically classified in the *Chironomidae* family. In Europe, *Prodiamesinae* subfamily contains four genera: *Monodiamesa*, *Odontomesa*, *Propsilocerus* and *Prodiamesa* (Moller, 2013). Four species have been described in Europe in the *Prodiamesa* Kieffer, 1906, genus: *P. olivacea* (Meigen, 1818), *P. rufovittata* Goetghebuer, 1932, *P. delphinensis* Serra-Tosio, 1964 and *P. bureschi* Michailova, 1977 (Moller, 2013).

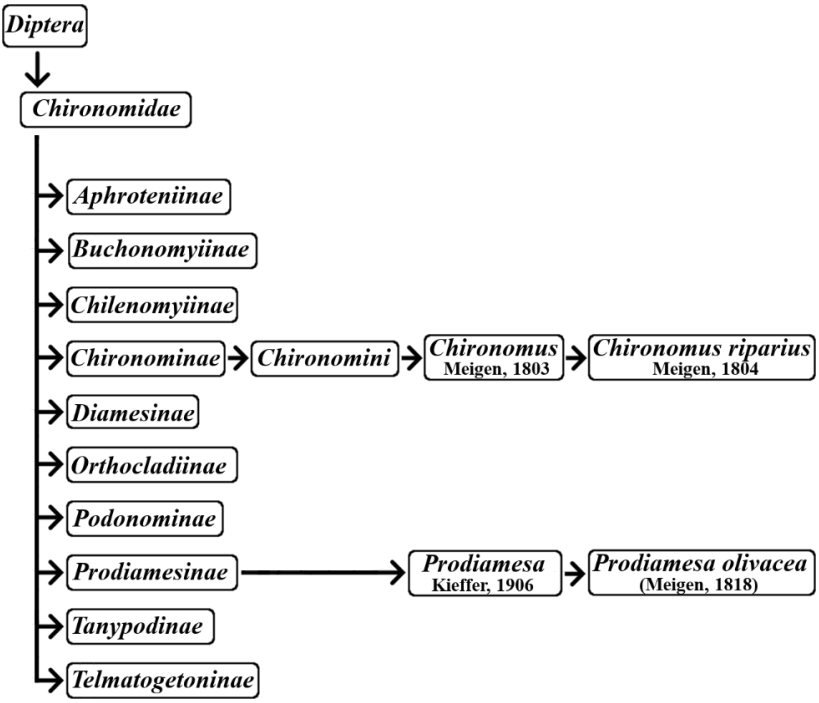


Figure 2. Phylogenetic classification of *P. olivacea* and *C. riparius*.

P. olivacea larvae are easy to distinguish from the rest of *Chironomidae* species, due to their characteristic pattern on the ventral side of the head capsule, containing ventromental plates with long hairs (Sundermann *et al*., 2007). The size of the head capsule is very variable, probably under the influence of environmental factors (Moller, 2013). In 1993, Schmid reported a head length of 338-586 µm in third instar larvae and 652-866 µm in fourth instar larvae. Larval size can be up to 15 mm long. Previous studies suggest that there is a general tendency for larger larvae in the late winter-early spring period and for smaller larvae in months of high temperatures such as July or August for third and fourth instar larvae (de Bisthoven *et al*., 1992; Gómez-Sande, 2018). Compared to *C. riparius*, the

most physiologically remarkable difference is the lack of haemoglobin, which gives the larvae a characteristic white colour.

2.4.2. Distribution, microhabitat and water type

P. olivacea larvae can be found in Atlantic Regions of the Northern Hemisphere at temperate latitudes and, generally in organically enriched water (de Jong *et al*., 2014). Larvae of this *Prodiamesinae* subfamily are rarely found on stones or among vegetation, as they are bottom inhabitants. They are found in flowing water with organic silt and sand with coarse and fine detritus (Moller, 2013). Since their survival is subjected to a combination of several factors such as considerable high oxygen levels, food availability, current velocity or temperature, individuals have been found at, as a maximum, 30 cm in depth where aerated surface water regularly penetrate (Williams and Hynes, 1974) and in stretches with a slow flow, where enough decomposing organic material is available (Moller, 2013).

2.4.3. Biology and physiology

As all chironomids, the life cycle of *P. olivacea* consists of four stages of different length: egg, larva (with four different instars), pupa and imago (Figure 3).

Figure 3. *Prodiamesa olivacea* life cycle. From left to right: egg mass, fourth instar larva and imago male (courtesy of Janet Graham www.flickr.com/photos/130093583@N04/).

The annual emergence has been recorded from March until October-November, and it is more abundant during January-April. (Servia *et al*., 1998; Moller, 2013). In general terms, there are two generations, emerging in spring and late summer (de Bisthoven *et al*., 1992), although in many cases, one generation is absent or very small. It is not clear the reason why under certain circumstances, there is only one generation; it might be due to poor oxygen supply (summer months) or the absence of larvae and food as a result of spates in winter (Moller, 2013).

As stated before, food availability and oxygen levels limit *P. olivacea* survival. Therefore, they try to find the balance between saprobity, in terms of intensity of decomposition, and oxygen supply, as they do not tolerate anaerobiosis inherent to places with much decomposing organic material. Besides, owing to the lack of haemoglobin and since as temperature rises, oxygen consumption increases more quickly than oxygen supply, *P. olivacea* survive better in polluted streams in winter (Moller, 2013).

2.4.4. Use in ecotoxicology

The number of studies working on this *Chironomidae* is scarce and limited since, to date, nobody has been able to maintain a *P. olivacea* culture under laboratory conditions, which limits research experimentation to natural populations.

As far as my knowledge goes along the available literature, the restricted number of studies in *P. olivacea* have focused on the analysis of morphological deformities, and cytogenetic alterations of natural populations as biomonitoring in aquatic ecosystems; but none of them has covered molecular parameters such as enzyme activity or transcription (Ilkova *et al*., 2018, 2017; Michailova *et al*., 2003; Mylnikov and Zhirov, 2016; Servia *et al*., 2000, 1998). The sensitivity of the *P. olivacea*

genome showed on these works points this aquatic dipteran as a good candidate for detecting the presence of genotoxic compounds in aquatic basins and for evaluating their genotoxic effects (Ilkova *et al*., 2018). Therefore, the identification and use of molecular biomarkers in *P. olivacea* could complement and add valuable information to freshwater monitoring and risk assessment studies in terms of early effects and organism response.

Taking all of this into consideration, biomonitoring of water quality using natural populations of *P. olivacea* might be a perfect complement in those cases where other chironomids such as *C. riparius* may be too tolerant, and some contaminant-induced effects might be minimised.

2.5. Water pollution: emerging contaminants

Water is an essential constituent of life. Owing to the industrial development and globalisation in the last few decades, a great variety of substances that damage the environment and health, have accumulated and form part of different ecosystems. Aquatic environments are especially vulnerable as they function as contaminant receptors where pollutants accumulate and integrate into a cycle that involves water, sediments and living organisms. These contaminants can have a direct toxic effect and/or bioaccumulate in the trophic chain causing adverse effects on ecosystems, aquatic biota and human health. These emerging pollutants (EPs), also known as contaminants of emerging concern (CECs), include among others drugs, pesticides, plasticisers, cosmetics, cleaning and personal care products (PCPs), as well as metabolites derived from these compounds (Rosenfeld and Feng, 2011).

According to the NORMAN network, at least 700 substances categorised into 20 classes have been identified in the European aquatic environment (www.norman-network.net). The main characteristic of

these CECs is that they do not need to be constantly present in the environment to cause adverse effects, due to their high transformation and removal rates (Barceló and López, 2008). Even though EPs are heterogeneous substances in terms of their chemical structure and use, all of them have in common toxic effects to aquatic organisms. Feminisation, hermaphroditism, or their action as endocrine disruptors are just a few examples of the harmful impacts that CECs have in aquatic environments (Grieshaber *et al*., 2018; Park *et al*., 2019).

The possible severe effects on humans and wildlife from exposure to chemicals that can interfere with the endocrine system are a growing interest in environmental toxicology in the last decades. Endocrine-disrupting chemicals/compounds (EDCs) have been described by the World Health Organization (WHO) as "*exogenous substances or mixtures that alter function(s) of the endocrine system and consequently cause adverse severe effects in an intact organism or its progeny or (sub)populations*" (Combarnous, 2017). Exposure to EDCs has reported several harmful effects including reproductive dysfunctions, developmental deformities, increased cancer risk, or disturbance in the immune and nervous system, among others (Park *et al*., 2010; Qiu *et al*., 2016; Ramakrishnan and Wayne, 2008; Sifakis *et al*., 2017; Zhang, C. *et al*., 2014). EDCs, also called, environmental hormones, are thought to cause adverse effects by different mechanisms (Figure 4) (Combarnous, 2017):

A) Direct interaction of EDCs with hormone nuclear receptors leading to stimulation (agonism) or inhibition (antagonism) of transcriptional activity.

B) Stimulation or inhibition of endogenous hormones biosynthesis.

C) Stimulation or inhibition of endogenous hormones degradation.

D) Stimulation or inhibition of endogenous hormone-binding proteins leading to decreased or enhanced circulating hormone availability.

Of around 85000 known chemical products, approximately 1000 are recognised as potential EDCs including plasticisers, flame retardants, pesticides, industrial and natural chemicals, synthetic medicaments or natural hormones and so forth (Street *et al*., 2018). Although some EDCs adverse effects can be detected (e.g. morphological changes), most of them are subtle and difficult to analyse, such as behavioural changes, which ultimately affect reproduction.

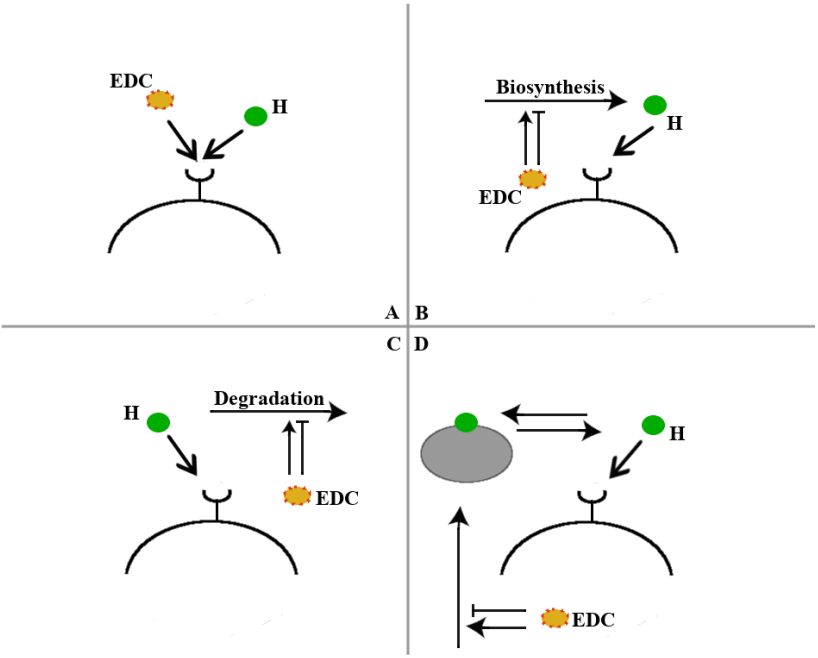


Figure 4. EDCs mechanisms of action. **A)** Direct EDC interaction with a hormone nuclear receptor leading to stimulation or inhibition of transcription, **B)** and **C)** stimulation or inhibition of endogenous hormone biosynthesis (B) or degradation (C) and **D)** stimulation or inhibition of endogenous hormone-binding protein, altering circulating hormone availability.

Because of this, it is of especial interest the detection of molecular targets of endocrine action that allow to detect xenobiotic effects on the hormonal system and to explain possible changes and consequences that could take place in organisms exposed to them. Examples of emerging contaminants of high concern with endocrine-disrupting capacity are benzyl butyl phthalate (BBP), bisphenol A (BPA) or benzophenone 3 (BP3).

2.5.1. Benzyl Butyl Phthalate (BBP)

In 2008, Benzyl butyl phthalate was classified as a substance of very high concern (SVHC) and included on the EC candidate list of substances for authorisation. The Regulation (EC) No 1272/2008 classifies the substance as toxic to reproduction and with endocrine-disrupting properties and it is included in the 1B category. Moreover, according to the harmonised classification and labelling approved by the EU, this phthalate it is classified as very toxic to aquatic life with long-lasting effects (chronic and acute 1), and may damage the unborn child (Category 2) and is suspected of damaging fertility (Category 3) (Table 1). In addition, BBP is included in Annex XIV of REACH which contains a list of SVHCs requiring authorisation before its use, and some uses of this substance are restricted under Annex XVII of REACH.

BBP is an organic colourless oily liquid substance at standard temperature and pressure. It can reach the environment via various routes: from production sites due to release to the atmosphere, wastewater effluents and air treatment plants, leaches from municipal landfills or waste incineration (Kaland, 1998).

Table 1. Summary of BBP relevant information that is expected to figure in safety data sheets.

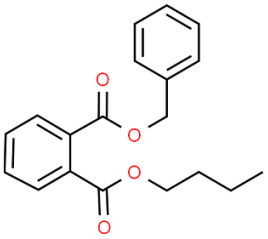
Benzyl Butyl Phthalate (BBP)		
Hazard pictogram	GSH08  GSH09 	Serious health hazard. Hazardous to the environment.
Hazard statements	H400 H360 H410	Very toxic to aquatic life. It may damage fertility or the unborn child. Very toxic to aquatic life with long-lasting effects.
Precautionary statements	P501 P405 P391 P308+P313 P281 P273 P201 P202	Dispose of contents/container in accordance with local/regional /national/international regulations. Store locked up. Collect spillage. If exposed or concerned: Get medical advice/ attention. Use personal protective equipment as required. Avoid release to the environment. Obtain special instructions before use. Do not handle until all safety precautions have been read and understood.

More than 92 % of the production of this plasticiser in the European Union has been used in the poly(vinyl chloride) (PVC) industry and the manufacture of many plastic consumer products such as conveyor belts, wall covering, artificial leather, traffic cones, vinyl gloves, cosmetics, car

care products, adhesives, carpet tiles, or shoe uppers. The European Chemicals Bureau (EU RAR, 2007) reported a BBP production of 45000 tonnes/year in the period 1994-1997, and an industrial use of 19500 tonnes/year for 2004 in the European Union. Although, there has been a considerable decline in BBP consumption in recent years due to its labelling as T; R61-62 (toxic) and N; R50-53 (dangerous for the environment). Chemical and physical properties are detailed in Table 2.

Table 2. Chemical and physical properties of BBP (NCBI).

Benzyl Butyl Phthalate (BBP)	
EC / List no.	201-622-7
CAS no.	85-68-7
Mol. formula	$-C_{19}H_{20}O_4$
Mol. Weight	312.4 g/mol
Melting point	-35 °C
Boiling point	370 °C
Vapour pressure	8.25×10^{-6} mmHg at 25 °C
Water solubility	2.69 mg/L at 25 °C
Log Kow	4.73



BBP is present everywhere in the environment: surface, ground, and drinking water as well as in sediments, sludge or other wastewater media (Table 3). It may be released to the environment from many industrial and municipal sources and it is estimated that 2.2 tonnes/year are liberated to the aquatic environment because of spillages during its distribution.

Table 3. Summary of BBP maximum concentrations (Clark *et al.*, 2003).

	Canada	US	Europe	Japan/ Asia
Surface water (µg/L)	84	66	13.9	-
Groundwater (µg/L)	-	38	0.24	-
Drinking water (µg/L)	2.8	38	< 0.1	-
Other wastewater media (µg/L)	84	449	30	1.5
Sediments (mg/kg)	370	5.5	18.2	0.02
Sludge (µg/kg)	1.40×10^4	-	2.10×10^5	-

BBP is a human-made phthalate ester that is ubiquitous in the environment. Photodegradation in the air is the major abiotic degradation process of this phthalate, whose half-life is estimated to be around 35 hours (EU RAR, 2007). On the contrary, biodegradation rates increase after 10 days and final biodegradation can take place in sludges and sediments at anaerobic conditions. Since BBP has a high bioconcentration factor (135-633 l/kg), it is considered to have a high bioaccumulation potential, which facilitates its persistence in the trophic chains, even more in those organisms with a limited BBP metabolising capacity (EU RAR, 2007).

This phthalate can be absorbed orally, dermally, and through inhalation. Several *in vivo* and *in vitro* studies have reported a large selection of harmful effects regarding BBP exposure in different organisms such as earthworms, rats, fish or even humans. Some examples of BBP toxic effects include systemic effects with body weight loss and organ weight gain (Aso *et al*., 2005), irritation and allergic responses (Kolarik *et al*., 2008), endocrine effects including morphological and functional organ alterations, altered expression, activation of key hormones such as testosterone or thyroid hormones, and even activation of estrogen receptors (ERs) and peroxisome proliferator-activated receptors (PPARs) (Harries *et al*., 2000; Lampen *et al*., 2003; Nakagomi *et al*., 2017). BBP also has developmental and reproductive consequences like female endometriosis, decreased pregnancy rates, and live births (Reddy *et al*., 2006; Sun and Li, 2022; Tyl *et al*., 2004). There have been described organ and skeletal malformations as a consequence of developmental instability (Ema *et al*., 1995) and finally, gene expression alteration, which may lead to increased susceptibility to carcinogenesis (Moral *et al*., 2007).

Finally, although there is not much available information in terms of the pro-oxidant character of this synthetic chemical, Song and collaborators reported in 2019 a direct relationship between higher BBP doses and higher generation of reactive oxygen species (ROS) in *Eisenia fetida*.

2.5.2. Bisphenol A (BPA)

In 2017, bisphenol A was one of the 205 substances listed by ECHA as SVHCs and it was included in the candidate list for authorisation. Besides, some uses of BPA were restricted under REACH Annexe XVII, and the substance is currently included in the community rolling action plan (CoRAP) to prioritise its evaluation process.

The Regulation (EC) No 1272/2008 classifies the substance as toxic for reproduction (Category 1B), skin sensitiser, and with endocrine-disrupting properties. According to the harmonised classification and labelling approved by the EU, this substance may damage fertility (Category 2), causes severe eye damage, and may cause an allergic skin reaction and respiratory irritation. Additionally, the classification provided by companies to ECHA identifies that BPA may damage fertility or the unborn child and that it is toxic to aquatic life with long-lasting effects (chronic 2 and 3) (Table 4).

Table 4. Summary of BPA relevant information that is expected to figure in safety data sheets (ECHA).

Bisphenol A (BPA)			
Hazard pictogram	GSH05		Corrosive Serious health hazard. Hazardous to the Environment.
	GSH07		
	GSH08		
	GSH09		
Hazard statements	H318 H317 H360 H335 H411		Causes serious eye damage. May cause an allergic skin reaction. May damage fertility or the unborn child. May cause respiratory irritation. Toxic to aquatic life with long lasting effects.

Table 4. Continuation

Bisphenol A (BPA)		
Precautionary statements	P261 P280 P302+P352 P305+P351+P338 P308+P313 P273 P201 P202	Avoid breathing dust/fume/gas /mist/vapours/spray. Wear protective gloves/protective clothing/eye protection/face protection. If on skin, wash with plenty of water. If in eyes, rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If exposed or concerned, get medical service/attention. Avoid release to the environment.

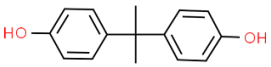
BPA is an industrial organic white solid chemical at standard temperature and pressure that has been used since 1957. 64 % of the world's BPA demand is used in the manufacture of polycarbonate plastic, 34 % for epoxy resins, and the rest for thermal paper (Almeida *et al*., 2018). Polycarbonate plastics are present in a wide variety of common reusable user goods such as food containers, water bottles and kitchen utensils as well as in medical devices, automotive parts or impact-resistant safety equipment. This chemical is also present in epoxy resins that act as a protective layer inside some metal-based food and beverage cans, bottle tops or water supply pipes.

BPA is one of the chemicals with the highest production volume worldwide, with a production of more than 4 million tons in 2016 (Almeida *et al*., 2018). It is projected that this production will reach 7.3 million tons by the end of 2023 (Global Bisphenol A market Report 2018, 2020). BPA release to the environment is likely to occur due to the indoor and outdoor use of long-life materials such as electronic equipment, flooring, furniture,

or metal, wooden and plastic construction and building materials. It is estimated that approximately 100 tons per year are released into the atmosphere (Rubin, 2011). BPA half-life in the environment is short, 4 days under aerobic conditions in freshwater (Klečka *et al*., 2001), but its presence in ecosystems is persistent due to its widespread utilisation and unceasing liberation through wastewater, landfill leachates, waste incineration and the natural decomposition of plastic materials. Therefore, many organisms are exposed almost continuously to this dangerous substance and cannot avoid exposure. Since BPA can leach into food from the protective internal epoxy resin coatings, diet is the primary source of BPA exposure for most people. Its chemical and physical properties are detailed in Table 5.

Table 5. Chemical and physical properties of BPA (NCBI).

Bisphenol A (BPA)	
EC / List no.	201-245-8
CAS no.	80-05-7
Mol. formula	$-C_{15}H_{16}O_2$
Mol. Weight	228.29g/mol
Melting point	156-157°C
Boiling point	360.5 °C
Vapour pressure	4 x 10^{-8} mmHg at 25 °C
Water solubility	300 mg/L at 25 °C
Log Kow	3.32



Owing to the multiple harmful effects of this chemical and its capacity to migrate from packaging into food, the use of BPA is being controlled by restricting its use to protect human health. On the one hand, in 2016 the EC restricted the use of BPA in thermal papers present in receipts, parking

tickets, or aeroplane boarding passes, among others. On the other hand, since June 2011 BPA has been banned from infant feeding bottles across the EU and currently, also in the EU, there is a limitation in the amount of BPA that is allowed to be released from toys for children up to three years and from any toy that is intended to be placed in a child's mouth. Finally, in December 2021, the European Food Safety Authority (EFSA) published a re-evaluation of the tolerable daily intake (TDI) and proposed to reduce the TDI from 4 µg/kg bw/day to 0.04 ng/kg bw/day.

Several research studies have pointed out the ability of BPA to interact with different types of hormone receptors involved in the regulation of the endocrine system and other systems of the organism. These receptors include nuclear and membrane-bound ERs, androgen receptors, thyroid receptors, glucocorticoid receptors and PPARs (MacKay and Abizaid, 2018). In addition, multiple works provide evidence of the endocrine-disrupting properties of BPA on numerous vertebrates (Crain *et al*., 2007), leading to alterations in multiple parameters such as reproduction, development and factors related to the immune and nervous system (O'Connor and Chapin, 2003). Finally, there are clear evidences pointing out the pro-oxidant character of this plasticiser showing the induction of ROS upon BPA exposures (Gassman, 2017; Guo, J. *et al*., 2017).

Even though the bibliography is full of studies focused on BPA toxic effects on vertebrates, scattered information is available on the biological effects of this EDC in invertebrates. Developmental and reproduction effects have been observed in the earthworms *E. fetida* and *Dendrobaena veneta* (Verdú *et al*., 2018). Superfeminization has been described in the mollusc *Marisa cornuarietis*, where BPA exposures led to additional female organs, enlarged sex glands and increased fecundity among other effects (Oehlmann *et al*., 2006, 2000). BPA endocrine effects have also been seen in other molluscs (de Andrade *et al*., 2017; Morales *et al*., 2018),

crustaceans (In *et al*., 2019; Plahuta *et al*., 2015) and insects (Maria *et al*., 2019; Weiner *et al*., 2014).

2.5.3. Benzophenone 3 (BP3)

Benzophenone 3, also called oxybenzone, is an ultraviolet radiation (UVR) filter that is under assessment by the ECHA and de CoRAP as an endocrine-disrupting compound. It is a substance classified as very toxic to aquatic life with long-lasting effects (Table 6).

Until 2018, the only regulation concerning the use of BP3 has been the percentage of the amount of this UVR filter that can be used as an active ingredient in sunscreens (Annex VI of Regulation (EC) No 1233/2009). While in Korea or the US maximum allowable levels of BP3 are up to 5-6 %, European levels are as high as double (10 %) (EEC Directive, 1983; Korea Food and Drug Administration, 2012; US FDA Department of Health and Human Services, 2013). In 2018, Hawaii was the first state in the US banning sales of sunscreens containing oxybenzone, among others UVR filters, due to concerns of coral reef bleaching (Narla and Lim, 2019). Since then, similar legislation has been approved and discussed in different states, nature reserve areas and the EU.

Table 6. Summary of BP3 relevant information that is expected to figure in safety data sheets (ECHA).

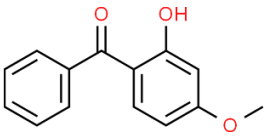
Benzophenone 3 (BP3)			
Hazard pictogram	GSH07		Health hazard. Hazardous to the Environment.
	GSH09		
Hazard statements	H400 H411		Very toxic to aquatic life with long lasting effects.
Precautionary statements	P273 P391 P501		Avoid release to the environment. Collect spillage and dispose of contents/ container to an approved waste disposal plant.

BP3 is a pale greenish-yellow powder substance at standard temperature and pressure that absorbs and scatters the sun's harmful ultraviolet (UV) rays. It is an organic UV filter that is photostable, lipophilic and potentially bioaccumulative in biota and sediments. Due to its chemical and physical properties (Table 7), BP3 is widely used in sunscreens and many other PCPs, including cosmetics, lipsticks, hair conditioners and sprays or insect repellents (Liao and Kannan, 2014). In the last decades, the presence of organic UV filters in aquatic ecosystems have been detected due to the global use of PCPs (Campos *et al*., 2019). The relative high BP3 log-Kow value suggests its slow biodegradation, that has been estimated in by about 4 % after 28 days in water (Chemicals Inspection and Testing Institute, 1992). It also tends to adsorb to suspended solids and sediments and has a low volatilisation potential from water surfaces. The half-life in surface water of this UVR filter has

been estimated at a few weeks in winter and 7-9 times higher in winter (Vione *et al*., 2013). All these properties make BP3 a perfect threat to the environment, where several harmful effects have been reported.

Table 7. Chemical and physical properties of BP3 (NCBI).

Benzophenone 3 (BP3)	
EC / List no.	205-031-5
CAS no.	131-57-7
Mol. formula	$-C_{14}H_{12}O_3$
Mol. Weight	228.24 g/mol
Melting point	65.5 °C
Vapour pressure	6.62 x 10^{-6} mmHg at 25 °C
Water solubility	3.7 mg/L at 25 °C
Log Kow	3.79



Oxybenzone is released into the environment in two primary ways: directly from sloughing off during recreational activities, or indirectly via wastewater treatment plant effluents (WWTP). BP3 concentrations registered in freshwater ranged from 125 ng/L in lake Hüttnersee (Switzerland) to 620 ng/L in outdoor swimming pools of South Bohemian (Czech Republic) (Grabicova *et al*., 2013; Poiger *et al*., 2004), or even higher levels in beaches of densely populated resort areas. Recently, it has been calculated that almost 11 % of the BP3 production in the US reaches WWTPs, while 3 % is emitted through WWTPs (Wang and Kannan, 2017). Levels of this UV filter in WWTPs ranged from < 5 up to 10400 ng/L, with the highest concentration recorded in wastewater at San Diego, US (Loraine and Pettigrove, 2006). BP3 and other benzophenones have also been detected in sediments of Chinese and American rivers and sludge of Chinese WWTPs in the range of ng/gr (Zhang, Z. *et al*., 2011).

UV filters have been detected in aquatic environmental samples (Fent *et al*., 2010; Grabicova *et al*., 2013; Sánchez-Quiles and Tovar-Sánchez, 2015; Tarazona *et al*., 2010), tap water and treated sewage sludge (Díaz-Cruz *et al*., 2012; Gago-Ferrero *et al*., 2011). Concerns have been raised over the safety of BP3 due to its endocrine-disrupting capacities. *In vitro* and *in vivo* studies have shown the antiestrogenic and antiandrogenic activity of BP3 in rodents and fish. BP3 binding affinity to ERs (ERα and ERβ) have been reported in *in vitro* studies, but also in fish like *Danio rerio* or in immature rats where BP3 exposure led to changes in the profile expression of estrogen-related genes and an increase in uterine weights (Blüthgen *et al*., 2012; Molina-Molina *et al*., 2008; Schlumpf *et al*., 2001; Schreurs *et al*., 2002). On the other hand, developmental and reproductive toxicity has been reported for aquatic vertebrate and invertebrate organisms such as algae *Desmodesmus subspicatus*, crustacean *D. magna* or fish *O. latipes* (Coronado *et al*., 2008; Sieratowicz *et al*., 2011) and studies working on *Carassius auratus* and *Mytilus galloprovincialis*, have demonstrated the capacity of BP3 to increase the production of ROS, leading to oxidative stress (Bordalo *et al*., 2020; Liu *et al*., 2015). As of last, in 2008 Danovaro *et al*. discovered for the first time the negative impact of oxybenzone to corals leading to bleaching and death. Downs *et al*. extended that work, in 2016, identifying the UV filter as a phototoxic, genotoxic and endocrine disruptor chemical (Danovaro *et al*., 2008; Downs *et al*., 2016).

Despite invertebrates play an important role in global biodiversity, studies on the possible toxic effects upon BP3 exposure are still scarce in these organisms. The available bibliography focus on the effects at the organism level, reports alterations in reproduction, development and survival of invertebrates such as the bivalve *M. galloprovincialis*, the crustacean *Siriella armata* or the echinoderm *Paracentrotus lividus* (Paredes *et al*., 2014). Having said this, there is a clear knowledge gap

concerning the mode of action of this compound at the cellular and molecular level. Additionally, investigations on invertebrates are necessary to fully understand the potential effects of UV filters in aquatic ecosystems.

Although the number of ecotoxicological studies focused on deciphering the molecular effects of these three chemicals in invertebrates is still scarce, there has been described molecular alterations in genes involved in relevant pathways. It has been reported the downregulation of genes related to critical cellular processes such as stress response, hormonal pathways, ribosome biogenesis, energy metabolism and detoxification activities in laboratory larvae of *C. riparius* exposed to BBP (Herrero *et al*., 2016, 2015) in contraposition to the gene overexpression seen in natural populations of this chironomid exposed to the plasticiser (Herrero *et al*., 2014). Moreover, Planelló *et al*. described the induction of stress and endocrine-related genes upon *C. riparius* BBP exposures (Planelló *et al*., 2011). Finally, recently Song and Zhou described the deregulation of detoxification genes in earthworms (Song and Zhou, 2019).

Significant changes in genes involved in the immune system have been reported in individuals of *M. galloprovincialis* and in the mud crab *Macrophthalmus japonicus* exposed to BPA (Canesi *et al*., 2007b; Park *et al*., 2019). In addition, Canesi also reported a deregulation in the redox balance and an increase in the expression of *ER* in contraposition to a downregulation of metallothionein genes which may have implications for mussel health (Canesi *et al*., 2007a, 2007b). Endocrine system disruption has been described in several BPA-exposed species. Upregulation of endocrine-related genes was reported in the freshwater mudsnail *Potamopyrgus antipodarum* (Stange *et al*., 2012), *Physa acuta* (Morales *et al*., 2018), in midges (Hwang *et al*., 2010; Morales *et al*., 2020), crustaceans (In *et al*., 2020, 2019) and moths such as *Sesamia nonagrioides*

(Kontogiannatos *et al*., 2015), while decreased gene levels were described in *E. fetida* (Novo *et al*., 2018), *Spodoptera littoralis* (Maria *et al*., 2019) and copepods (Hwang *et al*., 2010). Different alterations have been described for the cell stress pathway upon this xenobiotic. While lower levels of *HSPs* genes were reported in individuals of *Gammarus fossarum* (Schirling *et al*., 2006), increased genes expression have been described for different species of *Chironomus* (Lee *et al*., 2006; Martínez-Paz *et al*., 2014; Morales *et al*., 2011; Planelló *et al*., 2008), *P. acuta* (Morales *et al*., 2018) and *C. elegans* (Zhou *et al*., 2016). In addition to increased stress-related gene expression, exposures of this soil nematode to BPA have triggered the transcriptional induction of genes involved in the detoxification system (Ficociello *et al*., 2021). Similar results have been described in *D. magna* (Kim *et al*., 2019) or the mussel *Perna viridis* (Juhel *et al*., 2016), in contraposition to the inhibition reported for *C. riparius* (Martínez-Paz *et al*., 2012).

As last, the knowledge concerning the toxic effects of BP3 is very scarce. It has been described that BP3 provokes the deregulation of the cell stress and detoxification pathway in *C. riparius* (Martín-Folgar *et al*., 2018; Martínez-Guitarte, 2018). A research work with the coral species *Stylophora pistillata* pointed out the ability of this UV filter to disrupt the endocrine system and to damage the DNA (Downs *et al*., 2016).

2.6. Approach to experimental design

Biomarkers play an important role in environmental assessment and therefore it does not surprise the increasing interest that their use in ERA is having in the last decades. Biomonitoring of freshwater environments and the evaluation of ecosystems health status are essential in the development of effective protection strategies for the environment, human health and sustainable development. In this sense, one of the

research lines in toxicology is the identification of early warning signals or biomarkers that reflect adverse biological responses in a wide variety of organisms owing to exposure to anthropogenic environmental toxins. The most promising feature of biomarker investigation in terms of ERA is that these early indications include both time and concentration.

The term of biomarker has evolved along the time from the first definition in 1990 given by McCarthy and Shugart as "*measurement at the molecular, biochemical or cellular level in either wild populations from contaminated habitats or in organisms experimentally exposed to pollutants that indicate that the organism has been exposed to toxic chemicals and the magnitude of the organism's response to the contaminant*" to the basic and actual one given by the FDA-NIH previously mentioned.

Biomarkers are widely used for monitoring the effects of contaminants in freshwater ecosystems and to assess their health status via aquatic organisms' health. They are also used to test whether altered ecotoxicological parameters recover after a punctual contamination episode either naturally or after a bioremediation process (Cajaraville *et al*., 2000). Since biomarker responses integrate a wide array of environmental, toxicological and ecological factors suspected to be altered by environmental contaminants, they are potent tools, although sometimes complex to interpret (van der Oost *et al*., 2005). Therefore, for a successful ERA, it is indispensable a good understanding of the mechanisms underlying biomarker responses. In this sense, the presence of certain xenobiotics in the environment may modulate the expression of specific genes, and the analysis of gene expression profiles constitute a robust tool in ecotoxicology studies. The huge boom of molecular techniques in recent decades has impulse a new approach in environmental sciences in terms of risk assessment, integrating molecular and ecotoxicological responses. Environmental genomics combines gene

expression studies at different molecular levels (genome, transcriptome, proteome or metabolome) in natural populations, which allows a better understanding of the underlying mechanistic responses of organisms to xenobiotics exposure.

In this thesis, an experimental model based on the characterisation and validation of selected molecular parameters in *P. olivacea* has been used to study early time alterations produced by BBP, BPA or BP3 exposure, as well as the molecular effects owing to temperature changes.

In this sense, quantitative Polymerase Chain Reaction (qPCR), also called real-time PCR (RT qPCR) is a high sensitivity molecular technique that allows quantification of mRNA from genes transcribed at very low levels (Panti *et al*., 2011). This tool provides early warning of toxic stress or detoxification processes (Forbes *et al*., 2006; Piña *et al*., 2007). Although changes in protein profiles are ultimately responsible for physiologic effects in the organisms, early detection of differences in gene expression profiles in terms of RNA levels constitutes an excellent approximation to analyse possible hazardous effects due to exposure to toxic compounds.

One of the main mechanisms used by insects to fight against adverse conditions is represented by biotransformation routes that confer metabolic resistance. In this sense, antioxidant enzymes are crucial in the defence of the organisms against different stressors. In this sense, aquatic organisms and insects contain a vast pool of antioxidant enzymes that exhibit multiple antioxidant defences (such as catalase), as well as small antioxidants molecules (e.g. glutathione), each of which differs in contaminant sensitivity. As multiple available works suggest, antioxidant defences can be quantitatively altered depending on contaminant exposure, which has led to their exploitation as field biomarkers (Borges *et al*., 2018; Clark, 1989; Faheem and Lone, 2017; Kumari *et al*., 2014; Terriere, 1984).

2.6.1. Heat-shock proteins and cellular stress response

The cellular stress response comprises "*an orchestrated induction of key proteins that form the basis for the cell's protein repair and recycling system*" (Sanders and Dyer, 1994). This response can be activated by a wide variety of factors including hypoxia, anoxia, and also the presence of some metals, xenobiotics or UVR, among others.

Many stress-induced proteins have been described over time, and heat-shock proteins (HSPs) were the first of them, reported by Ferruccio Ritossa in 1962 in *Drosophila buschii* larvae submitted to abrupt temperature changes (5-15 °C) (Ritossa, 1962). Although HSPs is the classical name of these proteins, nowadays, they are well known to be induced by other stressors including anoxia, salinity stress, xenobiotics and oxidative stress in general (Amiard-Triquet *et al*., 2013); consequently, they can be found renamed as stress proteins (Newman, 2014). The fact that genes coding for HSPs are present in all organisms studied to date and, numerous stressors can induce them explains why these proteins have been widely explored in natural and anthropogenic environmental stress conditions such as seasonal temperature variations, hypoxia or chemical contamination (Feidantsis *et al*., 2020; Muñiz-González *et al*., 2021; Park *et al*., 2020).

HSPs are the most conserved proteins in eukaryotes and prokaryotes, and they are named and classified in families based on their molecular weight (in kilodaltons, kDa): high molecular weight family, HSP90 family, HSP70 family, HSP60 family, and the small or low molecular weight HSP family (Desai *et al*., 2010). As summarised in Figure 5, under stress conditions, these proteins play an essential role as molecular chaperones in the intracellular environment by assisting correct folding of nascent and stress-accumulated misfolded proteins, avoiding protein aggregation or triggering selective degradation of misfolded or denatured proteins (Gupta *et al*., 2010). Although there are basal cellular levels of these

proteins in normal conditions (constitutive expression), their transcription is rapidly induced upon any scenario that can confer a threat to the organism (stress-inducible expression). HSPs induction involves a family of heat-shock transcription factors (HSFs) that bind the heat-shock elements (HSEs) of the *HSPs* genes and mediate their transcription (Zhang, Y. *et al*., 2011). Interestingly, unlike other HSPs, low molecular weight HSPs have no known constitutive function and are only inducible during stress conditions (Ciocca *et al*., 1993).

HSPs play a central role in cellular protein homeostasis and repair in response to stress and harmful conditions. Since the induction of these stress proteins is the signal of exposure to conditions that alter intracellular proteins and given that their overexpression represents a molecular mechanism to manage stress, HSPs can be valuable indicators of stress responses in a diversity of organisms and circumstances (Matarredona *et al*., 2020; Mota *et al*., 2019; Niu *et al*., 2020). Therefore, these proteins have been largely used as biomarkers for the early detection of environmental changes and ecological risks in ecosystem biomonitoring.

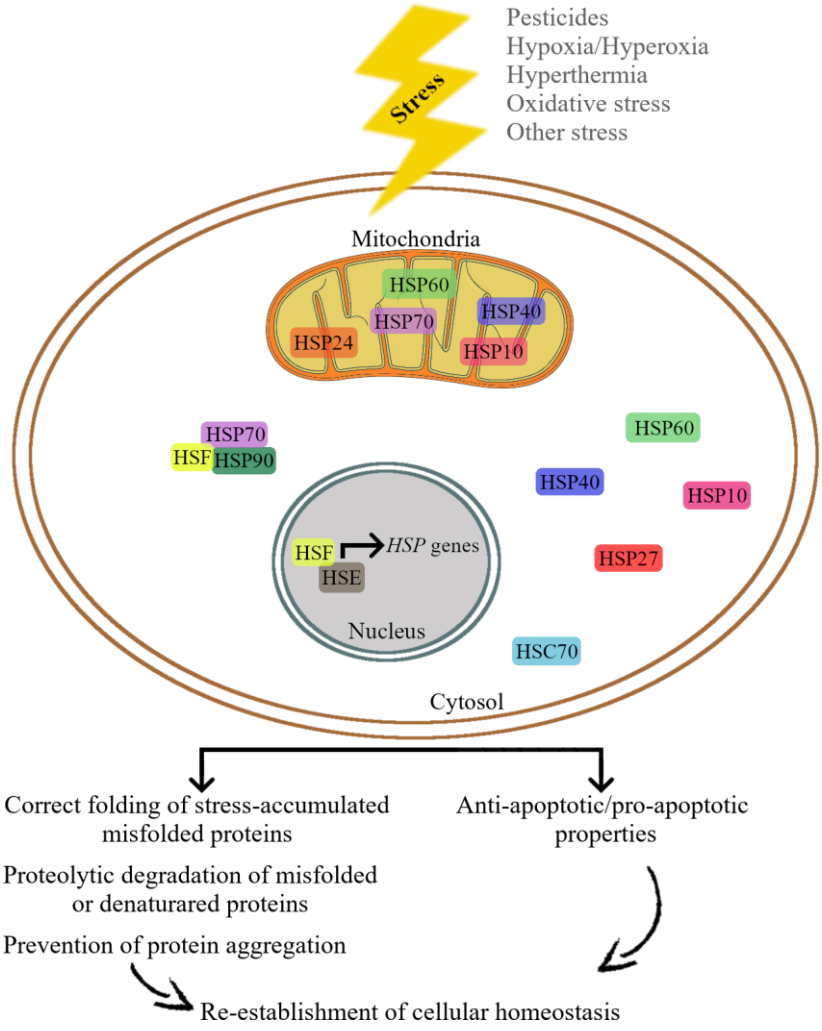


Figure 5. Schematic representation of HSPs induction under stress conditions. Different types of stressors such as pesticides, hypoxia, hyperoxia, hyperthermia or oxidative stress among others trigger the transcription of different HSPs genes to re-establish the cellular homeostasis.

To accomplish the defensive role, each HSP has different cellular functions. The main roles and cell location of the stress proteins analysed in this thesis are detailed below.

HSP90 (cytosolic)

The HSP90 is the most abundant cytosolic HSP family in eukaryotic cells (Gupta *et al*., 2010). As a molecular chaperone, HSP90 is involved in the correct maturation and activation of several key intracellular proteins like kinases, transcription factors, ribosomal proteins or even steroid hormone receptors (Jackson, 2013). HSP90, in cooperation with multiple co-chaperones, orchestrates crucial cellular processes including cell cycle control, cell survival, apoptosis, hormone signalling and other pathways. In addition to its chaperone function and regarding cell signalling through the maturation of key hormone receptors, HSP90 is involved in cytoskeleton arrangement and maintenance. Moreover, since HSP90 modulates DNA structure and contributes to RNA synthesis and processing, the percentage of nuclear *Hsp90* increases upon heat stress (Hoter *et al*., 2018). It confers resistance to thermal and osmotic stress as well as to metal contamination. Finally, in the last years, this cytosolic chaperone has been largely studied in cancer therapy as a target for anti-cancer drugs, due to the high sensitivity of cancer cells to HSP90 inhibitors (Hoter *et al*., 2018). Cytosolic chaperones HSP90 and HSC70 often function together and, according to some authors, they might be considered as parts of a more extensive multi-chaperone system (Young *et al*., 2003).

HSC70 (cytosolic)

The heat-shock cognate protein 70 is a constitutively expressed molecular chaperone that is mildly induced during stress situations. As a chaperone, it plays an essential role in protein homeostasis (folding,

maturation, translocation, assembly, disassembly, differentiation and degradation), maintaining their normal functions and structures. HSC70 cooperates and interacts with numerous co-chaperones and many other molecules, and it regulates various cellular functions. Among others, HSC70 affects and modulates intracellular trafficking of ion channels, liposome aggregation, membrane protein folding and polypeptide translocation, nucleocytoplasmic transport, lysosomal degradation of intracellular proteins, steroid receptor maturation and signalling, or apoptosis. HSC70 relocates in the nuclei when cells are exposed to stress conditions such as heat-shock or oxidative stress (Liu *et al*., 2012).

HSP70 (cytosolic)

The HSP70 family represents one of the most highly conserved classes of heat-shock proteins and, the first to be highly induced under a wide variety of stress conditions (Gupta *et al*., 2010). The HSP70 chaperone system has housekeeping and quality control functions in the cell as these 70-kDa proteins assist: protein folding and assembly (including the ecdysone hormone receptor), refolding of misfolded and aggregated proteins, translocation of secretory proteins and organelles to the membrane, control of regulatory proteins or differentiation and cell death, among other processes (Gehring, 1998; Mayer and Bukau, 2005). They confer resistance to thermal stress, xenobiotics, heavy metals, osmotic shock (salinity), hypoxia, UVR, hydrogen peroxide, or apoptosis (de Jong *et al*., 2008; Mayer and Bukau, 2005).

HSP60 (mitochondrial)

The HSP60 is a mitochondrial chaperone that has an essential role in the assembly of oligomeric proteins within organelles and protein translocation (Lewis *et al*., 1999). In addition to protein homeostasis,

HSP60 has been implicated in cell survival, intracellular protein trafficking, and in peptide-hormone and apoptosis signalling pathways (Cappello *et al*., 2008). Induction of *Hsp60* gene has been reported upon a variety of chemical and physical stresses, including heat-shock and exposure to heavy metals or xenobiotics (Gupta *et al*., 2010).

HSP27 (cytosolic)

The small heat-shock protein 27 is a multidimensional protein that acts as a chaperone and as an antioxidant. It plays a crucial role in apoptosis inhibition, actin cytoskeletal remodelling, and in the resistance to cellular stress conditions including cytotoxic drugs, oxidative stress and heat-shock (Ciocca *et al*., 1993; Vidyasagar *et al*., 2012). As a chaperone, it prevents the aggregation of damaged or unfolded proteins and polypeptides by facilitating their proper refolding. Moreover, HSP27 acts as an antioxidant, given that it reduces intracellular levels of ROS by raising glutathione levels and decreasing iron levels. In addition, HSP27 is an anti-apoptotic agent under chemical stress conditions that interacts with mitochondrial and independent apoptosis pathways (Vidyasagar *et al*., 2012). As of last, this small HSP regulates actin cytoskeletal dynamics by promoting actin polymerisation and functioning as actin capping protein (Huot *et al*., 1996).

2.6.2. Endocrine pathway

The endocrine system of invertebrates is a cell-cell communication that involves a complex network of hormone signalling that affect target cells. These endocrine processes use a wide variety of hormones to regulate growth, development, metabolism and other physiological parameters (Hartenstein, 2006). The endocrine system of insects is an important link between the environment and various physiological and

developmental events, constituting the most well-known and studied hormone system because of the economic and ecological importance of these invertebrates (deFur, 2004). Development and metamorphosis from egg to adult in holometabolous insects, including Diptera, are processes mainly regulated by two types of hormones: ecdysone and juvenile hormone (JH) (Riddiford, 1993).

Ecdysteroid and JH signalling pathways with their respective hormones are the most well-studied routes in insects. In general, for all the insects studied to date, it has been reported that these two signalling pathways work in a synergistic way to regulate development and metamorphosis (Noriega, 2014; Riddiford, 2012). Holometabolous insects undergo different larval stages before reaching the pupa phase, when severe physiological and morphological changes occur, leading to the sexual maturity of the individual and the emergence of the active adult stage. Stage transitions are preceded by peaks in ecdysone release. Additionally, the timing of ecdysone liberation, as well as the presence or absence of JH, will condition moulting and metamorphosis (Berger *et al*., 1992; Riddiford, 1978; Talbot *et al*., 1993).

A wide variety of changes in gene expression are involved in the control of insect developmental transitions. These changes are under the control of several key hormones and neuropeptides, but the master regulator is the steroid hormone ecdysone (Yamanaka *et al*., 2013). All these processes, summarised in Figure 6, are initiated in response to external stimuli through the secretion of prothoracicotropic hormone (PTTH), which is produced by two pairs of lateral neurosecretory brain cells. PTTH secretion stimulates the prothoracic gland (PG) in the haemolymph, where ecdysone is primarily synthesised from cholesterol molecules. This hormone is initially inactive and is converted into its active form 20-hydroxyecdysone (20E) in the peripheral tissues and fat body by a pool of ecdysteroidogenic genes (Miyakawa *et al*., 2018; Riddiford, 2012). The

classical knowledge around ecdysone control release, points out that, PTTH-producing neurons integrate and evaluate environmental and developmental stimuli in order to determine when the progress to the next developmental stage takes places. Nevertheless, new evidences have shown that multiple factors, in addition to PTTH, act on the PG to control ecdysteroidogenesis, suggesting that PG is the "decision-making centre" of developmental transitions (Yamanaka *et al*., 2013). Along with ecdysone, JH has primary roles in regulating development and reproductive maturation.

JH is a hormone unique to insects, synthesised in the *corpora allata* gland (CA) in the brain and dispersed into the haemolymph to act at peripheral tissues. It is converted into its most commonly active form, JH III by a series of enzymes (Goodman and Cusson, 2012; Miyakawa *et al*., 2018; Riddiford, 2012). JH biosynthesis is regulated by a wide variety of factors, including 20E and JH itself (Goodman and Cusson, 2012). Moreover, the juvenile hormone epoxide hydrolase (JHEH) is involved in JH catabolism by reducing JH titers in critical developmental periods (Goodman and Cusson, 2012).

In peripheral target cells, ecdysone and JH bind to specific receptors. 20E binds to a nuclear receptor superfamily member, the ecdysone receptor (EcR). This receptor forms a heterodimer with the ultraspiracle (USP) protein, an orphan nuclear receptor for which no endogenous ligand has been unambiguously stabilised (Goodman and Cusson, 2012). The hormone-activated EcR acts as a transcription factor by binding to hormone response gene promoters, thus triggering a cascade of changes in the expression of numerous downstream genes responsible for the major insect developmental transitions (Yao *et al*., 1993, 1992). Although USP does not contribute to the intrinsic function of EcR, it is an obligatory heterodimeric partner of the receptor required for both high-affinity ligand and DNA binding, as it acts as an allosteric effector (Browning *et al*.,

2007). Moreover, USP has been described as the JH receptor, as well as the methoprene-tolerant (Met) receptor (Riddiford, 2012). The nuclear receptor USP, an orthologue of the vertebrate retinoid X receptor (RXR), binds JH III and it has been suggested that this interaction may be necessary for the dimerisation of USP:EcR (Iwema *et al*., 2009).

Insect reproductive maturation from larvae to adult is controlled by an interplay between 20E and JH levels. JH exerts a negative or inhibitory action on the maturation process at the moult in insects which will result in a juvenile form (deFur, 2004). One of the described anti-metamorphic transcription factors that are transduced due to the JH signal is the Krüppel homolog 1 (*Kr-h1*) (Lozano and Belles, 2011). Thus, while simultaneous high levels of 20E and JH trigger moulting processes, high 20E levels combined with low JH levels lead to metamorphosis. A possible model of this 20E/JH interplay that considers larval growth, larval moulting and metamorphosis was described by Goodman and Cusson in 2012: 1) JH binds to homodimeric USP which interacts with JH specific response elements related to larval growth; 2) JH binds to USP:EcR and in the presence of 20E acts in concert with ecdysteroid-specific response elements to coordinate a larval moult and 3) in the absence of JH, 20E binds to USP:EcR and triggers metamorphosis.

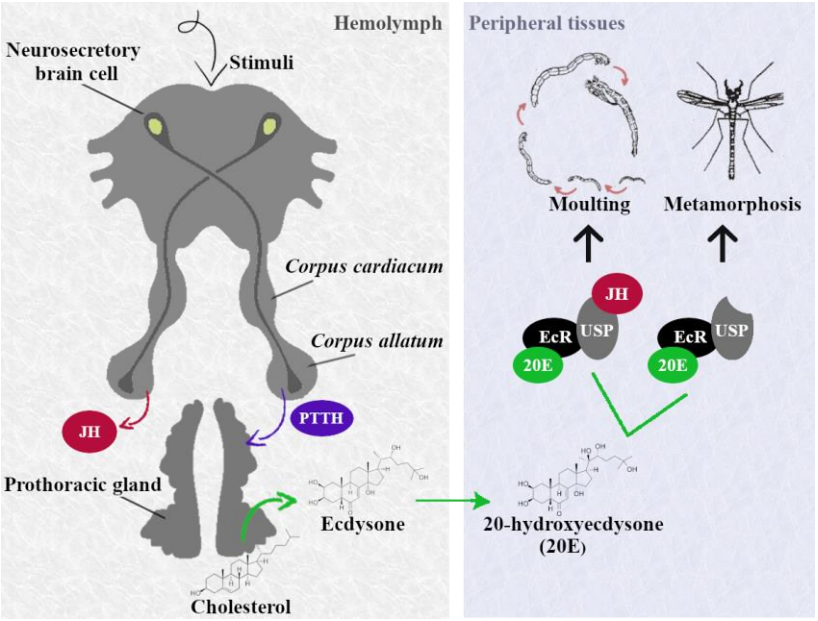


Figure 6. Insect ecdysone synthesis and JH/20E interplay in moulting and metamorphosis. In the account of external stimuli, PTTH is secreted by the neurosecretory brain cells and stimulates the prothoracic gland where ecdysone is synthetized from cholesterol. JH is synthesized by the *corpora allata* and secreted to the hemolymph. In the peripheral tissues, ecdysone is transformed into its active form 20E. Moulting and metamorphosis are subjected to an interplay between JH and 20E levels. 20E binds to the EcR and JH to the USP; while in the presence of both hormones, moulting takes place, when JH levels are low or it is absent, metamorphosis occurs. Figure modified from (Walker, 1987) and MacMillan Learning digital content (www.macmillanlearning.com).

In addition to EcR, other nuclear receptors are the estrogen-related receptors (ERR) that were identified based on sequence similarity to ER (Park and Kwak, 2010). Phylogenetic tree reconstruction proved that ERRs belong to the subgroup of steroid hormone receptors (Laudet, 1997),

revealing their similar structural and functional attributes to ERs, including binding to synthetic estrogenic ligands (Giguère, 2002). ERR and ER have overlapping affinities for both co-activators and DNA-binding sites, but they differ concerning their ligand binding and activation (Giguère, 2002).

Ecdysteroids are steroid hormones that play an essential role in development, growth, larval moulting and reproduction in *Chironomidae* species. Since chironomids undergo a full hormonally controlled metamorphosis added to the fact that aquatic organisms are directly exposed to pollutants, their endocrine system is the most vulnerable to environmental hormones (Kabir *et al*., 2015). These organisms are a powerful tool and of particular interest in ecotoxicology studies to assess possible contaminants endocrine effects. To date, several works have reported endocrine-disrupting molecular effects on *C. riparius* under different stress conditions such as metal, xenobiotic or antibiotic contamination, which evidence this aquatic dipteran as an excellent predictive model and useful in risk assessment (Arambourou *et al*., 2020, 2019; Herrero *et al*., 2018; Nair and Choi, 2012; Ozáez *et al*., 2016; Park and Kwak, 2018, 2010).

2.6.3. Detoxification mechanisms and oxidative stress

Throughout evolution, organisms have developed different mechanisms to adapt to the changing environment. Continuous chemical exposure is one of the most common threats that living organisms have to cope with. Most xenobiotics are lipophilic, and the only way to get rid of them is through metabolism. Metabolic biotransformation is a detoxification mechanism consisting of a chain of reactions that increase the water solubility of the compound and facilitates its excretion in three phases (modification, conjugation and excretion). Phase I reactions

consist of a chemical modification by introducing functional groups, while Phase II enzymes conjugate metabolites from Phase I with endogenous substrates increasing their water solubility and thereby facilitating their excretion in Phase III (Figure 7) (Amiard-Triquet *et al*., 2013).

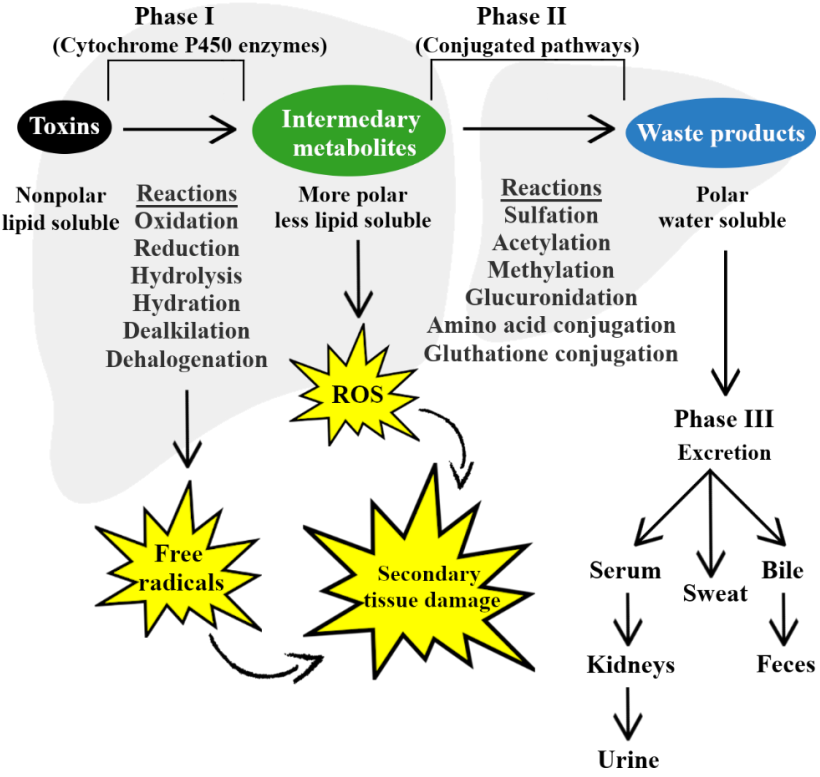


Figure 7. Phases of cellular detoxification system of xenobiotics. Cytochrome P450 enzymes are involved in biotransformation phase I by metabolising the compounds to make them more polar and less lipid soluble. Phase II conjugation reactions make the intermediary metabolites polar and water soluble to facilitate their cellular excretion in phase III. Free radicals and ROS are formed as a consequence of biotransformation reactions triggering secondary tissue damage.

In Phase I, the compound to metabolise undergoes a chemical modification by introducing polar functional groups through different chemical reactions such as oxidation, reduction and hydrolysis. Among all the enzymes involved in this biotransformation phase, cytochrome P450 (P450) superfamily oxidases are the primary catalytic system that metabolises endogenous and exogenous substrates. If Phase I metabolites are polar enough, they are excreted in the feces. Nevertheless, most of these products are difficult to eliminate, and they undergo Phase II biotransformation reactions.

Phase II reactions involve conjugation of the xenobiotic or its Phase I metabolite with endogenous ligands, usually large and polar chemical groups or compounds such as amino acids or sugars. A variety of transferases catalyses these conjugations. The result of this phase is a conjugated metabolite with higher molecular weight and more water-soluble than the original xenobiotic, which makes it less reactive and easier to excrete. One of the most important reactions is the conjugation with glutathione (GSH) catalysed by glutathione S-transferases (GSTs).

Phase II conjugates and their metabolites are excreted from cells in Phase III biotransformation processes. Their anionic groups act as affinity target for a wide range of transmembrane transporters, including the multidrug resistance-associated proteins (MRP) and the p-glycoprotein.

P450 and GST form part of two multifunctional detoxifying enzyme groups that are involved, respectively, in Phase I and Phase II reactions, metabolising a wide variety of compounds including xenobiotics and endogenous substrates (Ali *et al*., 2019; Cochrane and LeBlanc, 1986; Feyereisen, 1999; Scott and Wen, 2001; Wang *et al*., 2015).

Despite their name, P450s are formally not cytochromes. They are heme-thiolate proteins specialised in the metabolism of endogenous substrates, such as steroid hormones or lipids, and xenobiotic

detoxification (Feyereisen, 2012). CYP genes constitute one of the oldest and largest families of genes, with representatives in all living organisms: bacteria, protists, plants, fungi and animals (Feyereisen, 1999; Werck-Reichhart and Feyereisen, 2000). Their great diversity explains existing variations in biotransformation and xenobiotic toxicity among different species and also among individuals (Brattsten *et al*., 1986; Scott, 1999). P450s are best known for their NADPH-dependent monooxygenase role, catalysing the transfer of one atom of molecular oxygen to a substrate, and reducing the other to water. These low substrate-specific enzymes also catalyse at least 60 different chemical reactions (Feyereisen, 2012). Regarding the spectrum of P450s substrates, these cytochromes show plasticity and permissiveness that ensures the metabolisation of any xenobiotic. Insect's genomes carry more than a thousand *CYP* genes. They evolved from a common ancestral gene, and have been classified into five insect-specific families (*Cyp6*, *Cyp9*, *Cyp12*, *Cyp1*8 and *Cyp28*) and one family that includes sequences from vertebrates (*Cyp*4) (Arensburger *et al*., 2010; Feyereisen, 1999).

In addition to their fundamental role in detoxification, P450s are involved in basic physiological processes such as synthesis, activation and degradation of endogenous hormones including ecdysteroids and juvenile hormone (Feyereisen, 2012; Gilbert and Warren, 2005; Kayser *et al*., 1997); although in the response of these specific genes there must be an interaction between chemical exposure and endocrine functions (Le Goff *et al*., 2006).

Studies focus on the biological processes underlying toxification and detoxification have allowed unravelling the toxicity of many chemical substances. Molecular analyses of P450 transcript levels upon xenobiotic exposures have revealed a variation in the expression levels of these genes (Hao *et al*., 2014; Zhou *et al*., 2010). A similar response was described in

natural and laboratory populations of *C. riparius* (Herrero *et al*., 2017, 2015; Lencioni *et al*., 2016; Martínez-Guitarte, 2018; Planelló *et al*., 2013).

Although many *Cyp450* genes have been described in insects, particularly in dipterans, the fact is that these genes have not been so well explored in chironomids along the time. Studies characterising and evaluating *Cyp450* genes response in these non-biting midges are relatively new due to the growing interest on these species as a powerful tool to understand underlaying molecular mechanisms of toxicity and the mode of action of certain xenobiotics (Martínez-Guitarte, 2018; Martínez-Paz *et al*., 2012; Nair *et al*., 2013).

GSTs are a diverse superfamily of multifunctional proteins ubiquitously found in both eukaryotes and prokaryotes (Sheehan *et al*., 2001). They play a central role in cellular detoxification of toxic compounds including insecticides, plant secondary metabolites and organic hydroperoxides (Ranson and Hemingway, 2005) by conjugating these chemicals with a wide variety of electrophilic compounds (Board and Menon, 2013). These transferases are involved in intracellular transport, hormone biosynthesis, and protection against oxidative stress (Enayati *et al*., 2005). GSTs are classified according to their location within the cell: microsomal, cytosolic and mitochondrial (Hayes *et al*., 2005; Sheehan *et al*., 2001). To date, there is no data about mitochondrial GSTs in insects, and most of the works have been focused on the cytosolic ones, which in addition, are involved in bioremediation processes and cellular protection against ROS (Creaney *et al*., 1995; Escartín and Porte, 1996; Nair and Choi, 2011; Tjalkens *et al*., 1998).

The increasing interest in the study of insect's GSTs has primarily focused on their role in insecticide resistance as several works have linked insecticide resistance to higher GST activity levels and the production of different isoforms (Che-Mendoza *et al*., 2009; Hernandez *et al*., 2018;

Huang *et al*., 1998; Prapanthadara *et al*., 1993; Ranson and Hemingway, 2005; Sanil *et al*., 2014; Vontas *et al*., 2001). Besides, several works have pointed out in chironomid larvae that this enzymatic system takes part in the biotransformation of polycyclic aromatic hydrocarbons (PAHs) and other organic pollutants (Martínez-Paz *et al*., 2012; Park *et al*., 2010; Rakotondravelo *et al*., 2006; Yuen and Ho, 2001).

As of last, it is important to consider that while biotransformation reactions usually represent detoxification, they can also lead to the formation of ROS and free radicals, triggering oxidative stress (Macherey and Dansette, 2008).

Oxidative stress is a cellular phenomenon that occurs as a result of a physiological imbalance between the levels of antioxidants and oxidants (free radicals or reactive species) in favour of oxidants (Ighodaro and Akinloye, 2018). There are many different forms of ROS: singlet oxygen (O_2•), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and superoxide (O_2^-•). To cope with oxidative stress and to prevent damaging effects, the levels of protective antioxidants increase in the organism. Defences against ROS include scavenger compounds like the glutathione system (GSH/GSSG) and metallothionein (MT), and enzymes with antioxidant activities such as superoxidase dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) or glutathione reductase (GR) (Dröge, 2002). Figure 8 summarises pathways involved in ROS production, different strategies that organisms use to deal with them and the detrimental cellular effects of ROS.

SOD catalyses the dismutation of two molecules of superoxide anion (O_2^-•) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). There are three SOD forms coded by different genes: iron superoxide dismutase (*FeSOD*), which is commonly found in prokaryotes and chloroplasts of some plants; copper-zinc superoxide dismutase (*Cu/ZnSOD*), which is

predominant in eukaryotes and localised mainly in the cytosol but also chloroplasts and peroxisomes; and finally, manganese superoxide dismutase (*MnSOD*), which is present in prokaryotes and the mitochondria matrix of eukaryotes (Ighodaro and Akinloye, 2018). It has been proved that while mutations in *FeSOD* and *Cu/ZnSOD* does not compromise survival, *MnSOD* is essential for the survival of all aerobic organisms from bacteria to humans, and it is even found in many anaerobes as cell protection agents during exposure to aerobic conditions (Miriyala *et al*., 2012; Ravindranath and Fridovich, 1975).

CAT is an enzyme found in all aerobic organisms. It catalyses the decomposition of H_2O_2 to water and oxygen as cellular protection against the toxic effects of this peroxide (Valavanidis and Vlachogianni, 2019). CAT is abundant in the peroxisomes but is absent in the mitochondria, where the reduction of H_2O_2 to water and lipid peroxides to their corresponding alcohols is carried out by GPx (Ighodaro and Akinloye, 2018).

GPx protects cells from oxidative damage stress by removing H_2O_2 and inhibiting lipid peroxidation mainly in the mitochondria and sometimes in the cytosol (Gill and Tuteja, 2010). Besides, in the glutathione peroxide reaction, glutathione (GSH) is oxidised to glutathione disulphide (GSSG), which can be converted back to GSH by GR in an NADPH-consuming process (Figure 8). Specifically, phospholipid-hydroperoxide glutathione peroxidase (PHGPx) is an antioxidant enzyme in the GPx family that reduces phospholipid hydroperoxides and maintains the integrity of biomembranes (Nair *et al*., 2012).

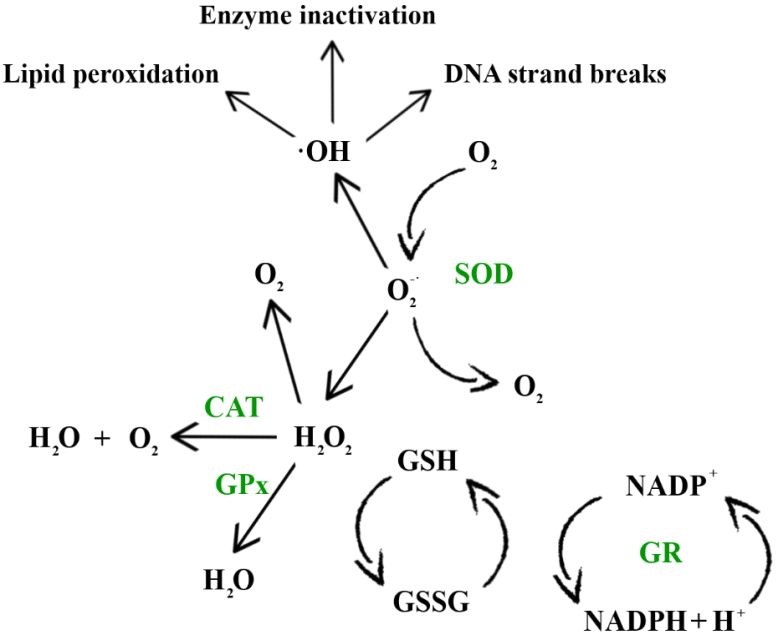


Figure 8. Reactive oxygen species and the protective action of antioxidants. Biotransformation process can lead to the formation of ROS and as last, to cellular oxidative stress. Singlet oxygen ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), the hydroxyl radical (·OH) and superoxide ($O_2^{\cdot-}$) are just few examples of form of ROS. Different strategies are activated in the organism to cope with these harmful radicals such as antioxidant enzymes that include superoxidase dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) or glutathione reductase (GR) (Modified from Kappus, 1985).

The fact that SOD and CAT activities are similar or higher in invertebrates than in vertebrates points out the critical role that these antioxidant defences have in the protection of aquatic organisms (Livingstone *et al*., 1992; Regoli *et al*., 2002).

In general terms, available literature has reported a remarkable increase of transcriptional levels in genes encoding *SOD*, *CAT* and *GPx*

enzymes upon exposure to a wide variety of stress conditions, such as metals or xenobiotics, in different organisms including *C. riparius* (Frat *et al*., 2021; Nair *et al*., 2012; Nair and Chung, 2015; Park *et al*., 2012; Viganò *et al*., 2020). Although changes in mRNA levels have a similar impact, as expected, at the enzymatic level changes are smoother and even sometimes unaffected compared to control (Di *et al*., 2017; Jiang *et al*., 2017; Lee *et al*., 2008; Peng *et al*., 2013).

Ecotoxicological studies combining transcriptional and enzymatic levels of genes involved in detoxification are scarce to date. These multilevel approaches could be a powerful tool in freshwater monitoring and risk assessment and are of substantial interest in the knowledge of the mode of action of pollutants.

2.6.4. Immune system

The overall function of the immune system is to prevent or limit infection. It can be affected by a variety of conditions, substances and agents such as radiation, immunosuppressive drugs or parasites. Also, growing evidence indicates that common environmental contaminants can adversely affect normal immune function in humans and wildlife (Desforges *et al*., 2016; Fisk *et al*., 2005; Selgrade, 2007; Silva dos Santos *et al*., 2018).

In contrast to the complexity of the vertebrate immune system, its relative simplicity in invertebrates, based mainly on nonspecific innate immune defences, makes it a potentially sensitive and accessible tool in the biomonitoring and risk assessment of environmental contaminants (Galloway and Depledge, 2001). The nonspecific innate immune response is the first line of defence against potential pathogenic organisms. It includes phagocytosis, cytotoxic activity, inflammatory reactions and

soluble mediators such as enzymes, antimicrobial peptides and cytokine-like molecules (Amiard-Triquet *et al*., 2013).

In the last decades, the number of studies working on the validation of experimental approaches and methodologies to assess the deleterious effects of xenobiotics on the immune system has grown considerably. These studies focus on the identification of immunological responses that are sensitive to a broad spectrum of xenobiotics to define immunological biomarkers (Amiard-Triquet *et al*., 2013). Furthermore, the immune system of insects has been widely explored since it confers a potential target in studies related to plant-insect interaction (Lampert, 2012; Yoon *et al*., 2019).

Insects have developed a complex network of cellular and humoral responses to protect themselves from pathogens and foreign substances. Although both responses share the same signalling pathways, they are activated by different stimuli (Marmaras and Lampropoulou, 2009). While the cellular immune response is engaged almost immediately upon infection and includes phagocytosis, nodulation and encapsulation, the humoral immune response takes place several hours later and includes the production of antimicrobial peptides (AMPs), activation of prophenoloxidase and production of reactive oxygen and nitrogen species (ROS-RNS) (Rosales, 2017; Tsakas and Marmaras, 2010).

The insect larval fat body is the major site of intermediate metabolism and is analogous to the vertebrate liver. In addition to being a target tissue for insect hormones such as JH or ecdysone, the fat body is also a site of response to microbial infection, where characterised immune genes are induced and encode AMPs that are released into the haemolymph (Tsakas and Marmaras, 2010). Both immune responses start with the recognition of the pathogen by the pattern recognition proteins/receptors (PRPs). The most characterised PRPs are C lectins, peptidoglycan recognition proteins

(PGRPs) or hemolin, among others (Bettencourt *et al*., 1997; Michel *et al*., 2001). Pathogen recognition triggers an orchestrated immune response that includes: 1) signalling pathways that activate genes encoding AMPs; 2) the proteolytic activation of the prophenoloxidase cascade, which contributes to killing pathogens through the formation of ROS and 3) phagocytosis, typically accompanied by melanin production and melanisation of nodules and capsules (James and Xu, 2012) (Figure 9). Thus, both humoral and cellular responses are not separate entities but comprise interdependent defensives forces.

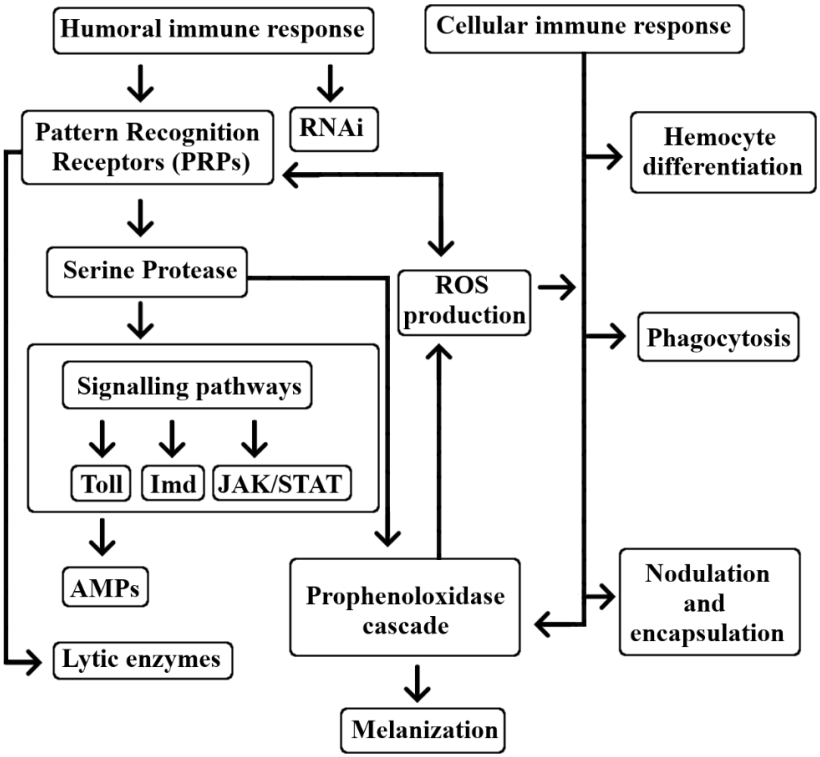


Figure 9. Summary of insect immune response (Modified from James and Xu, 2012).

The humoral immune response mainly involves the release of AMPs by the fat body of the insect via three different pathways: 1) Toll; 2) immune deficiency (Imd) and 3) the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. While the first two signalling pathways were firstly discovered in *D. melanogaster* (Ashok, 2009; Lemaitre *et al*., 1995), the JAK/STAT pathway was the first recognised as important in regulating multiple processes of human immunity (O'Shea and Plenge, 2012). Toll pathway has a dual function in immunity and developmental processes, whereas Imd functions exclusively in immunity. Both are interconnected and work together synergistically (Viljakainen, 2015). On the other hand, JAK/STAT along with the JUN N-terminal kinase pathway (JNK) and RNA interference also contribute to immunity and are essential to viral defence, but they are considered multipurpose pathways (Ferrandon *et al*., 2007). These pathways are evolutionarily conserved across insect taxa, and several immune genes have been annotated and characterised along the time (Table 8).

Table 8. Summary of insect immune system pathways and involved genes (Viljakainen, 2015).

Pathway	Gene
Recognition	*C-type lectin, Eater, Dscam, PGRP, βGRP, TEP*
TOLL (Signalling)	*Spätzle, Toll, MyD88, tube, pelle, cactus, dorsal*
Imd (Signalling)	*imd, FADD, Dredd, IAP2, TAK1, Tab2, IKKβ/ird5, IKKγ/key, Relish*
JAK/STAT (Signalling)	*domeless, hopscotch (JAK), Stat92E*

Table 8. Continuation

Pathway	Gene
JNK (Signalling)	*JNK/basket, hemipterous, Jra/Jun*
Effector	*defensin, other AMPs, lysozyme, NOS*
Modulator	*cSP, serpin*
Melanization	*PPO*
RNA interference	*Ago-2, Dcr-2*

In the last years, the study of the interaction between insect immunity and xenobiotics or temperature shifts is of great interest, as climate change and pollution are two of the main environmental problems. A good understanding of insect immunity and pesticide interaction, as well as the mechanism of immunotoxic action of chemicals, is essential for several reasons, such as reducing mortality in beneficial insects, controlling insect vectors of human diseases, or improving methods for controlling pest insects in agriculture (Blanco-Sánchez *et al*., 2021; James and Xu, 2012). In addition, temperature plays a key role in insect immune system, as it has an influence on host-pathogen interactions and can affect the efficiency of insect reaction to pathogen invasion (Ferguson and Sinclair, 2020; Linder *et al.*, 2008). Finally, in terms of ecotoxicology, it can modify toxicant's effect and the organism's response to it (Heugens *et al*., 2001). Related to this, there is an increasing number of reports showing that this interdependency between insect immune response and temperature is due to HSPs. On the one hand, infection triggers the expression of HSPs and, on the other hand, animals exposed to heat-

shock have their resistance to pathogens affected (Adamo, 2010; Altincicek *et al*., 2008).

Owing to the increasing knowledge around the insect immune system; in the last years, the ecotoxicological use of the immune response as a biomarker is attracting the interest of researchers for aquatic ecosystem health evaluation and risk assessment. These studies have been commonly performed on vertebrate species due to the in-depth knowledge related to their immunity and the capability to isolate organs relevant to the immune system such as liver or gills. Numerous studies have shown clear deregulation towards upregulation of transcriptional levels of immune system genes upon xenobiotic or endocrine-disputing chemical exposure in aquatic vertebrate species (Eder *et al*., 2008; Jiang, J. *et al*., 2015; Jin *et al*., 2010; Park *et al*., 2019; Qiu *et al*., 2016).

Regarding insect "immunomarkers", studies concerning changes at the transcriptional level are really limited and scarce. It is predominant the study of immunocompetence parameters such as phagocytic activity or immune cells activity and viability (Donaghy *et al*., 2016; Fournier *et al*., 2000; Gagné *et al*., 2008; Verdú *et al*., 2018). Recently, in our laboratory, two immune-related genes (*defensin* and *PO1*) have been identified in *C. riparius* (Muñiz-González and Martínez-Guitarte, 2018). In line with vertebrate data, deregulation of mRNA levels of immune-related genes was observed upon xenobiotic exposure (Martín-Folgar and Martínez-Guitarte, 2019; Muñiz-González and Martínez-Guitarte, 2020, 2018).

2.6.5. Energy metabolism

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plays a crucial role in the glycolytic pathway. It catalyses the reversible conversion of glyceraldehyde-3-phosphate (G3P) to 1,3-biphosphoglycerate in the presence of NAD+ and inorganic phosphate.

Until the mid-1990s, no possible extraglycolytic functions were attributed to this enzyme, and it was commonly used as a housekeeping gene in gene expression and protein studies (Nicholls *et al*., 2012). Given the important role of this enzyme, the gene encoding it, it is expressed at constitutively high levels in most tissues and cells. Because of this, it has been traditionally used as a reference gene for quantitative and semiquantitative PCR analyses, as well as in real-time PCR studies (Carnahan *et al*., 2013; Sirakov *et al*., 2013). Recently, new GAPDH roles have been discovered. It has been described that this enzyme far away from been used as a reference gene, it is a protein with multiple functions in cytoplasm, reticulum, mitochondria and nucleus. In addition to glycolysis, some examples of cellular processes in which GAPDH is involved are apoptosis, oxidative stress, regulation of the cytoskeleton, membrane fusion and transport, DNA repair, nuclear RNA export or activation of neurons transcription among others (Glaser *et al*., 2002; Kosova *et al*., 2017; Morgenegg *et al*., 1986; Nakajima *et al*., 2017; Nicholls *et al*., 2012; Tristan, 2011; White and Garcin, 2016).

Due to the wide variety of processes that GAPDH takes part in, its involvement in metabolic and physiologic pathways, and the numerous studies that have described its regulation upon different specific conditions, it is essential to check this gene prior considering as a reference gene in gene expression and protein studies.

2.6.6. The use of natural populations in ecotoxicology

Traditionally, ERA studies are based on the analysis of a selection of life-history traits in inbreed or single clonal laboratory populations to study potential hazard effects of contaminants. The use of laboratory populations ensures standardisation of laboratory procedures, which is of great interest in terms of chemicals regulation but might be of little

relevance in terms of predicting the potential of a natural population to evolve resistance and to adapt to environmental perturbations (Forbes and Depledge 1996).

Despite its practical utility, traditional ERA approaches used to evaluate the possible effects of chemical substances are limited to the study of doses/concentration individuals' responses. This limitation remains in the inability to answer more complex questions, which is essential in the last instance, for a better description of real contamination consequences on natural environments. To cover this gap, ecotoxicology makes use of biological monitoring, also called biomonitoring, which consists of recording quantitative and qualitative effects on living organisms caused by chemical substances (Lagadic, 2002). Biomonitoring includes the analysis of the state of the environment at different levels (individuals, species, populations and communities) to understand changes whose origin may be due to short or long periods of chemical exposure even below analytical detection limits or after chemical exposure has ceased (Pretti and Cognetti-Varriale, 2001).

Biomonitoring requires bioindicators, living organisms or communities of organisms that have particular requirements concerning to a known set of physical and chemical variables (Gerhardt, 2002) and that present a high sensitivity to environmental changes (Capó, 2002). Besides, contaminant accumulation in these organisms is manifested much earlier than in abiotic samples (Spahn and Sherry, 1999). In summary, data obtained testing wild populations can be considered more suitable for prediction of chemical stress (Lencioni *et al*., 2016), and it is a key tool for the development of effective environmental control and management programs.

Considering all this information, this thesis focuses on the use of natural populations of *P. olivacea* and *C. riparius* to study the toxic effect

of BBP, BPA or BP3 as emergent contaminants, and also on the effect of temperature shift. Moreover, this thesis studies the viability of the use of new characterised biomarkers in *P. olivacea*, a non-model chironomid whose molecular parameters have never been explored before in ecotoxicology.

HYPTOHESIS & OBJECTIVES

3. HYPOTHESIS AND OBJECTIVES

This thesis was based on the hypothesis that some non-model organisms rarely used in toxicity studies, such as *Prodiamesa olivacea*, could represent an excellent sentinel species for ecotoxicological risk assessment, especially in aquatic ecosystems. The work of this thesis represents the first molecular approach that has been made related to the effects that BBP, BPA and BP3 have on natural populations of *P. olivacea* as well as the first comparative study between this species and *C. riparius*, an aquatic dipteran that counts with standardised tests for the evaluation of water and sediment toxicity. Moreover, this has been the first time that the effects of acute heat-shock treatments have been analysed in natural populations of both aquatic dipterans.

The specific objectives to be achieved are:

- ✓ Identification and *de novo* characterization of *P. olivacea* genes related to cell stress response, endocrine system, biotransformation and oxidative response, immune system and energy metabolism.
- ✓ Molecular response assessment in gene and enzyme biomarkers of natural populations of *P. olivacea* 4th instar larvae exposed to BBP, BPA and BP3.
- ✓ Analysis of acute heat-shock effects in *P. olivacea* using biomarkers related to cell stress response and the immune system.
- ✓ Comparative analysis of the differential responses of *P. olivacea* and *C. riparius* to xenobiotic exposure and heat-shock.

MATERIALS & METHODS

4. MATERIALS AND METHODS

4.1. Testing animals and sampling site

All assays performed in this work have been done with natural populations of fourth instar aquatic larvae of the midge *Chironomus riparius*, Meigen, 1804 and *Prodiamesa olivacea* (Meigen, 1818).

Individuals of both species were collected from a selected area of the River Sar (Galicia, Spain) across the town Bertamiráns (A Coruña, Spain; 42°51'22.99"N 8°38'53.58"W) (Figure 10) to study the effect of emerging contaminants and heat-shock. The study area was selected due to the wide variety of studies made at that site, which provides us a good knowledge of its chemical and physical characteristics as well as its faunistic composition (Cobo *et al*., 1989; Cobo and González, 1991; Planelló *et al*., 2013; Servia *et al*., 2004). The sampling site was situated under the bridge that joins Bertamiráns with A Condomiña. This point is located 19 km from the source of the Sar River and 4 km downstream of the Silvouta WWTP. This plant treats urban and industrial wastes from Santiago de Compostela (a city with a population close to 100000 inhabitants), and it often operates over its capacity affecting the water quality of the River Sar. This stretch of the River is one of the most polluted in Galicia (Prego *et al*., 2008) and it is characterised by high levels of organic matter (Díaz-Fierros, 2000) with relatively high population densities of chironomids throughout the whole year, mostly *C. riparius* and *P. olivacea* (Servia *et al*., 1998).

Figure 10. Geographical location of *P. olivacea* and *C. riparius* natural populations. Sampling site of natural populations of both species of chironomids used in the research. Bertamiráns, A Coruña, Spain (42°51'22.99"N 8°38'53.58"W).

Sampling was done on May 6th and 7th, 2016 and it was performed choosing the most appropriate microhabitats for these organisms, that is to say, places of fine sediment, very low current velocity and accumulated organic matter (Figure 11, left).

Harvesting was carried out with a small hand net (15 cm diameter, 20 cm deep, 250 µm mesh size). Collected 4th instar larvae of *C. riparius* and *P. olivacea* were classified and washed by using a sieve (250 µm mesh size). The following physical and chemical parameters were measured

in situ at the sampling point: temperature, pH, total dissolved solids (TDS), percentage of organic matter, conductivity, oxygen saturation and dissolved oxygen. A portable multimeter Crison MM40+ and the oximeter YSI 550A was used. Mean values can be found in Table 9.

Table 9. Physical and chemical properties measured at the sampling point (River Sar, Bertamiráns, A Coruña, Spain, May 2016).

Measured parameter	Mean value
T (°C)	16.4
pH	6.8
Total dissolved solids (mg/L)	145.8
Organic matter (%)	2.3
Conductivity (µS/cm)	228
Oxygen saturation (%)	84.1
Dissolved oxygen (mg/L)	6.16

To minimise transport impact on larvae, samples were stored into 5-litre hermetic polystyrene containers with sediment and water of the sampling point before their transfer to the laboratory at the Hydrobiological Field Station "Encoro do Con" (Estación de Hidrobioloxía, EHEC), Pontevedra (Galicia, Spain). All samples were labelled at the same sampling time, and during their transport to the laboratory they were light preserved and kept at constant temperature (15 °C).

In the EHEC laboratory, the content of the containers with the larvae was mixed up and distributed into buckets where organisms were kept for 2 h with continuous aeration until their selection for the toxicity assays (Figure 11, right). The determination of the instar larvae was made by comparing the size of the head capsules among individuals of the same

sampling. 4th instar larvae of *P. olivacea* and *C. riparius* were selected and exposed to the different testing conditions (BBP, BPA, BP3 or heat-shock). Immediately after completing the exposures, individuals of the same species were separated into groups of five larvae in vials, and frozen down at -80 °C. For later use in our laboratory at the UNED Faculty of Science, samples were transferred in dry ice.

Figure 11. Field and laboratory work for sample selection. On the left, sampling of *P. olivacea* and *C. riparius* in the selected area of the Sar River. On the right collected organisms kept at continuous aeration on the laboratory of the Hydrobiological Field Station "Encoro do Con", Pontevedra, until their selection for the toxicity assays.

4.2. Chemical compounds

One aim of this doctoral thesis is to deep into the knowledge of possible harmful effects of three chemicals: benzyl butyl phthalate (BBP, CAS No. 85-68-7, purity ≥ 98 %), bisphenol A (BPA, CAS No. 80-05-7, purity ≥ 99 %) and benzophenone-3 (BP3, CAS No. 131-57-7, purity ≥ 98 %). These compounds meet the following selection criteria:

- ✓ Remarkable toxicity. Several harmful effects have been reported for the selected compounds in different ecotoxicological models and at different levels (molecular, cellular).
- ✓ Endocrine-disrupting properties. Endocrine-disrupting activities have been previously described for the three chemicals.
- ✓ Recurrent persistence in the aquatic environment. The compounds of study are frequently found in aquatic environments, and due to their physical-chemical properties, they are known to tend to deposit and to accumulate in sediments, the preferred habitat of the chironomids used in this research.

Two of the three assessed chemical compounds, BBP and BPA, are largely used as plasticiser mainly in the PVC industry. They are also used as additives in plastics and other products like cosmetics and, since they are not chemically attached to the plastic, they easily release. This characteristic, together with their worldwide use and their potentially detrimental effects on human health, has raised a growing public concern in the last decades. BP3 is an organic UV filter broadly used and therefore continuously released into the aquatic environment, where it tends to accumulate in biota and sediments. Feeding activity, cell viability, growth or endpoints related to reproduction have been described to be affected by this UV filter. In the last few years, all these deleterious effects, in addition to its endocrine activity, have given rise to new studies focusing on the mode of action and the possible toxic effects of BP3.

The main physical-chemical characteristics of BBP, BPA and BP3, as well as their relevant ecotoxicological information, have been previously described in this work (Introduction chapters 2.5.1, 2.5.2 and 2.5.3).

4.3. Reagents and enzymes

Salts, acids, inorganic bases, organic compounds and reagents used for the buffers and culture medium were purchased from leading brands in the chemical industry. The main reagents and enzymes used in this thesis are listed in Table 10.

Table 10. Main reagents and enzymes used in this thesis.

Reagent	Brand
Chloroform	Sigma-Aldrich (Madrid, Spain)
Ethanol	Roche (Basel, Switzerland)
Ethidium bromide	Sigma-Aldrich (Madrid, Spain)
Glutathione reduced (GSH)	Sigma-Aldrich (Madrid, Spain)
Glutathione reductase (GR)	Sigma-Aldrich (Madrid, Spain)
iScript™ Advanced cDNA Synthesis Kit for RT-qPCR	Bio-Rad (California, USA)
Isopropanol	Panreac (Barcelona, Spain)
Phenol:Chloroform:Isoamyl alcohol	Fluka (Madrid, Spain)
Quantimix Easy Kit	Biotools (Madrid, Spain)
Reagents for polyacrylamide gels	Bio-Rad (California, USA)
RNase-free DNase	Roche (Basel, Switzerland)
Superoxide dismutase (Ransod) assay kit	Randox (Northern Ireland, United Kingdom)
TRIzol®	Invitrogen (Madrid, Spain)
1-Chloro-2,4-dinitrobenzene (CDNB)	Sigma-Aldrich (Madrid, Spain)
5PRIME Phase Lock Gel Light tubes	Quantabio (Massachusetts, USA)

4.4. Exposure conditions of study

This doctoral thesis aims to assess the impact of exposure of larvae of natural populations of *C. riparius* and *P. olivacea* to two different stress conditions: xenobiotics and heat-shock.

4.4.1. Exposure to environmental contaminants

4.4.1.1. Selection of study concentrations

The environmental risk assessment of a substance evaluates the likelihood that adverse ecological effects result from their exposure. The evaluation is done based on the analysis of chemical exposure and the effects on relevant environmental compartments (e.g. soil, sediment, surface water, etc.). The exposure assessment considers two parameters:

- Predicted environmental concentration (PEC). The expected concentration of a substance in the environment considering the amount initially present, its distribution and rates of environmental degradation and removal.
- Predicted no-effect concentration (PNEC). It is the concentration of a substance in any environment below which adverse effects will mostly not occur during long or short them exposure.

PEC and PNEC are combined in order to characterise the risk so that when substance exposure is higher than the concentration without effect (PEC/PNEC > 1), there is an environmental risk. The most recent risk assessment carried out by the European Union on BBP and BPA, reported in general PEC/PNEC ratios below 1 for all the possible scenarios (surface water, sediment, atmosphere, etc.) (EU RAR, 2007, 2008). Although there is not a risk assessment from the EU regarding BP3, PEC/PNEC ratios

below 1 for this UV filter in surface waters have been reported in the literature (Krzeminski *et al*., 2017; Langford *et al*., 2015).

For the acute toxicity test, solutions of BBP, BPA and BP3 were purchased from Sigma-Aldrich, and dissolved in analytical grade EtOH at a concentration of 10 g/L to provide a stock solution of each compound that were stored in the dark at 4 °C.

Test solutions were freshly made from the stock in culture medium at a 0.01 % v/v EtOH final concentration, which was previously tested not to affect the analysed biomarkers.

4.4.1.2. Acute toxicity studies

Selected 4th instar larvae of both chironomid species were independently exposed to 1 µg/L of the three testing substances (BBP, BPA or BP3) for 4 h and 24 h (Figure 12). Nominal concentrations of 1 µg/L of each xenobiotic were chosen following previously published data obtained in *C. riparius* (Herrero *et al*., 2016; Nair *et al*., 2013; Ozáez *et al*., 2016).

Forty-five individuals of *P. olivacea* and *C. riparius* larvae were exposed to each chemical diluted in 50 mL of culture medium in 100 mL glass vessels. Each experimental condition was tested in triplicate in separate glass beakers (n_{total} = 135 larvae/condition). As stated before, each condition and experiment was accompanied by a negative control (only culture medium) and solvent control (EtOH 0.01 % v/v) in both 4 and 24-hour experiments, to ensure that EtOH does not alter the studied parameters. The culture medium was not changed during the treatments, larvae were not fed, and exposures were maintained at room temperature (RT) (around 20 °C).

Once the exposure was over, larvae survival was analysed for each condition. All those colourless and/or immobile larvae even after their stimulation were considered death. After counting, two pools of five alive larvae were collected from each glass per experimental condition, dried, and frozen at -80 °C in 2 mL freezing vials until use. A total of three pools of five larvae per experimental condition were studied in gene expression analyses by real-time qPCR. Five larvae per condition were used for enzymatic determinations (n_{total} = 15 larvae/condition).

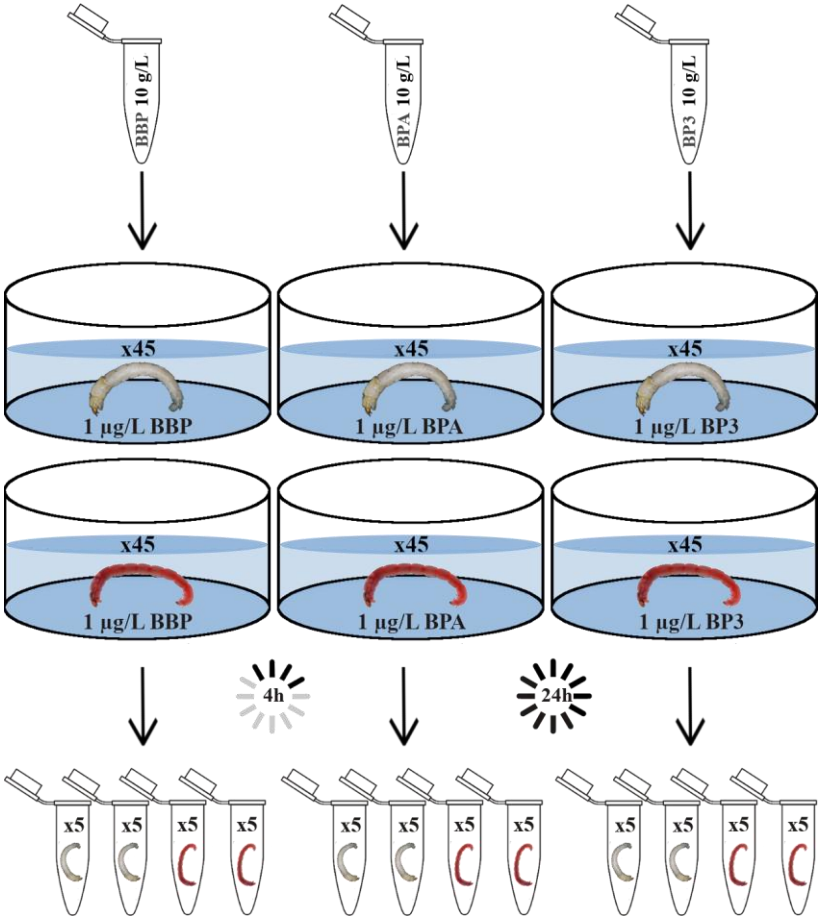


Figure 12. Protocol for the exposure of *P. olivacea* and *C. riparius* to environmental contaminants. Forty-five individuals of 4th instar larvae of both chironomids collected in the River Sar were exposed in 100 mL glass vessels to 1 µg/L of BBP, BPA and BP3 respectively diluted in 50 mL of culture medium at 0.01 % EtOH. Testing solutions were freshly made from the stock solution. After 4 h and 24 h of exposures, two pools of five alive larvae of each specie and testing condition were collected and frozen at -80 °C until their use.

4.4.2. Exposure to heat-shock

For heat-shock exposures, forty-five individuals of *C. riparius* and *P. olivacea* were respectively exposed to 35 and 39 °C for different times in preheated and aerated 50 mL culture medium in 100 mL glass vessels. On one hand, for 35 °C assay, larvae were exposed during 30, 60, 120 minutes and 120 minutes followed by 2 h at RT. On the other hand, 39 °C exposures were performed during 30, 60 minutes and 60 minutes followed by 2 h at RT. As a negative control, larvae were kept at RT for the same time. Neither food nor substrate was provided during exposures. For each temperature and time, a total of three independent experiments were carried out (n_{total} = 135 larvae/condition). Immediately after the heat-shock treatment, five larvae of each glass vessel were collected, dried, and frozen in 2 mL freezing vials at -80 °C until use. Similar to the acute toxicity assays, a total of two pools of five larvae were studied per heat-shock condition in gene expression analyses.

4.5. *De novo P. olivacea* transcriptome sequencing

4.5.1. Sequencing

Due to the great potential of *P. olivacea* as sentinel organism for ecotoxicity studies, we sequenced *de novo* the whole transcriptome of this chironomid to have a full battery of data for downstream applications.

As described in section 4.8.1, total RNA of ten *P. olivacea* 4th instar larvae collected in the River Sar was extracted, purified, mixed and resuspended in free-nucleases water and send to Macrogen (South Korea) for transcriptome sequencing.

In this study, a *de novo* whole transcriptome assembly was performed to reconstruct the transcript sequences of *P. olivacea* without a reference genome. However, it is worth to mention that this kind of study without a reference genome represents a computational challenge with a random component (Grabherr *et al*., 2011; Haas and Zody, 2010), which may result in an imperfect assembly, particularly with short reads (Schliesky *et al*., 2012).

Three cDNA libraries were constructed by Macrogen following TruSeq Stranded mRNA (Illumina) protocol and sequenced on an Illumina HiSeq 4000 System using a 100 cycles paired-ended protocol.

Briefly, the poly-A containing mRNA molecules were purified using poly-T oligo attached magnetic beads. After two rounds of purification, mRNA was fragmented into small pieces using divalent cations under elevated temperature. Cleaved RNA fragments were copied into first-strand cDNA using reverse transcriptase and random hexamer primers as templates. Using DNA polymerase and RNase H, the second strand cDNA was synthesised by removing the RNA template and synthesising a replacement strand incorporating dUTP instead of dTTP to generate a double-strand cDNA. AMPure XP beads were used to separate the double-strand cDNA from the second strand reaction mix. At the end of this process, blunt-ended cDNA was constructed, and end repair of the overhangs resulting from fragmentation into blunt ends was performed using an End Repair (ERP) mix. A single "A" nucleotide was added to the 3' ends of the blunt fragments to prevent them from ligating to each other during the adapter ligation reaction. In addition, ligation of multiple indexing adapters to the ends of the double-strand cDNA was carried out, to prepare them for hybridisation onto a flow cell. Finally, PCR amplification was used to selectively enrich those DNA fragments that have adapter molecules on both ends and to create the final cDNA library template.

4.5.2. *De novo* assembly and annotation

A reference transcriptome was assembled by integrating the RNA-seq reads obtained from three libraries. Before the assembly, sequence quality was checked by FastQC (v0.11.7) (Andrews, 2010) and low-quality reads (Phred value < 33) as well as adaptors, were removed using Trimmomatic (v0.38) program (Bolger *et al*., 2014). Trimmed reads were *de novo* assembled into transcript contigs using Trinity software (Grabherr *et al*., 2013) with default parameters.

For assembled genes, the longest contigs were filtered and clustered into the non-redundant transcripts using CD-HIT-EST (v4.6) program (Huang *et al*., 2010), so the transcripts were clustered into unigenes. To identify proteins with high sequence similarity and for putative functional annotation, all unigenes composed of more than 200 bp were annotated by BLASTx against several known protein sequence databases: Kyoto Encyclopedia of Genes and Genomes (KEGG_v20170706), NCBI nucleotide (NT), Pfam (v31.0), Gene Ontology (GO_v20150407), NCBI non-redundant Protein (NR), UniProt (v20170706) and EggNOG (v4.5.1) using BLASTn; an E-value cutoff of 1e-5 was used. Finally, to assign gene ontology (GO) terms, Blast2GO (v4.1) program was used (Conesa *et al*., 2005).

4.6. Database searching and *P. olivacea* phylogenetic analysis

A phylogenetic study was performed from the isolated gene sequences of the *de novo P. olivacea* transcriptome for a better gene characterisation and to evaluate the relationship between proteins that encoded the selected genes of study and homologous proteins in other insects and arachnids.

The nucleotide sequences of *P. olivacea* study genes were analysed through the Basic Local Alignment Search Tool (BLAST+_v2.10.1) (Altschul *et al*., 1990) available at the National Centre for Biotechnology Information of the United States (NCBI), ChromasPro (version 2.1.7) and SnapGene® (GSL Biotech, version 5.1)

On the one hand, for phylogenetic analysis, sequences alignments of the different *P. olivacea* proteins and related insect species were performed with MAFFT (v7.0) software (Katoh and Standley, 2013). Phylogenetic trees were generated using the Molecular Evolutionary Genetics Analysis (MEGA_v6.0) (Tamura *et al*., 2013) through the UPGMA method (Sneath and Sokal, 1973). The bootstrap consensus tree inferred from 1000 replicates (Felstein, 1985) and the evolutionary distances were computed using the Poisson correction method (Zuckerland and Pauling, 1965). All positions containing gaps and missing data were eliminated.

On the other hand and for each *P. olivacea* characterised protein, all the compiled data was used to draft a schematic diagram containing protein domain structures with functional motifs using DOG (v2.0) software (Ren and Xue, 2011).

4.7. Primers for real-time qPCR amplification

4.7.1. *Prodiamesa olivacea* primer design and validation

Most of the DNA primers used in this thesis for the amplification of the selected genes were designed using Primer3 (version 0.4.0) software (Untergasser *et al*., 2012) based on the *de novo P. olivacea* transcriptome obtained in our laboratory. Given that real-time qPCR was performed with an annealing temperature of 58 °C, the primers were designed with a melting temperature (Tm) between 62.5 - 63.5 °C. Oligonucleotides were synthesised by Stab Vida (Portugal). The lyophilised primers were diluted

to a concentration of 100 µM following the manufacturer's instructions. For each gene, a mix of 10 µM of forward and reverse primers was prepared and kept at -20 °C until use. In addition, since *P. olivacea* and *C. riparius* are evolutionarily close, the study of gene expression of some genes of *P. olivacea* were performed with *C. riparius* specific oligonucleotides that were already on use in our laboratory.

All primer pairs were tested for *P. olivacea* by real-time qPCR under the same conditions as the performed gene expression analyses (described later in 4.8.3 section). Adequate positive and negative controls were included in the PCR reaction whose total volume was 20 µL per well. Each well contained:

- ✓ 10 µL of Quantimix Easy Master Mix
- ✓ 2 µL of a mix of *P. olivacea* cDNA
- ✓ 1.2 µL of forward and reverse mix of primers
- ✓ 6.8 µL of nuclease-free water

To check the size and quality of the fragments, 10 µL of each amplification product were run in 9 % acrylamide gel with 30 % acrylamide/bis-acrylamide solution 29:1, 10 % ammonium persulfate w/v and 100 % TEMED at 85 V for 2 h in 1x TGE buffer (40 mM Tris-HCl (pH 8.5)), 200 mM glycine and 2.5 mM EDTA. 6x DNA loading dye (Thermo Fisher, US) was added to the samples and they were run in parallel with a GeneRuler™ 100 bp DNA ladder (Thermo Fisher, USA). Electrophoresis was done at RT at a constant voltage in a Mini-PROTEAN® Tetra vertical electrophoresis cell equipment (Bio-Rad). Gels were stained with ethidium bromide (Sigma-Aldrich) (0.5 µg/mL) and visualised on the Chemigenius transilluminator by using GeneSnap (version 6.05) software (both from SynGene, England).

The remaining volume of the amplification product (10 µL) was sent to Stab Vida for sequencing only if the size of the obtained electrophoresis bands matched with the expected ones according to the design of primers. Finally, Sanger sequencing chromatograms were interpreted by using ChromasPro (version 2.1.7) (Technelysium Pty Ltd), and identities of the fragments were verified with the sequencing BLAST tool (Agarwala *et al*., 2018; Altschul *et al*., 1990).

4.7.2. *Chironomus riparius* primers

C. riparius is a model organism daily used in our laboratory, therefore, except for the immune system, we did not have to design and test any pair of primers, as they were already validated and on use in our research team. However, since transcriptional alterations in the immune system of *C. riparius* have been little explored so far in ecotoxicological terms, we did not have available tested primers for the amplification of immune response genes of our interest. Thus, we tested and validated *P. olivacea* specific immune system oligonucleotides in the midge *C. riparius*, following the same procedure described in point 4.7.1.

Amplification efficiencies and correlation coefficients for each pair of primers in both species were calculated as specify in the *Tips, Tricks & Best Practices: The Ultimate qPCR assay design guide* Bio-Rad bulletin #6894. For all genes, the efficiencies were between 90 - 105 % ($R^2 > 0.980$).

Primer sequences of *P. olivacea* and *C. riparius* used for the amplification of complementary DNA (cDNA) to study alterations at the molecular level due to BBP, BPA, BP3 or heat-shock exposures, are detailed in Tables 11 and 12, respectively.

Table 11. Primers used for real-time qPCR amplification of the studied genes in *P. olivacea*. 5'-3' forward (F) and reverse (R) sequences, base pair (bp) length of the amplified product and reference to the original published paper when corresponds.

Gene	Primer sequences	Amplified product length
cdc37	**F** GACTGATGAAGAGAAAGAGCAGCAT **R** CTCGTGGGTCACATTTTAGTTGC	278 bp
hsp70	**F** ACTTGAACCAGTTGAGCGT **R** TTGCCACAGAAGAAATCTTG	130 bp (Morales *et al*., 2014)
hsc70	**F** CGTGCTATGACTAAGGACAA **R** GCTTCATTGACCATACGTTC	210 bp (Planelló *et al*., 2011)
hsp60	**F** GGCTCTTAGCACATTGGTGGTT **R** TCTCTCCTTTGCCCTTCAACAG	248 bp
hsp27	**F** CCTGTCAATTTTGGCTCACCA **R** TTCATCAGCTCTCTCACCATGC	171 bp
HSF	**F** TGGCAGTAAGGAGCAACAGGA **R** CGAACAAGCAACACCACTCG	103 bp
CAT	**F** CGCCAAGGGTGAAGAAGTTTT **R** CCAACAGGAATCAACGGGAAT	270 bp (Llorente *et al*., 2020)
MnSOD	**F** GTCACCCGAGAAGTCAAATCCTT **R** TGCCAAACAATGGAACGAGA	227 bp (Llorente *et al*., 2020)
PHGPx	**F** TGCATGACCAGGATTTCCCTA **R** TTCTGAGCCTTCCCGTTCTG	203 bp (Llorente *et al*., 2020)
Cyp4g15	**F** GACATTGATGAGAATGATGTTGGTG **R** TAAGTGGAACTGGTGGGTACAT	232 bp (Martínez-Paz *et al*., 2012)

Table 11. Continuation (I)

Gene	Primer sequences	Amplified product length
Cyp6a14-like	**F** TTGTTGGCGTTGGAATTGTTC **R** TCGGATCATGCTTTTCGTTAAAG	213 bp (Llorente *et al*., 2020)
Cyp6a2-like	**F** CCTCCTGCAACAATTATCAATCG **R** CGCCGAATGGGAGTAAGACA	217 bp (Llorente *et al*., 2020)
EcR	**F** TGCTCTGCTTACTGCCATCG **R** CAGCCACATCCCAAACTTCTTC	278 bp
Kr-h1	**F** CTCCCCTCATGCTCGATTTG **R** CGTTTTGTTGATTACGCTGGTG	175 bp
JHEH	**F** ACCGCAAATACCAAAGTCTATCCA **R** CGAGAATCCGTAACCCACCA	156 bp
TOLL	**F** ATGTGAAATGGGGCGACAAA **R** GATGGCGGTCTTTACGAGTCC	137 bp
JAK/ hopscotch	**F** TTACCGCCAACTCAACACGA **R** ATGTCCCATCACCGTTCCAG	175 bp
PGRP	**F** CCAGTGCCCAACAATCATCA **R** GCCATCTCCTCCAACACAGAA	247 bp
C-type lectin	**F** GAACGGCAAGAGACGATACAAGA **R** CCCCTGTGTTGAGGATACGG	176 bp
GAPDH	**F** GGTATTTCATTGAATGATCACTTTG **R** TAATCCTTGGATTGCATGTACTTG	110 bp (Park and Kwak, 2009)
Actin	**F** GATGAAGATCCTCACCGAACG **R** CGGAAACGTTCATTACCG	201 bp (Martínez-Guitarte *et al*., 2007)
26S	**F** TTCGCGACCTCAACTCATGT **R** CCGCATTCAAGCTGGACTTA	220 bp (Planelló *et al*., 2011)

Table 12. Primers used for real-time qPCR amplification of the studied genes in *C. riparius*. 5'-3' forward (F) and reverse (R) sequences, base pair (bp) length of the amplified product and reference to the original published paper when corresponds.

Gene	Primer sequences	Amplified product length
hsp70	**F** CATGTGAACGAGCCAAGAGA **R** TTGCCACAGAAGAAATCTTG	300 bp (Lee *et al*., 2006)
hsc70	**F** CGTGCTATGACTAAGGACAA **R** GCTTCATTGACCATACGTTC	239 bp (Planelló *et al*., 2011)
hsp60	**F** TGCTGTCCTTAAAGTCGGTGG **R** TCCACCACCAGCAACGATTC	160 bp (Martín-Folgar *et al*., 2018)
hsp27	**F** TCAACACACAGGACCG **R** ATCCTTTATTGGTGATTAATTATG	202 bp (Martínez-Paz *et al*., 2014)
HSF	**F** TGGCAGTAAGGAGCAACAGGA **R** CGAACAAGCAACACCACTCG	103 bp
CAT	**F** CGTGATCTTCGTGGTTTTGCTG **R** GGATTGGATCGCGGATGAAG	100 bp (Nair *et al*., 2011)
MnSOD	**F** CTGATGCACTCCAAAAAGCA **R** AACTCCAACAGCAGCGACTT	86 bp (Park *et al*., 2012)
PHGPx	**F** AAGTGTGGTTACACAGCTAAGCATT **R** GATATCCAAATTGATTACACGGAAA	112 bp (Nair *et al*., 2012)
Cyp4g15	**F** GACATTGATGAGAATGATGTTGGTG **R** TAAGTGGAACTGGTGGGTACAT	340 bp (Martínez-Paz *et al*., 2012)

Table 12. Continuation (I)

Gene	Primer sequences	Amplified product length
EcR	**F** AGACGGTTATGAACAGCC **R** CGAGCCATGCGCAACATC	240 bp (Planelló *et al*., 2008)
Kr-h1	**F** CCCTCGAGCTAACTCCACCC **R** GCTGCAATGTTTGACTGGTT	170 bp (Aquilino *et al*., 2016)
TOLL	**F** ATGTGAAATGGGGCGACAAA **R** GATGGCGGTCTTTACGAGTCC	137 bp
JAK/ hopscotch	**F** TTACCGCCAACTCAACACGA **R** ATGTCCCATCACCGTTCCAG	175 bp
PGRP	**F** CCAGTGCCCAACAATCATCA **R** GCCATCTCCTCCAACACAGAA	247 bp
C-type lectin	**F** GAACGGCAAGAGACGATACAAGA **R** CCCCTGTGTTGAGGATACGG	176 bp
GAPDH	**F** GGTATTTCATTGAATGATCACTTTG **R** TAATCCTTGGATTGCATGTACTTG	110 bp (Park and Kwak, 2009)
Actin	**F** GATGAAGATCCTCACCGAACG **R** CGGAAACGTTCATTACCG	201 bp (Martínez-Guitarte *et al*., 2007)
26S	**F** TTCGCGACCTCAACTCATGT **R** CCGCATTCAAGCTGGACTTA	220 bp (Planelló *et al*., 2011)

4.8. Gene expression analysis

4.8.1. RNA extraction

Total RNA was extracted using an acid guanidinium thiocyanate-phenol-chloroform based method, performed with a commercial kit (TRIzol®) designed to isolate high-quality total RNA, DNA and proteins

from cells and tissues. It was not necessary to adapt the RNA extraction protocol that is routinely used in our laboratory for *C. riparius,* as it worked perfectly in *P. olivacea* larvae, and it reported high and good RNA quantification data. For the two chironomid species used in this thesis, RNA extraction was carried out as follows.

Pools of five frozen larvae were transferred from the freezing vials to 1.5 mL Eppendorf tubes, where they were homogenised in 500 µL TRIzol® by using a hand-held tissue homogeniser (VWR®, US). The tubes were kept in dry ice during homogenisation. Once all the samples were homogenised, they were defrosted in ice and centrifugated at 12000 rpm / 4 °C for 10 minutes.

The supernatant was transferred to a clean 1.5 mL Eppendorf tube, 100 µL of chloroform was added, mixed vigorously and kept at RT for 3 minutes. Afterwards, samples were centrifugated at 12000 rpm / 4 °C for 15 minutes.

The aqueous phase of each sample, where RNA was contained, was transferred to a new clean 1.5 mL Eppendorf tube to precipitate the genetic material by adding 250 µL of isopropyl alcohol (0.5 % v/v), mixed gently and kept at RT for 30 minutes. After this time, samples were centrifugated at 12000 rpm / 4 °C for 15 minutes and the supernatant was discarded.

The RNA pellet contained in the Eppendorf tubes was washed by adding 500 µL of cold 70 % v/v EtOH, and it was centrifugated at 10000 rpm / 4 °C for 5 minutes. The supernatant was discarded, and precipitated RNA was resuspended in 44 µL nuclease-free water. To eliminate possible rest of DNA, samples were treated with RNase-free DNase. 1 µL of DNase and 5 µL of Buffer 10x were added to 44 µL samples for a total volume of 50 µL. Samples were incubated at 37 °C for 40 minutes.

After incubation, 150 µL of nuclease-free water and 200 µL of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each sample. The total volume of 400 µL was transferred to a Phase Lock Gel tubes and centrifugated at 10000 rpm / 4 °C for 15 minutes. Finally, the supernatant was collected, and RNA was checked for quality and quantity by absorbance spectrophotometry at 260 nm using a Biophotometer (Eppendorf, Germany).

RNA samples were stored at -80 °C until use.

4.8.2. Retrotranscription

Transcriptional levels of the genes of study were determined by quantitative real-time PCR (qPCR) using cDNA as template. To do that, mRNA contained in the total RNA extracted from samples of *C. riparius* and *P. olivacea* exposed to xenobiotics and heat-shock were retrotranscribed to cDNA in a thermocycler.

Retrotranscription of mRNA was done using the commercial kit iScript™ Advanced cDNA Synthesis Kit for RT-qPCR and following the manufacturer's instructions. For each condition and sample, 7 µg of isolated RNA was reverse transcribed using an MJ Mini and a C1000™ Thermal Cycler (both from Bio-Rad) with a 47 °C / 20 minutes heat cycle and a 95 °C / 1 minute inactivation cycle.

The obtained cDNA was quantified by absorbance spectrophotometry at 260 nm using a Biophotometer and it was diluted to work in all samples with an average concentration of 70-80 ng/µL.

cDNA samples were stored at -20 °C until use.

4.8.3. Real-time qPCR

The study genes were amplified by qPCR in a CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad) using the Quantimix Easy Kit.

To minimise pipetting error and ensure homogeneity in all PCR components, a master mix PCR was prepared for each gene and plate. Negative controls were included and the total volume per well was 10 µL composed of:

- ✓ 5 µL of Quantimix Easy Master Mix
- ✓ 2 µL of cDNA (*P. olivacea* or *C. riparius* exposed to experimental conditions)
- ✓ 1.2 µL of forward and reverse mix of primers of the gene of study
- ✓ 1.8 µL of nuclease-free water

Genes encoding actin and the 26S ribosomal subunit were used as endogenous reference genes. The statistical validation of the stability of the reference genes was performed by means of CFX Manager 3.1 software (Bio-Rad), using an interactive test for pairwise variation, according to Vandesompele *et al*., 2002. To normalise the gene expression results with respect to the selected endogenous reference genes, genes of study and reference genes were amplified in the sample plate of qPCR and under the same conditions. Fragments of these reference genes were amplified using the same pair of primers designed for *C. riparius*, and their identities were confirmed by Sanger sequencing by Stab Vida company. The qPCR program was run in the thermal cycler, as shown in Figure 13.

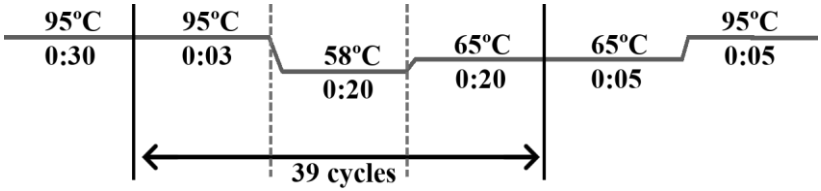


Figure 13. PCR program run for amplification of genes of study by real-time quantitative PCR (RT qPCR). Thermal cycle was programmed at 95 °C for 30" (seconds) for an initial denaturation, followed by 39 cycles of 3" at 95 °C for denaturation, 20" at 58 °C for primer annealing and an extension of 20" at 65 °C. After the amplification cycles, the program finished with 5" at 65 °C and 95 °C.

Six genes were included per 96-well plate (2 reference genes and 4 genes of study). Each sample was run in duplicate wells and for each experimental condition studied (with three biological replicates), three technical replicates were carried out.

4.8.3.1. Real-time qPCR data analysis

The mRNA level of each target gene was normalised against the expression of the two reference genes. A variation of the $2^{-\Delta\Delta Ct}$ (Livak) method (Livak and Schmittgen, 2001) was used to analyse relative changes in gene expression with CFX Maestro™ Software 1.0 (version 4.0.2325.0418) (Bio-Rad).

The Livak method assumes that both target and reference genes are amplified with efficiencies near 100 % and within 5 % of each other. Therefore, before gene amplification, the efficiencies of each pair of primers for both species were calculated and checked. To do that, amplification of diluted series of a mix of cDNA of *C. riparius* or *P. olivacea* were performed and the cycle threshold (Ct) standard curve was analysed.

All efficiencies were near 100 % and within 5 % of each other (data not shown).

The cycle threshold (Ct) value is defined as the number of cycles required for the fluorescent signal to cross the threshold (for example, background level). Ct levels are inversely proportional to the amount of the target gene in the sample. The lower the Ct level, the greater the amount of target gene in the sample.

The fold change in the target gene relative to endogenous reference genes was determined for each exposure condition by:

$$\text{Fold change} = 2^{\Delta\Delta Ct}; \text{ where } \Delta\Delta Ct = (Ct_{\text{reference gene}} - Ct_{\text{target gene}})$$

Finally, exposure conditions tested (BBP, BPA, BP3 and heat-shock) were compared against control samples to determine possible changes in expression levels of the genes of study.

4.9. Antioxidant response analysis

4.9.1. Protein extraction and quantification

Antioxidant response analysis was performed following the same protocols for both species used in this thesis. The same procedure used for RNA extraction was followed for tissue homogenisation. Pools of five frozen larvae were transferred from the freezing vials to 1.5 mL Eppendorf, where they were homogenised in 500 µL of TRIzol® using a hand-held tissue homogeniser.

With a first centrifugation at 500 rpm / 4 °C for 15 minutes, the post-mitochondrial supernatant (PMS) preparation to be used in the enzymatic determination was obtained. The supernatant was collected, and a second centrifugation at 12000 rpm / 4 °C for 30 minutes was performed.

The supernatant where PMS was contained was collected in new tubes and stored at -80 °C until use.

To determine the quantity of protein present in all samples, a commercial kit BCA Protein Assay was employed. The BCA Protein Assay is a copper-based total protein quantification method that relies on the reaction of bicinchoninic acid (BCA) with copper cations (Cu^{+}) to form the water-soluble BCA/copper complex, which exhibits a strong linear absorbance at 562 nm with increasing protein concentrations.

Following the manufacturer's instructions, samples were incubated with the buffers contained in the kit for 30 minutes, and absorbances were measured at 562 nm. A standard curve was developed using bovine serum albumin (BSA), and sample data were interpolated to obtained protein quantity present in all of them.

4.9.2. Protective antioxidants determination

All antioxidant enzyme activities (SOD, CAT, GST, GPx, GR) and non-enzymatic antioxidant (total content of GSH), were evaluated during an abroad internship in the Ecotoxicology and Animal Physiology Laboratory of the University of Aveiro (Portugal). Antioxidant determination were performed from the PMS extracts obtained from three pools of five larvae for each experimental condition, as described previously.

All measurements were carried out in a SpectraMax 190 UV/Vis microplate reader (Molecular Devices, US) at a constant temperature of 25 °C, and all experiments contained blank samples as an internal control. A total of three independent replicates were performed for each experimental condition on each enzymatic determination.

4.9.2.1. Superoxidase dismutase (SOD)

Superoxide dismutase was assayed using a superoxide dismutase (Ransod) commercial kit. The method employs xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD activity is measured by the degree of inhibition of this reaction considering that one unit of SOD causes 50 % inhibition of the rate of INT reduction under the conditions of the assay.

As recommended by the manufacturer, before measuring SOD levels and to ensure the correct performing of the kit, sample dilutions were checked so that the % of inhibition falls in a range between 30 % and 60 %. *P. olivacea* samples were 10-fold diluted, and *C. riparius* samples were 10-fold or 7-fold diluted, as required. Following the manufacturer's instructions, kit's reagents were added to each sample in the specified order in a 96-well plate and SOD activity was measured at 505 nm for 3 minutes 30 seconds. A series of blank samples were added as internal control and a standard curve was developed.

Results were expressed as SOD units · mg protein^{-1}.

4.9.2.2. Catalase (CAT)

Catalase activity was assayed with the Claiborne method as described by Giri *et al*., (1996). In a preliminary CAT absorbance test, we concluded that neither *P. olivacea* nor *C. riparius* samples needed to be diluted, as absorbance test data accomplished protocol requirements.

Briefly, the assay mixture consisted of 0.190 mL phosphate buffer (0.05 M, pH 7.0) with 0.19 µL H_2O_2 (0.010 M) and 0.010 mL of PMS in a final volume of 0.2 mL. Change in absorbance was measured in appropriate 96-well UV-transparent microplates (UV-Star® flat-bottom microplates,

Greiner Bio-One GmbH, Germany) spectrophotometrically at 240 nm for 3 minutes.

CAT activity was calculated in terms of µmol H_2O_2 consumed · min^{-1} · mg $protein^{-1}$ using a molar extinction coefficient (ε) of 43.5 $M^{-1} \cdot cm^{-1}$.

4.9.2.3. Glutathione S-transferase (GST)

GST activity was determined using CDNB (1-chloro-2,4-dinitrobenzene) as a substrate, following the method of Habig *et al*., 1974. *P. olivacea* and *C. riparius* samples were checked to test the most suitable dilution; the chosen dilution for *P. olivacea* was 5-fold and for *C. riparius* 5-fold and 10-fold as required.

The assay mixture consisted in 0.1 mL PMS and 0.17 mL GSH (0.0018 M) prepared in phosphate buffer (0.2 M, pH 7.9). The reaction was initiated by addition of 0.03 mL of CDNB (0.01 M), and the increase in absorbance was recorded in UV microplates at 340 nm for 5 minutes.

GST activity was calculated as nmol CDNB conjugate formed · min^{-1} · mg $protein^{-1}$ using a molar extinction coefficient (ε) of 9.6 $nM^{-1} \cdot cm^{-1}$.

4.9.2.4. Glutathione peroxidase (GPx)

GPx activity was measured in PMS according to the method described by Mohandas *et al*., in 1984 and modified in 1998 by Athar and Iqbal. None of the samples of both species needed to be diluted.

Assay mixture consisted in 0.09 mL phosphate buffer (0.05 M, pH 7.0), 0.03 mL ethylenediaminetetraacetic acid (EDTA; 0.010 M), 0.03 mL sodium azide (NaN_3; 0.010 M), 0.03 mL glutathione reductase (GR; 2.4 U mL^{-1}), 0.03 mL reduced glutathione (GSH; 0.010 M), 0.03 mL nicotinamide adenine dinucleotide phosphate-oxidase (NADPH; 0.0015 M), 0.03 mL of

PMS and the reaction was initiated by addition of 0.03 mL H_2O_2 (0.0025 M). Oxidation of NAPDH to $NADP^+$ was recorded in UV microplates at 340 nm for 5 minutes.

GPx activity was calculated in terms of nmol NADPH oxidised · min^{-1} · mg $protein^{-1}$ using a molar extinction coefficient (ε) of 6.22 x 10^3 M^{-1} · cm^{-1}.

4.9.2.5. Glutathione reductase (GR)

GR activity was assayed by the method of Cribb *et al*., (1989), with some modifications. This assay determines the GR activity indirectly by measuring the NADPH disappearance associated with a reduction of oxidised glutathione (GSSG) catalysed by GR. In this case, *P. olivacea* samples needed to be diluted 50-fold and *C. riparius* PSM samples, 75-fold.

The assay mixture contained 0.050 mL of sample and 0.250 mL of the reaction medium that was composed of: phosphate buffer (0.05 M, pH 7.0), NAPDH (0.0002 M), glutathione disulphide (GSSG; 0001 M), and diethylenetriaminepentaacetic acid (DTPA; 0.0005 M).

GR activity was determined in UV microplates by measuring the oxidation of NADPH at 340 nm for 5 minutes and calculated as nmol NAPDH oxidised · min^{-1} · mg $protein^{-1}$ using a molar extinction coefficient (ε) of 6.22 x 10^3 M^{-1} · cm^{-1}.

4.9.2.6. Total glutathione content (GSHt)

For the quantification of the total GSH content, PMS was precipitated with trichloroacetic acid (TCA; 12 % m/v) for 1 h and then centrifugated at 10000 rpm / 4 °C for 5 minutes. GSHt was determined from deproteinated PMS by adopting the enzymatic recycling method using GR excess, by which the sulfhydryl group of GSH reacts with 5,5-dithiobis-(2-

nitrobenzoic acid) (DTNB; Ellman's reagent) and produces a yellow coloured 5-thio-2-nitrobenzoic acid (TNB) (Baker *et al*., 1990; Tietze, 1969). The rate of TNB production is directly proportional to the concentration of GSH in the sample.

Prior to quantification, *P. olivacea* samples were 50-fold diluted and *C. riparius* 75-fold according to their concentrations.

The assay mixture consisted of 0.2 mL sodium phosphate buffer (0.143 M, pH 8), EDTA (0.0063 M), DTNB (0.001 M), NADPH (0.00034 M) and 0.04 mL of deproteinated PMS. The reaction was initiated by addition of 0.04 mL GR (8.5 U ml^{-1}). Formation of TNB was measured at 415 nm for 7 minutes.

GSHt was expressed as nmol TNB formed · min^{-1} · mg $protein^{-1}$ using a molar extinction coefficient (ε) of 14.1 x 10^3 M^{-1} · cm^{-1}.

4.10. Data processing and analysis

Data compilation from the different assays developed in this thesis and graphics were performed in Microsoft® Excel 2013 for Microsoft Office 365 MSO ProPlus (Microsoft Corporation, Washington, US). The final version of graphics and figures were processed with Adobe Photoshop CS6 (v13.0 x64) and Gimp (v2.10.12).

To analyse statistically significant differences between samples and exposure conditions, statistical analyses were carried out using IBM Statistics SPSS 25.0 for Windows (IBM, New York, US) and RStudio (v1.1.463) (RStudio Team, 2016). Mean and median were calculated respectively as the average and the middle of a data set, while the standard deviation represented the squared root of the variance.

Regarding the survival studies, the Student t vas used to check the statistically significant differences ($p \leq 0.05$) between the different experimental conditions.

In terms of transcriptional and antioxidant response analysis, for each parameter of study normality and homoscedasticity of data were checked by Shapiro-Wilk and Levene tests, respectively. When data accomplished normality and homoscedasticity, the normalised levels of gene transcripts and enzyme were analysed with ANOVA followed by Bonferroni's *post hoc* test. In contraposition, when data were not homogeneous or normally distributed, differences in gene and enzyme expression were evaluated using the nonparametric Kruskal-Wallis test followed by a pairwise Mann-Whitney-Wilcoxon *post hoc* test. In all cases, p-values ≤ 0.05 and ≤ 0.1 were used as cutoffs for statistical significance.

RESULTS

5. RESULTS

5.1. *P. olivacea de novo* transcriptome: sequencing, assembly and analysis of RNA-seq data

The cDNA of the reference transcriptome was assembled by pooling the data from three libraries, built from the whole body-RNA of *P. olivacea* larvae collected in the Sar River. After trimming, the *de novo* RNA sequencing rendered a total of 60.25 million raw-reads with a GC content of 42.13 % and a ratio of reads with a Phred quality score over 30 (Q30) of 92.15 %. Data were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB35133.

After removing low-quality adaptors and reads, the assembly of the high-quality reads reported a total of 34900 contigs with an N50 length of 1334 bp and an average transcript length of 808 bp. From the total number of transcripts BLASTx annotated 22413 unigenes against different protein sequence databases.

Among them, the GO categorisation identified 62 GO terms within three categories: "Biological process" (22 terms; 29%), "Molecular function" (21 terms; 22%) and "Cellular component" (19 terms; 18%) (Figure 14A). The largest group was *biological process* with 9938 unigenes, and the smallest group was *cellular component*, with 6334 genes.

The most enriched terms for *biological process* were *metabolic* (22 % of the total number of sequences classified under this category), *biological regulation* (19 %), and *single-organism process* (15 %). For *molecular function*, the dominant categories were *binding* (43 %) and *catalytic activity* (33 %). Finally, *cell part* comprised 38 % of the sequences under *cellular component*, followed by *organelle* (15 %) (Figure 14B).

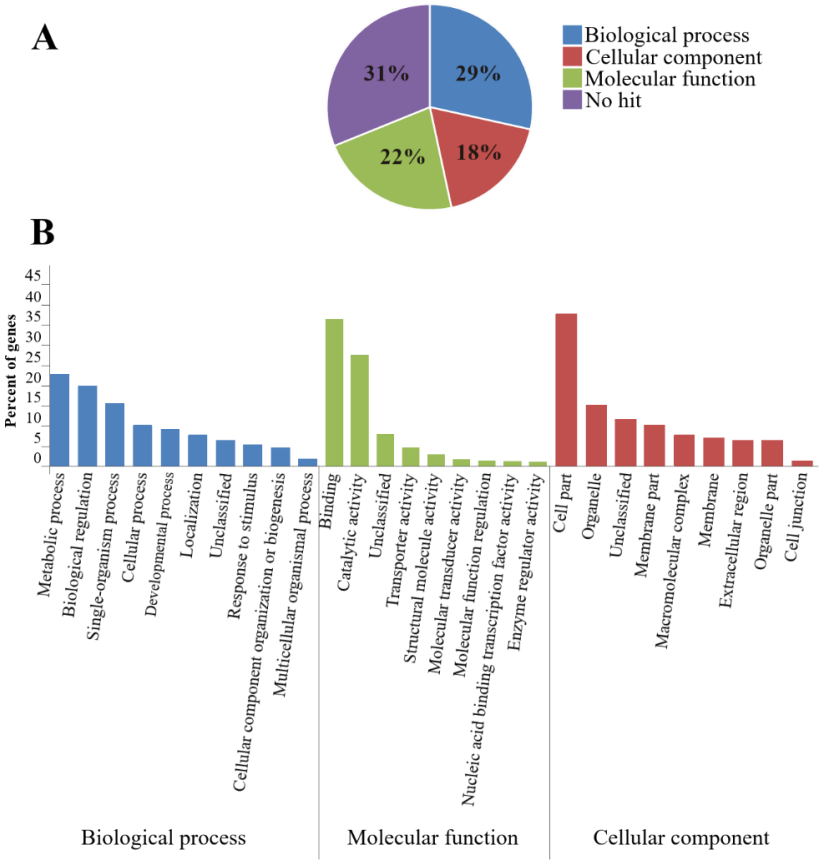


Figure 14. GO unigene categories of the *P. olivacea de novo* transcriptome. A) Unigenes were annotated into three main categories: biological process (blue), molecular function (green) and cellular component (red); 31 % of the sequences reported no hit (purple). **B)** Most represented terms for each category shown as a percentage of the total number of sequences in decreasing order.

5.2. Molecular characterisation of biomarkers in *P. olivacea*

A total of 19 *P. olivacea* genes involved in different relevant pathways were *de novo* identified and characterised in this research study. The molecular characterisation of selected biomarkers of interest in this non-model organism started with a systematic search in the transcriptome. This search rendered different sequences with open reading frames (ORFs) for their corresponding proteins. Those sequences were identified, analysed and deposited in the National Centre for Biotechnology Information (NCBI) database, GenBank. A table summary of all *de novo* characterised genes with their corresponding ORF and protein length as well as their database accession number can be found at the end of this section (Table 17). Finally, a phylogenetic study was conducted to evaluate the relationship between each described protein and homologous proteins in other species and to confirm the identity of each *de novo* biomarker characterised in this chironomid.

5.2.1. Cell stress response genes

A total of six genes involved in cell stress response were characterised and their sequences were registered at the GenBank database: *Heat-shock protein 27* (*Hsp27,* MW273341), *Heat-shock protein 60* (*Hsp60*, MW273342), *Heat-shock protein 70* (*Hsp70*, MW273351), *Heat-shock protein 70 cognate* (*Hsc70*, MW273352), *Hsp90 co-chaperone Cdc37* (*Cdc37*, MW273343) and *Heat-shock factor* (*HSF*, MW289589). A systematic search in our *P. olivacea* transcriptome rendered sequences with complete ORFs for their corresponding proteins. As described previously, a phylogenetic study including homologous proteins from other arthropods was accomplished to confirm the identity of each *de novo* characterised protein in this nonbiting midge (Figure 15, right; Table 13). Relevant domains of each ORF are presented in the left side of Figure 15.

Heat-shock protein 27 (HSP27)

The transcriptome search rendered a 911 bp cDNA contig corresponding to an ORF of 588 bp coding for a protein of 195 amino acids (aa). *P. olivacea* HSP27 protein shared a 76 % of sequence identity with the Chironomidae *Clunio marinus*, 49 % with *Anopheles darlingi* and 43 % with *Aedes aepypti*, *Drosophila albomicans* and *Nasonia vitripennis* (Table 13). In the C-terminal, the protein contains a characteristic stretch of 80 aa residues related to the so-called α-crystallin domain (ACD), a defining feature of the small stress-induced protein family (Kriehuber *et al*., 2010) (Figure 15A).

Heat-shock protein 60 (HSP60)

A 1722 bp ORF coding for HSP60 protein was included in a 2308 bp length contig. The ORF coded for a protein with 573 residues and a characteristic HSP60 chaperonin domain (groEL, aa 22-555) involved in productive folding of proteins (Lin and Rye, 2006) (Figure 15B). It shared 89 % of identity with *C. riparius* and more than 80 % with other selected dipteran, lepidopteran, hymenopteran and hemipteran species (Table 13).

Heat-shock protein 70 (HSP70)

The 3920 bp *P. olivacea Hsp70* cDNA contig isolated from its transcriptome included an ORF of 2535 bp encoding for an 844 aa complete protein. At the amino acid level, this protein shared 66 % and 63 % of identity with *A. aegypti* and *Anopheles albimanus* and more than 50 % with other representative insects such as *Drosophila mojavensis* or *Apis florea* (Table 13). The structural analysis of the protein revealed a HSP70 conserved domain covering positions from 3 to 700 aa (Figure 15C).

Heat-shock protein 70 cognate (HSC70)

The *P. olivacea Hsc70* cDNA was a 2528 bp contig that contained a 1929 bp ORF coding for a 642 aa complete protein. It showed more than 75 % identity with all the species included in the phylogenetic study, such as *C. riparius, A. aegypti, Halyomorpha halys*, *B. mori, N. vitripennis* or even *Ixodes scapularis*, species used as outgroup (Table 13). Due to its involvement in a wide range of diverse cellular processes and constitutive expression, its high degree of conservation among different species is not surprising, as shown in the phylogeny analysis. BLAST revealed an HSP70 conserved domain covering practically the total protein (10-614) (Figure 15D).

Heat-shock protein 90 co-chaperone Cdc37 (CDC37)

A 1400 bp length contig contained the complete ORF composed of 1116 bp and encoding a 371 aa HSP90 co-chaperone CDC37 protein. The protein contained a conserved Cdc37 amino-terminal domain (1-119), a middle domain (162-268) that interacts with HSP90 and a carboxy-terminal (285-361) domain (Figure 15E). It shared 76 % identity with *C. marinus* and an average of 60 % identity with species like *N. vitripennis*, *B. mori* or *H. halys* (Table 13).

Heat-shock factor (HSF)

The complete ORF for HSF was 1665 bp in length and encoded a 554 aa protein with a characteristic HSF domain at the N-terminal (Figure 15F).

It shared 59 % identity with the dipteran commonly known as the sleeping chironomid (*Polypedium vanderplanki*), and 76 %, 65 % or 62 % with HSFs from *Anopheles sinensis, A. mellifera* or *B. mori*, respectively (Table 13).

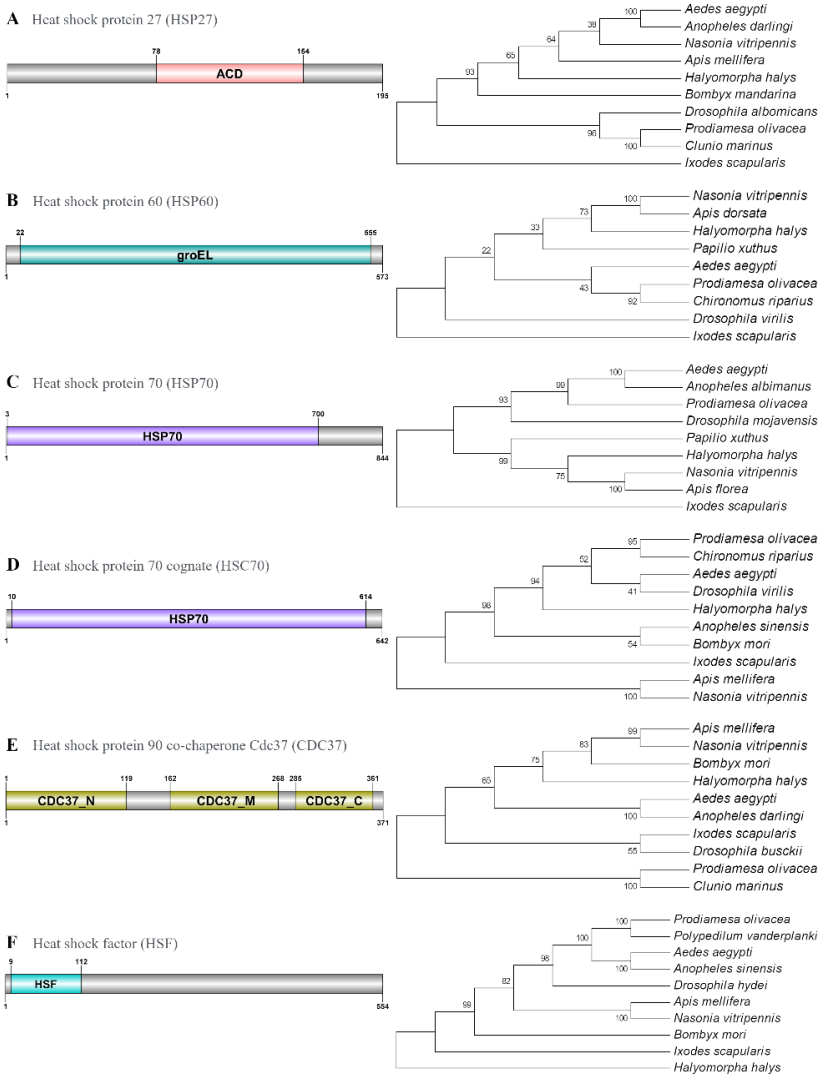


Figure 15

Figure 15. Cell stress response proteins of *P. olivacea* identified from its *de novo* transcriptome. Left) Diagram of proteins identified as putative mRNAs and their conserved domains: HSP27 **(A)**, HSP60 **(B)**, HSP70 **(C)**, HSC70 **(D)**, HSP90 co-chaperone CDC37 **(E)** and HSF **(F)**. ACD: alpha crystallin conserved protein domain; groEL: HSP60 chaperonin domain; HSP70: heat-shock protein 70 conserved domain; CDC37_N, CDC37_M and CDC37_C: amino-terminal, middle and carboxy-terminal conserved CDC37 domain; HSF: heat-shock factor. **Right)** Bootstrap consensus tree inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 40 % bootstrap replicates are collapsed. The evolutionary distances are in units of number of amino acid substitutions per site.

Table 13. Phylogenetic analysis of *P. olivacea* cell stress response proteins. Species, accession numbers, length and identity of the amino acid sequences used for the BLAST analysis and the phylogenetic trees of the identified cell stress response proteins in *P. olivacea*.

Gene	Species	Accession number	ORF length (aa)	Identity
Hsp27	*C. marinus*	CRK99494.1	198	76 %
	A. darlingi	ETN59632.1	208	49 %
	I. scapularis	EEC02529.1	163	47 %
	H. halys	XP_014274473.1	181	46 %
	A. aegypti	XP_001663499.2	192	43 %
	D. albomicans	XP_034104998.1	222	43 %
	N. vitripennis	XP_001600020.1	169	43 %
	B. mandarina	XP_028044206.1	188	42 %
	A. mellifera	XP_001120194.1	196	38 %

Table 13. Continuation (I)

Gene	Species	Accession number	ORF length (aa)	Identity
Hsp60	*C. riparius*	KA179261.1	564	89 %
	A. darlingi	ETN58825.1	574	86 %
	A. aegypti	XP_001661764.1	574	85 %
	N. vitripennis	XP_001600045.1	572	84 %
	A. dorsata	XP_006622784.1	570	83 %
	D. virilis	XP_002058167.1	573	83 %
	P. xuthus	KPJ02499.1	572	82 %
	H. halys	XP_014273033.1	573	82 %
	I. scapularis	EEC11387.1	544	54 %
Hsp70	*A. aegypti*	XP_021708352.1	839	66 %
	A. albimanus	XP_035793803.1	845	63 %
	D. mojavensis	XP_015018271.1	838	56 %
	P. xuthus	KPI91265.1	841	53 %
	A. florea	XP_003691374.1	866	53 %
	H. halys	XP_014280439.1	817	53 %
	N. vitripennis	XP_008202915.1	859	51 %
	I. scapularis	XP_029828017.1	624	34 %
Hsc70	*C. riparius*	ADL27420.1	644	87 %
	A. aegypti	XP_021709332.1	635	84 %
	D. virilis	XP_002056192.1	635	82 %
	H. halys	P_014275032.1	623	82 %

Table 13. Continuation (II)

Gene	Species	Accession number	ORF length (aa)	Identity
Hsc70	*B. mori*	NP_001296526.1	640	79 %
	A. sinensis	KFB46250.1	639	78 %
	I. scapularis	XP_029837103.1	635	77 %
	N. vitripennis	NP_001166228.1	655	76 %
	A. mellifera	NP_001153522.1	650	76 %
Cdc37	*C. marinus*	CRK96958.1	377	76 %
	D. busckii	XP_017843358.2	390	63 %
	N. vitripennis	XP_031782305.1	390	63 %
	A. aegypti	XP_001660603.1	375	61 %
	A. mellifera	NP_001229443.1	370	60 %
	A. darlingi	ETN58370.1	390	59 %
	B. mori	NP_001036991.1	372	59 %
	I. scapularis	XP_002414301.1	363	59 %
	H. halys	XP_014278874.1	363	58 %
HSF	*A. sinensis*	KFB51194.1	845	76 %
	A. aegypti	XP_021704330.1	725	73 %
	D. hydei	XP_023164880.1	700	70 %
	A. mellifera	XP_026299188.1	479	65 %
	B. mori	BAK26396.1	627	62 %
	N. vitripennis	XP_032455725.1	661	60 %
	P. vanderplanki	ADM13379.1	571	59 %
	I. scapularis	XP_029844053.1	630	56 %
	H. halys	XP_014271453.1	651	50 %

5.2.2. Endocrine system genes

Three genes involved in the hormonal pathway were characterised: *ecdysone receptor* (*EcR*, MW273344), *juvenile hormone epoxide hydrolase* (*JHEH*, MW273346), and *Kruppel homolog 1* (*Kr-h1*, MW273345). A systematic search of our *P. olivacea* transcriptome rendered sequences with complete ORFs for the EcR and JHEH corresponding proteins and an incomplete ORF for the Kr-h1 protein. Data regarding conserved protein domains and the phylogenetic study run to confirm the identity of each *de novo* characterised protein are presented in Figure 16 and Table 14.

Ecdysone receptor (EcR)

A 1614 bp ORF encoding for a 537 aa EcR protein was contained in a 2254 bp length contig. The complete protein comprised two characteristic nuclear receptor conserved domains: a DNA-binding domain (NR_DBD_EcR) from amino acid position 111 to 201 and a ligand-binding domain (LBD_EcR) from residues 277 to 509 (Figure 16A). *P. olivacea* EcR protein shared around 80 % of identity with homologous sequences from other selected dipterans such as *C. riparius*, *A. aegypti* or *A. sinensis* (Table 14).

Juvenile hormone epoxide hydrolase (JHEH)

A complete 1371 bp length ORF codified for a 456 aa JHEH protein. The protein contained an epoxide hydrolase conserved domain at the N-terminal region (55-166) (Figure 16B) and showed a 68 % amino acid identity with *C. marinus*, 62 % with *A. aegypti* and 58 % with *A. darlingi*, among other selected representative insects (Table 14).

Kruppel homolog 1 (Kr-h1)

An incomplete ORF encoding for Kr-h1 protein was found in the *P. olivacea de novo* transcriptome. The ORF was a 925 bp sequence coding a protein with 288 residues and a conserved zinc-finger double domain (zf-H2C2_2) (Figure 16C). The identified protein shared 80 % and 69 % identities with Kr-h1 of *C. marinus* and *A. albimanus*, respectively (Table 14).

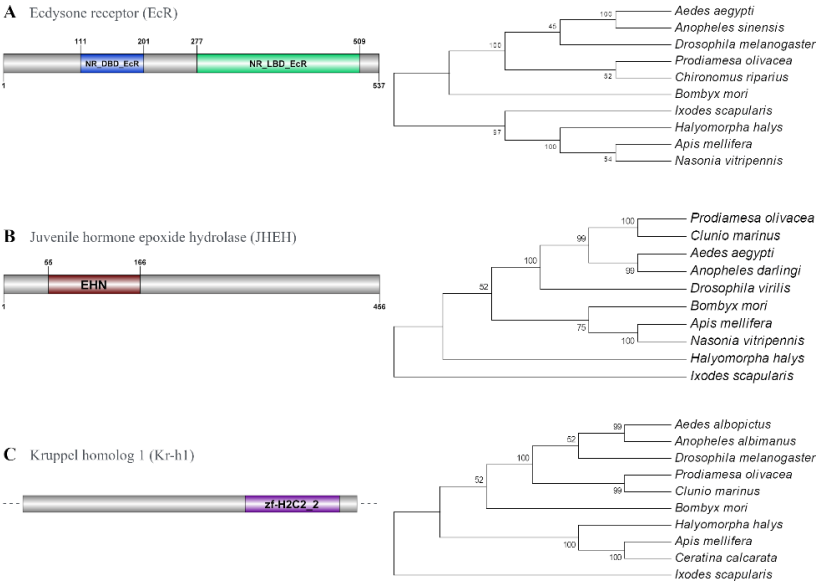


Figure 16. Hormonal pathway proteins of *P. olivacea* identified from its *de novo* transcriptome. Left) Diagram of proteins identified as putative mRNAs and their conserved domains: Ecdysone receptor (EcR) **(A)**, juvenile hormone epoxide hydrolase (JHEH) **(B)** and Kruppel homolog 1 (Kr-h1) **(C)**. NR_DBD_EcR and NR_LBD_EcR: nuclear receptor DNA-binding domain and ligand-binding domain respectively; EHN: Epoxide hydrolase N-terminus domain; zf-H2C2_2: Zinc-finger double domain. **Right)** Bootstrap consensus tree inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 40 % bootstrap replicates are collapsed. The evolutionary distances are in units of number of amino acid substitutions per site.

Table 14. Phylogenetic analysis of *P. olivacea* hormonal pathway proteins. Species, accession numbers, length and identity of the amino acid sequences used for the BLAST analysis and the phylogenetic trees of the identified hormonal pathway response proteins in *P. olivacea*.

Gene	Species	Accession number	ORF length (aa)	Identity
EcR	*A. aegypti*	A87394.1	675	81 %
	A. sinensis	KFB49673.1	481	80 %
	C. riparius	XP_021206380.1	539	79 %
	D. melanogaster	NP_724456.1	849	69 %
	B. mori	XP_021206380.1	515	61 %
	H. halys	XP_014281746.1	414	59 %
	I. scapularis	XP_002405625.1	433	59 %
	N. vitripennis	NP_001152828.1	577	58 %
	A. mellifera	BAF46356.1	567	57 %
JHEH	*C. marinus*	CRL02365.1	477	68 %
	A. aegypti	XP_001661591.1	458	62 %
	A. darlingi	ETN62698.1	516	58 %
	D. virilis	XP_002050951.1	562	54 %
	B. mori	BAF81491.1	461	49 %
	A. mellifera	XP_394922.3	456	48 %
	N. vitripennis	NP_001128399.1	470	48 %
	H. halys	XP_014293302.1	454	47 %
	I. scapularis	XP_029823841.1	624	43 %
Kr-h1	*C. marinus*	CRK96499.1	651	80 %
	A. albimanus	XP_035774676.1	891	69 %

Table 14. Continuation (I)

Gene	Species	Accession number	ORF length (aa)	Identity
Kr-h1	*B. mori*	BAL04727.1	361	68 %
	D. melanogaster	NP_477466.1	845	67 %
	C. calcarata	XP_017885185.1	495	66 %
	H. halys	XP_014292566.1	437	66 %
	A. mellifera	NP_001011566.1	500	65 %
	A. albopictus	XP_019548883.2	749	55 %
	I. scapularis	XP_029851311.1	331	33 %

5.2.3. Detoxification and oxidative stress genes

Six genes related to biotransformation and oxidative stress pathway were identified and characterised: *manganese superoxide dismutase* (*MnSOD*, MN862061), *catalase* (*CAT*, MN862062), *phospholipid-hydroperoxide glutathione peroxidase* (*PHGPx*, MN862064), *Cyp4g15* (MN862063*), Cyp6a14-like* (MN862066), and *Cyp6a2-like* (MN862065). While the systematic search across the *P. olivacea* transcriptome rendered sequences with complete ORF for five of them, one incomplete sequence was obtained for CYP6A2-like protein.

Manganese superoxide dismutase (MnSOD)

The complete ORF for *MnSOD* was 654 bp length and encoded a 217 aa protein with a conserved superoxide dismutase domain (SodA) from amino acid 22 to 217 (Figure 17A). It shared 87 % of sequence identity with *C. riparius*, 69 % with *A. aegypti* and 67 % with *A. darlingi* (Table 15).

Catalase (CAT)

An 1858 bp contig included a 1506 bp length ORF encoding a putative CAT protein with 501 aa residues and a highly conserved catalase domain at the N-terminal end (Figure 17B). Sequence analysis revealed that *P. olivacea* CAT protein shared more than 90 % of identity with the homologous protein of *C. riparius* and over 60 % with all the selected insect species for the phylogenetic analysis (Table 15).

Phospholipid-hydroperoxide glutathione peroxidase (PHGPx)

The ORF for *PHGPx* was 603 bp length and encoded a protein of 200 aa with a glutathione peroxidase family domain (GSH) at the C-terminal region (Figure 17C). It shared 88 %, 66 % and 65 % of amino acid identity with the PHGPx of *C. riparius*, *Melanaphis sachhari* and *N. vitripennis* respectively (Table 15).

P450 cytochromes

Three nucleotide sequences encoding different P450 cytochromes were identified; all of them presented a Cyp450 conserved domain. Two isolated ORFs were completed (Figure 17D, E), and one was incomplete (Figure 17F).

The CYP4G15 protein was composed of 568 aa and encoded by an ORF of 1707 bp. It shared more than 63 % of identity with representative dipteran, lepidopteran, hymenopteran and hemipteran species (Table 15).

The second complete ORF was 1491 bp length and codified for a CYP6A14-like protein with 496 aa residues. It had 65 % of identity with *C. marinus* and 48 % with *A. aegypti* and *A. darlingi* (Table 15).

Finally, the incomplete ORF had 1117 bp and encoded a 371 aa sequence corresponding to the 3' end of the protein CYP6A2-like sharing more than 50 % identity with other dipterans including *C. tentants* (Table 15).

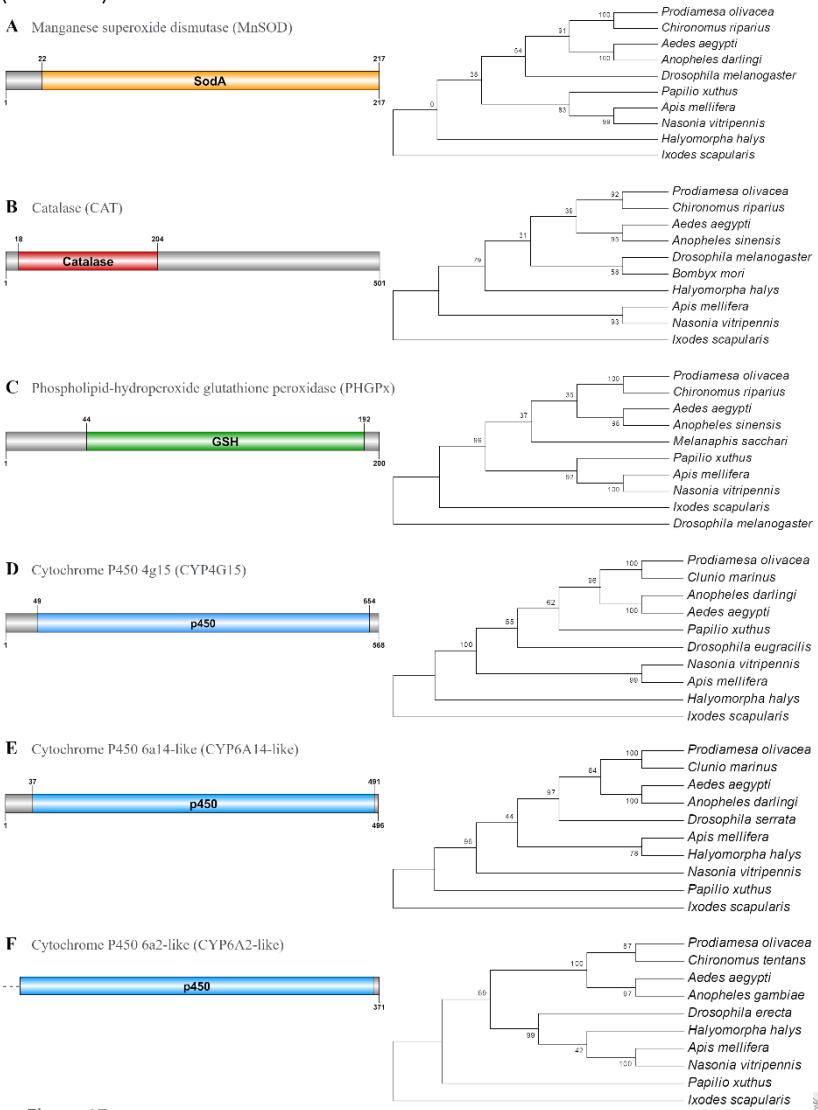


Figure 17

Figure 17. Detoxification and oxidative stress proteins of *P. olivacea* identified from its *de novo* transcriptome. Left) Diagram of the proteins identified as putative mRNAs and their conserved domains: manganese superoxide dismutase (MnSOD) **(A)**, catalase (CAT) **(B)**, phospholipid-hydroperoxide glutathione peroxidase (PHGPx) **(C)**, CYP4G15 **(D)**, CYP6A14-like **(E)** and CYP6A2-like **(F)**. SodA: Superoxide dismutase conserved protein domain; GSH: Glutathione peroxidase protein domain family; p450: Cytochrome p450 protein domain. **Right)** Bootstrap consensus tree inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 40 % bootstrap replicates are collapsed. The evolutionary distances are in units of number of amino acid substitutions per site.

Table 15. Phylogenetic analysis of *P. olivacea* biotransformation and oxidative stress proteins. Species, accession numbers, length and identity of the amino acid sequences used for the BLAST analysis and the phylogenetic trees of the detoxification and oxidative stress response proteins in *P. olivacea*.

Gene	Species	Accession number	ORF length (aa)	Identity
MnSOD	*C. riparius*	AFM78034.1	218	87 %
	A. aegypti	XP_001649850.1	219	69 %
	A. darlingi	ETN64551.1	219	67 %
	N. vitripennis	XP_001607380.1	220	67 %
	D. melanogaster	Q00637.3	217	66 %
	P. xuthus	XP_013179895.1	216	64 %
	A. mellifera	NP_001171519.1	218	61 %
	I. scapularis	XP_002403511.1	224	61 %
	H. halys	XP_014277433.1	217	59 %
CAT	*C. riparius*	AFV92463.1	308	91 %
	A. aegypti	XP_001663600.1	505	75 %
	A. sinensis	KFB50865	504	74 %

Table 15. Continuation (I)

Gene	Species	Accession number	ORF length (aa)	Identity
CAT	*D. melanogaster*	NP_536731.1	506	73 %
	B. mori	NP_001036912.1	507	70 %
	H. halys	XP_014281971.1	503	70 %
	A. mellifera	NP_001171540.1	513	66 %
	N. vitripennis	XP_008208479.2	615	65 %
	I. scapularis	XP_002400629.1	256	60 %
PHGPx	*C. riparius*	AFP50153.1	198	88 %
	M. sacchari	XP_025198754.1	194	66 %
	N. vitripennis	XP_003427378.1	191	65 %
	A. aegypti	XP_001653252.2	217	61 %
	P. xuthus	KPI94065.1	200	61 %
	A. mellifera	XP_006570759.1	196	59 %
	A. sinensis	KFB52143.1	213	59 %
	I. scapularis	XP_002407528.1	185	51 %
	D. melanogaster	NP_728868.1	198	49 %
Cyp4g15	*C. marinus*	CRK90412.1	564	86 %
	A. aegypti	XP_001648376.1	566	79 %
	A. darlingi	ETN64031.1	571	77 %
	P. xuthus	NP_001299286.1	556	72 %
	D. eugracilis	XP_017085043.1	563	71 %
	H. halys	NP_001351150.1	561	71 %
	N. vitripennis	NP_001165992.1	560	68 %
	A. mellifera	XP_006559403.1	533	63 %
	I. scapularis	XP_002413005.1	422	35 %

Table 15. Continuation (II)

Gene	Species	Accession number	ORF length (aa)	Identity
Cyp6a14-like	*C. marinus*	CRL05227.1	499	65 %
	A. aegypti	XP_001653674.1	493	48 %
	A. darlingi	ETN65662.1	500	48 %
	D. serrata	XP_020799030.1	509	43 %
	A. mellifera	XP_624795.3	510	39 %
	N. vitripennis	NP_001165999.1	512	39 %
	H. halys	XP_014276525.1	500	38 %
	P. xuthus	KPI92235.1	435	35 %
	I. scapularis	XP_002407452.1	518	27 %
Cyp6a2-like	*C. tentans*	ARO50438.1	499	56 %
	A. aegypti	XP_001652486.1	499	55 %
	A. gambiae	XP_558354.4	500	53 %
	N. vitripennis	NP_001165999.1	499	40 %
	D. erecta	XP_001970725.1	506	39 %
	H. halys	XP_024215789.1	500	38 %
	A. mellifera	NP_001035324.1	510	34 %
	P. xuthus	KPI92235.1	435	32 %
	I. scapularis	P_002401737.1	388	28 %

5.2.4. Immune system genes

Four genes related to the immune system were *de novo* characterised in *P. olivacea*: *Toll* (MW273350), *the peptidoglycan-recognition protein* (*PGRP*, MW273347), *C-type lectin* (MW273348) and *tyrosine-protein kinase hopscotch* (*JAK/hopscotch*, MW273349). Transcriptome searching rendered complete ORFs for three of them, while *JAK/hopscotch* was 5' end incomplete. A summary of data related to the conserved protein domains and the phylogenetic study performed for protein characterisation is available in Figure 18 and Table 16.

Toll

The contig of the isolated *P. olivacea Toll* gene was 3886 bp length, with a 3201 bp ORF encoding a 1066 aa protein. The encoded protein had two characteristic structures for gene encoding Toll-related proteins: a leucine-rich repeat conserved domain (LRR) from residues 259 to 563 and a Toll-interleukin 1-resistance domain (TIR) at the C-terminal domain (Bell *et al*., 2003; Toshchakov and Neuwald, 2020) (Figure 18A). It shared 55 % identity with *C. marinus* and over 30 % with Toll proteins of representative hemipteran and hymenopteran species such as *H. halys*, *N. vitripennis* or *Apis cerana* (Table 16).

Peptidoglycan-recognition protein (PGRP)

A 576 bp long cDNA corresponding to an ORF encoded a putative PGRP protein of 191 aa that contained a characteristic PGRP domain (23–163) (Figure 18B). This *P. olivacea* protein shared more than 70 % identity with *A. sinensis* and *Aedes albopictus* and over 35 % with homologous sequences from other insects (Table 16).

C-type lectin

P. olivacea C-type lectin characterised gene was encoded by an ORF composed of 585 bp. The 194 aa protein contained a C-type lectin conserved domain, and it exhibited 72 %, 67 % and 62 % identity at the amino acid level with *D. melanogaster*, *Apis dorsata* and *H. halys*, respectively (Figure 18C, Table 16).

Tyrosine-protein kinase hopscotch (JAK/hopscotch)

The *JAK/hopscotch* ORF was incomplete and had 3295 bp that encoded a 1097 aa sequence corresponding to the 3' end of the protein. It shared 67 % identity with *C. marinus* and around 35 % with other selected insects (Table 16). Protein BLAST analysis revealed three conserved domains characteristic of the *JAK/hopscotch* gene: Band 4.1 (B41), also known as ezrin/radixin/moesin (ERM) protein domain; serine/threonine protein kinase (SPS1); and protein tyrosine kinase domain (Pkinase_Tyr) (Figure 18D).

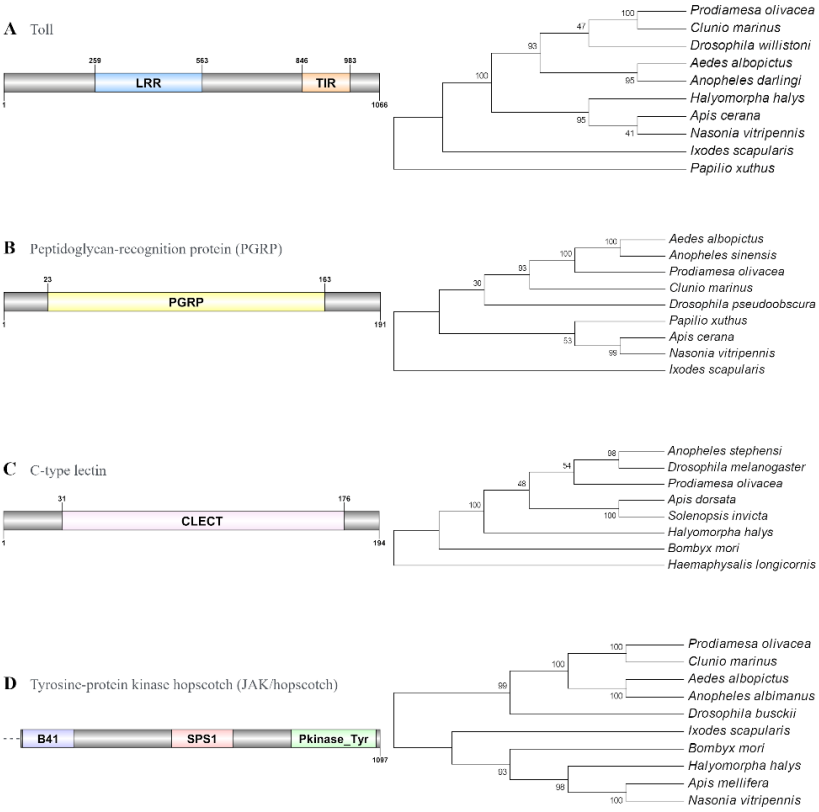


Figure 18. Immune system proteins of *P. olivacea* identified from its *de novo* transcriptome. **Left)** Diagram of the proteins identified as putative mRNAs and their conserved domains: Toll **(A)**, peptidoglycan-recognition protein (PGRP) **(B)**, C-type lectin **(C)** and tyrosine-protein kinase hopscotch (JAK/hopscotch) **(D)**. LRR: Leucine-rich repeat; TIR: Toll - interleukin 1 – resistance; PGRP: peptidoglycan recognition protein; CLECT: C-type lectin; B41: Band 4.1 homologues; SPS1: Serine/threonine protein kinase; Pkinase_Tyr: Protein tyrosine kinase. **Right)** Bootstrap consensus tree inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 40 % bootstrap replicates are collapsed. The evolutionary distances are in units of number of amino acid substitutions per site.

Table 16. Phylogenetic analysis of *P. olivacea* immune system proteins. Species, accession numbers, length and identity of the amino acid sequences used for the BLAST analysis and the phylogenetic trees of the immune system response proteins in *P. olivacea*.

Gene	Species	Accession number	ORF length (aa)	Identity
Toll	*C. marinus*	CRL07112.1	1075	55 %
	A. albopictus	XP_029710631.1	1129	40 %
	D. willistoni	XP_002072704.1	1095	40 %
	H. halys	XP_014288082.1	1051	38 %
	N. vitripennis	XP_001604577.1	1050	36 %
	A. darlingi	ETN66715.1	1162	34 %
	A. cerana	XP_016908796.1	1069	34 %
	I. scapularis	XP_029836730.1	1086	30 %
	P. xuthus	KPI94117.1	1343	23 %
PGRP	*A. sinensis*	KFB50650.1	189	73 %
	A. albopictus	XP_019552025.2	188	71 %
	C. marinus	CRL04926.1	186	55 %
	D. pseudoobscura	XP_001361529.3	191	48 %
	P. xuthus	KPJ05906.1	203	47 %
	N. vitripennis	XP_001605218.1	198	46 %
	A. cerana	XP_016922186.1	193	45 %
	I. scapularis	XP_029839737.1	259	36 %

Table 16. Continuation (I)

Gene	Species	Accession number	ORF length (aa)	Identity
C-type lectin	*D. melanogaster*	NP_001027437.1	194	72 %
	A. stephensi	XP_035900914.1	190	70 %
	A. dorsata	XP_006612866.1	188	67 %
	S. invicta	XP_025991456.1	187	65 %
	H. halys	XP_014280166.1	184	62 %
	B. mori	XP_021207206.1	188	27 %
	H. longicornis	BAU51440.1	535	20 %
JAK/hopscotch	*C. marinus*	CRK93974.1	1126	67 %
	A. albopictus	XP_029708892.1	1136	40 %
	A. albimanus	XP_035781041.1	1161	35 %
	A. mellifera	XP_006567751.1	1160	35 %
	N. vitripennis	XP_001602854.2	1141	35 %
	B. mori	XP_004922391.1	1114	34 %
	H. halys	XP_024215788.1	1093	34 %
	D. busckii	XP_017853154.1	1199	32 %
	I. scapularis	XP_029845111.1	1142	32 %

Table 17. Summary of the *de novo* characterised *P. olivacea* genes. Involved pathway, gene name, ORF and protein lengths of the *de novo* characterised *P. olivacea* genes as well as their corresponding database accession number.

Pathway	Gene	ORF length (bp)	Protein length (aa)	Accession number
Cell stress response	*Hsp27*	588	195	MW273341
	Hsp60	1722	573	MW273342
	Hsp70	2535	844	MW273351
	Hsc70	1929	642	MW273352
	Cdc37	1116	371	MW273343
	HSF	1665	554	MW289589
Hormonal	*EcR*	1614	537	MW273344
	JHEH	1371	456	MW273346
	Kr-h1	925 Incomplete	288 Incomplete	MW273345
Detoxification and oxidative stress	*MnSOD*	654	217	MN862061
	CAT	1506	501	MN862062
	PHGPx	603	200	MN862064
	Cyp4g15	1707	568	MN862063
	Cp6a14-like	1491	496	MN862066
	Cyp6a2-like	1117 Incomplete	371 Incomplete	MN862065
Immune system	*Toll*	3201	1066	MW273350
	PGRP	576	191	MW273347
	C-type lectin	585	194	MW273348
	JAK/ hopscotch	3295 Incomplete	1097 Incomplete	MW273349

5.3. Effects of xenobiotic exposure on larvae of natural populations of *P. olivacea*

Three ecotoxicological parameters were assessed in parallel on natural populations of *P. olivacea* exposed to BBP, BPA and BP3 for 4 h and 24 h: survival, transcriptional deregulation activity of selected genes and antioxidant response.

To deepen into the toxicity and to expand the knowledge of the mode of action of these xenobiotics, as well as to establish new biomarkers of toxicity, five pathways were studied at the molecular level by choosing key genes involved in them:

1) Cell stress response: *Hsp27*, *Hsp60*, *Hsp70* and the cognate form, *Hsc70*; 2) Endocrine system: *EcR*, *Kr-h1* and *JHEH*; *3)* Biotransformation and oxidative stress: *MnSOD*, *CAT*, *PHGPx*, *Cyp4g15*, *Cyp6a14-like* and *Cyp6a2-like*; 4) Immune system: *PGRP*, *C-type lectin*, *Toll* and *JAK/hopscotch* and *5)* Energy metabolism: *GAPDH.*

At the end of chapter 5.3, it is attached a summary table of transcriptional effects of BBP, BPA and BP3 in natural populations of *P. olivacea* (Table 21) and *C. riparius* (Table 22). Also, a summary of the comparative study performed between the aquatic midges is included (Table 23).

5.3.1. BBP

5.3.1.1. Transcriptional alterations in the cell stress response

Among the four analysed genes involved in the cell stress response, *Hsp27* and *Hsp60*, which underwent a complete loss of transcriptional activity after 4 h and 24 h exposures to BBP, showed the most striking

differences compared to the control (Figure 19A, B). *Hsp70* was downregulated after both exposures time, especially at the shortest time (69 % below control values; no statistical significance) (Figure 19C). Finally, of all the cell stress genes, *Hsc70* experienced the fewest changes in transcript levels (Figure 19D).

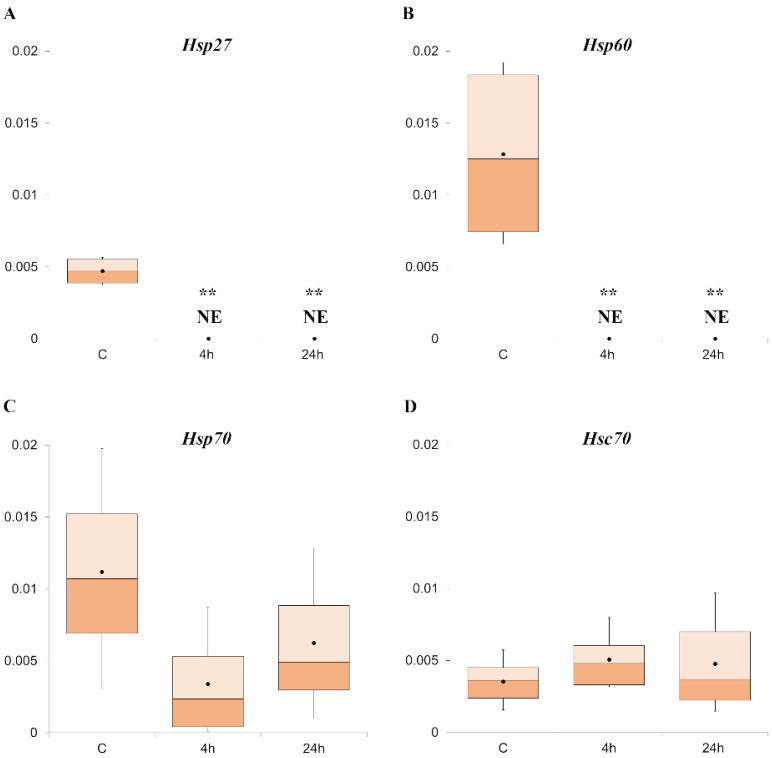


Figure 19. Transcriptional activity of *Hsp27*, *Hsp60*, *Hsp70* and *Hsc70* in *P. olivacea* larvae exposed 4 or 24 h to 1 µg/L BBP. Box and whisker plots represent the relative mRNA expression of *Hsp27* (A), *Hsp60* (B), *Hsp70* (C) and *Hsc70* (D) measured by RT qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control (C) values: $p \leq 0.05$ (**). NE: No expression.

5.3.1.2. Transcriptional alterations in the endocrine system

As showed in Figures 20A and 20C, BBP induced a time-dependent downregulation in the transcriptional activity of the hormone-related genes *EcR* and *JHEH*. This decline was significant for *JHEH*, reaching mRNA values below 60 % ($p = 0.045$) and 66 % ($p = 0.027$), compared to control after 4 and 24 h exposures (Figure 20C). In agreement with the literature available in different vertebrate and invertebrate species (Chen *et al*., 2014; Herrero *et al*., 2015; Moral *et al*., 2007), these results provide evidence of the potential of BBP as an endocrine-disrupting agent in the aquatic midge *P. olivacea*. Finally, no significant changes were observed regarding the transcription factor *Kr-h1*, whose levels were slightly higher after 4 h exposures and returned to control values after 24 h (Figure 20B).

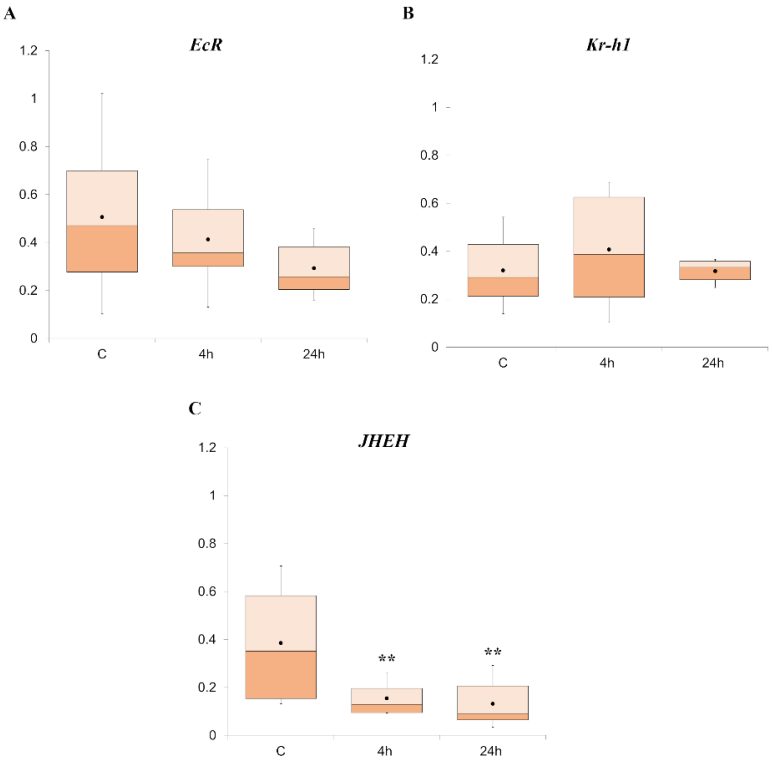


Figure 20. Transcriptional activity of *EcR*, *Kr-h1* and *JHEH* in *P. olivacea* larvae exposed 4 or 24 h to 1 µg/L BBP. Box and whisker plots represent the relative mRNA expression of *EcR* (A), *Kr-h1* (B) and *JHEH* (C) measured by RT qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control (C) values: $p \leq 0.05$ (**).

5.3.1.3. Transcriptional alterations in oxidative stress and biotransformation biomarkers

The transcriptional activity of all target genes involved in biotransformation and oxidative stress was substantially reduced under all the experimental conditions tested, except for *Cyp6a2-like* mRNA levels after 24 h exposure (Figure 21).

The strongest acute downregulations were found for *CAT* and *MnSOD* after 4 h (76 %; p = 0.001 and 60 %; p = 0.007 below control values, respectively) and the same trend was observed after 24 h exposures (45 %; p = 0.045 and 53 %, p = 0.035) (Figure 21A, B). Also, a clear time-dependent downregulation was observed for *PHGPx* (52 % and 72 %; p = 0.004) and *Cyp4g15* (42 % and 65 %; p = 0.029) (Figure 21C, D).

No changes were detected for the other two studied P450 cytochromes (*Cyp6a14-like* and *Cyp6a2-like*), the observed difference was that while in the case of *Cyp6a14-like* 24 h treatments triggered a downregulation in their levels (60 %; ns), *Cyp6a2-like* remained unchanged (Figure 21E, F).

Figure 21 (next page). Transcriptional activity of *MnSOD*, *CAT*, *PHGPx*, *Cyp4g15*, *Cyp6a14-like* and *Cyp6a2-like* in *P. olivacea* larvae exposed 4 or 24 h to 1 µg/L BBP. Box and whisker plots represent the relative mRNA expression of *MnSOD* (A), *CAT* (B), *PHGPx* (C), *Cyp4g15* (D), *Cyp6a14-like* (E) and *Cyp6a2-like* (F) measured by RT qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control (C) values: $p \leq 0.05$ (**).

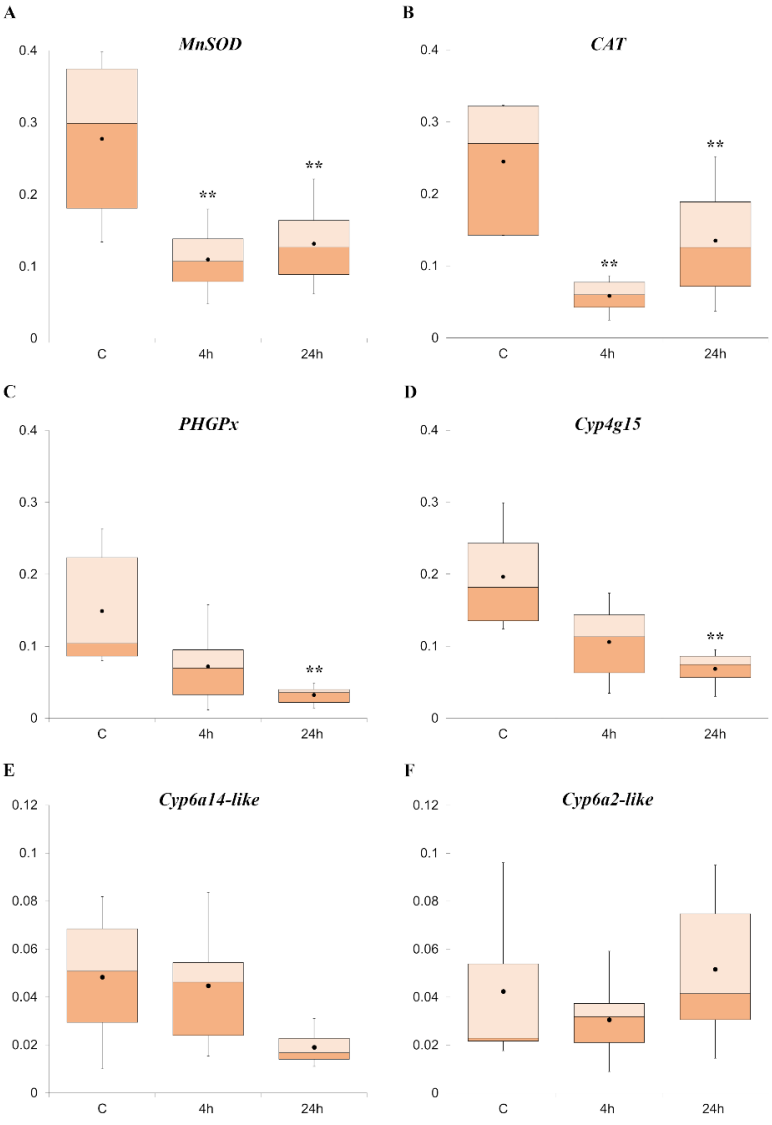

A
MnSOD
B
CAT
C
PHGPx
D
Cyp4g15
E
Cyp6a14-like
F
Cyp6a2-like
C
4h
24h
**

Figure 21

5.3.1.4. Transcriptional alterations in the immune system and the energy metabolism

As shown in Figure 22, the expression of three of the four analysed genes involved in the immune system response (*PGRP*, *Toll* and *JAK/hopscotch*) was significantly reduced after 24 h exposure to BBP with values 54 % ($p = 0.002$) and 62 % ($p = 0.008$) below control levels in the case of *Toll* and *JAK/hopscotch* respectively, and 40 % ($p = 0.0016$) lower in *PGRP* (Figure 22A, 22C, D). In contraposition to *Toll* and *JAK/hopscotch,* the xenobiotic exposure did not trigger a time-dependent downregulation in *PGRP*, whose transcript levels were significantly increased in 4 h treatments (54 %; $p = 0.063$). The expression of *C-type lectin* remained unchanged under both times of exposure (Figure 22B).

Finally, BBP had a time-dependent effect on the transcriptional activity of *GAPDH*, leading to a significant overexpression (70 %; $p = 0.028$) in 24 h exposures (Figure 22E).

Figure 22 (next page). Transcriptional activity of *PGRP*, *C-type lectin, Toll, JAK/hopscotch and GAPDH* in *P. olivacea* larvae exposed 4 or 24 h to 1 µg/L BBP. Box and whisker plots represent the relative mRNA expression of *PGRP* (A), *C-type lectin* (B), *Toll* (C), *JAK/hopscotch* (D) and *GAPDH* (E) measured by RT qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control (C) values: $p \leq 0.05$ (**), $p \leq 0.1$ (*).

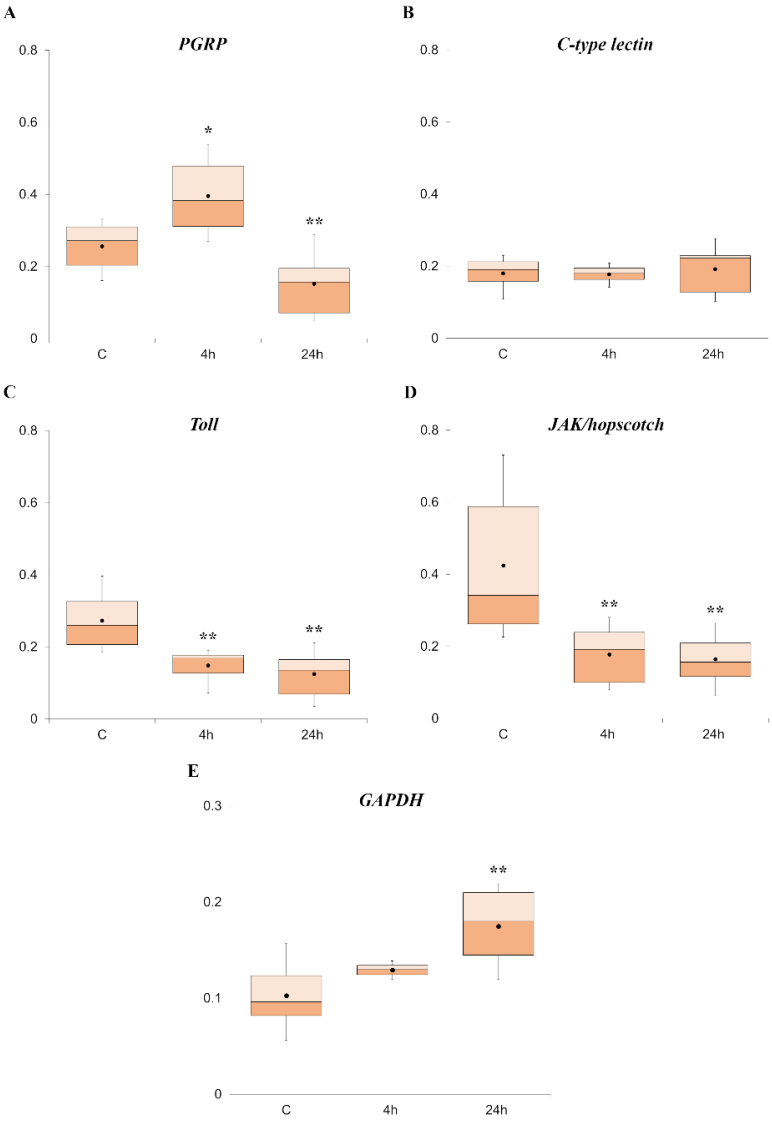

A
PGRP
0.8
0.6
0.4
0.2
0
*
**
C
4h
24h
B
C-type lectin
0.8
0.6
0.4
0.2
0
C
4h
24h
C
Toll
0.8
0.6
0.4
0.2
0
**
**
C
4h
24h
D
JAK/hopscotch
0.8
0.6
0.4
0.2
0
**
**
C
4h
24h
E
GAPDH
0.3
0.2
0.1
0
**
C
4h
24h

Figure 22

5.3.1.5. Pro-oxidant state

The pro-oxidant state of *P. olivacea* larvae exposed to BBP was assessed through enzymatic (SOD, CAT, GPx, GST and GR) and non-enzymatic (GSHt) assays to analyse the involvement of the antioxidant system modulation on the defence mechanism.

Significant inductions were observed in the activity of the measured enzymatic antioxidants after 4 h of BBP exposure, except for GR. In addition, it was detected a pattern of a peak of pro-oxidative response after 4 h exposures followed by a decrease in the antioxidant response after 24 h exposures (Figure 23A-E).

In 4 h exposures, SOD and CAT activities increased 45 % ($p = 0.004$) and 148 % ($p = 0.001$) respectively, over control values. After 24 h, these activities decreased but remained above control values, significantly for CAT (73 %; $p = 0.004$) (Figure 23A, B).

GPx activity was significantly ($p = 0.024$) higher after 4 h exposures, but temporal variations were not found in contraposition to SOD, CAT and GST activities. GPx values kept constant along the tested times, with values 60 % higher compared to the control (Figure 23C).

The most striking and statistically significant alterations were observed in GST activity, whose values compared to unexposed larvae increased by 40 % ($p = 0.002$) after 4 h and reached values below 66 % ($p = 0.001$) after 24 h of BBP exposure (Figure 23D).

Regarding the GSHt content, no differences were found at 4 h exposures, but its level was 48 % lower ($p = 0.094$) after 24 h of exposition (Figure 23F).

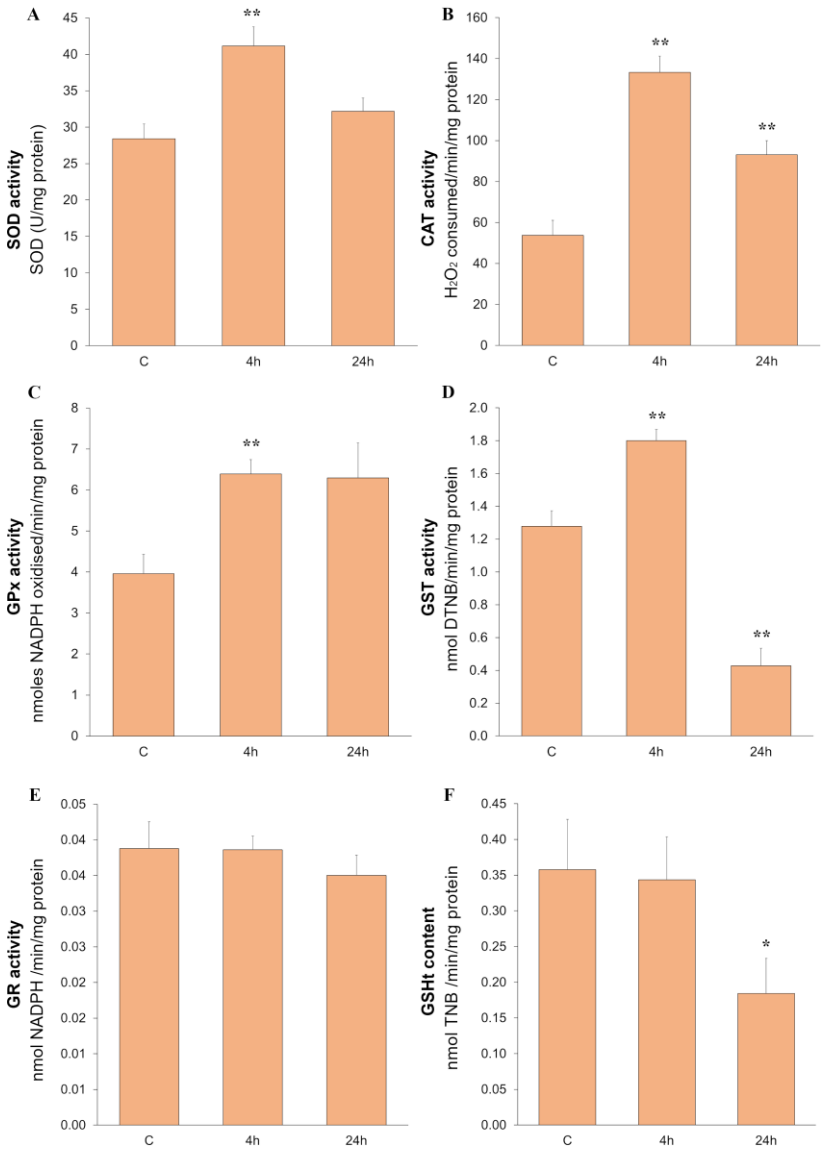

A
SOD activity
SOD (U/mg protein)
45
40
35
30
25
20
15
10
5
0
**
C
4h
24h
B
CAT activity
H2O2 consumed/min/mg protein
160
140
120
100
80
60
40
20
0
**
**
C
4h
24h
C
GPx activity
nmoles NADPH oxidised/min/mg protein
8
7
6
5
4
3
2
1
0
**
C
4h
24h
D
GST activity
nmol DTNB/min/mg protein
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
C
4h
24h
E
GR activity
nmol NADPH /min/mg protein
0.05
0.04
0.04
0.03
0.03
0.02
0.02
0.01
0.01
0.00
C
4h
24h
F
GSHt content
nmol TNB /min/mg protein
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
C
4h
24h

Figure 23

Figure 23. *P. olivacea* pro-oxidant state upon BBP exposure. Mean SOD (A), CAT (B), GPx (C), GST (D) and GR (E) activities, as well as GSHt (F) content in whole individuals of *P. olivacea* exposed 4 or 24 h to 1 µg/L BBP. Asterisks indicate significant differences with respect to control (C) values: $p \leq 0.05$ (**), $p \leq 0.1$ (*). Bars represent the standard error.

5.3.1.6. Summary of analysed effects

- BBP is a plasticiser that provoked early and severe downregulation of pathways that regulate essential insect pathways such as the cell stress response, the hormonal system, biotransformation and oxidative stress reactions or the immune system. In contrast, the energy metabolism was upregulated by xenobiotic exposure.
- *P. olivacea* larvae exposed to 1 µg/L BBP triggered strong repression and even total inhibition of transcriptional activity in genes involved in the cell stress response pathway.
- BBP induced a time-dependent downregulation of the hormonal-related genes *EcR* and *JHEH*.
- Our data show significant and strong inhibition of most of the analysed genes regarding the biotransformation and oxidative stress response. Despite in all cases, except for *Cyp6a2-like*, the transcript levels remained clearly below control values after 4 and 24 h of BBP exposure, *MnSOD* and *CAT* reported a mild recovery of their mRNA levels when compared to 4 h transcript values, opposed to the time-dependent downregulation of *PHGPx*, *Cyp4g15* and *Cyp6a14-like*.
- BBP altered the immune system by inhibiting *Toll* and *JAK/hopscotch*. *PGRP* activity was induced after 4 h of BBP exposure but repressed after 24 h.

- The energy metabolism was altered with the significant activation of *GAPDH*, after 24 h of BBP exposure.
- Regarding *P. olivacea* antioxidant system, BBP triggered higher levels of SOD, CAT or GPx activities. GST also increased after 4 h but dropped after 24 h. Finally, the xenobiotic led to a time-dependent downregulation of the total content of GSH.

5.3.1.7. *P. olivacea* and *C. riparius* comparative analysis

A comparative molecular analysis of the effects of BBP was performed between both species of aquatic dipterans to unravel whether there are any differences in its toxicity depending on the species. A total of 15 genes (*Hsp27*, *Hsp60*, *Hsp70*, *Hsc70*, *EcR*, *Kr-h1*, *MnSOD*, *CAT*, *PHGPx*, *Cyp4g15*, *PGRP*, *C-type lectin*, *Toll*, *JAK/hopscotch* and *GAPDH*) and 6 enzyme activities (SOD, CAT, GPx, GST, GR and total content of GSH) were analysed and compared.

5.3.1.7.1. Survival rates

1 µg/L BBP exposures triggered a significant ($p \leq 0.05$) increase in mortality after 24 h: 11.7 % in *P. olivacea* and 13.3 % in *C. riparius* (Table 18). No remarkable interspecific survival differences were found.

Table 18. Survival rates in *P. olivacea* and *C. riparius* 4th instar larvae exposed 4 or 24 h to 1 µg/L BBP. Results are expressed as the mean of three biological replicates ± standard deviation (n_{total} = 135 larvae/condition). Asterisks (**) indicate significant differences with respect to the corresponding control ($p \leq 0.05$).

	Species	Exposure time (h)	Control	BBP
Survival rate (mean ± sd)	*P. olivacea*	4	99.3 ± 0	96.8 ± 1.6
		24	100 ± 0	**88.3 ± 1.67****
	C. riparius	4	100 ± 0	100 ± 0
		24	100 ± 0	**86.7 ± 1.6****

5.3.1.7.2. Cell stress response

In contraposition to *P. olivacea*, transcription of *Hsp27* and *Hsp60* in *C. riparius* was not completely inhibited at any tested time, revealing a statistically significant species-dependent regulation (Figure 24A, B). Compared to control, *C. riparius* exposed to BBP showed a significant increase in *Hsp27* levels after 24 h and *Hsp60* values after 4 h.

Significant interspecific differences ($p = 0.071$) in the regulation of *Hsp70* were detected after 24 h exposures. While *C. riparius* showed a 248 % increase ($p = 0.004$) compared to control values, *P. olivacea* suffered a 44 % decrease, although not statistically significant (Figure 24C).

Although no remarkable interspecific differences were found in the regulation of the constitutive form (*Hsc70*), compared to their respective controls, *C. riparius* experienced a more severe increase (84 %) compared to *P. olivacea* (35 %) in 24 h exposures (Figure 24D).

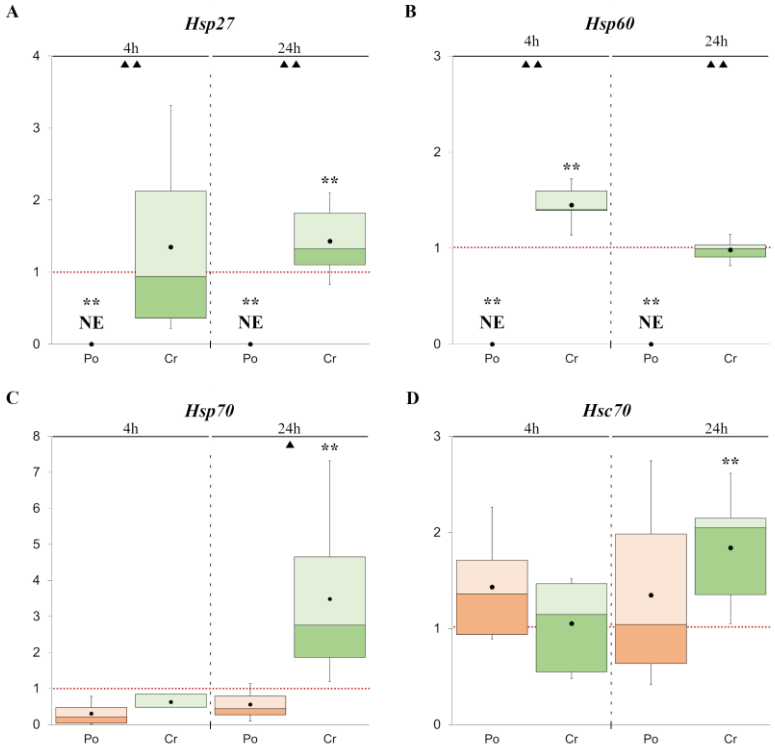


Figure 24. Transcriptional activity of *Hsp27*, *Hsp60*, *Hsp70* and *Hsc70* genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 4 or 24 h to 1 µg/L BBP. Box and whisker plots represent the relative mRNA expression of *Hsp27* (A), *Hsp60* (B), *Hsp70* (C) and *Hsc70* (D) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.05$ (**). Triangles indicate significant differences between species: $p \leq 0.05$ (▲▲), $p \leq 0.1$(▲). NE: No expression.

5.3.1.7.3. Endocrine system

BBP exposure led to species-specific deregulation of the hormonal genes analysed. For *EcR*, both species slightly repressed their transcriptional activity in 4 h exposures, an effect that became more pronounced (42 %) after 24 h in *P. olivacea* and, in contrast, turned into a marked overexpression (144 %, p = 0.026) in *C. riparius* (Figure 25A). BBP exposure did not alter the mRNA levels of the *Kr-h1* gene in *P. olivacea*, while *C. riparius* reached a 197 % (p = 0.004).

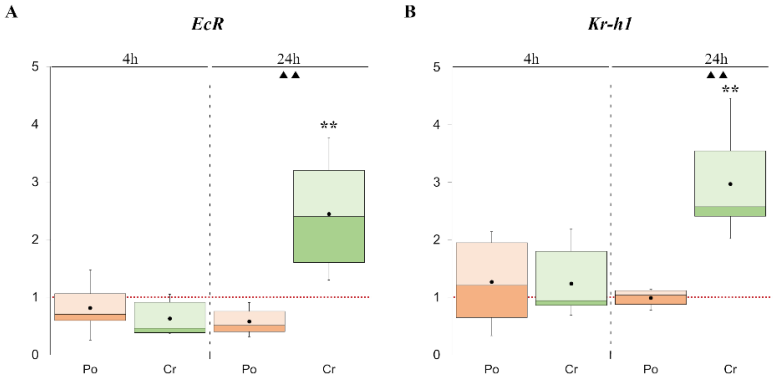


Figure 25. Transcriptional activity of *EcR* and *Kr-h1* genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 4 or 24 h to 1 µg/L BBP. Box and whisker plots represent the relative mRNA expression of *EcR* (A) and *Kr-h1*(B) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: p ≤ 0.05 (**). Triangles (▲▲) indicate significant differences (p ≤ 0.05) between species.

5.3.1.7.4. Biotransformation and oxidative stress

The biotransformation and oxidative stress pathway was differentially affected between both species of chironomids in all the scenarios tested.

Whereas *MnSOD* showed a significant downregulation in those individuals of *P. olivacea* exposed to BBP treatments during 4 and 24 h (60 %; p = 0.007 and 53 %; p = 0.035 respectively), *C. riparius* larvae were unaffected at 4 h and triggered a 68 % overexpression (p = 0.031) at 24 h (Figure 26A).

CAT showed similar results to those observed for *MnSOD*. The clear repression previously described in *P. olivacea* at 4 and 24 h of exposure to BBP (76 %; p = 0.001 and 45 %; p = 0.04) was attenuated in *C. riparius* larvae after 4 h (34 %; p = 0.021) and recovered control values after 24 h (Figure 26B).

Concerning the *PHGPx* gene, statistically significant differences were found between species for both exposure times (Figure 26C). *P. olivacea* showed a clear inhibition at both exposure times, significant after 24 h (79 % below control values; p = 0.004). However, in *C. riparius*, while 24 h led to an inhibition of the transcriptional activity of *PHGPx* (37 %; ns), an increase in mRNA levels was detected after the first 4 h (42 % above control, ns).

Finally, species-specific differential regulation of *Cyp4g15* gene was observed after BBP exposure. In contrast to the time-dependent decrease of *Cyp4g15* in *P. olivacea*, this gene's transcript levels in *C. riparius* remained stable compared to control values (Figure 26D).

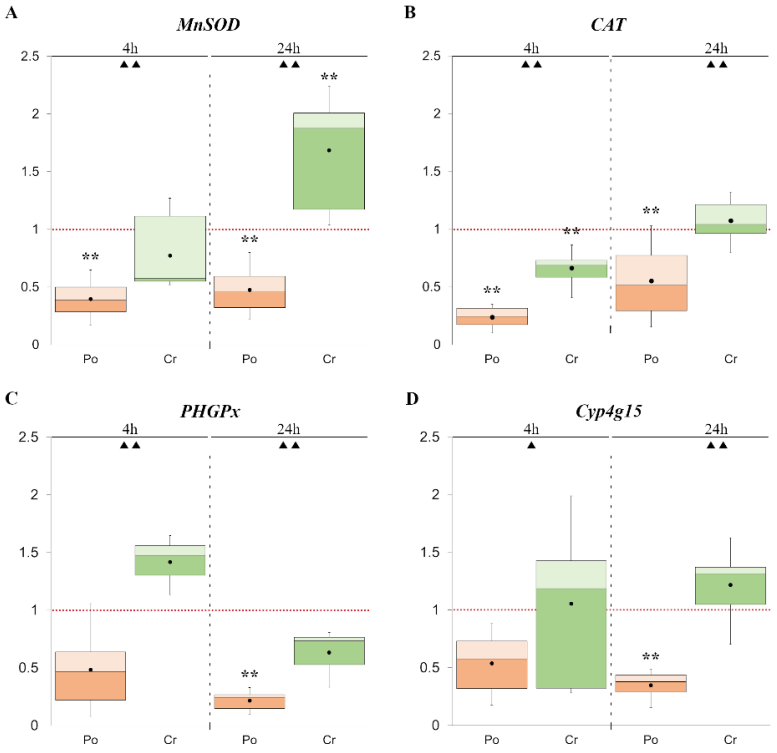


Figure 26. Transcriptional activity of *MnSOD*, *CAT*, *PHGPx* and *Cyp4g15* genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 4 or 24 h to 1 µg/L BBP. Box and whisker plots represent the relative mRNA expression of *MnSOD* (A), *CAT* (B), *PHGPx* (C) and *Cyp4g15* (D) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.05$ (**). Triangles indicate significant differences between species: $p \leq 0.05$(▲▲), $p \leq 0.1$ (▲).

5.3.1.7.5. Immune system and energy metabolism

Species-dependent transcriptional alterations were observed under BBP exposures.

C. riparius showed a marked upregulation of their *PGRP* mRNA levels at 4 h (225 %; p = 0.065) and at 24 h (160 %; p = 0.024), whereas in *P. olivacea* the increase detected at 4 h (54 % over control values; p = 0.063) turned into a marked downregulation at 24 h (40 % below control values; p = 0.016) (Figure 27A).

As described previously, transcript levels of *C-type lectin* remained unaltered in *P. olivacea*, whereas 4 h BBP treatments triggered a 79 % decrease (p = 0.016) in *C. riparius*, evidencing one more time a significant differential species-dependent regulation upon xenobiotic exposure (Figure 27B).

Toll and *JAK/hopscotch* deregulation showed differences between species. While BBP treatments led to a significant time-dependent drop in the transcriptional activity of both genes in *P. olivacea* (*Toll*: 46 % and 55 %; p = 0.002 for 4 and 24 h; *JAK/hopscotch*: 58 % and 61 %; p = 0.008, respectively), the same experimental conditions revealed no effects in *C. riparius* (Figure 27C, D). This species-dependent deregulation was significant in *Toll* after 24 h BBP exposure ($p \leq 0.1$) and in both tested times in *JAK/hopscotch* ($p \leq 0.05$).

As last, data revealed statistically significant differences in the regulation of the energy metabolism between species (Figure 27E). On the one hand, *GAPDH* transcript levels in the BBP-exposed *P. olivacea* larvae increased by 70 % (p = 0.028) after 24 h. On the other hand, BBP decreased *GAPDH* mRNA levels in *C. riparius* after 4 h (26 %; p = 0.029), while no effect was shown after 24 h.

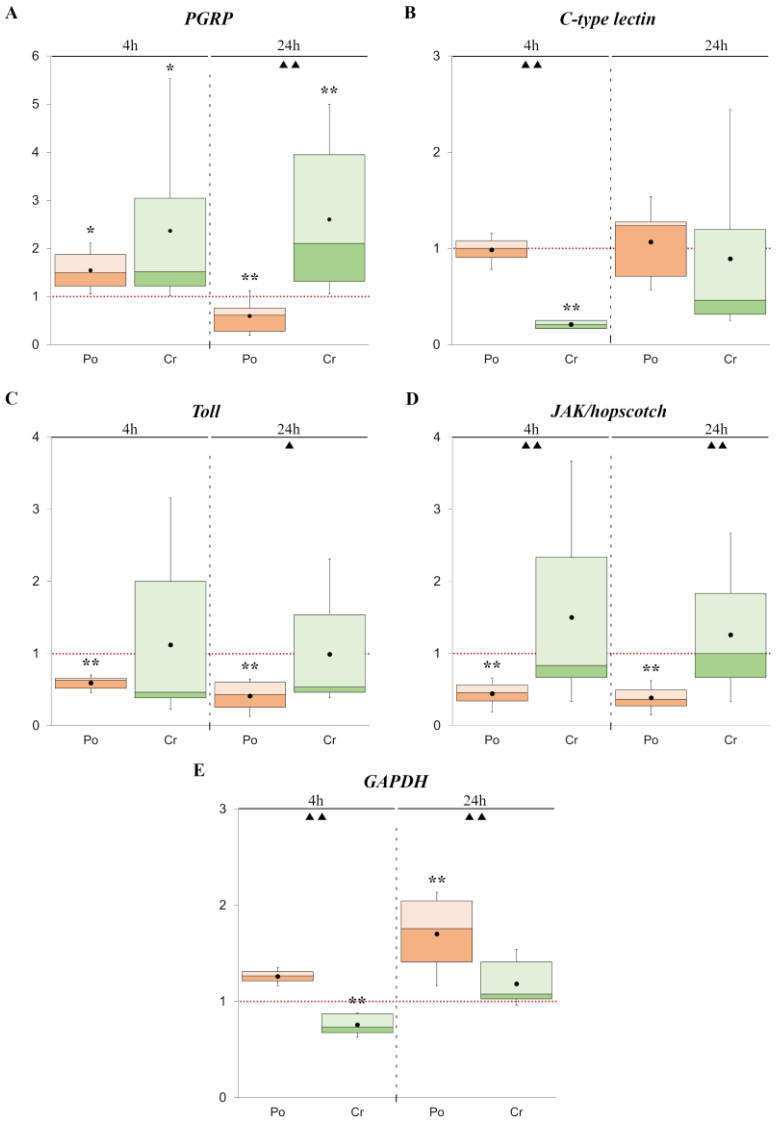

A
PGRP
4h
24h
B
C-type lectin
C
Toll
D
JAK/hopscotch
E
GAPDH
Po
Cr

Figure 27

Figure 27. Transcriptional activity of *PGRP*, *C-type lectin*, *Toll*, *JAK/hopscotch* and *GAPDH* genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 4 or 24 h to 1 µg/L BBP. Box and whisker plots represent the expression patterns of *PGRP* (A), *C-type lectin* (B), *Toll* (C), *JAK/hopscotch* (D) and *GAPDH* (E) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.05$ (**), $p \leq 0.1$ (*). Triangles indicate significant differences between species: $p \leq 0.05$ (▲▲), $p \leq 0.1$ (▲).

5.3.1.7.6. Pro-oxidant state

Concerning the antioxidant responses measured in BBP-exposed individuals, significant alterations between species were found in CAT, GPx, GST and GR activities as well as in the total GSH content (Figure 28).

SOD activity was the only enzymatic antioxidant measured that did not report significant alterations inter-species. It is worth mentioning the differences found in the activity of this enzyme between *P. olivacea* and *C. riparius* untreated larvae, revealing higher basal levels in the model species compared to the non-model (Figure 28A). Besides, 24 h treatments led to significant downregulation of SOD activity in *C. riparius* while *P. olivacea* remained close to control levels (Figure 28A).

CAT activity after the 4 h treatments was significantly altered ($p \leq 0.05$) in a species-dependent manner as shown in Figure 28B, the activity of this enzyme increased in both species, but the response in *P. olivacea* (45 %; $p = 0.002$) was stronger than in *C. riparius* (11 %; ns).

Regarding GPx activity, significant inter-species alterations were observed after 4 h ($p \leq 0.1$) and 24 h ($p \leq 0.05$) BBP exposures. The response of *P. olivacea* was faster, as after 4 h its level increased by 61 % ($p = 0.024$) while *C. riparius* showed no change (Figure 28C). Nevertheless, compared

to their corresponding control values, the maximum increase in activity was recorded in *C. riparius* (75 %; $p \leq 0.004$) after 24 h.

In terms of GST and GR activities and the total GSH content, the differences detected between species in unexposed organisms were striking, revealing that *C. riparius* control larvae had notably lower activity of the aforementioned enzymatic and non-enzymatic antioxidants (Figure 28D-F). When comparing both species, significant alterations ($p \leq 0.05$) were detected in the activity of GST and GR after 4 h BBP treatments. On the one hand, the exposure of *P. olivacea* to BBP triggered a 41 % increase ($p = 0.002$) in GST activity after 4 h followed by a 66 % decrease ($p = 0.004$) after 24 h, while the trend shown in *C. riparius* was opposite and considerably moderate (Figure 28D). On the other hand, while GR activity in *P. olivacea* remained close to control levels, it slightly decreased in *C. riparius* after 4 h BBP exposure and significantly increased ($p = 0.03$) after 24 h (Figure 28E).

Finally, differential effects between species ($p \leq 0.05$) were found regarding the total GSH content after 24 h treatments (Figure 28F): 49 % below control values ($p \leq 0.083$) for *P. olivacea* and 200 % above control values ($p \leq 0.042$) for *C. riparius*.

Figure 28 (next page). *P. olivacea* and *C. riparius* pro-oxidant state upon BBP exposure. Mean values of SOD (A), CAT (B), GPx (C), GST (D) and GR (E) enzyme activities, as well as GSHt (F) content in whole individuals of *P. olivacea* and *C. riparius* exposed 4 or 24 h to 1 µg/L BBP. Asterisks indicate significant differences with respect to respective control values: $p \leq 0.05$ (**), $p \leq 0.1$ (*). Triangles indicate significant differences between species: $p \leq 0.05$ (▲▲), $p \leq 0.1$ (▲). Bars represent the standard error.

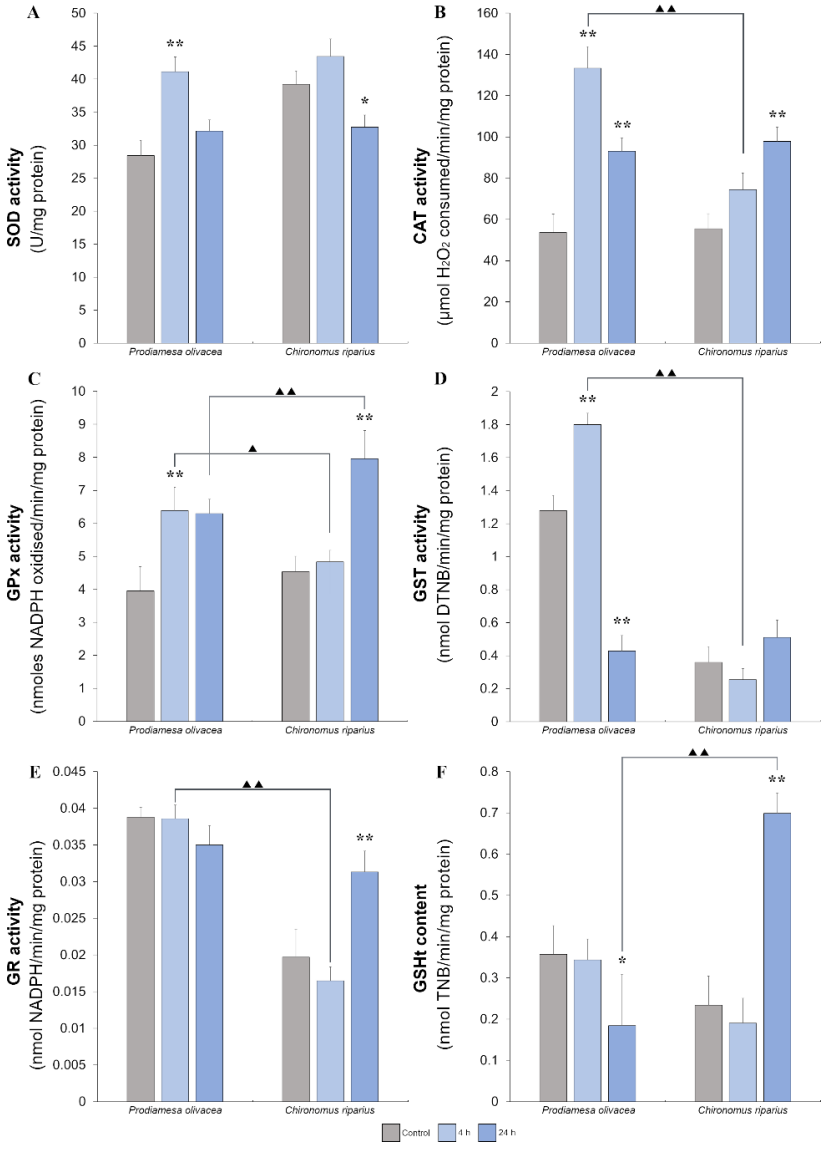

A
SOD activity
(U/mg protein)
B
CAT activity
(µmol H2O2 consumed/min/mg protein)
C
GPx activity
(nmoles NADPH oxidised/min/mg protein)
D
GST activity
(nmol DTNB/min/mg protein)
E
GR activity
(nmol NADPH/min/mg protein)
F
GSHt content
(nmol TNB/min/mg protein)
Prodiamesa olivacea
Chironomus riparius
Control
4 h
24 h

Figure 28

5.3.1.7.7. Comparative summary of the inter-specific effects of BBP

- BBP exposure reduced the survival of both species significantly after 24 h, although no inter-specific differences were found.
- In the comparative analysis of transcriptional activity between *P. olivacea* and *C. riparius*, this plasticiser differentially altered genes involved in regulatory pathways such as the cell stress response, the hormonal route, biotransformation and oxidative stress processes, the immune system and the energy metabolism.
- BBP induced significant species-dependent alterations in the cell stress response of both dipterans. It triggered a general downregulation and inhibited the transcription of *P. olivacea* cell stress genes, whereas it stimulated their transcription in *C. riparius*.
- BBP affected the hormonal pathway of both species in opposite ways in the longest time tested. On the one hand, the ecdysone receptor was time-dependently downregulated in *P. olivacea*, while 24 h exposures in *C. riparius* increased its transcription. On the other hand, BBP had no significant effect on *Kr-h1* in *P. olivacea* larvae but led to its time-dependent upregulation in *C. riparius*.
- Those genes involved in the biotransformation and oxidative stress response were acutely inhibited in *P. olivacea* after BBP exposure, whilst *C. riparius* showed less severe effects and, additionally, increased the transcription of genes such as *MnSOD*, *CAT* or *Cyp4g15* after 24 h.

- In general, BBP compromised in *P. olivacea* the transcription of genes involved in the immune system such as *Toll* or *JAK/hopscotch*, in contrast to *C. riparius*, whose mRNA levels remain unaltered or even increased for *PGRP*. Moreover, while BBP did not affect the transcription of *C-type lectin* in *P. olivacea*, it significantly downregulated the transcription of this gene in *C. riparius* after 4 h.
- *GAPDH* transcription increased time-dependently in *P. olivacea*, while in *C. riparius* it was downregulated after 4 h of BBP exposure and slightly upregulated after 24 h.
- Regarding the comparative study of the pro-oxidant status, the differences between the unexposed larvae of both species in terms of the basal activity of the analysed antioxidants are noteworthy. While SOD activity was lower in *P. olivacea* than in *C. riparius*, GST and GR activities and the total GSH content were significantly higher in the non-model organism.
- 4 h BBP exposures had more severe increase effects on the enzymatic antioxidant activities (SOD, CAT, GPx and GST) of *P. olivacea* than *C. riparius*. CAT and GPx underwent similar effects in both species at 24 h exposures, while SOD, GST and GR showed opposite trends.
- In 24 h exposures, BBP led to a drop in total GSH content in *P. olivacea* but resulted in a 200 % increase in *C. riparius*.

5.3.2. BPA

5.3.2.1. Transcriptional alterations in the cell stress response

Compared to the control samples, two of the four analysed genes coding for the cellular stress pathway reported significant alterations upon *P. olivacea* larvae BPA exposures. The most significant differences were observed in *Hsp27*, whose transcription was inhibited immediately after the two tested times (Figure 29A). Despite *Hsp60* mRNA levels remained constant after 4 h, 24 h exposure to BPA led to a 53 % increase ($p = 0.062$) (Figure 29B).

Hsp70 was induced by 205 % (ns) after 4 h of BPA exposure and dropped by 45 % (ns) after 24 h (Figure 29C). Finally, xenobiotic exposure did not affect the mRNA levels of *Hsc70*, which remained similar to those found in the untreated control group (Figure 29D).

5.3.2.2. Transcriptional alterations in the endocrine system

For the transcriptional activity study of genes involved in the endocrine system, significant alterations in the expression profile of the hormone receptor *EcR* and *JHEH* were observed. An increase of 220 % ($p = 0.073$) was detected in the mRNA levels of *EcR* after 4 h BPA treatment, followed in time by a sharp decrease reaching values below 56 % ($p = 0.073$) compared to control samples (Figure 30A). A time-dependent downregulation was observed for the *JHEH* gene; *P. olivacea* larvae treated with BPA showed a 66 % decrease ($p = 0.021$) after 24 h (Figure 30C). No differences were detected in the *Kr-h1* gene, whose mRNA levels remained close to controls (Figure 30B).

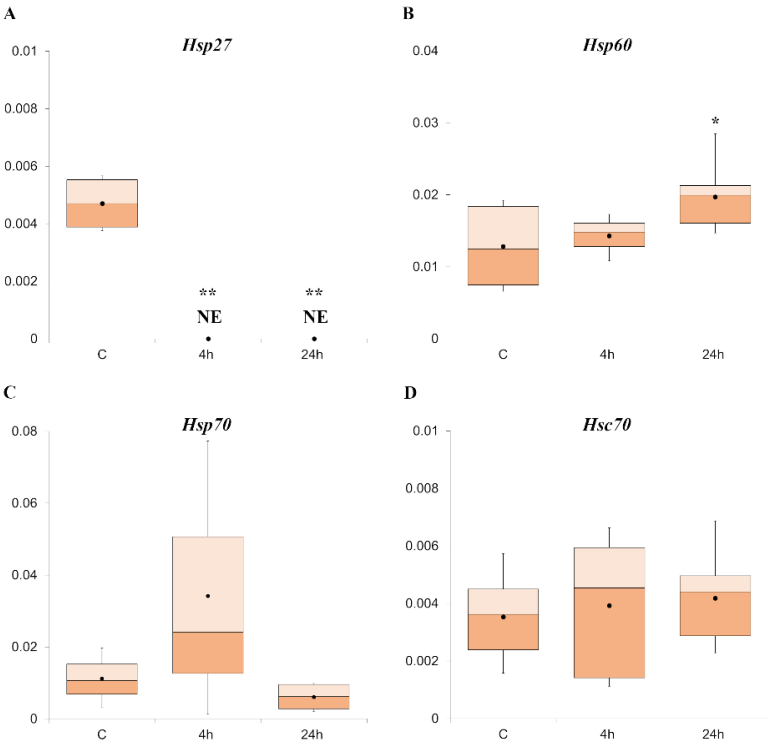


Figure 29. Transcriptional activity of *Hsp27*, *Hsp60*, *Hsp70* and *Hsc70* in *P. olivacea* larvae exposed 4 h or 24 h to 1 µg/L BPA. Box and whisker plots represent the relative mRNA expression of *Hsp27* (A), *Hsp60* (B), *Hsp70* (C) and *Hsc70* (D) measured by RT qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control (C) values: $p \leq 0.05$ (**), $p \leq 0.1$ (*). NE: No expression.

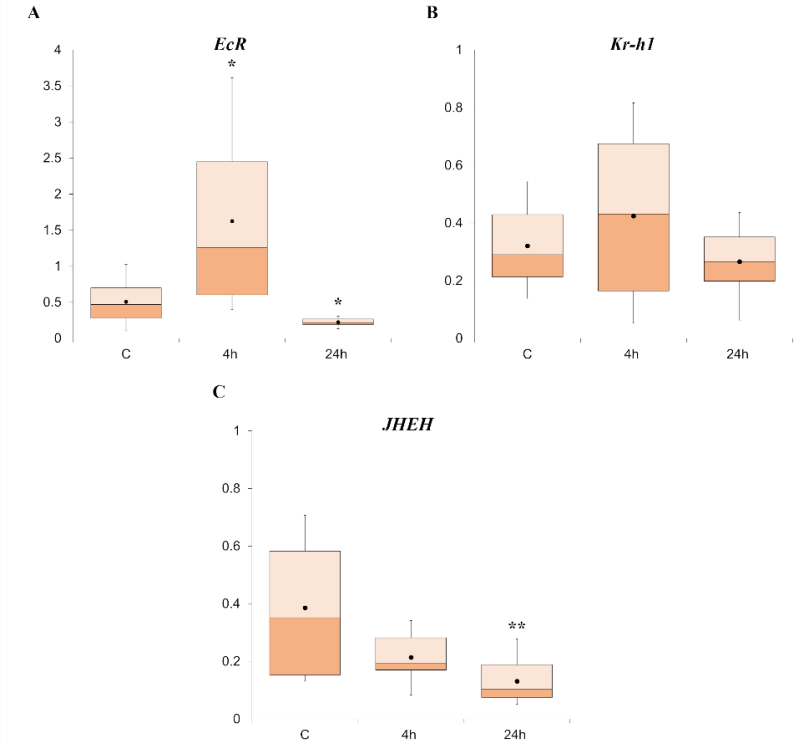


Figure 30. Transcriptional activity of *EcR*, *Kr-h1* and *JHEH* genes in *P. olivacea* larvae exposed 4 h or 24 h to 1 µg/L BPA. Box and whisker plots represent the relative mRNA expression patterns of *EcR* (A), *Kr-h1* (B) and *JHEH* (C) measured by RT qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control (C) values: $p \leq 0.05$ (**), $p \leq 0.1$ (*).

5.3.2.3. Transcriptional alterations in oxidative stress and biotransformation biomarkers

P. olivacea BPA exposure led to differential responses in four of the six biotransformation and oxidative stress analysed genes (*MnSOD*, *CAT*, *PHGPx* and *Cyp4g15*) (Figure 31).

A similar tendency in the change of transcript levels of *MnSOD*, *PHGPx* and *Cyp4g15* were observed across the tested times. 4 h BPA exposures showed a response towards significant gene downregulation: 50 % for *MnSOD* ($p = 0.073$), 53 % for *PHGPx* ($p = 0.065$) and 74 % for *Cyp4g15* ($p = 0.016$), followed by an increase of the mRNA levels after 24 h but always remaining lower than control values (Figure 31A, 31C-D). Besides, after 24 h BPA exposures *Cyp4g15* transcript level decreased below 56 % ($p = 0.01$).

BPA caused a significant time-dependent downregulation of the *CAT* gene reaching values of 37 % ($p = 0.083$) and 73 % ($p = 0.003$) lower than control (Figure 31B).

No statistically significant responses were detected for *Cyp6a14-like* and *Cyp6a2-like* genes (Figure 31E, F). While the transcript levels of *Cyp6a2-like* remained unaltered at both analysed times, 24 h treatment caused a *Cyp6a14-like* 46 % (ns) downregulation.

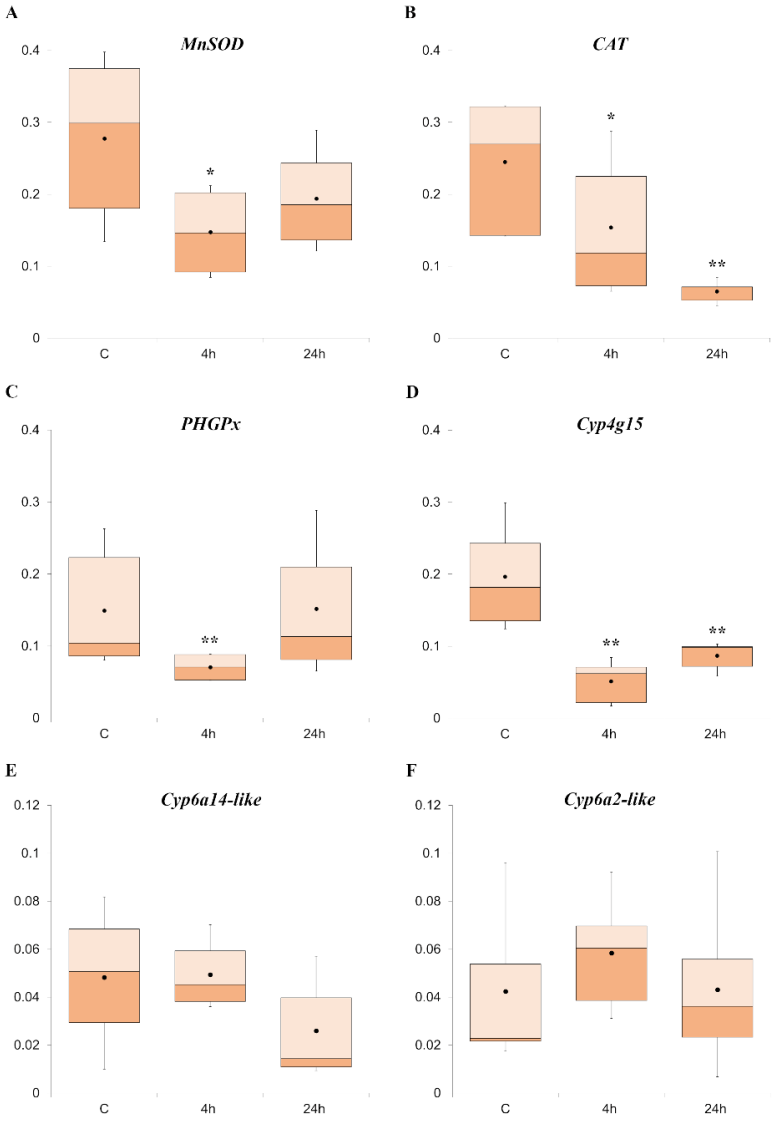

A
MnSOD
0.4
0.3
0.2
0.1
0
*
C
4h
24h
B
CAT
0.4
0.3
0.2
0.1
0
*
**
C
4h
24h
C
PHGPx
0.4
0.3
0.2
0.1
0
**
C
4h
24h
D
Cyp4g15
0.4
0.3
0.2
0.1
0
**
**
C
4h
24h
E
Cyp6a14-like
0.12
0.1
0.08
0.06
0.04
0.02
0
C
4h
24h
F
Cyp6a2-like
0.12
0.1
0.08
0.06
0.04
0.02
0
C
4h
24h

Figure 31

Figure 31. Transcriptional activity of *MnSOD*, *CAT*, *PHGPx*, *Cyp4g15*, *Cyp6a14-like* and *Cyp6a2-like* in *P. olivacea* larvae exposed 4 h or 24 h to 1 µg/L BPA. Box and whisker plots represent the relative mRNA expression of *MnSOD* (A), *CAT* (B), *PHGPx* (C), *Cyp4g15* (D), *Cyp6a14-like* (E) and *Cyp6a2-like* (F) measured by RT qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control (C) values: $p \leq 0.05$ (**), $p \leq 0.1$ (*).

5.3.2.4. Transcriptional alterations in the immune system and the energy metabolism

Exposure of *P. olivacea* individuals to BPA modulated the transcriptional expression of genes involved in the immune system response in different ways. While 4 h exposures triggered a significant overexpression of the *PGRP* gene with values above 120 % ($p = 0.014$) compared to control levels, *C-type lectin* activity clearly decreased under the same experimental conditions (Figure 32A, B). The mRNA levels of both genes returned to control values after 24 h.

Regarding *Toll* and *JAK/hopscotch*, obtained data showed similar changes in both immune system genes (Figure 32C, D). There were no significant differences after 4 h BPA exposures, but after 24 h treatments there was a significant downregulation of 74 % ($p = 0.001$) and 64 % ($p = 0.003$) below control levels, respectively.

Finally, no significant variations were detected in the activity of *GAPDH*, which remained constant along the two tested times (Figure 32E).

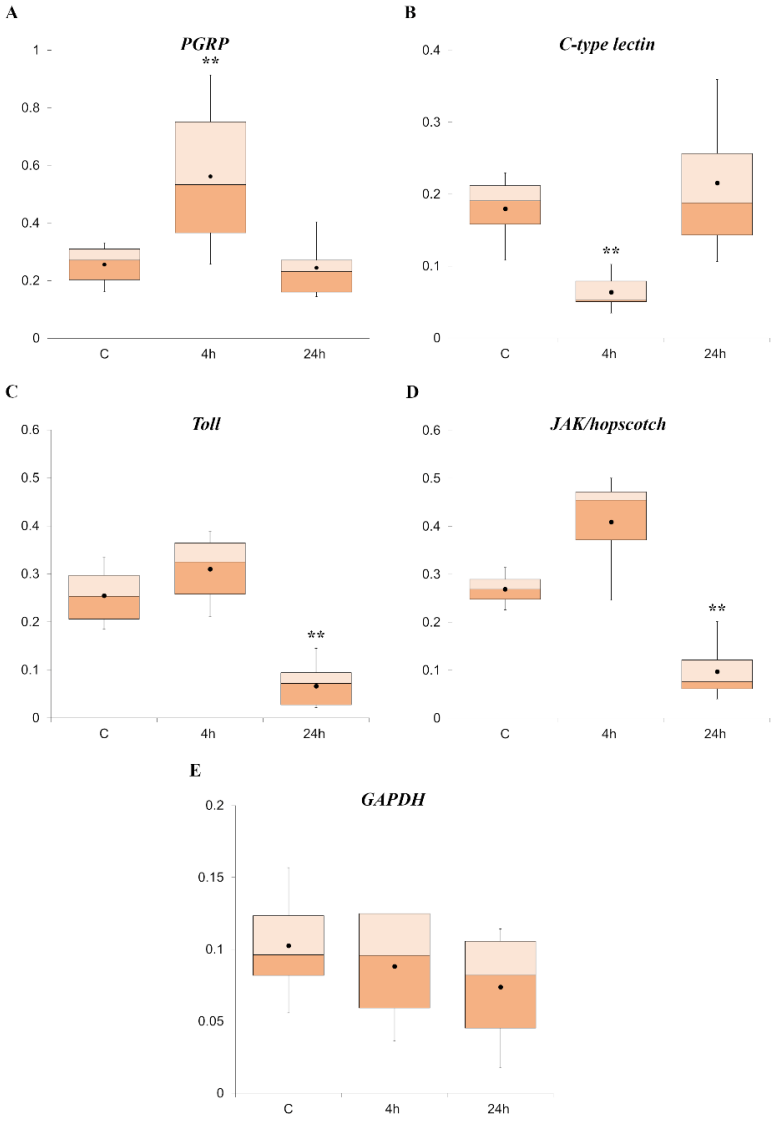

A
PGRP
**
1
0.8
0.6
0.4
0.2
0
C
4h
24h
B
C-type lectin
0.4
0.3
0.2
0.1
0
**
C
4h
24h
C
Toll
0.6
0.5
0.4
0.3
0.2
0.1
0
**
C
4h
24h
D
JAK/hopscotch
0.6
0.5
0.4
0.3
0.2
0.1
0
**
C
4h
24h
E
GAPDH
0.2
0.15
0.1
0.05
0
C
4h
24h

Figure 32

Figure 32. Transcriptional activity of *PGRP*, *C-type lectin*, *Toll*, *JAK/hopscotch* and *GAPDH* in *P. olivacea* larvae exposed 4 h or 24 h to 1 µg/L BPA. Box and whisker plots represent the relative mRNA expression of *PGRP* (A), *C-type lectin* (B), *Toll* (C), *JAK/hopscotch* (D) and *GAPDH* (E) measured by RT-qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control (C) values: $p \leq 0.05$ (**).

5.3.2.5. Pro-oxidant state

Antioxidant responses were measured in whole individuals of *P. olivacea* exposed to BPA for 4 and 24 h. On the one hand, concerning the enzymatic antioxidants analysed, SOD and GST activities were significantly upregulated after 4 h BPA exposure, while the remaining antioxidants (CAT, GPx and GR) reported lower activities compared to their corresponding controls, significantly for GR ($p = 0.067$) (Figure 33A-E). Compared to untreated larvae, 24 h BPA exposures reported higher activities for SOD (50 %; $p = 0.005$) and CAT (53 %; $p = 0.017$). A similar trend to increase was observed for GPx and GR activities, although not significant.

Finally, under the same experimental conditions, the GST activity was the only enzymatic antioxidant response analysed that was significantly repressed (59 % decrease compared to control; $p = 0.003$).

On the other hand, considering total glutathione (GSHt) content measures, no differences were found when comparing the control with 4 and 24 h BPA exposures (Figure 33F).

Figure 33 (next page). P. olivacea pro-oxidant state upon BPA exposure. Mean SOD (A), CAT (B), GPx (C), GST (D) and GR (E) activities, as well as GSHt (F) content in whole individuals of P. olivacea exposed 4 or 24 h to 1 µg/L BPA. Asterisks indicate significant differences with respect to control (C) values: $p \leq 0.05$ (**), $p \leq 0.1$ (*). Bars represent the standard error.

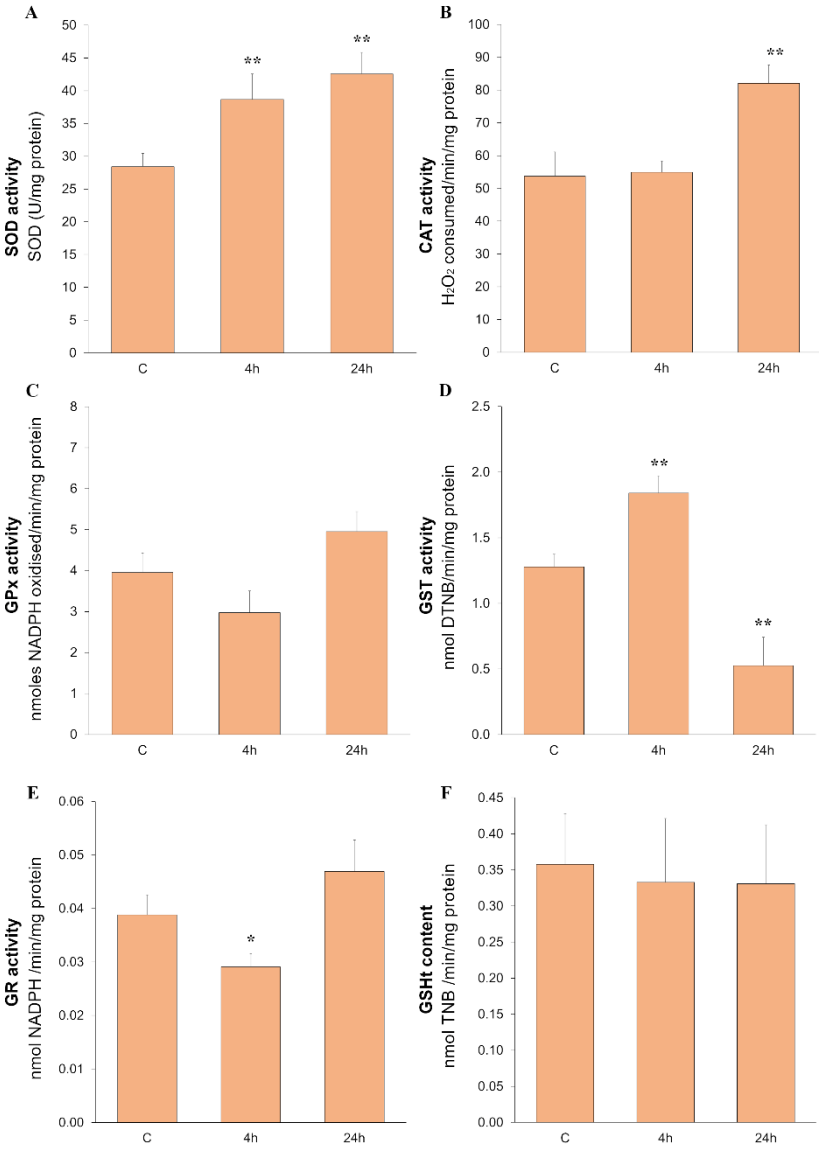

A
SOD activity
SOD (U/mg protein)
50
45
40
35
30
25
20
15
10
5
0
**
**
C
4h
24h
B
CAT activity
H2O2 consumed/min/mg protein
100
90
80
70
60
50
40
30
20
10
0
**
C
4h
24h
C
GPx activity
nmoles NADPH oxidised/min/mg protein
8
7
6
5
4
3
2
1
0
C
4h
24h
D
GST activity
nmol DTNB/min/mg protein
2.5
2.0
1.5
1.0
0.5
0.0
**
**
C
4h
24h
E
GR activity
nmol NADPH /min/mg protein
0.06
0.05
0.04
0.03
0.02
0.01
0.00
*
C
4h
24h
F
GSHt content
nmol TNB /min/mg protein
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
C
4h
24h

Figure 33

5.3.2.6. Summary of analysed effects

- BPA provoked mixed effects in genes involved in the cell stress response. While the expression of *Hsp27* was silenced, *Hsp60* was time-dependent upregulated. Moreover, whilst the transcript levels of *Hsp70* were decreased after 24 h, an early overexpression was seen in 4 h BPA exposed *P. olivacea*.

- BPA was responsible for endocrine-related effects in *P. olivacea* because, it induced the ecdysone receptor after 4 h and repressed it in 24 h exposures. Similarly to this *EcR* repression, *JHEH* was time-dependent downregulated.

- BPA led to a significant overall repression of the biotransformation and oxidative stress pathway. This repression was more severe at 4 h in *SOD*, *PHGPx* and *Cyp4g15* than at 24 h, while the transcriptional decrease was time-dependent in *CAT*.

- The endocrine disruptor had miscellaneous effects on the immune system. On the one hand, BPA activated the immune response at early times since the transcription of genes such as *PGRP*, *Toll* and *JAK/hopscotch* was increased at 4 h. On the other hand, the response was compromised at 24 h exposures considering *Toll* and *JAK/hopscotch* values drop. In contraposition, the *C-type lectin* gene was remarkably inhibited at 4 h BPA.

- BPA increased the activity of SOD and GST in contraposition to GPx, CAT and GR, whose levels decreased after 4 h BPA exposures. 24 h treatments stimulated the activity of antioxidant enzymes such as SOD, CAT, GPx and GR, while GST was repressed. BPA did not modify the total content of GSH.

5.3.2.7. *P. olivacea* and *C. riparius* comparative analysis

A comparative molecular analysis was performed between *P. olivacea* and *C. riparius* individuals exposed to BPA to elucidate possible differences in the toxicity of this xenobiotic depending on the species. As stated above in BBP study, total of 15 genes and 6 enzyme activities were analysed and compared.

5.3.2.7.1. Survival rates

Regarding the survival study, the non-model aquatic dipteran was more sensitive than the model one in 4 and 24 h BPA exposures (Table 19). After 24 h, a significant reduction in survival was noticed in *P. olivacea* exposed to 1 µg/L BPA (15 %); meanwhile, *C. riparius* larvae did not report significant mortality. Finally, 4 h exposures compromised *P. olivacea* larvae survival (3.3 %; ns) while *C. riparius* showed no mortality.

Table 19. Survival rates in *P. olivacea* and *C. riparius* 4th instar larvae exposed 4 or 24 h to 1 µg/L BPA. Results are expressed as the mean of three biological replicates ± standard deviation (n_{total} = 135 larvae/condition). Asterisks (**) indicate significant differences with respect to the corresponding control ($p \leq 0.05$).

	Species	Exposure time (h)	Control	BPA
Survival rate (mean ± sd)	*P. olivacea*	4	99.3 ± 0	96.7 ± 3.3
		24	100 ± 0	**85 ± 4.47****
	C. riparius	4	100 ± 0	100 ± 0
		24	100 ± 0	93.3 ± 4.4

5.3.2.7.2. Cell stress response

Comparative analyses of transcriptional activity revealed significant inter-species differences in three of the four analysed biomarkers involved in the cell stress response (Figure 34).

Firstly, the transcription of *Hsp27* was time-dependent upregulated in *C. riparius* individuals exposed to BPA, reaching values up to 219 % ($p = 0.05$) after 24 h compared to its corresponding control, while the same experimental conditions led to a complete loss of gene expression in *P. olivacea* (Figure 34A). This revealed significant species-dependent regulation upon BPA exposures at 4 and 24 h.

Secondly, statistical analysis detected differences between the performance of both species in regulating the transcription of *Hsp60* and *Hsc70* after 24 h exposures. While *Hsp60* mRNA levels of both species were time-dependent upregulated, the overexpression was stronger in *C. riparius* than in *P. olivacea* even more after 24 h treatment (Figure 34B). The most striking difference was found in *Hsc70*, whose transcript levels in *C. riparius* were boosted significantly, reaching values above 536 % ($p = 0.001$) control levels, while the same gene remained unaltered in *P. olivacea* regardless of xenobiotic exposure (Figure 34D).

By last, although significant inter-species differences were not reported, at 4 h exposure *Hsp70 C. riparius* gene experienced a 1490 % ($p = 0.019$) expression increase compared to its corresponding control in contraposition to *P. olivacea* that reported a mild 205 % (ns) increase (Figure 34C).

5.3.2.7.3. Endocrine system

BPA exposures led to species differential responses in the transcription of the analysed biomarkers involved in ecdysone response pathways: the hormonal receptor, *EcR*, and the late response gene, *Kr-h1* (Figure 35).

While no changes were detected in the *EcR* transcript levels of *C. riparius* after 4 h treatments, the *P. olivacea* receptor experienced a strong and quick overexpression reporting values of 220 % ($p = 0.073$) over its corresponding control (Figure 35A). In contraposition, mRNA values dropped below control levels after 24 h BPA exposure in *P. olivacea*, whereas in *C. riparius* the gene was 259 % increased ($p = 0.01$).

Kr-h1 expression remained close to control levels in the non-model aquatic dipteran, whereas in *C. riparius* the gene was significantly time-dependent upregulated (81 %; $p = 0.004$ and 280 %; $p = 0.001$) (Figure 35B).

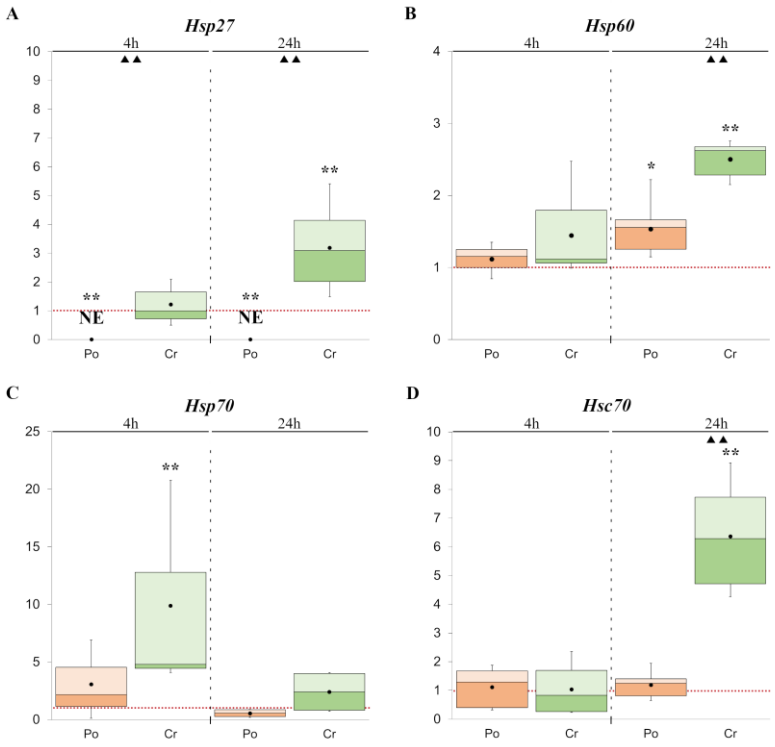


Figure 34. Transcriptional activity of *Hsp27*, *Hsp60*, *Hsp70* and *Hsc70* genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 4 or 24 h to 1 µg/L BPA. Box and whisker plots represent the expression patterns of *Hsp27* (A), *Hsp60* (B), *Hsp70* (C) and *Hsc70* (D) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.05$ (**), $p \leq 0.1$ (*). Triangles (▲▲) indicate significant differences ($p \leq 0.05$) between species. NE: No expression.

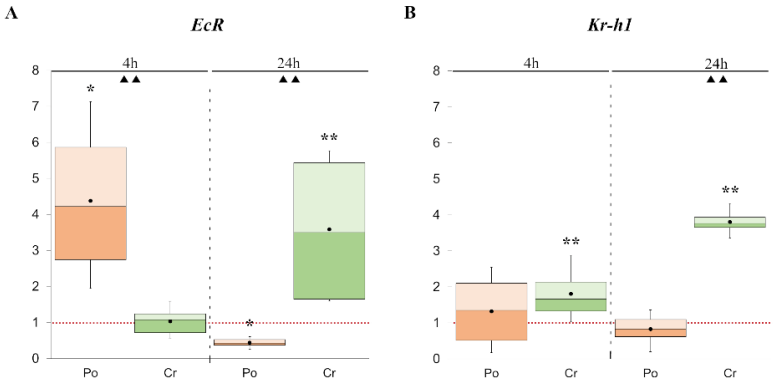


Figure 35. Transcriptional activity of *EcR* and *Kr-h1* genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 4 or 24 h to 1 µg/L BPA. Box and whisker plots represent the expression patterns of *EcR* (A) and *Kr-h1*(B) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.05$ (**), $p \leq 0.1$ (*). Triangles (▲▲) indicate significant differences ($p \leq 0.05$) between species.

5.3.2.7.4. Biotransformation and oxidative stress

Regarding the biotransformation and oxidative stress pathway, the comparative analysis between the two species upon exposure to BPA reported statistically significant differences for *MnSOD*, *CAT* and *Cyp4g15* (Figure 36).

While *P. olivacea* suffered the downregulation of *MnSOD* at both tested times (46 %; p = 0.073 and 30 %, ns), *C. riparius* larvae were unaffected at 4 h and triggered overexpression at 24 h (143 %; p = 0.01) (Figure 36A).

CAT showed similar results to those observed for *MnSOD*. The tendency to a significant downregulation already described in *P. olivacea* after 4 h (45 %; p = 0.083) and 24 h (38 %; p = 0.003) of BPA exposure, was maintained in *C. riparius* at the shortest time of treatment. Furthermore, the overexpression detected in *C. riparius* (37 %; p = 0.083) in the 24 h experiments, contrasted with obtained data in *P. olivacea* (Figure 36B).

Differential effects between species were also found in the regulation of *Cyp4g15*. A general *Cyp4g15* downregulation was found in those *P. olivacea* individuals exposed 4 and 24 h to the plasticiser (74 %; p = 0.014 and 56 %; p = 0.011 respectively). While a downregulation in a similar magnitude was reported for *C. riparius* after 4 h exposure, compared to controls, the mRNA transcript levels were 71 % upregulated after 24 h BPA exposures (Figure 36D). These data further show species differences in the transcriptional regulation of specific genes involved in insect relevant pathways upon xenobiotic exposure.

Finally, no differences were detected between the two species in the regulation of *PHGPx* (Figure 36C).

Figure 36 (next page). Transcriptional activity of *MnSOD*, *CAT*, *PHGPx* and genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 4 or 24 h to 1 µg/L BPA. Box and whisker plots represent the expression patterns of *MnSOD* (A), *CAT* (B), *PHGPx* (C) and *Cyp4g15* (D) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: p ≤ 0.05 (**). Triangles indicate significant differences between species: p ≤ 0.05 (▲▲).

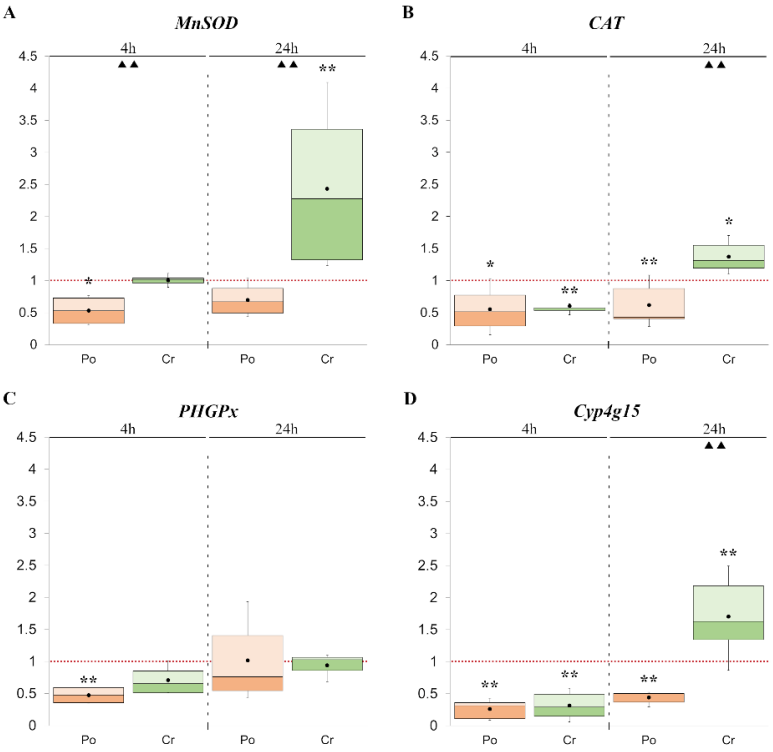


Figure 36

5.3.2.7.5. Immune system and energy metabolism

Species-dependent responses upon BPA exposure were found in the transcriptional regulation of *PGRP*, *Toll* and *JAK/hopscotch*, genes involved in the immune system pathway.

While increased expression levels of *PGRP* were observed in *P. olivacea* larvae after 4 h treatments (120 %; p = 0.014), mRNA levels remained unaltered *in C. riparius*. Meanwhile, expression levels returned to control

values in *P. olivacea* after 24 h, while *C. riparius* showed significant overexpression of 359 % ($p = 0.029$) (Figure 37A).

Statistically significant ($p \leq 0.05$) species-dependent differential effects were found in *Toll* at both exposure times (Figure 37C). Although not significant, while 4 h BPA exposure led to a 21 % gene overexpression in *P. olivacea*, *C. riparius* gene was 45 % inhibited. Moreover, 24 h exposures provoked a significant 74 % downregulation in the gene levels of *P. olivacea* ($p = 0.001$) in contraposition to *C. riparius*, whose *Toll* transcription was 70 % upregulated.

Significant differences were found between the two dipterans in the regulation of the *JAK/hopscotch* gene transcription after exposure to BPA for 4 h (Figure 37D). The increase in transcript levels in *P. olivacea* after 4 h exposures corresponded with the downregulation observed in *C. riparius* at the same time, while opposite effects were observed after 24 h experiments (64 % decrease; $p = 0.003$ for *P. olivacea* and 86% increase; ns for *C. riparius*).

C-type lectin was the only analysed gene involved in the immune system that did not report significant alterations between both insect species (Figure 37B).

Lastly, 24 h exposures revealed inter-specific differences in the regulation of the *GAPDH* gene, which is related to energy metabolism (Figure 37E). While mRNA levels were reduced by 21 % in *P. olivacea* (ns), *C. riparius* reported an 83 % overexpression ($p = 0.088$).

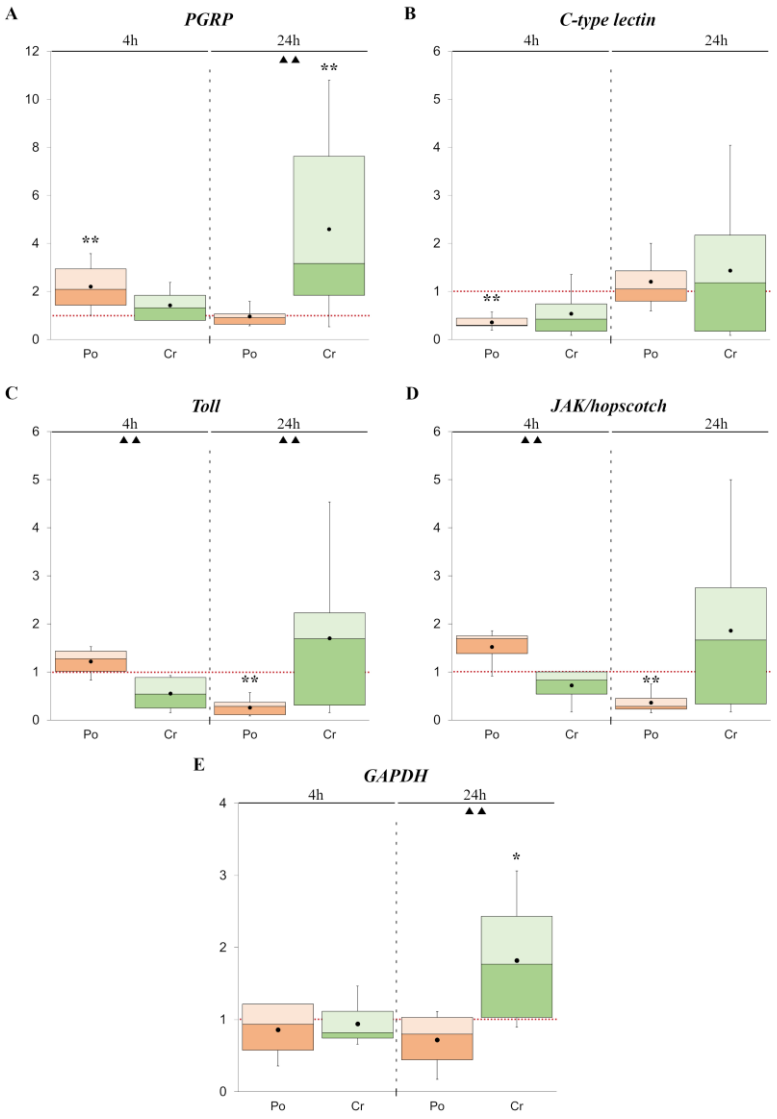

A
PGRP
4h
24h
12
10
8
6
4
2
0
**
▲▲
**
Po
Cr
Po
Cr
B
C-type lectin
4h
24h
6
5
4
3
2
1
0
**
Po
Cr
Po
Cr
C
Toll
4h
24h
▲▲
▲▲
6
5
4
3
2
1
0
**
Po
Cr
Po
Cr
D
JAK/hopscotch
4h
24h
▲▲
6
5
4
3
2
1
0
**
Po
Cr
Po
Cr
E
GAPDH
4h
24h
▲▲
4
3
2
1
0
*
Po
Cr
Po
Cr

Figure 37

Figure 37. Transcriptional activity of *PGRP*, *C-type lectin*, *Toll, JAK/hopscotch* and *GAPDH* genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 4 or 24 h to 1 µg/L BPA. Box and whisker plots represent the expression patterns of *PGRP* (A), *C-type lectin* (B), *Toll* (C), *JAK/hopscotch* (D) and *GAPDH* (E) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.05$ (**), $p \leq 0.1$ (*). Triangles indicate significant differences between species: $p \leq 0.05$ (▲▲).

5.3.2.7.6. Pro-oxidant state

Respecting the comparative evaluation of the antioxidant system of both species upon BPA exposure, significant alterations were found in SOD, CAT, GPx, GST and GR activity (Figure 38A-E).

SOD activity increased time-dependently in *P. olivacea* larvae, while a significant downregulation in activity was detected in *C. riparius* after 24 h (Figure 38A).

Regarding CAT activity, *C. riparius* experienced a 78 % ($p = 0.011$) decrease in contrast to *P. olivacea* whose enzyme levels remained unaltered after 4 h BPA exposure, (Figure 38B). Additionally, the activity of this enzyme returned to control levels after 24 h in exposure in *C. riparius*, whilst a 53 % increase ($p = 0.017$) was reported in *P. olivacea* under the same experimental conditions.

GPx and GST activities were species-dependently regulated ($p \leq 0.05$) at 4 h of BPA exposure. A 25 % (ns) decrease in GPx activity observed in *P. olivacea* larvae in contrast to the significant 76 % ($p = 0.026$) increase detected in *C. riparius* (Figure 38C). Also, after 4 h, a 44 % ($p = 0.006$) increase in GST activity was observed in *P. olivacea* compared to a 68 % ($p = 0.044$) decrease reported in *C. riparius* (Figure 38D). After 24 h, the

activity of this enzyme returned to control values in *C. riparius*, while the results showed a 59 % ($p = 0.003$) decrease in *P. olivacea*.

GR activity also revealed significant ($p \leq 0.05$) differences between species upon 4 and 24 h BPA treatments (Figure 38E). On the one hand, activity of this enzyme in *P. olivacea* was repressed after the shortest exposure time, while it remained unaltered in *C. riparius*. On the other hand, longer exposures triggered GR induction in both species, higher in *C. riparius* (39 %; $p = 0.028$) compared to *P. olivacea* (21 %, ns).

Finally, total GSH content did not show significant inter- or intra-specific differences. It is worth mentioning its 67 % (ns) increase in *C. riparius* after 24 h of exposure to BPA.

Figure 38 (next page). *P. olivacea* and *C. riparius* pro-oxidant state upon BPA exposure. Mean values of SOD (A), CAT (B), GPx (C), GST (D) and GR (E) enzyme activities, as well as GSHt (F) content in whole individuals of *P. olivacea* and *C. riparius* exposed 4 or 24 h to 1 µg/L BPA. Asterisks indicate significant differences with respect to respective control values: $p \leq 0.05$ (**), $p \leq 0.1$ (*). Triangles indicate significant differences between species: $p \leq 0.05$ (▲▲). Bars represent the standard error.

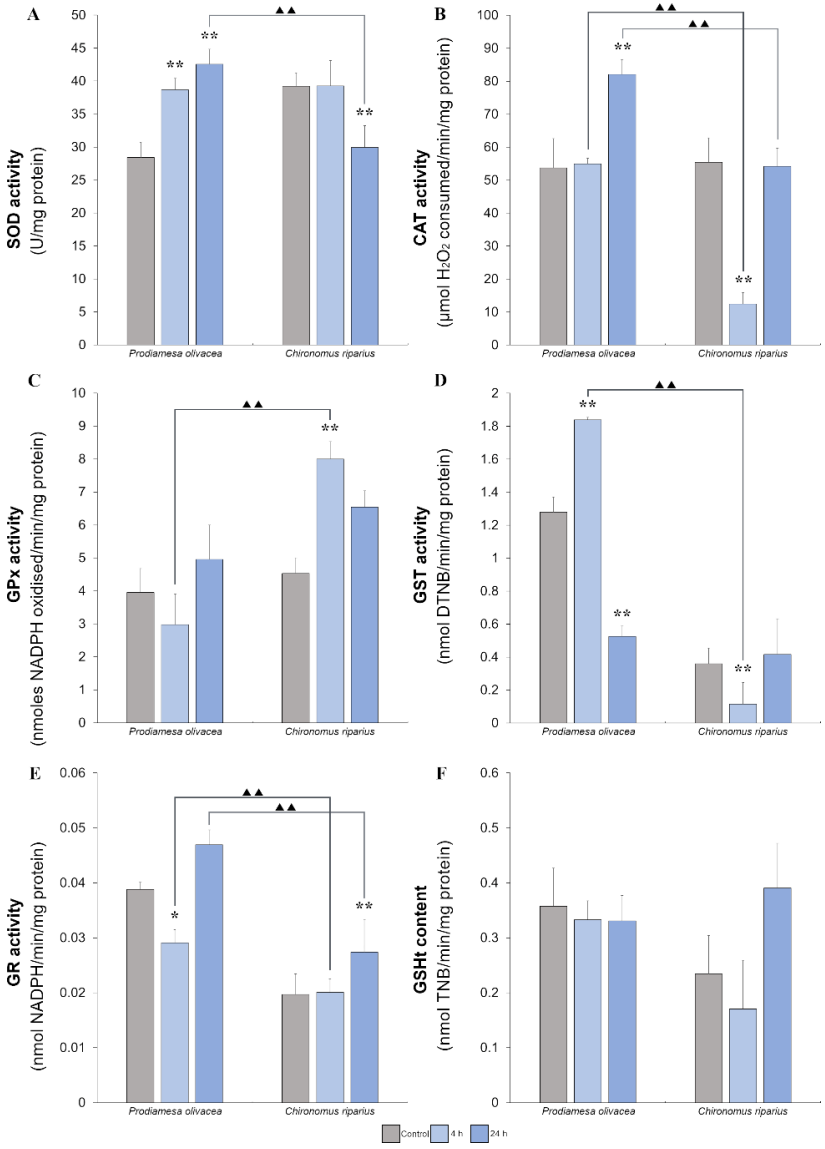

A
SOD activity
(U/mg protein)
B
CAT activity
(µmol H_2O_2 consumed/min/mg protein)
C
GPx activity
(nmoles NADPH oxidised/min/mg protein)
D
GST activity
(nmol DTNB/min/mg protein)
E
GR activity
(nmol NADPH/min/mg protein)
F
GSHt content
(nmol TNB/min/mg protein)
Prodiamesa olivacea
Chironomus riparius
Control
4 h
24 h

Figure 38

5.3.2.7.7. Comparative summary of the inter-specific effects of BPA

- Although BPA did not have a differential effect on the survival of both species in all tested scenarios, it significantly reduced the viability of *P. olivacea* larvae after 24 h.
- *Hsp27*, *Hsp60* and *Hsc70*, were time-dependently activated in *C. riparius* larvae exposed to BPA, in contrast to *P. olivacea*, whose transcript levels were completely lost (*Hsp27*), slightly increased (*Hsp60*) or unaltered (*Hsc70*). Moreover, although without significant differences between species, BPA triggered a stronger induction of *Hsp70* in *C. riparius* than in *P. olivacea* after 4 h.
- BPA caused an early upregulation of the ecdysone receptor in *P. olivacea*, while this transcriptional upregulation occurred after 24 h of exposure in *C. riparius*. *Kr-h1*, a late-response hormone gene, was upregulated in both species after 4 h, whereas after 24 h mRNA levels recovered control values in *P. olivacea* and *C. riparius* experienced a strong upregulation.
- BPA produced a strong inhibition of the biotransformation and oxidative stress pathway in *P. olivacea* under both tested times, in contrast to the general upregulation reported in *C. riparius* after 24 h.
- BPA had different effects on the modulation of the immune system of both species. While, in *P. olivacea* it enhanced *Toll* and *JAK/hopscotch* transcription after 4 h, and inhibited it after 24 h, in *C. riparius*, it had the opposite effect. mRNA levels of *C-type lectin* underwent similar changes in both aquatic dipterans. Finally, *PGRP* was strongly overexpressed after 24 h in *C. riparius* while remaining unaltered in *P. olivacea.*

- In contraposition to *C. riparius*, energy metabolism in *P. olivacea* tended to be downregulated in 24 h exposures.
- Analysis of the pro-oxidant status of both insects revealed significant differences between them in all enzymes tested. BPA produced stronger alterations in *P. olivacea*.
- BPA produced opposite modifications and in a different magnitude of CAT and GR enzyme activities after 4 and 24 h. While CAT levels remained unaltered at 4 h in *P. olivacea*, *C. riparius* experienced an increase in contrast to the decrease reported at 24 h in *P. olivacea* and the returned to control values of *C. riparius*. At 4 h, GPx and GST activities were species-dependently modified. While GPx was increased in *P.* olivacea, *C. riparius* reported a decrease in their GPx levels. In addition, *P. olivacea* GST levels were decrease in contrast to the *C. riparius* measured increase. Moreover at 24 h exposures, SOD activity was reversely altered by BPA, while it produced an increase in *P. olivacea*, it led to a decrease in *C. riparius.*
- The plasticiser did not significantly affect the total GSH content under any of the experimental condition tested.

5.3.3. BP3

5.3.3.1. Transcriptional alterations in the cell stress response

The analysis of transcriptional alterations in the cell stress response of *P. olivacea* larvae exposed to BP3 for 4 and 24 h revealed significant effects in almost all tested scenarios (Figure 39).

On the one hand, the expression of *Hsp27* and *Hsp70* was strongly downregulated (*Hsp27*: 93 % at 24 h; p = 0.034; *Hsp70*: 97 % at 4 h and 99 % at 24 h; p = 0.1) or even lost, as seen for *Hsp27* after 4 h BPA exposure (Figure 39A, 39C). In practice, these high repressions imply a gene turning off with the consequent presumed loss of protein production.

On the other hand, the exposure to the xenobiotic triggered in the individuals of *P. olivacea* a significant overexpression of *Hsp60* transcripts (205 %; p = 0.014 and 177 %; p = 0.003 at 4 and 24 h respectively) (Figure 39B). This increase was also observed in the cognate form *Hsc70* in 24 h exposures, while mRNA levels remained close to control values at 4 h (Figure 39D).

5.3.3.2. Transcriptional alterations in the endocrine system

The expression levels of *EcR* and *JHEH*, were time-dependently downregulated after BP3 exposures compared to non-exposed individuals (Figure 40A, 40C). This decrease was statistically significant (80 %; p = 0.003) for *JHEH* after 24 h treatments.

The reported variation in the transcript levels of *Kr-h1*, with a 45 % (p = 0.082) increase, was opposite to that of *EcR* and *JHEH* (Figure 40B). In contraposition, longer exposures repressed the transcription of this gene to values 42 % below control levels.

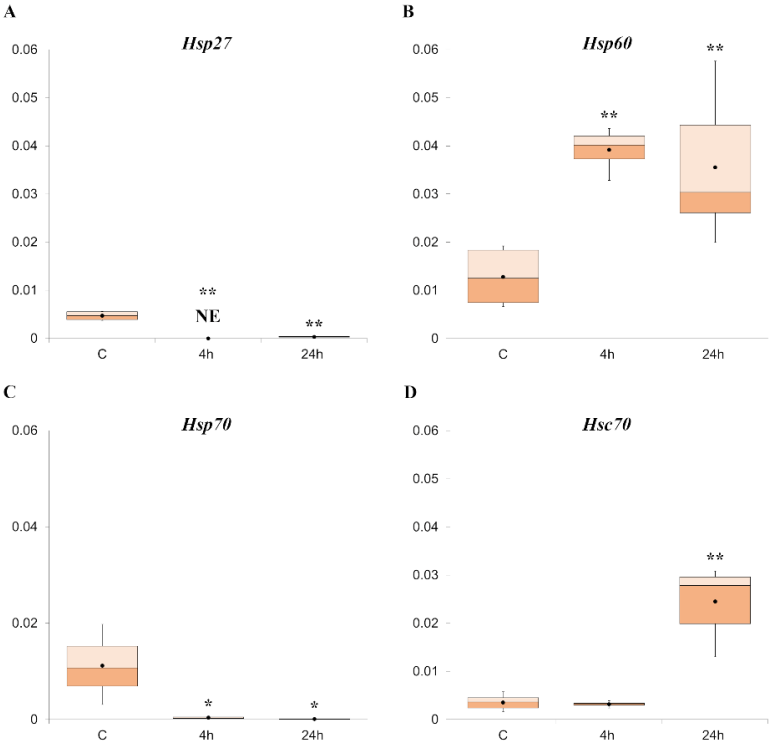


Figure 39. Transcriptional activity of *Hsp27*, *Hsp60*, *Hsp70* and *Hsc70* in *P. olivacea* larvae exposed 4 or 24 h to 1 µg/L BP3. Box and whisker plots represent the expression patterns of *Hsp27* (A), *Hsp60* (B), *Hsp70* (C) and *Hsc70* (D) measured by RT qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control (C) values: $p \leq 0.05$ (**), $p \leq 0.1$ (*). NE: No expression.

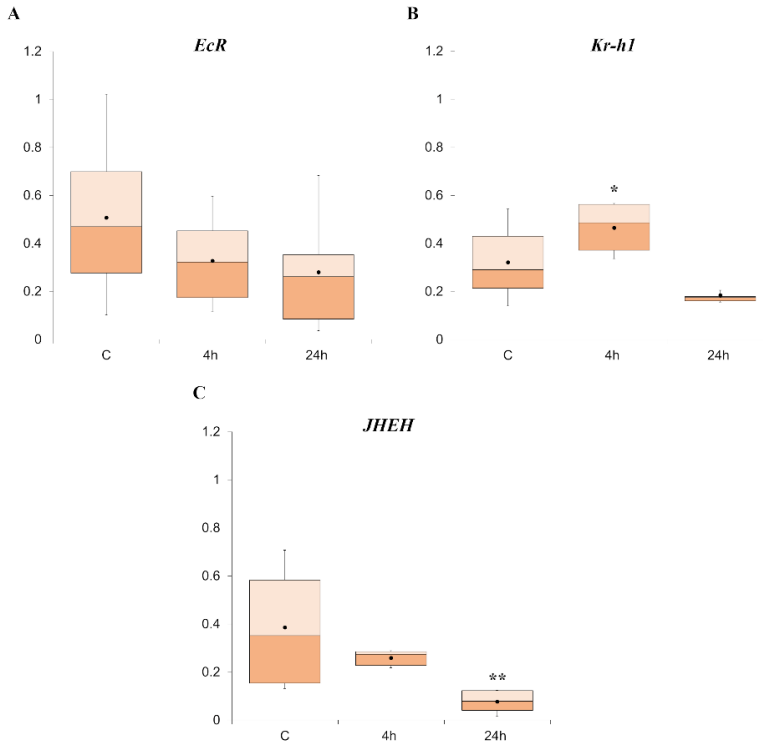


Figure 40. Transcriptional activity of *EcR*, *Kr-h1* and *JHEH* in *P. olivacea* larvae exposed 4 or 24 h to 1 µg/L BP3. Box and whisker plots represent the expression patterns of *EcR* (A), *Kr-h1* (B) and *JHEH* (C) measured by RT qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.05$ (**), $p \leq 0.1$ (*).

5.3.3.3. Transcriptional alterations in oxidative stress and biotransformation biomarkers

BP3 larval exposure did not have too many repercussions in the regulating genes involved in biotransformation and oxidative stress in *P. olivacea* (Figure 41).

Significant differences were found in *CAT* and *Cyp4g15*. *CAT* was 38 % ($p = 0.083$) downregulated in 4 h exposures, but the most striking deregulation was found in 24 h exposures, where a 232 % overexpression ($p = 0.034$) was induced (Figure 41B). *Cyp4g15* showed similar responses to those observed for *CAT,* significant only after 24 h BP3 (53 % increase; $p = 0.05$) (Figure 41D).

No significant effects for the other four studied genes could be detected: *MnSOD*, *PHGPx*, *Cy6a14-like* and *Cyp6a2-like*. The activity of *MnSOD* remained slightly below control levels while *PHGPx* was unaltered at 4 h exposure and increased at 24 h (Figure 41A, 41C). Regarding the other two cytochromes, *Cyp6a14-like* 4 h exposure showed values close to controls, whilst an 80 % (ns) downregulation was reported for 24 h treatments (Figure 41E). At last, a 38 % (ns) decrease in the *Cyp6a2-like* mRNA levels was noticed for the shortest exposure, followed by a 232 % (ns) overexpression at BP3 4h (Figure 41F).

Figure 41 (next page). Transcriptional activity of *MnSOD*, *CAT*, *PHGPx*, *Cyp4g15*, *Cyp6a14-like* and *Cyp6a2-like* in *P. olivacea* larvae exposed 4 or 24 h to 1 µg/L BP3. Box and whisker plots represent the expression patterns of *MnSOD* (A), *CAT* (B), *PHGPx* (C), *Cyp4g15* (D), *Cyp6a14-like* (E) and *Cyp6a2-like* (F) measured by RT qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.05$ (**), $p \leq 0.1$ (*).

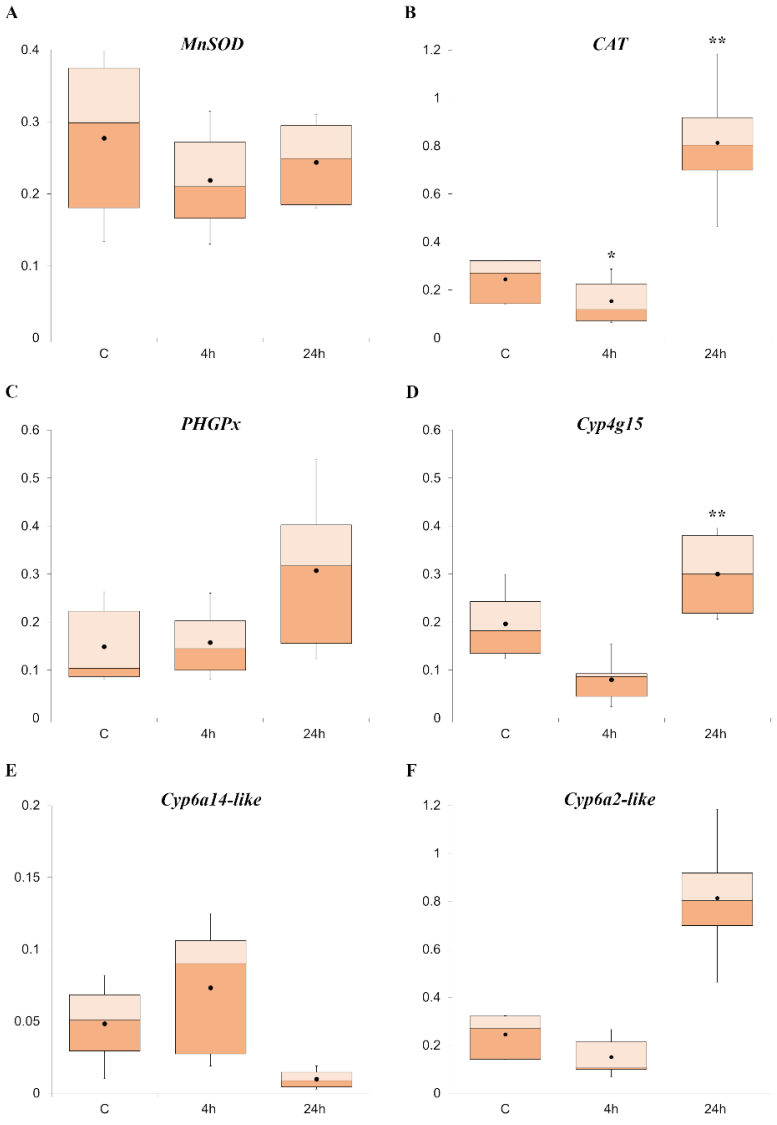

A
MnSOD
0.4
0.3
0.2
0.1
0
C
4h
24h
B
CAT
1.2
1
0.8
0.6
0.4
0.2
0
**
*
C
4h
24h
C
PHGPx
0.6
0.5
0.4
0.3
0.2
0.1
0
C
4h
24h
D
Cyp4g15
0.6
0.5
0.4
0.3
0.2
0.1
0
**
C
4h
24h
E
Cyp6a14-like
0.2
0.15
0.1
0.05
0
C
4h
24h
F
Cyp6a2-like
1.2
1
0.8
0.6
0.4
0.2
0
C
4h
24h

Figure 41

5.3.3.4. Transcriptional alterations in the immune system and the energy metabolism

BP3 treatments significantly compromised the transcription of half of analysed genes involved in the immune system (Figure 42A-D). Moreover, significant differences were found in the energy metabolism upon the UV filter exposure (Figure 42E).

The expression of *PGRP* and *C-type lectin* remained unaltered and close to control levels regardless of BP3 exposure (Figure 42A, B). However, the gene transcription of *Toll* and *JAK-hopscotch* was statistically significant time-dependent downregulated, reaching up to 94 % and 93 % decrease ($p = 0.011$, both) respectively after 24 h (Figure 42C, D).

Finally, mRNA level of the *GAPDH* gene, related to energy metabolism, was significantly decreased compared to controls (50 %; $p = 0.083$) after 24 h of BP3 exposure (Figure 42E).

Figure 42 (next page). Transcriptional activity of *PGRP*, *C-type lectin, Toll, JAK/hopscotch* and *GAPDH* in *P. olivacea* larvae exposed 4 or 24 h to 1 µg/L BP3. Box and whisker plots represent the expression patterns of *PGRP* (A), *C-type lectin* (B), *Toll* (C), *JAK/hopscotch* (D) and *GAPDH* (E) measured by RT qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.05$ (**), $p \leq 0.1$ (*).

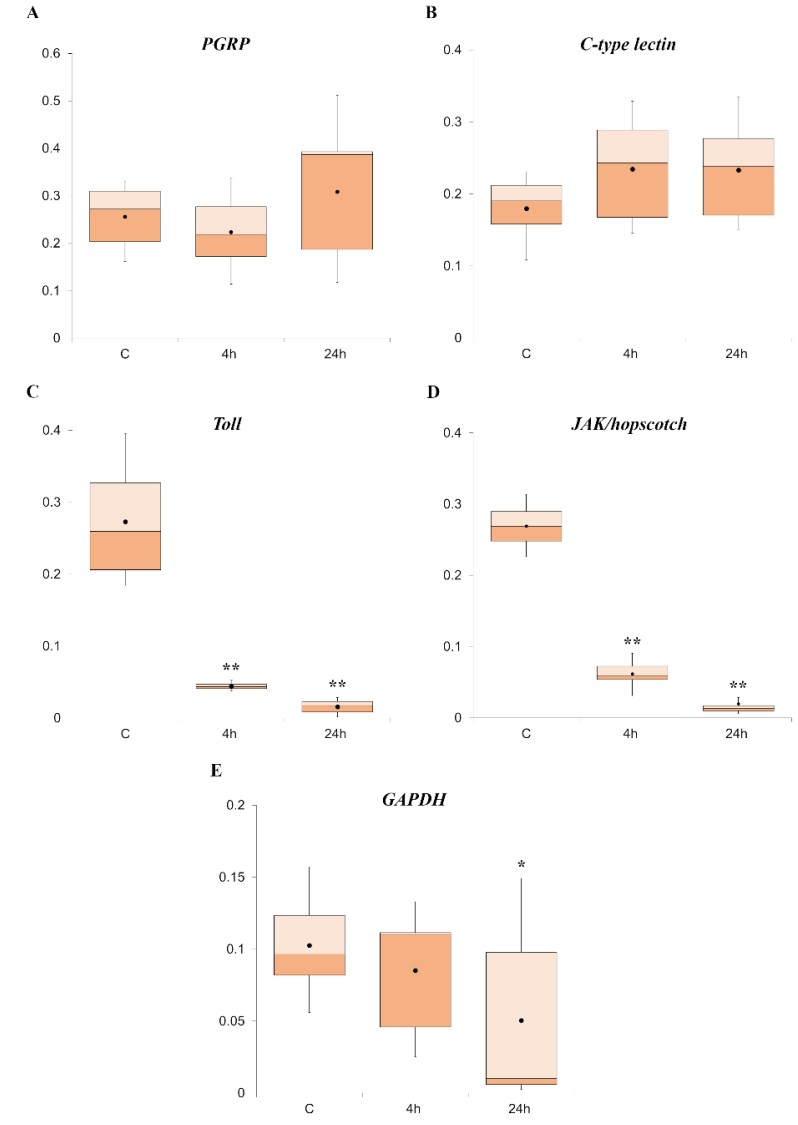

A
PGRP
0.6
0.5
0.4
0.3
0.2
0.1
0
C
4h
24h
B
C-type lectin
0.4
0.3
0.2
0.1
0
C
4h
24h
C
Toll
0.4
0.3
0.2
0.1
0
**
**
C
4h
24h
D
JAK/hopscotch
0.4
0.3
0.2
0.1
0
**
**
C
4h
24h
E
GAPDH
0.2
0.15
0.1
0.05
0
*
C
4h
24h

Figure 42

5.3.3.5. Pro-oxidant state

Concerning enzymatic antioxidant responses (Figure 43A-E), significant differences were found in SOD and CAT activities at 4 h exposures and GST activity at both tested times. Compared to control levels, 4 h BP3 exposure led to a significant increase in the expression of SOD (37 %; $p = 0.007$), CAT (80 %; $p = 0.003$) and GST (46 %; $p = 0.016$) (Figure 43A, B, D). In contraposition, while SOD and CAT experienced a slight decrease in their activity after 24 h exposures but remained over control levels, GST values dropped significantly up to 53 % ($p = 0.001$) below untreated larvae. GPx activity levels increased 49 % (ns) after 24 h BP3 exposure (Figure 43C), while GR activity remained stable among conditions (Figure 43E).

Regarding GSHt content, *P. olivacea* larvae reported significantly lower values than controls (55 % decrease; $p = 0.065$) at 4 h, in contrast to the 65 % ($p = 0.028$) increase at 24 h (Figure 43F).

Figure 43 (next page). *P. olivacea* pro-oxidant state upon BP3 exposure. Mean SOD (A), CAT (B), GPx (C), GST (D) and GR (E) activities, as well as GSHt (F) content in whole individuals of *P. olivacea* exposed 4 or 24 h to 1 µg/L BP3. Asterisks indicate significant differences with respect to control values: $p \leq 0.05$ (**), $p \leq 0.1$ (*). Bars represent the standard error.

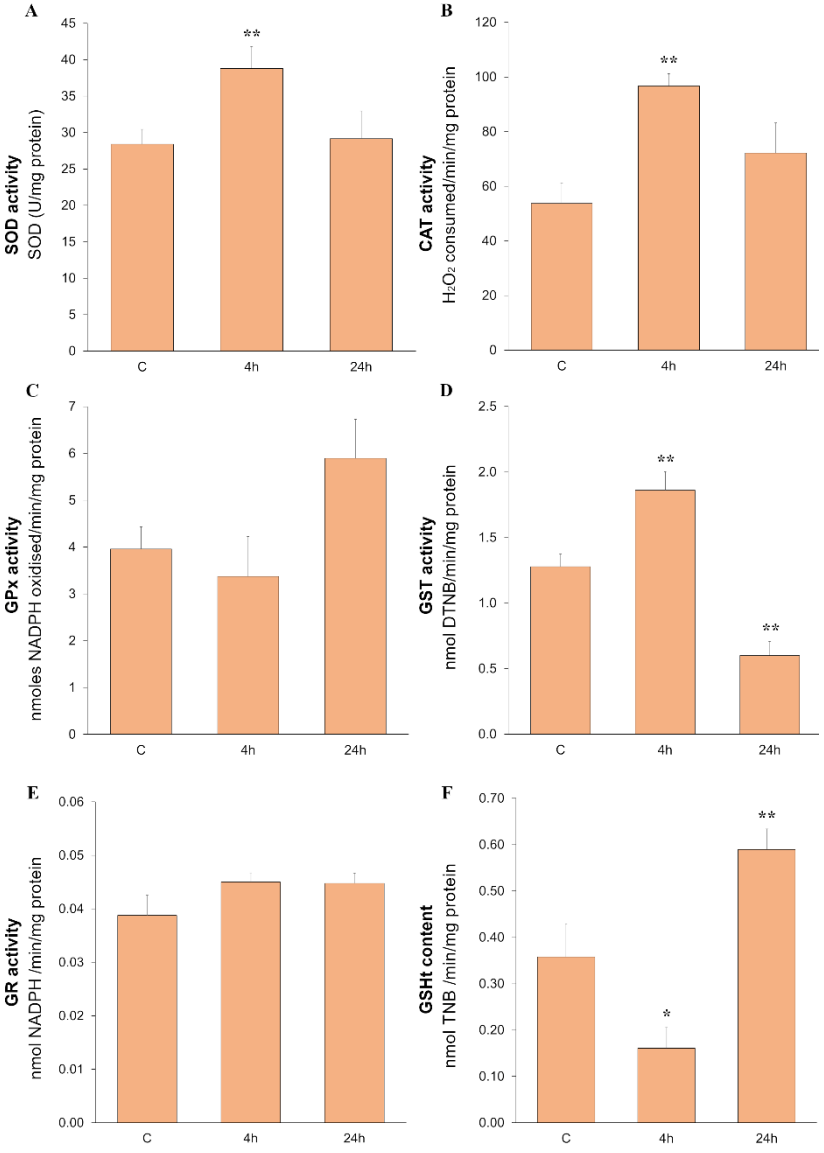

A
SOD activity
SOD (U/mg protein)
45
40
35
30
25
20
15
10
5
0
**
C
4h
24h
B
CAT activity
H2O2 consumed/min/mg protein
120
100
80
60
40
20
0
**
C
4h
24h
C
GPx activity
nmoles NADPH oxidised/min/mg protein
7
6
5
4
3
2
1
0
C
4h
24h
D
GST activity
nmol DTNB/min/mg protein
2.5
2.0
1.5
1.0
0.5
0.0
**
**
C
4h
24h
E
GR activity
nmol NADPH /min/mg protein
0.06
0.05
0.04
0.03
0.02
0.01
0.00
C
4h
24h
F
GSHt content
nmol TNB /min/mg protein
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
*
**
C
4h
24h

Figure 43

5.3.3.6. Summary of analysed effects

- BP3 altered the cell stress response significantly in different ways. It inhibited the transcription of *Hsp27* and *Hsp70* while stimulating *Hsp60* and the cognate form, *Hsc70*.
- Although *Kr-h1* was significantly upregulated after 4 h BP3 exposure, the hormonal route was mostly downregulated, evidenced by the time-dependent decrease in *EcR* and *JHEH* transcripts.
- Generally, the biotransformation and oxidative stress response was activated after 24 h BP3 exposure, while a shorter treatment produced a mild downregulation of this response. *Cyp6a14-like* underwent opposite alterations since its transcription was enhanced at 4 h exposures in contrast to the 80 % decrease shown at 24 h BP3 treatments.
- to 4 h exposure triggers its transcription in contraposition to 24 h BP3 that decreased the mRNA levels up to 80 % below compare to controls.
- BP3 inhibited significantly and in a time-dependent way the expression of *Toll* and *JAK/hopscotch*, relevant immune-related genes.
- BP3 repressed the energy metabolism of *P. olivacea* larvae significantly after 24 h.

5.3.3.7. *P. olivacea* and *C. riparius* comparative analysis

A comparative analysis of changes in the transcriptional and enzymatic activity was performed in individuals of *P. olivacea* and *C. riparius* exposed to 4 and 24 h BP3 to clear up possible inter-species differences in the toxicity of this xenobiotic.

5.3.3.7.1. Survival rates

Concerning the survival study, while the number of individuals of both species remained unaltered in 4 h exposures BP3; *P. olivacea* was more sensitive than *C. riparius* in 24 h treatments (Table 20).

Compared to their respective controls, a 15 % ($p \leq 0.05$) reduction in survival of the non-model aquatic dipteran was observed, while the model one decreased by 4.2 % ($p \leq 0.05$). In addition, the response was different ($p \leq 0.05$) between the two species.

Table 20. Survival rates in *P. olivacea* and *C. riparius* 4th instar larvae exposed 4 or 24 h to 1 µg/L BP3. Results are expressed as the mean of three biological replicates ± standard deviation (n_{total} = 135 larvae/condition). Asterisks (**) indicate significant differences with respect to the corresponding control ($p \leq 0.05$), and (+) corresponds to significant differences for the same condition between the two species ($p \leq 0.05$).

	Species	Exposure time (h)	Control	BP3
Survival rate (mean ± sd)	*P. olivacea*	4	99.3 ± 0	100 ± 0
		24	100 ± 0	**85 ± 0**+**
	C. riparius	4	100 ± 0	100 ± 0
		24	100 ± 0	**95.8 ± 3.3**+**

5.3.3.7.2. Cell stress response

On the one hand, while exposure to the xenobiotic caused downregulation and even the absence of *Hsp27* and *Hsp70* transcriptional activity in *P. olivacea*, in *C. riparius Hsp27* mRNA levels remained unaltered, and *Hsp70* levels increased significantly after 4 h (60 %; p = 0.019) and 24 h (242 %; p = 0.013) (Figure 44A, C).

On the other hand, BP3 did not alter the transcript levels of *Hsp60* in *C. riparius*, whilst a strong upregulation of up to 205 % (p = 0.014) and 177 % (p = 0.003) was reported in those individuals of *P. olivacea* exposed during 4 and 24 h, respectively (Figure 44B).

Finally, particularly remarkable is the induction of a constitute gene, *Hsc70*, in both species upon BP3 exposures (Figure 44D). 4 h exposure led to a 30 % (p = 0.042) increase in the gene transcription in *C. riparius* (Figure 44D). Moreover, BP3 triggered a stronger overexpression in *P. olivacea* after 24 h (594 %; p = 0.003), compared to the mild increase observed in *C. riparius* (54 %; p = 0.003). These data evidence one more time differences between species in the transcriptional response upon the ultraviolet filter exposure.

5.3.3.7.3. Endocrine system

With respect to the transcription of gene coding the ecdysone receptor, it was time-dependent inhibited in *P. olivacea* while the *EcR* gene was overexpressed under the same experimental conditions in *C. riparius* reaching a 63 % increase (p = 0.025) after 24 h (Figure 45A). In contraposition, the previously described overexpression of *Kr-h1* after 4 h BP3 in *P. olivacea*, did not take place in *C. riparius*, whose mRNA gene levels remained close to control levels (Figure 45B). Moreover, compared to untreated larvae, the transcription of this late response gene

experienced a 42 % (ns) decrease in *P. olivacea* larvae at 24 h, whereas *C. riparius* experienced a 101 % (p = 0.001) gene overexpression.

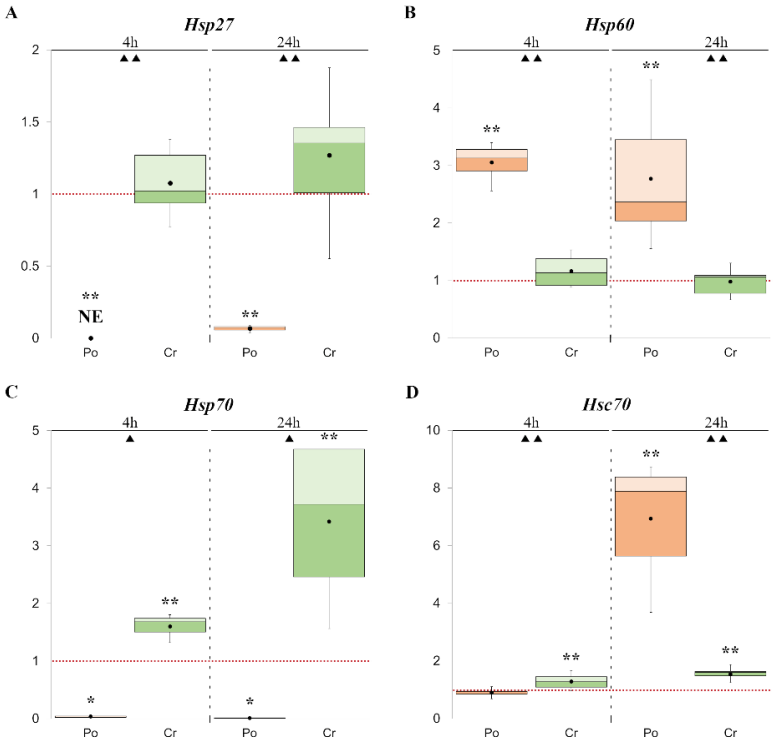


Figure 44. Transcriptional activity of *Hsp27*, *Hsp60*, *Hsp70* and *Hsc70* genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 4 or 24 h to 1 µg/L BP3. Box and whisker plots represent the expression patterns of *Hsp27* (A), *Hsp60* (B), *Hsp70* (C) and *Hsc70* (D) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: p ≤ 0.05 (**), p ≤ 0.1 (*). Triangles indicate significant differences between species: p ≤ 0.05 (▲▲), p ≤ 0.1(▲). NE: No expression.

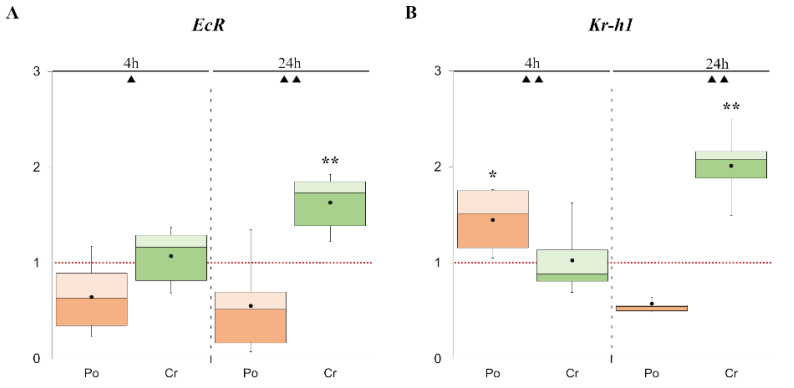


Figure 45. Transcriptional activity of *EcR* and *Kr-h1* genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 4 or 24 h to 1 µg/L BP3. Box and whisker plots represent the expression patterns of *EcR* (A) and *Kr-h1*(B) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.05$ (**), $p \leq 0.1$ (*). Triangles indicate significant differences between species: $p \leq 0.05$ (▲▲), $p \leq$

5.3.3.7.4. Biotransformation and oxidative stress

Significant differences between species were found in three of the four analysed genes involved in biotransformation and oxidative stress processes (Figure 46).

Regarding *MnSOD*, while transcript levels remained slightly below control levels in *P. olivacea*, *C. riparius* experienced a time-dependent overexpression up to 77 % ($p = 0.031$) over control values (Figure 46A). In both tested times, statistical analyses detected differences between the performance of both species ($p \leq 0.05$).

Differential effects between species were found regarding *CAT* mRNA levels ($p \leq 0.1$) and *Cyp4g15* ($p \leq 0.05$) after 4 h BP3 treatments. On the one hand, the significant changes already described in *P. olivacea* after 4 h and 24 h treatments for the *CAT* gene were not reflected in the case of *C. riparius* larvae whose mRNA levels remained close to control values (Figure 46B). On the other hand, the *Cyp4g15* inhibition detected in *P. olivacea* after 4h treatment did not occur in *C. riparius* larvae whose transcript levels remained unchanged (Figure 46D).

Finally, no differences were detected between the two species for *PHGPx* under BP3 exposures (Figure 46C).

Figure 46 (next page). Transcriptional activity of *MnSOD*, *CAT*, *PHGPx* and *Cyp4g15* genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 4 or 24 h to 1 µg/L BP3. Box and whisker plots represent the expression patterns of *MnSOD* (A), *CAT* (B), *PHGPx* (C) and *Cyp4g15* (D) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.05$ (**), $p \leq 0.1$ (*). Triangles indicate significant differences between species: $p \leq 0.05$ (▲▲), $p \leq 0.1$(▲).

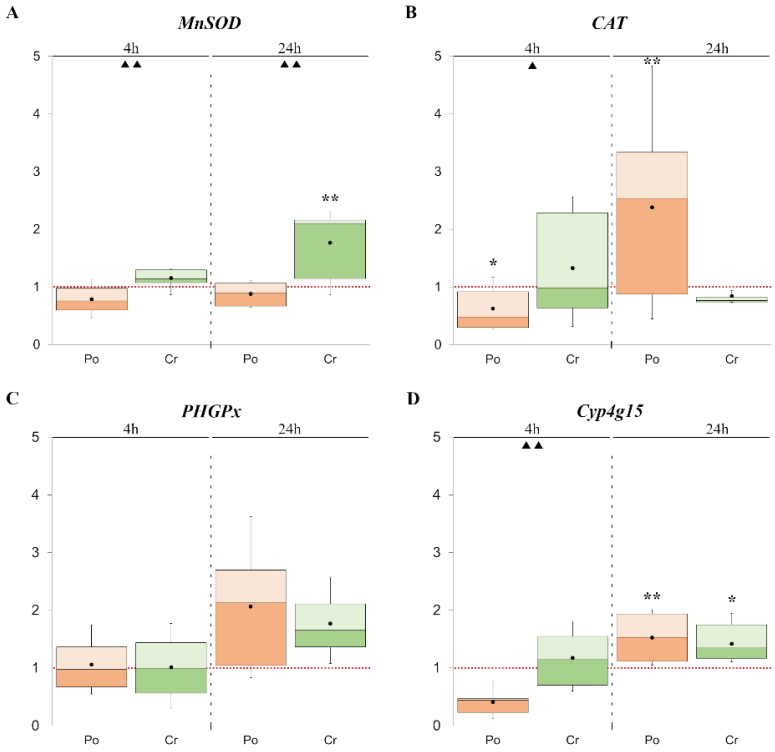


Figure 46

5.3.3.7.5. Immune system and energy metabolism

Species-dependent responses upon BP3 exposure were found in the transcriptional regulation of genes involved in the immune system pathway (*PGRP*, *C-type lectin*, *Toll* and *JAK/hopscotch*) and the energy metabolism (*GAPDH*) (Figure 47).

Regarding *Toll* and *JAK/hopscotch* genes, although mRNA expression levels of both biomarkers varied similarly upon 4 h xenobiotic exposure, the inhibition response was stronger in *P. olivacea* (84 % for *Toll* and 77 % for *JAK/hopscotch;* p = 0.011 both) than in *C. riparius* (52 % for *Toll* and 20 % for *JAK/hopscotch*; ns). Moreover, while the repression remained along the tested times for *P. olivacea*, transcript levels of both genes returned to control values in *C. riparius* larvae.

PGRP levels were unaltered in *P. olivacea* in contraposition to *C. riparius*, whose molecular analysis revealed a time-dependent gene upregulation up to 173 % (p = 0.003) after 24 h (Figure 47A). Significant differences in the regulation of this gene were found at 4 h (p ≤ 0.1) and 24 h (p ≤ 0.05).

C-type lectin reported differences in regulation between species after 4 h treatments (Figure 47B). As described previously, xenobiotic exposure did not alter the transcription of this gene in *P. olivacea* but led to a 58 % (ns) decrease in *C. riparius* after 4h.

Finally, significant changes between both dipterans were detected in the regulation of the *GAPDH* gene upon BP3 24 h. As seen in Figure 47E, its mRNA level in *P. olivacea* was decreased by a 51 % (p = 0.083), in contrast to the 22 % (ns) increase reported in *C. riparius*.

Figure 47 (next page). Transcriptional activity of *PGRP*, *C-type lectin*, *Toll*, *JAK/hopscotch* and *GAPDH* genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 4 or 24 h to 1 µg/L BP3. Box and whisker plots represent the expression patterns of *PGRP* (A), *C-type lectin* (B), *Toll* (C), *JAK/hopscotch* (D) and *GAPDH* (E) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: p ≤ 0.05 (**), p ≤ 0.1 (*). Triangles indicate significant differences between species: p ≤ 0.05 (▲▲), p ≤ 0.1(▲).

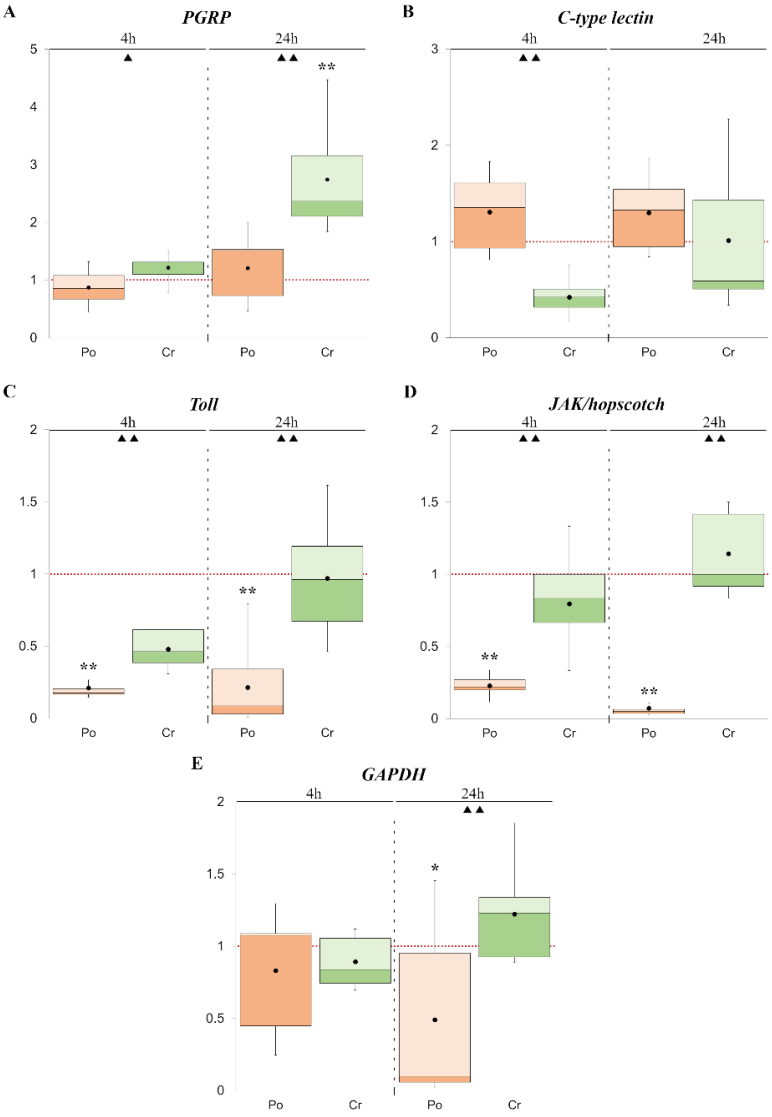

A
PGRP
4h
24h
B
C-type lectin
C
Toll
D
JAK/hopscotch
E
GAPDH
Po
Cr

Figure 47

5.3.3.7.6. Pro-oxidant state

The comparative analysis between species revealed significant inter-species differences in the regulation of CAT, GPx and GST activities at 4 h BP3 exposures. Moreover, GR activity was differentially altered at both tested times (Figure 48).

The alteration of SOD activity, although not statistically significant, followed opposite tendencies between species. While the activity was time-dependent decreased in *C. riparius*, *P. olivacea* had a peak of activity after 4 h BP3 exposure, followed by recovery of control values after 24 h (Figure 48A).

CAT and GST activities were significantly upregulated in those individuals of *P. olivacea* exposed to BP3 for 4 h (80 %; $p = 0.003$ and 46 %; $p = 0.0016$ respectively). Conversely, *C. riparius* activities decreased up to 75 % ($p = 0.014$) and 60 % ($p = 0.044$) below control values (Figure 48B, D). The CAT activity returned to control values in both species after 24 h. Nevertheless, compared to their respective controls, GST activity was 53 % ($p = 0.001$) decreased in *P. olivacea* while 50 % (ns) increase in *C. riparius* at the longest tested time.

Measurement of GPx activity revealed species-dependent regulation at 4 h BP3 exposure (Figure 48C). While the activity of this enzyme slightly decreased (ns) in *P. olivacea*, *C. riparius* increased its activity up to 56 % ($p = 0.059$) above control values.

GR reported differential responses between species at both tested times (Figure 48E). The activity of this enzyme remained unaltered in *C. riparius*, in contraposition to *P. olivacea*, whose levels were upregulated.

Finally, no statistically significant differences between species were found in the total GSH content due to BP3 exposures (Figure 48F).

It is worth mentioning that observed alterations in GSHt content after 4 h exposures were stronger in *P. olivacea* (55 % decrease; p = 0.065) than in *C. riparius* (25 % decrease; ns), while 24 h exposures led to higher increases in *C. riparius* (223 %; p = 0.003) than in *P. olivacea* (65 %; p = 0.028).

Figure 48 (next page). *P. olivacea* and *C. riparius* pro-oxidant state upon BP3 exposure. Mean values of SOD (A), CAT (B), GPx (C), GST (D) and GR (E) enzyme activities, as well as GSHt (F) content in whole individuals of *P. olivacea* and *C. riparius* exposed 4 or 24 h to 1 µg/L BP3. Asterisks indicate significant differences with respect to respective control values: p ≤ 0.05 (**), p ≤ 0.1 (*). Triangles indicate significant differences between species: p ≤ 0.05 (▲▲). Bars represent the standard error.

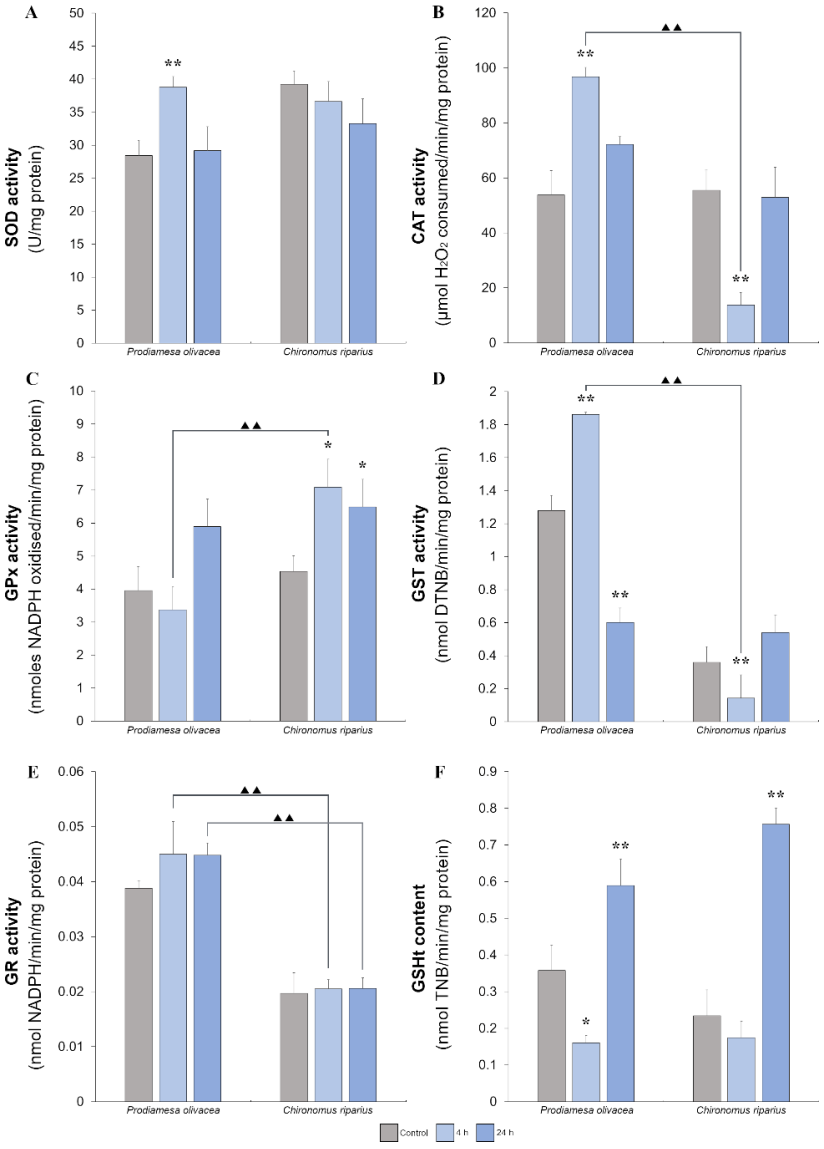

A
SOD activity
(U/mg protein)
B
CAT activity
(µmol H2O2 consumed/min/mg protein)
C
GPx activity
(nmoles NADPH oxidised/min/mg protein)
D
GST activity
(nmol DTNB/min/mg protein)
E
GR activity
(nmol NADPH/min/mg protein)
F
GSHt content
(nmol TNB/min/mg protein)
Prodiamesa olivacea
Chironomus riparius
Control
4 h
24 h

Figure 48

5.3.3.7.7. Comparative summary of the inter-specific effects of BP3

- BP3 caused higher mortality in *P. olivacea* than in *C. riparius* after 24 h.
- The UV filter triggered significant species-dependent alterations in the cell stress response of both chironomids. On the one hand, while it had no effect on the transcript levels of *Hsp27* and *Hsp60* in *C. riparius*, it produced a drastic inhibition of *Hsp27* and overexpression of *Hsp60* in *P. olivacea*. On the other hand, while *Hsp70* mRNA levels dropped by more than 96 % in *P. olivacea*, the model organism underwent a time-dependent upregulation due to BP3 exposure. Finally, *Hsc70* was more overexpressed in *P. olivacea* than in *C. riparius*.
- BP3 had differential effects between species in terms of modulation of the hormone system. It downregulated the ecdysone receptor in the non-model dipteran while triggering a time-dependent increase in the model one in the *EcR* and *Kr-h1* genes. *P. olivacea Kr-h1* gene was upregulated after 4 h BP3 exposure and downregulated after 24 h.
- Regarding the biotransformation and oxidative stress pathway, BP3 had species-dependent effects on the regulation of *MnSOD*, *CAT* or *Cyp4g15*, but not *PHGPx*. *MnSOD* levels remained slightly below control values in *P. olivacea*, whilst there was a time-dependent upregulation in *C. riparius.* Additionally, shortest treatments tended to downregulate *CAT* and *Cyp4g15* gene activities in the non-model insect, no alterations were detected in *C. riparius*. Moreover, the early downregulation in *P. olivacea* turned into sharp gene overexpression after 24 h of BP3

exposure, while mRNA levels in the model organism did not experience alterations.

- Both aquatic dipterans underwent differential alterations on immune system genes due to BP3 exposure. On the one side, *Toll* and *JAK/hopscotch* were strongly downregulated in *P. olivacea*, while the response of *C. riparius* was milder or absent. On the other side, the UV filter did not affect *PGRP* and *C-type lectin* expression in *P. olivacea*, whilst it triggered clear upregulation in the transcript levels of *PGRP* after 24 h and downregulation of *C-type lectin* after 4 h in *C. riparius* larvae.
- The energy metabolism was unaltered in *C. riparius*, in contraposition to *P. olivacea*, whose *GAPDH* transcriptional activity decreased after 24 h exposure.
- The comparative analysis of the antioxidant system revealed significant differences between species in the alteration of CAT, GPx and GST activities after 4 h, and GR activity after 4 and 24 h. In contrast to *C. riparius*, CAT and GST strongly increased their activities in *P. olivacea* BP3-exposed larvae. Inversely, GPx activity was downregulated in *P. olivacea* after 4 h, while it was upregulated in the model midge.
- No differences between species were found in the total GSH content due to BP3 exposure.

Table 21. Summary table of the effects of BBP, BPA and BP3 in *P. olivacea* on relevant pathways. Summary table of the transcriptional changes that BBP, BPA and BP3 (1 µg/L) have on relevant pathways in natural populations of fourth instar larvae *P. olivacea* exposed during 4 and 24 h. Changes with respect to control values in the expression of key genes involved in the cell stress response, hormonal pathway, biotransformation, oxidative stress, immune system and energy metabolism are codified as follow, red: statistically significant downregulation (dark: $p \leq 0.05$, light: $p \leq 0.1$), green: statistically significant upregulation (dark: $p \leq 0.05$, light: $p \leq 0.1$), white: no significant changes were detected. NE: No expression.

P. olivacea	Cell stress response								Hormonal pathway						Biotransformation and oxidative stress											
	Hsp27		*Hsp60*		*Hsp70*		*Hsc70*		*EcR*		*Kr-h1*		*JHEH*		*MnSOD*		*CAT*		*PHGPx*		*Cyp4g15*		*Cyp6a14 -like*		*Cyp6a2 -like*	
BBP	NE	NE	NE	NE																						
BPA	NE	NE																								
BP3	NE																									
	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h

Table 21. Continuation

P. olivacea	Immune system and energy metabolism									
	PGRP		*C-type lectin*		*TOLL*		*JAK/ hopscotch*		*GAPDH*	
BBP										
BPA										
BP3										
	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h

Table 22. Summary table of the effects of BBP, BPA and BP3 in *C. riparius* on relevant pathways. Summary table of the transcriptional changes that BBP, BPA and BP3 (1 µg/L) have on relevant pathways in natural populations of fourth instar larvae *C. riparius* exposed during 4 and 24 h. Changes with respect to control values in the expression of key genes involved in the cell stress response, hormonal pathway, biotransformation, oxidative stress, immune system and energy metabolism are codified as follow, red: statistically significant downregulation (dark: $p \leq 0.05$, light: $p \leq 0.1$), green: statistically significant upregulation (dark: $p \leq 0.05$, light: $p \leq 0.1$), white: no significant changes were detected.

C. riparius	Cell stress response								Hormonal pathway				Biotransformation and oxidative stress							
	Hsp27		*Hsp60*		*Hsp70*		*Hsc70*		*EcR*		*Kr-h1*		*MnSOD*		*CAT*		*PHGPx*		*Cyp4g15*	
BBP																				
BPA																				
BP3																				
	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h

Table 22. Continuation

C. riparius	Immune system and energy metabolism									
	PGRP		*C-type lectin*		*TOLL*		*JAK/ hopscotch*		*GAPDH*	
BBP										
BPA										
BP3										
	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h

Table 23. Summary table of the comparative effects of BBP, BPA and BP3 in *P. olivacea* and *C. riparius* on relevant pathways. Comparative transcriptional changes that BBP, BPA and BP3 (1 µg/L) have on relevant pathways in natural populations of fourth instar larvae *P. olivacea* and *C. riparius* exposed during 4 and 24 h. Comparative inter-species changes in the expression of key genes involved in the cell stress response, hormonal pathway, biotransformation, oxidative stress, immune system and energy metabolism. Triangles indicate significant differences between species: $p \leq 0.05$ (▲▲), $p \leq 0.1$(▲).

P. olivacea vs *C. riparius*	Cell stress response								Hormonal pathway				Biotransformation and oxidative stress							
	Hsp27		*Hsp60*		*Hsp70*		*Hsc70*		*EcR*		*Kr-h1*		*MnSOD*		*CAT*		*PHGPx*		*Cyp4g15*	
BBP	▲▲					▲				▲▲		▲▲	▲▲	▲▲	▲▲	▲▲	▲▲	▲▲	▲	▲▲
BPA	▲▲	▲▲		▲▲	▲			▲▲	▲▲	▲▲		▲▲	▲▲	▲▲		▲▲				▲▲
BP3	▲▲	▲▲	▲▲	▲▲	▲	▲	▲▲	▲▲	▲	▲▲	▲▲	▲▲	▲▲	▲▲	▲				▲▲	
	4h	24h	4h	24h	4h	24h	4h	24h	4h	24h	4h	24h	4h	24h	4h	24h	4h	24h	4h	24h

Table 23. Continuation

P. olivacea vs *C. riparius*	Immune system and energy metabolism									
	PGRP		*C-type lectin*		*TOLL*		*JAK/ hopscotch*		*GAPDH*	
BBP		▲▲	▲▲			▲	▲▲	▲▲	▲▲	▲▲
BPA										
BP3	▲	▲▲	▲▲		▲▲	▲▲	▲▲	▲▲		▲▲
	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h

5.4. Analysis of the effects of heat-shock on natural populations of *P. olivacea*

In this thesis, we have studied changes in the expression profile of HSPs (*Hsp27*, *Hsp60*, *Hsp70*, *Hsc70*, *Cdc37* and *HSF*) and immune system genes (*PGRP*, *C-type lectin*, *Toll* and *JAK/hopscotch*) of *P. olivacea* larvae exposed to thermal stress. 4th instar larvae of *P. olivacea* were exposed to 35 °C and 39 °C for different times (30', 60' and 120' at 35 °C and 30' and 60' at 39 °C) and the molecular response was analysed by real-time qPCR. In addition, at both temperatures, an exposure condition of 1 and 2 h respectively, followed by 2 h at RT was added to study possible transcriptional recovery responses afterwards heat-shock.

Finally, a comparative analysis between *P. olivacea* and *C. riparius*, where heat-shock response is well described (Martín-Folgar *et al*., 2015; Martínez-Paz *et al*., 2014; Muñiz-González and Martínez-Guitarte, 2020), was performed to assess possible differential responses depending on the species and hypothesize differences in the adaptation of both aquatic dipterans. Tables 24, 25 and 26 that can be found at the end of this chapter summarise all the data.

5.4.1. Analysis of transcriptional alterations of heat-shock genes

Alterations in the expression levels of five genes coding for HSPs were evaluated in both study insects under two different extreme temperatures and at different times. Besides, to study the interconnection between the heat-shock response and the immune system, which will be described in later chapters, transcriptional changes of *HSF* were measured at 30' and 60'and HSF.

5.4.1.1. Effects of 35 °C heat-shock in the cell stress response of *P. olivacea*

In terms of survival, no mortality was seen for any of the tested conditions at 35 °C. mRNA levels of *Hsp27*, *Hsp60*, *Hsp70*, *Hsc70* and the *Hsp90* co-chaperone *Cdc37* were measured in larvae of *P. olivacea* exposed to 35 °C for 30', 60', 120' and 120' followed by 2 h at RT.

The transcriptional analysis revealed a general upregulation of the five HSPs measured (Figure 49A-E). In contraposition, *HSF* was significantly ($p \leq 0.1$) inhibited, reaching values of expression up to 87 % and 68 % below control levels after 30' and 60' respectively (Figure 49F).

Transcription of the smallest HSP studied, *Hsp27*, was enhanced in the account of heat exposure at the three tested times without the recovery period (Figure 49A). Statistical analysis revealed a significant upregulation after 1 h, reaching mRNA values 195 % ($p = 0.045$) higher than those of unexposed *P. olivacea* larvae. In contraposition, 2 h of exposure followed by 2 h at RT triggered a 69 % ($p = 0.014$) decrease of *Hsp27* transcript levels below control values. It is worth mentioning that *Hsp27* was the only HSP gene to reach expression values lower than control levels when the organisms were kept at RT after the heat-shock.

Hsp60 was significantly upregulated in all the tested conditions (Figure 49B). The highest peak of expression was reached after 35 °C for 1 h, whose mRNA levels experienced a 911 % ($p = 0.004$) increase. After the hour of heat treatment, the activity of this HSP was slightly decreased. Nevertheless, *Hsp60* transcript levels remained up to 749 % and 721 % ($p = 0.004$ both) higher than control values after 120' and 120' followed to 2 h at RT, respectively.

Concerning the expression of *Hsp70*, exposure of *P. olivacea* organisms to 35 °C triggered a clear ($p = 0.004$) time-dependent upregulation (5375 %, 12681 % and 15905 % respectively) (Figure 49C). However, when

the larvae were left to recover at RT during 2 h, this gene's expression was striking downregulated compared to the expression after 2 h, although it kept 978 % higher than the non-exposed sample.

The constitutive form, *Hsc70*, was overexpressed ($p \leq 0.05$) in all the tested times (Figure 49D). From the exposures without a recovery period, the highest peak of expression was reached after 1 h of 35 °C with mRNA levels up to 570 % ($p = 0.025$) higher than control values. Nevertheless, the highest *Hsc70* transcript levels were measured in those organisms left to recover at RT after the temperature shock with values up to 760 % ($p = 0.02$) compared to unexposed ones.

Concerning *Cdc37* (*Hsp90* co-chaperone) while 30' and 2 h of exposure did not report significant changes in its expression levels, after 1 and 2 h followed by a recovery period, mRNA levels significantly increased, reaching values up to 137 % ($p = 0.027$) and 114 % ($p = 0.057$) respectively compared to control levels (Figure 49E).

Figure 49 (next page). Transcriptional activity of *Hsp27*, *Hsp60*, *Hsp70*, *Hsc70, Cdc37* and *HSF* in *P. olivacea* larvae exposed 35 °C heat-shock for 30', 60', 120' and 120' followed by 2 h of recovery period at RT. Box and whisker plots represent the expression patterns of *Hsp27* (A), *Hsp60* (B), *Hsp70* (C), *Hsc70* (D), *Cdc37* (E) and *HSF* (F) measured by RT qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control (C) values: $p \leq 0.05$ (**), $p \leq 0.1$ (*).

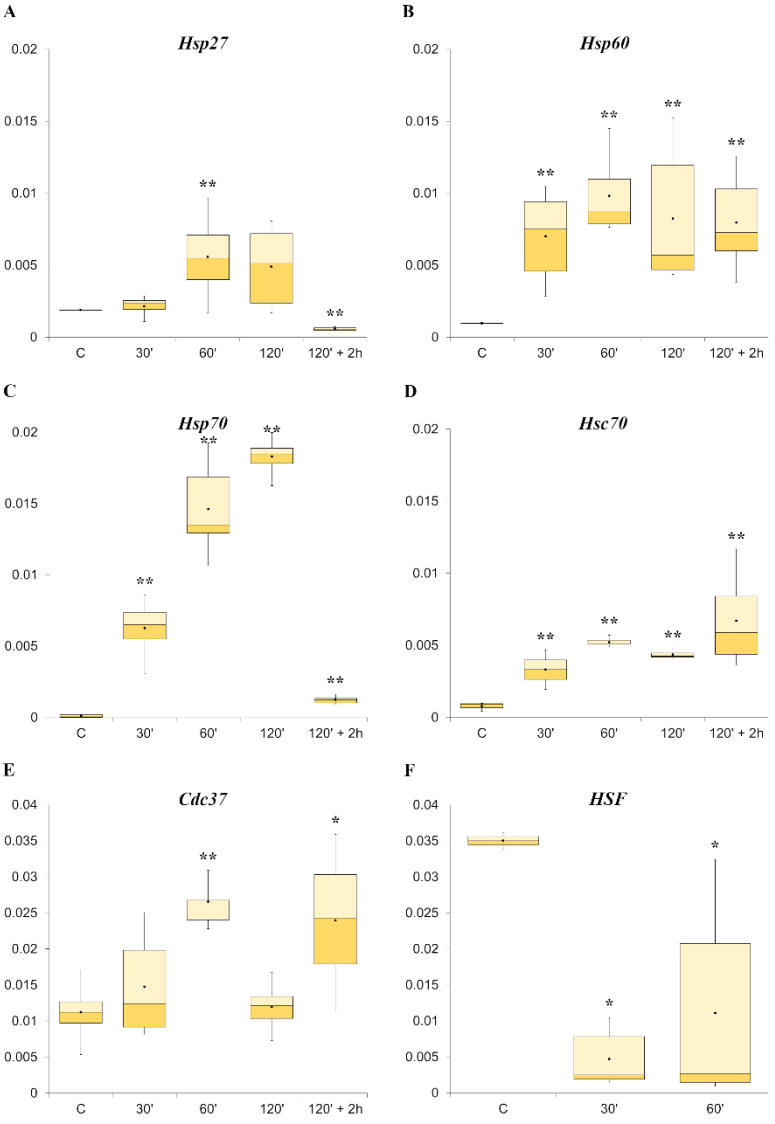


Figure 49

5.4.1.2. Effects of 39 °C heat-shock in in the cell stress response of *P. olivacea*

In this case, since the temperature was extreme and *P. olivacea* larvae did not survive longer than an hour, the exposures were performed at 30', 60' and 60' followed by 2 h of a recovery period. Although the survival rate was 100 %, organisms' generalised loss of mobility of organisms was sighted after 1 h of heat-shock.

A general upregulation of *Hsp60*, *Hsp70*, *Hsc70* and *Cdc37* was seen in the account of 39 °C heat-shock (Figure 50B-E). As reported in the 35-degree results, the expression of *HSF* gene was repressed up to 90 % ($p = 0.04$) and 80 % ($p = 0.024$) below control levels after 30' and 60' respectively (Figure 50F).

39 °C exposures led to a statistically significant ($p \leq 0.05$) downregulation of *Hsp27* mRNA levels (Figure 50A). The highest decrease was observed after 1 h of exposure (57 % below control values; $p = 0.045$).

Hsp60, *Hsp70*, and *Hsc70* were significant time-dependent upregulated ($p \leq 0.05$), reaching the highest values after leaving the organisms to recover for 2 h after being exposed for 2 h at 39 °C. The most striking increase in expression was measured for *Hsp70*, whose transcript levels were overexpressed up to 4868 % ($p = 0.008$) (Figure 50C). The constitutive form, *Hsc70* achieved values 518 % higher ($p = 0.004$) than control larvae (Figure 50D) and *Hsp60* 87 % ($p = 0.017$) (Figure 50B).

Heat-shock led to a slight decrease of the *Cdc37* mRNA levels after 30' of exposure, but after 1 h and even after the recovery period, *P. olivacea* individuals experienced an increase in the gene activity of 48 % (ns) and 124 % ($p = 0.048$) compared to control values (Figure 50E).

Figure 50 (next page). Transcriptional activity of *Hsp27*, *Hsp60*, *Hsp70*, *Hsc70*, *Cdc37* and *HSF* in *P. olivacea* larvae exposed 39 °C heat-shock for 30', 60' and 60' followed by 2 h of recovery period at RT. Box and whisker plots represent the expression patterns of *Hsp27* (A), *Hsp60* (B), *Hsp70* (C), *Hsc70* (D), *Cdc37* (E) and *HSF* (F) measured by RT qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control (C) values: $p \leq 0.05$ (**).

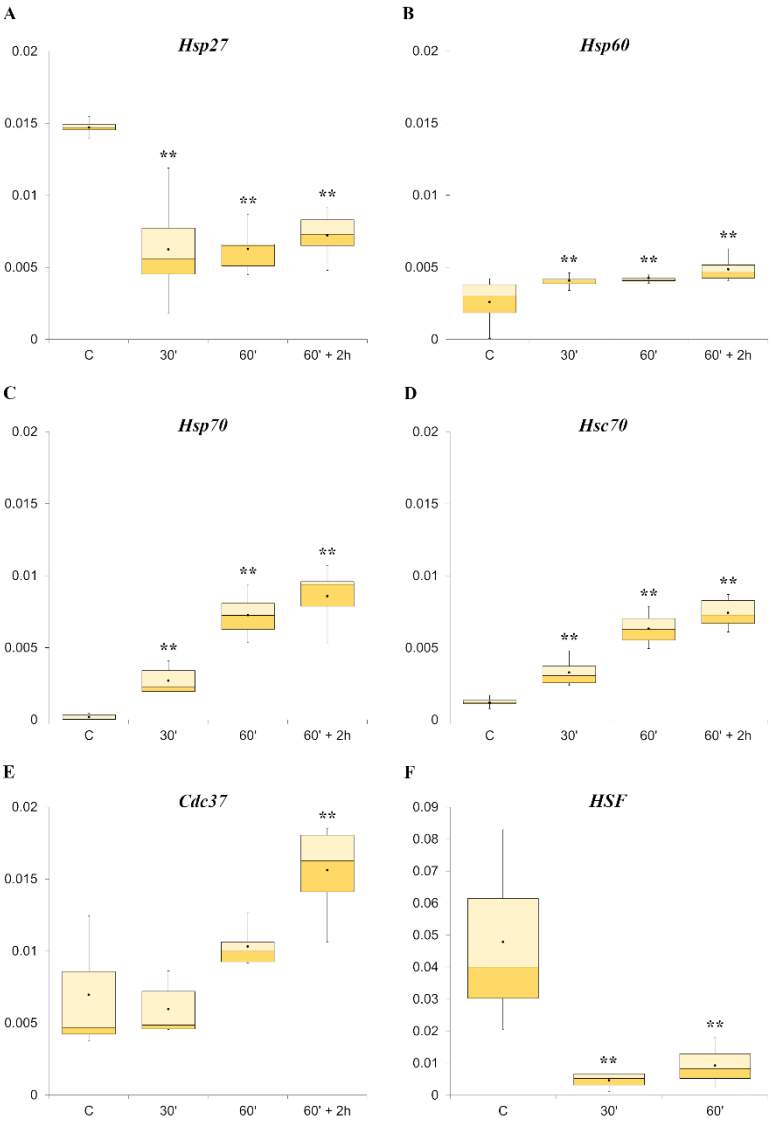

A
Hsp27
0.02
0.015
0.01
0.005
0
**
**
**
C
30'
60'
60' + 2h
B
Hsp60
0.02
0.015
0.01
0.005
0
**
**
**
C
30'
60'
60' + 2h
C
Hsp70
0.02
0.015
0.01
0.005
0
**
**
**
C
30'
60'
60' + 2h
D
Hsc70
0.02
0.015
0.01
0.005
0
**
**
**
C
30'
60'
60' + 2h
E
Cdc37
0.02
0.015
0.01
0.005
0
**
C
30'
60'
60' + 2h
F
HSF
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
**
**
C
30'
60'

Figure 50

5.4.1.3. Summary of the effects of heat-shock in the cells stress response of *P. olivacea*

- The survival of *P. olivacea* larvae was not affected after 2 h and shorter period times at 35 °C.
- 35 °C heat-shock triggered a general upregulation of cell stress tested genes, *Hsp27*, *Hsp60*, *Hsp70*, *Hsc70* and *Cdc37* after 30', 60' and 120' exposures.
- When *P. olivacea* larvae were allowed to recover at RT after 35 °C heat-shock, the transcript levels of *Hsp60*, *Hsp70*, *Hsc70* and *Cdc37* remained significantly above control levels in contrast to *Hsp27*, which was the only gene showing significantly lower values compared to its respective control.
- Compared to control, an 87 % and 68 % significant inhibition of the mRNA levels of *HSF* took place after 30' and 60' exposures to 35 °C, respectively.
- 39 °C heat-shock activated in a striking and significant time-dependent way the transcription of *Hsp60*, *Hsp70* and *Hsc70* after 30', 60' and 60' followed by 2 h of a recovery period.
- 35 and 39 °C exposures led to a generalised *Hsp90* co-chaperone *Cdc37* overexpression.
- *Hsp27* and *HSF* were significantly repressed when *P. olivacea* was exposed to 39 °C for 30' and 60' and even after the 2-h recovery period in the case of *Hsp27*.
- At 35 °C and 39 °C, the lowest values of *HSF* were reached after the first 30' of exposure.

5.4.1.4. *P. olivacea* and *C. riparius* comparative analysis

To study possible differences in tolerance and adaptation between the model species, *C. riparius*, and the non-model organism, *P. olivacea,* a heat-shock comparative analysis between these two aquatic dipterans was performed. 4th instar larvae of both species were exposed to the same temperature shift and time conditions, and changes in transcriptional activity of *Hsp27*, *Hsp60*, *Hsp70*, *Hsc70* and *HSF* were statistically analysed. No mortality was observed, as survival was 100 % in both organisms.

5.4.1.4.1. Effects of 35 °C heat-shock in the cell stress response

Molecular data revealed significant differences between species in the transcriptional regulation of genes involved in the cell stress response. Although compared to their respective controls, both insect species experienced changes following the same tendency in most analysed biomarkers; the magnitude of these changes is completely different between them.

Statistical analysis revealed significant ($p \leq 0.05$) species-dependent alterations in all the 35 °C heat-shock exposures of study in the five HSPs analysed genes (*Hsp27*, *Hsp60*, *Hsp70*, *Hsc70* and *HSF*) (Figure 51). Aside from the case of *HSF*, the temperature shock of both chironomids triggers a generalised activation of the transcription of cell stress genes. The most striking difference was found in the magnitude of these changes. Compared to their respective controls, the increase in *Hsp60* and *Hsc70* mRNA levels was much higher in *P. olivacea* than in *C. riparius,* while the opposite parallelism was measured for *Hsp27* and *Hsp70*. Finally, the *HSF* gene was reverse transcriptional regulated when comparing both species at the shortest tested time.

The transcription of *Hsp27* increased after 30', 60' and 120' in both species but significant ($p \leq 0.05$) differences were found between them in all the study scenarios since the magnitude of change was completely different (Figure 51A). Compared to their respective controls, gene upregulation in response to heat-shock was stronger in the model organism (3526 %; $p = 0.009$, 11013 %; $p = 0.005$, and 9209 %; $p = 0.005$ after 30', 60' and 120' respectively 120') than in *P. olivacea* (14 %; ns, 195 %; $p = 0.045$, and 159 %; ns, increase after 30', 60' and 120' respectively). Moreover, when both larvae were allowed to recover for 2 h after 2 h of 35 °C heat-shock, *C. riparius* transcript levels remained over control values (415 % increase; $p = 0.005$), while *P. olivacea* experienced a 69 % decrease ($p = 0.014$). In both species, the highest peak of expression was reached after the first hour of temperature shock.

Concerning the expression of *Hsp60*, although 35 °C exposures led to a notable increase in gene expression, significant ($p \leq 0.05$) species-dependent differences were found in all the tested conditions between both organisms of study (Figure 51B). The genetic response of the non-model organism was stronger than the response measured in larvae of the model aquatic dipteran. Compared to non-exposed larvae, the highest peak of transcript levels was reached after 1 h of temperature shock, but it corresponded to a 911 % increase ($p = 0.004$) in *P. olivacea* compared to a 169 % increase ($p = 0.016$) in *C. riparius*.

In terms of the *Hsp70* gene, contrary to what was seen for the *Hsp60* transcriptional alterations, the first two tested times, 30' and 60' triggered a higher increase in the mRNA levels of *C. riparius* (11333 %; $p = 0.010$, and 29875 % increase; $p = 0.005$ respectively) than in *P. olivacea* (5375 % and 12681 %; $p = 0.004$ both) (Figure 51C). After the first hour at 35 °C, the *Hsp70* transcript levels of *C. riparius* tended to decrease but always remained over control values, while the highest expression in *P. olivacea*

(15905 %; p = 0.004) was reached at the second hour of heat-shock. Finally, after the recovery period, the mRNA levels of the model ecotoxicology insect returned to control values, while in *P. olivacea* persisted increased more than 978 %.

Regarding the cognate gene *Hsc70*, the highest transcript levels were measured in larvae of the non-model organism upon 35 °C heat exposure (Figure 51D), and significant ($p \leq 0.05$) differences were found between both species in all study conditions. In addition, the gene response was earlier in *C. riparius* than in *P. olivacea* since the highest peak of mRNA values was reached after 1 h in the first mentioned species (358 %; p = 0.005), while in *P. olivacea* it was achieved after the recovery period (761 %; p = 0.020).

Finally, as mentioned previously, statistical analysis revealed significant ($p \leq 0.05$) species-dependent *HSF* transcriptional regulation after 30' 35 °C exposure (Figure 51E). While the gene transcription was repressed ($p \leq 0.1$) in *P. olivacea* to 86 % and 68 % below control levels, in *C. riparius*, the same exposure conditions led to a non-significant 16 % upregulation after 30' and just a 9 % downregulation after 60'. These data evidence one more time the differences between both aquatic dipteran species in terms of gene alterations upon exposure to stress conditions.

Figure 51 (next page). Transcriptional activity of *Hsp27*, *Hsp60*, *Hsp70*, *Hsc70* and *HSF* genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 35 °C heat-shock for 30', 60', 120' and 120' followed by 2 h of recovery period at RT. Box and whisker plots represent the expression patterns of *Hsp27* (A), *Hsp60* (B), *Hsp70* (C), *Hsc70* (D) and *HSF* (E) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.05$ (**), $p \leq 0.1$ (*). Triangles indicate significant differences between species: $p \leq 0.05$ (▲▲).

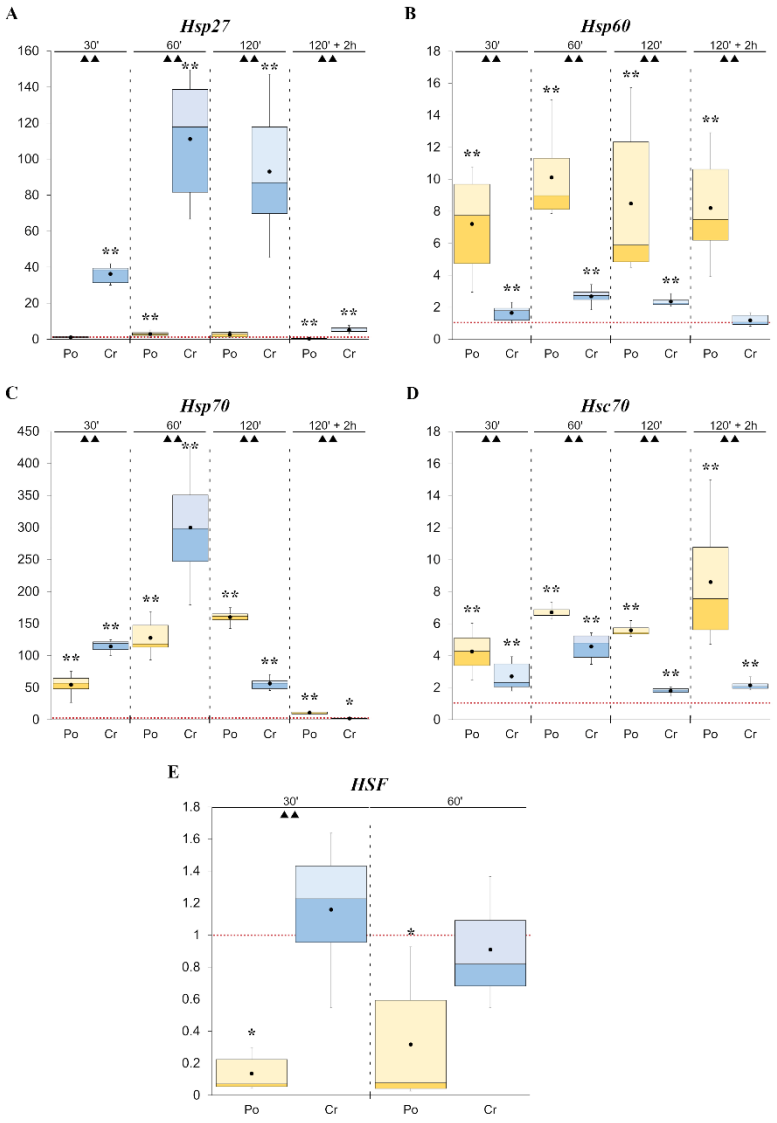


Figure 51

5.4.1.4.2. Effects of 39 °C heat-shock in the cell stress response

In this case, although the survival rate of both insect species was 100 %, at 39 °C exposures *C. riparius* larvae showed a generalized loss of mobility and colouring in 60' exposures. Even though a loss of mobility was seen in *P. olivacea*, the lack of haemoglobin impeded visualising any colour change due to the characteristic white colour of the larvae.

Although transcriptional changes of both chironomids followed similar tendencies in the account of 39 °C exposures, significant ($p \leq 0.05$) species-dependent differences were found in most of the study scenarios due to differences in the response's magnitude, as it was described for 35 °C heat-shock (Figure 52).

Hsp27 and *HSF* were regulated in oppositely ways, revealing significant species-dependent differences. Whilst heat-shock inhibited the transcription of both genes *in P. olivacea*, the same conditions of study enhanced the gene expression in *C. riparius* (Figure 52A, 52E). On the one hand, *Hsp27* transcript levels were decreased up to 57 % ($p = 0.045$) below control values in *P. olivacea* after 1 h, while in *C. riparius* the same condition was translated into a 7912 % ($p = 0.005$) gene upregulation. On the other hand, the transcription of *HSF* was downregulated in *P. olivacea* larvae (90 %; $p = 0.04$, and 80 %; $p = 0.024$ respectively after 30' and 60') in contraposition to the gene overexpression quantified in *C. riparius* (110 %; $p = 0.05$, and 138 %; $p = 0.031$).

39 °C exposures led to an upregulation of the mRNA levels of *Hsp60* in both species (Figure 52B). Statistical analysis revealed significant ($p \leq 0.05$) differences inter-species at the conditions of 60' and 60' followed by 2 h of recovery time. These differences were based on the different magnitude of the changes in the transcript levels. Under the same temperature shock treatment, *C. riparius* showed a higher increase

response (130 % and 152 % respectively at 60' and 60' + 2 h; p = 0.016 for both) than *P. olivacea* (64 %; p = 0.05, and 87 %; p = 0.017).

In light of *Hsp60* data, the mRNA levels of *Hsp70* were upregulated in both species, but *C. riparius* reached significantly higher transcript levels than *P. olivacea* (Figure 52C). In addition, the response was faster in *C. riparius* since the highest peak of expression was measured after 1 h (41,289 % increase; p = 0.016), while the maximum *Hsp70* levels were quantified after the recovery period (4,868 % increase; p = 0.008) in *P. olivacea*. Significant species-dependent differences were found after 30' and 60' 39 °C exposures.

Finally, significant (p ≤ 0.05) differences between both species were found in the transcriptional regulation of the constitutive form, *Hsc70*, after 60' and 60' followed by 2 h of recovery period (Figure 52D). 39 °C triggered a time-dependent gene upregulation. Compared to their respective controls, larvae of the non-model organism reached higher mRNA levels (up to 518 % increase; p = 0.004 after the recovery period) than the model aquatic insect of study under the same premises (277 % increase; p = 0.009).

Figure 52 (next page). Transcriptional activity of *Hsp27*, *Hsp60*, *Hsp70*, *Hsc70* and *HSF* genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 39 °C heat-shock for 30', 60' and 60' followed by 2 h of recovery period at RT. Box and whisker plots represent the expression patterns of *Hsp27* (A), *Hsp60* (B), *Hsp70* (C), *Hsc70* (D) and *HSF* (E) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: p ≤ 0.05 (**). Triangles indicate significant differences between species: p ≤ 0.05 (▲▲).

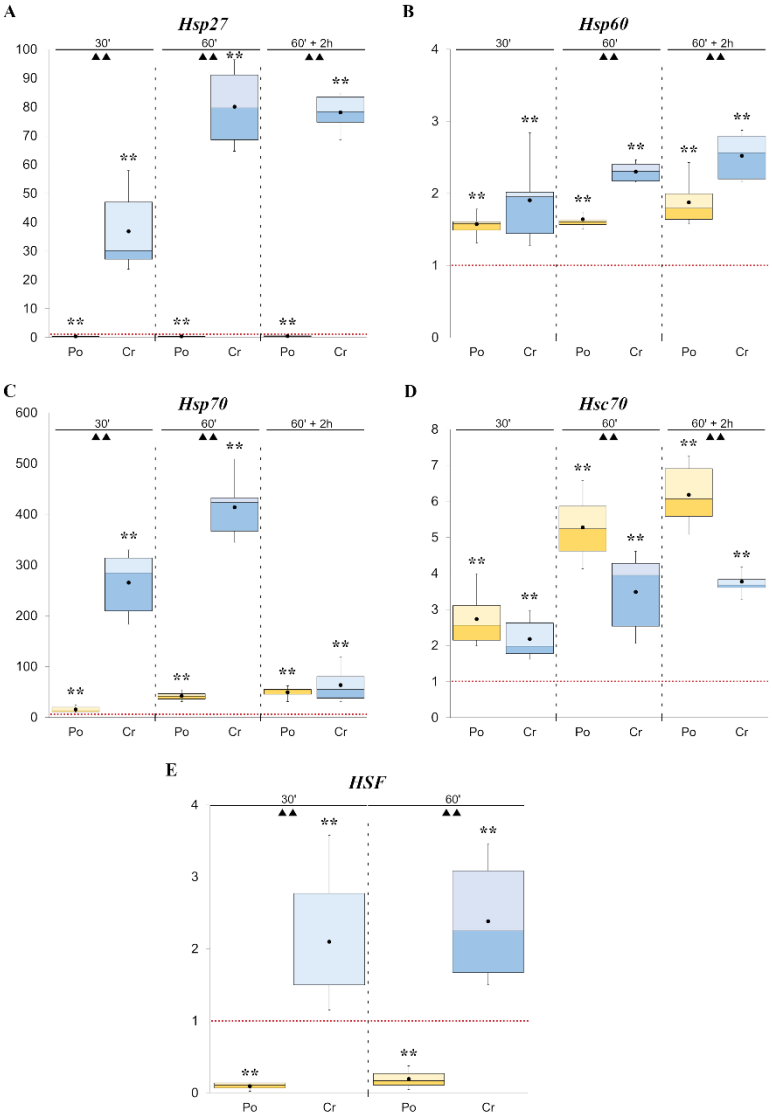


Figure 52

5.4.1.4.3. Comparative summary between species of the effects of temperature shift in the cell stress response

- At the studied conditions, 35 °C and 39 °C heat-shock did not affect the survival of *P. olivacea* and *C. riparius*.
- Heat-shock exposures triggered similar tendencies in the cell stress gene response of both species. In general, the response of *C. riparius* was stronger and faster than *P. olivacea*.
- 35 °C exposures produced an overexpression of *Hsp27*, *Hsp60*, *Hsp70* and *Hsc70* in *C. riparius* and *P. olivacea* after 30', 60' and 120'. There was a general tendency towards an acute and earlier increase in gene transcription in *C. riparius* compared to *P. olivacea* whose changes in mRNA levels were later and lower.
- *HSF* expression was clearly repressed in *P. olivacea* after 30' and 60' at 35 °C and 39 °C. In contraposition, larvae of *C. riparius* exposed to 39 °C overexpressed the transcription of *HSF* evidencing one more time a clear species-dependent regulation upon heat-shock.
- 39 °C exposure triggered an increase in the transcription of *Hsp27* in *C. riparius* (up to 7913 % increase at 60'), while in *P. olivacea* it inhibited the gene transcript levels.
- 39 °C heat-shock induced an overexpression of *Hsp60, Hsp70* and *Hsc70* in *C. riparius* and *P. olivacea*. Higher *Hsp60* and *Hsp70* mRNA levels were measured in larvae of the insect model compared to *P. olivacea*. In contrast, *Hsc70* expression reached higher values in the non-model aquatic dipteran than the model one.

5.4.2. Analysis of transcriptional alterations of immune system-related genes

Taking into account the molecular analysis obtained data related to transcriptional changes in the cell stress response of both chironomids and the relationship between heat-shock and the immune system pathways; 30' and 60' exposures at both temperatures were chosen to perform a transcriptional study to assess possible modifications in the expression levels of immune-related genes.

5.4.2.1. Effects of 35 °C heat-shock in the immune system response of *P. olivacea*

The transcription of three of the four analysed genes involved in the immune system was clearly inhibited in 4th instar larvae of *P. olivacea* exposed to 35 °C for 30' and 60' (Figure 53).

PGRP was the only analysed gene whose transcription was increased, although no significant, upon temperature shift with a mean of expression increase of 33 % (Figure 53A).

Concerning the other pattern recognition analysed protein (PRP), C-type lectin, a time-dependent gene transcription downregulation was detected, reaching values of 28 % (ns) and 45 % (ns) below control levels after 30' and 60' (Figure 53B).

Finally, 35 °C led to a significant ($p \leq 0.05$) drop in the transcriptional activity of *Toll* and *JAK/hopscotch* genes causing a remarkable downregulation of around 97 % compared to those non-exposed *P. olivacea* larvae (Figure 53C, D).

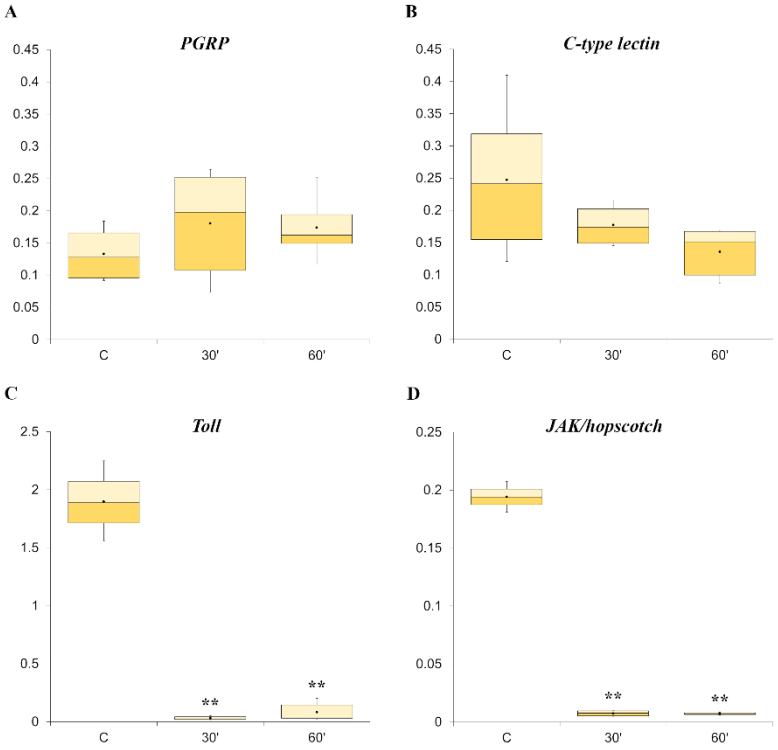


Figure 53. Transcriptional activity of *PGRP*, *C-type lectin*, *Toll* and *JAK/hopscotch* in *P. olivacea* larvae exposed 35 °C heat-shock for 30' and 60'. Box and whisker plots represent the expression patterns of *PGRP* (A), *C-type lectin* (B), *Toll* (C) and *JAK/hopscotch* (D) measured by RT qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control (C) values: $p \leq 0.05$ (**).

5.4.2.2. Effects of 39 °C heat-shock in the immune system response of *P. olivacea*

Statistical analysis revealed significant ($p \leq 0.05$) inhibition of the transcription of *C-type lectin*, *Toll* and *JAK/hopscotch* in *P. olivacea* organisms exposed to 39 °C for 30' and 60' (Figure 54B-D). Since mRNA values remained close to control levels after 30' and slightly increased after 1 h of exposure, no statistically significant responses could be detected for *PGRP* (Figure 54A).

As described for *C-type lectin* at 35 °C, a time-dependent transcriptional downregulation was seen for the transcript levels of this gene in account of 39 °C exposures (Figure 54B). In this case, the inhibition was stronger, reaching values lower than 86 % ($p = 0.016$) and 90 % ($p = 0.037$) compared to control levels.

Regarding the analysed genes involved in the immune system signalling pathways, *Toll* and *JAK/hopscotch*, the tendency seen at 35 °C towards a strong downregulation was observed at 39 °C as well (Figure 54C, D). The transcriptional activity of both genes was inhibited ($p \leq 0.05$) up to 98 % compared to those non-exposed larvae of *P. olivacea*.

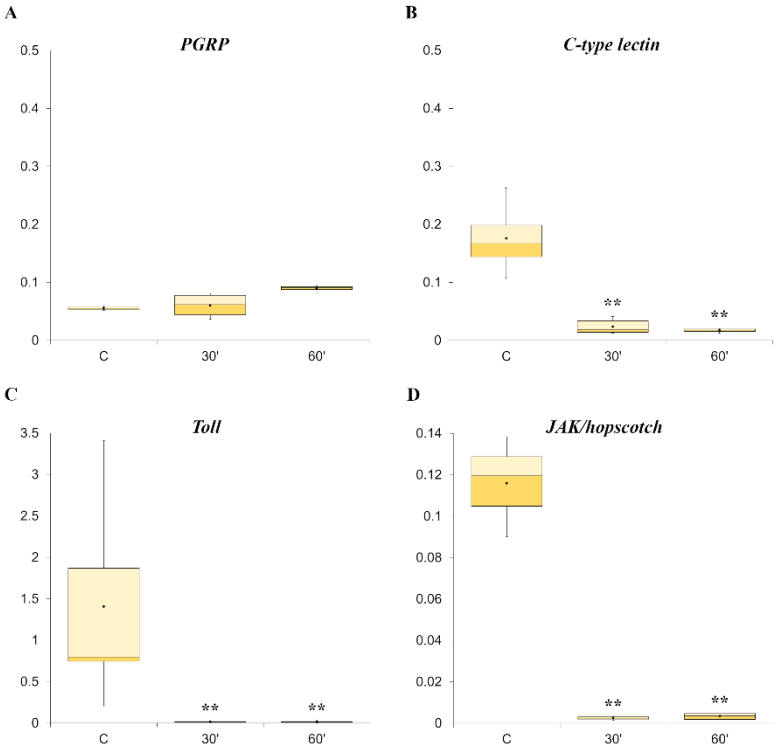


Figure 54. Transcriptional activity of *PGRP*, *C-type lectin*, *Toll* and *JAK/hopscotch* in *P. olivacea* larvae exposed 39 °C heat-shock for 30' and 60'. Box and whisker plots represent the expression patterns of *PGRP* (A), *C-type lectin* (B), *Toll* (C) and *JAK/hopscotch* (D) measured by RT qPCR. The box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control (C) values: $p \leq 0.05$ (**).

5.4.2.3. Summary of the effects of heat-shock in the immune system response of *P. olivacea*

- 35 °C and 39 °C *P. olivacea* larvae exposures triggered a downregulation close to inhibition with values of expression around 98 % of the transcription of genes involved in the signalling process of the immune system: *Toll* and *JAK/hopscotch*.
- The transcriptional activity of *C-type lectin* was time-dependent repressed in *P. olivacea* larvae exposed to both acute temperature conditions. Compared to control, the downregulation was stronger and statistically significant at 39 °C.
- Although no significant alterations were found in the *PGRP* gene, there was a tendency towards upregulation of the transcript levels when *P. olivacea* organisms were exposed to 35 °C and 39 °C.

5.4.2.4. *P. olivacea* and *C. riparius* comparative analysis

In correlation to previous chapters, a comparative transcriptional analysis was performed between *C. riparius* and *P. olivacea* to study possible differences inter-species in the regulation of genes involved in the immune system response in the account to heat-shock exposures.

5.4.2.4.1. Effects of 35 °C heat-shock in the immune system response

From the four analysed genes involved in the response of the immune system, in two of them (*Toll* and *JAK/hopscotch*), there were significant ($p \leq 0.05$) species-dependent alterations at both tested times while in the other two genes related to PRPs (*PGRP* and *C-type lectin*), significant changes ($p \leq 0.1$) were obtained at the most prolonged exposure to 35 °C (Figure 55).

On the one hand, although the temperature shift produced a transcriptional activation (ns) of the *PGRP* gene in both species of aquatic dipterans, the *C. riparius* response was stronger (134 % and 157 % increase after 30' and 60' respectively) than the response measured in *P. olivacea* (36 % and 30 % increase) (Figure 55A).

On the other hand, the transcription of *C-type lectin*, *Toll* and *JAK/hopscotch* genes were stimulated in those larvae of *C. riparius* exposed to 35 °C in contraposition to the inhibition of transcription previously described in *P. olivacea*. While the *C-type lectin* activity was a 49 % (ns) increase in 4th instar larvae of *C. riparius* exposed to 35 °C for 1 h, the same condition triggered a 45 % (ns) gene inhibition in *P. olivacea* (Figure 55B). The most striking significant differences were found in *Toll* and *JAK/hopscotch* genes since the temperature shock led to a 97 % reduction of the transcript levels in the non-model insect of study in comparison to *C. riparius*, whose mRNA levels were upregulated up to 97 % (Toll; ns) and 137 % (JAK/hopscotch; $p = 0.05$) after de longest tested time (Figure 55C D).

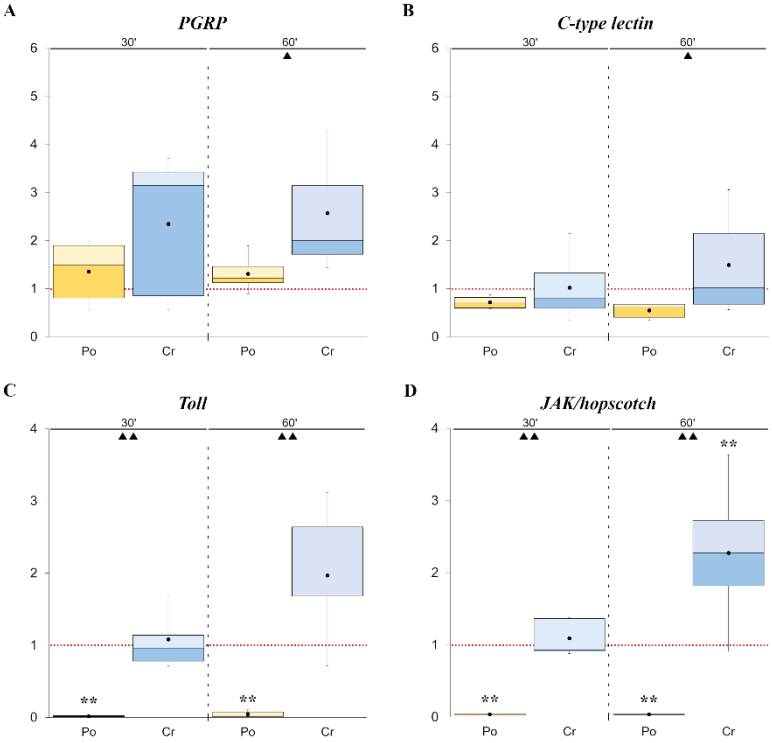


Figure 55. Transcriptional activity of *PGRP*, *C-type lectin*, *Toll* and *JAK/hopscotch* genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 35 °C heat-shock for 30' and 60'. Box and whisker plots represent the expression patterns of *PGRP* (A), *C-type lectin* (B), *Toll* (C) and *JAK/hopscotch* (D) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.05$ (**). Triangles indicate significant differences between species: $p \leq 0.05$ (▲▲), $p \leq 0.1$(▲).

5.4.2.4.2. Effects of 39 °C heat-shock in the immune system response

In correlation to previously described molecular data, 39 °C heat-shock induced species-dependent alterations in the activity of immune response genes of both chironomids of study. Statistical analyses revealed significant ($p \leq 0.05$) differences among species at both tested times in *C-type lectin*, *Toll* and *JAK/hopscotch* and after 30' in *PGRP* (Figure 56).

On one side, although the temperature shift increased the transcription of *PGRP* in both species, the magnitude of the response along 30' and 60' exposures was stronger in *C. riparius* (105 % and 79 % respectively; ns) than in *P. olivacea* (9 % and 60 %; ns) (Figure 56A).

On the other side, the activity of the other three analysed immune-system genes, *C-type lectin*, *Toll* and *JAK/hopscotch*, was striking repressed in *P. olivacea* larvae, as seen previously, whilst under the same premises of heat stress conditions, the transcription of these genes was noteworthy upregulated in *C. riparius*. Compared to their respective controls, *C-type lectin* was 86 % ($p = 0.016$) and 90 % ($p = 0.037$) decreased in *P. olivacea* in contraposition to the 436 % ($p = 0.023$) and 256 % ($p = 0.023$) increase quantified in *C. riparius* (Figure 56B). As of last, the transcription of *Toll* and *JAK/hopscotch* dropped up to 97 % below control levels in *P. olivacea* organisms while it was 155 % ($p = 0.041$) and 115 % ($p = 0.065$) enhanced in *C. riparius* for *Toll* gene and 92 % ($p = 0.082$) and 144 % ($p = 0.026$) for *JAK/hopscotch* (Figure 56C, D). These data evidence one more time a clear and significant inter-species transcriptional modification upon the same stressing scenarios.

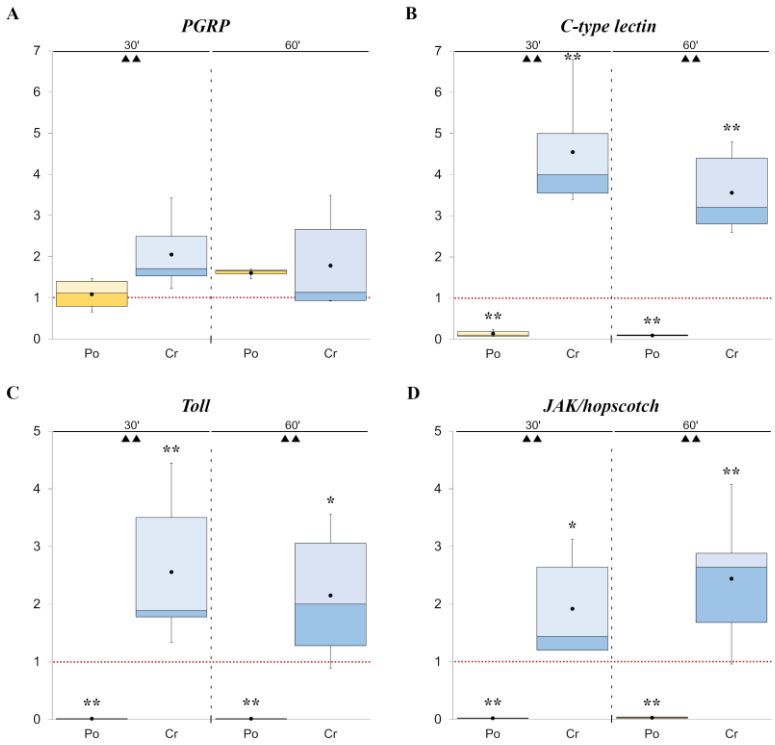


Figure 56. Transcriptional activity of *PGRP*, *C-type lectin*, *Toll* and *JAK/hopscotch* genes in natural populations of *P. olivacea* (Po) and *C. riparius* (Cr) larvae exposed 39 °C heat-shock for 30' and 60'. Box and whisker plots represent the expression patterns of *PGRP* (A), *C-type lectin* (B), *Toll* (C) and *JAK/hopscotch* (D) measured by RT qPCR. Results were normalised to control values (horizontal red dotted line). Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; the mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.05$ (**), $p \leq 0.1$ (*). Triangles indicate significant differences between species: $p \leq 0.05$ (▲▲).

5.4.2.4.3. Comparative summary between species of the effects of temperature shift in the immune system response

- 35 °C and 39 °C heat-shock produced significant differences between *P. olivacea* and *C. riparius* in the expression of genes involved in the signalling process of the immune system. While a strong transcriptional inhibition, up to 97 % below control values, was produced in *Toll* and *JAK/hopscotch* genes of *P. olivacea* larvae, a generalized overexpression was reported under the same premises in *C. riparius*.

- On the one hand, among the two PRP analysed genes, 35 °C produced significant ($p \leq 0.1$) species-dependent alterations at the longest tested times in the expression of *C-type lectin*, while higher temperature (39 °C), was translated into differences between species at 30' and 60'. In summary, temperature shift produced a generalised *C-type lectin* downregulation in *P. olivacea* whilst enhancing the gene transcription in *C. riparius*.

- On the other hand, the other PRP analysed gene, *PGRP*, was upregulated in both species upon exposure of the larvae to heat-shock. Nevertheless, the transcription activation was stronger in *C. riparius* than in *P. olivacea*, reporting significant differences at 35 °C 60' and 39 °C 30'.

Table 24. Summary table of the effects of heat-shock in *P. olivacea* on the cell stress and immune system response. Transcriptional changes that heath-shock have on the cell stress and immune system response in natural populations of fourth instar larvae *P. olivacea* exposed to 35 and 39 °C for 30', 60', 120' and 120' followed by 2 h at room temperature. Changes with respect to control values in the expression of key genes involved in the cell stress response and immune system are codified as follow, red: statistically significant downregulation (dark: p ≤ 0.05, light: p ≤ 0.5), green: statistically significant upregulation (dark: p ≤ 0.05, light: p ≤ 0.5), white: no significant changes were detected.

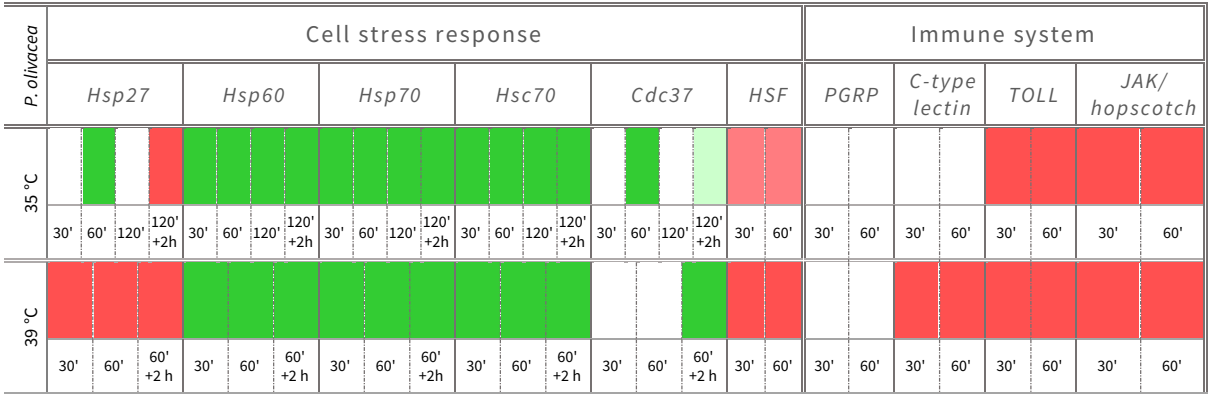


Table 25. Summary table of the effects of heat-shock in *C. riparius* on the cell stress and immune system response. Transcriptional changes that heath-shock have on the cell stress and immune system response in natural populations of fourth instar larvae *C. riparius* exposed to 35 and 39 °C for 30', 60', 120' and 120' followed by 2 h at room temperature. Changes with respect to control values in the expression of key genes involved in the cell stress response and immune system are codified as follow, red: statistically significant downregulation (dark: $p \leq 0.05$, light: $p \leq 0.5$), green: statistically significant upregulation (dark: $p \leq 0.05$, light: $p \leq 0.5$), white: no significant changes were detected.

C. riparius	Cell stress response																Immune system									
	Hsp27				*Hsp60*				*Hsp70*				*Hsc70*				*HSF*		*PGRP*		*C-type lectin*		*TOLL*		*JAK/ hopscotch*	
35 °C																										
	30'	60'	120'	120' +2h	30'	60'	120'	120' +2h	30'	60'	120'	120' +2h	30'	60'	120'	120' +2h	30'	60'	30'	60'	30'	60'	30'	60'	30'	60'
39 °C																										
	30'	60'	60' +2 h		30'	60'	60' +2 h		30'	60'	60' +2h		30'	60'	60' +2 h		30'	60'	30'	60'	30'	60'	30'	60'	30'	60'

Table 26. Summary table of the comparative effects of heath-shock in *P. olivacea* and *C. riparius* in the cell stress and immune system response. Comparative transcriptional changes that heath-shock have in the cell stress and immune system response in natural populations of fourth instar larvae *P. olivacea* and *C. riparius* exposed to 35 and 39 ºC for 30', 60', 120' and 120' followed by 2 h at room temperature. Comparative inter-species changes in the expression of key genes involved in the cell stress response and immune system. Triangles indicate significant differences between species: $p \leq 0.05$ (▲▲), $p \leq 0.1$(▲).

P. olivacea vs *C. riparius*	Cell stress response																		Immune system							
	Hsp27				*Hsp60*				*Hsp70*				*Hsc70*				*HSF*		*PGRP*		*C-type lectin*		*Toll*		*JAK/ hopscotch*	
35 °C	▲▲	▲▲	▲▲	▲▲	▲▲	▲▲	▲▲	▲▲	▲▲	▲▲	▲▲	▲▲	▲▲	▲▲	▲▲	▲▲	▲▲			▲		▲	▲▲	▲▲	▲▲	▲▲
	30'	60'	120'	120' + 2h	30'	60'	120'	120' +2h	30'	60'	120'	120' + 2h	30'	60'	120'	120' + 2h	30'	60'	30'	60'	30'	60'	30'	60'	30'	60'
39 °C	▲▲	▲▲	▲▲		▲▲	▲▲	▲▲		▲▲	▲▲			▲▲	▲▲	▲▲	▲▲	▲▲	▲▲	▲▲		▲▲	▲▲	▲▲	▲▲	▲▲	▲▲
	30'	60'	60' +2 h		30'	60'	60' +2 h		30'	60'	60' +2 h		30'	60'	60' +2 h		30'	60'	120'	120' + 2h	30'	60'	30'	60'	30'	60'

DISCUSSION

6. DISCUSSION

The assessment of ecosystem health is a complex process that requires a multidisciplinary approach and a variety of biological indicators to provide a detailed overview of the alterations that may be taking place. In this research, we have studied: 1) the molecular effects of three xenobiotics, benzyl butyl phthalate (BBP), bisphenol A (BPA) and benzophenone 3 (BP3) and 2) the effect of heat-shock. In aquatic ecotoxicology, model species, such as *C. riparius*, are largely used since they have a broad scientific consensus that allows the assessment of environmental disturbances from molecular mechanisms to population dynamics. However, many other species could also be considered as good bioindicators, providing a more global vision of the effects that pollution can cause in organisms that are chronically exposed in natural scenarios. In the present study, we analyse *P. olivacea* as a sentinel non-model organism to study the health status of aquatic ecosystems. One of the main difficulties to work with a non-model organism is the lack of available information. To date, the significant gap of *P. olivacea* genetic information has made it difficult to use in this type of study. In ecotoxicological risk assessment, it is essential to identify and validate biomarkers routinely used in gene expression studies to analyse ecosystems health.

6.1. *P. olivacea de novo* transcriptome

RNA-seq is a cost-effective and powerful tool for obtaining many functional genes in non-model organisms. In recent years, the number of different nature research projects involving transcriptomic analyses has sharply increased due to the versatility and variety of applications of this technique. In the present research study, we have generated for the first time a *de novo* reference assembled transcriptome of *P. olivacea* consisting of 34900 contigs with an N50 length of 1334 bp and

22413 annotated unigenes distributed in 62 GO terms. From the 62 GO terms, 22 of them corresponded to biological processes, 21 to molecular function and 19 to cellular components. Our results are in the value ranges of published dipteran transcriptomic studies.

Recently, Wei *et al*. (2020) published the assembly of the transcriptome of *Chironomus dilutus* as part of an ecotoxicogenomic approach. The *de novo* assembling revealed 36797 unigenes with an average length of 1168 bp, which is in accordance with our data. A similar range of transcripts was reported by Chen *et al*. (2014) in the malaria vector (*A. sinensis*) that notified 38504 contigs from which 26650 unigenes were annotated in 62 GO terms and with an N50 length of 711 bp. Also, a previous transcriptome sequencing of *Anopheles funestus* released by Crawford and collaborators (2010) described 46987 contigs with an N50 length of 1140 bp and a total of 7567 unigenes annotated in 119 GO categories.

Finally, more than 29600 unigenes from which 21650 were categorised into 60 subcategories in the vegetable pest, *Delia antiqua*, with an average N50 length of 818 bp (Zhang, Y.J. *et al*., 2014).

The *P. olivacea* GO assignment matches the transcriptome of these three animal species, in which binding, cell, metabolic processes and biological regulation were the most representative groups. Contrary to these studies, there was no genome reference for *P. olivacea*, which comprised a challenge for the *de novo* assembly and annotation. Nevertheless, the fact that 50 % of the contigs are longer than 1334 bp, added to the number of found genes that take part in a broad range of functions (e.g. biological and metabolic processes, immune system, development and growth) and the moderate proportion of unigenes with no hit on the GO categorisation (31 %), evidences the quality of the obtained data. This *de novo* transcriptome provides a valuable resource for future molecular analysis and assemblies of closely related species.

6.2. Xenobiotic exposure

6.2.1. Cell stress response

A wide variety of responses are activated when organisms are exposed to environmental disturbances that can imbalance their physiological parameters with the dangerous effects that this can have on them. Insects survive under a broad range of environmental stressors, including xenobiotics, metals, metalloids, temperature, radiation, desiccation, and osmotic and infection stress. Among others, this stress tolerance is due to the rapid activation of the cellular stress response, which comprises the synthesis of a diverse suite of heat-shock proteins (HSPs). Under normal conditions, these HSPs facilitate the folding and assembly of newly synthesised proteins, while under conditions of environmental stress, they protect target proteins and repair denatured proteins (Gupta *et al*., 2010).

There are no previous data regarding the effects of BBP, BPA or BP3 on genes related to cell stress response in *P. olivacea*. In this work, BP3 showed the most toxic effects since the four analysed genes were statistically significant deregulated. Significant transcription repression of *Hsp70* was observable after both exposures times as well as in *Hsp27*, where even a complete loss of expression was notified after BP3 4 h exposures. Our results are contrary to those observed in laboratory cultures of *C. riparius* exposed to higher concentrations of this UV filter for 24 h, where the compound led to an increase in the transcription of these two genes (Martín-Folgar *et al*., 2018; Ozáez *et al*., 2014). In the previously mentioned work, Martín-Folgar and collaborators (2018) also reported no transcriptional changes in the *Hsp60* gene in *C. riparius* while our results pointed out an acute induction of this gene at both tested times together with the induction of the cognate form *Hsc70* after 24 h BP3 exposures. Although some heat-shock proteins, especially *Hsp90* and *Hsc70*, are required for ecdysone receptor activity to contribute to the steroid cell

signalling (Gehring, 1998), the decrease of *EcR* levels under BP3 treatments evidence the protective role of *Hsc70* in the context of the study.

It is striking the effects that BBP provoked in *P. olivacea*: a complete loss of *Hsp27* and *Hsp60* expression combined with the *Hsp70* decrease and no changes in the *Hsc70.* These data partially differ from the data obtained by Herrero *et al*. (2014) on natural populations of *C. riparius* whose *Hsp70* transcript levels were sharply upregulated in contraposition to the unaltered levels of *Hsc70* after 24 h of BBP exposure. However, the same author reported no changes in the expression of *Hsp70* of laboratory cultures of *C. riparius* exposed to the same stress conditions as natural populations (Herrero *et al*., 2015). This evidences that responses observed in laboratory populations of model organisms may not be extrapolated to natural scenarios. In addition and contraposition to *P. olivacea*, *Hsp27* levels of *C. riparius* cultures remained unchanged when exposed to the phthalate (Herrero *et al*., 2015).

In *D. melanogaster*, *Hsp27* has been associated with starvation resistance, and in some species starvation has been reported to influence the expression of some Hsp genes (Hao *et al*., 2007; Nguyen *et al*., 2017; Tian *et al*., 2018). Inhibition of *Hsp27* has been reported in *Musca domestica* after 6 h starvation, while induction of *Hsp60, Hsc70* and *Hsp90* in a starvation-time dependent way was reported in *Pteromalus puparum* (Hymenoptera) (Tian *et al*., 2018; Wang *et al*., 2012). Moreover, 24 and 48 h starvation tests led to an *Hsp90* increase and no significant changes in *Hsp70* in the lepidopteran *Ectomyelois ceratoniae* (Farahani *et al*., 2020). These findings suggest that responding expression of Hsp genes to starvation depend on Hsp types and starvation duration. Although the non-exposed *P. olivacea* individuals were submitted to the same starvation times as those exposed to xenobiotics, one possible explanation of the scenarios where no expression of *Hsp27* and *Hsp60* was

detected could be the combination of two stressors: starvation and xenobiotic exposure. This supposition is in line with our hypothesis of the high sensitivity of this non-model dipteran which may be translated into an incapacity of activating the cellular stress response in the account of multiple stressors of different nature. As of last, *Hsp60* plays an active role in folding proteins in mitochondria using ATP, and its inactivation has resulted in embryonic lethality in mice (Boshoff, 2015; Christensen *et al*., 2010). Obtained *P. olivacea* data showed for the first time the complete loss of expression of this gene in the account of BBP exposures which may affect the proper protein folding in the mitochondria affecting the health status of the natural population severely.

From the three tested xenobiotics, BPA was the one that triggered less significant changes in the cell stress response of the non-model insect of study. In contraposition to the *Hsp27* upregulation reported in laboratory cultures of *C. riparius* (500 µg/L BPA), individuals of *P. olivacea* exposed to BPA experienced a completely silencing of gene transcription after 4 and 24 h (Martínez-Paz *et al*., 2014). The levels of *Hsc70* resulted unaltered, which is in line with similar results observed in *C. riparus* exposed to higher concentrations of BPA (Morales *et al*., 2011) and in contraposition to the upregulation reported in *C. tentants* (Lee *et al*., 2006). As of last, *Hsp70* induction has been reported in larvae of *M. galloprovincialis* exposed to 1 µg/L BPA after 24 and 48 h, which is in agreement with the *Hsp70* peak reported in *P. olivacea* exposed during 4 h, and in contrast to the values lower than controls after 24 h (Balbi *et al*., 2016).

In terms of the cellular stress response, there are no previous published data that report comparable *P. olivacea* results. This work shows for the first time novel and interesting data regarding the cell stress response in *P. olivacea*. Bibliography highlights a general induction of *Hsp* genes regardless of the stress condition and the invertebrate organism of study. For example, individuals of *C. riparius* exposed to reclaimed water

experience higher transcript levels of a pool of analysed *Hsp* (Planelló *et al*., 2020), desiccation and starvation slightly increase basal *Hsp* expression on the forest ant *Aphaenogaster picea* (Nguyen *et al*., 2017) and xenobiotic exposure induce Hsp pathway activation in the moth *Sesamia nonagrioides* (Michail *et al*., 2012).

HSPs are proteins candidates to play a significant role in the adapting natural populations to their environments (Harada and Goto, 2017). The generalised downregulation of the cell stress response in individuals of *P. olivacea* exposed to BBP, BPA and BP3 could compromise the tolerance and resistance of this species and their adaptation. Having said this, this no extreme tolerance of *P. olivacea* can be used as a double-edged sword in ecotoxicological studies and risk assessments where model organisms might be too tolerant, and some effects may be minimised.

The comparative analysis between species revealed opposite responses in natural populations of the model organism *C. riparius* exposed to the same stress conditions as *P. olivacea*. *C. riparius* cell stress response was mildly activated when exposed to the three xenobiotics. In addition, while BP3 reported the most toxic effects in *P. olivacea*, *C. riparius* transcript levels of *Hsp27* and *Hsp60* remained closed to control values in contraposition to the significant and quick *Hsp70* and *Hsc70* activation. Many HSPs are constitutively expressed, leading to a permanent stock of HSP proteins to deal with various processes (Chen, B. *et al*., 2018). This partial cell stress response activation may be due to the high tolerance of this dipteran that is already adapted to different stress conditions, and therefore since the cell stress response is energetically expensive, it limits the cellular stress response. We could conclude that energy saving has probably been used in favour of other molecular pathways.

These novel results suggest that BBP, BPA and BP3 may compromise the cell stress response of the non-model *P. olivacea* in different ways. They provide valuable information about cell stress response in this species and highlight that despite being an aquatic dipteran closely related to *C. riparius*, the magnitude and way of activation of the cellular response is different between both species. The inability to properly activate the transcription of cell stress-related genes together with the loss of their expression may minimise *P. olivacea* tolerance. In other words, the quick and acute activation of the cellular stress pathway in *C. riparius* compared to *P. olivacea* may confer a high resistance and adaptability of the model organism under certain circumstances. This high tolerance could minimise certain harmful xenobiotic effects when using only *C. riparius* for ecosystem health assessment. This work provides for the first time new potential biomarkers of the cell stress response in *P. olivacea* that could be useful for water monitoring and complement *C. riparius* data obtained in natural scenarios.

6.2.2. Endocrine system

Hormonal control is essential for the coordination and regulation of many aspects of the developmental process of insects. Their endocrine system is one of the best studied, and the ecdysone genomic response has been largely characterised in model organisms such as *Drosophila* spp. or *Chironomus* spp. Moreover, the endocrine system is an important link between the target cell and the environment, and it is axiomatic that environmental factors are major players in their regulation. In this work, we have characterised for the first time in *P. olivacea* the effects of BBP, BPA and BP3 on three key genes involved in the control of insect development: *EcR*, *Kr-h1* and *JHEH*.

Ecdysteroids tightly regulate development, growth, moulting and metamorphosis in insects. From an endocrine point of view, metamorphosis is regulated by two types of hormones: moulting and juvenile hormone (JH). One of the most active moulting hormones, 20E, plays an essential role in coordinating developmental transitions by interaction with a heterodimeric nuclear hormone receptor complex encoded by *EcR* and *Usp* genes (Uryu *et al*., 2015). The induction of the nuclear receptor *EcR* activates a cascade of hormonal effector genes that regulate cellular changes associated with moulting and metamorphosis. In addition, *EcR* activation triggers an autoregulatory loop that increases the receptor protein level in response to hormone ligand (Koelle *et al*., 1991; Spindler *et al*., 2009).

In contraposition, JH plays a crucial repressive role in insect metamorphosis since it modulates the quality of the moult, from an immature stage when it is present to the adult when it is absent (Lozano and Belles, 2011). JH signalling activates the transcription of *Kr-h1*, a zinc finger anti-metamorphic transcription factor that represses the expression of 20E primary-response genes to prevent 20E-induced metamorphosis. JH titers are regulated at critical times, and its degradation mainly occurs by the activation of juvenile hormone esterase (JHE) and/or juvenile hormone epoxide hydrolase (JHEH), which lead to a signalling decrease to permit metamorphosis (Goodman and Cusson, 2012).

Until date, there are no previous data concerning the effects of BBP, BPA and BP3 on the endocrine system of *P. olivacea*. Transcriptional *EcR* study showed that BBP and BP3 triggered a time-dependent downregulation on the nuclear receptor. This BBP hormonal antagonist effect is in contraposition to the dose-dependent agonist BBP action reported in *C. riparius* exposed during 24 h (Herrero *et al*., 2015; Planelló *et al*., 2011). Nevertheless, Herrero and collaborators (2015) showed that

longer BBP exposures led to a significant *EcR* repression meaning that BBP acts as a hormone antagonist in such conditions. This work in *C. riparius* reveals a time-dependent response of the nuclear receptor with a strong repression level after 48 h while similar inhibition was reached in *P. olivacea* after 24 h, which may be due to the high sensitivity of this aquatic dipteran.

In contrast to the antagonist UV effect reported in *P. olivacea* individuals, previous studies demonstrated a weak BP3 effect in the transcription of the ecdysone receptor in *C. riparius* larvae exposed during 24 h. However, it affects the transcription of different hormonal-related genes, mimicking ecdysone's action (Ozáez *et al*., 2016, 2014, 2013). There are no other studies in the literature assessing the hormonal effect of BBP and BP3 in insects from a molecular point of view. Finally, our *P. olivacea* BPA treatments showed a clear 4 h *EcR* induction followed by mRNA values dropping below control levels after 24 h. It seems that similar to what Herrero *et al*. (2015) reported for BBP, in *P. olivacea* BPA acts in very short exposure times as a hormone agonist while it has an opposite effect after 24 h. A general BPA agonist effect has been reported in midges and moths (Kontogiannatos *et al*., 2015; Planelló *et al*., 2008) in contraposition to the antagonist effects described in crustaceans (Kim *et al*., 2019), earthworms (Novo *et al*., 2018) and in copepods (Hwang *et al*., 2010) at longer exposures as well as in lepidopteran (Maria *et al*., 2019).

While neither BBP nor BPA triggered changes in the mRNA levels of the anti-metamorphic transcription factor *Kr-h1* in *P. olivacea*, the UV filter increased its expression after 4 h followed by a gene expression drop after 24 h. Despite until date, there is no data regarding the effect of these xenobiotics in the expression of *Kr-h1* in insects, Aquilino *et al*. (2016) reported a gene upregulation in *C. riparius* in fourth instar larvae exposed to a fungicide, while other works have reported no gene changes in the

event of ultraviolet filters (Muñiz-González and Martínez-Guitarte, 2018) and ibuprofen treatments in this model organism (Muñiz-González, 2021).

As of last, our data showed that the transcription of *JHEH* was time-dependent repressed regardless of the xenobiotic of study in *P. olivacea* exposures. In terms of ecotoxicological risk assessment, to date, little attention has been paid to this gene as a biomarker, and there is no literature related to insect transcriptional *JHEH* changes in the account of xenobiotics exposure. Nevertheless, the number of pest management studies pointing out the juvenile hormone epoxide hydrolase as a promising target has surged in the last years. Increased levels of this gene have been reported in the thiamethoxam resistant strain of *Aphis gossypii* (Ullah *et al*., 2021), in contraposition to unaltered *JHEH* mRNA levels in resistance to acetamiprid (Zhang, H. *et al*., 2020). These cases reported in the same species with different neonicotinoid insecticides suggest that the regulation of *JHEH* depends on the nature of the substance that individuals are exposed to. Therefore, an exhaustive and individual assessment should be done for a robust conclusion. Because of the critical role in the regulation of JH in insects, *JHEH* should be considered of great interest in risk assessment since it may be helpful for a better understanding of the mechanisms of action of certain xenobiotics in terms of hormonal pathway deregulation.

Our results suggest that the three xenobiotics tested, BBP, BPA and BP3 interfered with the endocrine system of *P. olivacea* through interaction with the ecdysone receptor and, consequently, on the ecdysone-mediated response of this aquatic dipteran. While BBP and BP3 acted as a time-dependent antagonist, BPA behaved as an ecdysone-mimetic in very short-term exposures instead of longer acute treatments where the nuclear receptor is significantly downregulated. These novel findings suggest that BBP and BP3 might impair the time development due to the combination of *EcR* and *JHEH* repression, which may be

translated into a longer persistence of the juvenile forms. Moreover, from all the study scenarios, the most developmental delay may be expected in those larvae exposed to the UV filter during 4 h due to the combination of the three anti-metamorphic pressures they appear to be subjected (*EcR* and *JHEH* downregulation in addition to *Kr-h1* increase).

The results of this study identify the endocrine-disrupting ability of BBP, BPA and BP3 on natural populations of *P. olivacea* and add new potential molecular biomarkers for freshwater monitoring of endocrine disruption.

The comparative analysis between *P. olivacea* and *C. riparius* highlights significant differences in the effects that BBP, BPA and BP3 have in the endocrine system of these two chironomids. An important difference is that all three xenobiotics act as hormone agonists in *C. riparius* after 24 h of exposure, instead of the ecdysteroid-antagonist effect of BBP and BP3 reported in *P. olivacea* fourth instar larvae. In addition, while BBP and BPA exposures did not affect the transcription of the late response gene *Kr-h1*, *C. riparius* overexpressed this gene after 24 h of the three treatments. This differential endocrine response of natural populations of closely related species indicates that nearby species may show different sensitivity to the same study scenarios in essential pathways such as the hormonal system. These differences may have a different impact on the growth, development and reproduction of these aquatic dipterans. In terms of risk assessment, our findings evidence the need for complementary studies to model organism ecotoxicological approaches to have a broader view of xenobiotic effects in chronically exposed natural populations of insects.

6.2.3. Biotransformation and oxidative stress

ROS are formed in those organisms exposed to a variety of toxic chemicals and play an essential role in the occurrence of oxidative stress in terms of the disturbance in the pro-oxidant and antioxidant balance in favour of oxidants (Macherey and Dansette, 2008). The accumulation of ROS in the cells can involve potential toxicity since they can damage a variety of essential macromolecules such as nucleic acids, lipids and proteins. The evolution of the antioxidant defence system allows organisms to prevent oxidative damage caused by toxic substances and to maintain body homeostasis (Ighodaro and Akinloye, 2018). Detoxification enzymes are critical components of this system, and their genes are expected to respond to toxic chemicals exposure.

The genes *MnSOD*, *CAT* and *PHGPx* are part of the first line of defence in the antioxidant response since they act to suppress or prevent the formation of free radicals or reactive species in cells (Ighodaro and Akinloye, 2018). This collection of antioxidants, together with the cytochrome P450 monooxygenases, have an essential and indispensable role in maintaining the normal metabolism of organisms. MnSOD is an enzyme located in the mitochondria and participates in the oxidative stress response. It catalyses the conversion of the singlet oxygen (O_2•) or superoxide anion radical (O_2^-•) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Ighodaro and Akinloye, 2018). Catalase, an enzyme abundant in the peroxisomes, breaks down H_2O_2 into water and molecular oxygen. Finally, GPx reduces the H_2O_2 generated in the mitochondria into water and lipid peroxides (Ighodaro and Akinloye, 2018).

P450 monooxygenases constitute a diverse enzymatic system involved in the phase I detoxification mechanism and, therefore, in the oxidative stress response. *Cyp4g15* encodes a monooxygenase enzyme mainly present in the nervous system of insects (Maïbèche-Coisne *et al*., 2000). It is involved in the catabolism of xenobiotics like drugs, pesticides and

plant toxins, as well as in the anabolism of endogenous compounds (Scott, 1999). CYP6A2 is a cytochrome abundant in the Malpighian tubules whose ability to metabolise pesticides has been recognised (Amichot *et al*., 2004; Wang *et al*., 2004). In addition, the *Cyp6a14* gene, together with *Cyp6a2*, has been described as cytochrome P450-mediated detoxification resistance genes since their expression has been reported to be significantly increased in resistant insects compared to susceptible ones (Oppert *et al*., 2015; Xi *et al*., 2015).

Finally, various of transferases take part in the phase II detoxification mechanism. GSTs play a central role in detoxifying endogenous and xenobiotic compounds. and they are also involved in key processes such as intracellular transport, biosynthesis of hormones and protection against oxidative stress (Enayati *et al*., 2005). In addition to GSTs, GR and the levels of GSHt constitute the antioxidant defence. Since variations in the antioxidant defence at the mRNA and enzymatic level can be very sensitive in revealing a pro-oxidant condition, they constitute a powerful molecular tool in terms of oxidative stress biomarkers.

Our own previously published paper was the first work that described the effects of BBP, BPA and BP3 in natural populations of *P. olivacea* on genes related to the detoxification mechanism (Llorente *et al*., 2020). In addition, there are no previous data regarding the effects of these xenobiotics at the enzyme activity level in this aquatic dipteran.

BBP was the xenobiotic that reported the most toxic effects on most of the analysed biomarkers since it induced significant repression after 24 of exposure, observable even after only 4 h in some cases (*MnSOD* and *CAT*). Similarly, reported data in laboratory larvae of *C. riparius* described the repression of detoxification-involved genes after 48 h of plasticiser exposure (Herrero *et al*., 2015). That said, it is important to mention the temporal difference in gene repression between Herrero's work and our

results, due to in the work of 2015, *C. riparius* 24 h BBP exposure did not alter the mRNA levels of *Cyp4g15* and *PHGPx*. Notwithstanding, as it was described previously in this manuscript for the cell stress response, a similar work on BBP previously carried out on natural populations of the model aquatic dipteran reported increased levels of *Cyp4g15*, which differs from the laboratory data obtained and our results in *P. olivacea* (Herrero *et al*., 2014). This evidences one more time the differential response between laboratory cultures and natural populations exposed to the same stress conditions and highlights that those effects observed in laboratory cultures of model organisms may not be always extrapolated to natural scenarios.

Preliminary work with earthworms showed the formation and accumulation of ROS in a BBP dose-dependent manner (Song *et al*., 2019). The increased enzyme activity of SOD, CAT and GPx in fourth instar larvae of *P. olivacea* exposed to this xenobiotic could be interpreted as a first oxidative stress response related to the sharp increase of ROS content derived from BBP exposure. These results are consistent with other works in invertebrate species such as gastropods (Aljahdali and Alhassan, 2020), mussels (Boudjema *et al*., 2014), clam (Zhang, H. *et al*., 2014) and scallops (Guo, R. *et al*., 2017), whose SOD and CAT activity increased when exposed to heavy metals and mixtures of polyaromatic hydrocarbons.

Moreover, the accumulation of ROS may lead to the inability to trigger gene transcription and the inactivation of functional proteins. This could explain the reported significant downregulation of genes involved in the first barrier of the detoxification mechanism in *P. olivacea*. In line with previous enzymatic studies, the fact that GST increased in the short term, followed by a drop below control values after 24 h, could be due to the active interaction of phase I enzymes, which decreases the impact of the pollutant by weakening the induction of GST (Ma *et al*., 2017; Song *et al*., 2018). Finally, the depletion of the total content of GSH in those

individuals of *P. olivacea* exposed to BBP during 24 h could be the result of xenobiotic and ROS conjugation reactions resulting in GSH consumption which may decrease the defence potential leading to oxidative stress. In this regard, our data are consistent with previous results in bivalves that showed a correlation between xenobiotic exposure and a decrease in GSH total levels (Jiang, F. *et al*., 2015; Sureda *et al*., 2011).

Overall, the combination of enzyme activity and transcriptional gene data suggests that *P. olivacea* larvae exposed to BBP undergo oxidative stress derived from ROS formation. On the one hand, this stress leads to an acute increase of phase I and phase II detoxification enzymes to counteract oxidative alterations, while on the other hand, the accumulation of ROS impedes the transcriptional activation of detoxification involved genes.

The pro-oxidative characteristics of BPA have been largely reviewed in numerous cell lines (Guo, J. *et al*., 2017; Porreca *et al*., 2016) and crustaceans (Zhang, Y. *et al*., 2020), where ROS levels increased when exposed to the endocrine disruptor. In addition, several studies have reported *in vivo* and *in vitro* genotoxic BPA effects in human HeLa cells and aquatic sentinel species such as *Daphnia*, *C. tentants* and *C. riparius* (Martínez-Paz *et al*., 2013; Park and Choi, 2007).

In our work, *P. olivacea* BPA exposures triggered similar responses than those observed and discussed before for BBP since general repression on the activity of detoxification genes was reported. Previous results in other organisms described a BPA-induced downregulation of *Cyp4g15* in *C. riparius* (Martínez-Paz *et al*., 2012), or *CAT* in the fish *Oryzias javanicus* (Woo *et al*., 2014). BPA also inhibited the expression of *SOD* in Atlantic salmon kidney cells (Yazdani *et al*., 2016) and the mollusc *M. galloprovincialis* (Balbi *et al*., 2016) in contraposition to the results

obtained in the nematode *C. elegans* (Zhou *et al*., 2016). In contrast to the reported data on *M. galloprovincialis* by Balbi and collaborators, transcriptional *CAT* levels dropped down in natural populations of *P. olivacea* when exposed to BPA. As of last, our data revealed an acute and significant downregulation in the mRNA levels of *PHGPx* after 4 h exposures ,while another work with *Daphnia* exposed to higher BPA concentrations, described transcript levels increase after 24 h exposures and the repression was seen at longer exposures (48 h) (Kim *et al*., 2019). This mixture of data might reflect that the transcriptional activity of certain detoxification genes could be species-specific, and therefore a particular study should be performed to better characterise xenobiotic effects on the detoxification mechanisms.

At the enzymatic level, the detoxification response of *P. olivacea* larvae exposed to BPA was less acute after 4 h of exposure, and the enzyme system took longer to respond. Since the pro-oxidative and genotoxic effects of BPA have been largely described, the hypothesis of the absence of ROS formation and, therefore, an absence of acute detoxification response could be discarded. In addition, while we may conclude that hydrogen peroxide (H_2O_2) was the direct mediator of oxidative stress in BBP exposures due to the increase in the enzymes that catalyse its decomposition (CAT and GPx), following the same line of thought, we might say that BPA exposures trigger the formation of superoxide anions. In (Lee and Choi, 2007), the authors discussed the high tolerance of *C. riparius* to various environmental stresses derived from their enzyme analyses that revealed GPx downregulation in addition to CAT and GST upregulation in the account of 24 h BPA exposures. Our data could conclude that the detoxification system of natural populations of *P. olivacea* takes longer to increase its activity to prevent harmful effects derived from BPA exposures. Therefore, this species' tolerance and capacity of adaptation might be compromised.

When exposed to BBP and BPA, *P. olivacea* vulnerability could be especially intensified considering the significant drop observed in *MnSOD*, *CAT*, *PHGPx* and *Cyp4g15* gene expression, presumably linked to the ROS accumulation. Besides, this generalised gene transcription inhibition observed in all the analysed pathways (cell stress response hormonal, biotransformation, and immune system) is probably an adaptative mechanism that larvae undergo to survive until the source of damage is over.

Concerning the UV filter BP3, a limited number of studies have been found in the existing literature on its effects of exposure in the detoxification system in other species. In our work, BP3 showed utterly different effects on the studied genes in *P. olivacea* since *CAT*, *PHGPx* and *Cyp4g15* showed a time-dependent upregulation in 24 h exposures which contrast with their drop at the transcriptional level induced by BBP and BPA. Our result agrees with the data reported in *Danio rerio*, whose *CAT* and *PHGPx* transcriptional levels increased in contraposition to a drop in *SOD* mRNA levels after BP3 48 h exposures (Rodríguez-Fuentes *et al*., 2015). A study by Martínez-Guitarte and colleagues (2018) performed in *C. riparius* larvae exposed to BP3 reported no effect on genes encoding different P450 cytochromes, in contrast to the activation of phase II-related genes.

Based on available literature, the pro-oxidative characteristics of this UV filter have not been dealt with in depth. A study with the coral species *S. pistillata* indicated its genotoxic effects and evidenced DNA damages (Downs *et al*., 2016). Based on the pro-oxidant state of *P. olivacea* larvae exposed to BP3, we could conclude that the filter triggered a ROS production since SOD, CAT and GST activity were increased after 4 h of exposure. In addition, the acute decrease in the total content of GSH evidences the high activity of conjugation reactions that are taking place. Previous work reported no changes in GST, CAT and GPx activities in

individuals of *M. galloprovincialis* (Bordalo *et al*., 2020) and the midge *C. riparius* (Campos *et al*., 2017) exposed to different concentrations of BP3, hypothesising that the stress induced by the UV filter was not high enough to increase enzyme activity in these species. Finally, phase I enzyme activities usually are generally recovered to control values at longer exposures, and phase II transferase was repressed. One possible explanation could be that the exposure of *P. olivacea* to BP3 provokes an acute increase in ROS production that is efficiently autoregulated with a drastic activation of the detoxification system. Moreover, this autoregulation could be linked with the important signalling function that ROS have under specific conditions (Nilangekar and Shravage, 2018). We may hypothesize that after 24 h, enzyme activities recover to control values, as this mechanism is energy-consuming for the individuals. Nevertheless, the rise in GSH total content at the longest exposure is in line with similar responses reported in mussels, where this response was interpreted as an indicator of oxidative stress (Dafre *et al*., 2004).

In summary, in light of transcriptional and enzyme activity data, we could conclude that BP3 exposures lead to ROS production that is counterbalanced by an enzymatic activity increase of phase I and phase II detoxification enzymes. However, the UV exposure does not produce significant changes at the transcriptional level in most of the analysed biomarkers.

Interestingly, mRNA levels of *Cyp6a2-like* and *Cyp6a14-like* genes remained unaltered in all tested scenarios (apart from BP3 24h). Since increased levels of *Cyp6a2* and *Cyp6a14* genes has been described as the cause of resistance to insecticides, as mentioned earlier, this invariability in the transcript levels could be one possible explanation of the sensitivity of *P. olivacea* to polluted environments.

One limitation of our research is that the lipid peroxidation was impossible to analyse since study samples were already homogenised when the pro-oxidant state analysis was performed. This is because it was not initially planned to include this experimental approach in the scope of this thesis and, consequently, all samples were homogenised for subsequent transcriptional measurements. Further research into lipid peroxidation levels under the different xenobiotic exposure conditions would be interesting to extend the knowledge and the degree of harmful effects these compounds have in *P. olivacea* individuals.

This is the first time that a molecular multi-level ecotoxicological assessment has been performed in natural populations of *P. olivacea*. These novel results suggest that BBP, BPA and BP3 may compromise the detoxification and oxidative stress responses in different ways. The study of the combination of transcriptional and enzyme activity changes provides valuable information about the detoxification mechanisms in the non-model species *P. olivacea*, and also about its ability to metabolise these compounds and trigger an adaptative response against toxic exposures in natural scenarios. Additionally, this study reveals new potential biomarkers of toxicity in *P. olivacea* that could be useful in water quality monitoring and complements *C. riparius* data obtained in natural scenarios. Our observations provide a broader view of possible molecular effects of natural stressors in these organisms, as well as novel information about crucial detoxification mechanisms.

Time-dependent alterations were observed in the comparative study of the effects of BBP, BPA and BP3 on exposed 4th instar larvae. The response of the studied genes differed depending on the species and the pollutant. In general, *C. riparius* showed a transcriptional and enzyme activity upregulation after 24 h exposure to any of the xenobiotics tested. In contrast, BBP and BPA caused a time-dependent inhibition in *P. olivacea* gene activity. In addition, *P. olivacea* showed an acute

generalised increase in the pro-oxidant state after 4 h exposures compared to *C. riparius*. This can be linked to the higher sensitivity of the non-model dipteran and the need for an acute enzymatic activity response to counteract the ROS production derived from xenobiotic exposure. A critical aspect of aerobic life is the potentially dangerous effects derived from the imbalance between antioxidant defences and oxidative forces. DNA damage, protein and enzymatic inactivation and peroxidation of cells components are some examples of deleterious effects reported in aquatic organisms as a consequence of a pro-oxidative state (Winston and Giulioz, 1991). Our comparative transcriptional study reveals that regardless of the ROS production, *C. riparius* is able to respond to pollutants by activating phase I and phase II reactions. In contrast, compounds could compromise the detoxification capacity in *P. olivacea* at longer times. The induction observed in *C. riparius* in detoxification genes may be the first step towards response against oxidative effects of BBP and BPA. This could be translated into a higher capacity to respond to possible harmful effects derived from a pro-oxidative state. On the other hand, the gene repression observed in *P. olivacea* could point out that these larvae do not activate the detoxification machinery, suggesting a lower capacity to respond to a potentially deleterious pro-oxidative state provoked by xenobiotic exposures.

It is worth mentioning the differences in the enzyme activities between controls since *P. olivacea* showed remarkably higher GST, GR and total content of GSH in the non-exposed individuals. Glutathione is involved in cellular protection against oxidative stress and numerous xenobiotics (Lushchak, 2011). In addition, Pedrosa *et al*., (2017) proposed that higher levels of GSH might provide a competitive advantage in native environments. Following our hypothesis of the higher tolerance of *C. riparius* compared to *P. olivacea*, these results seem contradictory.

This apparent lack of correlation could be explained by the need for *P. olivacea* to have higher basal enzyme activity to respond to drastic and unexpected oxidative stress conditions where *C. riparius* would not have any problem surviving. In addition, this effort in maintaining high basal enzyme levels to survive entails an energetic cost that cut back the energy investment for other essential processes such as development or growth.

6.2.4. Immune system and energy metabolism

The immune system prevents and limits infection. Unlike mammals, insects lack adaptative immunity, and their immune response is based mainly on nonspecific innate immune defences which comprise cellular and humoral responses. In the last term, humoral immunity triggers the induction of several immune genes that encode antimicrobial peptides (AMPs). Among these genes, *PGRP* and *C-type lectins* are receptors involved in recognising the pathogen via both innate responses. In addition, *Toll* and *JAK/hopscotch* are involved, among others, in the signalling cascades that regulate the expression of AMPs of the humoral immune response (Tsakas and Marmaras, 2010).

There has been a growing interest in using these genes as targets in the biomonitoring and risk assessment of environmental contaminants in recent years. These studies have been performed on vertebrate species due to the in-depth immunity knowledge and the capability to isolate organs relevant to this system, while invertebrate studies are much scarcer. Until the date, apart from the work of Zhang, Y. and collaborators (2020), there are no previous data regarding the effects of BBP, BPA and BP3 on genes related to immunity in any aquatic invertebrate species. This is the first time that transcriptional alterations upon the three xenobiotics of studies are described in natural populations of *P. olivacea*. In addition, recent works have suggested a close connection and interplay

between metabolic control and insect immune system regulation (Buchon *et al*., 2014; Sackton *et al*., 2017; Unckless *et al*., 2015). In the light of these findings, transcriptional alterations of *GAPDH* were also assessed in those organisms exposed to the three xenobiotics of study since GAPDH is an enzyme involved in energy production through glycolysis and also in multiple cellular processes such as apoptosis, oxidative stress, DNA repair or metabolic and physiologic pathways (Tristan, 2011).

In the present work, BBP was the xenobiotic that triggered the most transcriptional deregulation of the immune system in *P. olivacea* larvae. 4 h exposures led to an upregulation of the gene coding for the peptidoglycan recognition protein (PGRP), while after 24 h exposures, the transcription dropped below control values. *Toll* and *JAK/hopscotch* expression were time-dependent inhibited in contraposition to data obtained for *GAPDH* mRNA levels that followed the opposite tendency. These results are consistent with previous studies in crustaceans exposed to different phthalate esters, where the immune response was reduced in high concentration treatments (Chen and Sung, 2005; Su *et al*., 2017). This immune suppression derived from phthalate exposures has also been reported in the fish species *Pelteobagrus fulvidraco* (Mo *et al*., 2019), contrary to data obtained in *Oryzias melastigma* under similar xenobiotic exposures (Yin *et al*., 2021). This highlights the complexity of the immune system response and the fact that nearby species may show different sensitivity to the same study conditions. Finally, regarding *GAPDH* transcription, the metabolic activation reported in *P. olivacea* upon BBP exposure substantiates previous findings in the already mentioned work of Yin and collaborators (2021) on the marine medaka. This increase in the enzyme transcription could be a consequence of stressful condition exposures, which led to an increase in energy demand to tackle possible harmful repercussions.

Regarding the effects of BP3 on larvae of *P. olivacea*, while no changes were reported in any of the analysed genes encoding for recognition proteins, *Toll* and *JAK/hopscotch* were, once again, downregulated in a time-dependent manner, reaching average mRNA values lower than those of BBP exposures. Moreover, the transcription of *GAPDH* was also inhibited along the two tested times in contraposition to the obtained data for BBP treatments. These findings differ from the only available analogous work described in *C. riparius* exposed to two different ultraviolet filters where immune genes were unaffected regardless of single and binary UV filter mixture treatments (Muñiz-González and Martínez-Guitarte, 2018). According to the obtained time-dependent fall in *GAPDH* transcription, it appears that BP3 exposures may imply metabolic dysregulation which in the last instance could influence a wide variety of processes in *P. olivacea* such as energy metabolism, larvae growth or fecundity among others. In addition, since exposure to stress situations entails an increase in energy demand to maintain homeostasis, the inactivation of GAPDH reduces the ability of the organism to maintain optimal functioning conditions. Considering all these assumptions, the evidence from BP3 exposures suggests that the UV filter may have detrimental consequences at the individual and population level of *P. olivacea* as and the tolerance and adaptation of this species to changing environments.

Slightly different immune-related gene transcriptional alterations were reported when individuals of *P. olivacea* were exposed to BPA. On the one hand, 4 h exposures upregulated the expression of *PGRP* in contraposition to the inhibition of *C-type lectin*, although in both cases, the alterations were transient as values were recovered similar to control levels after 24 h. On the other hand, while 24 h BPA exposures led to acute repression of *Toll* and *JAK/hopscotch* genes as was previously reported for BBP and BP3 treatments, these genes showed a trend towards increasing after 4 h BPA exposures. This data differs from the *Toll* upregulation

reported in *M. galloprovincialis* and a salmon kidney cell line after 24 and 48 h of treatment (Balbi *et al*., 2016; Yazdani *et al*., 2016). However, generalised immunosuppression has been reported in other species, such as the mud crab *Macrophthalmus japonicus* or the crayfish *Procambarus clarkii* (Park *et al*., 2019; Zhang, Y. *et al*., 2020). In contraposition to these results, enhanced immune response in *in vitro* and *in vivo* mouse models and zebrafish embryos has been demonstrated (Sowers *et al*., 2020; Xu *et al*., 2013). Since many studies using a wide variety of species and models reveal conflicting results, until the date, there is not a clear consensus about the immune-suppressing or immune-stimulating effects of BPA. Finally, the energy metabolism analysed biomarker was unaltered in both case studies.

This study provides for the first time new putative biomarkers genes related to the immune system in *P. olivacea* that could be useful in ecotoxicological studies and risk assessment. Our results revealed the susceptibility of the immune system in this aquatic dipteran to three environmental xenobiotics widely distributed: BBP, BPA and BP3. In addition, they provide valuable information about the immune system modulation of this chironomid when exposed to chemicals of different nature and bring forward possible explanations of the hypothesised sensitivity of this species.

In the comparative study between the two dipterans, species-dependent alterations were observed in 4th instar larvae exposed to BBP, BPA and BP3. The most marked observation to emerge from the data comparison was the nearly unaltered or light tendency to increase the transcript levels of *Toll* and *JAK/hopscotch* genes in *C. riparius* regardless of the pollutant of study. Interestingly, transcription of *PGRP* was activated in *C. riparius* in a time-dependent manner in all three study scenarios, while different results were observed in *P. olivacea*, as previously was discussed. The fact that xenobiotic exposure activated the

recognition immune *PGRP* gene but did not trigger a downstream immune system response may be explained due to the high tolerance of *C. riparius*. In line with the high dipteran tolerance theory, pollutant exposures are not high enough damaging to trigger a complete immune system response, and larvae do not need to invest energy in this cost-demanding process.

From a biological point of view, an interesting question to address is whether these dipterans detect the xenobiotics as toxic and therefore activate their immune system response or whether this immune response is a side effect due to a cheated immune system. Due to until the date this system related to chemical exposure has been very little explored in insects, this question opens a great and a tough line of discussion. The main emerged hypothesis is that these compounds have not been detected as toxic by the immune system since the transcriptional activity of *PGRP* and *C-type lectin*, recognition-immune genes, is not strongly altered. Nevertheless, the evident inhibition of the signalling pathway in *P. olivacea* could be linked to the disturbance of the antioxidant system caused by exposure to xenobiotics. In line with this theory, some studies have linked the chemical exposure to the production of ROS, oxidative stress and, as last, suppression of the immune system (Tao *et al.*, 2016; Zhang, Y. *et al.*, 2020). Moreover, the lack of transcriptional alterations reported in immune system signalling genes in *C. riparius* could be explained by the capacity of this aquatic dipteran to counteract efficiently the ROS imbalance triggered by xenobiotic exposures.

As of last, hormones can affect innate and adaptive, humoral and cell-mediated immune responses, and an imbalance in hormone levels can lead to immune-mediated diseases (Yin *et al*., 2021). The differences observed between both chironomids in the regulation of immune response could be linked to the differential hormonal pathway alteration produced by these compounds.

This work outlines the differential responses in natural populations of closely related species exposed to the same stressful conditions. In addition, it highlights the need for complementing water quality monitoring *C. riparius* obtained data in those scenarios where the high tolerance of the aquatic midge may be minimising or declining possible detrimental effects leading to inconclusive conclusions.

As it has been previously reported in laboratory cultures and natural populations of *C. riparius* (Herrero *et al*., 2014, 2015), it is plausible that the origin of the larvae may influence the obtained results. This emphasizes the need of carrying out studies with different populations to get a more realistic approach to the effects of contaminants. The implementation of *P. olivacea* laboratory cultures would allow to carry out studies in different populations in order to define more precisely the effect targets that are altered by the exposure to these xenobiotics. In addition, it would help to unravel the type of response that they trigger depending on the particular circumstances of a real scenario, when larvae are chronically exposed to complex mixtures of contaminants.

6.2.5. Survival rates

Xenobiotics exposure spanned from 4 to 24 hours at a concentration of 1 µg/L. This concentration was chosen regarding previous published works in *C. riparius* (Herrero *et al*., 2016; Nair *et al*., 2013; Ozáez *et al*., 2016). BBP and BP3 concentrations of study are below to those recorded in the environment, and specifically in water resources over the world, while BPA is closed to concentrations found in freshwater (Clark *et al*., 2003; Höhne and Püttmann, 2008; Sánchez-Quiles and Tovar-Sánchez, 2015; Wu and Seebacher, 2020).

While none of the 4 h treatments had effect in *P. olivacea* and *C. riparius* larval survival, the mortality of both chironomids was significantly increased upon BBP and BP3 after 24 h of exposures. Moreover, 24 h BPA treatments also compromised *P. olivacea* survival.

BBP obtained data are in accordance with the reduction in survival detected in natural populations of *C. riparius* (Herrero *et al*., 2014) but in contrast to the lack of significant mortality reported in laboratory cultures of *C. riparius* under identical conditions, where the LC50 was stablished at $2.7 \cdot 10^4$ µg/L (Herrero *et al*., 2015). These lack of mortalities has also been detected for other invertebrate species such as *Chironomus tentants*, *C. elegans*, *Hyalella Azteca*, *Lumbriculus variegatus* or *Moina macrocopa* (Call *et al*., 2001a, 2001b; Kwon *et al*., 2011; Wang *et al*., 2011).

The lack of *C. riparius* significant mortality upon BPA exposure agrees with previously reported data in this and other model organisms, such as *C. elegans* or *Spodoptera littoralis* (Martínez-Paz *et al*., 2012, 2013; Maria *et al*., 2019; Hyun *et al*., 2021). This lack of significant mortality could be linked with the proposed strong pro-survival effects of this xenobiotic which in combination with other cytotoxic agents promote adaptative responses (Dobrzyńska and Radzikowska, 2013; Gassman *et al*., 2015; Gassman and Wilson, 2016; LaPensee *et al*., 2009). In contraposition to *C. riparius* and available bibliography, *P. olivacea* experienced a significant decrease in survival after 24 h of BPA exposure which supports our hypothesis of the higher sensitivity of this non-model organism.

Particularly in invertebrates, there is little information regarding the toxicity of BP3 in terms of survival rates. The last years, the most common studies regarding UV filters are in combination with microplastic or temperature changes exposures. The significant increase in mortality seen after 24 h BP3 exposures in *P. olivacea* and *C. riparius* differs to the lack of survival effects in *C. riparius*, the crustacean *D. magna* and the

bivalve *M. galloprovincialis* exposed to even higher BP3 doses (Bordalo *et al*., 2020; Boyd *et al*., 2021; Ozáez *et al*., 2013; Song *et al*., 2021). The reason for these differences could be the influence in the origin of larvae since, as we have mentioned previously, differences in toxicity among data obtained in laboratory cultures and natural populations have been reported. This emphasizes the need of carrying out studies with different populations to get a more realistic approach to the effects of contaminants.

As of last, the inter-species survival differences and the higher sensitivity of *P. olivacea* larvae, may be linked to the generalised strong transcriptional downregulation in essential pathways such as cell stress response, biotransformation and oxidative stress system or immune system, among others. In contraposition, the ability of *C. riparius* to successfully activate the detoxification system or the cell stress response results in a lower mortality rate and a high tolerance and survival capacity under the same stress conditions.

6.3. Heat-shock exposure

The evident global change is a prominent environmental problem that involves different processes, including climate change, which implies an increase in the mean temperatures of most areas of the planet, such as freshwater ecosystems. Temperature is one of the most critical environmental factors that affect metabolism, physiology, behaviour and life-history of ectotherms (Angilletta, 2009). In addition, the temperature is a physical factor that can alter the distribution of toxicants and the response of the organisms to them (Marchand and Haddad, 2017; Nadal *et al*., 2015). As poikilothermic organisms, many life-history parameters are influenced in insects as a cause of environmental temperature changes. Moreover, cold or heat stress frequently results in changes in metabolic rate, energy consumption, development and innate immune response (Sinclair *et al*., 2013; Williams *et al*., 2012). The influence of high temperature on different life-history parameters such as survival, mating, offspring production and even resistance to adverse conditions has been addressed in different insects (Abbes *et al*., 2015; Chen, H. *et al*., 2018; Jang and Lee, 2018; Zeng *et al*., 2018).

Alongside other factors, extreme temperatures trigger insect immune responses, and the positive relationship between temperature and immune responses has been described in the literature (Catalán *et al*., 2012; Shamakhi *et al*., 2019; Wojda, 2017). For all of this, the performance of individuals to adapt to temperature changing environments may have a direct impact on their survival, adaptation and tolerance. Therefore, knowledge about how insects react and adapt to global warming is one of the main challenges in predicting future biodiversity trends.

Although new data has become available recently (Guo, L. *et al*., 2018; Guo, S.N. *et al*., 2018; Harada and Goto, 2017; Muñiz-González and Martínez-Guitarte, 2020), there is a clear knowledge gap at the molecular level effects in invertebrates. Considering that global warming is one of

the most detrimental aspects of climate change and is expected to increase the temperature of freshwater ecosystems, it is essential to expand the awareness of the molecular effects that temperature shifts can have on aquatic insects.

On the one hand, the direct relationship between an increase in the expression of heat-shock proteins (HSPs) and exposure to environmental stresses such as extreme temperatures, hypoxia, infection or osmotic and oxidative stress has been demonstrated. HSPs act as molecular chaperones facilitating the proper folding of proteins during their synthesis, processing, degradation and translocation across cellular membranes (Wojda, 2017). Under non-stress conditions, there is a pool of these and their constitutive forms due to their essential protein folding function. In insects, four major families of these proteins are recognised: small heat-shock proteins (sHSPs), HSP60, HSP70 and HSP90.

On the other hand, the critical role of these stress proteins in immune responses has been established. In 2002, Matzinger exposed the danger model, which proposes that those HSPs proteins, among others, released from damaged host tissues can act as a danger/damage signal co-stimulating the immune response (Matzinger, 2002). Invertebrates rely on the innate immune system, which consists of both cellular and humoral components. Both responses are triggered by the recognition of PRPs, including C lectins and PGRP, as potential hazards. While cellular response involves phagocytosis and encapsulation, the humoral response involves the secretion of AMPs through the activation of three signalling pathways: Toll, Imd and JAK/STAT.

Finally, pathways responsible for the reaction of heat-shock and infection overlap and both involve HSF transcription. HSF binds to the promoters of HSP genes, and as a result of HSF activation, there is an acute increase in the transcription of these genes, but also immune genes are

transcribed. In the last few years, there has been an increasing number of reports underlying the interdependency between heat-shock and insect immune response showing that HSPs proteins play an essential role in the insect's immune response. On the one side, infection activates the expression of HSPs, and on the other side, exposure of organisms to heat-shock affects their pathogen's resistance (Adamo, 2010; Altincicek *et al*., 2008)

In this part of the work, we have analysed at the transcriptional level the cell stress response and the modulation of the immune system of 4th instar larvae of *P. olivacea* exposed to acute thermal stress (35 and 39 ºC for different times). In addition, to study possible differences in the tolerance to thermal shock, we have compared the transcriptional responses induced by the temperature shift in the non-model species *P. olivacea* with those gene expression changes obtained in the model organism *C. riparius* exposed to the same conditions of the study.

6.3.1. Cell stress response

As was mentioned throughout this thesis, the cell stress response of organisms comprises an orchestrated response where a wide variety of mechanisms are activated to mitigate the disturbances that can imbalance physiological parameters. HSPs are stress-response proteins essential for cell viability and are well known to respond to high temperatures, among other environmental stressors.

No previous data have been described concerning the effect that high temperatures have on the transcriptional expression of *HSPs* genes in *P. olivacea*. Exposures to 35 ºC were made up to 2 h, while the longest 39 ºC time was 1 h since this temperature seemed too extreme for *P. olivacea* and longer treatments were not viable in survival terms.

In this work, similar alterations were found when organisms were exposed to 35 and 39 ºC, since the transcription of *Hsp60*, *Hsp70*, *Hsc70* and the *Hsp90 co-chaperone Cdc37* was upregulated in comparison to controls, while *HSF* was strongly repressed. Nevertheless, considering 39 ºC data, we may hypothesise that the highest temperature could have more severe effects since the activation grade is minimised compared to the lower high extreme temperature. This data highlights *P. olivacea* sensitivity since although the cell stress response is activated at both temperatures, under extremely high temperatures a possible failure in the effective activation of this response could seriously compromise its adaptation and survival. Indeed, a work in the cold-stenothermal chironomid *Pseudodiamesa branickii* reported that although the organism can survive short-term heat shocks, at extreme temperatures the ability to survive was limited to a very short period of exposure (7 h at 32 ºC) (Bernabò *et al.*, 2011). This limited tolerance to extreme temperatures agrees with our obtained data since 39 ºC exposures needed to be shortened due to the mortality rate of *P. olivacea*.

In addition, from the already *HSPs* mentioned enhanced genes, only the transcript levels of *Hsp70* were recovered close to control values in those animals that were left to recover at room temperature during 2 h after 2 h 35 ºC temperature shock in contraposition to the rest of the conditions were mRNA values kept increasing. One possible explanation for these results may be that temperature shift is so aggressive for *P. olivacea* larvae that, even after a 2-hour recovery period, the transcription of genes involved in the cell stress response needs to be activated to maintain the organism's homeostasis and ensure its survival. Our data is in line with the available bibliography since the increase of HSPs expression in the account of heat exposures have been largely described in a wide variety of animals such as fungi (Mota *et al*., 2019),

molluscs (Feidantsis *et al*., 2020), arachnids (Niu *et al.*, 2020), insects (Chen *et al*., 2019; Lencioni *et al*., 2013; Martín-Folgar *et al*., 2015; Martínez-Paz *et al*., 2014; Shu *et al*., 2020) or fishes (Guo, S.N. *et al*., 2018; Yang *et al*., 2016) among others.

A remarkable result to merge from 35 ºC exposures is that the transcription of *Hsp60*, *Hsc70* and *Cdc37* reached the maximum levels after an hour of exposures, and after 2 h it tended to decrease followed by an increase during the recovery period without heat pressure. Interestingly, this tendency has been recently described in the hymenopteran *Bombus terrestris* (Blasco-Lavilla *et al*., 2021). This pattern might be related to the negative feedback on the regulation mechanism of Hsp expression and agrees with the idea that this downregulation during acclimatation to heat stress may be a mechanism to reduce the costs of a heat response in favour of fecundity and development (Hoffmann *et al*., 2003).

In contraposition, the activation of *Hsp70* followed a time-dependent increased tendency along all the testes times, reaching the maximum peak of more than 15900 % of upregulation after 2 h of exposition and sharply switched to transcript levels that, although still high (up to 977 % increase), were closer to control values after 2 h at RT. One possible explanation could be the central role that Hsp70 proteins play in the cellular network of molecular chaperones and folding catalysts. This upregulation may be a clear link with the high protein synthesis that the organisms might be experienced and the need to a proper protein folding regulation. Organism's survival is subjected to their correct functioning and transcriptional regulation upon different stressors. This could explain the maintained high time-dependent overexpression over the heat pressure and the quick recovery of mRNA values relatively closer to control levels immediately after stressor removal. In addition, it is important to bear in mind that the organisms have to find a balance

between activating the stress response to survive and the energy consumption that this process requires.

Finally, compared to the other heat stress analysed genes, it is worth mentioning the basal high transcript levels of the co-chaperone *Cdc37,* and thus the higher mRNA values reached when the animals were exposed to 35 ºC. The Hsp90 / Cdc37 complex stands at the hub of many intracellular signalling networks, and its effects have been described beyond the housekeeping protein folding pathways into the regulation of a wide range of cellular processes (Calderwood, 2015). Among others, *Cdc37* plays an essential role in cell growth pathways, which could explain of the high levels of this gene in unexposed animals.

As of last, while both temperature shift treatments led to a strong downregulation of the *HSF*, a remarkable difference between both exposure conditions was the deregulation of the small HSP, *Hsp27*. *HSF* inhibition results differ from some published studies (Klumpen *et al*., 2017; Niu *et al*., 2020; Xie *et al*., 2014), although the fact is that until the date, the number of available papers studying transcriptional *HSF* changes in animals exposed to heat-shock is limited. Instead, this gene is a key target under constant study in the genetic improvement of plants and important crops (Duan *et al*., 2019; Panzade *et al*., 2021; Wan *et al*., 2019). Considering that HSF is essential for mediating *HSP* transcription, of these findings refute our hypothesis about the sensitivity of *P. olivacea* since, under high extreme temperature conditions, its heat-shock response is compromised.

Although the initial impression of *P. olivacea* obtained data is an apparent contradiction with *HSF* downregulation and *HSPs* upregulation, these findings are in line with the work of Harada and Burton (2020). In the mentioned study, the heat response of the copepod *Tigriopus californicus* was upregulated in terms of *HSPs* transcript levels even in those *HSF-1*

knockdown groups. One reason for this non-correlated HSF-HSPs interplay may be the absence of HSEs in the transcription start site of certain *HSPs* genes. Moreover, although we cannot completely assure this, based on the sequence alignments and domains, we may say that the *HSF P. olivacea* characterised gene corresponds to the isoform 1 (*HSF-1*). Given that *Hsp90* and *Hsp70* negatively regulate *HSF-1* activation via an autoregulatory loop (Leach *et al*., 2012), this could explain the obtained acute *HSF* transcriptional repression in our study.

Finally, while *Hsp27* was upregulated in 35 ºC exposures, its transcription was significantly repressed in 39 ºC treatments. A generalise increase in the transcription of small *HSPs* has been described in the account of insects heat exposure and, specifically in *C. riparius* under the same exposure conditions as our study, which matches with 35 ºC results (Guo, H. *et al*., 2018; Martín-Folgar *et al*., 2015; Martínez-Paz *et al*., 2014; Singh and Tiwari, 2016; Zhang *et al*., 2016). However, this is the first time that a significant *Hsp27* downregulation has been reported in the account of heat stress exposure. We may hypothesise that 39 ºC exposures are such extreme conditions for *P. olivacea* larvae that the transcription of certain *HSPs* is compromised, which may ultimately impact the adaptation and survival of this species in natural scenarios. There are few examples of stronger heat responses of heat-acclimated insect species in the literature compared to unacclimated individuals (Gu *et al*., 2019; Quan *et al*., 2020). Our results have raised an interesting future research line that could be focused on the differential expression of acclimated and non-acclimated *P. olivacea* larvae to further study the thermal tolerance of this dipteran.

As last, although an acute and early increase in *HSPs* transcript levels is a defensive strategy that all organisms trigger against different stressors, it is essential to keep in mind that changes in mRNA expression may not be strictly linked to changes in protein activity. In this sense, the latter effect is extremely important since transcript levels may experience

a quick up/down-regulation, whereas their corresponding protein levels may rise slowly but persist longer (Feidantsis *et al*., 2020). Moreover, we cannot exclude the possibility that the over- or underexpression of chaperones may influence the stability of the proteins and their half-life. In a broader view, we propose that further research should be undertaken to determine the protein activity at longer times. Having said this, *P. olivacea* data have proven that *HSPs* transcript levels can be used as an early indicator of acute thermal stress in addition to *HSF* gene expression

According to the comparative inter-species study, the cell stress response upon heat-shock seems to be conserved in most of the analysed genes since a generalised upregulation took place. Nevertheless *C. riparius* and *P. olivacea* comparative analysis reported significant differences in the magnitude response between aquatic dipterans. In general, *C. riparius* showed a more acute and stronger upregulation of the transcript levels of the *HSPs* analysed genes at both study temperatures compared to *P. olivacea*. In addition, while the transcription of *HSF* was significantly silenced in *P. olivacea* individuals, the expression of this factor was upregulated in *C. riparius*. These differences might reflect a divergent thermal tolerance between both non-biting midges and emphasise the high tolerance that the model organism has under extreme conditions.

6.3.2. Immune system

Given that HSPs can act as a damage/danger signal co-stimulating the immune response (Matzinger, 2002), added to the fact that extreme temperatures trigger immune responses in insects, makes the immune system an exceptional pathway to study the effects of heat-shock exposures.

Despite this interest, no one has studied the modulation of the immune system response at the transcriptional level in the non-model species *P. olivacea* experienced when they are subjected to high temperatures. In the present work, albeit both heat-shocks led to a generalise acute repression of the immune system response, 39 ºC produced the most harmful effects since three of the four analysed biomarker genes were significantly repressed. It is worth noting that the downregulation took place in a really short period, such as 30 minutes. According to previous literature (Wojda, 2017), upregulation of immune genes in insects has been observed under thermal stress as a consequence of an interaction between immune and heat-shock responses. However, our data agree with Chen *et al*., (2019), interesting work with *Ostrinia furnacalis* (Lepidoptera), where they show that immune system genes were downregulated in heat-shock while *HSP* genes were upregulated under the same conditions. This inverse relationship between heat-shock and immune system response has also been described in the honey bee *A. mellifera* (McKinstry *et al*., 2017). This antagonistic response could be the evidence of two hypotheses: 1) a trade-off between the thermal and the immune response, agreeing with the high costs of activating the immune system, or 2) a more sophisticated/evolved system that better detects the dangers and triggers the most adequate and specific response for each of them.

Nevertheless, the comparative study between both chironomids evidenced completely opposite tendencies, showing overexpression of the immune system in those individuals of *C. riparius* exposed to heat-shock. This capacity of activating both responses could be an explanation of the high tolerance of this aquatic midge, whose rapid adaptation is already described and evidenced (Foucault *et al*., 2018; Pfenninger and Foucault, 2020), in contraposition to *P. olivacea* who needs lower extreme conditions in order to survive.

The interplay between the heat-shock and immune response have been evidenced in some works where it is proven how the cellular stress response confers insect protection against different viral and fungal infections (McMenamin *et al*., 2020; Merkling *et al*., 2015; Richards *et al*., 2017; Wrońska and Boguś, 2020). This link can directly impact the survival and adaptation of the species. The fact that *C. riparius* can activate a much more acute and faster response at the transcriptional level than *P. olivacea* could suggest lower responsiveness of the non-model organism to a potentially harmful context. These differences may reflect the ability of each species to adapt to altered environments. In addition, since both chironomids share habitat, the differential adaptative responses to the same stimulus may have a direct impact at the ecological level and may imply a way of competing.

In the line of the study of Richards and colleagues (2017), where they report an HS-induced increase in lepidopteran larval survival against fungal infection, an interesting future field of research could be the study of the transcriptional responses of chironomids adapted to extreme cold and hot temperatures and whether the response differs and confers any kind of advantage to survive.

In summary, the work described herein indicates that in contraposition to cell stress transcriptional modulation, the immune system response genes involved in recognition (*C-type lectin*) and signalling (*Toll* and *JAK/hopscotch*) pathways are significantly inhibited in those individuals of *P. olivacea* that are subjected to acute and short temperature shift treatments. The data obtained give us essential information concerning the effect of heat-shock on the ongoing expression of cell stress and immune-relevant genes. These findings are of especial interest since temperature changes may affect larval susceptibility to further scenarios where an effective and quick immune response could be the key for survival and adaptation. In addition, the

differential responses detected between *P. olivacea* and *C. riparius* provide novel information on the effects of thermal shock on aquatic midges and reflect the differences in the ability of each species to adapt to altered environments through heat-shock and immune system responses. Taken together, these differential expression changes could compromise in a species-specific way the ability of each population to manage the same changing conditions, which could interfere differently in the population viability.

CONCLUSIONS

7. CONCLUSIONS

1. The present work represents the first research in *P. olivacea* using high-throughput technologies. The assembled *de novo* transcriptome represents a valuable genomic resource for future applications, such as identifying potential molecular biomarkers to deepen the knowledge of the physiological effects of multiple scenarios of interest.

2. A total of nineteen genes involved in critical metabolic pathways have been identified and characterised for the first time in *P. olivacea* and could be used as molecular biomarkers of exposure and early effect to assess the health status of aquatic ecosystems and shed light on the mechanisms of toxic action of chemicals of interest.

3. Among the three xenobiotics tested, BBP is the most toxic for *P. olivacea* 4th instar larvae, leading to quick and robust inhibition of genes related to cell stress, immune system, biotransformation and oxidative stress.

4. BBP and BPA induce the greatest oxidative stress in *P. olivacea* by increasing the activity of phase I and phase II detoxification enzymes, thereby counteracting the accumulation of ROS that silences gene transcription.

5. Exposure of *P. olivacea* to BBP, BPA and BP3 leads to xenobiotic-specific differences in the modulation of gene transcription, illustrating the potential of the analysed genes to be used as sensitive molecular biomarkers in this species.

6. All three compounds interfere with the endocrine system of *P. olivacea*, triggering an ecdysteroid-antagonist effect in long-term studied exposures. These results show for the first time in this

non-model species the ability of BBP, BPA and BP3 to alter the endocrine signalling pathway by inhibiting *EcR* and *JHEH* genes expression, key elements for metamorphosis and development.

7. The comparative transcriptional analysis between *C. riparius* and *P. olivacea* shows differential species-specific gene regulation: xenobiotic exposure triggers early and strong transcriptional activation in *C. riparius*, whereas it leads to downregulation in *P. olivacea*.

8. The inter-species comparative study at molecular level, supports our hypothesis of higher stress tolerance of *C. riparius* compared to *P. olivacea*, reflecting possible differences in the ability of each species to adapt to altered environments.

9. Heat-shock (35 and 39 °C) drastically activates the cell stress response and inhibits the immune system in *P.* olivacea, whereas both responses are activated in *C. riparius*. These effects might compromise the adaptative capacity and survival of *P. olivacea* and emphasise the high tolerance of *C. riparius* to extreme environments.

10. Testing species with different susceptibility to environmental perturbations and combining molecular tools is a strategy that could lead to more robust environmental risk assessments and a better understanding of the risks that pollutants or other abiotic factors may pose to aquatic biota.

11. The differential responses detected between *P. olivacea* and *C. riparius* provide novel information on the harmful effects of BBP, BPA, BP3 and heat-shock on these aquatic midges and highlight the potential of *P. olivacea* to be considered as a suitable sentinel organism for ecotoxicity studies in natural scenarios.

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8. REFERENCES

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APPENDICES

Appendix I.

Published research papers directly related to the present work:

- **Llorente, L.**, Herrero, Ó., Aquilino, M., Planelló, R., (2020). *Prodiamesa olivacea*: *de novo* biomarker genes in a potential sentinel organism for ecotoxicity studies in natural scenarios. Aquat. Toxicol. 227, 105593. doi:10.1016/j.aquatox.2020.105593.

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- **Llorente, L.**, Herrero, Ó., Aquilino, M., Planelló, R. *De novo* characterisation and expression of heat-shock and immune genes in natural populations of P*rodiamesa olivacea* (Diptera) fourth instar larvae exposed to heat stress. Aquat. Toxicol. AQTOX-D-22-00239 (Under review).

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Prodiamesa olivacea: *de novo* biomarker genes in a potential sentinel organism for ecotoxicity studies in natural scenarios

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ABSTRACT

Along with traditional ecotoxicological approaches in model organisms, toxicological studies in non-model organisms are being taken into consideration in order to complement them and contribute to more robust approaches. This allows us to figure out the complexity of the exposures involved in natural ecosystems. In this context, in the present research we have used the model species *Chironomus riparius* (Chironomidae, Diptera) and the non-model species *Prodiamesa olivacea* (Chironomidae, Diptera) to assess the aquatic toxic effects of acute 4-h and 24-h exposures to 1 $\mu g L^{-1}$ of three common environmental pollutants: butyl benzyl phthalate (BBP), bisphenol A (BPA), and benzophenone 3 (BP3). Individuals of both species were collected from a contaminated river (Sar) in Galicia (Spain).

Regarding *Chironomus*, there are four OECD standardized tests for the evaluation of water and sediment toxicity, in which different species in this genus can be used to assess classical toxicity parameters such as survival, immobilization, reproduction, and development. In contrast, *Prodiamesa* is rarely used in toxicity studies, even though it is an interesting toxicological species because it shares habitats with *Chironomus* but requires less extreme conditions (e.g., contamination) and higher oxygen levels. These different requirements are particularly interesting in assessing the different responses of both species to pollutant exposure.

Quantitative real-time PCR was used to evaluate the transcriptional changes caused by xenobiotics in different genes of interest. Since information about *P. olivacea* in genomic databases is scarce, its transcriptome was obtained using *de novo* RNAseq. Genes involved in biotransformation pathways and the oxidative stress response (*MnSOD*, *CAT*, *PHGPx*, *Cyp4g15*, *Cyp6a14-like* and *Cyp6a2-like*) were *de novo* identified in this species. Our results show differential toxic responses depending on the species and the xenobiotic, being *P. olivacea* the dipteran that showed the most severe effects in most of the studied biomarker genes.

This work represents a multi-species approach that allows us to deepen in the toxicity of BBP, BPA, and BP3 at the molecular level. Besides, it provides an assessment of the tolerance/sensitivity of natural populations of model and non-model insect species chronically exposed to complex mixtures of pollutants in natural scenarios. These findings may have important implications for understanding the adverse biological effects of xenobiotics on *P. olivacea*, providing new sensitive biomarkers of exposure to BBP, BPA, and BP3. It also highlights the suitability of *Prodiamesa* for ecotoxicological risk assessment, especially in aquatic ecosystems.

1. Introduction

Water pollution can be caused in many ways, including direct sources such as effluent outfalls (from factories, waste treatment plants, etc.), and indirect sources in which contaminants enter the aquatic systems from soils or groundwater systems and the atmosphere via rainwater. Substances such as fertilizers, pesticides, herbicides, and a wide variety of toxic chemicals enter the dynamics of the ecosystems, disrupt the physical or biological components of the aquatic environments, and can lead to shifts in the viability of their populations. Among the polluting substances, those that are most persistent in the environment often give rise to a particular concern, since the effects resulting from their exposure can be prolonged over time. In this regard, butyl benzyl phthalate (BBP), bisphenol A (BPA), and benzophenone 3 (BP3) are ubiquitous contaminants whose presence in the environment is expected for decades.

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Appendix II.

Other published research papers during the development of the thesis:

- Arambourou, H., Planelló, R., **Llorente, L.**, Fuertes, I., Barata, C., Delorme, N., Noury, P., Herrero, Ó., Villeneuve, A., Bonnineau, C., (2019). *Chironomus riparius* exposure to field-collected contaminated sediments: From subcellular effect to whole-organism response. Sci. Total Environ. 671. doi:10.1016/j.scitotenv.2019.03.384.

- Arambourou, H., **Llorente, L.**, Moreno-Ocio, I., Herrero, Ó., Barata, C., Fuertes, I., Delorme, N., Méndez-Fernández, L., Planelló, R., (2020). Exposure to heavy metal-contaminated sediments disrupts gene expression, lipid profile, and life history traits in the midge *Chironomus riparius*. Water Res. 168, 115165. doi:10.1016/j.watres.2019.115165.

- Planelló, R., Herrero, O., García, P., Beltrán, E.M., **Llorente, L.**, Sánchez-Argüello, P., (2020). Developmental/reproductive effects and gene expression variations in *Chironomus riparius* after exposure to reclaimed water and its fortification with carbamazepine and triclosan. Water Res. 178, 115790. doi:org/10.1016/j.watres.2020.115790.

- Blanco-Sánchez, L., Planelló, R., **Llorente, L.**, Díaz-Pendón, J.A., Ferrero, V., Fernández-Muñoz, R., Herrero, Ó., de la Peña, E., (2021). Characterization of the detrimental effects of type IV glandular trichomes on the aphid *Macrosiphum euphorbiae* in tomato. Pest Manag. Sci. 77, 4117–4127. doi:10.1002/ps.6437.

- Frat, L., Chertemps, T., Elise, E., Bozzolan, F., Dacher, M., Planelló, R., Herrero, Ó., **Llorente, L.**, Moers, D., Siaussat, D., (2021). Single and mixed exposure to cadmium and mercury in *Drosophila melanogaster*: Molecular responses and impact on post-embryonic development. Ecotoxicol. Environ. Saf. 220. doi:10.1016/j.ecoenv.2021.112377.

- Marçal, R., **Llorente, L**., Herrero, O., Planelló, R., Guilherme, S., Pacheco, M., (2021). Intergenerational patterns of DNA methylation in *Procambarus clarkii* following exposure to genotoxicants: A conjugation in past simple or past continuous? Toxics 9, 271. doi:doi.org/10.3390/toxics9110271.

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Chironomus riparius exposure to field-collected contaminated sediments: From subcellular effect to whole-organism response

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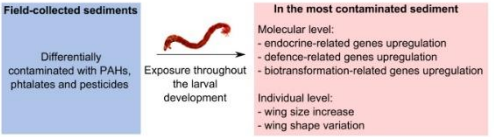
HIGHLIGHTS

- Chironomids larvae were exposed to three field-collected sediments differentially contaminated
- After 15 days of exposure, effects were measured from the molecular to the whole-organism response
- Differential gene expression and wing shape variation were observed in the group exposed to the most contaminated sediment
- This approach contributes to improved assessment of the risk associated with complex mixture exposure

GRAPHICAL ABSTRACT



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ABSTRACT

The toxicity of three field-collected sediments differentially contaminated with pesticides, heavy metals, phtalates and polycyclic aromatic hydrocarbons (PAHs), was assessed in *Chironomus riparius*. For this purpose, *C. riparius* larvae were exposed throughout their entire life cycle to sediments collected in three sites along the Saulx river in France, and the toxic effects were measured at different levels of biological organization: from the molecular (lipidomic analysis and transcriptional variations) to the whole organism response (respiration rate, shape markers and emergence rate). In the sediment characterized by an intermediate level of contamination with PAHs and phtalates, we detected an increase of the cell stress response and delayed emergence of males. In the group exposed to the most contaminated sediment with PAHs, phtalates and pesticides, genes related to endocrine pathways, cell stress response and biotransformation processes were overexpressed, while female wing shape was affected. Field-collected sediment exposure did not induce significant effects on mentum shape markers or on the lipid profile. The present study provides new insights into the multilevel effects of differentially contaminated sediments in insects. This integrative approach will certainly contribute to improved assessment of the risk that complex mixtures of pollutants pose to the aquatic ecosystem.

1. Introduction

Anthropogenic activities release a wide range of chemical compounds into aquatic systems. Exposure to this complex mixture of chemicals can affect the health of aquatic animals from molecules,

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Exposure to heavy metal-contaminated sediments disrupts gene expression, lipid profile, and life history traits in the midge *Chironomus riparius*

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ABSTRACT

Despite the concern about anthropogenic heavy metal accumulation, there remain few multi-level ecotoxicological studies to evaluate their effects in fluvial ecosystems. The toxicity of field-collected sediments exhibiting a gradient of heavy metal contamination (Cd, Pb, and Zn) was assessed in *Chironomus riparius*. For this purpose, larvae were exposed throughout their entire life cycle to these sediments, and toxic effects were measured at different levels of biological organization, from the molecular (lipidomic analysis and transcriptional profile) to the whole organism response (respiration rate, shape markers, and emergence rate). Alterations in the activity of relevant genes, as well as an increase of storage lipids and decrease in membrane fluidity, were detected in larvae exposed to the most contaminated sediments. Moreover, reduced larval and adult mass, decrease of larval respiration rate, and delayed emergence were observed, along with increased mentum and mandible size in larvae and decreased wing loading in adults. This study points out the deleterious effects of heavy metal exposure at various levels of biological organization and provides some clues regarding the mode of toxic action. This integrative approach provides new insights into the multi-level effects on aquatic insects exposed to heavy metal mixtures in field sediments, providing useful tools for ecological risk assessment in freshwater ecosystems.

1. Introduction

Human activities release a wide range of chemical compounds into aquatic systems, and exposure to this complex mixture can affect the health of aquatic animals at the molecular, tissue, organ and whole organism level, even contributing to population decline (Floury et al., 2013; Vörösmarty et al., 2000). Insects are key elements for the functioning of freshwater ecosystems, as they sustain higher trophic levels and contribute to the carbon cycle in streams and rivers. Certain developmental stages within their life cycle are particularly vulnerable to environmental stressors, and toxic exposures during these critical periods may have irreversible consequences (Weis, 2014). Indeed, early-life exposures could translate into phenotypic variations in adults, which can affect their fitness. In line with this, developmental abnormalities after exposure to chemical compounds have been reported in insects, particularly in chironomid larvae (Di Veroli et al., 2014; Martinez et al., 2003).

The current study focuses on the Oiartzun River (Basque Country, Spain), in the Natural Park of Aiako Harria. Study sites are located downstream the Arditurri mines, a complex of abandoned old Pb/Zn mines, characterized by a gradient of heavy metal contamination in sediments along the river (Méndez-Fernández,

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Developmental/reproductive effects and gene expression variations in *Chironomus riparius* after exposure to reclaimed water and its fortification with carbamazepine and triclosan

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Apical endpoints

ABSTRACT

The potential benefits of reclaimed water (RW) uses for environmental enhancement and restoration could become adverse impacts if RW does not meet the quality criteria that ensure wildlife preservation. RW can contain complex mixtures of micropollutants that may accumulate in sediment after environmental uses and affect benthic fauna. Therefore, we designed this study to assess the effects of RW on a sediment insect species used mainly in ecotoxicology (*Chironomus riparius*). Whole organism effects and gene expression were measured in a water sediment system after spiking RW as overlying water, which was renewed 3 times during the test. Development rate, emergence rate and fecundity were monitored after the 21-day exposure. Endocrine-related genes (*EcR, ERR, E75, Vtg*), cellular stress genes (*hsp70, hsc70, hsp24, hsp10*) and biotransformation genes (*gp93, GSTd3, GPx, cyp4g*) were assessed in larvae after the 10-day exposure. The experimental design also included single or binary fortifications of both test medium and RW, obtained by adding two emerging pollutants: carbamazepine (100 μg/L CBZ) and triclosan (20 μg/L TCS). The chemical characterisation of RW showed that 20 of the 23 screened emerging pollutants fell within the detection limit, 10 exceeded 0.01 μg/L (including CBZ) and three exceeded 0.1 μg/L (hydrochlorothiazide, atenolol, ibuprofen). The analytical measures of sediment (day 21) and overlying water (days 7, 14 and 21) were taken to know the water-sediment distribution of CBZ and TCS added to fortifications. CBZ distributed mainly in overlying water (110–164 μg/L and 73–100 μg/kg), while TCS showed a higher affinity to sediment (2.8–5.1 μg/L and 36–55 μg/kg). RW had significant effects in molecular terms (*Vtg, hsp70, hsc70*), but had no significant effects on the whole organism. Nevertheless, the single RW fortifications impaired both the development rate and fecundity, while the binary RW fortification impaired only fecundity. The most marked increase in *EcR* expression was observed for the binary RW fortification. *Hsps, GSTd3* and *cyp4g* showed a similar tendency to that observed for *EcR* and *Vtg* in the binary and single RW fortifications. The binary mixture (CBZ and TCS together) in RW was toxic, but not in the medium tests. Therefore, the major concern of RW uses is apparently related to the interactivity between this complex matrix and any other pollutants possibly present in the environment where RW is applied. Our results underscore the need for raising awareness about RW effects, which can be achieved by ecotoxicological testing.

1. Introduction

It is widely recognised that effect-based methods and chemical analyses are complementary tools to assess water quality, especially to address mixture issues. Pollutants co-occur at low concentrations in mixtures of many chemicals, which spells concerns

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Characterization of the detrimental effects of type IV glandular trichomes on the aphid *Macrosiphum euphorbiae* in tomato

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Abstract

BACKGROUND: Glandular trichomes are essential in plants' defence against pests however, the mechanisms of action are not completely understood. While there is considerable evidence of feeding and movement impairment by trichomes, the effect on other traits is less clear. We combined laboratory and greenhouse experiments with molecular analysis to understand how glandular trichomes affect the behavior, population growth, and the expression of biomarkers involved in detoxification, primary metabolism, and developmental pathways of the aphid *Macrosiphum euphorbiae*. We used two isogenic tomato lines that differ in the presence of type IV glandular trichomes and production of acylsucroses; i.e.,*Solanum lycopersicum* cv. 'Moneymaker' and an introgressed line from *Solanum pimpinellifolium* (with trichomes type IV).

RESULTS: Type IV glandular trichomes affected host selection and aphid proliferation with aphids avoiding, and showing impaired multiplication on the genotype with trichomes. The exposure to type IV glandular trichomes resulted in the overexpression of detoxication markers (i.e., *Hsp70*, *Hsp17*, *Hsp10*); the repression of the energetic metabolism (*GAPDH*), and the activation of the ecdysone pathway; all these, underlying the key adaptations and metabolic trade-offs in aphids exposed to glandular trichomes.

CONCLUSION: Our results demonstrate the detrimental effect of glandular trichomes (type IV) on the aphid and put forward their mode of action. Given the prevalence of glandular trichomes in wild and cultivated Solanaceae; and of the investigated molecular biomarkers in insects in general, our results provide relevant mechanisms to understand the effect of trichomes not only on herbivorous insects but also on other trophic levels.

Supporting information may be found in the online version of this article.

Keywords: acylsucroses; glandular trichomes; molecular biomarkers; physiological responses; *Solanum pimpinellifolium*; *Solanum lycopersicum*

1 INTRODUCTION

Insect herbivory is a critical component in the coevolution of plants and insects.[1] To deal with phytophagous insects, plants show different defensive strategies (e.g., morphological and chemical adaptations) that impair insect movement, feeding and reproduction.[2] Herbivorous insects have developed morphological, behavioral and metabolic adaptations that enable them to deal (temporarily or permanently) with plant defenses.[3] Glandular trichomes, i.e., epidermal structures widely conserved across the plant kingdom,[4] play a key role in the defense of plants against herbivorous insects by producing allelochemicals that potentially affect insect pests behavior and life-cycle.[5] To date, the mechanisms by which glandular trichomes affect herbivore insects are not completely understood. While there is considerable evidence

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Single and mixed exposure to cadmium and mercury in *Drosophila melanogaster*: Molecular responses and impact on post-embryonic development

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ABSTRACT

Heavy metals, like many other chemical elements, are naturally present in the environment; however, the concentrations of these metals in various environmental matrices have increased through their intensive use in many human activities (such as industry, mining and agriculture). Among the heavy metals, cadmium (Cd) and mercury (Hg) induce a wide variety of defects in animals. While the effects of these heavy metals have been widely documented, a single exposure paradigm is typically used. Few studies have focused on evaluating combined exposure to these metals. However, in the environment, animals are confronted with a plethora of substances simultaneously; thus, the presence and origin of such substances must be determined to reduce the sources of contamination. Using the model of the fruit fly *Drosophila melanogaster*, for which many tools are readily available, we investigated how different concentrations of Cd and Hg in single and combined exposures impact post-embryonic development. In parallel, we evaluated the extended expression pattern of 38 molecular targets used as potential biomarkers of exposure through qPCR. Our results showed that both metals caused developmental delays and mortality in dose-dependent responses. Both metals were able to deregulate genes involved in hormonal control, general stress, and oxidative stress. Importantly, we confirmed synergistic interactions between Cd and Hg. Our results indicate the importance of assessing several biomarkers and their kinetics in mixtures. *Drosophila* represents a useful model for monitoring the toxicity of substances in polluted environments.

1. Introduction

Heavy metals and many others chemical elements are naturally present in environment, with cadmium (Cd) and mercury (Hg) being particularly widespread (Wu et al., 2016). These two metals are intensively used in several human activities, including non-ferrous metal refining and household waste incinerators, resulting in their being discharged in industrial waste and wastewater; consequently, their concentrations are high in various environmental matrices (Caballero-Gallardo et al., 2016; Wu et al., 2016). Cd and Hg are toxic compounds that induce a wide variety of defects in animals. Depending on their mode of action and route of exposure, Cd and Hg can bioaccumulate, induce protein denaturation, disrupt essential enzymes (e.g., DNA methyltransferase), and even damage cell membranes by oxidative stress. These molecular effects disrupt critical functions, including locomotion, digestive activity, and the nervous system (Genchi et al., 2020). Over the last 10 years, Cd and Hg have also been identified as potential endocrine disruptors (EDCs) in vertebrate and invertebrate models, altering the expression of hormone receptor genes and circulating hormone levels, along with disrupting the production in the endocrine gland (Iavicoli et al., 2009; Planelló et al., 2010; Rana, 2014). Yet, the analysis of heavy metals impacts needs refining to

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Intergenerational Patterns of DNA Methylation in *Procambarus clarkii* Following Exposure to Genotoxicants: A Conjugation in Past Simple or Past Continuous?

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Abstract: Epigenome is susceptible to modulation by environmental pressures—namely, through alterations in global DNA methylation, impacting the organism condition and, ultimately, reverberating on the phenotype of the subsequent generations. Hence, an intergenerational study was conducted, aiming to clarify the influence of genotoxicants on global DNA methylation of the crayfish *Procambarus clarkii*. Two subsequent generations were exposed to the herbicide penoxsulam (Px; 23 $\mu g \cdot L^{-1}$) and to the genotoxicant model ethyl methanesulfonate (EMS; 5 $mg \cdot L^{-1}$). Px did not induce changes in DNA methylation of adult crayfish (F_0). However, the hypomethylation occurring in unexposed F_1 juveniles demonstrated that the history of exposure per se can modulate epigenome. In F_1 descendants of the Px-exposed group, methylome (hypermethylated) was more affected in males than in females. EMS-induced hypomethylation in adult females (F_0), also showed gender specificity. In addition, hypomethylation was also observed in the unexposed F_1 crayfish, indicating an intergenerational epigenetic effect. The modulatory role of past exposure to penoxsulam or to EMS also showed a dependency on the crayfish developmental stage. Overall, this research revealed that indirect experiences (events occurring in a predecessor generation) can have an impact even greater than direct experiences (present events) on the epigenetic dynamics.

Keywords: crustacean; epigenetics; methylome; intergenerational; genotoxic; pesticides; penoxsulam; epigenotoxicology

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1. Introduction

Ecotoxicological research has been mostly centered on temporally restricted assessments at individual and sub-individual levels, which can represent a limitation in terms of representativeness, keeping in view the requirement to predict the actual ecological impact of contamination. In this context, the implementation of inter- and transgenerational studies can represent a valuable advance toward the elucidation of processes able to produce deleterious effects at higher organizational levels (e.g., population), thereby increasing the ecological relevance. This approach has been settled mainly through reproductive (e.g., developmental abnormalities, reproductive success) [1] and growth/survival endpoints [2]. More recently and following the conceptualization of epigenetic inheritance [3], the use of epigenetic markers emerged as a novel and promising strategy, offering suitable information on the diagnosis and prediction of ecotoxicological impacts. This is substantiated by the assumption that epigenetic changes

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