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Transcriptomic and biochemical analysis of *Procambarus clarkii* upon exposure to pesticides: Population-specific responses as a sign of pollutant resistance?

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ABSTRACT

The effects that anthropogenic stressors may have on modulating species' plasticity has been relatively unexplored; however, it represents a scientific frontier that may offer insights into their ability to colonize new habitats. To explore the advantage that inhabiting polluted environments may offer to invasive species, we selected the crayfish *Procambarus clarkii*, a species that can colonize and thrive in a wide range of aquatic environments, including heavily polluted ones. Here, we studied the molecular and physiological responses of crayfish when experimentally exposed to a pesticide mix of azoxystrobin and oxadiazon at sublethal concentrations. We compared these responses in three isolated crayfish populations in Southern France that are established in areas with different pollution levels: i) Camargue, seasonally affected by pesticide pollution; ii) Bages-Sigean, impacted all year-round by domestic effluents and; iii) Salagou, a more pristine site. Gene expression analyses revealed that the response to the pesticide mix was the strongest in the Camargue crayfish. In this population, a total of 88 differentially expressed genes (DEGs) were identified in hepatopancreas and 78 in gills between exposed and control laboratory groups. Among genes that were differentially expressed and successfully annotated, those involved in stress response, DNA repair, immune response, and translation and transcription processes stand out. Interestingly, the hepatopancreas responded mainly with up-regulation, while the gills showed down-regulation. Our results demonstrate population-specific responses to pesticide stress in populations with different life-history of exposure to pollutants. The high regulation of the aforementioned mechanisms indicates that they play a crucial role in the adaptation of this invasive species to polluted environments.

1. Introduction

Exposure to harmful levels of chemical pollutants has led to the evolution and tolerance in populations of several marine species (Reid et al., 2016; Hamilton et al., 2017; Whitehead et al., 2017; Oziolor et al., 2019). Although there is still a lack of evidence on the physiological basis for these adaptations, it is suspected that they may rely on processes involving absorption, distribution, and excretion of the chemical in question (Hamilton et al., 2017). While rapid evolution driven by pollutants has only been vaguely studied in aquatic invertebrates, research on killifish has provided insights into the key features enabling rapid evolutionary rescue in degraded environments. Studies analyzing killifish exposure to industrial pollutants revealed impacts on nucleotide diversity and several molecular structures (Reid et al., 2016; Hamilton et al., 2017; Whitehead et al., 2017), and multiple metabolic pathways (Reid et al., 2016; Oziolor et al., 2019). Potential adaptive mechanisms include enhanced antioxidant responses and increased capacity for DNA and tissue repair (Hamilton et al., 2017). These findings suggest that the evolutionary influence of anthropogenic stressors, as selective agents, is a widespread phenomenon (Whitehead et al., 2017). Nonetheless, there is very limited information on how these stressors may affect the tolerance, adaptation, and rapid evolution of aquatic invertebrates, let

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alone those that are highly invasive. This knowledge gap is of key relevance to fully understanding the impact that anthropogenic pollution could have on the ability of invasive species to rapidly adapt to new environments. This is particularly relevant in the current global context, where freshwater bodies and many other ecosystems, are seriously impacted by human activities derived from industrial and agricultural processes that release massive amounts of contaminants into the environment, including pesticides.

Here, we address the adaptation of aquatic invertebrates to pesticide exposure using the invasive crayfish *Procambarus clarkii* as a study model. This invasive species is well known for its ability to colonize a wide range of aquatic environments with different levels of water quality. It can disperse widely, tolerate environmental extremes, has generalist and opportunistic feeding habits (Gherardi and Barbaresi, 2007), and is more resistant to diseases than most of its native counterparts (Collas et al., 2007). It is native to the North of Mexico and the United States, but its highly adaptive nature has driven it to be well-established throughout Europe, Asia, Africa, North America and South America. It was first introduced in Western France in 1974, and by the mid-1990s it had established populations in 36 of the 96 metropolitan France counties (departments), particularly around the Mediterranean coastal areas (Meineri et al., 2013). Previous studies with this species showed that responses to specific pesticides differed according to the water quality of the environment where the individual originated. Some of these responses included differences in respiration rates, hydro-osmotic balance, and the activity of digestive proteases and lipases (Raffalli et al., 2024). In this study, we analyzed and compared the gene expression and enzymatic responses in gills and hepatopancreas (midgut) of the same populations following a 96-h laboratory exposure to a pesticide cocktail containing azoxystrobin and oxadiazon at sublethal concentrations.

In the last decades, most studies in ecotoxicology have focused on the analysis of well-known biochemical parameters to measure the impact of toxicants on organisms. However, the impact may extend beyond these stress mechanisms, and by limiting the focus of the research, we could be missing the bigger picture. For this reason, in this study, we analyzed the entire set of genes that are transcribed in response to toxicant exposure. We investigated whether gene expression in the hepatopancreas and gills differ between these three populations and which pathways were most impacted by a laboratory exposure to the pesticide mix. Furthermore, to complement gene expression data, we targeted antioxidant and detoxification enzymatic responses by monitoring the activity of physiological markers that are good stress indicators in marine and aquatic invertebrates. We focused our analyses on gills and hepatopancreas due to their role in crustacean toxicology: gills are the primary organ of respiration and osmoregulation, and the first barrier of exposure to water-borne chemicals (Burnett and McMahon, 1985) whereas the hepatopancreas, being part of the digestive system, is another major entry route for toxicants while playing a major role in detoxification processes (White and Rainbow, 1986; Liu X et al., 2021). For this purpose, crayfish from three different populations were collected in Southern France, these being at: i) the brackish Camargue wetland system, which is seasonally affected by the presence of at least 30 pesticides, including azoxystrobin and oxadiazon (Cheiron and Bricault, 2020). These two pesticides are regularly detected, namely in the water drainage canals from the surrounding rice fields (Munaron et al., 2020). Since 2011, azoxystrobin and oxadiazon have reached up to 3 and 17 times the Environmental Quality Standard (EQS). However, the concentrations vary depending of the season and year, with the highest peaks occurring from March to September (CGEDD, 2011); ii) the brackish Bages-Sigean lagoons, regularly impacted by pesticides together with domestic effluents and; iii) the Salagou lake, a more pristine freshwater site. These three populations originate from the same invasion route through southern Spain in the mid-1970s (Oficialdegui et al., 2019). We hypothesized that crayfish with life-long exposure to varying pulses of environmental chemicals would exhibit different responses to additional pollutant stressors. Consequently, we anticipated that the Camargue and Bages-Sigean populations would show a distinct gene expression fingerprint compared to the Salagou one, which likely had not previously faced exposure to these pollutants. Furthermore, we also hypothesized that the antioxidant and detoxification responses would be lower in populations that formerly and repeatedly encounter pesticides in their natural environment.

2. Methods

2.1. Animal sampling and maintenance

Using land nets, crayfish were collected from three populations in the South of France : i) the Fumemorte canal in the Camargue area (43°30′52.6″N, 4°40′02.1″E), with an environmental salinity of 24–33 g/L and degraded environmental quality from March to September, due to the intense agricultural activity primarily consisting of rice fields in the area, and hence affected by pesticide pollution; ii) Bages-Sigean wetlands (43°07′30.7″N, 3°01′20.9″E) (from Bages-Sigean region), an area with a salinity of 26–40 g/L and presenting degraded water quality throughout the year, associated to urban and domestic discharges and; iii) Salagou lake (43°39′45.0″N, 3°22′20.5″E), a freshwater body (<1 g/L) presenting an overall good environmental quality (Fig. 1).

2.2. Experimental design

After field collection, animals were transported to the laboratory where they were acclimated to freshwater for 4 months in large aquaria. A total of 90 individuals (1:1 sex ratio) were used for this study: 15 individuals (5 per population) were distributed in each of six 20-L aquaria. Within the aquaria, each individual was placed in an immersed glass jar, closed by a glass wire net to individually identify each animal and avoid the negative impact of social conflicts among them. Animals were allowed to acclimate to the new housing conditions (i.e. the glass jars) for 1 week before the start of the experiments. After this time, half of the animals remained undisturbed for control purposes while the other half were exposed to a mixture of 2 pollutants: azoxystrobin (95 μg/L, Sigma-Aldrich 31697) and oxadiazon (30 μg/L, Sigma-Aldrich 33382) for 96 h. These concentrations are 100 times higher than the EQS-MAC (maximum acceptable concentration) according to the environmental quality standards for these two pesticides (EFSA, 2010a, 2010b). The EQS-MAC was used because peaks of azoxystrobin and oxadiazon occur seasonally rather than year-round. Furthermore, because a preliminary test determined that the concentration of the pesticides decreased by about 10% every 24h, 80% of the water along with the corresponding doses of pollutants was daily renewed to ensure a constant chemical concentration throughout the 4-day experimental time. The chemicals

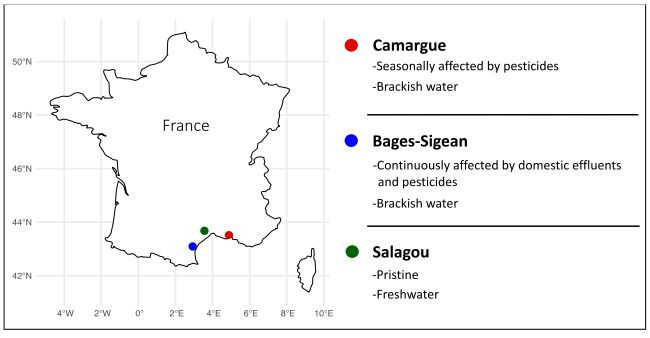

Fig. 1. Sampling areas of crayfish, *Procambarus clarkii* analyzed in this study. The color of the circles represents the populations: Red for Camargue, blue for Bages-Sigean and green for Salagou. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

were diluted in methanol for their delivery in the corresponding aquaria, while controls received the equivalent amount of methanol. The total concentration of methanol in each aquarium was 0.018 ml/L, which is below the lowest concentration known to cause and effect due to chronic exposure to methanol (Kaviraj et al., 2004). During crayfish acclimation to laboratory conditions and during the biocide exposures, water temperature was maintained at 20 °C (± 0.4 °C) with a photoperiod of 12:12-h. All animals were maintained in recirculated and dechlorinated tap water.

2.3. Sample collection

After the 96 h-exposure, animals were removed from the aquaria and euthanized following RSPCA policies: here, crayfish were immediately anaesthetized through air-chilling method, i.e. placing the animals in a −20 °C chamber for 15 min, rendering them insensitive to stimuli. After this time, animals were euthanized by removal of the frontal part of the cephalothorax and the tissues dissected. Hepatopancreas and gills samples were flash-frozen in liquid nitrogen and stored −80 °C for enzyme analyses. They were also stored in microtubes containing RNAlater® Stabilization Solution (Ambion, Inc., Texas, USA) and subsequently stored at −80 °C for gene expression analyses. All experiments were conducted in accordance with valid international, European and National laws, applying the principles of replacement, reduction and refinement.

2.4. RNA extraction

About 30 mg of hepatopancreas and gill tissue previously stored at −80 °C in RNAlater® were used for RNA extraction. Cell lysis was performed in 0.6 ml of lysis buffer RLT provided in the Qiagen Kit (Qiagen, Hilden, Germany) with beta-mercaptoethanol and using ceramic beads (Precellys® Keramik-kit, PeqLab, Erlangen, Germany), with 16 s shaking at 6500 rpm. Samples were then centrifuged at 16,000 g for 10 min and then transferred into 2 ml microtubes. Total RNA was isolated using RNAeasy Mini spin columns (Qiagen, Germany) following the manufacturer's instructions. RNA quantity was analyzed using a NanoDrop One spectrophotometer (Thermo Fisher Scientific) and quality was determined by microfluidic electrophoresis in a Bioanalyzer (Agilent Technologies, USA).

2.5. RNAseq normalized cDNA libraries and Ilumina sequencing

The construction of cDNA libraries from 64 individuals was done by Macrogen Europe (Amsterdam, Netherlands) following the TruSeq stranded mRNA sample protocol. To verify the size of PCR enriched fragments, the template size distribution was checked by running on an Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip. Additionally, the prepared libraries were quantified using qPCR according to the Illumina qPCR Quantification Protocol Guide. Finally, to calculate the library sample concentration we also used Roche's Rapid library standard Quantification solution and calculator. Only libraries with a concentration over 10 nM were used. Subsequently, the libraries were sequenced on an Illumina NovaSeq 6000 sequencer by Macrogen Europe with a throughput of 80 M reads per sample (100 bp paired-end).

2.6. Differential gene expression analysis and functional annotation

Trimmomatic software in paired-end mode was used for quality filtering of the raw RNA reads and adapter filtering. Reads with a quality below 28, and a length less than 40 bp were discarded. After the filtering process, clean reads were mapped onto *P. clarkii* genome assembly (Reference sequence Genbank: PRJNA727411) (Xu et al., 2021), using bwa-mem2 (Vasimuddin et al., 2019) with default parameters. Alignments were then filtered to discard unmapped reads, and we used featureCounts (Liao et al., 2014) to obtain a table of read count per transcript. Differential gene expression was assessed using the Bioconductor R package DESeq2 (Love et al., 2014), with an alpha of 0.001. Functional annotation of the differentially expressed transcripts was performed using the fasta protein file of the transcripts from the genome, and the BeeDeeM pipeline (https://github.com/pgdurand/BeeDeeM) on Uniprot and Swissprot databases.

2.7. Biochemical analyses

A portion of the hepatopancreas and gills were homogenized in 100 mM phosphate buffer in a 1:5 (w:v) ratio using the Precellys keramik-kit (MP, Germany), performing 2 cycles of 15 s shaking and a 20 s pause in between. The buffer used for hepatopancreas (100 mM phosphate buffer) also contained 150 mM KCl and 1 mM EDTA. The homogenates were centrifuged at 10,000g for 20 min at 4 °C and the resulting supernatants were stored at −80 °C for further biochemical determinations. Protein quantification was performed after Bradford (1976) with bovine serum albumin as standard (A9418, Sigma Aldrich).

Antioxidant capacity was estimated through the catalase (CAT) and glutathione reductase (GR) enzyme activities, both being crucial enzymes involved in the antioxidant defense machinery while being good biomarkers of pollutant exposure in aquatic invertebrates (e.g. Nandi et al., 2019; Hossen et al., 2023). Detoxification and biotransformation were assessed as glutathione-*S*-transferase (GST) and carboxylesterase (CE) enzyme activities. CAT activity was measured in hepatopancreas at 240 nm following the method described by Aebi (1984). GR activity in hepatopancreas supernatants was measured at 340 nm during 3 min adapted from the method described by Carlberg and Mannervik (1985). GST activity was measured in both hepatopancreas and gills homogenates at 340 nm for 3 min following the method described by Habig et al. (1974). Carboxylesterase (CE) activity in hepatopancreas and gills was measured using p-nitrophenyl acetate p-NPA (N8130, Sigma Aldrich) and p-nitrophenyl butyrate p-NPB (N9876, Sigma) as substrate at 405 nm for 3 min following the protocol described by Hosokawa and Satoh (2001). Furthermore, neurotoxicity was assessed as acetylcholinesterase (AChE) activity as well as through the potential to interfere with molting by means of N-acetyl-β-D-glucosaminidase (NAGase) activity. The inhibition of AChE activity is one of the most frequently adopted biomarkers for pesticide-induced neurotoxicity but also for other environmental chemicals (see review by Fu et al., 2018) and exploited for pest control monitoring purposes (Casida and Durkin, 2013; Lignot et al., 1998). Environmental chemicals such heavy metals (e.g. Zhang et al., 2008; Han and Wang, 2009; Rivera-Ingraham et al., 2021), hydrocarbons (Zhang et al., 2008), pharmaceuticals (e.g. Rhee et al., 2013) or even herbicides like the ones here tested (e.g. Kovačević et al., 2023) inhibit AChE. NAGase activity has been suggested as a good moult biomarker in ecotoxicological approaches as it is affected by a wide variety of environmental pollutants (e.g. Lin et al., 2005; Zhang et al., 2010; Mesquita et al., 2015).

AChE was measured in hepatopancreas and gills by using acetylthiocholine as substrate and the kinetics of the metabolite formed with DTNB was read at 412 nm for 5 min following the protocol described by Ellman et al. (1961). NAGase activity was determined using 4-nitrophenyl N-acetyl-β-D-glucosaminide as substrate and spectrophotometrically recording the formation of 4-nitrophenol at 410 nm also for 5 min (Rollin et al., 2023). All enzymatic activities were expressed per mg of protein content, as measured using the Bradford (1976) method and bovine serum albumin (BSA) (0.05–0.5 mg/mL) as standard. All activities were measured with an Infinite200 TECAN spectrophotometer (Tecan, Männendorf, Switzerland) at 25 °C using the Magellan kinetic mode v6.0.

2.8. Statistical analyses

For statistical analyses of enzymatic activities, RStudio version 2023.03.0 software was used. Shapiro's test was used to verify normal

distribution and homogeneity of variances of biochemical data. One-way ANOVAs were used to compare populations within each condition. Differences among groups were subsequently identified by pairwise comparisons using Tukey's post-hoc test. To test difference among treatments and controls from every population the non-parametric Wilcox test was applied. The level for statistical significance was set at $P < 0.05$.

3. Results

3.1. Differential gene expression

A multidimensional Principal Components Analysis (PCA) based on the gene expression analysis of the three crayfish populations clearly identified hepatopancreas and gill tissues in specific clusters (Fig. 2A), with PC1 explaining 72.85% of the variance. Population's dispersion can be observed in PC2, PC3, and PC4 which altogether explained 7.2% of the variance (Fig. 2A and B).

In the hepatopancreas, a heatmap analysis of the differentially expressed genes (DEG) corroborated that when comparing all three populations simultaneously, the difference between pesticide-exposed and control crayfish is not evident (Fig. 3A). However, when analyzing each population separately, we observed a clear difference upon exposure to the pesticide mixture, with the most distinct pattern shown by Camargue crayfish, seasonally encountering pesticides in their natural environment (Fig. 3B, C, D). In this tissue, DEG under the pesticide mixture also differed among populations: Camargue showed 88 DEG (52 up-regulated and 36 down-regulated genes) (Fig. 3B), far exceeding the changes experienced by Bages-Sigean crayfish (5 up-regulated and 4 down-regulated) and Salagou ones (6 up-regulated and 1 down-regulated) (Fig. 3C and D). However, not all DEGs were functionally annotated: only 55 genes out of 88 in Camargue, 6 out of the 7 in Salagou and 7 out of the 9 in Bages-Sigean.

For the particular case of Camargue crayfish, among the genes that were differentially expressed in the hepatopancreas and successfully annotated, those involved in stress response (6 genes up- and 4 down-regulated), DNA repair (4 up and 2 down), immune response (8 up and 2 down), and translation and transcription processes (3 up and 3 down) stand out (Table 1). In contrast to Camargue crayfish, the Salagou specimens up-regulated only two genes related to stress and immune response in the hepatopancreas (Table 1).

Contrarily to the results obtained for the hepatopancreas, differential gene expression in gills can easily separate the experimentally exposed and control individuals (Fig. 4A). As observed in hepatopancreas, the gills of Camargue crayfish exhibited a higher number of DEG under pesticide mixture exposure in comparison with the unexposed controls (Fig. 4B).

Out of the 78 DEGs identified in gills of Camargue crayfish, 55 were annotated. Among the 78 DEGs, 67 genes were down-regulated. Genes involved in stress response, DNA repair, immune response, and translation and transcription were mostly down-regulated, except for a zinc finger protein and elongation factor, which were up-regulated and are involved in translation and transcription (Table 1). For crayfish from Bages-Sigean no DEG analysis was possible, since only 2 samples met the library quality requirements for sequencing.

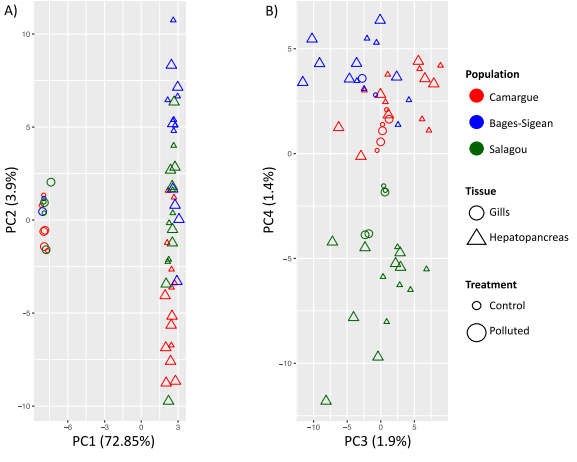

Fig. 2. PCA of differentially expressed genes between tissues and populations. Circles and triangles represent gill and hepatopancreas results, respectively, and their size differs according to treatment (smaller for control animals and larger for pollutant-exposed animals). Different colors represent the three different populations considered: red for Camargue, blue for Bages-Sigean, and green for Salagou. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Biochemical assessments: antioxidant and detoxification activities

Among all the enzymatic determinations carried out, statistical differences between controls and pesticide-exposed animals were detected in hepatopancreas (Fig. 5D, F, 5G) but not in gills (Fig. 6). Neither CE activities measured with both substrates (pNPA or pNPB) nor AChE were affected by the 96 h treatment in either population; although, on average, hydrolysis rates in hepatopancreas were 80% higher than in gills (Figs. 5C and 6C), as it corresponds to the main metabolic organ. A higher metabolic responsiveness in hepatopancreas than in gills coincides with the enhanced transcriptomic changes also observed in the hepatic tissue.

Regarding antioxidant activities (Fig. 5D, E, F), only the antioxidant enzyme GR responded to pesticide mixture exposure with Salagou animals (originally collected from the pristine freshwater site) showing a two-fold higher GR activity than the other two more polluted populations (Fig. 5D). In contrast, detoxification phase II GST (Fig. 5F), only showed significant changes in those animals collected from Camargue (i.e. pesticide polluted site): when exposed to the mixture under laboratory conditions, Camargue animals responded by increasing their GST activity by 20%.

NAGase activity (Fig. 5G) was similar in all control animals regardless of their origin with an average of 11.07 ± 7.3 nmol min^{-1}· mg $protein^{-1}$. However, when experimentally exposed to pesticides for 96 h, Salagou animals showed a two-fold increase in NAGase activity, while crayfish from Camargue and Bages-Sigean decreased their activities by 17 and 20%, respectively.

Overall, crayfish from Bages-Sigean showed a decrease in pNPA-CE (Fig. 5A), pNPB-CE (Fig. 5B) and GST (Fig. 5F) activities in hepatopancreas when exposed to the pesticide mixture for 96 h while specimens from Camargue and Salagou showed an increase in these detoxification activities. Crayfish from Bages-Sigean experienced an increase in AChE activity while the other two populations showed a decrease in activity under laboratory exposure to the pesticide mixture (Fig. 5C) (for numerical values of Figs. 5 and 6 see Table 1 in supplementary materials).

4. Discussion

Aquatic organisms are often exposed to harmful chemicals and the selection pressures associated with such exposures often lead to the evolution of tolerance levels, a phenomenon that has been well-addressed in insects and their resistance to pesticides (reviewed by Hawkins et al., 2018). However, the effects of anthropogenic stressors, such as pesticide inputs, on the plasticity of invasive aquatic species has not yet been addressed. Under laboratory conditions, we here addressed this subject using a transcriptomic and metabolic approach to determine the differential response of three populations of the invasive crayfish *P. clarkii* (differing in their environmental quality and historical

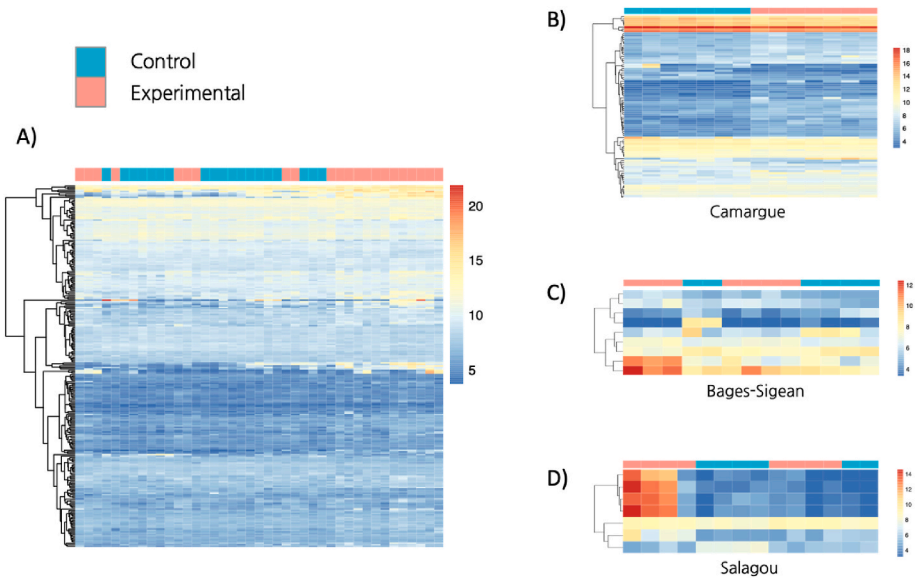

Fig. 3. Heatmaps showing differentially expressed genes (DEGs) in the hepatopancreas of crayfish after 96-h pesticide mixture exposure in animals from the three studied populations: A) General overview; B) Camargue; C) Bages-Sigean; D) Salagou.

background of chemical pollution) to a mix of two largely used pesticides (i.e. azoxystrobin and oxadiazon).

4.1. Differential gene expression analysis revealed a population-specific response

The most significant result derived from the differential gene expression analysis is that the response to the pesticide mix was highly population-dependent. In this study, we hypothesized that when chronically exposed to a certain extent of environmental pollutant loads, crayfish would exhibit particular responses upon facing a new chemical challenge. Consequently, we anticipated that the Camargue and Bages-Sigean crayfish populations would show a distinct response to those from the Salagou, likely to be more vulnerable as they were less exposed to anthropogenic pollutants. Our results corroborated this hypothesis; however, they also revealed a very distinct response pattern between the Camargue and Bages-Sigean crayfish populations, which encounter different types of pollutants at different exposure times.

The response of the Camargue crayfish (seasonally exposed to pesticide inputs) was by far, the most pronounced, mostly characterized by a down-regulation of genes in gills, which we attributed to being due to the tissues being the first barrier of contact with the chemical. Both up-and down-regulation was recorded in the hepatopancreas as a consequence of the pesticide challenge. A high number of down-regulated stress genes has been observed in crustaceans exposed to cadmium (Liu X et al., 2021) and low temperatures (Yang et al., 2022). For other phyla such as Mollusca, reports have equally shown down-regulation as a response to pesticide exposure (e.g. Cao et al., 2019). Former studies have contrasted gene expression levels between natural populations of killifish from polluted and unpolluted sites (e.g. Fisher and Oleksiak, 2007) or, in response to heavy metal cadmium (Cd^{2+}) in a laboratory scenario (e.g. Liu X et al., 2021). However, to the best of our knowledge, this is the first study to address differential gene expression in populations from differentially polluted natural sites upon new pesticide load under laboratory conditions.

4.2. Tissue-specific response

Crayfish from Salagou (reference site) exhibited a higher DEG response in gills than in hepatopancreas while those from Camargue showed similar levels of DEGs in both tissues. Also, contrary to Camargue crayfish, the response of Salagou gills was mainly up-regulation. Likewise, the number of modulated DEGs in the freshwater prawn *Macrobrachium rosenbergii* upon Cd exposure was significantly higher in the gills than in the hepatic tissue (Liu X et al., 2021). Moreover, Liu X et al. also reported that with over exposure time the number of DEGs in gills decreased while in the hepatopancreas increased. These results suggest that the gills act as the initial site and transient storage organ during short-term exposure, with the contaminant gradually being transferred from the gills to the hepatopancreas. Following this premise, it can be anticipated that crayfish from Camargue and Salagou populations may metabolize pesticide inputs at different rates, leading to delayed hepatopancreas responses after 96 h of exposure in the most traditionally exposed Camargue population. This is partially confirmed by the fast and significant response in NGAase and GR activities in crayfish from the reference site upon pesticide exposure. However, in order to fully validate this hypothesis, it would be necessary to follow the responses in both crayfish populations in a longer time frame. Another differential trait between these two contrasted populations is that, in gills, Camargue crayfish showed more down-regulated genes than up-regulated, contrarily to Salagou crayfish for which most of the DGEs were up-regulated. This could also be related to the rate at which gills, the first organ exposed to these pollutants, responded.

4.3. Expression of defense/stress-related genes

Although the Camargue crayfish population showed the highest differential gene expression in hepatopancreas and gills, in terms of enzymatic responses, only the detoxification GST activity was statistically enhanced in hepatopancreas. These results were confirmed with the annotation of the DEGs. The particular antioxidant and detoxification gene expression and enzymatic response did not increase as expected; however, we demonstrated that other important defense mechanisms at the gene expression level such as stress response, DNA repair, immune response, and translation and transcription processes, were altered. The Camargue crayfish population differentially responded with a large number of up-regulated genes in the hepatopancreas when exposed to the pesticide mixture under laboratory conditions, particularly those involved in stress response, DNA repair, immune response, and translation and transcription, while in the gills, these processes are mainly down-regulated. These results also suggest that although both tissues of Camargue crayfish are highly regulated, the

Table 1
List of regulated genes involved in stress response, DNA repair, immune response and translation and transcription according to tissue and crayfish population. Positive values indicate up-regulation, and negative values indicate down-regulation.

Camargue population				
Hepatopancreas				
Transcript	**NCBI reference protein sequence**	**log2FoldChange**	**Type of response**	
Insulin-like growth factor-binding protein 7	XP_045588282.1	+1.259	Stress response & DNA repair	GO:0034599: Cellular response to oxidative stress GO:0006977: DNA damage response
Heat shock cognate 70 kDa protein	XP_045595297.1 XP_045625695.1 XP_045625697.1	−1.036 −0.776 −0.833	Stress response	GO:0009408: Response to heat GO:0034620: Cellular response to unfolded protein GO:0034976: Response to endoplasmic reticulum stress
Hemocyte protein-glutamine gamma-glutamyltransferase-like	XP_045598315.1	+1.507	Stress response	GO:0009611: Response to wounding GO:0050832: Defense response to fungus
LOW QUALITY PROTEIN: nitric oxide synthase-like protein	XP_045601142.1	+2.481	Stress response	GO:0034614: Cellular response to reactive oxygen species GO:0006979: Response to oxidative stress
Thioredoxin-like protein 1	XP_045601804.1	−0.654	Stress response & DNA repair	GO:0034599: Cellular response to oxidative stress GO:0006281: DNA repair GO:0006979: Response to oxidative stress
DNA damage-inducible transcript 4-like protein	XP_045623842.1 XP_045623843.1	+1.238 +2.332	Stress response & DNA repair	GO:0006974: DNA damage response GO:0006281: DNA repair GO:0033554: Cellular response to stress
Protein spaetzle 4-like	XP_045624048.1	−2.313	Stress response & Immune response	GO:0006952: Defense response GO:0009611: Response to wounding GO:0008063: Toll signaling pathway GO:0006955: Immune response
Vascular endothelial growth factor receptor 1-like	XP_045625733.1	+1.744	Stress response	GO:0008285: Negative regulation of cell proliferation GO:0007169: Transmembrane receptor protein tyrosine kinase signaling pathway
Chorion peroxidase-like	XP_045583397.1	+1.746	Stress response	GO:0006979: Response to oxidative stress GO:0033554: Cellular response to stress
Histone H1-delta-like	XP_045591925.1	+1.259	DNA repair	GO:0006974: DNA damage response GO:0006303: Double-strand break repair via non-homologous end joining
SUMO-activating enzyme subunit 1-like	XP_045597860.1	−0.505	DNA repair	GO:0006302: Double-strand break repair GO:0006301: Postreplication repair
E3 ubiquitin-protein ligase TRIM9-like	XP_045603701.1	+0.521	DNA repair	GO:0043161: Proteasome-mediated ubiquitin-dependent protein catabolic process
Folate receptor beta-like	XP_045601829.1	−1.889	Immune response	GO:0050776: Regulation of immune response GO:0009408: Response to heat
Astakine isoform X2 like	XP_045617935.1	+1.577	Immune response	GO:0006955: Immune response GO:0045087: Innate immune response
Beta-1,3-glucan-binding protein-like	XP_045588931.1	+2.115	Immune response	GO:0008233: Peptidase activity GO:0050830: Defense response to Gram-positive bacterium GO:0006952: Defense response
Leukocyte elastase inhibitor-like	XP_045596624.1	+2.57	Immune response	GO:0004866: Endopeptidase inhibitor activity GO:0009611: Response to wounding GO:0050727: Regulation of inflammatory response
Pulmonary surfactant-associated protein D-like	XP_045605160.1	+1.321	Immune response	GO:0006955: Immune response GO:0008009: Chemokine activity GO:0030203: Glycosaminoglycan metabolic process
Integrin alpha-8-like	XP_045614929.1	+1.300	Immune response	GO:0007229: Integrin-mediated signaling pathway GO:0050900: Leukocyte migration
CLIP domain-containing serine protease -like	XP_045621670.1	+1.741	Immune response	GO:0008236: Serine-type peptidase activity GO:0006955: Immune response GO:0006952: Defense response
Angiotensin-converting enzyme-like	XP_045622842.1	+7.521	Immune response	GO:0002003: Angiotensin maturation GO:0006955: Immune response
Vascular endothelial growth factor receptor 1-like	XP_045625733.1	+1.744	Immune response	GO:0001525: Angiogenesis GO:0050900: Leukocyte migration
Translation elongation factor 2-like	XP_045596644.1	−0.609	Translation and transcription	GO:0003746: Translation elongation factor activity
Eukaryotic translation initiation factor 3 subunit B-like	XP_045600183.1	−0.741	Translation and transcription	GO:0003743: Translation initiation factor activity
Transcription initiation factor TFIID subunit 1-like	XP_045583537.1	+1.488	Translation and transcription	GO:0006352: DNA-templated transcription initiation
Mediator of RNA polymerase II transcription subunit 19-like	XP_045615251.1	−0.681	Translation and transcription	GO:0006351: DNA-templated transcription
Elongator complex protein 6-like isoform X1	XP_045597767.1	+2.100	Translation and transcription	GO:0003723: RNA binding

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Table 1 (*continued*)

Camargue population				
Hepatopancreas				
Transcript	**NCBI reference protein sequence**	**log2FoldChange**	**Type of response**	
mRNA decay activator protein ZFP36L1-like isoform X1	XP_045596521.1	+0.693	Translation and transcription	GO:0006402: mRNA catabolic process
Gills				
Endochitinase-like	XP_045623478.1 XP_045600716.1	−5.344 −1.380	Stress response & Immune response	GO:0009409: Response to cold GO:0006032: Chitin catabolic process GO:0008063: Toll signaling pathway GO:0050832: Defense response to fungus
Proline-rich extensin-like protein EPR1	XP_045626046.1	−1.703	Stress response	GO:0006950: Response to stress GO:0009651: Response to salt stress
E3 ubiquitin-protein ligase RNF12-B-like	XP_045618706.1	−4.486	Stress response & DNA repair	GO:0006950: Response to stress GO:0006281: DNA repair GO:0006974: DNA damage response GO:0016567: Protein ubiquitination
Chitin deacetylase -like	XP_045604353.1 XP_045619312.1 XP_045590550.1	−2.533 −1.494 −1.948	Stress response & Immune response	GO:0009409: Response to cold GO:0006952: defense response GO:0008063: Toll signaling pathway GO:0010200: Response to chitin
Transcription initiation factor TFIID subunit 13-like	XP_045591906.1	−6.405	DNA repair & Translation and transcription	GO:0003684: Damaged DNA binding GO:0006352: DNA-templated transcription initiation GO:0005669: Transcription factor TFIID complex GO:0006367: Transcription initiation at RNA polymerase II promoter
Cyclin-dependent kinase inhibitor 1-like	XP_045598155.1	−1.414	DNA repair & Translation and transcription	GO:0003684: Damaged DNA binding GO:0006281: DNA repair GO:0051726: Cellular cycle regulation GO:0006974: DNA damage response GO:0003676: Nucleic acid binding GO:0006355: Regulation of DNA-templated transcription
Lysosomal aspartic protease-like	XP_045611751.1	−6.241	Immune response	GO:0004190: Aspartic-type endopeptidase activity GO:0006915: Apoptotic process GO:0006952: Defense response
Protein spaetzle 4-like	XP_045624048.1	−1.059	Immune response	GO:0008063: Toll signaling pathway GO:0006955: Immune response GO:0006952: Defense response
Anti-lipopolysaccharide factor-like	XP_045583628.1	−1.745	Immune response	GO:0006952: Defense response
Zinc finger protein 271-like	XP_045593849.1	+1.054	Translation and transcription	GO:0006351: DNA-templated transcription GO:0003677: DNA binding
Heterogeneous nuclear ribonucleoprotein A3 homolog 1-like	XP_045583862.1	−2.364	Translation and transcription	GO:0003676: Nucleic acid binding GO:0000398: mRNA splicing, via spliceosome
Translation initiation factor IF-2-like	XP_045592091.1 XP_045596149.1	−3.940 −3.153	Translation and transcription	GO:0006413: Translational initiation
Ribosome-binding protein 1-like	XP_045592090.1	−1.256	Translation and transcription	GO:0006412: Translation
Elongation factor 1-alpha-like	XP_045605276.1	+4.369	Translation and transcription	GO:0006414: Translational elongation
Salagou Population				
Hepatopancreas				
Transcript	**NCBI reference protein sequence**	**log2FoldChange**	**Type of response according to enrichment analysis**	
Delta-1-pyrroline-5-carboxylate synthase-like	XP_045599855.1	+3.481	Stress response	GO:0003842: 1-pyrroline-5-carboxylate dehydrogenase activity.
Pseudohemocyanin-2-like	XP_045606900.1 XP_045606902.1 XP_045606903.1 XP_045606904.1	+10.50 +9.29 +11.45 +10.342	Immune response	GO:0006952: Defense response GO:0019826: Oxygen transport
Gills				
Calmodulin-like	XP_045598052.1	+2.107	Stress response	GO:0005509: Calcium ion binding GO:0005516: Calmodulin binding
Digestive cysteine proteinase	XP_045606835.1 XP_045613782.1 XP_045616603.1	+8.076 +20.780 +5.361	Stress response	GO:0006508: Proteolysis GO:0006952: Defense response
WAP four-disulfide core domain protein 2-like	XP_045603563.1	+2.363	Immune response	GO:0006952: Defense response
Probable chitinase 10	XP_045625648.1	+8.229	Immune response	GO:0006032: Chitin catabolic process GO:0006952: Defense response
Chitinase-3-like protein 1	XP_045626131.1	+6.457	Immune response,	GO:0006032: Chitin catabolic process GO:0006952: Defense response

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Table 1 (*continued*)

Salagou Population				
Gills				
Transcript	**NCBI reference protein sequence**	**log2FoldChange**	**Type of response according to enrichment analysis**	
Hematopoietic prostaglandin D synthase-like	XP_045584031.1 XP_045584251.1	+4.503 −2.383	Immune response	GO:0006693: Prostaglandin metabolic process GO:0006952: Defense response
Protein Skeletor, isoforms B/C-like isoform X2	XP_045605289.1	+1.450	Translation and transcription	GO:0006351: DNA-templated transcription GO:0006355: Regulation of DNA-templated transcription
Bages-Sigean Population				
Hepatopancreas				
Transcript	**NCBI reference protein sequence**	**log2FoldChange**	**Type of response according to enrichment analysis**	
Daf-12-interacting protein 1-like	XP_045599075.1	+1.656	Stress response	GO:0009267: Cellular response to starvation GO:0042594: Response to starvation GO:0033554: Cellular response to stress GO:0009628: Response to abiotic stimulus
Arylalkylamine N-acetyltransferase-like 2	XP_045586733.1	−3.022	Stress response	GO:0006970: Response to osmotic stress GO:0007585: Respiratory gaseous exchange by respiratory system GO:0007623: Circadian rhythm
Facilitated trehalose transporter Tret1-2 homolog	XP_045588008.1	+1.563	Stress response	GO:0006970: Response to osmotic stress GO:0009651: Response to salt stress GO:0042538: Hyperosmotic salinity response
Origin recognition complex subunit 3-like	XP_045593094.1	−1.091	DNA repair Translation and transcription	GO:0006270: DNA replication initiation GO:0003688: DNA replication origin binding GO:0031297: Replication fork processing GO:0003677: DNA binding GO:0006355: Regulation of DNA-templated transcription
Low density lipoprotein receptor adapter protein 1-A-like	XP_045606366.1	+0.793	Immune response	GO:0006955: Immune response

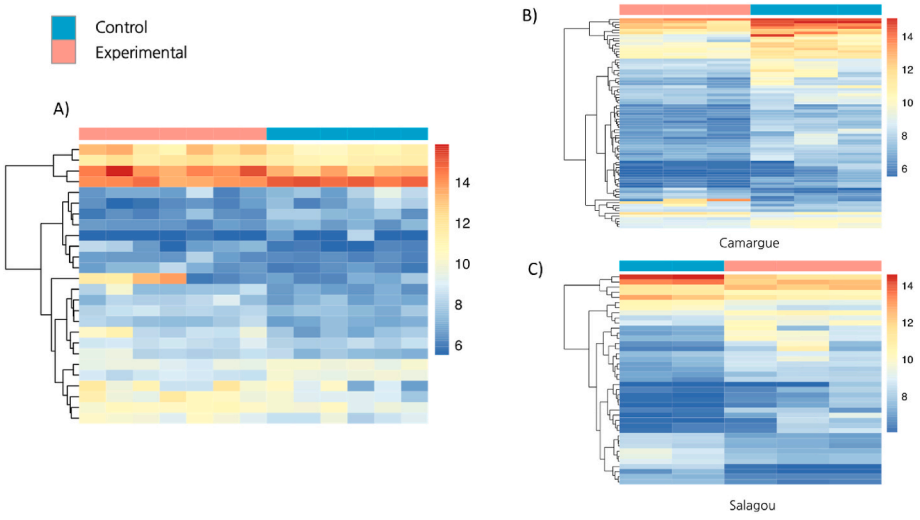

Fig. 4. Heatmaps showing differentially expressed genes (DEGs) in gills of crayfish after 96-h pesticide mixture exposure in animals from the three studied populations: A) General overview, and from two of the studied populations B) Camargue; C) Salagou.

midgut gland up-regulates genes involved in stress response, DNA repair, and immune response, while the gills down-regulate them. In contrast, Salagou individuals, although also exhibiting a high response in these metabolic processes, they took place primarily in the gills, and they are mostly up-regulated.

Interestingly, out of the three crayfish populations considered, only those from the pristine site (i.e. Salagou) did not show an induction of DNA repair-related genes in response to pesticide exposure in the laboratory. Normally, the activation of DNA-damage-inducible genes is expected to confer protection preventing genotoxicity (Papathanasiou and Fornace, 1991). We therefore hypothesize that this is their role in the case of the Camargue and Bages-Sigean crayfish and it could be seen as an adaptation to the periodical exposure to pesticides that they face in their natural environment. We attribute this response of DNA

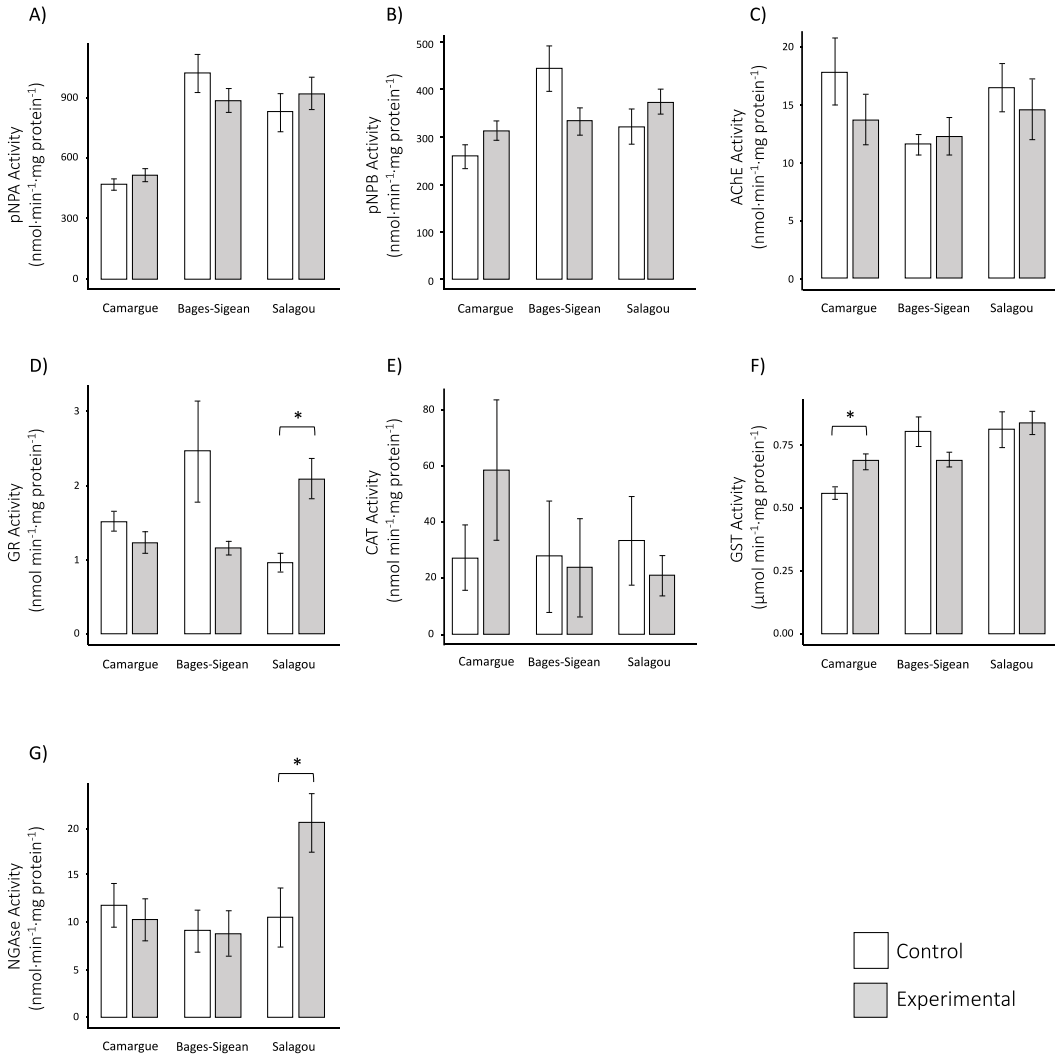

Fig. 5. Activity of carboxylesterase using p-nitrophenyl acetate (p-NPA) and using p-nitrophenyl butirate (p-NPB), acetylcholinesterase (AChE), glutathione reductase (GR), catalase (CAT), glutathione-S-transferase (GST), and NAGase (NAG) in hepatopancreas for each of the three populations considered. Error bars represent standard deviation. The * represents statistically significant values at $p \leq 0.05$.

damage-inducible related genes to the presence of oxadiazon in the pesticide cocktail, given its demonstrated genotoxic potential even at low concentrations (Zanjani et al., 2017). In aquatic species, regularly exposed to harmful levels of chemicals, some of the potential adaptive mechanisms of defense include enhancement of antioxidant responses, and increased capacity for DNA and tissue repair (Hamilton et al., 2017).

4.4. *Expression of chitinase-related genes with a possible role in the immune response*

Our results on the regulation of the chitinase or chitin related genes are also worth highlighting. These were highly down-regulated in the gills of Camargue crayfish (pesticide polluted) but highly up-regulated in the gills of Salagou crayfish (reference site). This enzyme not only degrades chitin during growth, development and the molting processes in arthropods, but it also plays a key role in the immune responses and regulation (e.g. Niu et al., 2018; Liu M et al., 2021). In other crustaceans like the tiger shrimp (*Penaeus monodon*) the expression of chitin genes has been detected in several tissues although the highest levels have been found in gills and hepatopancreas. It has been suggested that some chitinase-related genes may be involved in the innate immune responses in *P. clarkii* by modulating the toll pathway (Liu M et al., 2021). Furthermore, chitinase in crustacean species has been observed to strongly respond to cadmium stress (Yang et al., 2024), and gene expression levels significantly increased under ammonia-N stress (Zhou et al., 2018). As mentioned previously, we found differentially expressed chitinase genes in the gills of Camargue and Salagou crayfish. In Camargue crayfish, two chitinase genes were down-regulated in the gills while being up-regulated in the Salagou crayfish: it is particularly interesting that in the gills of Salagou crayfish, they were expressed 300 and 87 times more in the pesticide exposed animals than in controls, which makes it a very sensitive response. In fact, this gene up-regulation is also confirmed at the enzymatic level by a two-fold increase in NGAase activity, an enzyme involved in chitin degradation during the molting process (Rollin et al., 2021). Out of all experimental crayfish, none of them were in their molting period during or after the experimental exposure took place, leading us to believe that this response is not due to the natural molting process. Hence, we attribute the chitinase response in gills to an immune response triggered by the pesticide exposure. The up-regulation of this protein is particularly significant in Salagou individuals, which also exhibited a significant up-regulation of pseudo hemocyanin-like proteins, albeit in the hepatopancreas. Hemocyanin-like proteins are crucial immune proteins in arthropods (Decker and Jaenicke, 2004; Yan et al., 2011), and their expression has

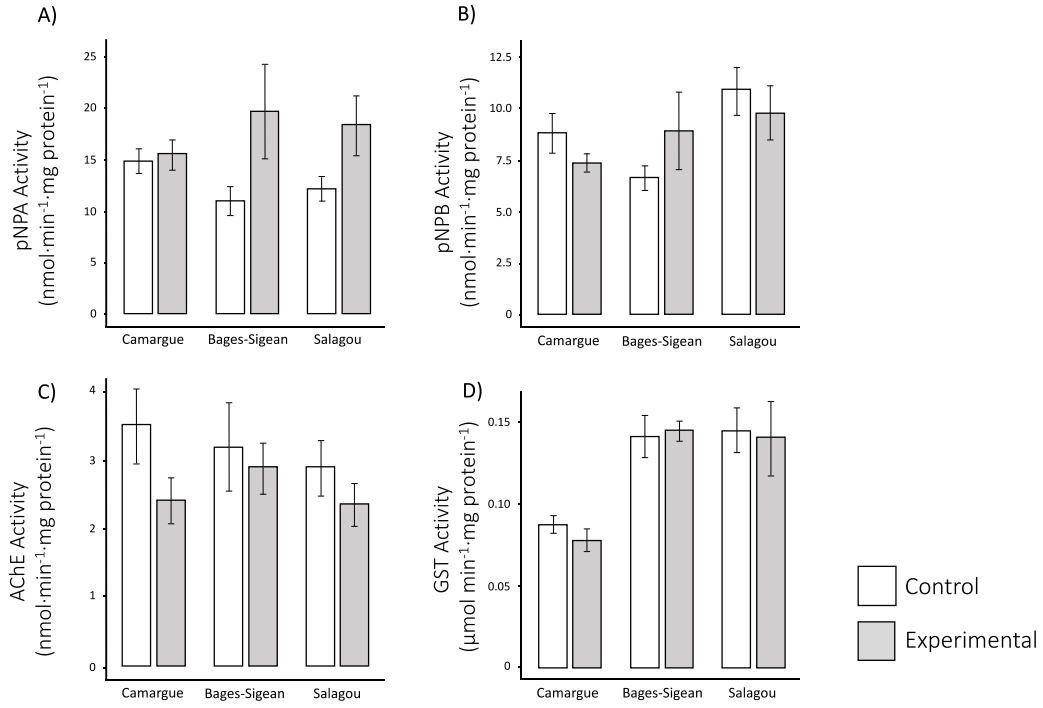

Fig. 6. Activity of carboxylesterase (CE) using p-nitrophenyl acetate (p-NPA) and using p-nitrophenyl butyrate (p-NPB) as substrates, acetylcholinesterase (AChE) and glutathione-S-transferase (GST) in gills for each of the three populations considered. Error bars represent standard deviation.

been shown to increase significantly following cadmium exposure (Liu X et al., 2021). These findings further support the hypothesis that individuals from Salagou (reference site) initiate an immune response in the gills and hepatopancreas, even if this entails a distinct set of proteins compared to those shown in crayfish from the pesticide-exposed site of Camargue.

4.5. Enzymatic responses: effects on antioxidant defenses, detoxification processes and health biomarkers upon exposure to a pesticide mixture

It is well recognized that the presence of xenobiotics, including pesticides, induces the production of reactive oxygen and nitrogen species (RONS), compromising antioxidant defenses and, eventually, leading to oxidative stress (e.g. Sule et al., 2022). In this study, as a consequence of a 96 h exposure to the pesticide mixture, only crayfish from the Camargue population displayed an increase in GST activity in hepatopancreas. In a former study by Uçkun and Öz (2021), exposure to one of the two pesticides (azoxystrobin) caused enhanced GST activity in another crayfish species. An interesting work by Kovačević et al. (2023) shows the temporal dynamics of exposure to azoxystrobin in a terrestrial invertebrate. Although the study by Kovačević et al. (2023) considered pollutant concentrations per kilogram of soil, their lowest concentrations could be comparable to the concentrations used here per liter of water (despite a likely different bioavailability). They observed that CAT and GST activities increased during the first three days of exposure, but then decreased from day five onwards. However, most reports agree that exposure to herbicides causes a decrease in antioxidant defenses, leading to oxidative stress.

CEs are also potential biomarkers of pesticide exposure, as they are an important family of enzymes involved in the metabolism of xenobiotic and endogenous compounds for a wide variety of organisms, including crustaceans (Wheelock et al., 2008; Nos et al., 2021). In this study, we did not find a significant change in CE activities in the hepatopancreas or gills with either of the two substrates used. However, since the hydrolysis rates with pNPA substrate exceeds that of pNPB by approx. 50% in almost all cases, it is likely that pNPA is more adequate substrate for CE measurements in this crayfish species.

In this study, the polluted populations of Bages-Sigean and Camargue, responded to pesticide exposure in the laboratory by decreasing their NGAse activity. Existing literature indicates that pollutants such as heavy metals (i.e. Cd or Zn) (e.g. Mesquita et al., 2015; Rollin et al., 2023), herbicides (e.g. Glyphosate) or drugs (e.g. pentoxifylline) can cause a decrease in NAGase activity in crustaceans (Rollin et al., 2023). It has been also observed in the European green crab *Carcinus maenas* that NAGase activity decreases as a response to heavy metals (Cd in particular) and this response is dependent on the quality of their natural environment. In this sense, *C. maenas* from a relatively pristine site, shows NAGase activity inhibition when exposed to Cd while crabs from a moderately polluted site do not. Our results do not align with those with *C. maenas*, as Salagou crayfish (more pristine area) experienced a two-fold increase in NAGase activity when exposed to the pesticide input. Likewise, Rollin et al. (2023) observed a 35% increase as a response to the fungicide dithiocarbamate in the marine prawn *Palaemon serratus*. All in all, even if increases in NAGase activity as a response to pollutants are not unheard of, decreases in activity rates have been most frequently documented.

In the present study, we did not observe a significant inhibition of the AChE activity in either gills or hepatopancreas. Contrasting with our results, another crayfish species, *Astacus leptodactylus*, experienced an increase in AChE activity upon exposure to azoxystrobin (Uçkun and Öz, 2021). Although the role of AChE in innerved tissues is mostly neural transmission, in others it can have an immune role as acetylcholine is a signaling molecule initiating this process (Giordani et al., 2023).

5. Conclusions

Our results showed population-specific responses to pesticide stress in populations with different chemical backgrounds. Crayfish populations used to dealing with pesticides in their environment respond to this stress by highly regulating genes involved in DNA repair, immune response, and translation and transcription mechanisms, suggesting these play a crucial role in coping with pesticide exposure. However,

other populations naïve to the pesticides, did not develop the same mechanism of response. Altogether, we can corroborate our hypothesis that life-history of exposure to pollutants determines a particular response upon facing a new chemical challenge. These differences also contrasted between the hepatopancreas and gills of crayfish from the studied populations. Our results highlight the importance of considering physiological plasticity and tolerance to pollutants as being population-specific, especially for risk assessments of invasive species. These population-specific responses suggest a rapid adaptation to anthropogenic stressors that is well worth further investigation, particularly through a multigeneration evolution experiment to determine whether this adaptation is plastic or genetic.

Finally, although this study focuses mainly on pesticides, the regulation of genes associated with stress response, RNA processing, transcription regulation, DNA and tissue repair, and immune response has also been observed in other aquatic species exposed to a wide range of chemical contaminants including metals, other pesticides and PAHs (i.e. Pilcher et al., 2014; Hamilton et al., 2017; Liu X et al., 2021). Interestingly, the same genes are also regulated under salinity and temperature stress (i.e. Rivera-Ingraham and Lignot, 2017; Jeyachandran et al., 2023; Martínez-Alarcón et al., 2024). Therefore, genetic adaptations at the population level that involves these processes may have the potential to enhance tolerance not only to a wide range of pollutants, but also to salinity and temperature as stressors.

Altogether, this study highlights the importance of studying not only stress-related enzymes but also regulatory processes involved in the stress responses. Focusing solely on specific enzymes may overlook critical aspects of how organisms adapt to stress at the molecular level.

CRediT authorship contribution statement

Diana Martínez-Alarcón: Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Celine M.O. Reisser:** Writing – review & editing, Methodology, Formal analysis. **Montserrat Solé:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Jehan-Hervé Lignot:** Writing – review & editing, Investigation, Conceptualization. **Georgina Rivera-Ingraham:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation.

Ethics statements

No permits were required to conduct this study. However, it was conducted in accordance with the local legislation and institutional requirements. All animals were captured, manipulated and euthanized following the International Guiding Principles for Biomedical Research Involving Animals, issued by the Council for the International Organizations of Medical Sciences.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Diana Martinez-Alarcon reports financial support was provided by European Commission. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2025.120963.

Data availability

The raw sequences and associated metadata generated for this study can be found on the online repository ENA (European Nucleotide Archive) under the accession number PRJEB81785.

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