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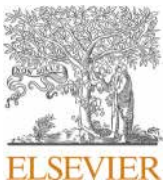

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# Is malathion oncogenic for mosquitoes? A transcriptomic and histological study of adults derived from malathion exposed larvae

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ABSTRACT

Malathion is a widely used pesticide with potentially oncogenic properties and may have deleterious effects on organism health and fitness. Although malathion use is now restricted in the European Union, it remains widely used for public health campaigns in other parts of the world, particularly for mosquito control. Understanding its sublethal and long-term effects is thus essential, both for evaluating its ecotoxicological impacts and for anticipating resistance mechanisms. However, empiric data on its effects in wild organisms - especially in invertebrates - remain limited. Here, we quantitatively investigated whether larval exposure to environmentally realistic concentrations of malathion could affect mosquito tissue structure and gene expression profiles of adult *Aedes aegypti* (yellow fever mosquitoes), using both RNA-seq and histological approaches. Results show no neoplastic or pre-neoplastic lesions in adults exposed to malathion during larval development, contrary to previous studies in other organisms showing carcinogenic effects of malathion. However, our differential gene expression analyses revealed significant changes in genes related to mitochondrial function, energy metabolism, and detoxification pathways, suggesting significant physiological impacts of malathion in adults after early-life pesticide exposure. Notably, females exhibited stronger transcriptomic responses than males, including the upregulation of genes involved in detoxification (e.g., P450 cytochromes), olfactory perception, and stress response, with potential consequences for resistance mechanisms. Our findings underscore the ability of mosquitoes to mount transient molecular responses to environmental pollutants, potentially contributing to the long-term selection of metabolic resistance traits – an outcome with important implications for vector control strategies.

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## 1. Introduction

It is increasingly recognized that the planet's ecosystems are exposed to growing levels of toxic and mutagenic pollution driven by human activity (Dujon et al., 2021; Giraudeau et al., 2018). Theoretically, this is expected to trigger an upsurge in physiological dysregulation and oncogenic processes in multicellular organisms due to the incapacity of physiological and anti-cancer defenses - evolved in low toxic and oncogenic environments -to effectively counteract contemporary environmental risks, i.e. an evolutionary mismatch (Aktipis and Nesse, 2013). Hereafter, oncogenic effects are defined as cellular or tissue-level alterations indicative of abnormal proliferation, tumor-like growths, dysplasia, or dysregulated gene expression related to, for instance, cell cycle and apoptosis. As recently proposed by Dujon et al. (2024), such predictions are not straightforward as several phenomena may come into play (Dujon et al., 2024). For instance, excessive exposure to toxic oncogenic substances may first trigger heightened activation of physiological and anti-cancer defenses. This can effectively hinder physiological alterations and the development of cancers, resulting in the absence of cellular damage and visible tumors, even though individuals may, in turn, suffer increased energy demand and physical exhaustion due to the sustained activation of defenses. Natural selection may also favor changes in life-history traits and physiological adjustments within populations exposed to polluted environments, leading to an increase in tolerance or resistance mechanisms at the expense of reproduction or other traits (Arnal et al., 2017; Boutry et al., 2022). In such circumstances, cellular damage and tumors may not have sufficient time to fully develop. Over longer periods, some species may evolve additional physiological and anti-cancer defenses (Boutry et al., 2020; Sulak et al., 2016; Vittecoq et al., 2018), so that any apparent increase in cancers incidence may only be temporary on an evolutionary scale. The effects of increased exposure to toxic molecules and oncogenic factors on wildlife are thus multifaceted, encompassing immediate physiological responses to long-term evolutionary adaptations that are difficult to predict. Currently, these processes are not well understood, especially in the context of increasing pollution and gaining a better understanding of them is crucial (Dujon et al., 2024; McAloose and Newton, 2009).

One of the most potentially oncogenic factors in anthropized ecosystems is organic pollution by pesticides. According to the Atlas of Pesticides (2022, https://eu.boell.org/sites/default/ files/2023–04/ pesticideatlas2022_ii_web_20230331.pdf), pesticide consumption stands at 4 million tones globally, representing an 80 % increase in the last 20 years. There is substantial evidence on a relationship between exposure to pesticides and an increased incidence of chronic diseases such as Parkinson's or childhood leukemia, liver and breast cancer, diabetes, and endocrine disorders in humans. However, the underpinning mechanisms and the effects on wildlife are still poorly known.

According to the European Environmental Agency, malathion is one of the pesticides that most often exceeds national thresholds in European surface waters (European Environment Agency2024, https://www.eea.europa.eu/en/analysis/indicators/pesticides-in-rivers-lakes-and). Malathion is an organophosphate insecticide widely used in agriculture, domestic pest control, and vector control programs. It has been banned in Europe in 2008 but is still widely used in several parts of the world. In the context of public health, malathion is routinely used in mosquito control strategies, particularly against *Aedes aegypti* (yellow fever mosquito), a major vector of arboviruses, including dengue, Zika, chikungunya, and yellow fever (Tomé et al., 2014). Given its continued use in integrated vector management programs worldwide, malathion remains a highly relevant compound for investigating its potential unintended physiological effects on animal populations. It is classified by the WHO as "probably carcinogenic" for humans (Group 2 A, https://www.iarc.who.int/wp-content/uploads/2018/07/MonographVolume112-1.pdf) (Calaf et al., 2021). It also poses risks to animal health including humans (Badr, 2020; Bastos et al., 2020; Cook et al., 2005; Deka and Mahanta, 2016), particularly under prolonged exposure. Scientific research has suggested that chronic exposure to malathion, even at levels well below those causing acute poisoning (lethal dose estimated at 246–471 mg/kg, depending on the animal species, (Faragó, 1967; Jush and Mille, 1978)), may be associated with long-term health issues, including certain types of cancer such as prostate cancer, as well as impacts on the endocrine and immune systems (Anjitha et al., 2020; Bonner et al., 2007; Calaf and Echiburú-Chau, 2012; Calaf and Roy, 2008; Yang et al., 2020). However, the molecular and cellular effects of malathion at environmentally realistic concentrations in the natural environment remain debated.

Pesticide pollution is especially problematic in wetlands (Girones et al., 2021; Li et al., 2022). It is therefore crucial to understand the effects of such pollution on cancer-related processes in species inhabiting these habitats, particularly insects (Baines et al., 2021). Malathion disrupts the nervous system of insects, leading to paralysis and death (Xue, 2008). However, its other potential effects on invertebrates remain poorly known. While numerous studies have explored the effects of malathion on insect viability and development, no study to our knowledge has explored its potential oncogenicity in this group. Aside from *Drosophila* (Gong et al., 2021; Martorell et al., 2014), available data on insect physiology, gene expression and cancer development in insects are very limited (Arnal et al., 2020). Extensive research in *Drosophila melanogaster* has helped characterize several molecular hallmarks of oncogenesis, including the dysregulation of tumor suppressor genes, activation of oncogenic signaling pathways, and disruption of cell polarity and apoptosis (Gonzalez, 2013; Sonoshita and Cagan, 2017). Indeed, while insects do not develop cancer in the classical sense observed in vertebrates—such as tumor vascularization or metastasis—the underlying cellular pathways leading to uncontrolled proliferation and dedifferentiation are probably evolutionarily conserved (Martorell et al., 2014).

In addition, it is not clear whether early-life exposure to such pesticide can influence the later physiological dysregulation and oncogenic risk in adult insects. This also raises an intriguing possibility: tumorigenic processes in insects might not necessarily be rare but could frequently occur during larval or pupal stages. However, because these life stages are tightly linked to developmental processes such as molting and metamorphosis, any disruption caused by uncontrolled cell proliferation or tissue disorganization might result in developmental arrest or death before reaching the adult stage. This could represent a powerful natural filter eliminating tumor-bearing individuals early, thereby limiting the detection of cancer in adult insect populations. It is thus particularly relevant to study the potential effects of malathion in adult mosquitoes that were exposed to sublethal concentrations during their larval stages in their natural contaminated aquatic environment.

In this study, we focused on yellow fever mosquitoes, *A. aegypti,* a species commonly found in pesticide-contaminated aquatic systems and known for its significant impact on human health. Due to its widespread distribution and its anthropophilic behavior, it is a key target in vector control programs. Moreover, this species frequently develops resistance to pesticide treatments used to limit their spread around urban areas. Investigating the transcriptomic and physiological responses to malathion in *A. aegypti* will therefore help clarify its potential oncogenic effects in non-target species and may reveal mechanisms contributing to cellular damage, oncogenesis and/or resistance evolution.

We used an experimental approach to investigate the consequences in adult *A. aegypti* mosquitoes of a pre-exposure to malathion during the larval aquatic stage. Larvae have been exposed to environmentally realistic concentrations of malathion dissolved in water, simulating moderate to severe stress conditions potentially found in wetlands. Using a combination of histological and RNA-seq analyses in whole adults, we aimed to provide valuable insights into the impact of early-life malathion exposure on the health and physiology of adult mosquitoes and to improve our understanding of evolutionary responses to mutagenic pollution in contaminated ecosystems.

## 2. Material and methods

### 2.1. Development of Aedes aegypti in a stressful environment

Based on preliminary results (see Supplementary Material), we reared yellow fever mosquitos' larvae *Aedes aegypti* Bora Bora strain during 6–7 days - which correspond to the typical duration of the larval stage period - under chemically stressful conditions, *i.e.* larvae rearing water with 0.03 mg/L of malathion, 1.4 mg of food per larvae (Novo-Prawn®, fish food) and an ambient temperature of 25 °C. This specific insecticide concentration was selected because it provides pollutant exposure without exceeding the sublethal threshold for mosquitoes. The median lethal dose ($LD_{50}$) and the 95 % lethal dose ($LD_{95}$) for malathion in the breeding environment of *A. aegypti* (Bora Bora strain) are 0.065 mg/L and 0.119 mg/L, respectively (Goindin et al., 2017). Since our focus in on the adult stage, this concentration enables us to maintain realistic exposure conditions while avoiding significant larval mortality. Control larvae were reared under the same conditions (25 °C and 1.4 mg of food per larva), but without malathion.

The malathion concentration used in this study is higher than average environmental levels reported in mosquito habitats. However, such concentrations may be encountered locally in contaminated breeding sites near agricultural areas or in pesticide-treated environments (European Environment Agency 2024, https://www.eea.europa.eu/en/analysis/indicators/pesticides-in-rivers-lakes-and). This study was therefore designed as a proof-of-concept to assess whether larval exposure to malathion could have long-term consequences on adult tissue organization.

The experimental setup consisted of 25 larvae per cup (200 ml of water per cup), with a total of 50 cups (25 for the control group and 25 for malathion-exposed group), representing a total of 1250 individuals.

Pupae from both treatments were transfered from the cups (control and malathion) into mosquito cages. Just after the emergence, males and females were separated into different cages, fed with a 10 % sugar solution, and maintained under observation for up to 3 weeks at 27°C. After this period (either 2 or 3 weeks), male and female adults were anesthetized and split into two groups by condition: half were fixed *in toto* in 4 % formalin for histological analysis (stored in cassettes within 400 ml plastic tubes), and the other half were stored individually in 1.5 ml Eppendorf tubes at −80°C for transcriptomic analysis (see Table 1).

**Table 1**
Summary of the experimental design and the number of adult mosquito samples proceeded in histological and frozen for transcriptomic analysis.

| Treatment | Sex | Adult Age (in weeks) | Number of samples | Type of Analysis |
|---|---|---|---|---|
| Malathion | Male | 2 | 50 | Histology |
| Malathion | Female | 2 | 35 | Histology |
| Negative Control | Male | 2 | 20 | Histology |
| Negative Control | Female | 2 | 20 | Histology |
| Malathion | Male | 3 | 50 | Histology |
| Malathion | Female | 3 | 35 | Histology |
| Negative Control | Male | 3 | 20 | Histology |
| Negative Control | Female | 3 | 20 | Histology |
| Malathion | Male | 2 | 50 | Transcriptomic |
| Malathion | Female | 2 | 50 | Transcriptomic |
| Negative Control | Male | 2 | 50 | Transcriptomic |
| Negative Control | Female | 2 | 41 | Transcriptomic |
| Malathion | Male | 3 | 50 | Transcriptomic |
| Malathion | Female | 3 | 50 | Transcriptomic |
| Negative Control | Male | 3 | 43 | Transcriptomic |
| Negative Control | Female | 3 | 50 | Transcriptomic |

### 2.2. RNA extraction

For each of the 8 experimental conditions - defined by treatment (malathion vs control), sex (male vs female) and age (2 weeks vs 3 weeks), −13 whole mosquitoes were randomly sampled from the individuals set aside for transcriptomic analysis and pooled, resulting in a total of 24 pools (see Supplementary Table S1). Each pool was stored at −80°C in 700 μL of QIAzol® Lysis Reagent (QIAGEN) in a 2 ml SafeLock microtube until further processing.

Tissue disruption was performed mechanically by adding a 2 mm stainless bead to each sample and using a TissueLyser (QIAGEN) for 2 cycles of 2 min at 20 Hz. After incubation for 5 min at room temperature, 140 μL of chloroform was added to the homogenate, followed by vigorous shaking for 15 s and a 3-min incubation at room temperature. Phase separation of the lysate was achieved by centrifugation at 12,000 x g for 15 min at 4°C. The upper aqueous phase (~ 350 μL) containing RNA was carefully transferred to a clean 2 ml microtube. Total RNA was then purified using the RNeasy® Mini QIAcube Kit (QIAGEN) and the QIAcube automated workstation (QIAGEN), according to the manufacturer's protocol, to ensure reproducibility and standardization of RNA extraction. DNA was removed by on-column DNAse digestion. RNA was eluted in 30 μL of RNase-free water and samples were stored at −80°C until sequencing. The concentration and purity of RNA in each sample was measured using a NanoDrop™ ND-1000 spectrophotometer (Thermo Scientific). RNA concentrations ranged from 163.96 to 926.56 ng/μL.

### 2.3. RNA sequencing

The 24 cDNA libraries were prepared using the Stranded Total RNA Prep kit, following ribosomal RNA depletion using the Ribo-Zero Plus rRNA Depletion Kit (both from Illumina). For each library, 500 ng of purified RNA was used as input, and 11 cycles of enrichment PCR were performed. Constructed indexed libraries were assessed for quality and quantification using a LabChip® GX Touch™ (Revvity) and a Qubit™ fluorometer with the Qubit™ dsDNA HS Assay kit (Thermo Fisher Scientific). All libraries were validated and pooled using 300 ng of cDNA per sample (Supplementary Table S2).

Sequencing was performed on an Illumina® NovaSeqTM platform, with a 100 bp paired-end configuration. Raw sequencing reads were first processed with RCorrector v1.04 (https://anaconda.org/bioconda/rcorrector/files?version=1.0.4) with default settings and -rf configuration to correct sequencing errors (Song and Florea, 2015). Uncorrectable reads were removed using the FilterUncorrectabledPEfastq tool (https://github.com/harvardinformatics/TranscriptomeAssemblyTools/blob/master/FilterUncorrectabledPEfastq.py). The output reads were further processed for adapter removal and trimming with TrimGalore! v0.6.4 (https://github.com/FelixKrueger/TrimGalore) with default parameters. Read quality was assessed before and after read trimming with FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Raw RNA-seq data have been deposited in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/ bioproject/) under the BioProject accession number PRJNA1169358. Individual SRA numbers are displayed in Supplementary Table S3. RNA extraction and sequencing were performed by the ACOBIOM company (France).

### 2.4. Quantification of gene expression

Read were mapped against the annotated reference genome of *Aedes aegypti* AaegL5.0 (NCBI RefSeq Assembly GCF_002204515.2) (Matthews et al., 2018). Reference genome files were downloaded from VectorBase

(release 66) https://vectorbase.org/vectorbase/app/downloads/Current_Release/ AaegyptiLVP_AGWG/. Mapping was performed using STAR v2.7.8a (Dobin et al., 2013). Gene-level read counts were generated using HTSeq-count v0.9.1 (Anders et al., 2015) with the mode set to *union* and the –outFilterMultimapNmax option to 20. We applied a pre-processing step on the count matrix to remove genes with fewer than 10 aligned reads in fewer than two samples per condition. Differential gene expression analysis were performed using the DESeq2 R package (Love et al., 2014). Count normalization was performed through the Relative Log Expression method (RLE) implemented in DESeq2 (Anders and Huber, 2010). Fold changes between two conditions were considered significant when the adjusted p-value (Padj), corrected for multiple testing using the Benjamini–Hochberg procedure to controls the false discovery rate (FDR), was $< 0.05$. Redundancy analysis (RDA) was performed on RLE-normalized counts, transformed as log2(RLE normalized counts + 1) values. Enrichment analysis of Gene Ontology (GO) terms was performed using Fisher's Exact Test. Fold enrichment of KEGG pathways was calculated as the percentage of differentially expressed genes in this pathway divided by the percentage of genes in this pathway in the *A. aegypti* genome with Benjamini-Hochberg test. Enrichments were retained as significant with p-values$< 0.05$.

### 2.5. Histology

Formalin-fixed whole individuals were individually included in an aqueous gel (Histogel®, MM France), then dehydrated and embedded in paraffin blocks (one animal per block). Longitudinal histological sections were cut at 3.5 µm and stained with hematoxylin, eosin, and saffron (HES). For each mosquito, six serial section levels were evaluated using a Leica DM2000 microscope (Leica Microsystems, Germany) at various magnifications (×25 to ×630). All anatomical structures were examined to detect potential lesions or development abnormalities and to compare the overall histological condition between exposed and unexposed individuals (Supplementary Figure S1).

## 3. Results

### 3.1. No evidence of histological lesions

Histological analyses revealed no tumoral lesions in any of the different tissues and organs examined, in either the malathion-treated or control groups. Likewise, no preneoplastic changes - such as atypical hyperplasia or dysplasia - were identified in the evaluated tissue sections. A concise histological atlas is available in Supplementary Figure S1.

### 3.2. Malathion exposure induces modifications in expression profiles

In total, sequencing of the 24 pools (corresponding to 312 individuals) yielded between 31.88 and 47.09 million clean paired reads per sample, of which 93.06 % - 95.63 % successfully mapped to the *A. aegypti* reference genome (Table 2).

The Redundancy Analysis (RDA), performed on the transformed normalized count matrix, showed the very strong effect of sex on the first dimension, explaining 71.16 % of the variance. An effect of age was observed on the second dimension, accounting for 2.17 % of the variance (Fig. 1A). On the third axis (only 1.29 % of the variance), we observed the effect of malathion treatment on individuals (Fig. 1B). According to these results, the expression profiles were analyzed separately for differential expression according to sex and age.

Differential expression analysis using DESeq2 was performed separately for each group, comparing mosquitoes exposed to malathion with their respective controls. In 2-week-old mosquitoes, 49 genes were differentially expressed (Padj$<0.05$) between exposed and control females (9 upregulated, 40 downregulated) and 83 in males (13 upregulated, 70 downregulated) (Fig. 2A and for the complete list of genes and log2FC values see Supplementary Table S4). In 3-week-old mosquitoes, we found 79 differentially expressed genes (DEGs) in exposed females (68 upregulated, 11 downregulated), and only 1 in males (downregulated). These results may suggest that the duration of gene expression dysregulation following malathion exposure differs between sexes, with potentially shorter effects in males. However, given the limited number of time points, this interpretation should be taken with caution. In addition, very few DEGs were shared between the different conditions (Fig. 2B).

### 3.3. Relevant biological functions in adult mosquitoes are disrupted following sub-lethal exposure to malathion during the larval stage

Of the 199 DEGs identified in at least one condition, 132 were

**Table 2**
RNAseq sequencing and STAR mapping metrics in males and females mosquitoes in control conditions or exposed to malathion (early exposure of larvae).

| Attributes | | | Nbrraw reads (2x).$10^6$ | Nbrclean reads (2x).$10^6$ | GC % | % reads mapped uniquely | % reads mapped to multiple locations | Total % reads mapped |
|---|---|---|---|---|---|---|---|---|
| **Controls** | *Female* | FCS2–1 | 42.22 | 38.97 | 49 | 16.81 | 78.39 | 95.20 |
| | | FCS2–2 | 43.49 | 40.03 | 48 | 17.41 | 76.98 | 94.39 |
| | | FCS2–3 | 44.59 | 40.96 | 48 | 17.42 | 77.94 | 95.36 |
| | | FCS3–1 | 44.18 | 40.94 | 48 | 16.09 | 79.14 | 95.23 |
| | | FCS3–2 | 42.10 | 39.03 | 49 | 16.66 | 78.48 | 95.14 |
| | | FCS3–3 | 41.23 | 38.33 | 49 | 15.94 | 78.45 | 94.39 |
| | *Male* | MCS2–1 | 46.08 | 41.14 | 48 | 19.45 | 74.77 | 94.22 |
| | | MCS2–2 | 39.45 | 35.00 | 48 | 19.51 | 73.91 | 93.42 |
| | | MCS2–3 | 43.54 | 38.87 | 48 | 19.38 | 73.68 | 93.06 |
| | | MCS3–1 | 43.38 | 38.79 | 48 | 18.80 | 74.61 | 93.41 |
| | | MCS3–2 | 39.17 | 34.80 | 47 | 20.72 | 72.87 | 93.59 |
| | | MCS3–3 | 47.58 | 42.58 | 47 | 20.77 | 72.62 | 93.39 |
| **Treated** | *Female* | FTS2–1 | 48.47 | 44.82 | 49 | 15.69 | 79.38 | 95.07 |
| | | FTS2–2 | 50.90 | 47.09 | 49 | 17.04 | 77.99 | 95.03 |
| | | FTS2–3 | 46.08 | 42.64 | 49 | 15.35 | 79.94 | 95.29 |
| | | FTS3–1 | 42.65 | 39.40 | 48 | 17.14 | 78.49 | 95.63 |
| | | FTS3–2 | 43.75 | 40.51 | 49 | 15.42 | 80.21 | 95.63 |
| | | FTS3–3 | 44.71 | 41.29 | 49 | 15.91 | 79.31 | 95.22 |
| | *Male* | MTS2–1 | 43.35 | 38.54 | 47 | 21.12 | 72.69 | 93.81 |
| | | MTS2–2 | 43.58 | 38.79 | 47 | 20.19 | 73.12 | 93.31 |
| | | MTS2–3 | 45.32 | 40.35 | 48 | 19.93 | 73.23 | 93.16 |
| | | MTS3–1 | 35.79 | 31.88 | 48 | 19.59 | 74.40 | 93.99 |
| | | MTS3–2 | 45.32 | 40.43 | 47 | 22.14 | 70.94 | 93.08 |
| | | MTS3–3 | 41.29 | 36.86 | 47 | 20.14 | 73.47 | 93.61 |

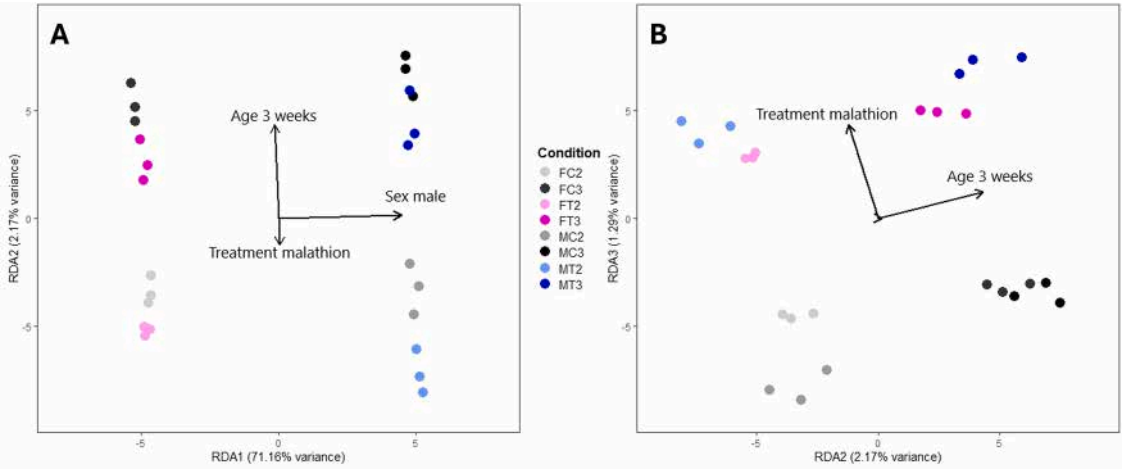


**Fig. 1.** Redundancy analysis (RDA) showing overall significance of the model with sex, age and treatment (malathion vs control) as factors contributing to variation in gene expression (ANOVA with 1000 permutations, p-value<0.001). RDA was performed on RLE normalized counts transformed in log2 (RLE normalized counts + 1) values. The amount of variance in percent accounted for by each dimension is included in brackets.

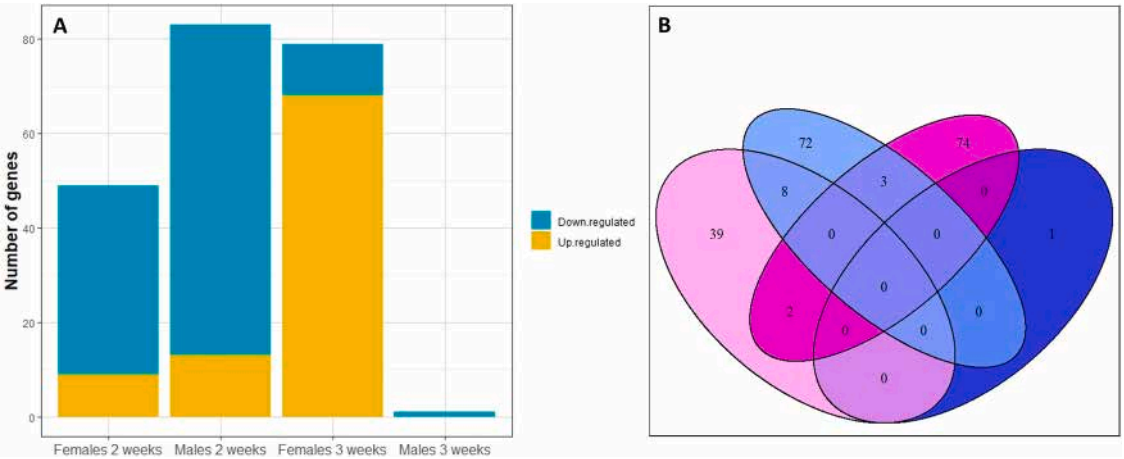


**Fig. 2.** (A) The number of genes differentially expressed (DESeq2 results with Padj<0.05) in malathion-treated mosquitoes varied according to sex and age. Yellow: Upregulated genes; Blue: Downregulated genes. (B) Venn diagrams of differentially expressed genes in malathion-exposed mosquitoes. Only a few genes are shared between the different conditions.

annotated and used for the biological interpretation of expression profiles (for gene annotations see Supplementary Table S4).

The results of the enrichment analysis (Fisher's exact test) are shown in Fig. 3. Although a few DEGs were shared among to the different conditions - i.e females 2 weeks (F2W), males 2 weeks (M2W), females 3 weeks (F3W) and males 3 weeks (M3W) - as shown in Fig. 2B, enrichment analysis revealed that malathion treatment induced biological changes common to both M2W and F2W groups. Specifically, GO term enrichment of "Cellular Components" category showed a significant overrepresentation of genes encoding proteins located in the mitochondria (GO: 0005739) (specifically associated with the inner membrane, GO: 0005743) in both M2W and F2W exposed to malathion (Fig. 3A, the list of genes associated with each enriched GO term and their log2FC values compared to the controls are shown in the Supplementary Table S5). Enrichment in "Biological Processes" terms (Fig. 3B, Supplementary Table S5) further supported these findings. Two GO terms were shared between M2W and F2W groups, "generation of precursor metabolites and energy" (GO: 0006091) and "nucleobase-containing small molecule metabolic process" (GO: 0006139). Additionally, "carbohydrate derivative metabolic process" (GO: 1901135) was specifically enriched in F2Ws, while "transmembrane transport" (GO: 0055085) was enriched in M2Ws. All of these enriched "biological processes" cluster genes encoding mitochondrial proteins (Supplementary Table S5) and are indicative of mitochondrial functions such as energy production. Interestingly, the expression of the entire set of genes associated with these enriched GO families was downregulated in malathion-treated mosquitoes, suggesting a depletion of mitochondrial activities. To gain further insight into the metabolic and signaling pathways affected by malathion exposure, we conducted KEGG pathway enrichment analysis (Fig. 4). Additionally, "glycolysis/gluconeogenesis" was enriched in F2Ws, while "pyruvate metabolism," "fatty acid degradation," and "starch and sucrose metabolism" were enriched in M2Ws, suggesting a broader suppression of energy metabolism in both groups. Consistent with the GO enrichment results, we found two significantly enriched KEGG pathways - "oxidative phosphorylation" and "TCA cycle" - shared between F2Ws and M2Ws (and again pointing to a depletion in mitochondrial activity. In addition, "glycolysis/gluconeogenesis" was enriched in F2Ws, while "pyruvate metabolism", "fatty acid degradation" and "starch and sucrose metabolism" were enriched in M2Ws, suggesting a broad reduction of energy metabolism.

Enrichment analysis could not be performed for the M3W group due to the identification of only one DEG in this group. However, this gene also encoded a mitochondrial protein (aconitate hydratase) and was downregulated, consistent with a potential impact on mitochondrial

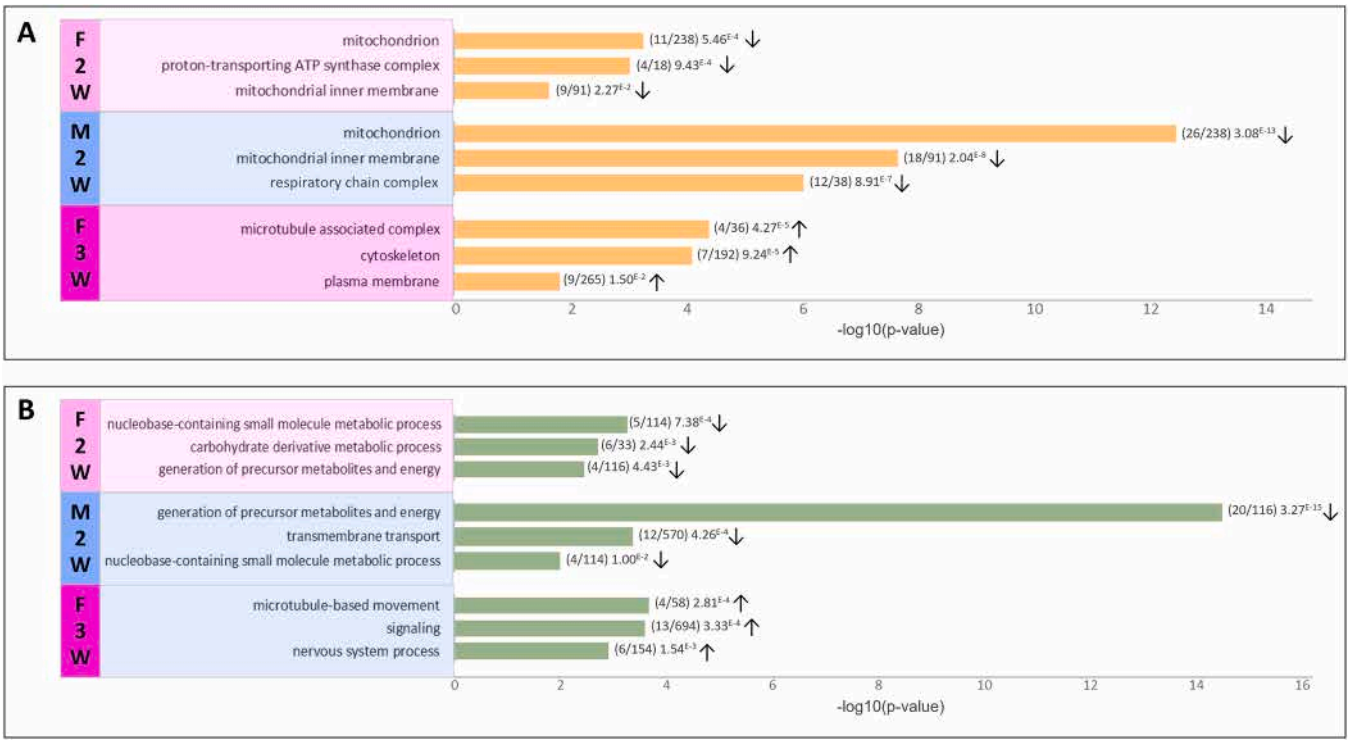


**Fig. 3.** Enrichment analysis of GO terms in Differentially Expressed Genes between malathion treated and control mosquitoes calculated by Fisher's exact test. A) GO terms from Cellular Component ontology are in yellow; B) GO terms from Biological Process ontology are in green. The number of DEGs associated with the GO term compared to the number of genes in the *Aedes aegypti* background associated with the same GO term and p-values are reported next to each bar. ↑: upregulated genes; ↓: downregulated genes. F2W, 2-week-old females; M2W, 2-week-old males; F3W, 3-week-old females. We reported only GO terms with a p-value $< 0.05$ (Fisher's Exact Test).

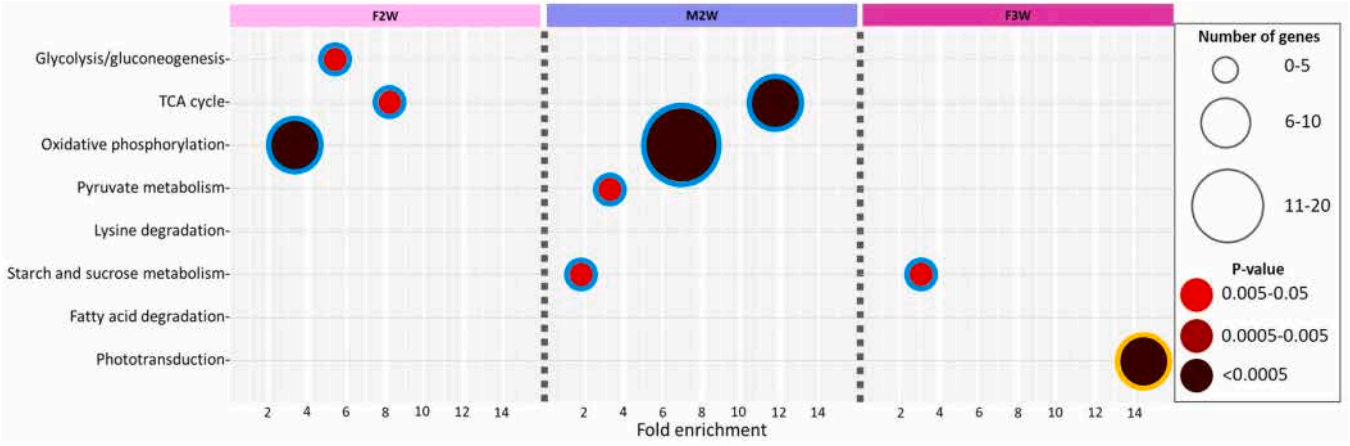


**Fig. 4.** KEGG pathway enrichment analysis of differentially expressed genes between malathion-treated mosquitoes and controls. Blue circled dots: downregulated genes; Yellow circled dots: up-regulated genes. Fold enrichment was calculated as the percentage of differentially expressed genes in this pathway divided by the percentage of genes in this pathway in the *A. aegypti* genome. We reported Benjamini-Hochberg adjusted p-values.

function.

In contrast, the transcriptional response to malathion in F3W differed markedly from that observed in other groups. None of the DEGs in F3Ws encoded for mitochondrial proteins. Instead, in the "cellular components" category, F3Ws showed enrichment for genes associated with the "cytoskeleton" (GO: 0005856), particularly the "microtubule associated complex" (GO: 0005875), and "plasma membrane" proteins (GO: 0005886) (Fig. 2A). In the "biological processes" category, enriched terms included "microtubule-based movement" (GO: 0007018), "signaling" (GO: 0023052) – mainly opsins - and "nervous system process" (GO: 0050877) (Fig. 2B). KEGG pathway enrichment analysis (Fig. 4) further specified that "phototransduction" was the "signaling" process upregulated in F3Ws. Notably, a depletion of "starch and sucrose metabolism" pathway was also observed in F3Ws, similar to M2Ws, potentially indicating a reduction in energy metabolism in this group as well.

### 3.4. Specific genes are dysregulated in adult mosquitoes after larval malathion exposure

RLE-normalized expression levels of all annotated DEGs are shown in Fig. 5. As suggested by the enrichment analyses, several genes encoding

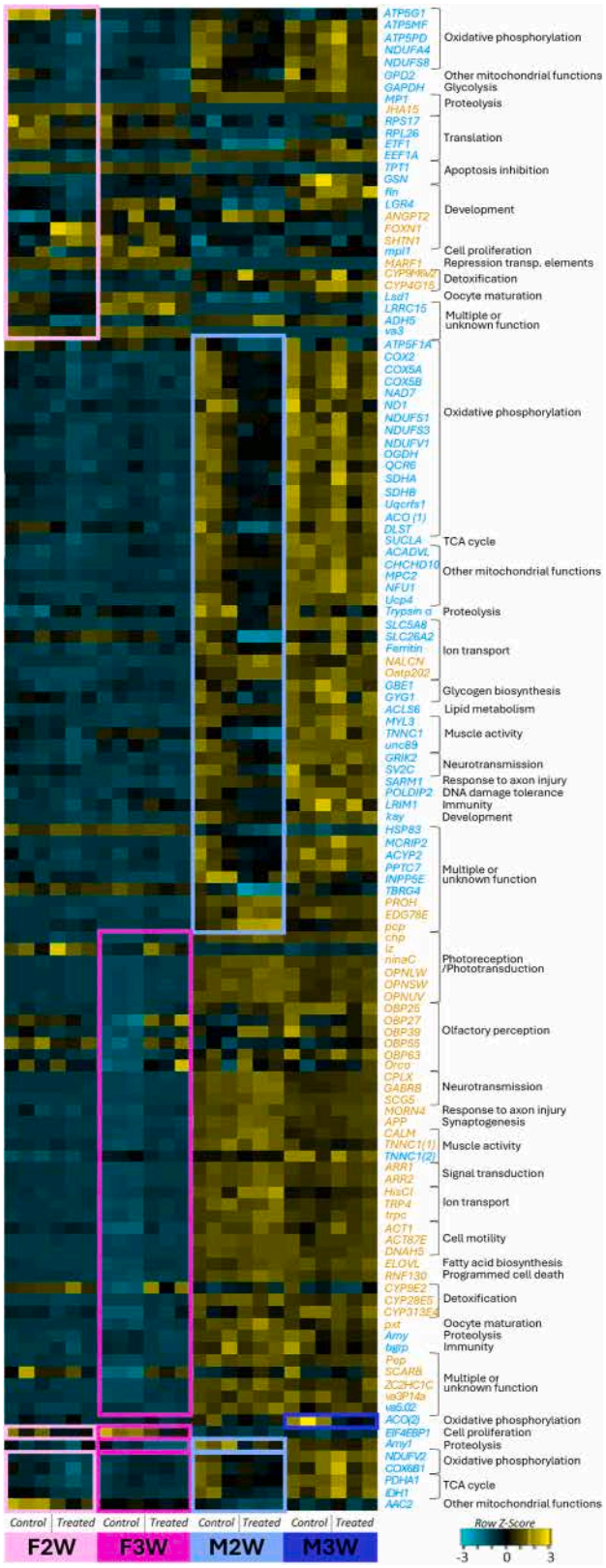


**Fig. 5.** RLE normalized expression levels in control and malathion-treated mosquitoes of all annotated Differentially Expressed Genes with Z-scores, calculated as Gene expression value in sample of interest - Mean expression across all samples / Standard Deviation. F2W: 2-week-old females; F3W: 3-week-old females; M2W: 2-week-old males; M3W: 3-week-old males.

mitochondrial genes (Supplementary Table S4) were downregulated in 2-week-old malathion-treated mosquitoes compared to untreated controls, suggesting a reduction of mitochondrial activity. In females, genes related to oxidative phosphorylation (*ATP5G1, ATP5MF, ATP5PD, COX6B1, NDUFA4, NDUFS8, NDUFV2*), TCA cycle (IDH1, PDHA1) and other mitochondrial functions (*AAC2, GPD2*) were all downregulated. In males, downregulated genes included oxidative phosphorylation-related genes (*ATP5F1A, COX2, COX5A, COX5B, COX6B1, NAD7, ND1, NDUFS1, NDUFS3, NDUFV1, NDUFV2, OGDH, QCR6, SDHA, SDHB* and *Uqcrfs1*), TCA cycle-related genes (*ACO, DLST, IDH1, PDHA1* and *SUCLA*) (), and genes involved in various mitochondrial functions (*AAC2, ACADVL, CHCHD10, MPC2, NFU1* and *Ucp4*). The only DEG in M3Ws was also a TCA cycle-related gene (*ACO*) and was downregulated. Five DEGs were shared among the other 3 groups (F2Ws, M2Ws and F3Ws), and all were downregulated. They encoded mitochondrial genes involved in oxidative phosphorylation (*COX6B1* and *NDUFV2*), TCA cycle (*IDH1* and *PDHA1*) and other mitochondrial functions (*AAC2*). Although the enrichment analyses did not reveal a significant dysregulation of mitochondrial pathways in F3Ws, this shared downregulation suggests that a reduction in mitochondrial activity may still be occurring in 3-week-old females.

Additional genes related to other biologically relevant processes were also dysregulated in malathion-treated mosquitoes, although these processes were not significantly enriched. For example, we found dysregulation of genes associated with proteolysis in all groups, such as *MP1* (downregulated) and *JHA15* (upregulated) in F2Ws, *Amy1* and *trypsin-alpha* (both downregulated) in M2Ws and *Amy1* in F3Ws.

DEGs involved in developmental processes (*fln* and *LGR4* downregulated and *ANGPT2, FOXN1* and *SHTN1* upregulated in F2Ws; *kay* downregulated in M2Ws) were only observed in 2-week-old mosquitoes.

Some biological showed sex-specific patterns of dysregulation. In females, genes involved in cell death (*GSN* and *TPT1* downregulated in F2Ws; *RNF* upregulated in F3Ws), cell proliferation (*mpl* in F2Ws and *EIF4EBP1* in F3Ws) and oocyte maturation (*Lsd1* in F2Ws and *pxt* in F3Ws) were differentially expressed, suggesting that malathion exposure may affect female gametogenesis. Notably, P450 cytochromes - putatively involved in pesticide detoxification - were significantly upregulated only in females (*CYP9M6v2* and *CYP4G15* in F2Ws, and *CYP9E2*, *CYP28A5* and *CYP313A4* in F3Ws) suggesting a stronger detoxification response in females.

Other biological processes were represented by DEGs in both M2Ws and F3Ws, but not in F2Ws. We observed DEGs related to ion transport (*NALCN* and *Oatp202*, upregulated, and *ferritin*, *SLC5A8* and *SLC26A2*, downregulated in M2Ws; and *HisCl, TRP4* and *trpc* upregulated in F3Ws), muscle activity (*MYL3, TNNC1* and *unc89* downregulated in M2Ws, and *CALM* and *TNNC1(gene1)* upregulated and *CALM(2)* downregulated in F3Ws), neurotransmission (*GRIK2* and *SV2C*, downregulated in M2Ws, and *CPLX, GABR* and *SCG5* upregulated in F3Ws), response to axon injury (*SARM1*, downregulated in M2Ws, and *MORN4* upregulated in F3Ws), and immunity (*LRIM1*, downregulated in M2Ws, and *bgrp* downregulated in F3Ws).

In contrast, some gene expression changes were specific to individual groups. In F2Ws, we found a dysregulation of genes related to translation (*EEF1A, EIF4EBP1, ETF1, RPL26* and *RPS17* downregulated), glycolysis (*GAPDH* downregulated) and repression of transposable elements (*MARF1* upregulated), and in M2Ws, genes involved in glycogen biosynthesis (*GBE1* and *GYG1*, downregulated), lipid metabolism (*ACSL6*, downregulated) and DNA damage tolerance (*POLDIP2*, downregulated). F3Ws displayed a distinct transcriptional profile characterized by upregulation of genes involved in photoperception and phototransduction (*chp, lz, ninaC, OPNLW, OPNSW*, *OPNUV*), olfactory perception (*OBP25, OBP27, OBP39, OBP55, OBP63, Orco*), synaptogenesis (*APP*), fatty acid biosynthesis (*ELOVL*), intracellular signal transduction (*ARR1, ARR2*), and cell motility (*ACT1, ACT87E, DNAH5*).

## 4. Discussion

The rise in anthropogenic pollution in ecosystems, particularly aquatic ones, is increasing globally, making it crucial to understand and predict how living organisms will cope with the influx of toxic and mutagenic substances (Baines et al., 2021; Sepp et al., 2019). This situation can create a mismatch between heightened physiological damage, cancer risk and the effectiveness of physiological defenses developed during the course of evolution in less polluted environments (Aktipis and Nesse, 2013; Boutry et al., 2020). As proposed by Dujon et al., 2024, abnormal exposure to toxic and mutagenic agents can initially lead to an overactivation of physiological and anticancer defenses (*e.g.* (Fortunato et al., 2021)), followed by physiological adjustments, changes in life history traits (Boutry et al., 2022), and even the selection of additional anticancer defenses in the long term (Dujon et al., 2024; Vazquez et al., 2018). Beyond these general predictions, a crucial aspect to consider in understanding these phenomena is also the taxonomic identity of the species and their ecology, as these factors can lead to differences in intrinsic vulnerability and/or exposure to cancer risk (Vincze et al., 2022; Vittecoq et al., 2013).

Our results first showed that no known genes related to cancer risk/development – i.e members of major oncogenic signaling pathways (Sanchez-Vega et al., 2018) - have been identified (either up or downregulated) in malathion-treated yellow fever mosquitoes (*Aedes aegypti*). In the histological study, no signs of tumorous cells or precancerous lesions were found in the malathion-exposed group. There could be several explanations for these results. First the concentrations of malathion used in this study may have not been sufficient to trigger oncogenic responses in exposed insects, and it is also possible that certain effects went undetected due to the limited sample size. In addition, mosquitoes are short-lived species and may be unlikely to live long enough for developing oncogenic processes even though currently exposed to highly mutagenic substances. This is the case for *A. aegypti* mosquitoes, which have a lifespan of approximately 6 weeks in laboratory. Furthermore, species that have existed for eons near mutagenic substances are potentially expected to be naturally more resistant to the increasing cancer risks generated by current pollution (Vittecoq et al., 2018). Additionally, the exposure to malathion occurred at the larval stage, which means that the potential effects on tissues and genetic transcription may not be as pronounced or detectable at the adult stage, when the analyses were conducted. This timing of exposure and analysis could thus mask imminent effects of malathion. Finally, it is important to distinguish between sources of pollution and their effects, which can vary in mutagenicity and potential oncogenicity but may also cause other health issues likely to decrease individual fitness in the short term rather than through cancer. For example, malathion is recognized as a carcinogen, meaning it has the potential to cause cancer (in humans (Calaf et al., 2021)). However, its health impacts extend beyond carcinogenicity. Exposure to malathion can also lead to a range of other health problems, including respiratory issues (Abdo et al., 2021), and neurological damage (Elmorsy et al., 2022; Salama et al., 2015) in humans, and disruptions to the endocrine system in fish (Lal et al., 2013; Ortiz-Delgado et al., 2019). Therefore, it is important to consider both its carcinogenic risks and the broader spectrum of health effects when evaluating its potential to influence the evolutionary trajectory of species in polluted ecosystems. Thus, predicting how wildlife will respond to anthropogenic pollution and the contribution of oncogenic processes to these responses is complex and primarily requires experimental studies at this moment.

Beyond cancer, toxic pollutants such as malathion can potentially impact wildlife health and invertebrate populations by producing important changes in organism physiology and behavior (Legradi et al., 2018). In this study, we showed that malathion triggered important changes in gene expression (up and downregulation) linked to several physiological functions, suggesting adjustments of adult metabolism following insecticide exposure in larvae. A couple of genes encoding enzymes involved in the cellular signal transduction, muscle activity, photoperception/phototransduction (*e.g. OPNLW, OPNSW*), neurotransmission, olfaction (*e.g.* OBP and Orco) and detoxification (*e.g.* P450) were significantly upregulated, hence suggesting a global stress response, as well as neurological and physiological changes of the mosquitoes after exposure to malathion (Sanders and Martin, 1993). Although it is difficult to predict the physiological consequences of these DEGs, some of the upregulated genes found in our study encode for enzymes and proteins having important biological function in mosquitoes.

First, important changes in metabolism-related changes were triggered by the exposure of larvae to malathion, mostly at the age of 2 weeks. For instance, results show important changes in the expression levels of genes related to energy regulation, i.e. mitochondrial proteins, metabolites, oxidative phosphorylation, glycogenesis (in F2W and M2W), as well as proteolysis (F2W) and fatty acid biosynthesis (M2W). These changes suggest substantial metabolic shift, consistent with previous studies showing a reallocation of energy towards physiological defenses and/or among life-history traits in pollution-exposed organisms - compensation strategy, *i.e.* (Gandar et al., 2017b, 2017a, 2016; Petitjean et al., 2023, 2019; Sokolova, 2013). While mitochondrial dysregulation is often linked to cancer development (Frezza and Gottlieb, 2009; Grasso et al., 2020), depletion of mitochondrial activity may also reflect a broader metabolic response to environmental stressors (Bouly et al., 2024), potentially serving as a short-term metabolism changes rather than an indication of oncogenic progression. Interestingly, since mitochondrial homeostasis and function are also deeply involved in regulating organismal aging (Sun et al., 2016), the observed mitochondrial dysregulation could indicate that early-life exposure to malathion may have long-term consequences on aging processes in mosquitoes. Future long-term studies should investigate whether such exposure impacts longevity, cellular senescence, or other markers of age-related decline.

Importantly, changes in some defense pathways (detoxication pathways in F2W and F3W) were observed in malathion exposed individuals. In our study five different P450s were upregulated after exposure to malathion, which may result in a greater ability of mosquitoes to tolerate insecticides used in vector control. Indeed, P450s are encoded by more than 100 *CYP* genes involved in detoxification, transport and cell metabolism (Strode et al., 2008). Previous transcriptomic studies showed that mosquito exposure to fluoranthene, copper, and imidacloprid induced over-transcription of various *CYP450*s (mainly *CYP6*, *CYP*9 and *CYP3* families) involved in the metabolism of a wide range of molecules and pesticides (Chandor-Proust et al., 2013; David et al., 2010).This suggests that long-term exposure to pollutants such as malathion may thus favor the selection of metabolic-based resistance in mosquitoes (Poupardin et al., 2012) and hence directly impact vector control interventions. This upregulation may enhance the ability of mosquitoes to tolerate insecticides commonly used in vector control. Female mosquitoes, which require blood for oogenesis, face additional metabolic challenges during blood digestion, including the generation of reactive oxygen species from the breakdown of hem (Strode et al., 2006). The observed higher expression of detoxification-related genes in females compared to males after malathion exposure could reflect an adaptive mechanism to manage these dual metabolic demands and mitigate potential toxicity, although further studies are needed to test this hypothesis.

Interestingly, transcriptomic results suggest potential molecular changes related to olfactory functions following malathion exposure. Indeed, several genes involved in olfactory perception were upregulated in *A. aegypti*, including *OBP25, OBP27, OBP39, OBP55, OBP63*, and *Orco*. The olfactory system is particularly vulnerable to neurotoxic contaminants such as malathion, because of the direct contact of olfactory sensory neurons with the environment (Legradi et al., 2018). Odorant binding proteins (OBPs) proteins have been reported to have multiple functions including host-seeking, feeding and reproduction.

They are globular proteins that play a pivotal role in insect olfaction by transporting semiochemicals through the sensillum lymph to odorant receptors (ORs) and Orco, which results in electrical signals sent to the insect brain (Zafar et al., 2022). Changes in the expression of several OBDs caused by malathion could thus have direct consequences on the chemosensory abilities of the mosquitoes to sense diverse sets of attractants and/or repellent molecules (Lombardo et al., 2017). Our study thus suggest that malathion could further influence specific behavioral features (*i.e.*, food, host and oviposition site preference, probing and seeking) and virus transmission capabilities (Sim et al., 2012), and future studies should test this hypothesis to refine current vector control strategies.

The ecological significance of tissue alterations or tumorigenic processes induced by larval malathion exposure in mosquitoes remains an open question. However, these effects could have multiple consequences at the population or ecosystem level. Tissue damage, altered metabolism, or impaired physiological functions may reduce mosquito longevity, reproductive success, or flight ability, ultimately affecting population dynamics. Alternatively, if certain tissue alterations confer enhanced survival or stress tolerance (e.g., resistance to immune challenges or pollutants), they could promote the persistence of individuals in contaminated environments, potentially selecting for more resilient but also more pathogen-compatible phenotypes. Moreover, in polluted habitats where both chemical exposure and pathogen circulation are frequent, interactions between pollution-induced tissue remodeling and vector competence may modulate disease transmission dynamics. Future studies should explore these eco-evolutionary consequences in more detail.

Although our results do not directly explore insecticide resistance mechanisms, it is tempting to speculate that tissue alterations induced by larval exposure to malathion could interact with resistance pathways. Previous studies have shown that chronic exposure to pollutants or insecticides can select for increased detoxification capacity, stress responses, or tissue repair mechanisms in mosquitoes (Prud'homme et al., 2017). Such adaptive processes might overlap with those involved in maintaining or restoring tissue homeostasis following damage, potentially leading to cross-resistance phenomena. Further research is needed to explore whether selection for tissue resilience or altered cellular dynamics could contribute, directly or indirectly, to the evolution of insecticide resistance in polluted environments. Similarly, one may anticipate that such effects of malathion might influence key mosquito life-history traits. Tissue remodeling, inflammation, or metabolic disturbances could potentially affect survival, fecundity, or immune responses, thereby altering disease transmission dynamics. Moreover, if exposure to pollutants such as malathion during larval stages becomes common in contaminated environments, it may shape mosquito population structure, longevity, or interactions with pathogens. These possible eco-epidemiological consequences warrant further investigation.

Nevertheless, our study has some limitations that deserved to be discussed. First, future work should investigate whether the consequences of larval malathion exposure on adult mosquitoes might extend to subsequent generations. Changes in gene expression, tissue organization, or physiological stress during development could have transgenerational impacts through epigenetic inheritance or altered parental investment. Such effects have been reported in other invertebrate models exposed to pollutants and deserve further investigation in mosquitoes. Second, even if our results reveal tissue alterations and gene expression changes consistent with early tumorigenic processes, these findings remain preliminary and further research is required to establish a direct link between larval malathion exposure and tumor development in mosquitoes.

By combining toxicological exposure during larval development with histological and transcriptomic analyses in adults, our study bridges two traditionally distinct fields: environmental toxicology and pathology. It highlights how chemical stressors in early life can produce long-lasting tissue remodeling, blurring the line between reversible toxic effects and the emergence of stable pathological-like structures.

Taken together, our results emphasis the strong capacity of malathion to interfere with the metabolism and physiology of mosquitoes of public health importance. The fact that gene expression appears transitory and age-dependent may suggest different evolutionary processes involved in mosquito response to a xenobiotic (plasticity over selection). It is important to emphasize that while changes in gene expression can provide strong indications of physiological processes, they may not necessarily translate to functional or phenotypic outcomes. Therefore, the interpretations provided here should be confirmed through integrative approaches including proteomics, metabolomics, functional and behavioral assays. Further work would also be needed to assess how insect exposure to a xenobiotic may select for multiple defensive pathways including those involved in pesticide resistance/tolerance. Despite important differences across time and sexes, results point out the key role of changes in cell proliferation, regulation, detoxication and neurological processes in the responses of insects to pesticides, with expected consequences for behavior and ultimately on fitness in the wild. The identification of key pathways involved in pesticide responses over time is particularly valuable from an evolutionary perspective, as it enables the detection of mechanisms underlying resistance and tolerance development in natural populations.

## CRediT authorship contribution statement

**Sophie LABRUT:** Writing – review & editing, Formal analysis. **Lisa JACQUIN:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Dujon Antoine:** Data curation, Writing – original draft, Writing – review & editing. **Erika BURIOLI:** Writing – original draft, Formal analysis, Data curation, Conceptualization. **Vincent CORBEL:** Writing – review & editing, Writing – original draft, Conceptualization. **Arnal Audrey:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Delphine NICOLAS:** Writing – review & editing. **Marie ROSSIGNOL:** Methodology. **Stéphane DUCHON:** Writing – review & editing. **Frédéric THOMAS:** Writing – review & editing, Writing – original draft, Conceptualization. **Jérôme ABADIE:** Writing – review & editing, Methodology. **Jordan MELIANI:** Writing – review & editing. **Beata UJVARI:** Writing – review & editing.

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## Declaration of Competing Interest

I have nothing to declare

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.118449.

## Data availability

Data will be made available on request.

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