Supplementary PCR Data Methods in brief

Hypothesis-driven dragging of transcriptomic data to analyze proven targeted pathways in *Rhinella arenarum* larvae exposed to organophosphorus pesticides.

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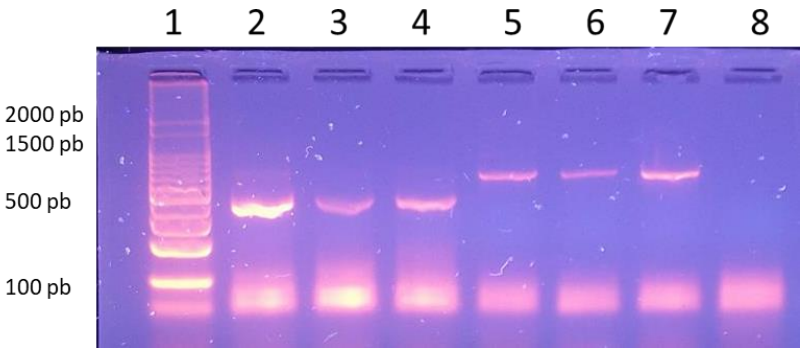
Workflow of PCR molecular studies to validate annotated transcripts and analyse gene expression in *R. arenarum*

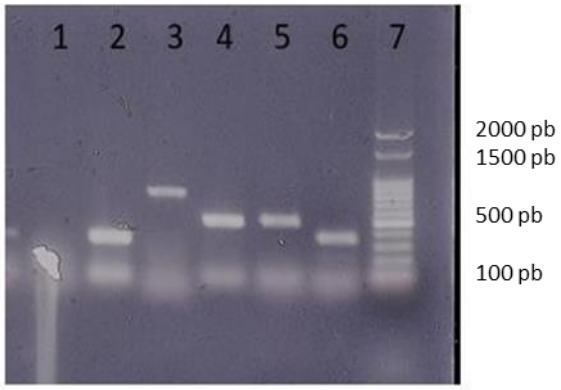
Based on the depurated transcript sequences annotated from the *R. arenarum* transcriptomic experiment [1], primers were designed using the tools Primer 3 and PrimerBLAST [2–4]. Considering that there are pathways known to be impacted by exposure to organophosphorus pesticides, primer design was focused on the polyamine metabolism pathway, the antioxidant and detoxifying system, and transcription factors and signaling pathways. The following table shows the primers that were designed to validate the annotated transcripts through RT-PCR and product sequencing.

PRIMER	GENE NAME	PATHWAY	SEQUENCE
DCOR1_F DCOR1_R	Ornithine decarboxylase	Polyamine Metabolism (PM)	CTGGTAGGTCGAAACGCTCA ACTTTCCAGTTGACTCCGGC
PAOX_F PAOX_R	Acetyl spermidine/spermine oxidase	PM	AGGGCCATCTGCTCTTTCAC CGTGGACGAGGCTTGTAACT
AOC1_F AOC1_R	Diamine oxidase	PM	GAGGGCCTATTGGAGACGTG AATGCAGTGGACGACCTGTT
SMOX_F SMOX_R	Spermine oxidase	PM	TCCTTCCTGGAGAACCCCTTT TGTGGTACGAAAACGCGTCA
AMD1_F AMD1_R	S-adenosylmethionine decarboxylase proenzyme	PM	ATGAGGCCAAGACCGTCAAC CGTTGAACTGAGCGAACTGG
CATA_F CATA_R	Catalase	Antioxidant System (AS)	ACCAGAGAGATGGGTTCAG TGCCAGTTTGTCCAGTTCAG
GSHR_F GSHR_R	Glutathione reductase	AS	CCCATTTTGATAGCGACGGC GGCAGAGCACTTTTGACACC
SODC_F SODC_R	Superoxide dismutase	AS	GTCCAGTTGGGGCTCTTACC TGTGCTGGTACAGCAGTAAAC
GSTPI_F GSTPI_R	Glutathione S-transferase Pi	DS	CACCAGGTTGTAGTCGGCAA GGCAGCCCTCATAGACATGG
FOS_F FOS_R	Fos proto-oncogene	Transcription Factor (TF)	AGCATCGCTATGGGTCACAG ACTCCTGGGTTCCGGGATAA
NRF2_F NRF2_R	Nuclear factor erythroid 2-related factor 2	TF	AGAAGGAATTGTACCCGCCG GATGTCCCTGACCAAAGCCA
JUN-F JUN_R	Jun proto-oncogene	TF	CCGCTCGGCTTTTATCCTCT ACCCAGTTCCTCTGCCCTAA
JUNK_F JUNK_R	c-Jun N-terminal kinase	TF	TTGCTGCTGCTCACTCTTTG CGATCTGGAGTTTGTTCCT
ACTB_F ACTB_R	Beta Actin	Housekeeping (HK)	TCCTGACCCTGAAGTACCCC GGTGCCCATCTCCTGCTCGA

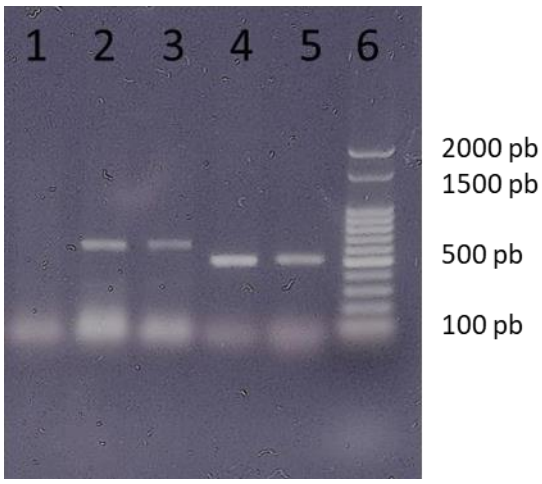
With these primers, RT-PCR were performed with R. arenarum samples.The following pictures show representative agarose gel electrophoresis of the selected genes, along with the product size in base pairs (bp):



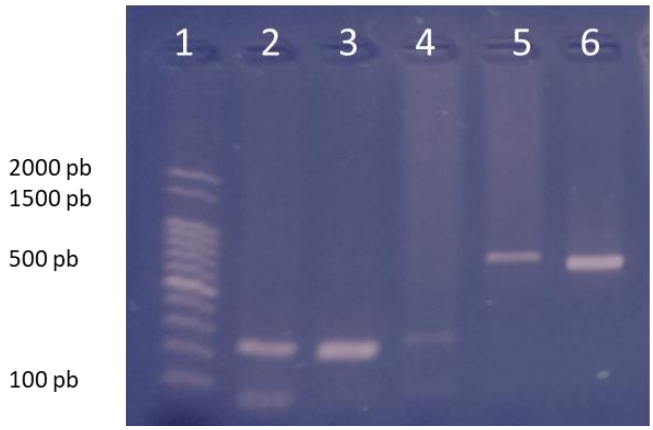
PCR amplification of ACTB and DCOR1. Line 1: molecular weight marker. Lines 2-4: ACTB amplified by RT-PCR from liver of adult *R. arenarum* individuals; 500 bp. Lines 5-7: DCOR1 amplified by RT-PCR from liver of adult *R. arenarum* individuals; 800 bp.



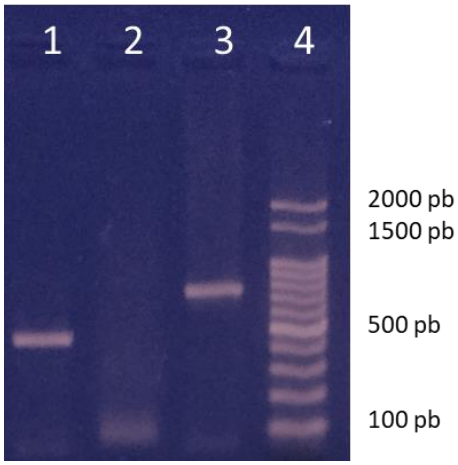
PCR amplification of JUN, PAOX, ACTB and CATA. Line 1: negative control. Line 2: JUN, 200 bp. Line 3: PAOX, 700 bp. Lines 4 and 5: ACTB, 500 bp. Line 6: CATA, 300 bp. Line 7: molecular weight marker.



PCR amplification of NRF2 and SODC. Line 1 negative control: Lines 2 and 3: NRF2, 600 bp. Lines 4 and 5: SODC, 500 bp. Line 6: molecular weight marker.

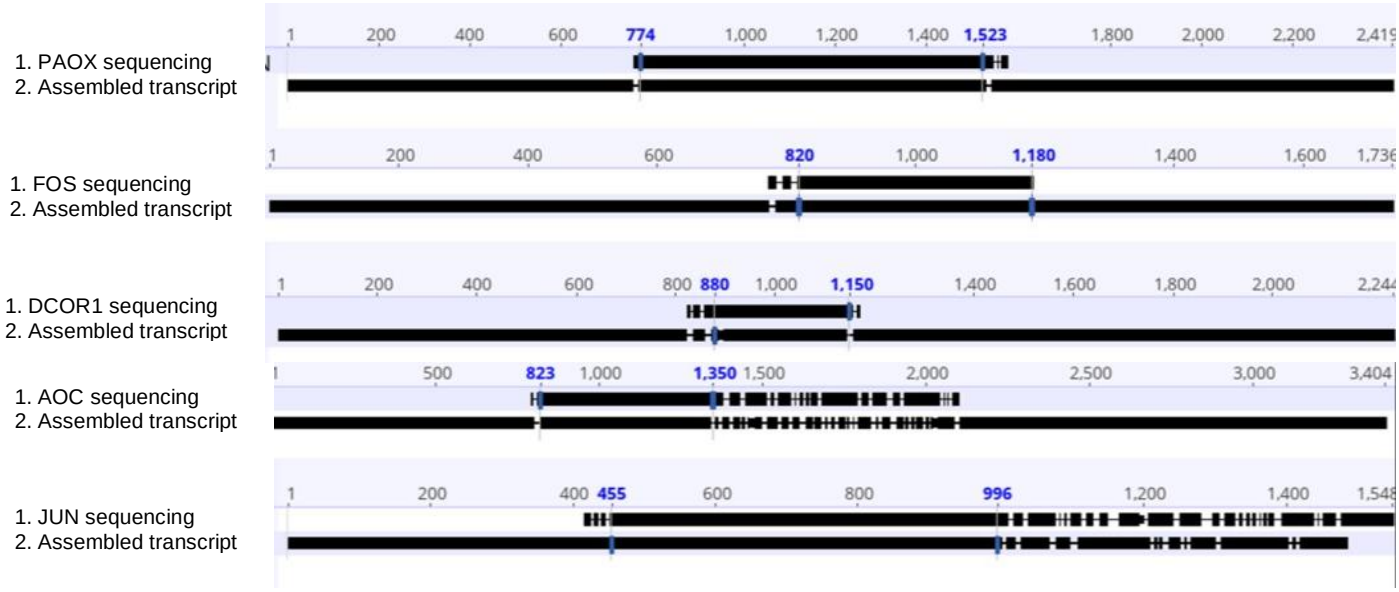


PCR amplification of JUN, AOC1 and GSTPI. Line 1: molecular weight marker. Lines 2 and 3:GSTPI, 200 bp. Line 5: c-JUN, 700 bp. Line 6: AOC1, 700 bp.



PCR amplification of AMD1 and JUN. Line 1: AMD1, 500 bp. Line 2: non amplified sample. Line 3: JUN, 700 bp. Line 4: molecular weight marker.

The genes that were successfully amplified by RT-PCR were sequenced and aligned using a free version of Geneious Prime. PAOX, FOS, DCOR1, AOC1 and JUN displayed 100% homology with the shown portions of the transcriptome-assembled transcripts (blue numbers in the accompanying figure). No homology was found between the PCR-amplified genes CATA, NRF2, SODC, AMD1 and GSTP and the transcriptome information.



Quantitative PCR analyses applied to selected *R. arenarum* transcripts

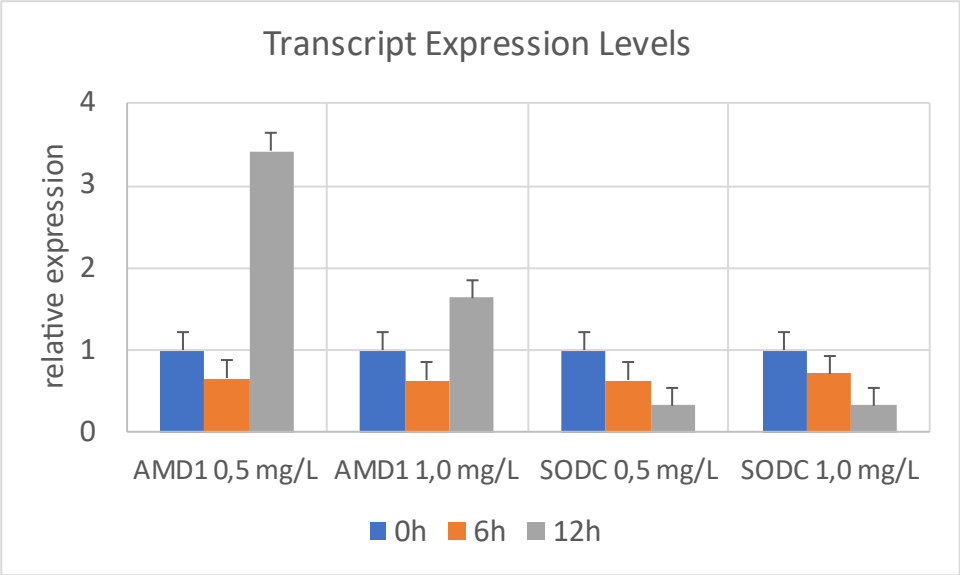
Our next task was to design new primers suitable for qPCR studies. The following table shows the designed primers:

PRIMER	GENE NAME	PATHWAY	SEQUENCE
PAOX_F PAOX_R	Acetyl spermidine/spermine oxidase	PM	CTGGAAACTGCTCACCATCTTCAC AAGGGCTACCAAGCTCTACTAGAC
AOC1_F AOC1_R	Diamine oxidase	PM	CTGCTGCTCCATCACTTG CTCTGCCAGGGTCGTAATAG
SMOX_F SMOX_R	Spermine oxidase	PM	TCCTTCCTTCCTGGAGAAC GAGGCTGAAACTGGCTATG
SPEE_F SPEE_R	Spermidine Synthase	PM	AAGACTCTGTTCCCTGTGGTAG ATGCTGCCCGATGAATGTTG
DCOR1_F DCOR1_R	Ornithine decarboxylase	PM	TCCGTAGACACCATCGTTGAC TGTTGCTTCAGCCTTCAC
AMD1_F AMD1_R	S-adenosylmethionine decarboxylase proenzyme	PM	ACCAGACCATCAGGAATACC CATGCGTCCCATGCAATAAG
SODC_F SODC_R	Superoxide dismutase	AS	GCAACGCCATCTTTGGAGG GCACTATCAACGGGCTGAC
FOS_F FOS_R	Fos proto-oncogene	TF	ACCACCTACACCACCTCGTTTGTC ACTGCTCGTTGCTGCTGCTTC
ACTB_F ACTB_R	Beta actin	HK	GCTGTGCTGTCCCTGTATG CAAGTCCAGACGCAGGATG
RL8_F RL8_R	Ribosomal protein L8	HK	GTGGCTATGAACCCTGTAGAA ACGACGAGCAGCAATAAGAC

We developed qPCR protocols with these primers to assess transcript expression in *R. arenarum*. Primer validation through standard curves and dynamic range calculation was carried out for the designed primers. Primers to amplify RL8, AMD1, ACTB and SODC were validated (see next table). Primers designed to amplify FOS, PAOX, DCOR1 AOC1, SMOX and SPEE could not be validated, as their expression levels were very low and amplification began between Ct 28 and Ct 33. Pre-amplification cycles and addition of DMSO were attempted, but no positive results were obtained.

PARAMETER	ACTB	AMD1	SODC
Slope	-2.191	-3.561	-3.173
Y-intercept	31.78	34.58	34.51
Efficiency	1.86	0.91	1.07
$R^{\wedge 2}$	0.986	0.996	0.984

Initial trials were developed to test the applicability of the *R. arenarum* transcriptome database in qPCR assays, using the validated primers and protocols for SODC and AMD1 genes using ACTB and RL8 as housekeeping genes in *R. arenarum* larvae exposed to chlorpyrifos. A pipeline similar in several aspects to the GeNorm algorithm, was used to analyse the expression results: First, geometric mean calculations for both HK were performed, using Ct values for each condition and replicate. After calculating delta Ct values using the calculated geometric mean HK value, we performed the geometric mean calculations on the sample replicates. We obtained an average standard deviation for the genes of interest from the mean deviation of their ranges of variation. We then normalized to control-zero values, as the delta-delta Ct, proceeding then to obtain the relative expression levels in each condition for the genes of interest. The results of this trial are shown in the following figure:



Our preliminary results support the conclussion that the *de novo* assembled *R. arenarum* transcriptome provides adequate information to choose a battery of potential housekeeping genes and further analyze gene expression by quantitative PCR on a series of genes from hypothesis-selected pathways.

References

1. Ceschin, D. G., Pires, N. S., Mardirosian, M. N., Lascano, C. I. & Venturino, A. The Rhinella arenarum transcriptome: de novo assembly, annotation and gene prediction. *Sci. Rep.* **10**, (2020).
2. Untergasser, A. *et al.* Primer3--new capabilities and interfaces. *Nucleic Acids Res.* **40**, e115 (2012).
3. Koressaar, T. & Remm, M. Enhancements and modifications of primer design program Primer3. *Bioinformatics* **23**, 1289-91 (2007).
4. Ye, J. *et al.* Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**, 134 (2012).