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Development of biomarker genes for endocrine disruption assessment in the non-model crustacean species *Gammarus fossarum*

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Introduction

- Proteins are the first functional target affected by contaminants, and constitute effective biomarkers.
- New technologies such as next generation sequencing and "shotgun" proteomics allow mining for molecular information in non-model species.
- From a RNAseq-derived transcriptome containing 218 574 assembled contigs, the use of the "proteogenomics" approach in the crustacean sentinel species *Gammarus fossarum* allowed the creation of a protein database containing **1873 species-specific proteins which** were **experimentally identified by mass spectrometry**.
- However, key proteins involved in hormonal regulation, that could be potential candidates for endocrine disruption biomarkers, but with very low levels, were not identified.

OBJECTIVE: Develop an alternative strategy for identifying key molecules in the hormonal system of *Gammarus fossarum* and propose candidate biomarkers genes/proteins of endocrine disruption in this species.

Methodology

1. Proposition of candidate genes involved in amphipod reproduction and molting (amphipod/crustacean/arthropod literature)

- **Enzymes from hormonal metabolism** . FaMET, JHE, cytP450
- **Hormonal receptors** . Met, RxR, EcR, allostatin A/B/C receptors
- **Regulators** . BR, Chd64, FKBP39, Hexamerin, CHH
- **Others** . Calreticulin, vasa, Dmrt, FEM-1, hemocyanin

2. Search in *Gammarus fossarum* RNAseq-derived transcriptome database (GFOSS) by sequence homology

BLAST Gammarus Results

Example RxR blastp

Gene name	GFOSS RNA
Ecdysone receptor	✓
Allostatin A, B, C receptors	☒
Methoprene-tolerant	☒
Broad-complex	✓
Chd64	☒
FKBP39	☒
Hexamerin	☒
Crustacean hyperglicemic hormone	✓

3. *In silico* phylogenetic validation of functional annotation

Construction of an homologous sequence dataset for each candidate (multigenic family):
- **Ortholog** sequences AND **Paralog** sequences in a diversity of arthropod species

Phylogenetic analysis:
- Sequence alignment
- Tree construction

4. Experimental functional validation and sensitivity to Endocrine Disruptors

iTaq™ Universal SYBR® Green Supermix - Bio-Rad / Reference genes: EF, GAPDH

a) Gene sequencing and primer testing for chosen candidates

Proposed candidate sequences:
RxR -> contig_83504 and sing307974
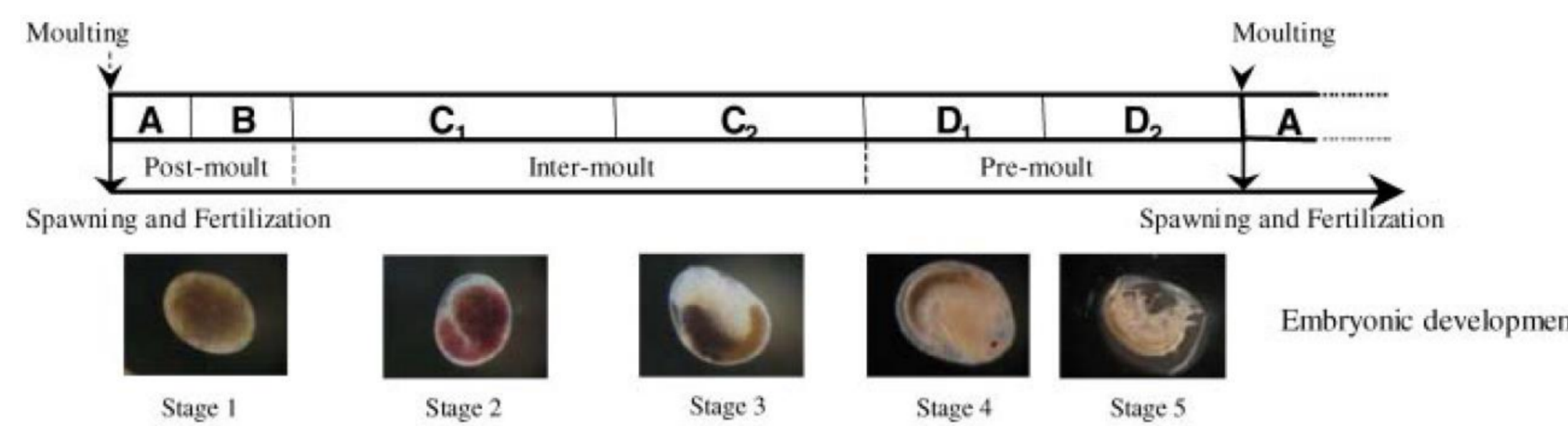**E75** -> contig_2900, contig_178180
BR -> contig_3641, contig_1452
EcR -> no contigs found in GFOSS

PCR Cloning Sequencing

b) Gene expression studies
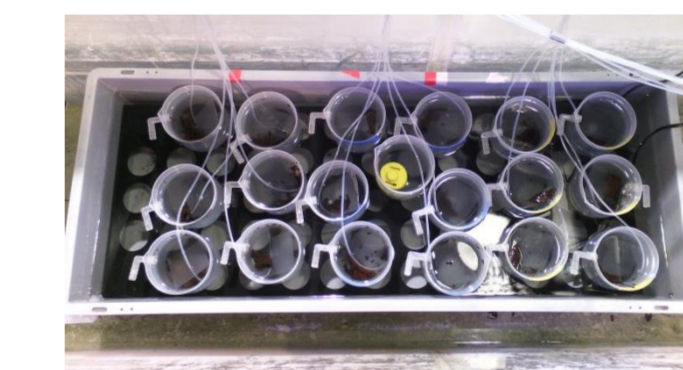
b.1) Female reproductive cycle

Moulting ... Moulting
A | B | C1 | C2 | D1 | D2 | A
Post-moult | Inter-moult | Pre-moult
Spawning and Fertilization ... Spawning and Fertilization
Embryonic development
Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5

b.2) Exposures to endocrine disrupting chemicals

Pyriproxyfen (PYR) 5 µg/L
Tebufenozide (TEB) 0,5 µg/L
Piperonyl Butoxide (PBO) 150 µg/L

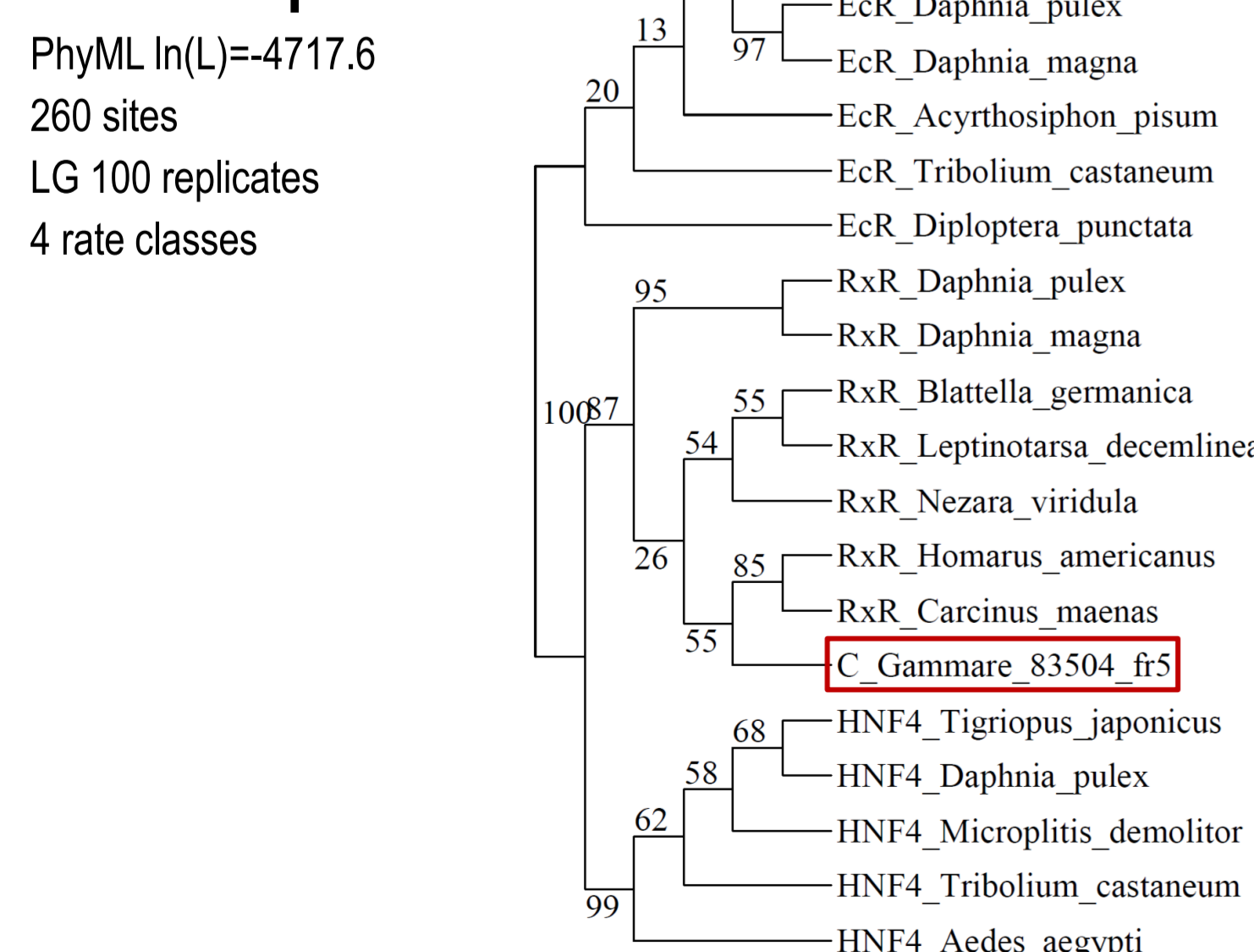WARNING PESTICIDES

Results

Phylogenetic validation

Gammarus fossarum_83

Crustaceans: RxR CarcInus maenas, RxR Homarus american, RxR Daphnia pulex
Insects: RxR Blattella german, RxR Nezara viridula, RxR Leptinotarsa dec
EcR: EcR Marpenensis jap, EcR Marpenensis magna, EcR Daphnia pulex, EcR Tribolium casta, EcR Acyrthosiphon pi, EcR Diploptera punct
HNF4: HNF4 Tribolium casta, HNF4 Microplitis dem, HNF4 Tigriopus japon, HNF4 Daphnia pulex

Example RxR
PhyML ln(L)=-4717.6
260 sites
LG 100 replicates
4 rate classes

```
        ┌ EcR_Marsupeaneus_japonicus
     52 ├ EcR_Neomysis_integer
   45├──┤
13 ├   │ 97┬ EcR_Daphnia_pulex
20 ┤   └───┴ EcR_Daphnia_magna
   │       ┌ EcR_Acyrthosiphon_pisum
   │       ├ EcR_Tribolium_castaneum
   │       └ EcR_Diploptera_punctata
   │   95┬ RxR_Daphnia_pulex
   │     └ RxR_Daphnia_magna
100┤87├ 55┬ RxR_Blattella_germanica
   │  54├ RxR_Leptinotarsa_decemlineata
   │    └ RxR_Nezara_viridula
   │  26├ 85┬ RxR_Homarus_americanum
   │    └   └ RxR_Carcinus_maenas
   │     55└ C_Gammare_83504_fr5
   │  68┬ HNF4_Tigriopus_japonicus
   │ 58├ HNF4_Daphnia_pulex
   │62├ HNF4_Microplitis_demolitor
   99└ HNF4_Tribolium_castaneum
      └ HNF4_Aedes_aegypti
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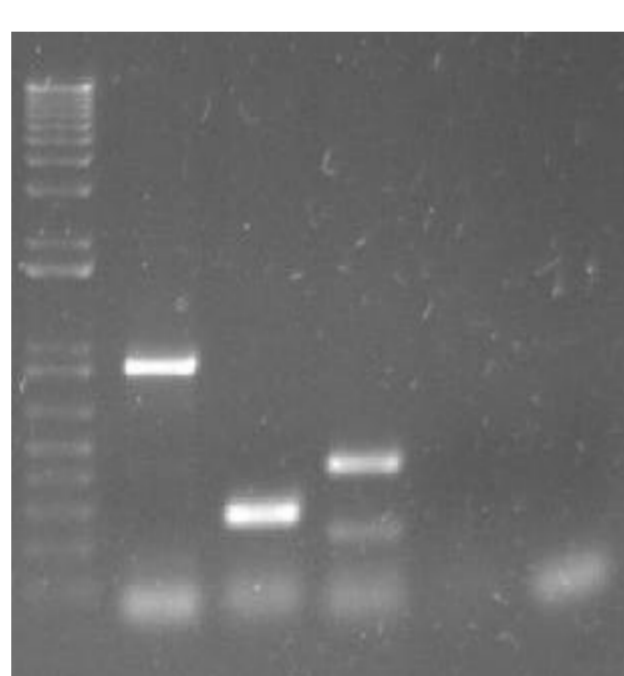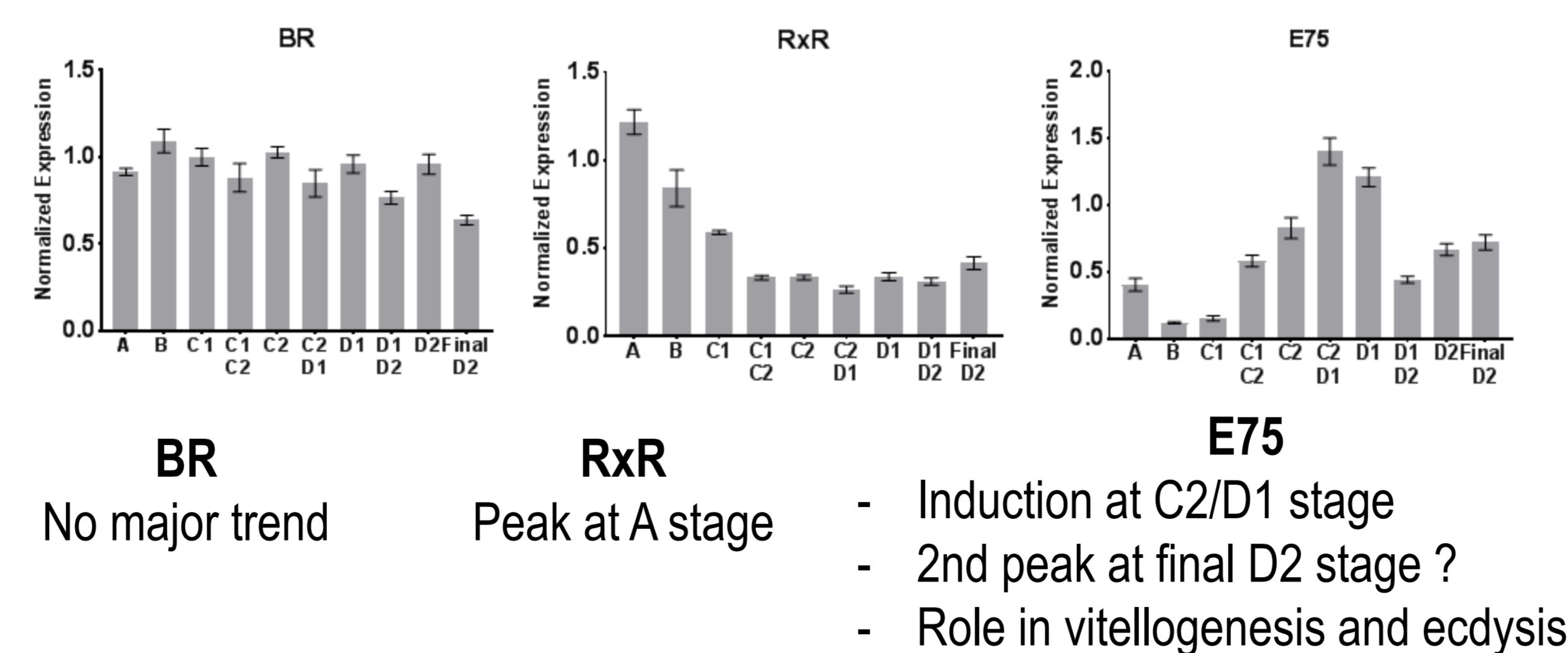
Experimental functional validation

a) Gene sequencing and primer testing for final candidates

RxR, E75, BR ✅   EcR ❌

- Specific *Gammarus fossarum* sequences for RxR E75 and BR
- Validated primers for subsequent qPCR studies

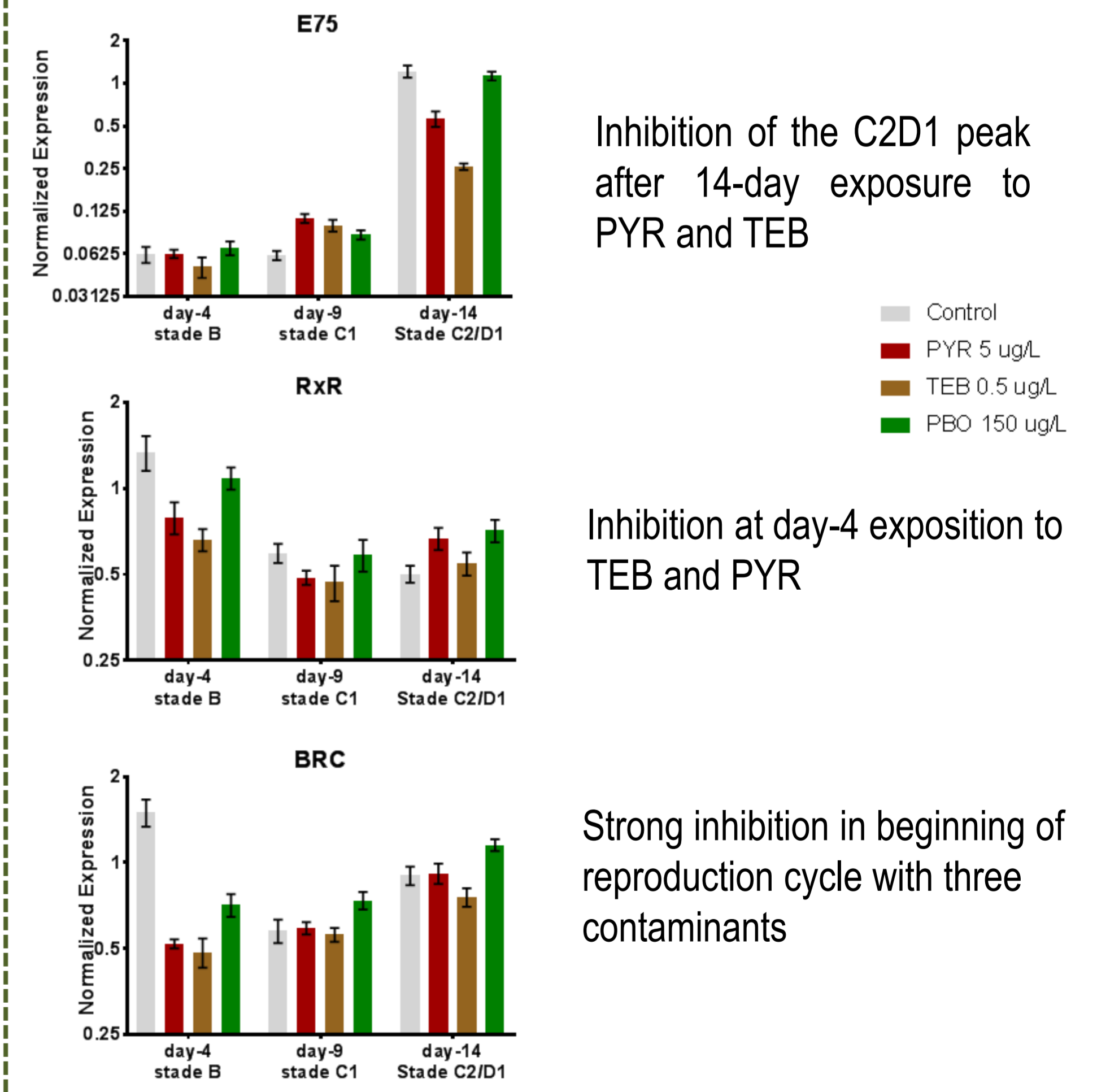**b) Gene expression during female reproductive cycle**

BR | RxR | E75 (Normalized Expression, stages A B C1 C2 D1 D2 Final D2)

BR
No major trend

RxR
Peak at A stage

E75
- Induction at C2/D1 stage
- 2nd peak at final D2 stage ?
- Role in vitellogenesis and ecdysis

c) Gene expression after exposure to endocrine disrupting chemicals

E75 (Normalized Expression; day-4 stage B, day-9 stage C1, day-14 stage C2/D1)

Control | PYR 5 ug/L | TEB 0.5 ug/L | PBO 150 ug/L

Inhibition of the C2D1 peak after 14-day exposure to PYR and TEB

RxR (Normalized Expression; day-4 stage B, day-9 stage C1, day-14 Stade C2/D1)

Inhibition at day-4 exposition to TEB and PYR

BRC (Normalized Expression; day-4 stade B, day-9 stade C1, day-14 stade C2/D1)

Strong inhibition in beginning of reproduction cycle with three contaminants

Conclusion & Perspectives

- Shotgun proteomics is very useful for the construction of protein libraries, but is limited by the complexity of the proteomes and low-level proteins are often missed
- With this alternative approach we managed to obtain specific sequences from key molecules of the hormonal system of *Gammarus fossarum* and assessed their role during female reproduction cycle
- Gene expression studies showed important roles for RxR and E75 in female molt cycle, and exposure to hormone-interfering insecticides demonstrated the sensitivity of candidates to ED contamination -> pertinent candidate biomarkers for future developments