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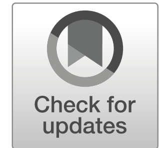
RESEARCH ARTICLE



Effects of low glyphosate-based herbicide concentrations on endocrine-related gene expression in the decapoda *Macrobrachium potiuna*

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Abstract

Glyphosate-based herbicides (GBH) are the most used herbicides worldwide and are considered as endocrine-disrupting compounds (EDC) for non-target organisms. However, effects of GBH on their endocrine systems remain poorly understood. Thus, the aim of this study was to assess the effects of low concentrations of Roundup WG® on growth and reproduction process molecules in both males and females of the decapod crustacean *Macrobrachium potiuna*, by the relative transcript expression levels of the ecdysteroid receptor (EcR), the molt-inhibiting hormone (MIH), and the vitellogenin (Vg) genes. Prawns were exposed to three concentrations of GBH (0.0065, 0.065, and 0.28 mg L^{-1}) for 7 and 14 days. The results revealed that only in males the three genes transcript levels were influenced by the GBH concentration, time of exposure, and the interaction between the concentrations and time of exposure, suggesting that males were more sensitive to GBH than females. For males, after 7 days of exposure at 0.065 mg L^{-1}, EcR and MIH were over-expressed, while the Vg expression was only over-expressed after 14 days. The present study highlighted that GBH impacted endocrine systems of *M. potiuna*. Moreover, EcR and MIH gene expressions could be promising EDC biomarkers of exposure in crustaceans. These results also indicate that GBH concentrations, considered secure by regulatory agencies, should be reviewed to minimize the effects on non-target organisms.

Keywords Roundup · Molt-inhibiting hormone · Vitellogenin · Ecdysteroid receptor · Endocrine disruption · Biomarker

Highlights
• GBH was able to upregulate the transcript expression levels of EcR and MIH.
• Males of *Macrobrachium potiuna* were more affected by the GBH exposure than females.
• Integrated biomarker response (IBR) shows an induction of biomarkers for males.

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Introduction

Glyphosate-based herbicides (GBH) are the most used herbicide worldwide. In the environment, glyphosate has been found not only in agriculture areas but also in sediments, surface, and groundwater (Freire et al. 2012; Gunarathna et al. 2018; Okada et al. 2018; Pinto et al. 2018), reaching in some cases considerable levels (e.g., 2024 μg L^{-1} for surface water). Furthermore, the herbicide has been detected in blood, urine, and maternal fluids in humans (Bolognesi et al. 2009; Conrad et al. 2017; Mills et al. 2017; Osten and Dzul-Caamal 2017). Although animals lack the metabolic pathway inhibited by glyphosate (Annett et al. 2014), some evidence suggests that GBH is toxic to these non-target organisms (Folmar et al. 1979; Tsui and Chu 2003; Kasuba et al. 2017; Hong et al. 2018; de Melo et al. 2019).

For GBH toxicity in animals, the most usual subacute and chronic end points are oxidative stress, genotoxicity, cytotoxicity, neurotoxicity, and teratogenicity (Kasuba et al. 2017; Bonfanti et al. 2018; Hong et al. 2018; Pereira et al. 2018).

Moreover, for vertebrates models and human cells, glyphosate is also considered as an endocrine-disrupting compound (EDC), mimicking the effect of sexual and growth hormones (Gasnier et al. 2009; Myers et al. 2016; Soso et al. 2007). Thus, molecules like aromatase, steroidogenic factor-1, and vitellogenin were used to investigate endocrine disruption caused by GBH exposure (Armiliato et al. 2014; Richard et al. 2005; Xia et al. 2013). However, to the best of our knowledge, only few studies have been focused on the assessment of GBH endocrine disruption on invertebrates and on the standardization of biomarkers for organisms exposed to this herbicide (Le et al. 2010; Omran and Salama 2013). For the shrimp *Caridina nilotica* (48–61 day post hatch), a 14-day exposure to a GBH formulation caused an increase in the cumulative number of molts (Mensah et al. 2012), suggesting that this exposure had a stimulatory effect on the neuroendocrine system of the shrimps. However, the adverse outcome pathway involved in the molt disturbance after EDC exposure is still poorly understood.

The lack of investigations on the EDC (e.g., glyphosate) effects on invertebrates may be due to the limited knowledge of their endocrine system (Tillmann et al. 2001). In crustaceans, both the X-organ/sinus gland complex (XO) and the Y-organ (YO) are relevant endocrine organs that control process of growth and reproduction (Deecaraman and Subramoniam 1983; Mazurová et al. 2008; Rodríguez et al. 2007). In fact, the YO produces and secretes the precursors of 20-hydroxyecdysone (20-HE). This active steroid is known by its role in the molting process regulation and is also required in the reproduction process (Mu and LeBlanc 2002). Additionally, evidence on caridean shrimps (*Macrobrachium* spp.) suggests that ecdysteroids also stimulate vitellogenesis during ovarian oocyte maturation, through induction of vitellogenin (Vg) synthesis (Souty et al. 1982; Subramoniam 2011). Ecdysteroid action in target cells is exerted through a member of the nuclear receptor superfamily, the intracellular ecdysteroid receptor (EcR) (Mazurová et al. 2008). On the other hand, the synthesis of ecdysteroids by YO is negatively controlled by the molt-inhibiting hormone (MIH), a crustacean hyperglycemic hormone produced and secreted by XO (LeBlanc 2007; Mazurová et al. 2008).

Regarding the effects of EDC on the endocrine system of crustaceans, Lafontaine et al. (2016b) have shown that the insecticide chlordecone was able to modulate the expression of Vg and vitellogenin receptor (VgR) genes on the prawn *Macrobrachium rosenbergii*. Similarly, Gismondi (2018) observed modulation of MIH and EcR genes expression on the freshwater amphipod *Gammarus pulex* exposed to a series of proven EDC for aquatic vertebrates. Furthermore, an adverse outcome pathway describing those different EcR agonists (e.g., ecdysone, 20-HE, pesticides) can lead to incomplete ecdysis and mortality (Song et al. 2017). These results suggest that known EDC for vertebrates could also disturb the endocrine system of crustaceans, but this hypothesis needs more investigation to be confirmed. Therefore, this study was aimed to assess the effects of low GBH concentrations on molecules involved in the endocrine system of the decapod *Macrobrachium potiuna*, by evaluating the relative expression levels of MIH, EcR, and Vg genes in the studied species. In addition, the present study has evaluated if such genes may be good candidates in crustaceans as biomarkers for EDC exposure. *M. potiuna* is commonly found in freshwater environments and is therefore sensitive to different xenobiotics that can reach these environments. Moreover, the species was already used as a model in low GBH concentration assessment, and the prawns exposed to GBH concentrations presented hepatopancreas histopathological and subcellular compartment alterations (de Melo et al. 2019).

Materials and methods

Animals

Male and female *M. potiuna* prawns (total length 25.99 ± 1 mm) were collected in a clear freshwater stream located in the Permanent Preservation Area of Parque Municipal do Maciço da Costeira, Santa Catarina Island (27° 36′ 57.89″ S, 48° 30′ 7.63″ W) in the months of June and July of 2018 and directly transferred to the laboratory. During a 7-day acclimation period, they were kept in a 20-L aquarium containing dechlorinated water at 26 ± 1 °C, with constant aeration and feeding every 2 days with commercial food for aquatic organisms. The sampling procedure adopted in this study was approved by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA; permanent license no. 15294-1/2008).

Experimental design

A GBH formulation, containing 720 g kg^{-1} glyphosate, Roundup WG® (Monsanto do Brasil Ltda) was used for the experiments. Three GBH dilutions (0.0065, 0.065, and 0.28 mg L^{-1}) were prepared in dechlorinated water, based on the amount of glyphosate contained in the GBH formulation. To evaluate the effects of each GBH concentrations, 144 males and 144 females were randomly divided into 4 groups and exposed for 7 and 14 days to 0.00 (control group—dechlorinated water), 0.0065, 0.065, and 0.28 mg L^{-1}. Males and females were exposed separately and placed in 8 different 6-L glass aquarium filled with 4 L of exposure media (2 aquarium per condition). Exposure media were totally renewed every 4 days in order to maintain the GBH concentration in the aquaria (Sinhorin et al. 2014). All concentrations used were based on permitted levels for Brazilian inland waters through resolution no. 357 (CONAMA 2005) and for tap water in the

USA by the United States Environmental Protection Agency (USEPA 2009). During the exposure, the prawns were fed every 2 days with commercial food and maintained under constant aeration, controlled temperature of 26 ± 1 °C pH of 7.23, and natural photoperiod. No mortality was observed during the experiments.

After 7 and 14 days of GBH exposure, 6 replicates for each gender and concentration (pool of 3 prawns for each replicate) were randomly collected from the aquaria. The Vg gene expression was measured in hepatopancreas since this organ is one of the main synthesis sites of Vg in freshwater prawn *Macrobrachium* sp., as observed in *M. rosenbergii* (Soroka et al. 2000). EcR gene expression was also measured in the hepatopancreas, and MIH gene expression was measured in the eyestalks. Each pool of organs was immediately placed in RNA*later*® (Sigma R0901) solution for stabilization and stored at − 80 °C until analysis. The analyses were carried out on pools of tissues in order to avoid interindividual variations.

Total RNA extraction and cDNA synthesis

Tissue was removed of the RNA*later*® and crushed in the homogenization buffer provided by the kit using a mechanical potter. Total RNA of each sample (6 replicates—pool of 3 organs per replicate per gender and concentration) was extracted using the NucleoSpin RNA kit (Macherey-Nagel, Germany), following the manufacturer's instructions. The RNA integrity was checked on a 1.5% agarose gel electrophoresis in TAE buffer (Tris 40 nM, acetic acid 1 mM, EDTA 40 mM) stained with GelRed, and the RNA concentrations were measured using a NanoDrop ND-1000 spectrometer (NanoDrop, USA). Synthesis of complementary DNA (cDNA) was performed with 1 μg of total RNA using the RevertAid First-Strand cDNA Synthesis kit (Thermo Scientific) with random hexamer primers, following the manufacturer's instructions.

Primers design and sequences analysis

EcR, MIH, and Vg primers were designed based on the alignment resulting (Multalin program—Corpet, 1988) from corresponding sequences available on GenBank database for *Macrobrachium* sp. and their conserved regions. Primers for Actin, EF1α (elongation factor 1 α), and RPL8 (ribosomal protein L8) were used as housekeeping genes due to their stabilities (Table S1). Due to the high conservation of the difference gene sequences, specific primers were directly designed and tested for amplification (Table 1).

PCR amplification were carried out on 300 ng of cDNA with the following reaction mixture: 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl2, 200 μM of dNTP mix, 2 μM of each primer, and 0.04 U/μL of HotStart Taq DNA Polymerase (Thermo Scientific). Several amplification conditions were tested: a 5-min initial denaturation at 95 °C; 40 cycles of heat denaturation at 95 °C for 30 s; annealing at 35, 40, 45, or 50 °C for 1 min; polymerization at 72 °C for 1 min; and a 5-min final extension at 72 °C. Then, when the expected fragment length was obtained, PCR products were sequenced in the GIGA Genomics platform (University of Liège, Belgium) in order to check the corresponding protein.

Each nucleotide sequence obtained was analyzed using BlastX programs (Basic Local Alignment Search Tool) from the NCBI site (National Center for Biotechnology Information). When the desired sequences were identified, specific primers were designed in order to be used for the quantitative real-time PCR. All obtained sequences in *M. potiuna* in this work were deposited in GenBank database (EcR: accession number MK273063; MIH: accession number MK273064; Vg: accession number MK273068; Act: accession number MK273067; EF1α: accession number MK273066; RPL8: accession number MK273065).

Semi-quantitative real-time PCR

Specific primers (EcR-F, EcR-R, MIH-F, MIH-R, Vg-F, Vg-R), allowing smaller fragments, were designed and used to amplify cDNA of EcR, MIH, Vg, and the three housekeeping genes: actin, EF1α, and RPL8 by semi-quantitative real-time PCR (Table 1). Optimal concentrations of primers were evaluated across two concentrations (150 and 300 nM), and seven dilutions of a cDNA mixture (1 μL cDNA of each sample per organ) between 5-fold and 320-fold were tested to estimate the PCR efficiency (E) for each primer (Table 1), calculated according to the slope estimated with the linear regression of the Ct value according to the logarithm of the cDNA amount ($E = 1–10$^(1/slope)).

After optimization, qPCR analyses were carried out with a CFX96 Touch™ Real-Time PCR Detection System, using 2 μL of diluted cDNA 1:20, 5 μL of iTaq TM Universal SYBR Green Supermix (BioRad, Netherlands), 2 μL of primer mix 300 nM, and 1 μL of ultrapure water. PCR program consisted of 15 min initial denaturation at 95 °C, followed by 49 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and polymerization at 65 °C for 5 s. The relative transcription expression levels of EcR, MIH, and Vg were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), considering the PCR efficiency, and normalized to the respective control condition (e.g., male or female). Briefly, for each sample, expression ratio of each gene was calculated according to each housekeeping gene and the three expression ratios obtained were averaged (resulting in one expression ratio per sample). The obtained average expression ratio was calculated for the control group and used to normalize gene expression in the exposed groups. Lastly, averages and standard deviations of normalized gene expression were calculated.

Table 1 Primers used to identify all the sequences in *Macrobrachium potiuna* and to evaluate relative transcript expression levels of EcR, MIH, and Vg by real-time PCR

Genes	Primers	Efficiency (%)	Amplicon length (bp)	Sequences (5′–3′)
MIH	MIH-F1		155	CATCATCAGCCTGTTTCTGGTCT
	MIH-R1			ATGAYATGGGCACCAGATGCAG
	MIH-F2		235	GCACGTCGGCCAGGTACC
	MIH-R2			GGCTCGTAGGATGCTCATCCA
Vg	Vg-F1		438	CATCCTGATGATGACACTTGGTC
	Vg-R1			CTGAAGGAGGAATCTGGTCTG
Actin	Actin-F1		121	TCTCCCACACTGGTCCCATC
	Actin-R1			GGTGTAGCCACGTTC GGTC
EF1α	EF1α-F1		144	CAAGATGGACAGCACTGAACC
	EF1α-R1			CATGTTGTCTCCGTTGAAACCAG
RPL8	RPL8-F1		125	GTATTGTTGCTGGTGGAGGTC
	RPL8-R1			CTCTACAGGGTTCATCGCTAC
EcR	EcR-F	99.3	142	GCCGTCTATCAGTGTAAATACGG
	EcR-R			TTCACCTGGCACTGGGACTC
MIH	MIH-F	96.7	90	TAGCAGATGCAGGAAGGACTG
	MIH-R			GTTGAGCTGTTCCACGTCAC
Vg	Vg-F	97.7	74	GCGAAAAGGTAAAGCACGGAGT
	Vg-R			CACGGCGCAAGAAATGTAATGC
Actin	Actin-F	96.7	91	GTCGTGACTTGACCGATTACC
	Actin-R			GATGTCACGCACGATTTCTCG
EF1α	EF1α-F	99.7	79	CTGCCTATGTCAAGAAAGTTGG
	EF1α-R			CATGTTGTCTCCGTTGAAACCA
RPL8	RPL8-F	98.3	78	CTGGACGTGCTTACCACAAG
	RPL8-R			TCTACAGGGTTCATCGCTACAC

Integrated biomarker response

Using the results obtained with the relative transcript expression levels of EcR, MIH, and Vg, the Integrated Biomarker Response (IBR) index was calculated considering GBH concentration, gender, and time of exposure and using the IBR calculation modified by Sanchez et al. (2013). Briefly, for each time of exposure, the ratio between the biomarkers data of the exposed groups and the means of those the control group were calculated. The obtained ratio (Yi) was logarithmically transformed yi = Log(Yi + 1), and the general means (μ) and standard deviation (s) were obtained. The transformed values were standardized by the formula Zi = (yi − μ)/*s*. The same procedure was applied for the control groups, standardizing the Z0 values. The differences between Zi and Z0 (*A*) were calculated, determining the value of the response of each biomarker for each gender, at each time and concentration of exposure. $A > 0$ represents biomarker induction and $A < 0$ represents biomarker inhibition. Finally, the IBR value for each gender and time of exposure were obtained by the sum of (*A*) values. The deviation of the investigated biomarkers in relation with the control groups was represented in radar type graphs.

Statistical analysis

After having tested normality and variances homogeneity (Shapiro and Bartlett tests), the significant differences among the means of the control and exposed groups were analyzed with, the one-way ANOVA followed by post hoc Tukey's test. A significance level of $p < 0.05$ was adopted. The results were evaluated in the program the GraphPad Prism 7.0 program (GraphPad Software, La Jolla, CA, USA), and data were represented by mean ± standard error (SE).

Results

Relative transcript expression levels of EcR

The results of the statistical analysis revealed that for males, the EcR relative transcript expression levels were upregulated after 7 days of exposure to 0.065 mg L^{-1} (Table 2), compared to the respective control and exposed groups ($p < 0.05$, 2.1-fold) (Fig. 1). In addition, after 14 days of exposure, a similar

Table 2 Statistical analysis (one-way ANOVA) evaluating the relative transcript expression levels of ecdysteroid receptor (EcR), molt-inhibiting hormone (MIH) and vitellogenin (Vg) in *Macrobrachium potiuna* exposed at three concentrations of GBH for 7 and 14 days

ANOVA	*p* values					
Source of variation	EcR		MIH		Vg	
	Male	Female	Male	Female	Male	Female
7 days	< 0.0001	0.2850	0.0115	0.3099	0.0002	0.6974
14 days	< 0.0001	0.0175	0.3756	0.0007	0.0091	0.06164

result was observed in males exposed to 0.28 mg L^{-1}, as compared to control ($p < 0.05$, 3.9-fold).

In females, no effect of GBH was observed after 7 days of exposure, compared to respective control ($p > 0.05$). However, females exposed for 14 days showed a significant decrease in the groups 0.0065 and 0.065 mg L^{-1} ($p > 0.05$) when compared to control group (Fig. 1).

Relative transcript expression levels of MIH

After 7 days of exposure, an upregulation ($p < 0.05$, 3.6-fold) was observed in males exposed to 0.065 mg L^{-1}, compared to the control group, while no difference (Table 2; $p > 0.05$) was observed after 14 days of exposure (Fig. 2). Additionally, the two higher concentrations showed a significant time of exposure effect ($p < 0.05$) (Fig. 2).

In females, no difference of MIH expression was observed after 7 days of exposure ($p > 0.05$), whatever the GBH concentration. Nevertheless, a downregulation ($p < 0.05$, 0.4-fold) was observed after a 14 days-exposure at 0.065 mg L^{-1}, as compared to control group (Fig. 2).

Relative transcript expression levels of Vg

The statistical analysis indicated that Vg gene expression in control males exposed for 7 days was different from the exposed groups (Table 2) ($p < 0.05$). Moreover, after 14-day exposure, a significant over-expression ($p < 0.05$, 10.5-fold) was measured in males exposed to 0.065 mg L^{-1}, as compared to the respective control (Fig. 3).

In females, no significant effect ($p > 0.05$) was observed, whatever the time and the concentration of exposure, except for a slight under-expression measured after 7 days of exposure to 0.065 mg L^{-1} (Table 2; Fig. 3). Moreover, this effect was time-dependent, since no under-expression was observed after 14 days of exposure ($p > 0.05$). On the contrary, a slight over-expression ($p > 0.05$) of Vg was observed at the two lowest GBH concentrations after 14 days of exposure.

Integrated biomarker response

The IBR index was calculated for each exposure time for both males and females (Fig. 4). At 7 days of exposure, it was

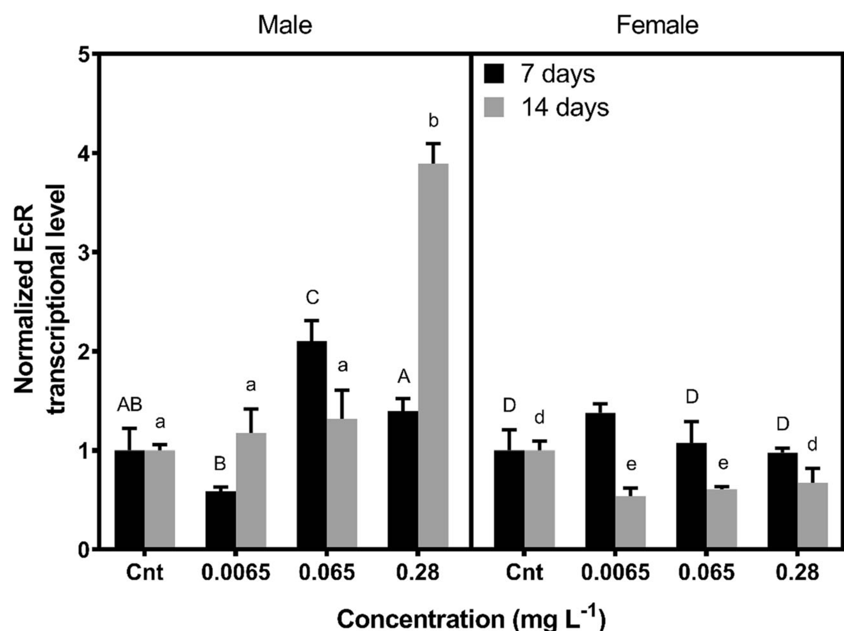

Fig. 1 Relative transcript expression levels of EcR (mean ± SE) in *Macrobrachium potiuna* males and females exposed to GBH for 7 and 14 days. Different capital letters denote statistical differences across the groups exposed for 7 days for males and females separately. Different lowercase letters denote statistical differences across the groups exposed for 14 days for males and females separately (Tukey's HSD test $P < 0.05$)

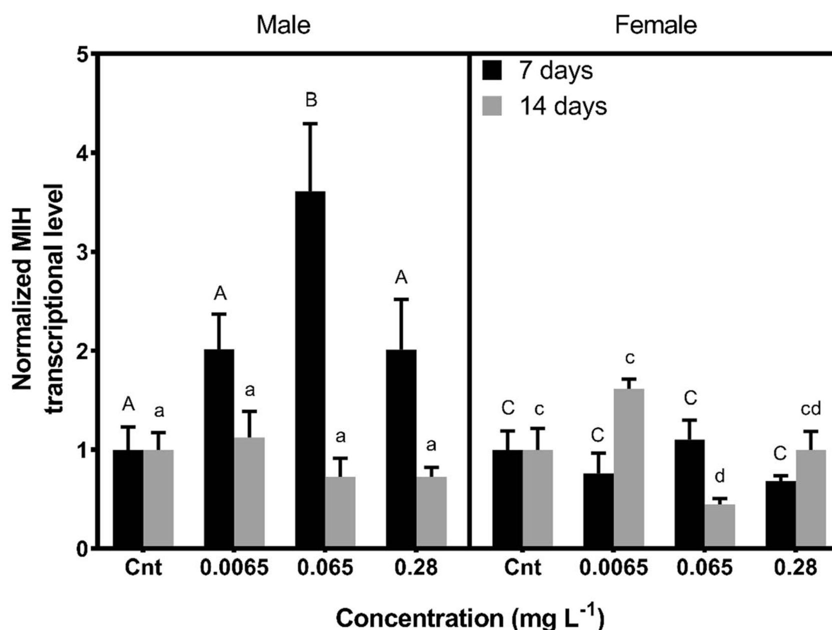

Fig. 2 Relative transcript expression levels of MIH (mean ± SE) in *Macrobrachium potiuna* males and females exposed to GBH for 7 and 14 days. Different capital letters denote statistical differences across the groups exposed for 7 days for males and females separately. Different lowercase letters denote statistical differences across the groups exposed for 14 days for males and females separately (Tukey's HSD test, $P < 0.05$)

observed an induction of EcR and MIH (areas of EcR and MIH more than 0) for the two higher concentrations in males (Fig. 4). However, after 14 days exposure, the only induction was observed for the EcR at the higher concentration (Fig. 4).

On the other hand, females exposed to GBH for 7 days presented values very similar to the control group. Conversely, females exposed for 14 days showed mostly induction of MIH at 0.0065 mg L^{-1} (Fig. 4).

The global IBR values, calculated considering EcR, MIH, and Vg results, for each concentration, gender, and time of exposure confirmed different modulations for males and females (Table 3). Although males and females show negative IBR values at 0.0065 mg L^{-1}, except females exposed for 14 days, the main differences were observed for the two higher GBH concentrations. Indeed, at 0.065 and 0.28 mg L^{-1}, males had positive IBR values, whereas females had negative values, regardless the time of exposure.

Discussion

Although, for vertebrates, glyphosate is known as an EDC, altering aromatase activity and modulating the profile of cortisol, testosterone, and 17 β-estradiol hormones (Gasnier et al. 2009; Myers et al. 2016; Soso et al. 2007), its effects on the crustaceans endocrine system remain uncertain. In order to

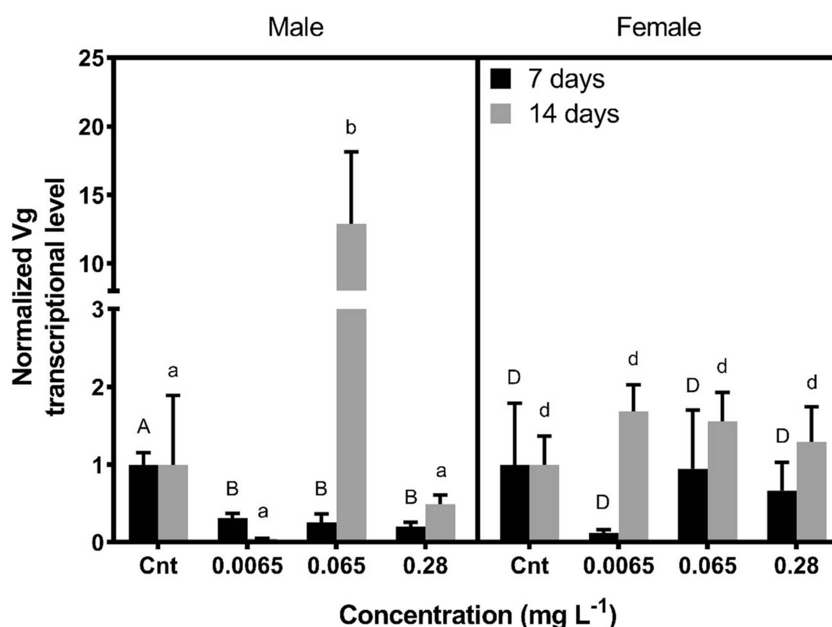

Fig. 3 Relative transcript expression levels of Vg (mean ± SE) in *Macrobrachium potiuna* males and females exposed to GBH for 7 and 14 days. Different capital letters denote statistical differences across the groups exposed for 7 days for males and females separately. Different lowercase letters denote statistical differences across the groups exposed for 14 days for males and females separately (Tukey's HSD test, $P < 0.05$)

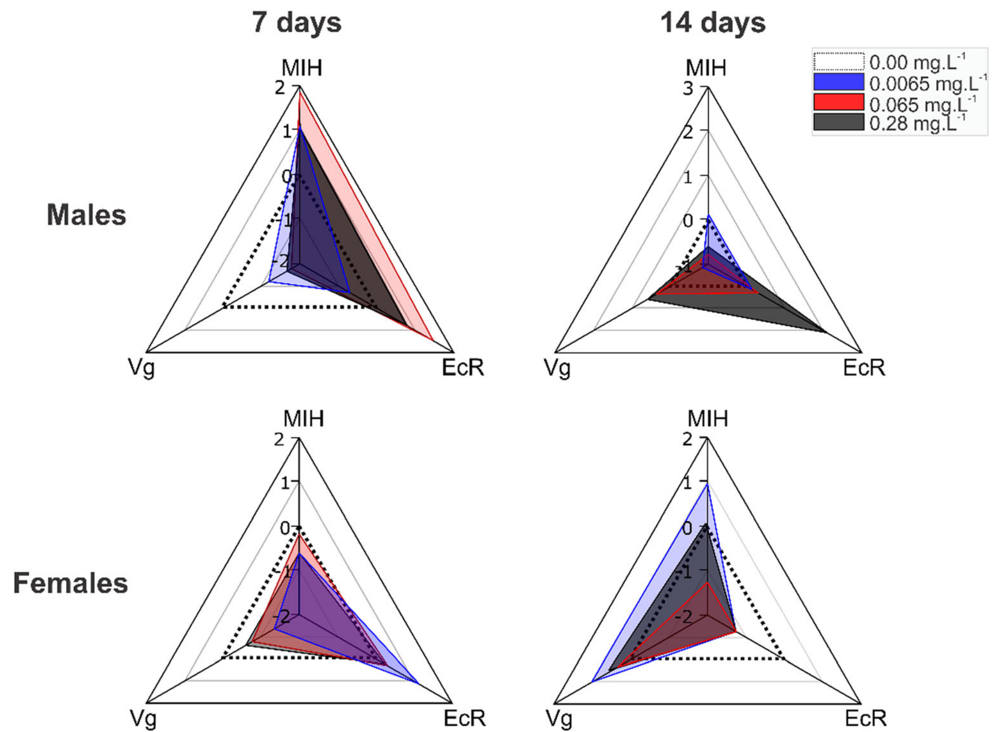

Fig. 4 Biomarker radar plots of *Macrobrachium potiuna* males and females exposed to GBH for 7 and 14 days. MIH molt-inhibiting hormone, EcR ecdysteroid receptor, Vg vitellogenin. Black dotted lines indicate the control group

investigate this issue, this study demonstrated, for the first time, the ability of GBH to modulate the expression of genes involved in the endocrine pathways of crustaceans.

Data presented here revealed that the EcR relative expression was modulated after GBH exposure, mostly in males, with no apparent concentration-dependent response. Although there are few studies on EDC effects on EcR gene expression, similar results were found in studies carried out on other invertebrates. Indeed, Planelló et al. (2011) observed an upregulation of EcR expression after butyl benzyl phthalate (BBP) exposure, without a clear concentration-dependent response, in the midge *Chironomus riparius.* Alternatively, Nair and Choi (2012), evaluating the EcR expression in the same model organism, observed a concentration-dependent response for midges exposed for 24 h to silver nanoparticles, but the organisms exposed for 48 h and 72 h showed downregulation regarding the concentration. In addition, the authors also observed an EcR upregulation at the lower concentration and lower time of exposure, while a longer exposure time was necessary to observe the same result in midges exposed to the highest nonylphenol concentration (Nair and Choi 2012). Similar results were observed in our study, which at 7 days of exposure, only the 0.065 mg L^{-1} was able to induce an upregulation in EcR expression levels, while at 14 days, such increase was observed only in prawns exposed to the highest concentration (0.28 mg L^{-1}). As stated by some authors, EDC can act at low rather than at high concentrations (Lafontaine et al. 2016a), explaining the results for 7 days, but a prolonged exposure to higher concentrations is also able to induce EcR upregulation.

Likewise, Planelló et al. (2008), Morales et al. (2014), and Martínez-Paz et al. (2017) found upregulation of the EcR mRNA expression on *C. riparius* larvae after bisphenol A, pentachlorophenol, and triclosan exposures. In freshwater amphipods, Gismondi (2018) found an EcR upregulation on males and females *Gammarus pulex* exposed to four EDC tested in vertebrates. However, our results were different concerning the females, since EcR expression of *M. potiuna* females was not influenced by the GBH exposure, as observed in *M. potiuna* males. These results can be explained by the fact that EcR is linked to the available ecdysteroids (20-HE), which are synthesized by the YO. Therefore, GBH could act on the YO, causing a 20-HE increase that will positively control the synthesis of EcR. In fact, Gismondi (2018) observed an upregulation of EcR transcript levels after exposure to 20-HE in *G. pulex*, confirming the link between the EcR and 20-HE. Nonetheless, the production of ecdysteroids by YO is controlled by the XO, especially by the MIH release. Consequently, our EcR upregulation observed could be

Table 3 The IBR index values for biomarkers of *Macrobrachium potiuna* exposed to GBH for 7 and 14 days. For the control groups, the IBR = 0

Concentration (mg L^{-1})	Male		Female	
	7 days	14 days	7 days	14 days
0.0065 mg L^{-1}	−0.78	−0.58	−0.81	0.32
0.065 mg L^{-1}	1.55	0.52	−0.62	−2.16
0.28 mg L^{-1}	0.15	2.09	−0.96	−0.62

caused by GBH acting on XO and particularly inhibiting the MIH synthesis.

Nevertheless, our results mainly highlighted an increase in MIH transcript levels after 7 days of exposure. Similar results were observed in previous studies on the EDC effects in crustaceans (Gismondi 2018; Mattson and Spaziani 1985) and were explained by EDC effect on XO. Therefore, the MIH results observed here could be described by a GBH effect on XO, responsible for the MIH synthesis and release. However, as explained above, MIH increase could inhibit YO, thus decreasing the 20-HE release and therefore the EcR. Such results were observed by Mattson and Spaziani (1985) in the crab *Cancer antennarius* and also can be explained for natural variations of EcR and MIH genes expression like in *Gammarus pulex* (Gismondi and Joaquim-Justo 2019). On the other hand, the increase in MIH expression levels could be due to a negative feedback control of the 20-HE. Therefore, a higher concentration of 20-HE could cause an increase in MIH synthesis, which could stop the ecdysteroid synthesis. This assumption is consistent with the increase in MIH expression levels observed in *G. pulex* and *C. antennarius* exposed to 20-HE (Gismondi 2018; Mattson and Spaziani 1986, respectively). Consequently, considering the MIH and EcR results obtained here in males, the assumption of a GBH effect mainly on YO (and therefore 20-HE synthesis) seems to be the more plausible.

Our results showed that the Vg expression levels were only affected in males exposed for 14 days at 0.065 mg L^{-1}, contrary to the females in which no difference was observed. These results are in contrast with those of Lafontaine et al. (2016b), who observed no significant differences for *Macrobrachium rosenbergii* males exposed to the insecticide chlordecone, while females exposed for 240 days showed an upregulation of the Vg expression levels. Moreover, GBH were able to stimulate female ovarian growth and maturation in the crab *Neohelice granulata*, indicating a probably increase of Vg synthesis in females (Avigliano et al. 2014; Avigliano et al. 2018). Besides, our results are consistent with our previous hypothesis, suggesting that GBH can increase 20-HE, once there is evidence suggesting that 20-HE induce Vg (Hyne 2011). Furthermore, Luo et al. (2015) observed Vg mRNA increase at 4–6 days after injection of MIH in the shrimp *Litopenaeus vannamei*. Thus, in our study, the increase of Vg transcript levels observed in males exposed to 0.065 mg L^{-1} for 14 days could be the consequence of the increase in MIH transcript levels observed in the same concentration in males exposed for 7 days. Additionally, GBH can act as an agonist of ecdysteroid receptor, binding to EcR, stimulating the Vg synthesis. In crustaceans, some compounds have been shown to act as an agonist or even antagonist by directly binding to hormone–receptor complex (LeBlanc 2007; Rodríguez et al. 2007).

For vertebrates exposed to EDC, Vg is commonly used as biomarker of exposure, as several studies observed an increase of Vg gene expression, indicating an estrogenic effect of the studied EDC (Donohoe and Curtis 1996; Flouriot et al. 1996; Hammond et al. 1979). However, the use of Vg as a biomarker of exposure for endocrine disruption in crustaceans is very questionable in the literature (Short et al. 2014; Boulangé-Lecomte et al. 2017). To our knowledge, only the studies of Sanders et al. (2005) in *Palaemon elegans* and Xuereb et al. (2011) in *G. fossarum* found significant differences in the Vg gene expression in males after EDC exposure. Therefore, the use of Vg as biomarker of EDC exposure in crustaceans should be carefully taken into account.

Otherwise, although the present work highlighted transcription disruption in males, no GBH effect was underlined in females. The lack of modulation in female transcript expression levels of EcR, MIH and Vg observed after 7 and 14 days of exposure is interesting and reinforces the ecotoxicological relevance of gender in pollutant toxicity assessment. We can assume that females possess a more robust regulation of their endocrine system, since they are responsible for the population maintenance. Furthermore, this could also be explained by a more efficient detoxification system in females. In fact, several studies observed higher antitoxic defense (antitoxic enzymes and antioxidant molecules) in females than males crustaceans (Gismondi et al. 2013; Horion et al. 2015; Sroda and Cossu-leguille 2011). Another explanation for the sexual differences observed could be the fact that although males and females have the same XO-YO-hepatopancreas axis system, the regulation could be different, due to the reproduction process, which is different in males and females. Since the crustacean hormonal process and regulation are not completely known for now, we could assume that females have a “better” regulation of their endocrine axis, leading to no GBH effect. Moreover, even though we tried to avoid the molt stage factor by making a pool of prawns, we cannot discard the possibility of our results be in part a reflex of the natural gene expression fluctuations during the molt cycle.

Thus, in further investigations, the assessment of antitoxic enzymes after GBH exposure in both males and females could be of great value to better understand the different gender responses, Moreover, the assessment of GBH effect in their gonads can provide valuable insights to understand how this widely used herbicide could compromise the reproduction of crustaceans. Indeed, Vg, MIH, and EcR expression level modulations observed in males during this study did not follow a classic concentration–response relationship (GBH effects mainly observed in the group 0.065 mg L^{-1}). However, as stated by some authors, EDC can act at low rather than at higher concentrations, probably by possible sublethal toxic effects at the higher concentrations (Jubeaux et al. 2012; Lafontaine et al. 2016a; Sanders et al. 2005; Welshons et al. 2003). As a result, all these observations led to conclude that GBH could be an EDC in crustaceans, as *M. potiuna*, by influencing 20-HE concentration or by behaving like 20-HE.

Moreover, as previously reported in the shrimp *Caridina nilotica*, GBH could disturb the molt cycle, increasing the cumulative number of molts (Mensah et al. 2012).

Conclusion

This study is the first one in measuring the relative transcription levels of some genes involved in the processes of molting and reproduction of crustaceans, after exposure to environmental relevant concentrations of GBH. The results showed modulation of these three genes in males but not in females, leading to believe that males are more sensitive to EDC than females.

Results led to conclude that GBH seems to be an EDC for crustaceans probably by mimicking the natural molt hormone (20-HE). In addition, as underlined by other studies, the use of Vg expression showed not to be the most reliable biomarker for EDC exposure in crustaceans, whereas the use of males seems to be more appropriate than females. Besides, although there have been few studies about the regulation of genes in aquatic organisms after exposure to EDC, our study offers potential gene biomarkers (EcR and MIH) for further genomic environmental investigations. In conclusion, the lack of information about the endocrine system of crustaceans makes it difficult to fully understand and explain the GBH adverse outcome pathways and their consequences at the population levels. Thus, future investigations are needed to characterize and better understand the natural fluctuations of endocrine system molecules and evaluate their intrinsic relationship with other molecules (e.g., 20-HE, chitobiase) involved in molting process. This reinforces also the need to find and develop new biomarkers of exposure for EDC in invertebrates to be complementary to those developed in vertebrates and having a global point of view of EDC effects on aquatic ecosystems.

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