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RESEARCH ARTICLE

Vitellogenin and vitellogenin receptor gene expression and 20-hydroxyecdysone concentration in *Macrobrachium rosenbergii* exposed to chlordecone

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Abstract Chlordecone is a persistent organochlorine pesticide widely used in Guadeloupe (French West Indies) to control the banana weevil *Cosmopolites sordidus*. Although it was previously highlighted that chlordecone may affect the reproduction and growth of vertebrate species, little information is available on the chlordecone effects in invertebrates. The present study investigated the effects of chlordecone on a hormone and a protein having key roles in reproduction and growth of the decapod crustacean *Macrobrachium rosenbergii*, by measuring the 20-hydroxyecdysone concentration, vitellogenin, and vitellogenin receptor gene expression, as well as the bioconcentration of chlordecone in exposed prawns. First, the results revealed that chlordecone was accumulated in *M. rosenbergii*. Then, it was found that Vg and VgR gene expression were increased in male and female *M. rosenbergii* exposed to chlordecone for 90 and 240 days, while the 20-hydroxyecdysone concentrations were decreased. This work suggests that chlordecone accumulates in prawn tissues and could affect key molecules involved in the reproduction and the growth of the invertebrate *M. rosenbergii*. However, many questions remain unresolved regarding the impacts of chlordecone on growth and reproduction and the signaling pathways responsible for these effects, as well as the potential role of confounding factors present in in situ studies.

Keywords *Macrobrachium rosenbergii* · Chlordecone · Vitellogenin · Vitellogenin receptor · 20-Hydroxyecdysone

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Introduction

Studies on the impacts of endocrine-disrupting compounds (EDCs) on wildlife have focused mainly on hormonal regulation related to reproduction and development processes which have critical roles in population dynamics (Arukwe and Goksøyr 2003; Gismondi and Thomé 2014; Jubeaux et al. 2012). Indeed, many pollutants in aquatic environment are able to interfere with the endocrine system of exposed organisms which could lead to dysfunctions of biological processes (e.g., growth, reproduction) (Hyne 2011). Endocrine disruption has been extensively described in aquatic vertebrates (Kortenkamp et al. 2011; LeBlanc 2007), and the best known marker is vitellogenin (Vg) which has been used as exposure biomarker in several vertebrate species due to its key role in the reproduction and its induction via the control of the estrogen receptor pathway (Jones et al. 2000; Sumpter and Jobling 1995; Tyler et al. 1996; Zhong et al. 2014). However, Thornton et al. (2003) suggested that ecdysozoans lost the steroid receptor family, and Thomson et al. (2009) underlined the absence of receptors of the 3A group (estrogen receptor) and the 3C group (androgen, progestogen receptors) in *Daphnia pulex*. Therefore, although invertebrates can also be exposed to known EDCs in vertebrates, it is still difficult to make assumptions about the

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impacts of EDCs on invertebrates using knowledge from vertebrate species (Scott 2013). Investigations are thus necessary to improve our understanding of the metabolic pathways involved in the disruption of the endocrine functions in invertebrates.

Chlordecone (CLD) was an insecticide commonly employed in the French West Indies (FWI) in Guadeloupe to control the banana weevil *Cosmopolites sordidus* from 1972 to 1993. Chlordecone is an organochlorine compound that acts by altering the sodium channels, essential for the transmission of nerve impulses in organisms (Guzelian 1982; Newhouse et al. 2009). This inhibition of the sodium channels induces an increase of the intracellular concentration of calcium which activates the contractile proteins and causes convulsions and death of the target insect. As CLD interferes with the nervous system, it is considered a neurotoxic substance.

A few years after its introduction, widespread pollution of soils, rivers, wild animals, and aquatic organisms was reported (Cavelier 1980; Snegaroff 1977). Indeed, because of its high Koc (soil organic carbon water partitioning coefficient), Kow (octanol–water partition coefficient), and its affinity for lipids, CLD is persistent in the environment and accumulates in the food web (Cabidoche and Lesueur-Jannoyer 2012). Since Hammond et al. (1979) demonstrated that CLD can bind to estrogen receptor in rat, many studies investigated the endocrine effects of CLD in various biological models. However, in aquatic ecosystems, endocrine effects of CLD were mainly studied in vertebrate models. For example, Donohoe and Curtis (1996) observed an increase of the Vg concentration in juvenile rainbow trout *Oncorhynchus mykiss* exposed for 33 weeks to CLD. Moreover, Curtis and Beyers (1978) observed a decrease of the oviposition of the teleost *Oryzias latipes* after 252 h of CLD exposure. Until now, few studies have been carried out in invertebrate models to investigate the toxic effects of CLD. Nevertheless, Gaume et al. (2014) highlighted that a CLD exposure caused the induction of genes involved in the anti-oxidative defense (e.g., catalase and glutathione peroxidase) or in the biotransformation process (i.e., cytochrome P450 and glutathione-S-transferase). Giusti et al. (2014) showed the reduction of the oviposition and the fecundity of the gastropod *Lymnaea stagnalis* exposed to CLD at environmental relevant concentrations. Finally, our previous study showed that CLD exposure in laboratory affected the 20-hydroxyecdysone concentration (i.e., molting hormone) and the chitobiase activity (i.e., molting enzyme), both controlled by the endocrine system, in the decapod *Macrobrachium rosenbergii*, suggesting that CLD could be an EDC in invertebrates (Lafontaine et al. 2016).

According to previously cited studies, CLD is suspected to be an EDC in invertebrates and could thus affect the reproduction and development. The present in situ study aimed to investigate the effects of an environmental concentration of CLD on the endocrine system of the decapod *M. rosenbergii*, which is one of the most important resources in Guadeloupe and which can be considered a good model for the wild *Macrobrachium spp.* living in freshwater ecosystems of these regions, by evaluating the relative expression of the vitellogenin protein (Vg) gene. As Vg needs receptors to cross the cell membrane and exert its role in the gonadal tissue, the relative expression of the vitellogenin receptor gene (VgR) was also assessed. In parallel, since the 20-hydroxyecdysone (20-HE) synthesis is linked to the induction of the Vg synthesis (Hyne 2011), the concentration of 20-HE was also assessed. Finally, to incriminate the observed effects to the CLD exposure, the CLD concentrations were measured in exposed and non-exposed prawns.

Materials and methods

In situ chlordecone exposure and sampling

Post-larvae of *M. rosenbergii* were provided by a hatchery farm (OCEAN-SA, Guadeloupe, FWI) in a geographic area free of CLD contamination. Pretests have previously been carried out to evaluate the presence of CLD in tissues of prawns from the hatchery farm, and results showed no contamination (concentrations below detection limit) (data not shown). Prawns were transferred into two farming ponds, naturally filled by rivers. The first, called "control site," was located in Pointe-Noire (North Basse-Terre, Guadeloupe: 16° 22 49 N, 61° 77 69 W), and the second one, called "contaminated site," was located in Saint-Claude (South Basse-Terre, Guadeloupe: 16° 01 93 N, 61° 68 37 W), which is a pond supplied by the Rivière Aux Herbes, a CLD-contaminated river (0.33 μg L^{-1} average measured between 2003 and 2008 by DIREN Guadeloupe (2003)). The farm based in Pointe-Noire is still in operation, while the Saint-Claude farm has had to cease operations because the CLD concentration in prawns was higher than the French and European maximum residue limit of 20 ng g^{-1} wet weight (Anon 2008; Ministère de l'Écologie du Développement durable et de l'Énergie 2015a). Water temperature, pH, and dissolved oxygen were measured throughout the experiment, which took place from March 2012 to November 2012 (Table 1). These values are in accordance with optimal water

Table 1 Water parameters of Pointe-Noire (control site) and Saint-Claude (contaminated site) measured during the 240 days of exposure

Parameter	Pointe-Noire	Saint-Claude
CLD concentration (μg L^{-1})	<LOD	0.33 ± 0.10
Temperature (°C)	27.64 ± 1.65	27.66 ± 1.76
pH	8.47 ± 0.47	8.00 ± 0.47
Dissolved oxygen (mg L^{-1})	7.18 ± 1.72	6.44 ± 2.46

temperature, pH, and dissolved oxygen commonly used in prawn farms (New 2002).

After 90 and 240 days of exposure, 20 females and 20 males were collected in each site. For each gender, five prawns (corresponding to five replicates) were used for the CLD bioconcentration assessments, five prawns (corresponding to five replicates) were used to measure the 20-HE concentration, and ten prawns (corresponding to ten replicates) were used to measure the vitellogenin (Vg) and the vitellogenin receptor (VgR) gene relative expression. The Vg gene expression was measured in hepatopancreas since this organ is one of the main synthesis sites of Vg in the giant freshwater prawn *M. rosenbergii* (Soroka et al. 2000), while the synthesis site of VgR is the gonadal tissue (Roth and Khalaila 2012). The CLD concentration was also measured in both control and exposed prawns. After sampling, individuals were transferred to the laboratory where hepatopancreas and gonadal tissue were immediately dissected, frozen in liquid nitrogen, and stored at −80 °C until analysis. Before dissections, body length and body weight were measured and no significant difference ($p > 0.05$ of the two-way ANOVA test) was observed between individuals from the control site and those of the contaminated site, taking into account time exposure (i.e., 90 days Pointe-Noire, 10.20 ± 0.52 cm and 10.21 ± 1.74 g; Saint-Claude, 10.96 ± 0.45 cm, 15.00 ± 3.16 g; 240 days Pointe-Noire, 12.03 ± 0.97 cm, 21.79 ± 4.18 g; Saint-Claude, 12.72 ± 0.74 cm, 23.42 ± 4.74 g). Individual sex was confirmed during dissections, thanks to the observation of gonadal tissues.

Chlordecone concentration in *Macrobrachium rosenbergii*

The CLD concentration was analyzed in *M. rosenbergii* tissues according to a method adapted from Debier et al. (2003), Guldner et al. (2010), and Multigner et al. (2010). Briefly, prawns were freeze-dried with a Benchtop 3 L Sentry Lyophilisator (VirTis, USA). The extraction of CLD was performed with a solvent mixture of n-hexane/dichloromethane (90:10; *v*/*v*; Biosolve Chimie, France) using an Accelerated Solvent Extractor (ASE 200) (Dionex, Thermo Scientific, USA), and a hexanic solution of PCB congener 112 (Dr. Ehrenstorfer, Germany) was added to the samples as a surrogate internal standard. After the extraction, samples were cleaned up with 98 % sulfuric acid (Merck, Germany) in order to remove organic matters (e.g., lipids, lipoproteins, carbohydrates). Then, a volume of 5 μL of nonane was added in the collected phase as a keeper; samples were evaporated under a gentle nitrogen stream and resuspended in n-hexane added with a solution of PCB 209 as an injection volume internal standard (Dr. Ehrenstorfer, Germany). In parallel of the sample extractions, a procedural blank (i.e., ASE extraction without biological matrix allowing to control the extraction and the clean-up procedure) and a quality control (QC) were carried out. The QC was performed to control the CLD recovery by using a CLD-free biological matrix (here, freeze-dried *Penaeus monodon*) spiked with a defined concentration of CLD in order to obtain a nominal concentration of 2.5 ng g^{-1} wet weight. The purified extracts, procedural blank and QC, were analyzed by high-resolution gas chromatography. The analytical parameters were described by Guldner et al. 2010 and Multigner et al. 2010. CLD was identified based on its retention time previously determined with a linear calibration curve (1.5 to 200 pg μL^{-1}, $r > 0.99$) established with CLD certified solutions (Riedel-de Haën, Germany). The quantification of CLD was achieved by means of the internal standard method. The CLD concentrations in each sample and in the QC were corrected with the percentage recovery of the surrogate PCB 112 and the initial sample weight. The recovery efficiency was within the limits recommended by SANCO (SANCO/12571/2013 European commission, n.d.). The limit of detection (LOD) was 0.02 ng g^{-1} wet weight and the limit of quantification (LOQ) was 0.06 ng g^{-1} wet weight. The CLD concentrations in *M. rosenbergii* were measured in five replicates per condition and the mean was calculated. The CLD concentrations were expressed in nanograms per gram of wet weight.

20-Hydroxyecdysone concentration

The concentrations of the 20-HE hormone and its derivatives (called "20-HE" in the following text, figures and tables) were measured by following the manufacturer's instructions of the enzyme immunoassay (EIA) kit (Cayman Chemical Company, USA), which were adapted to our biological organism (e.g., weight of tissue, solvent of homogenization, standard curve). The 20-HE concentration assessment was entirely described in Lafontaine et al. (2016).

Quantification of vitellogenin and vitellogenin receptor gene relative expression levels

Total RNA extraction and cDNA synthesis

Total RNAs were extracted from 30 mg of tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen, Germany), following the manufacturer's instructions. Quality of RNA was verified by electrophoresis on a 1.5 % agarose gel in TAE buffer (Tris 40 mM, acetic acid 1 mM, EDTA 40 mM) and visualization under UV light, and RNA concentrations were measured using a NanoDrop ND-1000 spectrometer (NanoDrop, USA). Synthesis of complementary DNA (cDNA) was performed with 150 ng of total RNA using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fisher Scientific) and random hexamer primers. The reverse transcription polymerase chain reaction (reverse-PCR) was performed at 42 °C for 60 min with a reaction mixture containing 10 mM Tris–HCl, 50 mM KCl, 5 mM MgCl2, 1 mM dNTP mix, 2.5 μM of

random hexamer primers, 2.5 U/μL of RevertAid MuLV RT, and 1 U/μL of RiboLock RNase Inhibitor.

Quantitative real-time PCR

Evaluation of Vg and VgR gene expression levels was carried out by using quantitative real-time PCR (RT-qPCR). Specific primers were designed to amplify the corresponding cDNAs according to Roth and Khalaila (2012) and the specific gene sequences of *M. rosenbergii* available in GenBank database (Vg: accession number AB056458.1; VgR: accession number GU454802.1) (Table 2). Actin (accession number AY626840.1) (Qiu et al. 2008) and 18S (accession number KM101531.1) (Roth and Khalaila 2012) fragments were tested as housekeeping genes.

RT-qPCR was performed in 384-well plates with an ABI PRISM 7900HT system (Applied Biosystems) using MESA GREEN qPCR MasterMix (Eurogentec). A total of three technical repeats were run for each cDNA gene and primer pair (Table 2). For each reaction, an equal amount of cDNA (4 μL of the cDNAs diluted 50-fold and prepared from 150 ng of total RNAs) was completed with a reaction mix containing 5 μL of MESA GREEN qPCR MasterMix and 2.5 pmol of each primer in a final volume of 10 μL. The following standard thermal profile was used: 2 min at 50 °C, 10 min at 95 °C, 40 repeats of 15 s at 95 °C, and 60 s at 60 °C. At the end of the 40 repeats, dissociation curves of the amplified products were established with the following thermal steps: 15 s at 95 °C, 15 s at 60 °C, and 15 s at 95 °C. The quality of the reactions was checked through the amplification and dissociation curves. In addition, reaction efficiencies were determined for each RT-qPCR using the LinRegPCR software v2013 (Ruijter et al. 2009). Mean reaction efficiencies were then determined for each primer pair from all reactions (>100 reactions; Table 2) and used to calculate relative gene expression levels using 18S as housekeeping gene for normalization with the qBase software (Biogazelle, Hellemans et al. 2007). The 18S was selected to normalize the data, as it displayed the highest stability across the sample set (no significant differences upon a three-way ANOVA test, with $p > 0.05$, taking into account "CLD exposure," "duration of exposure," and "gender" as factors).

Statistical analysis

Statistical analyses were carried out with the STATISTICA 10 Software (StatSoft 2012 USA). The data met normality and variance homogeneity which were tested with the Shapiro–Wilk and the Bartlett tests, respectively. First, a MANOVA test was performed in order to test the influence of gender, CLD, and duration of exposure on the whole measured parameters (Table 3). Then, to investigate the CLD bioaccumulation as well as the effects of CLD, gender, and duration of exposure on the relative expression of the Vg and VgR genes and the 20-HE concentration, data were analyzed using a three-way ANOVA test (Table 3), followed by Tukey's HSD post hoc tests which were performed to describe significant differences. A probability value of less than 0.05 was regarded as significant. The correlations between measured parameters were analyzed using the Pearson correlation coefficient.

Results and discussion

Chlordecone concentration in *Macrobrachium rosenbergii*

The CLD concentrations were measured in post-larvae provided by the hatchery, before the transfer in each farming pond (T0, Table 4). CLD concentrations were below the LOD, which confirmed that prawns were free of CLD contamination at the beginning of the experiment. The CLD concentrations were also measured in *M. rosenbergii* transferred in the control and the contaminated farms after 90 and 240 days of exposure (Table 4). The ANOVA results showed a significant influence of the exposure site ($p < 0.05$) on the CLD concentration in prawns. In control prawns, the very low concentrations of CLD detected could be explained by the bioaccumulation of trace amounts of CLD (lower than the LOQ in water, i.e., 0.01 μg L^{-1}) detected in the river supplying the Pointe-Noire farming pond (Bonan and Prime 2001; Ministère de l'Écologie du Développement durable et de l'Énergie 2015b). The results obtained from field studies are generally more environmentally realistic and ecologically meaningful than laboratory tests (Connon et al. 2012; Crane and Babut 2007), but they are difficult to interpret, because of several confounding factors (e.g.,

Table 2 Sequences and reaction efficiencies of each primer pair for each of the studied genes

Genes	Species	Primers		Efficiency (mean ± SD)
		Forward 5 –3	Reverse 5 –3	
Vg	*M. rosenbergii*	GCGAAAAGGTAAAG CACGGAGT	ACGGCGCAAGAAAT GTAATGC	1.957 ± 0.083
VgR	*M. rosenbergii*	CTCCCTTGACTACG TCTGCAAC	GCATTGCATCTTGA GGTCTCG	1.911 ± 0.068
18S	*M. rosenbergii*	TAGCAATTCGCCGT CGTTATTC	CTACCCCCGGAACT CAAAGACT	2.127 ± 0.141

Table 3 Univariate and multivariate analyses of variance (ANOVA/MANOVA) investigating variations in vitellogenin (Vg) and vitellogenin receptor (VgR) gene expressions and 20-HE concentrations according to chlordecone exposure (CLD), gender, and duration of exposure

MANOVA	*p* values		
Sources of variation			
Gender	<0.0001		
CLD	<0.0001		
Duration of exposure	0.06		
Gender × CLD	0.04		
Gender × duration of exposure	0.002		
CLD × duration of exposure	0.01		
Gender × CLD × duration of exposure	0.03		
ANOVA	*p* values		
Sources of variation	Vg gene	VgR gene	20-HE level
Gender	<0.0001	<0.0001	0.07
CLD	0.36	0.002	<0.0001
Duration of exposure	0.70	0.33	0.009
Gender × CLD	0.64	0.003	0.75
Gender × duration of exposure	0.41	0.51	<0.0001
CLD × duration of exposure	0.025	0.06	0.68
Gender × CLD × duration of exposure	0.011	0.03	0.02

season, other chemicals). However, the CLD accumulation observed here is consistent with the fact that CLD has a high potential of bioaccumulation in aquatic organisms, based on its physicochemical properties, even when concentrations are very low (ATSDR et al. 1995). As expected, a significant accumulation of CLD was observed in prawns sampled in the contaminated pond (Saint-Claude). This result is in agreement and of the same order than those of Monti (2007) who measured CLD concentrations of 2204 ng g^{-1} in *M. carcinus* and 1134 ng g^{-1} in *M. faustinum*, sampled in the Rivière aux Herbes, which is the river supplying the Saint-Claude pond, used as contaminated site in the present study. On the contrary, the CLD concentrations measured in *M. rosenbergii* in the present study were higher than those measured in the same organism in another in situ study (Gaume et al. 2014). This difference is probably due to the duration of exposure, since Gaume et al. (2014) studied a 96-h exposure compared to our study where prawns were exposed for 90 and 240 days. Nevertheless, results of these studies show clearly that the CLD is constantly accumulated in *M. rosenbergii*, especially after a long-term exposure.

20-Hydroxyecdysone concentration

The results revealed that 20-HE concentration was significantly influenced by the CLD exposure. Indeed, it was observed that 20-HE concentrations were from 1.3-fold to 1.9-fold lower in prawns exposed in the contaminated site compared to the control prawns, whatever the duration of exposure (Fig. 1). This decrease was significant in female *M. rosenbergii* exposed for 90 days and in males and females exposed for 240 days. Moreover, a significantly negative correlation between 20-HE concentrations and CLD concentrations measured in prawns was obtained ($p = 0.0019$, $r = -0.70$). A decrease of the 20-HE hormone in *M. rosenbergii* has already been observed in the presence of CLD in our previous study in laboratory conditions (Lafontaine et al. 2016). The same result obtained here supports the hypothesis that CLD could be an endocrine disruptor in invertebrates.

Chlordecone can be considered a steroid receptor ligand (Kavlock 1996), and various EDCs have been shown to act

Table 4 CLD bioconcentrations (mean ± S.D.), on a wet weight basis, measured in *Macrobrachium rosenbergii* sampled in the control (Pointe-Noire) and the contaminated site (Saint-Claude), as well as post-larvae, before the transfer in each farming pond (T0)

CLD concentration in prawns (ng g^{-1})		Pointe-Noire	Saint-Claude
T0		<LOD	<LOD
90 days	Males	9.09 ± 9.20	858.50 ± 92.09[a]
	Females	13.01 ± 3.79	646.51 ± 58.40[a]
240 days	Males	13.57 ± 4.04	767.03 ± 144.64[a]
	Females	9.04 ± 3.69	1247.39 ± 238.22[a]

[a] Significantly different values of CLD concentrations between control and contaminated samples. *LOD* limit of detection

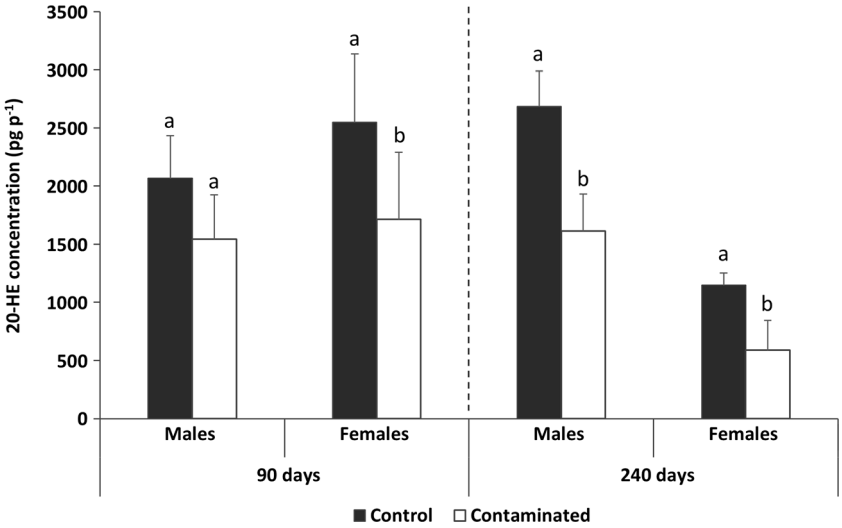

Fig. 1 20-HE concentrations (pg g^{-1}; mean ± S.D.) in hepatopancreas of male and female *Macrobrachium rosenbergii*, sampled after 90 and 240 days of exposure in control and chlordecone-contaminated sites. *Different letters above the bars* indicate significant differences for each duration of exposure (Tukey's HSD test, p values <0.05)

as ecdysteroid synthesis inhibitors or ecdysteroid receptors (EcR) antagonists in crustaceans (LeBlanc 2007), leading to a possible decrease of ecdysteroid concentrations (Forget-Leray et al. 2005; Rodriguez et al. 2007). Consequently, CLD could be responsible for the decrease in the 20-HE concentration by binding directly to EcR, as a consequence of a potential anti-ecdysteroidal activity (Zou and Fingerman 1999; Zou 2005). The secretion of 20-HE by the crustacean Y-organ is under negative control of the molt-inhibiting hormone (MIH), which is a hormone secreted by the X-organ/sinus gland complex (Rodriguez et al. 2007). The 20-HE hormone might have a feedback control on the production and/or release of MIH from the X-organ/sinus gland complex located in the eyestalk or on the ecdysteroid synthesis in Y-organs (Dell et al. 1999; Techa and Chung 2015). The presence of EcRs in eyestalks and Y-organs of decapod species has been demonstrated and supports these suggestions (Chang and Mykles 2011; Chung et al. 1998; Dell et al. 1999; Techa and Chung 2013). The binding of CLD on EcR in the X- or Y-organ could thus disrupt the 20-HE synthesis. However, as in other EDCs, CLD may interfere with the endocrine system by several mechanisms, at any step of the transduction pathway of the hormones (Hyne 2011; Rodriguez et al. 2007). Methyl farnesoate (MF), the secretion of which is inhibited by the mandibular organ-inhibiting hormone (MOIH) from the X-organ, stimulates the secretion of 20-HE (Rodriguez et al. 2007). The decrease of 20-HE concentration observed in exposed prawns could also be due to the disruption of the mandibular organ (through MOIH) following an interaction of CLD with the X-organ. These effects of CLD on the 20-HE concentration could therefore affect the organism growth and development in the long term but also the reproduction of prawns since the 20-HE is involved in the reproduction process (Hyne 2011; LeBlanc et al. 2000; Rodriguez et al. 2007; Snyder and Mulder 2001).

Expression of the vitellogenin gene

The results revealed that the expression of the Vg gene was significantly influenced by gender of prawns, as well as the interaction between gender, CLD concentration, and duration of exposure and the interaction between CLD concentration and duration of exposure (Table 3). The results showed that the expression of the Vg gene was not completely silent in males and that strong differences in Vg gene expression were observed between males and females ($p < 0.0001$). These results confirm the baseline level of Vg in male crustaceans as reported in the copepod *Tigriopus japonicus* (Lee et al. 2008) or in the amphipod *Gammarus fossarum* (Jubeaux et al. 2012). Moreover, no significant difference ($p > 0.05$) was detected between the Vg gene expression measured in CLD-exposed and control males after 90 days of exposure, although a trend of increase was observed after 240 days of exposure (Fig. 2a). Conversely, in females, a trend of decrease was observed after 90 days of exposure, while a significant increase ($p = 0.04$) of Vg gene expression was measured after 240 days of exposure, compared to respective controls (Fig. 2b). In addition, a significantly positive correlation ($p = 0.01$, $r = 0.70$) between Vg gene expression and the CLD concentrations measured in prawns was obtained, suggesting that a relationship exists between the CLD exposure and the modification of Vg gene expression in *M. rosenbergii*.

The increase of the Vg gene expression after a CLD exposure has been observed in some vertebrates, through the interaction with the estrogen receptor (Donohoe and Curtis 1996; Flouriot et al. 1996; Hammond et al. 1979). For example, an increase of the Vg synthesis in the juvenile trout *Oncorhynchus mykiss* fed during 33 weeks with CLD-contaminated food (0.4 mg kg^{-1} day^{-1}) was measured (Donohoe and Curtis 1996). However, Thornton et al. (2003) assumed the loss of the estrogen receptor in ecdysozoans, which invalidates the

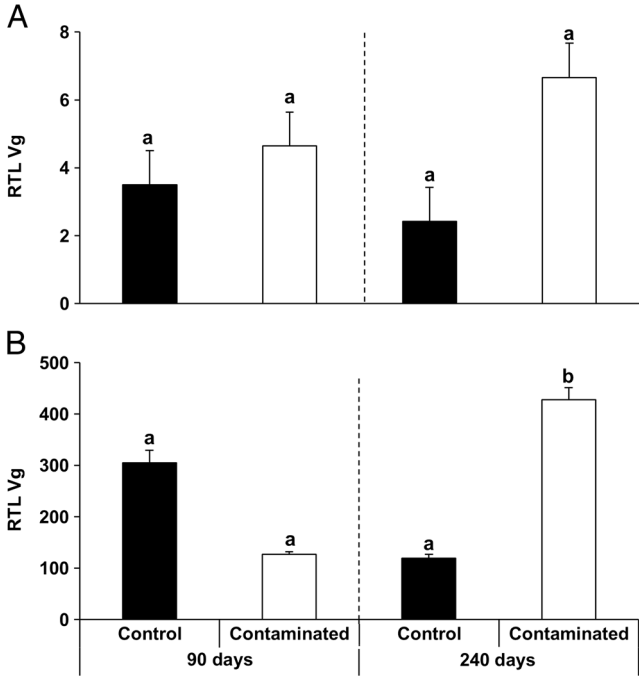

Fig. 2 Relative transcript levels (RTL) of the Vg gene in hepatopancreas of male (**a**) and female (**b**) *Macrobrachium rosenbergii*, sampled after 90 and 240 days of exposure in control and chlordecone-contaminated sites. Expression levels are relative to rRNA 18S and are the average of 10 replicates (means ± SE). *Different letters above the bars* indicate significant differences for each duration of exposure (Tukey's HSD test, p values <0.05)

hypothesis of an interaction between CLD and an estrogen receptor in *M. rosenbergii*. Nevertheless, previous investigations showed an increase of the Vg gene expression in crustaceans exposed to EDCs (e.g., xenoestrogen compounds) (Billinghurst et al. 2000; Ghekiere et al. 2006; Huang and Chen 2004; Huang et al. 2006; Oberdörster et al. 2000; Sanders et al. 2005). For example, in female shrimp *Neocaridina denticulata*, Vg gene expression was induced by exposure to chlordane at 0.001 and 0.01 μg L^{-1} (Huang et al. 2006), suggesting that chlordane may cause some reproductive impairments and alterations in endocrine functions.

As biological functions in crustaceans are hormonally dependent (Hyne 2011), our results could be explained by an interaction of the CLD with the endocrine system of *M. rosenbergii*. Shrivastava and Princy (2014) indicated that MIH inhibits the 20-HE synthesis in the Y-organ on the one hand and stimulates vitellogenesis in the hepatopancreas on the other hand. As all of the chemical insecticides, CLD is not species-selective with regard to targets of toxicity and it has neurotoxic properties since it interferes with the nervous system of the target organisms (Costa 2015). Therefore, CLD could affect the secretion of neurohormones in crustaceans by binding with key receptors such as steroid receptor (Kavlock 1996), or EcR, but no information is available regarding the potential interaction between CLD and EcR. However, CLD could interact with the X-organ of *M. rosenbergii*, through EcR, causing an increase of the synthesis and/or release of MIH, which would explain the decrease of the 20-HE concentration coupled with an increase of the Vg gene expression, observed here.

CLD exposure could also inhibit the gonad-inhibiting hormone (GIH) synthesis, resulting in an increase of the vitellogenin production. Another hypothesis could be that the increase of Vg expression would be due to an increase of the gonad-stimulating hormone (GSH) from the thoracic ganglion or the vitellogenin-stimulating ovarian hormone (VSOH) from the ovary, which seem to play a similar role as 17β-estradiol in egg-laying vertebrates (Hasegawa et al. 1993; Kusk and Wollenberger 2007). Finally, the last assumption is related to the low levels of 20-hydroxyecdyone (as observed here after CLD exposure) coupled with the secretion of GSH by the thoracic ganglion, which could initiate the production of vitellogenin (Hyne 2011). This assumption is supported by a negative, but not significant correlation between 20-HE concentration and Vg expression. The 20-HE/Vg relationship was previously investigated by Young et al. (1993) in *P. monodon*, who observed low 20-HE concentration when high Vg concentrations were measured.

Expression of vitellogenin receptor gene

The results revealed that the expression of the VgR gene was significantly influenced by gender and CLD exposure, as well as by the interaction between these two factors (Table 3). Moreover, the level of expression of the VgR gene was impacted by the interaction between gender, CLD exposure, and duration of exposure. In males (Fig. 3a) and females (Fig. 3b), the level of VgR expression was significantly increased in exposed prawns compared to respective controls, whatever the duration of exposure. These observations suggested that a relationship exists between the CLD exposure and the modification of VgR gene expression in both *M. rosenbergii* genders. Indeed, a strong significant positive correlation between VgR gene expression and the CLD concentrations in prawns was also observed ($p = 0.04$, $r = 0.64$) and supports this relationship. Moreover, the results obtained in males showed that, in addition to the Vg gene, male *M. rosenbergii* expressed also the gene of the vitellogenin receptor.

VgR is a receptor on the oocyte membrane having a high affinity for the Vg proteins and which mediates the endocytic process required for the internalization of Vg in oocytes by a receptor-mediated endocytosis (Raikhel and Dhadialla 1992; Sappington and Raikhel 1998; Tiu et al. 2008). The presence of this Vg-specific receptor on the oocyte membrane has been shown in invertebrates such as insect species (Raikhel and Dhadialla 1992; Sappington and Raikhel 1998) or shrimps *Penaeus monodon* (Tiu et al. 2008) and *M. rosenbergii* used in this study (Roth and Khalaila 2012). However, although VgR plays an important role in oocyte maturation through

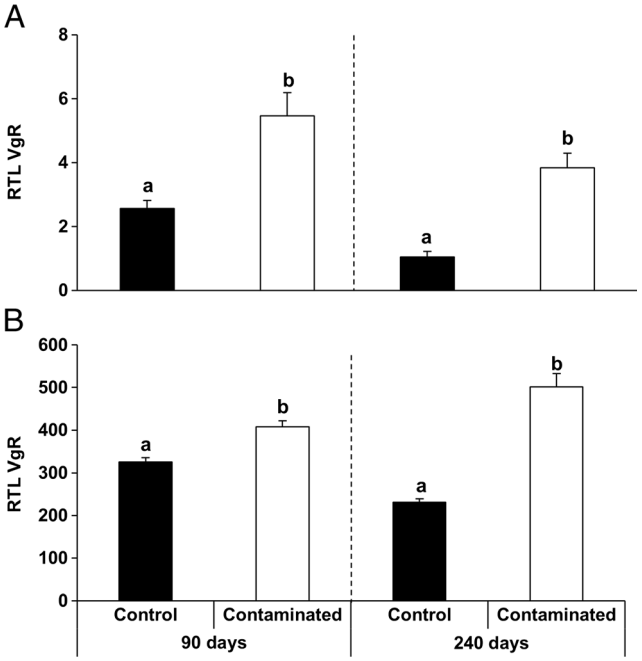

Fig. 3 Relative transcript levels (RTL) of the VgR gene in gonads of male (**a**) and female (**b**) *Macrobrachium rosenbergii* sampled after 90 and 240 days in control and chlordecone-contaminated sites. Expression levels are relative to rRNA 18S and are the average of 10 replicates (means ± SE). *Different letters above the bars* indicate significant differences for each duration of exposure (Tukey's HSD test, p values <0.05)

the Vg incorporation in the gonadal tissue (Tiu et al. 2008), only a few studies focused on VgR gene expression or VgR levels in crustaceans (Subramoniam 2011). Moreover, to our knowledge, no study has considered the modification of VgR gene expression in the presence of EDCs.

In the present study, CLD significantly increased the expression of the VgR gene in both genders, whatever the duration of exposure. So far, it is difficult to compare our results to previous studies because none dealt with the effects of EDCs on this receptor. However, the induction of VgR expression could be explained by the increase of the Vg expression as suggested by Tiu et al. (2008) in the shrimp *P. monodon*. In their study, the authors supposed that the VgR could be overexpressed in order to provide more receptors for increased amounts of Vg. This assumption is supported by a significant positive correlation ($p = 0.003$, $r = 0.88$) between Vg and VgR expressions. Otherwise, the induction of the VgR expression could be due to an effect of CLD similar to that of the MIH. As suggested above, CLD could be an agonist of MIH, resulting in both a decrease of 20-HE and an increase of Vg. The interaction between CLD and the X-organ could also inhibit the GIH synthesis, resulting in an increase of both Vg and VgR. In the same way, this increase of VgR expression could be the consequence of the synthesis of GSH by a direct action of CLD on the thoracic ganglion or indirectly for example through a disturbance of the serotonin. Indeed, in crustaceans, serotonin is identified as a neurotransmitter that stimulates release of some neurohormones such as crustacean hyperglycemic hormone (CHH) or MIH (Keller and Beyer 1968; Mattson and Spaziani 1985) and inhibits the release of GIH (Kulkarni and Glade 1991; Tinikul et al. 2008). Processes regulated by serotonin may become a target for environmentally relevant endocrine disruptors, such as insecticides because of their neurotoxic properties (Brooks et al. 2003; Henry et al. 2004; Mazurová et al. 2008). The assumption on the serotonin involvement in the Vg and VgR gene expressions is supported by the fact that Ruttanakorn et al. (2014) observed an increase of the Vg concentration in *M. rosenbergii*, after serotonin injection.

Our results suggest that CLD could act on endocrine pathways involved in the reproduction and molt processes. It could act through the X-organ, which is one of the main neuroendocrine organs in crustaceans, or through the Y-organ or thoracic ganglion, or several endocrine structures simultaneously.

The incorporation of Vg by VgR into the oocytes is essential to the reproduction process of the organism (e.g., survival and development of eggs) (Roth and Khalaila 2012). Although it was already observed in vertebrates that changes in Vg expression could cause disruptions of reproduction and population dynamics in the long term (Bosker et al. 2010; Kidd et al. 2007; Martín-Díaz et al. 2005), in invertebrates, this question is less advanced. However, it was observed in several crustacean species that a modification of the Vg gene expression causes reproduction impairments as well as growth disturbances (Hannas et al. 2011; Tokishita et al. 2006). Moreover, impairments in reproduction (i.e., infertility of females) have been related to a deficiency of VgR in *Drosophila melanogaster* females (Schonbaum et al. 1995). According to these previous studies and our results, it appears that CLD could disrupt the reproduction and/or development of the decapod crustacean *M. rosenbergii* through the modification of the molting hormone (20-HE) and Vg and VgR gene expression. However, due to the fact that this study was carried out in field conditions, confounding factors should be considered in further studies to incriminate the observed effects only to the CLD exposure.

Conclusion

The present study is the first to link the CLD effects on the 20-HE concentration and both Vg and VgR gene expressions in crustaceans. The results revealed that the expression of Vg and VgR genes in *M. rosenbergii* were modified following a CLD exposure and that CLD decreased the 20-HE concentration as previously observed in the laboratory experiment. Since Vg, VgR, and 20-HE are hormonally controlled, these observations suggest a potential disruption of the endocrine system of crustaceans after a CLD exposure. Consequently, CLD seems to be an EDC for *M. rosenbergii* and more generally for crustaceans. However, this

study only revealed a possible relation of putatively endocrine-disrupting effects of CLD in an environmentally relevant context, but did not focus on the underlying mechanisms. Further investigations are thus needed to explain how CLD acts on hormonal control and/or metabolic pathways, especially by focusing on the X/Y-organs, their receptors (e.g., ecdysteroid receptor), and their secreted hormones (e.g., MIH, GIH).

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