

2010

## Crustacean Endocrine Disruption Through a Pathway Involving Nuclear Receptors, Cyclic Nucleotides and Calcium Transporters

Laxminath Tumburu  
*Wright State University*

Follow this and additional works at: [https://corescholar.libraries.wright.edu/etd\\_all](https://corescholar.libraries.wright.edu/etd_all)



Part of the [Environmental Sciences Commons](#)

---

### Repository Citation

Tumburu, Laxminath, "Crustacean Endocrine Disruption Through a Pathway Involving Nuclear Receptors, Cyclic Nucleotides and Calcium Transporters" (2010). *Browse all Theses and Dissertations*. 1004.  
[https://corescholar.libraries.wright.edu/etd\\_all/1004](https://corescholar.libraries.wright.edu/etd_all/1004)

This Dissertation is brought to you for free and open access by the Theses and Dissertations at CORE Scholar. It has been accepted for inclusion in Browse all Theses and Dissertations by an authorized administrator of CORE Scholar. For more information, please contact [library-corescholar@wright.edu](mailto:library-corescholar@wright.edu).

CRUSTACEAN ENDOCRINE DISRUPTION THROUGH A PATHWAY  
INVOLVING NUCLEAR RECEPTORS, CYCLIC NUCLEOTIDES AND CALCIUM  
TRANSPORTERS

A dissertation submitted in partial fulfillment of the requirements for the degree of  
Doctor of Philosophy

By

LAXMINATH TUMBURU  
M.S., University of Charleston, SC, 2005

---

2010  
Wright State University

COPYRIGHT BY  
LAXMINATH TUMBURU  
2010

WRIGHT STATE UNIVERSITY  
SCHOOL OF GRADUATE STUDIES

August 6, 2010

I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Laxminath Tumburu ENTITLED Crustacean Endocrine Disruption through a Pathway involving Nuclear Receptors, Cyclic Nucleotides and Calcium Transporters BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

\_\_\_\_\_  
Michele G. Wheatly, Ph.D.  
Dissertation Director

\_\_\_\_\_  
Donald F. Cippolini, Ph.D.  
Director, Environmental  
Sciences Ph.D. Program

\_\_\_\_\_  
Andrew T. Hsu, Ph.D.  
Dean, School of Graduate  
Studies

Committee on  
Final Examination

\_\_\_\_\_  
Michele G. Wheatly, Ph.D.

\_\_\_\_\_  
Ronny Blust, Ph.D.

\_\_\_\_\_  
G. Allen Burton, Jr., Ph.D.

\_\_\_\_\_  
Christopher M. Gillen, Ph.D.

\_\_\_\_\_  
Courtney E.W. Sulentic, Ph.D.

## ABSTRACT

Tumburu, Laxminath. Ph.D., Environmental Sciences Ph.D. Program, Wright State University, 2010. Crustacean Endocrine Disruption through a Pathway involving Nuclear Receptors, Cyclic Nucleotides, and Calcium Transporters.

Endocrine disruption is a complex phenomenon in the sense that endocrine disrupting chemicals (EDCs) are known to act via multiple modes of action, but the mechanisms of actions are poorly understood. In crustaceans, calcium ( $\text{Ca}^{2+}$ ) apart from its role as a second messenger, is also a major constituent of the calcified exoskeleton which undergoes a periodic mineralization/demineralization process known as the molting cycle. Molt cycle is under the control of steroid hormones, ecdysteroids. EDCs disrupt this molting process via their interference with receptor-mediated ecdysteroid signaling. However, the hormonal regulation of  $\text{Ca}^{2+}$  flux in crustacean molting is poorly understood. Cyclic nucleotides –cyclic adenosine monophosphate (cAMP) and/or cyclic guanosine monophosphate (cGMP) –play an important role in ecdysteroidogenesis as well as in the regulation of  $\text{Ca}^{2+}$  transporters. These three components –nuclear receptors, cyclic nucleotides and  $\text{Ca}^{2+}$  transporters –are seemingly inter-connected, and can be affected by endocrine disruption. I investigated a mechanistic pathway involving these three components in the crustacean endocrine disruption in freshwater crayfish, *Procambarus clarkii*. I selected  $17\alpha$ -ethinyl-estradiol (EE2, a synthetic estrogen) and  $17\beta$ -estradiol (E2, a natural estrogen) as model EDCs. We determined the expression profiles of i) nuclear estrogen receptors (EcR: Ecdysone Receptor; RXR: Retinoid X Receptor), ii) cyclic nucleotides (cAMP and cGMP), and iii)  $\text{Ca}^{2+}$  transporters (SERCA: Sarco/Endoplasmic Reticulum  $\text{Ca}^{2+}$ -ATPase; CaM: Calmodulin; and PMCA: Plasma Membrane  $\text{Ca}^{2+}$ -ATPase) in the hepatopancreatic cells of *P. clarkii*. Finally, we

determined the chitinase (*Chi*) gene expression to substantiate our findings, as *Chi* expression represents the terminal events in ecdysteroid signaling. During the molt cycle of *P. clarkii*, all three components were differentially expressed. Significant upregulation of nuclear estrogen receptors, cyclic nucleotides, and Ca<sup>2+</sup> transporters was observed in the presence of environmentally relevant concentrations (100 ng/L and 500 ng/L) of EE2 and E2. *Chi* expression was significantly higher during postmolt stage, and when cray were fish exposed to E2 and EE2. Understanding the roles of these three components in a natural molt cycle will provide insights about pathways associated with ecdysteroid signaling. Determining the effects of EDCs on these components will aid in understanding the mechanisms behind the disruption of this signaling.

# TABLE OF CONTENTS

<b>I. INTRODUCTION AND PURPOSE.....</b>	<b>1</b>
A. ENDOCRINE DISRUPTION.....	1
B. ENDOCRINE DISRUPTION: HUMANS, VERTEBRATES AND NON-TARGET SPECIES .....	2
C. EVIDENCE OF ENDOCRINE DISRUPTION IN INVERTEBRATES: ECOLOGICAL RELEVANCE.....	2
D. ENDOCRINE DISRUPTION IN CRUSTACEANS .....	3
<i>D. 1. Crustacean endocrinology.....</i>	<i>4</i>
<i>D. 2. Endocrine disruption in crustaceans vis-à-vis molting.....</i>	<i>6</i>
E. CA <sup>2+</sup> AND ITS RELEVANCE TO CRUSTACEAN MOLTING.....	7
F. TRANSCELLULAR CA <sup>2+</sup> TRANSPORT.....	10
G. EFFECTS OF EDCs ON SERCA, PMCA AND CAM .....	11
H. ROLE OF CYCLIC NUCLEOTIDES IN ECDYSTEROID SIGNALING.....	15
I. ECDYSTEROID RECEPTORS.....	19
J. CHITINASE AS AN ECDYSTEROID-RESPONSIVE GENE.....	20
K. SIGNIFICANCE OF PROPOSED RESEARCH.....	21
L. SPECIFIC AIMS .....	22
<b>II. EXPERIMENTAL MODELS AND METHODS .....</b>	<b>25</b>
A. BIOLOGICAL MODELS .....	25
<i>A. 1. Crayfish as a model organism.....</i>	<i>25</i>
<i>A. 2. Hepatopancreas as a model epithelial tissue .....</i>	<i>26</i>
<i>A. 3. E2 and EE2 as model EDCs.....</i>	<i>26</i>
B. METHODS.....	28
<i>B. 1. Test species.....</i>	<i>28</i>
<i>B. 2. Partial cDNA cloning and sequencing of EcR, RXR, and Chitinase genes.....</i>	<i>28</i>
<i>B. 3. Molt cycle studies.....</i>	<i>31</i>
<i>B. 4. Exposure studies.....</i>	<i>31</i>
<i>B. 5. Quantitative Real Time-PCR(Q-RT-PCR).....</i>	<i>33</i>
<i>B. 6. Cyclic nucleotide assays.....</i>	<i>36</i>
<i>B. 7. E2 and EE2 sample analysis .....</i>	<i>37</i>
<b>III. RESULTS.....</b>	<b>41</b>
A. PARTIAL ECR, RXR, CHI DNA CLONING .....	41
B. MOLT CYCLE STUDIES .....	42
<i>B. 1. Expression of sex-steroid nuclear receptors.....</i>	<i>42</i>
<i>B. 2. Measurement of cyclic nucleotides.....</i>	<i>42</i>
<i>B. 3. Expression of calcium transporters.....</i>	<i>50</i>
C. EXPOSURE STUDIES .....	54
<i>C. 1. Hepatosomatic Index (HSI).....</i>	<i>54</i>
<i>C. 2. Expression of sex-steroid nuclear receptors .....</i>	<i>54</i>
<i>C. 3. Measurement of cyclic nucleotides.....</i>	<i>62</i>
<i>C. 4. Expression of calcium transporters.....</i>	<i>63</i>
D. CHITINASE GENE EXPRESSION.....	68
<i>D. 1. Molt cycle studies.....</i>	<i>68</i>
<i>D. 2. Exposure studies .....</i>	<i>74</i>
E. E2 AND EE2 SAMPLE ANALYSIS .....	79
<b>IV. DISCUSSION.....</b>	<b>82</b>
A. MOLT CYCLE STUDIES.....	82

<i>A. 1. Expression of nuclear receptors</i> .....	82
<i>A. 2. Measurement of cyclic nucleotides</i> .....	84
<i>A. 3. Expression of calcium transporters</i> .....	85
B. EXPOSURE STUDIES .....	86
<i>B. 1. Expression of sex-steroid nuclear receptors</i> .....	87
<i>B. 2. Measurement of cyclic nucleotides</i> .....	89
<i>B. 3. Expression of calcium transporters</i> .....	91
C. CHITINASE GENE EXPRESSION .....	93
<i>C.1. Molt cycle studies</i> .....	94
<i>C. 2. Exposure studies</i> .....	96
<b>V. CONCLUSION</b> .....	<b>99</b>
<b>REFERENCES</b> .....	<b>101</b>

## LIST OF FIGURES

<b>Figure 1.</b> i) Schematic representation of the location of the X-organ sinus gland complex and Y-organ in crustacean (shrimp) endocrine system. ii) A schematic diagram of the blue crab X-organ/Sinus gland complex and eyestalk neural ganglia; XO: X-organ, SG: Sinus gland, MT: Medulla terminalis, MI: Medulla interna, ME: Medulla externa, LG: Lamina ganglionaris. (Source: Lachaise et al., 1993, Watson et al., 2001). .....	5
<b>Figure 2.</b> Structure of crustacean ecdysteroids: (a) ecdysone, (b) 20-OH-ecdysone, (c) 3-dehydroxyecdysone (Source: Huberman 2000). .....	9
<b>Figure 3.</b> A working model for the apical-to-basolateral transcellular calcium transport and proposed subcellular storage in epithelial cells (gills, antennal gland and hepatopancreas) based on studies using isolated membrane vesicles. Pharmaceutical inhibitors are shown in parentheses. SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; CaBP, calcium-binding protein; CaM, calmodulin; Mit, mitochondria; Vac, vacuole; FW, freshwater; SW, seawater; and RyR, ryanodine receptor. (Source: Wheatly et al., 2002a).....	12
<b>Figure 4.</b> Steroid hormone formation and regulation in crustacean YO steroidogenic cells. HDL, high-density lipoprotein; ATP, adenosine triphosphate; cAMP, adenosine 3', 5'-cyclic monophosphate; C, cholesterol; MIH, molt-inhibiting hormone; R, receptor (source: Spaziani et al., 1989).....	16
<b>Figure 5.</b> Model for the regulatory role of cyclic nucleotides on crustacean ecdysteroidogenesis in Y-organ. ATP, Adenosine triphosphate; AC, adenylyl cyclase; Ca, calcium; cAMP, adenosine 3',5'-cyclic monophosphate; cGMP, guanosine 3',5'-cyclic monophosphate; CM, calmodulin; HDL, high-density lipoprotein; HDLR, HDL apoprotein receptor; MIH, molt-inhibiting hormone; MIHR, MIH receptor; PDE, phosphodiesterase; PKC, protein kinase C; PKCLR, PKC-linked receptor, PTK, protein tyrosine kinase (Spaziani et al., 1999). .....	18
<b>Figure 6.</b> 17 $\alpha$ -Ethinylestradiol engineered from endogenous hormone 17 $\beta$ -Estradiol..	30
<b>Figure 7.</b> Comparison of deduced partial amino acid sequence of crayfish hepatopancreatic EcR with those of other crustaceans. Shaded regions indicate conserved regions of the sequences .....	43
<b>Figure 8.</b> Comparison of deduced partial amino acid sequence of crayfish hepatopancreatic RXR with those of other crustaceans. Shaded regions indicate conserved regions of the sequences. ....	44
<b>Figure 9.</b> Comparison of deduced partial amino acid sequence of crayfish hepatopancreatic Chi with those of other crustaceans. Shaded regions indicate conserved regions of the sequences. ....	45
<b>Figure 10.</b> Q-RT-PCR assay for the expression of <i>Procambarus clarkii</i> PcEcR mRNA in hepatopancreas as a function of molting stage. The calibrator stage was intermolt. Values are represented as means $\pm$ SEM (n=3). Different letters above the error bars represent significant differences. Statistical analysis was performed using one-way ANOVA (p<0.05). .....	46
<b>Figure 11.</b> Q-RT-PCR assay for the expression of <i>Procambarus clarkii</i> PcRXR mRNA in hepatopancreas as a function of molting stage. The calibrator stage was intermolt.	

Values are represented as means±SEM (n=3). Different letters above the error bars represent significant differences. Statistical analysis was performed using one-way ANOVA (p<0.05). .....	47
<b>Figure 12.</b> Intracellular cAMP levels in hepatopancreas during a molt cycle of <i>Procambarus clarkii</i> . Results are expressed as mean±SEM (n=3). Different letters above the error bars represent significant differences. Statistical analysis was performed using one-way ANOVA (p<0.05). .....	48
<b>Figure 13.</b> Intracellular cGMP levels in hepatopancreas during a molt cycle of <i>Procambarus clarkii</i> . Results are expressed as mean±SEM (n=3). Different letters above the error bars represent significant differences. Statistical analysis was performed using one-way ANOVA (p<0.05). .....	49
<b>Figure 14.</b> Q-RT-PCR assay for the expression of <i>Procambarus clarkii</i> SERCA mRNA in hepatopancreas as a function of molting stage. The calibrator stage was intermolt. Values are represented as means±SEM (n=3). Different letters above the error bars represent significant differences. Statistical analysis was performed using one-way ANOVA (p<0.05). .....	51
<b>Figure 15.</b> Q-RT-PCR assay for the expression of <i>Procambarus clarkii</i> PMCA mRNA in hepatopancreas as a function of molting stage. The calibrator stage was intermolt. Values are represented as means±SEM (n=3). Different letters above the error bars represent significant differences. Statistical analysis was performed using one-way ANOVA (p<0.05). .....	52
<b>Figure 16.</b> Q-RT-PCR assay for the expression of <i>Procambarus clarkii</i> CaM mRNA in hepatopancreas as a function of molting stage. The calibrator stage was intermolt. Values are represented as means±SD (n=3) (Gao et al. 2009). .....	53
<b>Figure 17.</b> Hepatosomatic index (HSI; hepatopancreas weight [g]/body weight [g] * 100) of <i>Procambarus clarkii</i> following a 40 day exposure to nominal concentrations of 100 and 500 ng/L E2. No significant difference was observed between control and treatments (t-test, p>0.05) .....	55
<b>Figure 18.</b> Hepatosomatic index (HSI; hepatopancreas weight [g]/body weight [g] * 100) of <i>Procambarus clarkii</i> following a 40 day exposure to nominal concentrations of 100 and 500 ng/L EE2. No significant difference was observed between control and treatments (t-test, p>0.05) .....	56
<b>Figure 19.</b> Q-RT-PCR assay for the expression of crayfish <i>Procambarus clarkii</i> EcR mRNA in hepatopancreas exposed to nominal concentrations E2 for 40 days. Relative quantification (RQ) expressed as mean ±S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene, ΔCt) and finally calculated using ΔCt of control as calibrator. Different letters above the error bars represent significant differences from control. Statistical analysis was performed using one-way ANOVA (p<0.05). .....	58
<b>Figure 20.</b> Q-RT-PCR assay for the expression of crayfish <i>Procambarus clarkii</i> RXR mRNA in hepatopancreas exposed to nominal concentrations E2 for 40 days. Relative quantification (RQ) expressed as mean ±S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene, ΔCt) and finally calculated using ΔCt of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA (p<0.05). .....	59

- Figure 21.** Q-RT-PCR assay for the expression of crayfish *Procambarus clarkii* EcR mRNA in hepatopancreas exposed to nominal concentrations EE2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. No significant difference was observed between control and treatments (one-way ANOVA,  $p>0.05$ ) ..... 60
- Figure 22.** Q-RT-PCR assay for the expression of crayfish *Procambarus clarkii* RXR mRNA in hepatopancreas exposed to nominal concentrations EE2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ ). ..... 61
- Figure 23.** Intracellular cAMP levels in hepatopancreas of *Procambarus clarkii* exposed to nominal concentrations of 100 and 500 ng/L of E2. Results are expressed as mean $\pm$ SEM (n=3). Different letters above the error bars represent significant differences from control. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ )..... 64
- Figure 24.** Intracellular cGMP levels in hepatopancreas of *Procambarus clarkii* exposed to nominal concentrations of 100 and 500 ng/L of E2. Results are expressed as mean $\pm$ SEM (n=3). No significant difference was observed between the control and treatments (one-way ANOVA,  $p>0.05$ )..... 65
- Figure 25.** Intracellular cAMP levels in hepatopancreas of *Procambarus clarkii* exposed to nominal concentrations of 100 and 500 ng/L of EE2. Results are expressed as mean $\pm$ SEM (n=3). Different letters above the error bars represent significant differences control. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ )..... 66
- Figure 26.** Intracellular cGMP levels in hepatopancreas of *Procambarus clarkii* exposed to nominal concentrations of 100 and 500 ng/L of EE2. Results are expressed as mean $\pm$ SEM (n=3). No significant difference was observed between the control and treatments one-way ANOVA,  $p>0.05$ ). ..... 67
- Figure 27.** Q-RT-PCR assay for the expression of crayfish *Procambarus clarkii* SERCA mRNA in hepatopancreas exposed to nominal concentrations E2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ )..... 69
- Figure 28.** Q-RT-PCR assay for the expression of crayfish *Procambarus clarkii* PMCA mRNA in hepatopancreas exposed to nominal concentrations E2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator.

- Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p < 0.05$ ). ..... 70
- Figure 29.** Q-RT-PCR assay for the expression of crayfish *Procambarus clarkii* CaM mRNA in hepatopancreas exposed to nominal concentrations E2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p < 0.05$ ). ..... 71
- Figure 30.** Q-RT-PCR assay for the expression of crayfish *Procambarus clarkii* SERCA mRNA in hepatopancreas exposed to nominal concentrations EE2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p < 0.05$ ). ..... 72
- Figure 31.** Q-RT-PCR assay for the expression of crayfish *Procambarus clarkii* PMCA mRNA in hepatopancreas exposed to nominal concentrations EE2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. There was no significant difference between treatments and control (one-way ANOVA,  $p > 0.05$ ). ..... 73
- Figure 32.** Q-RT-PCR assay for the expression of crayfish *Procambarus clarkii* CaM mRNA in hepatopancreas exposed to nominal concentrations EE2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p < 0.05$ ). ..... 75
- Figure 33.** Q-RT-PCR assay for the expression of *Procambarus clarkii* Chi mRNA in hepatopancreas as a function of molting stage. The calibrator stage was intermolt. Values are represented as means  $\pm$ SEM (n=3). Different letters above the error bars represent significant differences. Statistical analysis was performed using one-way ANOVA ( $p < 0.05$ ). ..... 76
- Figure 34.** Q-RT-PCR assay for the expression of crayfish *Procambarus clarkii* Chi mRNA in hepatopancreas exposed to nominal concentrations E2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p < 0.05$ ). ..... 77
- Figure 35.** Q-RT-PCR assay for the expression of crayfish *Procambarus clarkii* Chi mRNA in hepatopancreas exposed to nominal concentrations EE2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M (n=3); it was performed by

normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta Ct$ ) and finally calculated using  $\Delta Ct$  of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p < 0.05$ ). ..... 78

## LIST OF TABLES

<b>Table 1.</b> Sequences of degenerate primers and annealing temperatures used for partial cDNA cloning of PcEcR, PcRXR, and PcChi. ....	34
<b>Table 2.</b> Primers and Optimal Conditions for Quantitative Reverse Transcriptase Polymerase Chain Reaction (Q-RT-PCR) .....	38
<b>Table 3.</b> Nominal, measured and effective concentrations of E2 in crayfish-held glass bowls during the 40-day exposure experiment. Concentrations (ng/L) were presented as mean values (n=3) with standard error. Percentage differences relative to nominal concentration are presented in brackets.....	80
<b>Table 4.</b> Nominal, measured and effective concentrations of EE2 in crayfish-held glass bowls during the 40-day exposure experiment. Concentrations (ng/L) were presented as mean values (n=3) with standard error. Percentage differences relative to nominal concentration are presented in brackets.....	81
<b>Table 5.</b> Summary of findings of the specific aims of this study. “↑” indicates significant increase; “↓” significant decrease; “↔” indicates control or no effect; “/” indicates non-significant increase. ....	98

## ACKNOWLEDGEMENTS

Although this dissertation is my individual effort to look into some unanswered questions associated with crustacean endocrine disruption pathways, it would not have been possible without the assistance and help of some amazing people I came across during the last five years here at the Wright State University. These include friends who instilled sanity and balance to my life in the midst of the challenges that I encountered while pursuing this research, and my dissertation committee members who displayed tremendous patience and friendliness with their expert advice and suggestions.

First and foremost, I would like to express my deepest gratitude and respect to my dissertation director, Dr. Michele Wheatly for her guidance, inspiration, and the opportunity. I am extremely fortunate to know and watch her, as a leader dealing with everyday administrative challenges, as a principle investigator guiding our laboratory research operations, and most importantly, as a person who deeply cares about her students, friends and family. I could not have wished for a better role model.

My sincere thanks to all my committee members: Drs. Allen Burton, Ronny Blust, Christopher Gillen, and Courtney Sulentic for their time and support. Despite his hectic schedule, Dr. Burton who is also the founder of ES Ph.D program has been very gracious to provide me with timely help whenever I needed. I thank Dr. Blust who is an external committee member from the University of Antwerp (Belgium), and a reknowned expert in ecophysiology, biochemistry and toxicology, for his time and invaluable suggestions with various aspects of this research. Special thanks to Dr. Gillen and Dr. Sulentic for always being there for me, and helping me with various aspects, ranging from my dissertation in specific to my career in general.

The most important part of my dissertation period is the time I spent in my laboratory with my colleagues and friends. I will never forget the good times I had with my lab crew: Dr. Yongping Gao (Ping), Zeenat Jamal, and Yang Gao. I learnt a lot from Ping, especially how to balance your life with family and work. I do not need to say how much I enjoyed knowing Zeenat and Yang; they have become my life-long friends. The long coffee hours and weekend get-togethers I had with Zeenat and Yang will remain forever as one the best moments of my life. I will miss their company, but hope to keep in touch.

I thank Dr. Don Cipollini, director of ES Ph.D program, for helping with the support for the Summer quarter, and also for his time in providing me with suggestions with regard to my post doctoral career prospects. Thanks to Ms. Cathy Kempf, coordinator of the program, for helping me with the administrative paperwork.

I thank all the faculty and staff of the biological sciences department. Thanks to Dr. David Goldstein, Gretchen Baumle, Jennifer Papadakis and all others at the biology office. Special thanks to James (Jim) Frisbie (Goldstein lab); I will never forget our coffee and lunch conversations. Good times!

Last but not the least, I thank all my friends, who although are not part of or directly related to my academic life, but were part of my social circle, making my life much more lively and interesting! Thank you!!

## **DEDICATION**

To Amma, Baba, Lallu and Bujji

# I. INTRODUCTION AND PURPOSE

## ***A. Endocrine disruption***

Disruption of endocrine mediated processes such as reproduction and development by environmental pollutants, (through anthropogenic and natural), occurs via the disruption of endocrine homeostasis, through mimicking or blocking the action of endogenous hormones (Colborn et al., 1993). These pollutants are classified as endocrine disrupting chemicals (EDCs). EDCs have been defined in broadly different ways. According to Kavlock et al. (1996) they are defined as “exogenous agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes”. The European Community (1997) defined an EDC as “an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function”. According to the U.S Environmental Protection Agency (U.S. EPA 1998), an endocrine disruptor is defined as “an exogenous chemical substance or mixture that alters the structure or function(s) of an endocrine system and causes adverse effects at the level of the organism, its progeny, populations, or subpopulations of organisms, based on scientific principles, data, weight-of-evidence, and the precautionary principle”. Given these broad definitions, it is obvious that the EDC-related effects occur through various complex mechanisms. These “exogenous chemical agents” mainly originate from municipal, industrial and agricultural sources, some of which are designed specifically to disrupt the normal endocrine function

(e.g. pesticides and oral contraceptives), but most of which produce inadvertent effects in non-target species as a byproduct of other functions (Clotfelter et al., 2004).

### ***B. Endocrine disruption: Humans, vertebrates and non-target species***

Over the last 50 years, substantial evidence has been mounting suggesting the role of the EDCs on the reproductive and developmental alterations in non-target species such as fish, other wildlife species, and even humans (Colborn et al., 1993). Although there is a high degree of uncertainty about the effects of EDCs on human populations (Safe 1995, vomSaal 1995), a mounting body of research implicates EDCs for their role in adversely affecting wildlife, both at the individual and population levels, caused by disruption of one or more endocrine systems (Ankley and Giesy 1998). Examples of these effects include: measureable amounts of vitellogenin in male fish; male-specific gonadopodia in female mosquitofish; delayed sexual maturation, reduced gonadal growth, and altered steroidogenic capacity in other fish species (white sucker); morphologic abnormalities and decreased reproduction in alligator populations in Lake Apopka (Florida); imposex (masculinization of female rockshell) and decreased reproductive success in some marine gastropod species (Ankley et al., 1998). The above-mentioned EDC-related effects clearly suggest their disruptive role in reproductive and developmental systems.

### ***C. Evidence of endocrine disruption in invertebrates: Ecological relevance***

Vertebrates and invertebrates possess functionally dissimilar growth, development and reproduction systems (e.g. different larval stages and associated metamorphosis in invertebrates, which are absent in vertebrates) and the associated endocrine systems

(deFur et al., 1999; Oetken et al., 2004). However, hormonal systems in invertebrates are not well understood, and therefore, endocrine disruption in invertebrates has received far less attention, which has favored vertebrates (fish in particular) as systematic groups for ecotoxicological research (Oetken et al., 2004). However, invertebrates constitute more than 95% of all known species in the animal kingdom, and are a significant part of global biodiversity, many of which are vital to the structure and function of an ecosystem (Oetken et al., 2004). Despite the fact that the invertebrates were not given enough attention, they provide some of the well-documented examples for the effects associated with endocrine disruption, although, most of these effects were documented at the organismic level in controlled laboratory bioassays. Initially, the laboratory studies for endocrine disruption were dominated by the documentation of the effects of an anti-fouling biocide Tributyltin (TBT) in mollusks and insect growth regulators (IGRs). Imposex (TBT: mollusks), and hyperrecdysionism as well as delaying post embryonic, nymphal-adult intermediates (IGRs: insects) are the most notable effects observed in these studies (Oetken et al., 2004). Other notable representative lab observations documenting the EDC-induced effects in invertebrates include: induction of spawning behavior in annelids (Beckmann et al., 1995), alterations in fecundity (Woin and Bronmark 1992), stimulation of spawning and inhibition of gonadal development (Kluytmans et al., 1988) in mollusks.

#### ***D. Endocrine disruption in crustaceans***

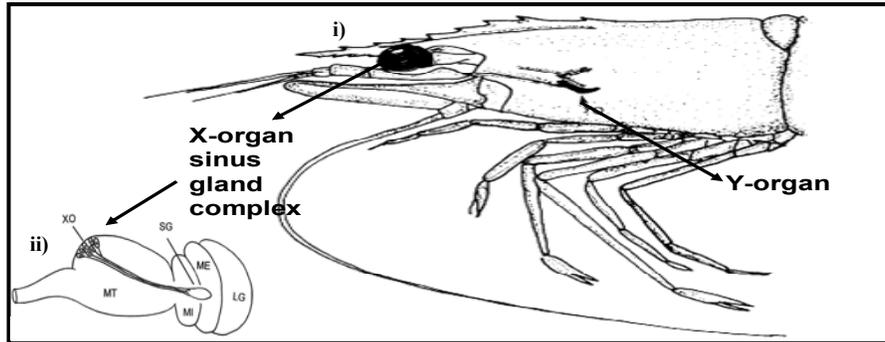
Crustaceans are one of the most ubiquitous groups among invertebrates, basically inhabiting every type of aquatic habitat (Rodriguez et al., 2007). In order to better understand the ecological effects of vertebrate EDCs, research efforts have gained

momentum on testing these chemicals on crustaceans (Hutchinson et al., 2000; Hutchinson 2002).

## **D. 1. Crustacean endocrinology**

In a nutshell, the crustacean endocrine axis is comprised of two neuroendocrine glands: i) the X-organ-sinus gland complex, and ii) the Y-organ (YO) (Figure. 1). The X-organ is part of the medulla terminalis in the eyestalk, and the sinus gland lies between the medulla externa and medulla interna in the eyestalks (Figure. 1, Rodriguez et al., 2007); the X-organ–sinus gland complex is located in homologous structures of the central nervous system in the head. The main hormones secreted by the sinus gland are the following: Molt inhibiting hormone (MIH), gonad inhibiting hormone (GIH), mandibular organ inhibiting hormone (MOIH), crustacean hyperglycemic hormone (CHH), several hormones that control pigment migration and neurodepressing hormone (NDH). While some hormones (MIH, GIH, and MOIH) have another endocrine gland as their target, the others have somatic tissues as targets. MIH, GIH, MOIH and CHH belong to a single family of peptides, exhibiting some cross reactivity (Keller 1992; Webster 1998; Chan et al., 2003).

The YO, also called the molting gland or ecdysal gland, consists of paired organs of ectodermal origin in the head region, and is homologous to the prothoracic organs of



**Figure 1.** i) Schematic representation of the location of the X-organ sinus gland complex and Y-organ in crustacean (shrimp) endocrine system. ii) A schematic diagram of the blue crab X-organ/Sinus gland complex and eyestalk neural ganglia; XO: X-organ, SG: Sinus gland, MT: Medulla terminalis, MI: Medulla interna, ME: Medulla externa, LG: Lamina ganglionaris. (Source: Lachaise et al., 1993, Watson et al., 2001).

insects. The molting hormone secreted by YO mediates several aspects of crustacean growth and reproduction (Quackenbush 1986, Chang et al., 1993). The specific molting hormones it produces and secretes are ecdysone and 3-hydroxyecdysone, which are converted to 20-hydroxyecdysone, the biologically active ecdysteroid (Figure. 2). MIH, which originates from the X-organ-sinus gland complex, exerts an inhibitory effect on the YO by raising the intracellular level of the second messenger cAMP; the decrease of circulating MIH at the beginning of premolt triggers the secretion of ecdysone by the YO. Thus, the synthesis and/or secretion of ecdysone by the YO appear to be inhibited by the MIH (Mattson and Spaziani 1986; Chang et al., 1993).

## **D. 2. Endocrine disruption in crustaceans vis-à-vis molting**

Several *in vivo* studies have focused efforts on understanding the effect of EDCs on crustacean molting. For example, molting was inhibited in *Daphnia magna* when they were exposed to diethylstilbestrol (DES), endosulfan, Polychlorinated biphenyl (PCB), Aroclor 1242, and diethyl phthalate (Zou and Fingerman 1997a; Zou and Fingerman 1997b); the above-mentioned EDCs also inhibited the chitinase activity in the epidermis of fiddler crab, *Uca pugilator* (Zou and Fingerman 1999a; Zou and Fingerman 1999b). Anderson et al. (2001) reported inhibition of molting in a copepod, *Acartia tonsa* when exposed to EDCs such as 17 $\alpha$ -ethinylestradiol, p-octylphenol, tamoxifen and flutamide; the authors hypothesized that these EDCs interact with the ecdysone receptor, raising the ecdysone threshold required to trigger molting. Snyder and Mulder (2001) reported delay in the onset of molting in female lobster, *Homarus americanus*, when exposed to heptachlor; the delay is correlated with reduced levels of circulating ecdysteroids. Heavy

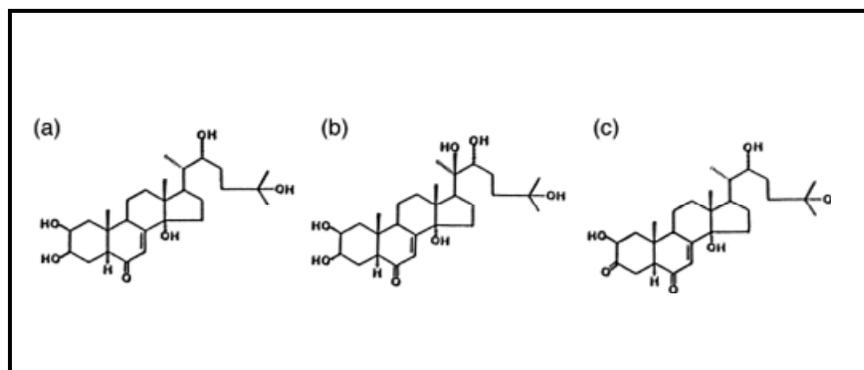
metals such as cadmium were reportedly taken up by several crustaceans through calcium channels or specific calcium transporting proteins (Bondgaard and Bjerregaard 2005; Norum et al., 2005). Although cadmium inhibited the molting in the eye-stalk ablated premolt adult crabs *Chasmagnathus granulatus*, the possibility of inhibition of calcium transport, and hence interference with the calcium resorption of the exoskeleton during the premolt, was ruled out (Norum et al., 2005). The study suggested that cadmium inhibited molting by inhibiting the ecdysone secretion by YO. The study however did not look into the role of various second messengers such as of cAMP (cyclic adenosine monophosphate), cGMP (cyclic guanosine monophosphate), and inositol-1, 4, 5 triphosphate (IP<sub>3</sub>) in the activation of ecdysone synthesis; the role of these second messengers vis-à-vis endocrine disruption and calcium dyshomeostasis will be discussed later. Although the role of second messengers in ecdysteroidogenesis has been established, their role in the EDC-induced endocrine disruption of molting is yet to be investigated; most studies to date report the EDC-induced changes in crustaceans by documenting the changes in ecdysteroid titers.

### ***E. Ca<sup>2+</sup> and its relevance to crustacean molting***

Crustacean growth and development involves post-embryonic periodic demineralization, replacement, and remineralization of the cuticle. This physiological event is called the molting cycle. It is unique, in that it is an endocrine-mediated process, and has gained importance in the field of crustacean biology and endocrinology, especially in the context of the associated effects of calcification/decalcification on Ca<sup>2+</sup> and CO<sub>2</sub> homeostasis (Cameron and Wood 1985; Wheatly et al., 2001). The functions of Ca<sup>2+</sup> range from intracellular signaling (subcellular: second messenger of hormonal

action) to the formation of mineralized tissues such as cuticle (organismic scale). At the organismic level, the majority (80%) of the whole body  $\text{Ca}^{2+}$  is stored in the cuticle, the remaining 20% is stored in the extra- and intra- cellular fluid pools (Wheatly et al., 2002a). At the cellular level, this state is imitated, in that 20%  $\text{Ca}^{2+}$  is stored in the cytosol at low concentration ( $10^{-7}$  M), and the remaining 80% is stored at higher concentration ( $10^{-3}$  M) in membrane-bound compartments that can rapidly exchange their  $\text{Ca}^{2+}$ , particularly the intracellular store, sarcoplasmic reticulum.

With their ubiquitous inhabitation of a variety of environments, and unique ability to withstand various physiological assaults on their osmoregulatory capabilities, crustaceans have a common goal of regulating  $\text{Ca}^{2+}$  concentrations in their blood and tissues (Ahearn et al., 2004). One such type of prominent physiological assault, which is a quintessential one for crustacean growth and development, is the molt cycle, where  $\text{Ca}^{2+}$  plays an intimate role in the remineralization of cuticle (Ahearn et al., 2004). Epithelial cells (hepatopancreas, gills, integument, and antennal gland) play an important role in regulating the  $\text{Ca}^{2+}$  homeostasis during the molt cycle. As 80% of the organismic  $\text{Ca}^{2+}$  resides in the exoskeleton, its periodic ecdysal replacement puts the animal at risk of facing massive tissular  $\text{Ca}^{2+}$  fluctuations in the internal concentration of this extremely important element (Ahearn et al., 2004). To minimize this risk, the solubilized  $\text{Ca}^{2+}$  from integumentary storage depots is transported through epithelial cells, without significantly altering the cytosolic  $\text{Ca}^{2+}$  concentration, and temporarily sequestered in labile cell and tissue locations where it can be quickly transported back for the remineralization of a new and uncalcified integument.



**Figure 2.** Structure of crustacean ecdysteroids: (a) ecdysone, (b) 20-OH-ecdysone, (c) 3-dehydroxyecdysone (Source: Huberman 2000).

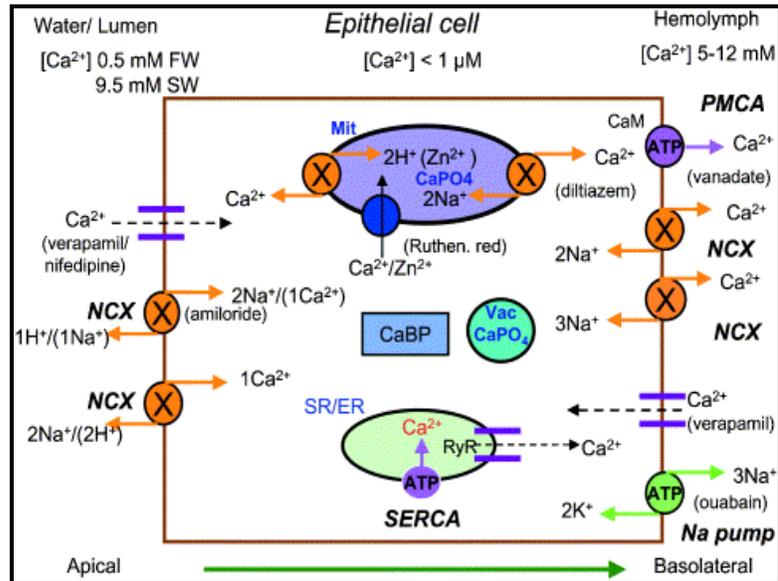
## ***F. Transcellular Ca<sup>2+</sup> transport***

Our laboratory's current working model for transcellular Ca<sup>2+</sup> transport in freshwater crustaceans (Figure. 3, Wheatly et al., 2002a,b) suggests that in Ca<sup>2+</sup>-transporting epithelia, Ca<sup>2+</sup> is passively transported across the apical membrane by means of low-affinity and high-capacity cation exchangers (sodium-calcium exchanger: NCX), and verapamil-sensitive Ca<sup>2+</sup>-channels. On the contrary, the Ca<sup>2+</sup> transfer across the basolateral membrane involves the combination of both passive and active transport systems. Active systems include a high-affinity and low-capacity CaM-dependant PMCA and NCX of variable stoichiometry, and passive systems include verapamil-sensitive Ca<sup>2+</sup>-channels similar to the ones found on the apical membrane. These transport systems are responsible for the vectorial transfer of Ca<sup>2+</sup> across the epithelial cell, both during normal intermolt osmoregulatory activity, when Ca<sup>2+</sup> requirements are minimal, and during the pre- and post-molt periods which involve massive Ca<sup>2+</sup> fluxes. The model (Figure. 3) also suggests the possible roles of organelles such as mitochondria, lysosomes, and endoplasmic reticulum (ER). The ER in epithelial cells, which acts as a Ca<sup>2+</sup> depot during the molt cycle, possesses an active calcium ATPase (SERCA) (Zhang et al., 2000; Chen et al., 2002; Hagedorn and Ziegler 2002; Hagedorn et al., 2003). SERCA facilitates Ca<sup>2+</sup> uptake from cytoplasm into the ER. Although their roles in Ca<sup>2+</sup> buffering (i.e. Ca<sup>2+</sup> storage and release) in crustacean molt cycle are well-documented, their relative roles in Ca<sup>2+</sup> translocation remain uncertain (Ahearn et al., 2004). The scope of this study, with regard to Ca<sup>2+</sup> transport, is limited to activities of SERCA and CaM-PMCA in their respective roles of Ca<sup>2+</sup> buffering and translocation in epithelial cells (hepatopancreas) during endocrine disruption. The mass transport of Ca<sup>2+</sup> from one

CaCO<sub>3</sub> storage site (e.g. exoskeleton) to another (e.g. gastrolith) across the transporting epithelial cells, is facilitated without significantly changing the intracellular Ca<sup>2+</sup> concentration (Wheatly et al., 2002a). Hence, the Ca<sup>2+</sup> transport across epithelial cells as a result of the molting cycle remains an excellent model system in understanding subcellular Ca<sup>2+</sup> dynamics (Wheatly et al., 2002a). It would be relevant to document the impact of E2 and EE2 (model EDCs of this study) on SERCA, CaM and PMCA (model Ca<sup>2+</sup> transporters of this study) in epithelial cells (hepatopancreas).

### ***G. Effects of EDCs on SERCA, PMCA and CaM***

The sarcoplasmic reticulum (SR, in muscular cells) or endoplasmic reticulum (ER in non-muscular cells) are well established organelles of calcium sequestration owing to their capacity and rapidity of Ca<sup>2+</sup> exchange with cytosol (Alvarez et al., 1999). Existing data suggests that the Ca<sup>2+</sup> uptake rates into crustacean SR are substantially higher than vertebrate SR (Van der Kloot 1969; Baskin 1971; Deamer 1973). Ca<sup>2+</sup> from the SR is released via the inositol 1,4,5-triphosphate receptor (IP<sub>3</sub>R) and the ryanodine receptor (RyR) Ca<sup>2+</sup> channel (McCormack and Cobbold 1991). It has recently been suggested that depletion of intracellular Ca<sup>2+</sup> stores or disturbance of the functioning of the ER is toxic to cells (Paschen and Frandsen 2001; Nguyen et al., 2002). Based on studies conducted so far, we can come to a general conclusion that EDCs have the potential to inhibit SERCA, i.e. preventing it from functioning in Ca<sup>2+</sup> uptake. For example, Kang et al. (2004) in their study have shown that catecholaminergic cell-line, CATH.a.cells, when exposed to a commercial PCB mixture Aroclor 1254, resulted in elevation of intracellular Ca<sup>2+</sup>.



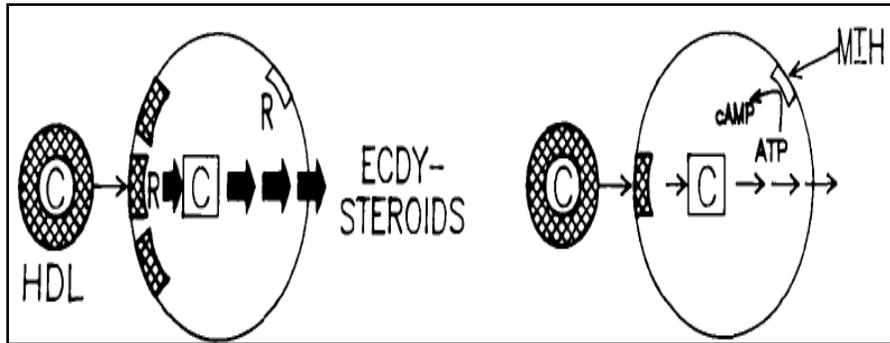
**Figure 3.** A working model for the apical-to-basolateral transcellular calcium transport and proposed subcellular storage in epithelial cells (gills, antennal gland and hepatopancreas) based on studies using isolated membrane vesicles. Pharmaceutical inhibitors are shown in parentheses. SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; CaBP, calcium-binding protein; CaM, calmodulin; Mit, mitochondria; Vac, vacuole; FW, freshwater; SW, seawater; and RyR, ryanodine receptor. (Source: Wheatly et al., 2002a)

This elevation in intracellular  $\text{Ca}^{2+}$  was attributed to release of ER  $\text{Ca}^{2+}$  via  $\text{IP}_3\text{R}$  and  $\text{RyR}$  channels. The activation of  $\text{IP}_3\text{R}$  and  $\text{RyR}$ , and the subsequent depletion of ER  $\text{Ca}^{2+}$  triggered various destructive processes leading to cell death. The elevation of intracellular  $\text{Ca}^{2+}$  may not solely be due to ER  $\text{Ca}^{2+}$  depletion due to  $\text{IP}_3\text{R}/\text{RyR}$  mediated  $\text{Ca}^{2+}$  release, but may also be due to the reduction in the rate of  $\text{Ca}^{2+}$  uptake by  $\text{SERCA}$ . Ogunbayo and Michelangeli (2007) in their study on rat cerebellar microsomes have shown that a commonly used flame retardant tetrabromobisphenol A (TBBPA) directly binds to the  $\text{SERCA}$ , thus reducing the affinity of the  $\text{SERCA}$  for  $\text{Ca}^{2+}$  binding. This resulted in the disruption of  $\text{Ca}^{2+}$  homeostasis within cells, and subsequent cytotoxicity. Earlier Inglefield and Shafer (2000) also reported the  $\text{IP}_3\text{R}$  mediated  $\text{Ca}^{2+}$  release from ER of developing rat cortical cells exposed to PCBs. This resulted in the initial increase in the intracellular  $\text{Ca}^{2+}$  concentration. However, they have also reported this  $\text{IP}_3\text{R}$  mediated  $\text{Ca}^{2+}$  release was followed by  $\text{Ca}^{2+}$  influx through store-operated  $\text{Ca}^{2+}$  channels (SOCs) and L-type voltage-gated calcium channels (L-Type VGCCs). This suggests a physical and functional coupling of  $\text{SERCA}$  pumps and the  $\text{Ca}^{2+}$  channels situated on the plasma membrane. Also, Khan et al. (2003) reported inhibition of  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release from ( $\text{IP}_3$ )-sensitive  $\text{Ca}^{2+}$  channels on porcine cerebellum and rat testicular microsomes, when exposed to alkylphenols. A number of factors could contribute to these contradictions, including different cell types used, maturity of cells at the time of testing, or different concentrations of the test chemical. Given the fact that crustacean SR/ER has greater capacity for  $\text{Ca}^{2+}$  uptake/storage, EDCs may have significant effect on the functionality of  $\text{SERCA}$  pumps, and hence the overall intracellular  $\text{Ca}^{2+}$  homeostasis.

PMCA is the basolateral plasma membrane calcium ATPase that moves  $\text{Ca}^{2+}$  against its electrochemical gradient from the cytosol into the hemolymph using hydrolysis of ATP (Gao and Wheatly 2004). The property of “higher-affinity and lower-capacity” for  $\text{Ca}^{2+}$  suggests that PMCA is a major  $\text{Ca}^{2+}$  efflux carrier, and fine tunes  $\text{Ca}^{2+}$  at lower intracellular concentrations. It is regulated by the  $\text{Ca}^{2+}$  signaling protein CaM which stimulates PMCA activity by binding to a C-terminal regulatory region (Gao and Wheatly 2004). Although there has been no substantial scientific evidence about the effect of an EDC on the PMCA functionality, it can be hypothesized that in the presence of EDCs, PMCA is activated, resulting in increased  $\text{Ca}^{2+}$  efflux across the basolateral membrane (cytosol to hemolymph/blood). Pottorf II and Thayer (2002) in their study on the rat dorsal root ganglion have reported that the most likely mechanism for the  $\text{Ca}^{2+}$ -induced increase in PMCA activity is a slow dissociation of calmodulin from the pump. Szemraj et al (2003) in their study on excitable (rat cortical synaptosomes) and non-excitable (human erythrocytes) tissues have shown that E2 can “non-genomically” stimulate the PMCA activity. Dick et al (2003) have observed that E2 increased PMCA activity in distal tubule kidney cell lines. Zylinska et al (2009) confirmed the findings by Szemraj et al (2003), and concluded that E2 and other steroids increased the PMCA activity, and this response depended on the cell type, PMCA isoforms expression profile, CaM presence, and the steroids structure. He et al (2004) in their study on fenvalerate (a synthetic pyrethroid, an EDC) exposure of human luteinizing-granulosa cells have observed a slow increase in intracellular  $\text{Ca}^{2+}$  and CaM. Thus the increase in intracellular  $\text{Ca}^{2+}$  and CaM may stimulate PMCA activity (i.e. increase in  $\text{Ca}^{2+}$  efflux).

## ***H. Role of cyclic nucleotides in ecdysteroid signaling***

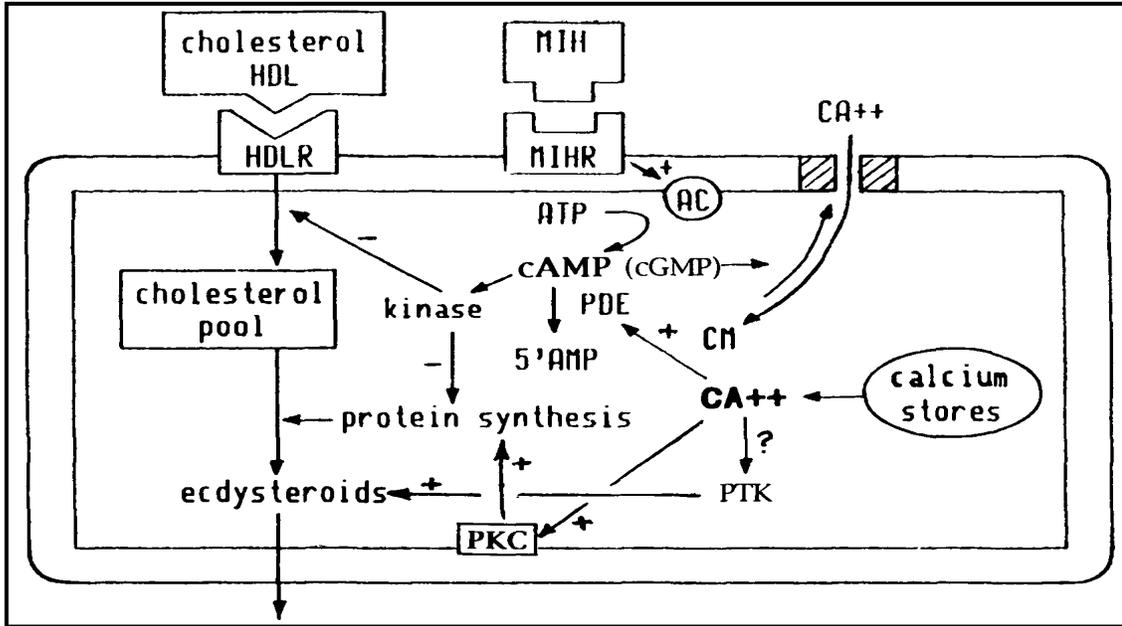
Cell signaling pathways are actively involved in the regulation of ecdysteroid synthesis (Spaziani et al., 1999; Han et al., 2006). Spaziani et al. (1999) observed that the rise in the cAMP (or cGMP in some other crustaceans) mediates in MIH-induced suppression of ecdysteroid synthesis (Figure. 4). The study also found that cAMP inhibits the receptor-mediated uptake of cholesterol (the obligate ecdysteroid precursor), by decreasing the number of receptor sites for the lipoprotein carrier of cholesterol. Also the same study observed the role of cellular free  $\text{Ca}^{2+}$ , which evidently stimulates ecdysteroid synthesis, and in turn antagonizes MIH action; this mechanism involved lowering of cAMP levels by enhancing phosphodiesterase (PDE) activity via CaM (Figure. 5). The study also notes that the  $\text{Ca}^{2+}$  dependant protein kinase (PKC) activity, and the subsequent ecdysteroid production is directly stimulated by  $\text{Ca}^{2+}$ . Nakatsuji et al. (2006) observed an increase in cyclic nucleotide phosphodiesterase (PDE) activity during the premolt stage, thereby antagonizing the inhibitory effect of MIH on ecdysteroid production; PDE is  $\text{Ca}^{2+}$ /CaM dependent, and regulates cAMP (cGMP) levels. Thus, ecdysteroid synthesis involves at least two signaling pathways: i) the MIH/cAMP (or cGMP) pathway, and ii) the  $\text{Ca}^{2+}$ /PKC pathway. However, it should be noted that most of these observations are from YO epithelial cells. It is also suggested that the  $\text{Ca}^{2+}$  clearance via SERCA and PMCA is modulated by cAMP; cAMP potentiates these  $\text{Ca}^{2+}$  pumps by PKA-mediated phosphorylation. For example, it was documented that cAMP potentiates SERCA in the cardiac cells by PKA-mediated phosphorylation of phospholamban protein (Brittsan and Kranias 2000).



**Figure 4.** Steroid hormone formation and regulation in crustacean YO steroidogenic cells. HDL, high-density lipoprotein; ATP, adenosine triphosphate; cAMP, adenosine 3', 5'-cyclic monophosphate; C, cholesterol; MIH, molt-inhibiting hormone; R, receptor (source: Spaziani et al., 1989).

However, this is specific to SERCA2 isoform, and has not yet been confirmed in crustacean cells. Also, it was observed that cAMP potentiates PMCA activity via a  $\text{Ca}^{2+}$ -dependant PKA-mediated phosphorylation; the PKA-phosphorylation increases the affinity of CaM binding to PMCA which increases the  $\text{Ca}^{2+}$  transport activity of the pump (Bruce et al. 2002). PKA-mediated regulation of PMCA may also help in the spatial shaping of cytosolic  $\text{Ca}^{2+}$  signals, which may play a key role in  $\text{Ca}^{2+}$  efflux in epithelial cells, where PMCA is predominantly distributed on the apical membrane (Bruce et al., 2003).

To summarize the above-mentioned “complex” observations in a nutshell, higher free cellular  $\text{Ca}^{2+}$  contributes to lower concentration of cyclic nucleotides (cAMP/cGMP), and thereby promotes ecdysteroidogenesis. Also, it is quite evident that cAMP, apart from its direct role in modulating the  $\text{Ca}^{2+}$  signaling machinery, can also regulate the long-term gene expression and synthesis of key proteins involved in  $\text{Ca}^{2+}$  signaling (Kuo et al. 1993). Thus, from the perspective of crustacean epithelial  $\text{Ca}^{2+}$  transport, it can be hypothesized that changes in cAMP levels might affect gene expression of SERCA, PMCA and CaM.



**Figure 5.** Model for the regulatory role of cyclic nucleotides on crustacean ecdysteroidogenesis in Y-organ. ATP, Adenosine triphosphate; AC, adenylyl cyclase; Ca, calcium; cAMP, adenosine 3',5'-cyclic monophosphate; cGMP, guanosine 3',5'-cyclic monophosphate; CM, calmodulin; HDL, high-density lipoprotein; HDLR, HDL apoprotein receptor; MIH, molt-inhibiting hormone; MIHR, MIH receptor; PDE, phosphodiesterase; PKC, protein kinase C; PKCLR, PKC-linked receptor, PTK, protein tyrosine kinase (Spaziani et al., 1999).

## ***I. Ecdysteroid receptors***

In crustaceans, ecdysteroid action in target cells manifests itself via intracellular ecdysteroid receptor (Mazurova et al., 2008). EcR belongs to a nuclear receptor family that regulates gene activities at the transcriptional level by interacting with ecdysteroids (Zou 2005; Asazuma et al., 2007; Mazurova et al., 2008). In the nucleus of a responsive crustacean cell, the regulation and expression of these genes is effective only when the crustacean EcR binds/hybridizes with another important nuclear receptor, the retinoid X receptor, forming a functional EcR/RXR heterodimer, which then binds to the DNA response elements of the genes (Wu et al., 2004; Zou 2005; Asazuma et al., 2007; Mazurova et al., 2008). Thus EcR/RXR heterodimerization, which is a vital component of ecdysteroid signaling, may be affected by EDCs as observed in some ecotoxicological studies documented so far. For example, Mu and Leblanc (2004) documented *in vitro* that synthetic EDCs such as pyriproxifen and fenoxicarb decreases the mRNA expression of EcR and RXR in the *Drosophila* Kc cells; the study proposed a mechanistic action where EcR gene expression is reduced due to the deprivation of its heterodimer partner (here, ultraspiracle, USP). Nishikawa et al. (2004) observed that EDCs such as tributyltin and triphenyltin (TPT), interacted with RXR of marine gastropod *Thais clavigera* (rock shell), inducing imposex, although the entire mode of action is unclear owing to lack of research on heterodimer partner and other coupling factors. Sternberg et al. (2008) confirmed that this imposex may be due to retinoid signaling initiated by TBT during an inappropriate time in females.

It is a well known fact that EDCs can affect ecdysteroid signaling in a ligand-dependent manner (direct binding to receptors EcR and RXR). However it can also be disrupted in a ligand-independent manner, such as receptor activation through membrane-bound receptor tyrosine kinase (RTK) pathways (Hatakeyama et al., 2002). These different pathways and mechanisms have not yet been characterized in crustaceans (Zou 2005). It would be worthwhile to see if EDCs accumulating in crustacean tissues (such as hepatopancreas) follow these different pathways and mechanisms in disrupting ecdysteroid signaling. In this study, I looked into a possible ecdysteroid signaling pathway, involving ecdysteroid receptors during the natural molt cycle and in the presence of EDCs, in crayfish.

#### ***J. Chitinase as an ecdysteroid-responsive gene***

During the crustacean molting process, chitinolytic enzymes such as chitinase and N-acetyl- $\beta$ -glucosaminidase (NAG) digest the chitinous exoskeleton (Zou and Fingerman, 1999). Chitinase, apart from its role in molting, is also involved in digestion of chitinous food, and defense against chitin-bearing pathogens (Proespraiwong et al., 2010). As such, chitinase is present in tissues such as hepatopancreas and epidermis, and is differentially expressed in crustacean species (Zou and Fingerman 1999; Tan et al., 2000; Zou and Bonvillain 2004). Although, as discussed earlier, several calcium transporters associated with transcellular  $\text{Ca}^{2+}$  transport were found to express differentially during molt cycle, understanding the hormonal regulation of  $\text{Ca}^{2+}$  flux in crustacean molt cycle has been limited. Thus, in order to substantiate the observations of this study that involves nuclear receptors, cyclic nucleotides and calcium transporters in

the proposed ecdysteroid signaling pathway, I decided to determine the expression pattern of chitinase gene during crustacean molt cycle, and in the presence of EDCs.

### ***K. Significance of proposed research***

Evidently EDCs are ubiquitous in the environment, and they pose risk to humans as well as wildlife. Studies involved in the ecological risk assessment of endocrine disruptors primarily focused on *in vivo* studies, to address effects at the population level, taking into account effects on survival and reproductive output. Such studies were primarily aimed at developing screening assays for regulatory purposes. To complement these studies it will be necessary to understand the EDC mode of action at the cellular level. The classical mode of action of ecdysteroid is the receptor-mediated control of gene activity, influencing transcription, and subsequently, protein synthesis. However, we now have some information, albeit not extensive, that these nuclear receptors can also be activated by ligand-independent pathways in vertebrates. The conservation of major signal transduction pathways in animals (such as insulin signaling pathways, Claeys et al., 2002), gives rise to the possibility of such ligand-independent pathways in crustaceans. Also, as discussed in the preceding sections, EDC effects can target nongenomic sites (Ca<sup>2+</sup> transporting proteins) involving second messengers such as cAMP and Ca<sup>2+</sup>. We know that cAMP and Ca<sup>2+</sup> levels affect the availability of receptor sites for cholesterol (an ecdysteroid precursor) uptake (Spaziani et al., 1999). Thus there is a probability that the EcR (and RXR) can be activated by ligand-dependent/independent, and genomic/nongenomic pathways. Thus investigations on the likelihood of EcR activation by EDCs in a ligand-dependent/independent and genomic/nongenomic manner would only be possible after the delineation of signaling

pathways leading to EcR activation. In this study, I made an effort to investigate endocrine disruption by delineating a previously unaddressed pathway, by testing the hypotheses described in the following section.

### **L. Specific aims**

As discussed earlier, in crustaceans,  $\text{Ca}^{2+}$  not only serves as an intracellular second messenger, but also as a major constituent of the calcified exoskeleton. As such it is involved in the cycle of mineralization/demineralization known as molting cycle. Molting is an endocrine-controlled process, under the control of steroid hormones, ecdysteroids. Numerous environmental chemicals (both natural and synthetic), that have been found to interfere with crustacean molting, have been categorized as EDCs. Studies have shown these EDCs can interfere with ecdysteroid signaling, thereby disrupting the natural molting process. However, progress in understanding the hormonal regulation of  $\text{Ca}^{2+}$  flux in crustacean molting has been limited. Also, evidence suggests that cyclic nucleotides such as cAMP and cGMP play an “indirect” role in ecdysteroidogenesis, and a direct role in  $\text{Ca}^{2+}$  clearance in cells. These observations suggests that the crustacean hormonal system, signaling machinery, and  $\text{Ca}^{2+}$  transporters, are all inter-related to some degree, since there appears to be a connection between ecdysteroid signaling and  $\text{Ca}^{2+}$  homeostasis. In this study, I have determined that molting is a function of ecdysteroid signaling vis-à-vis change in the expression of EcR and its heterodimer partner RXR, and in the presence of a natural and a synthetic EDC, the expression of these hormonal receptors can change in such a way that ends in the disruption of natural molt cycle. We have selected two EDCs, a synthetic estrogen 17 $\alpha$ -ethinyl-estradiol (EE2), and a natural estrogen 17 $\beta$ -estradiol (E2). The freshwater crayfish, *Procambarus clarkii*, which has

emerged as a non-mammalian/crustacean model to study the transcellular  $\text{Ca}^{2+}$  transport during the molt cycle, will serve as the model organism for this study. I hypothesize that in the  $\text{Ca}^{2+}$  transporting epithelial cells of crayfish, EE2 and E2 will increase the expression of nuclear receptors (EcR/RXR), leading to an increase in concentrations of cyclic nucleotides (cAMP and cGMP), and (concurrently) affecting  $\text{Ca}^{2+}$  homeostasis by increasing the expression of  $\text{Ca}^{2+}$  transporters (SERCA, PMCA, and CaM), thereby potentially disrupting the natural molt cycle. We utilized the epithelial cells from the hepatopancreatic tissues of crayfish in this proposed study.

**Specific Aim 1 (SA1):** Determine the gene expression of  $\text{Ca}^{2+}$  transporting proteins (SERCA, CaM, PMCA) and nuclear receptors (EcR and RXR) in epithelial cells during the molt cycle of crayfish. This will help in testing the hypothesis that the expression of these genes is differentially expressed as a function of molt cycle.

**Specific Aim 2 (SA2):** Determine the concentration of cyclic nucleotides, cAMP and cGMP, in epithelial cells during the molt cycle of crayfish. This will enable us to test the hypothesis that cyclic nucleotides play a major role in regulating ecdysteroidogenesis vis-à-vis molt cycle.

**Specific Aim 3 (SA3):** Determine the gene expression of  $\text{Ca}^{2+}$  transporting proteins (SERCA, CaM, PMCA) and nuclear receptors (EcR and RXR) in epithelial cells of crayfish exposed to EE2 and E2. We will test the hypothesis that EE2 and E2 affects

Ca<sup>2+</sup> transporting proteins (SERCA inhibition and CaM-PMCA activation), and attenuates nuclear receptors.

**Specific Aim 4 (SA4):** Determine the concentrations of cyclic nucleotides, cAMP and cGMP, in epithelial cells of crayfish exposed to EE2 and E2. We will test the hypothesis that EE2 and E2 increases the concentrations of cyclic nucleotides.

And finally,

**Specific Aim 5 (SA5):** Determine the expression *Chi* gene expression in epithelial cells during the molt cycle of crayfish, and when exposed to EE2 and E2. This will enable us to substantiate the findings from SA1-SA4, with a *Chi* gene that we assume is ecdysteroid-responsive in crayfish hepatopancreas.

## II. Experimental models and Methods

The following section describes the experimental models and methods used in addressing the objectives of this study.

### A. Biological Models

#### A. 1. Crayfish as a model organism

As mentioned earlier, the crustacean molting cycle has emerged as a non-mammalian model system to study  $\text{Ca}^{2+}$  homeostasis (Wheatly et al., 2002a) and our laboratory has traditionally the freshwater crayfish *P. clarkii*, as the species of choice because it resides in the hostile environment for  $\text{Ca}^{2+}$  balance (Chen et al., 2002). Also, as discussed earlier,  $\text{Ca}^{2+}$  (in addition to its crucial role as second messenger) not only plays a vital role in the crustacean molting cycle, but also plays an important role in the ecdysteroidogenesis. Thus, *P. clarkii* makes an ideal model crustacean to address the hypotheses of this research. Also, in aquatic ecosystems various aquatic animals are being used as indicator species for monitoring environmental quality, including *P. clarkii*. It is a bottom dweller and has been used in previous studies, both in the field and laboratory, as a bio-indicator of various environmental pollutants (Alcorlo et al., 2006). The abundance and widespread distribution, long life cycles, and relatively sedentary lifestyle makes it an ideal indicator species (Sanchez-Lopez et al., 2004). Therefore, it serves as a relevant model species in addressing all stated objectives of this study.

## **A. 2. Hepatopancreas as a model epithelial tissue**

Hepatopancreas is the main organ of storage and detoxification of xenobiotics in crustaceans, and is highly sensitive to physiological and environmental challenges (Johnston et al., 1998). It is a major storage and metabolic organ, and as such will undergo differential gene expression patterns during molt cycle (Chang 1995; Yudkovski et al., 2007). Endogenous ecdysones and exogenous ecdysone-mimicking EDCs can exert their effect by binding to intracellular receptors (e.g. EcR/RXR) within the target tissue (e.g. hepatopancreas) of crustaceans (Chang and O'Connor 1988; Tamone and Chang 1993; Lye et al., 2005). For example, Zou and Fingerman (1999b) documented that diethyl phthalate and 2,4,5-trichlorobiphenyl significantly inhibited the chitinase activity in hepatopancreas of the fiddler crab (*U. pugilator*); the study attributed it to the interaction of these EDCs with the ecdysteroid receptors in the hepatopancreas. Also, the hepatopancreas is one of four critical  $\text{Ca}^{2+}$  transporting epithelia in the mass transcellular  $\text{Ca}^{2+}$  transport during ecdysis. Therefore, it is an apt model epithelial tissue for this proposed study.

## **A. 3. E2 and EE2 as model EDCs**

E2 and EE2 were chosen as model EDCs for this proposed study. In the environment, natural and synthetic estrogens (such as E2 and EE2 respectively) have contributed to the pool of EDCs in the effluent water from sewage treatment plants (Larsson et al., 1999). E2 is a natural endogenous sex steroid hormone that regulates reproduction in many invertebrates and all classes of vertebrates (Cheek et al., 1997), whereas EE2 is a synthetic estrogen widely used in combination with other steroid hormones in oral contraceptive pills and contraceptive patches (Parrott and Blunt 2005,

Caldwell et al., 2008). Both E2 and EE2 have been detected and implicated in the estrogenic activity in sewage treatment effluents (and occasionally in surface waters) in U.S., U.K., Canada., Germany and elsewhere (Desbrow et al., 1998; Filby et al., 2006; Caldwell et al., 2008). These two compounds are known to exhibit a receptor-mediated mode of action in humans, and thus can exhibit similar actions in aquatic organisms that are known to have estrogen receptors similar to those found in mammalian systems (Gunnarsson et al., 2008). In fact due to its strong affinity to estrogen receptor, EE2 exhibits *in vitro* biological activities similar to that of the endogenous E2 (Pawlowski et al., 2004; Van den Belt et al., 2004). In fact, EE2 is biochemically engineered from E2 (Figure 6). Natural steroid estrogens such as E2 have a hydroxyl group at C-3 position, whereas the synthetic EE2 has an additional ethinyl group on C-17 position, making it much more resistant to biodegradation than E2 (Cluozot et al., 2009). E2 concentrations detected in the aquatic environment, depending on the proximity to the sewage and waste water treatment plants, had concentrations ranging up to 100 ng/L (Brion et al., 2004; Jukosky et al., 2008). Similarly, the EE2 concentrations detected in the environment ranged from 1 ng/L to 273 ng/L, (Dussault et al., 2009). These concentrations have been found to profoundly impact the physiological conditions of aquatic animals. Most of the ecotoxicological studies involving effects of E2 and EE2 are confined to reproductive health of fish (Hutchinson et al., 1999). However evidence suggests the presence of endogenous estrogens in crustaceans, giving rise to the possibility of environmental estrogens impacting crustaceans (Hutchinson et al. 1999). Moreover, sediments, which many benthic invertebrate organisms including crustaceans inhabit, concentrate EDCs in much higher quantities than in the water column, and so, could result in their significant

uptake by the organisms (Langston et al., 2005). Therefore, E2 and EE2 would make a good fit as model compounds for this study. One of the aims of this study is to determine whether the E2 and EE2 exposure of crayfish leads to disruption of endocrine mode of action, or a general toxic stress. For this, two environmentally relevant concentrations, one lower (100 ng/L) and one higher (500 ng/L) were chosen.

## **B. Methods**

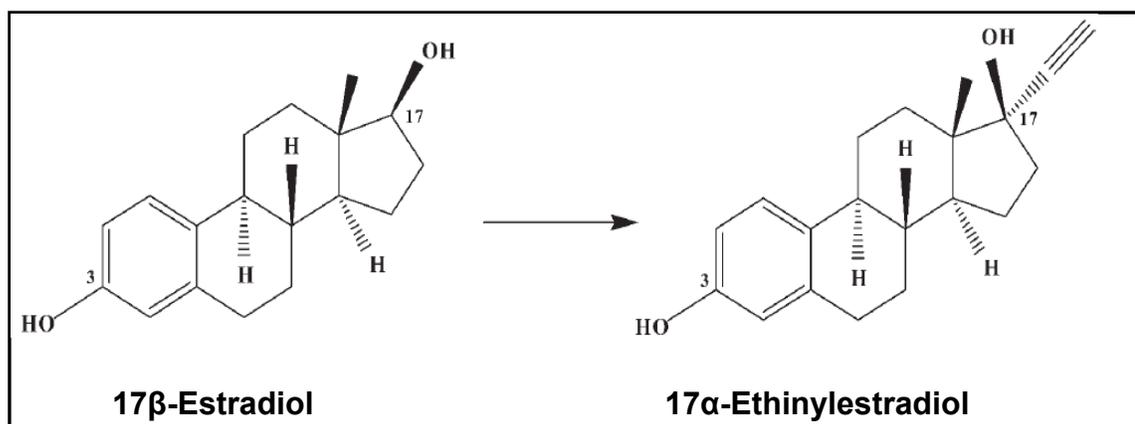
### **B. 1. Test species**

Crayfish *P. clarkii* (Girard) were obtained from Atchafalaya Biological Supply Co. (Raceland, LA), and maintained in 40 liter aquaria in filtered aerated water at room temperature (23°C) and a 16 h: 8 h light-dark cycle. Crayfish were fed everyday with cooked shrimp.

### **B. 2. Partial cDNA cloning and sequencing of EcR, RXR, and Chitinase genes**

Three cDNAs encoding EcR (*PcEcR*), RXR (*PcRXR*) and Chi-1 (*PcChi-1*) were partially cloned and sequenced from *P. clarkii* using PCR techniques. Following decerebration, hepatopancreae were removed from crayfish, snap-frozen in liquid nitrogen and stored at -80 °C. Total RNA was isolated by utilizing TRIzol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The resulting RNA (1 µg) was reverse transcribed for preparation of template cDNA using Superscript® II reverse transcriptase (Invitrogen) with oligo (dT)<sub>12-18</sub> as primer. For PCR targeting a 392 bp partial *PcEcR* cDNA fragment, a set of degenerate primers, *PcEcR*-dF1, 5'-CAAGGCDTGYTCMWSHGARGTB-'3 and *PcEcR*-dR, 5'-GCAYAYCTC WGAGTTBADRTTBCC-'3 for first PCR and *Pc*-ECR-dF2, 5'-

CMWSHGARGTBATG ATGYTBCGM-3 for nested PCR, were designed based on the conserved sequence of *Daphnia magna* EcR (GenBank accession no. AB274820), *Marsupenaeus japonicus* EcR (AB295492), *Gecarcinus lateralis* EcR (AY642975) and *Carcinus maenas* EcR (AY496927). A 484 bp cDNA fragment of *PcRXR* was amplified using the following degenerate primers, *PcRXR*-dF1, 5'-GTDGARTGGGCSAARCACATCCCM-3 and *PcRXR*-dR, 5'-DGGHGTRTCYCCAACHARYTTRAA-3 for first PCR and *PcRXR*-dF2, 5'-ATCCCMCAYYTYACRGAMYTKCCY-3 for nested PCR, designed based on the conserved sequence of *D. magna* RXR (GenBank accession no. ABF74729), *M. japonicus* RXR (BAF75376), *G. lateralis* RXR (AAZ20368) and *Celuca pugilator* RXR (AAC32789). A 586 bp cDNA fragment was amplified using the following degenerate primers, of *PcChi*-dF1, 5'-CCCGARGGYCARGCSCGSCGYGTG-3 and *PcChi*-dR, 5'-CGYGTGTGYTACTAYGAGGCSTGG-3 for first PCR and *PcChi*-dF2, 5'-GTTGCCCKCAGGTCRTACGTCAT-3 for nested PCR, designed based on the conserved sequence of *Fenneropenaeus chinensis* Chitinase 1 (GenBank accession no. ABB85237), *M. japonicus* Chitinase 1 (BAA12287), *P. monodon* Chitinase 1 (AAD40313), and *Litopenaeus vannamei* Chitinase 1 precursor (ACG60513). PCR was performed using a MJ Research thermal cycler (PTC 100, MJ Research, Watertown, MA) and Taq DNA polymerase (Invitrogen) with the PCR programs with initial denaturation of cDNA at 95 °C for 3 min followed by 35 cycles of amplification which include 95 °C for 45 s, annealing temperature specific to a primer for 30 s, 72 °C for 1min 30s and a final cycle at 72 °C for 10 min. The sequences of the primers for *PcEcR*, *PcRXR*, and *PcChi* and the annealing temperature used for cloning are represented in Table. 1.



*Figure 6. 17  $\alpha$ -Ethinylestradiol engineered from endogenous hormone 17  $\beta$ -Estradiol.*

PCR products were analyzed on a 1.0 % agarose electrophoresis gel with 0.5  $\mu\text{g mL}^{-1}$  of ethidium bromide in 1x TAE buffer (40  $\text{mmol L}^{-1}$  Tris, 40  $\text{mmol L}^{-1}$  sodium acetate and 1 $\text{mmol L}^{-1}$  EDTA, pH 7.2), and the DNA bands were visualized under the ultraviolet light. PCR products were ligated to PCR 2.1 vector (Invitrogen) and transformed into chemically competent TOPO10 cells (Invitrogen). Each clone was digested with appropriate restriction enzymes and subcloned for sequencing. The cDNA clones were sequenced by automated sequencing (ABI PRISM 377, 3100 and 3700 DNA sequencers, Davis Sequencing). Sequence homology was determined using a GenBank database search with the BLAST algorithm search (<http://www.ncbi.nlm.nih.gov/blast>).

### **B. 3. Molt cycle studies**

For studies involving natural molting cycle, hepatopancreas tissues were collected from decerebrated animals at various stages of natural molting cycle as outlined in earlier studies (Gao and Wheatly, 2004). Briefly, premolt status of animals was determined from the gastrolith index (McWhinnie, 1962), and postmolt status was classified in reference to the day of ecdysis (shedding). After dissection, the tissues were frozen immediately in liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$ , until further molecular assays were performed including gene expression analyses, and cyclic nucleotide quantifications.

### **B. 4. Exposure studies**

For exposure studies involving E2 and EE2, the crayfish were individually held for two weeks in 472 ml BPA-free glass bowls (Anchor Hocking Company, Lancaster OH) prior to the exposure. E2 and EE2 (98% minimum purity by HPLC) purchased from

Sigma-Aldrich (St. Louis, MO) were handled with good laboratory practices (i.e., gloves, eye protection, lab coat. etc), and guidelines mentioned in the material safety datasheet (MSDS) for each chemical. All liquid and solid waste were retained in appropriate containers and disposed of by the Department of Environmental Health and Safety, Wright State University. Intermolt juvenile crayfish ( $4.53 \pm 0.72$  g in weight) were exposed to nominal concentrations (100 and 500 ng/L) of E2 and EE2 under static-renewal conditions for a period of 40 days. Stock solutions (100 mg/L) for both chemicals were prepared in carrier solvent ethyl alcohol (200 proof- absolute, anhydrous ACS/USP grade, PHARMCO- AAPER (Brookfield, CT and Shelbyville, KY), and stored in the dark at 4 °C. The stock solution was subsequently diluted to standard solution (1 mg/L) using double distilled deionized water. The treatment solution—at the desired nominal concentration—was prepared daily by diluting the standard solution with reconstituted water, and used to expose (100 ml/bowl) crayfish held individually in glass bowls. All treatments including the solvent control, received the same amount of solvent, and the final concentration of the solvent was less than 0.0001%. All experiments were conducted in triplicate with a sample size of  $n = 3$ .

To confirm the E2 and EE2 estrogenic activity, water samples (50 ml/bowl) were collected from treatments on days 1, 20 and 40, and were stored at -80 °C for further analysis using commercially available enzyme-linked immunosorbent assays (ELISAs).

At the end of the 40-day exposure, hepatopancreae were harvested from the decerebrated crayfish, and hepatosomatic index (HSI) was calculated as follows: (liver weight/total body weight)  $\cdot 100$ . Following this, the tissue samples were snap-frozen in

liquid nitrogen and stored at -80 °C until further molecular assays which include gene expression analyses, and cyclic nucleotide quantifications.

## **B. 5. Quantitative Real Time-PCR(Q-RT-PCR)**

The specific aims SA1 and SA3 and SA5 proposed in this study require quantification of gene expression of Ca<sup>2+</sup> transporters, nuclear receptors, and molting gene Chitinase. Total RNA was isolated from the stored hepatopancreas samples isolated from the above-mentioned molt cycle and exposure studies, by utilizing TRIzol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Genomic DNA was removed from total RNA samples using the TURBO DNA-free kit (Ambion, Austin, TX). DNA-free total RNA (0.5 picograms) was reverse transcribed with random hexamers to create cDNA using Taqman Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The resulting cDNA was employed in PCR amplifications optimized with gene-specific primers (Table. 1) containing a fluorescent reporter molecule (SYBR Green PCR core reagents kit PE Applied Biosystems). Oligonucleotide primers for all the crayfish genes of interest for this study (*PcEcR*, *PcRXR*, *PcChi*, SERCA, PMCA and CaM) were chosen with the Primer Express™ software (PE Applied Biosystems) using sequence information obtained from clones that were identified earlier in this study (*PcEcR*, *PcRXR* and *PcChi*); primers for SERCA, PMCA and CaM were designed from the sequence information available from the previously published studies in our lab (Chen et al., 2002; Gao et al., 2009).

Gene	cDNA length (bp)	Annealing °C (s)	Forward Primer	Reverse primer
PcEcR	392	1° PCR 50 (30)	F1 CAAGGCDTGYTCMWSHGARGTB	GCAYAYCTCWGAGTTBADRTTBCC
		2° PCR 55 (30)	F2 CMWSHGARGTBATGTGYTBCGM	
PcRXR	484	1° PCR 50 (30)	F1 GTDGARTGGGCSAARCACATCCCM	DGGHGTRTCYCCA AKHARYTTRAA
		2° PCR 55 (30)	F2 ATCCCMCAYTTYACRGAMYTKCCY	
PcChi	586	1° PCR 62 (30)	F1 CCCGARGGYCARGCSCGSCGYGTG	CGYGTGTGYTACTAYGAGGCSTGG
		2° PCR 63 (30)	F2 GTTGCCCCCKAGGTCRTACGTCAT	

*Table 1. Sequences of degenerate primers and annealing temperatures used for partial cDNA cloning of PcEcR, PcRXR, and PcChi.*

The synthesized (Qiagen) primers were then used for Q-RT-PCR reactions. Primer concentrations were optimized and tested for amplification efficiency. The integrity of the cDNA was checked by the presence of a fragment of crayfish 18S rRNA gene. RNA 18S primers (sense 5'- TGGTGCATGGCCGTTCTTA-3' and antisense 5'- AATTGCTGGAGATCCGTCGAC-3') were designed (Table. 2) from *P. clarkii* 18S rRNA gene (GenBank accession no. AF436001). The reaction mixture (25  $\mu$ L in total) contained the following: 2.5  $\mu$ L of 10x SYBR GREEN PCR buffer, 3  $\mu$ L of 25 mM  $MgCl_2$ , 2  $\mu$ L of 12.5 mM dNTP mix (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, and 5 mM dTTP), 0.125  $\mu$ L of AmpliTaq Gold (5 U/ $\mu$ L), 0.25  $\mu$ L of AmpErase (1 U/ $\mu$ L), 2  $\mu$ L of template cDNA, and 1  $\mu$ L of each 10  $\mu$ M primers in water. Q-RT-PCR reactions were performed in 25  $\mu$ L sterile SmartCycler® tubes (Cepheid, Sunnyvale, CA) using the relative quantification  $\Delta\Delta C_t$  method on a Cepheid SmartCycler® real-time PCR machine (Cepheid, Sunnyvale, CA) with the following cycling conditions: 50 °C for 2 minutes, 95 °C for 10 min followed by 40 cycles of 95 °C for 15s and annealing temperature specific to a primer for 60s. Melting curve analysis was performed at the end of the PCR reaction. Threshold cycles ( $C_t$ ) were determined as the PCR cycle at which an increase in SYBR Green fluorescence above a baseline signal was first detected. For 18S rRNA reaction mix, 2  $\mu$ L of 0.1 $\times$  diluted cDNA were used. cDNA samples were analyzed in triplicate and fold changes relative to the selected calibrator condition (intermolt for the molting stage expression, and the control treatment for expression in the presence of E2 and EE2) were calculated based on the relative

quantification  $\Delta\Delta\text{Ct}$  method. Relative quantification (RQ) was performed by normalizing the Ct values of each sample gene with the Ct value of the endogenous control 18S rRNA gene ( $\Delta\text{Ct}$ ) and finally calculated using  $\Delta\text{Ct}$  of the control tissue/condition as a calibrator.  $\Delta\Delta\text{Ct}$  corresponds to the difference between the  $\Delta\text{Ct}$  of the gene of interest and the  $\Delta\text{Ct}$  of the endogenous control 18S rRNA. Fold change in expression was calculated as  $\text{RQ}=2^{(-\Delta\Delta\text{Ct})}$ . A number of controls were performed to ensure proper PCR amplification. Negative controls including no template controls and templates in which reverse transcriptase was not added to cDNA synthesis reactions were run with every assay.

## **B. 6. Cyclic nucleotide assays**

In order to directly address specific aims **SA2** and **SA4** of this proposed study, methods involving direct measurements of cAMP and cGMP were employed using commercially available enzyme immunoassay kits. Cyclic nucleotides (cAMP and cGMP) were extracted from hepatopancreas frozen (-80 °C) samples collected from the molt cycle and exposure studies. Without allowing samples to thaw, hepatopancreas tissues were homogenized on ice with a Pyrex® glass-glass tissue grinder (Corning Incorporated, Corning NY) in 5 volumes (mL of solution/gram of tissue) of 5% trichloroacetic acid (TCA) in water. Precipitated protein was removed by centrifugation at 1,500 x g for 10 minutes. TCA was removed by the triplicate extraction with water-saturated ether, and residual ether was evaporated by heating samples to 70 °C for five minutes. Supernatants from the tissue extraction assayed directly for the quantification of cyclic nucleotides, cAMP and cGMP, by enzyme-linked immunoassay (EIA) using antibodies obtained from Cayman Chemical (Ann Arbor, MI). Briefly, cAMP- and cGMP-acetylcholinesterase conjugate, cAMP- and cGMP-specific rabbit antibody, and

either standard or sample were added to each well of an EIA plate precoated with mouse monoclonal anti-rabbit IgG antibody and blocked with blocking proteins. After 18 h incubation at 25 °C, the plate was washed five times to remove all unbound reagents. Ellman's reagent (5,5-dithio-bis-2- nitrobenzoic acid) was then added to each well, and the plate was read at 412nm with a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA). The results are expressed as pmoles/g of tissue. Assays were performed in triplicate. The standard curve for the assay was prepared in the same matrix as the samples. For this, a sufficient amount of 5% TCA was prepared with ether in the same manner as used for sample extraction. The residual ether was removed by heating, and the remaining solution was used to prepare the standard curve. The sensitivity and specificity of this assay for cAMP were 3.1 pmol/mL and 100%, for cGMP 1 pmol/mL and 100%, respectively.

The intra- and interassay variation were <10%. Cross-reactivity of the cAMP and cGMP assays with other cyclic nucleotides was <0.01%.

## **B. 7. E2 and EE2 sample analysis**

To confirm the E2 and EE2 estrogenic activity, water samples (50 ml/bowl) collected on days 1, 20 and 40 from all treatments, were stored at -80 °C, for further analysis using commercially available ELISAs.

### **B. 7. a. E2 analysis**

For E2 analysis, the samples were triplicate extracted with 4X the sample volume of methylene chloride. The combined methylene chloride extracts were evaporated by heating to 30 °C under a gentle stream of nitrogen, and then reconstituted in 0.5 mL of

EIA buffer. The reconstituted samples were then assayed for the quantification of E2 by EIA using antibodies obtained from Cayman Chemical (Ann Arbor, MI).

Gene	Product length	Annealing °C (s)	Sense primer	Antisense primer
18S rRNA	105	55 (60)	TGGTGCATGGCCGTTCTTA	AATTGCTGGAGATCCGTCGAC
SERCA	105	60 (60)	TTGACTGCTGCTCTAGGTCTT	AGGGTTGAAGCCCAAGGCAGT
PMCA	105	55 (60)	GCAAGTGGTGGCTGTAAGTGGT	GGCCTCCTTTGCAACACTAGTA
CaM	103	55 (60)	CAACGAAGTGGACGCTGAC	GGCTTCCTGATCTCTTCCT
EcR	105	55 (60)	TATGTGGAGAATCGACGAATG	GCATATCTCTGAGTTTAGGTT
RXR	105	55 (60)	ACACGTAAGTACTACCTGAA	CAAAAAGAGATACTCTAGGCA
Chitinase	105	57 (60)	GACCCTGCCAAGAGGAAGAC	ACCTCGTTGTGCTGGGTACT

*Table 2. Primers and Optimal Conditions for Quantitative Reverse Transcriptase Polymerase Chain Reaction (Q-RT-PCR)*

Briefly, estradiol-acetylcholinesterase conjugate, estradiol antiserum, and either standard or sample were added to each well of an EIA plate precoated with mouse monoclonal anti-rabbit IgG antibody and blocked with blocking proteins. After one hour incubation at 25 °C, the plate was washed five times to remove all unbound reagents. Ellman's reagent was then added to each well, and the plate was read at 412nm with a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA). The results are expressed as ng/L of E2 concentration in water. Assays were performed in triplicate. The standard curve for the assay was prepared in the same matrix (EIA buffer) as the samples. The sensitivity and specificity of this assay were 19 ng/L and 100%, respectively. The intra- and interassay variation were <10%. Cross-reactivity of this E2 assay with other compounds such as estradiol-3-glucuronide, estrone, estradiol-17-glucuronide was <15%.

#### B. 7. b. EE2 analysis

For EE2 analysis, water samples were filtered through glass fiber filter (1µm pore diameter), diluted with methanol to obtain final concentration of 10% methanol. The filtered samples were then assayed for the quantification of EE2 by EIA using antibodies obtained from Abraxis (Warminster, PA). Briefly, EE2 antigen-enzyme conjugate and standard (or sample) were premixed and added to each well of an EIA plate to compete for limited number of binding sites of specific antibodies immobilized on the surface of the wells. After one hour incubation at 25 °C, the plate was washed four times to remove all unbound reagents. To initiate the chromogenic reaction, "color solution" was added to each well, and the plate incubated for 30 minutes at 25 °C. The reaction was stopped

by adding “stop solution”, and the plate was read at 450nm with a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA). The results are expressed as ng/L of EE2 concentration in water. Detection limits for this assay were between 50 and 3000 ng/L.

### III. RESULTS

#### **A. Partial EcR, RXR, Chi DNA cloning**

Primers and the RT-PCR conditions were successful in the amplification of discrete products (392 bp EcR, 484 bp RXR, and 586 bp Chi) from crayfish hepatopancreas cDNA. A BLAST search for the *PcEcR* revealed that the nucleotide sequence matched exclusively with the EcRs from Atlantic fiddler crab *C. pugilator* (90 %), black back land crab *G. lateralis* (90 %), green crab *C. maenas* (82 %), and kuruma prawn *M. japonicus* (70 %). The amino acid sequence (Figure 7) showed 90 % homology with *C. pugilator* and *G. lateralis*, 83 % with North Sea brown shrimp *Crangon crangon*, 82 % with *C. maenas*, and 70 % homology with *M. japonicus*.

A BLAST search for the *PcRXR* revealed that the nucleotide sequence matched exclusively with the RXRs from *G. lateralis* (93 %), *C. pugilator* (92 %), *M. japonicus* (89%), and *D. magna* (79 %). The amino acid sequence (Figure 8) showed 93 % homology with *G. lateralis*, 92 % with *C. pugilator* and *C. crangon*, 90 % with *C. maenas*, and 89 % homology with *M. japonicus*.

A BLAST search for the *PcChi* revealed that the nucleotide sequence matched exclusively with the Chitinase sequences from giant mud crab *Scylla serrata* (65 %), fleshy prawn *Fenneropenaeus chinensis* (62%), *Litopenaeus vannamei* and *M. japonicus* (61%). The *PcChi* amino acid sequence showing homology with these crustacean species are shown in Figure 9. These partial sequences (*PcEcR*, *PcRXR*, and

*PcChi*) provided crucial DNA sequence information required for designing primers for quantitative real-time PCR.

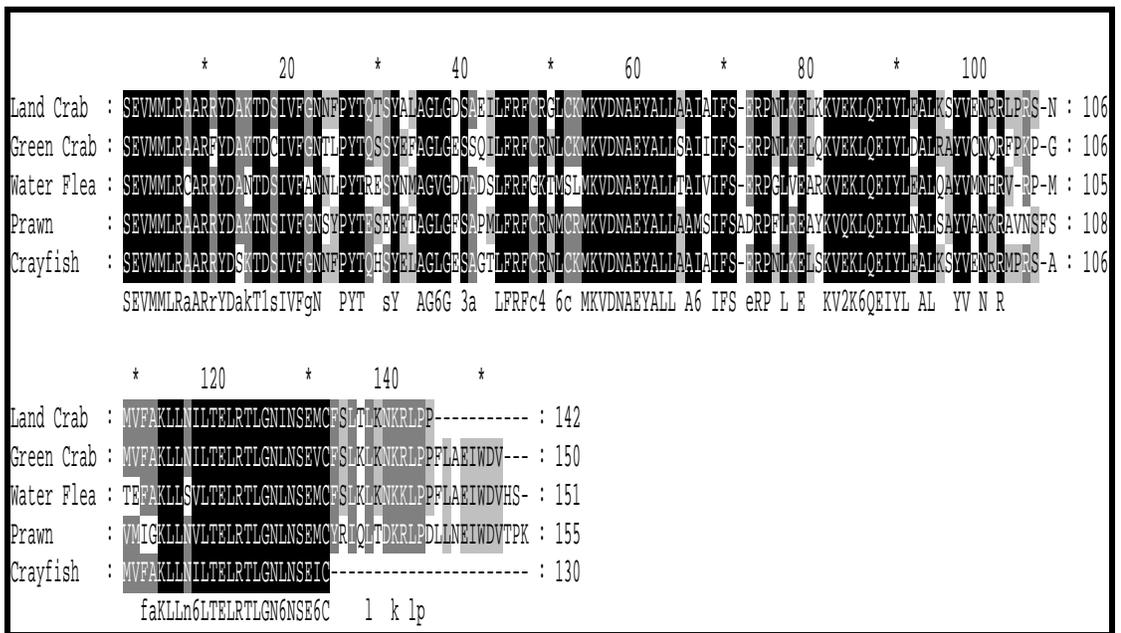
## **B. Molt cycle studies**

### **B. 1. Expression of sex-steroid nuclear receptors**

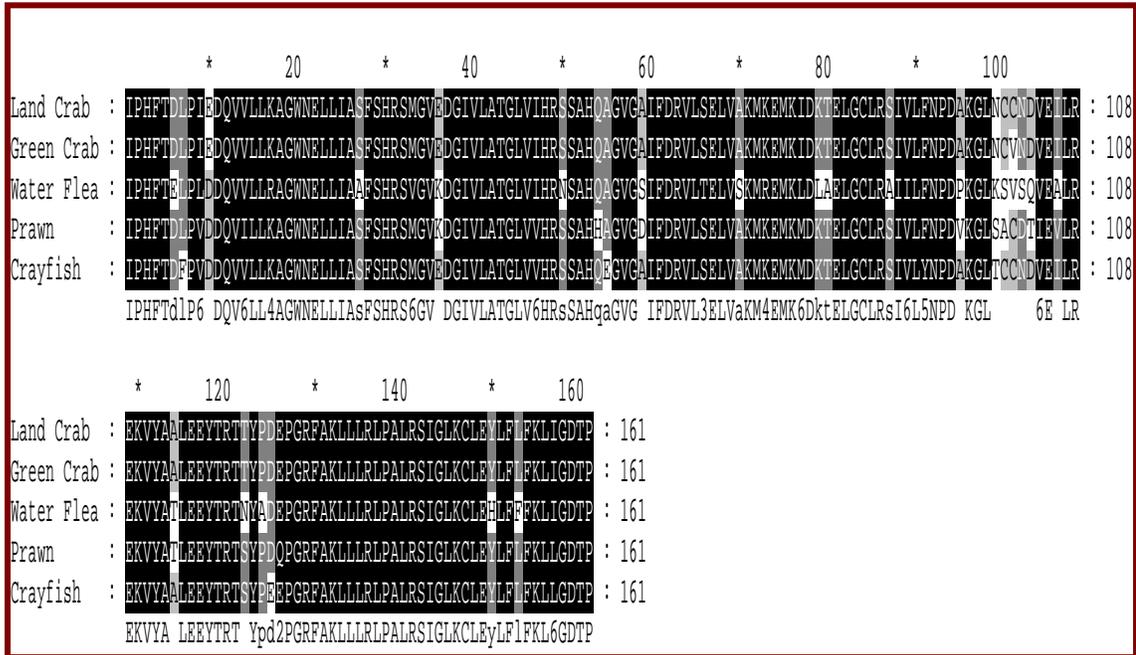
Total RNA extracted from hepatopancreas of *P. clarkii* in different molting stages (inter, pre, post) were subjected to Q-RT-PCR to investigate temporal expression levels of *PcEcR* and *PcRXR* associated with molt cycle. When compared to intermolt (used as a calibrator), the expression level of *PcEcR* increased significantly (>2- fold) during the premolt stage, but was recovered to intermolt levels in postmolt (ANOVA,  $p=0.000$ ) (Figure 10). Expression of *PcRXR* increased in premolt stage (>2-fold). The increase was however significantly higher (>4 fold) in the postmolt (ANOVA,  $p=0.000$ ) (Figure 11).

### **B. 2. Measurement of cyclic nucleotides**

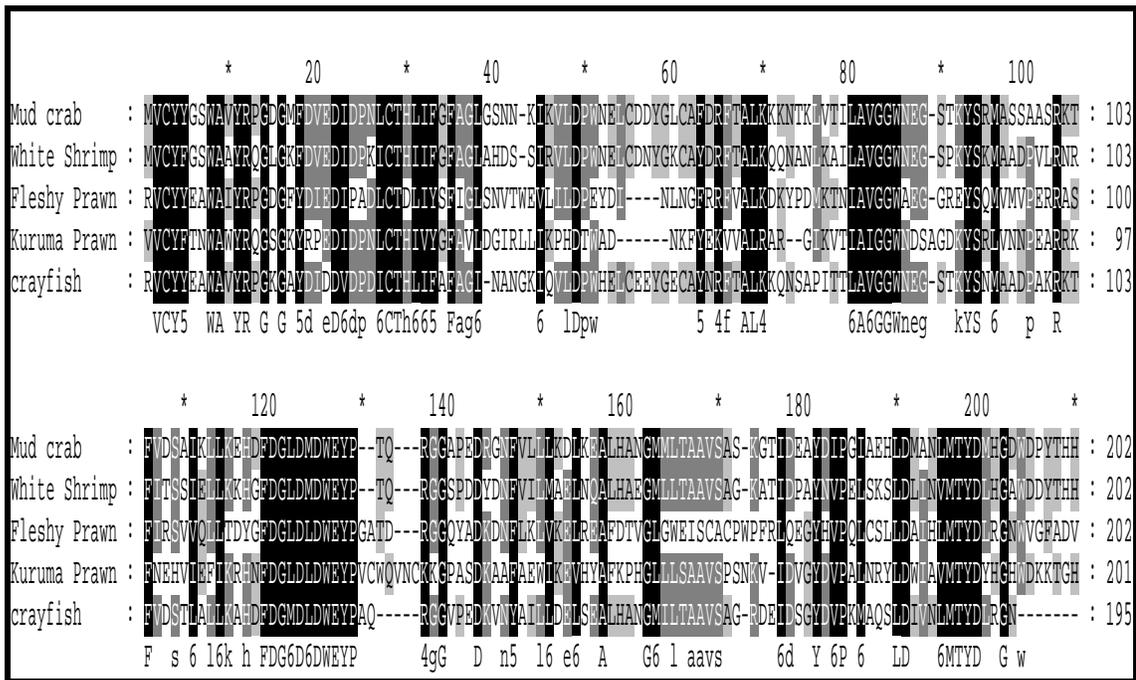
To investigate the effect of ecdysteroid fluctuations associated with molt cycle on intracellular cyclic nucleotide levels, I measured the cyclic nucleotide levels in crayfish hepatopancreas extracted from different molt stages (inter, pre, and post). The cAMP level was significantly higher (>300-fold) in premolt stage (ANOVA,  $p=0.000$ ) compared to inter and postmolt stage (Figure 12). The cGMP level at postmolt was significantly lower (Figure 13) when compared to intermolt (ANOVA,  $p=0.001$ ). There was no significant difference in cGMP level between pre- and postmolt hepatopancreas (ANOVA,  $p=0.069$ ).



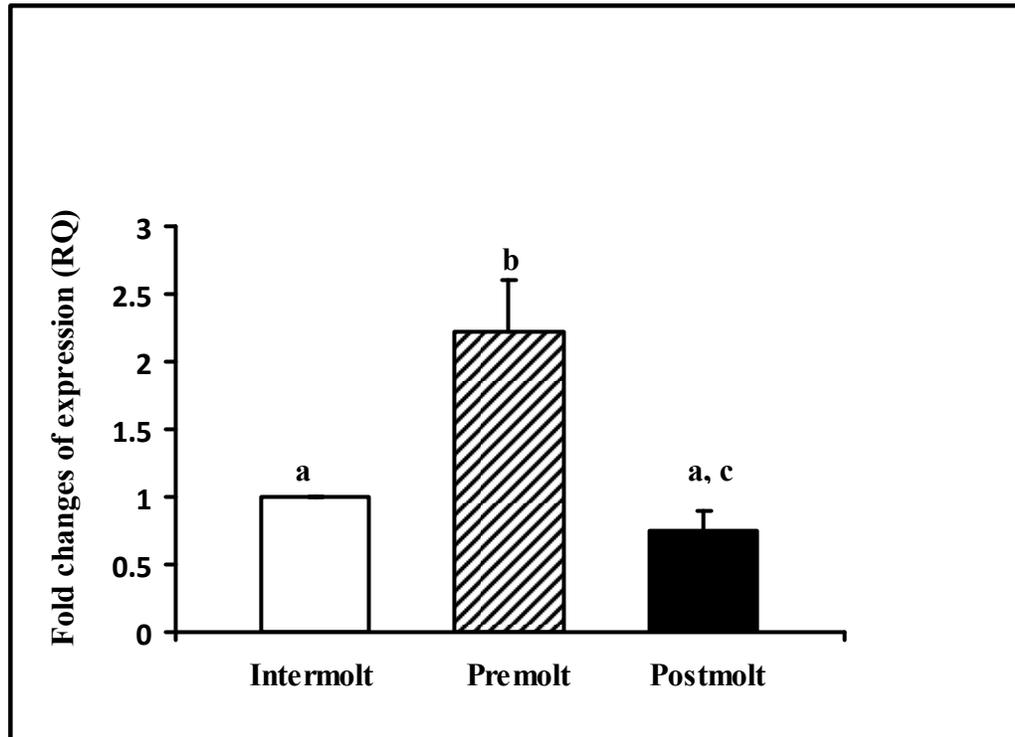
**Figure 7.** Comparison of deduced partial amino acid sequence of crayfish hepatopancreatic EcR with those of other crustaceans. Shaded regions indicate conserved regions of the sequences



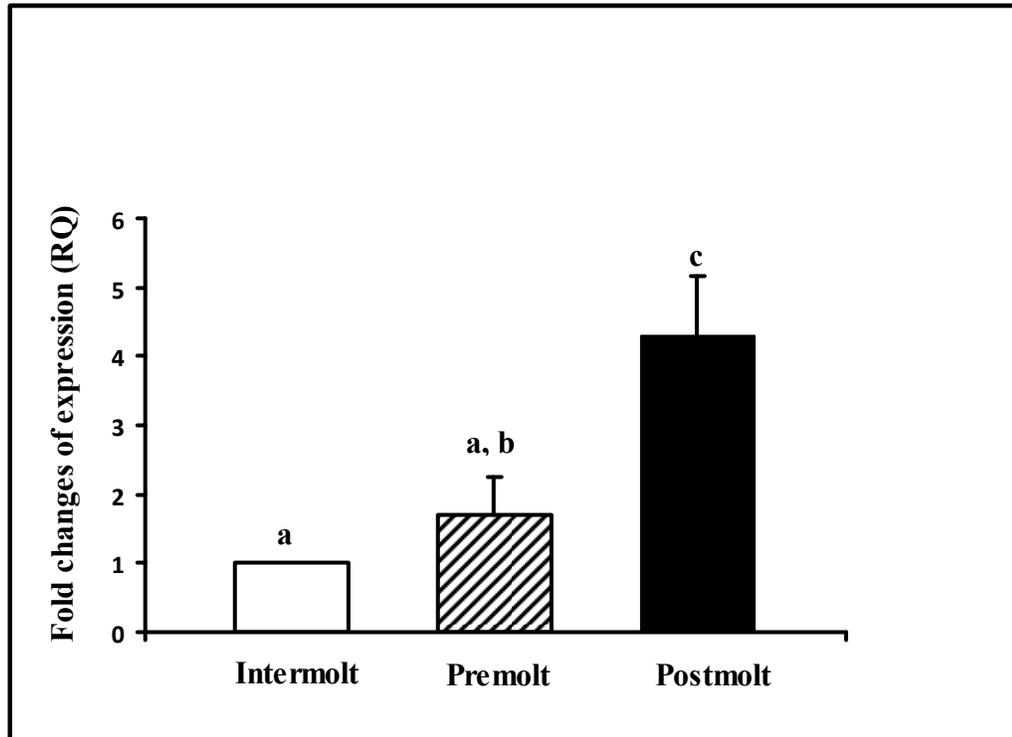
**Figure 8.** Comparison of deduced partial amino acid sequence of crayfish hepatopancreatic RXR with those of other crustaceans. Shaded regions indicate conserved regions of the sequences.



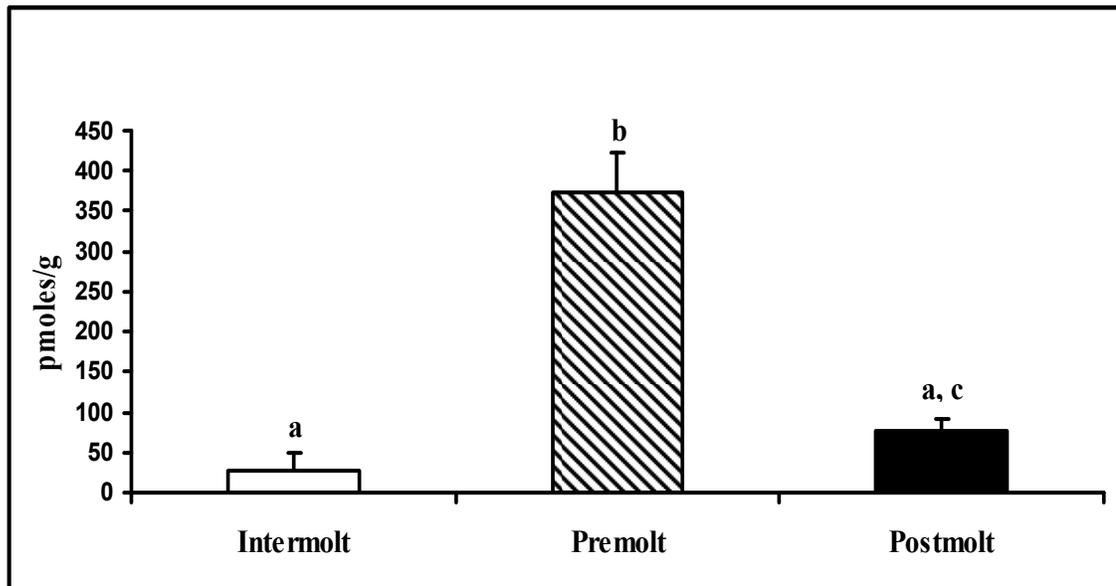
**Figure 9.** Comparison of deduced partial amino acid sequence of crayfish hepatopancreatic Chi with those of other crustaceans. Shaded regions indicate conserved regions of the sequences.



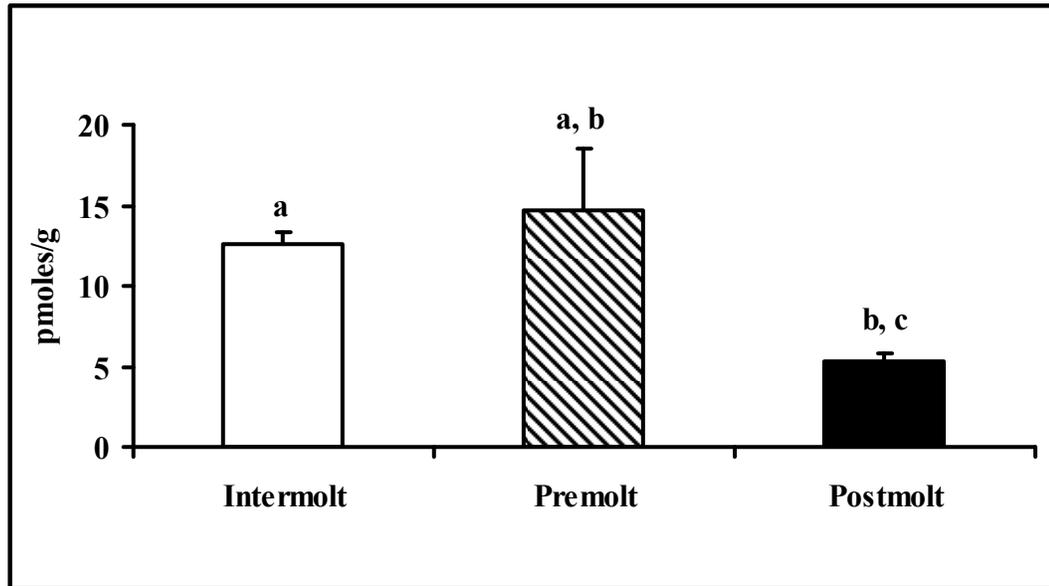
**Figure 10.** *Q*-RT-PCR assay for the expression of *Procambarus clarkii* PcEcR mRNA in hepatopancreas as a function of molting stage. The calibrator stage was intermolt. Values are represented as means $\pm$ SEM (n=3). Different letters above the error bars represent significant differences. Statistical analysis was performed using one-way ANOVA ( $p < 0.05$ ).



**Figure 11.** *Q*-RT-PCR assay for the expression of *Procambarus clarkii* PcRXX mRNA in hepatopancreas as a function of molting stage. The calibrator stage was intermolt. Values are represented as means $\pm$ SEM ( $n=3$ ). Different letters above the error bars represent significant differences. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ ).



**Figure 12.** Intracellular cAMP levels in hepatopancreas during a molt cycle of *Procambarus clarkii*. Results are expressed as mean $\pm$ SEM (n=3). Different letters above the error bars represent significant differences. Statistical analysis was performed using one-way ANOVA ( $p < 0.05$ ).

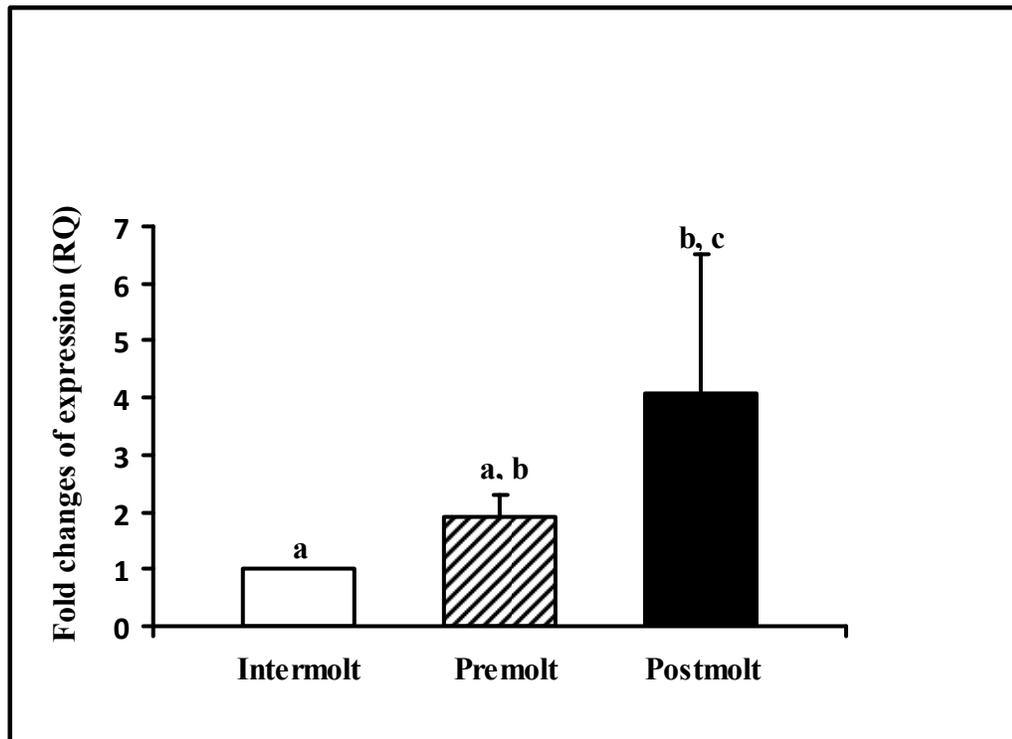


**Figure 13.** Intracellular cGMP levels in hepatopancreas during a molt cycle of *Procambarus clarkii*. Results are expressed as mean $\pm$ SEM (n=3). Different letters above the error bars represent significant differences. Statistical analysis was performed using one-way ANOVA ( $p < 0.05$ ).

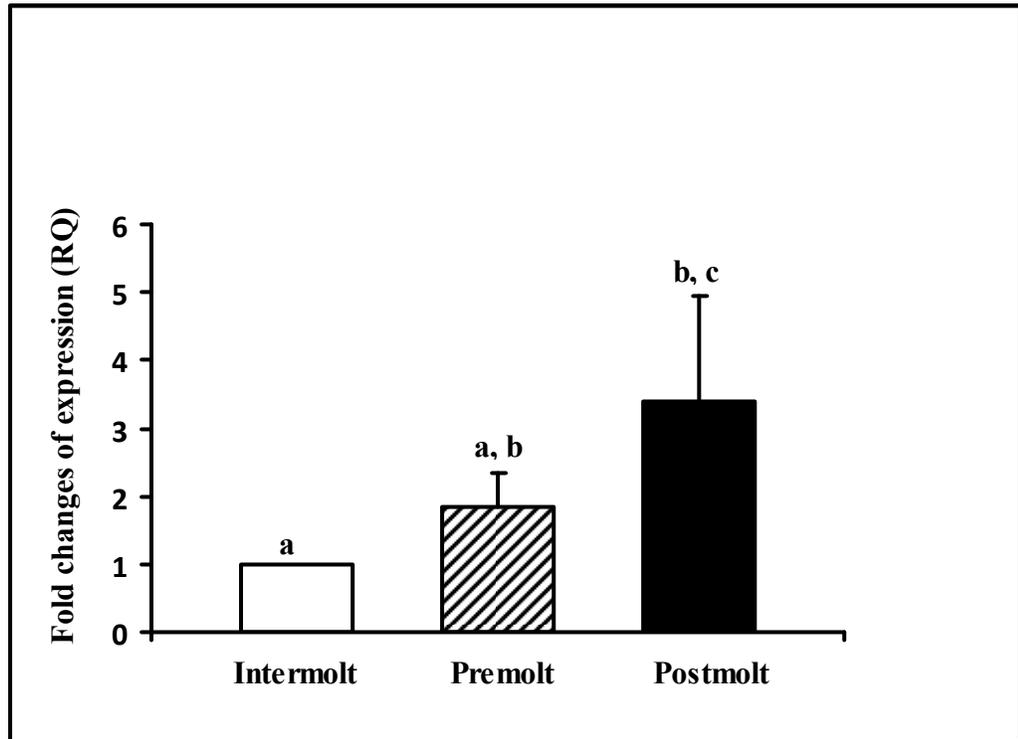
### **B. 3. Expression of calcium transporters**

Compared with baseline (intermolt), Q-RT-PCR data revealed a trend towards increased mRNA expression of all the three calcium transporters (SERCA, PMCA and CaM) in crayfish hepatopancreas during premolt (doubling), with a further doubling in postmolt.

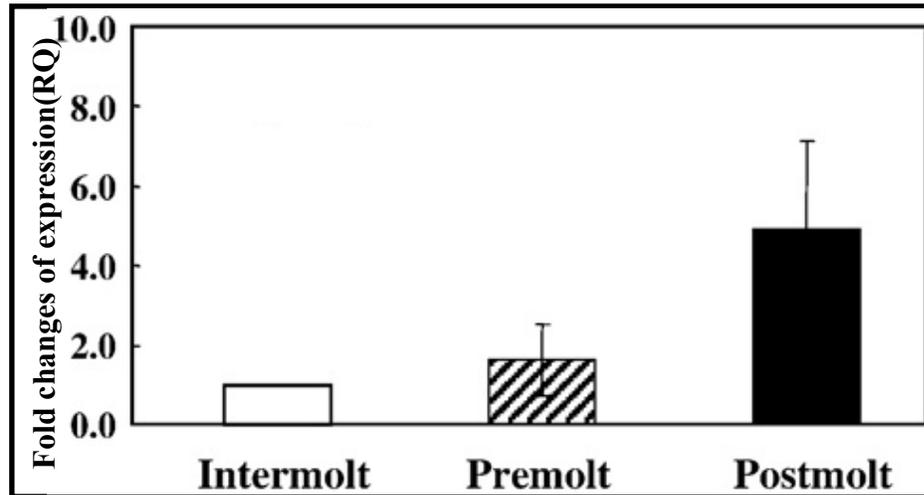
A 1.9-fold increase in SERCA mRNA expression was observed during premolt, and a 4.1-fold increase was observed during postmolt compared to intermolt (ANOVA,  $p=0.006$ ) (Figure 14). PMCA mRNA expression followed a similar trend when compared to intermolt, increasing 1.9-fold during premolt, and 4.0-fold during postmolt (ANOVA,  $p=0.004$ ) (Figure 15). Similarly, the expression profile for CaM gene which has previously been documented in our laboratory (Figure 16, Gao et al. 2009) followed an identical pattern, with ~2.0-fold increase during premolt and ~5.0-fold increase during postmolt.



**Figure 14.** Q-RT-PCR assay for the expression of *Procambarus clarkii* SERCA mRNA in hepatopancreas as a function of molting stage. The calibrator stage was intermolt. Values are represented as means $\pm$ SEM (n=3). Different letters above the error bars represent significant differences. Statistical analysis was performed using one-way ANOVA ( $p < 0.05$ ).



**Figure 15.** *Q*-RT-PCR assay for the expression of *Procambarus clarkii* PMCA mRNA in hepatopancreas as a function of molting stage. The calibrator stage was intermolt. Values are represented as means $\pm$ SEM ( $n=3$ ). Different letters above the error bars represent significant differences. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ ).



**Figure 16.** *Q-RT-PCR* assay for the expression of *Procambarus clarkii* CaM mRNA in hepatopancreas as a function of molting stage. The calibrator stage was intermolt. Values are represented as means $\pm$ SD ( $n=3$ ) (Gao et al. 2009).

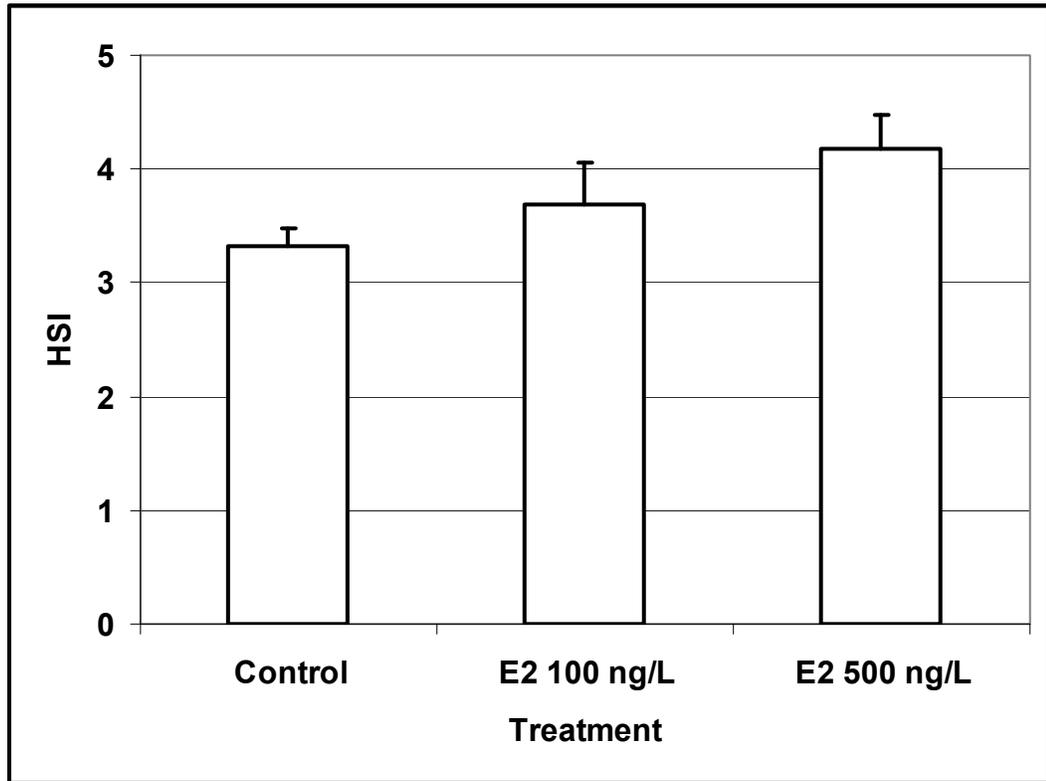
## **C. Exposure studies**

### **C. 1. Hepatosomatic Index (HSI)**

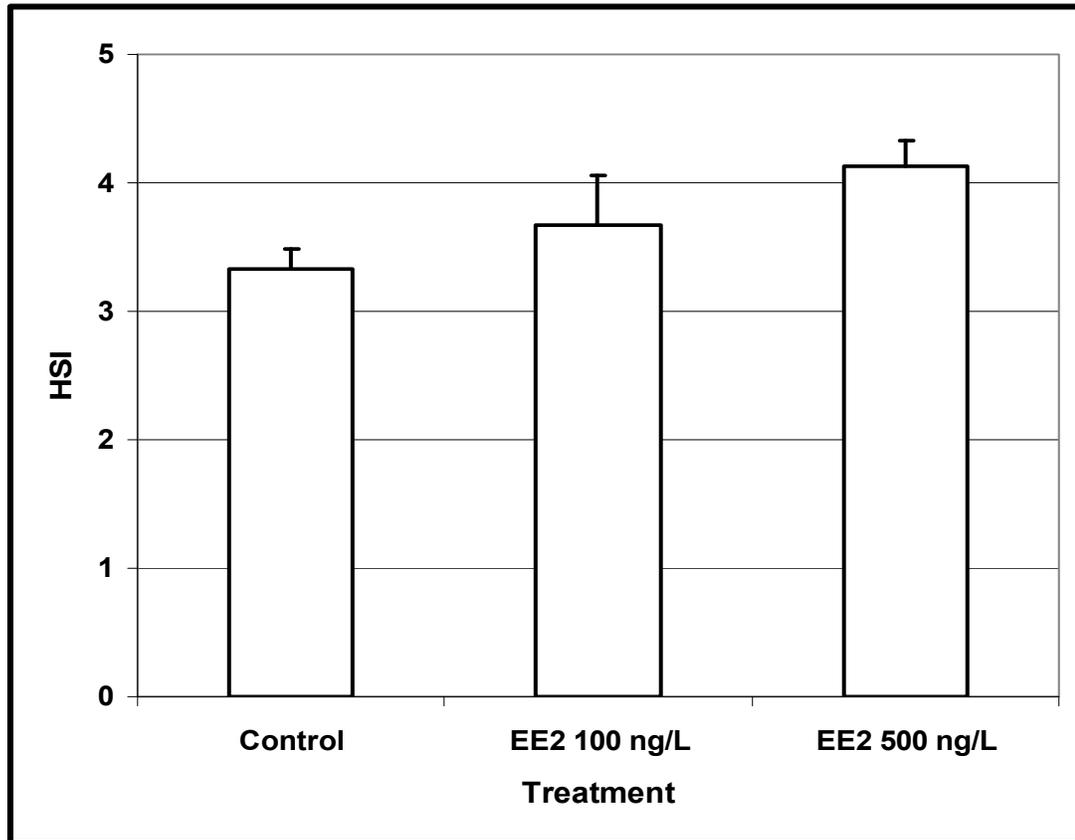
As expected, the HSI increased in the crayfish exposed to E2 and EE2, in contrast to the HSI in unexposed crayfish. Figure 17 compares the mean HSI of untreated crayfish with the ones exposed to 100 and 500 ng/L E2. As seen in the figure, animals exposed to 100 ng/L E2 had their hepatopancreas weights increased by 11% compared to the unexposed (control), and the ones exposed to 500 ng/L E2 had their liver weights increased by 26%. Figure 18 compares the mean HSI of untreated crayfish with the ones exposed to 100 and 500 ng/L EE2. Animals exposed to 100 ng/L EE2 had their liver weights increased by 11% compared to the unexposed (control), and the ones exposed to 500 ng/L EE2 had their liver weights increased by 24%.

### **C. 2. Expression of sex-steroid nuclear receptors**

Total RNA extracted from hepatopancreas of *P. clarkii*, exposed for 40 days to sublethal and nominal concentrations (100 and 500 ng/L) of E2 and EE2, were subjected to Q-RT-PCR to investigate the expression pattern of estrogen receptors *PcEcrR* and *PcRXR* in the presence of these EDCs.



**Figure 17.** Hepatosomatic index (HSI; hepatopancreas weight [g]/body weight [g] \* 100) of *Procambarus clarkii* following a 40 day exposure to nominal concentrations of 100 and 500 ng/L E2. No significant difference was observed between control and treatments (*t*-test,  $p > 0.05$ )



**Figure 18.** Hepatosomatic index (HSI; hepatopancreas weight [g]/body weight [g] \* 100) of *Procambarus clarkii* following a 40 day exposure to nominal concentrations of 100 and 500 ng/L EE2. No significant difference was observed between control and treatments (*t*-test,  $p > 0.05$ )

### **C. 2. a. E2 exposure**

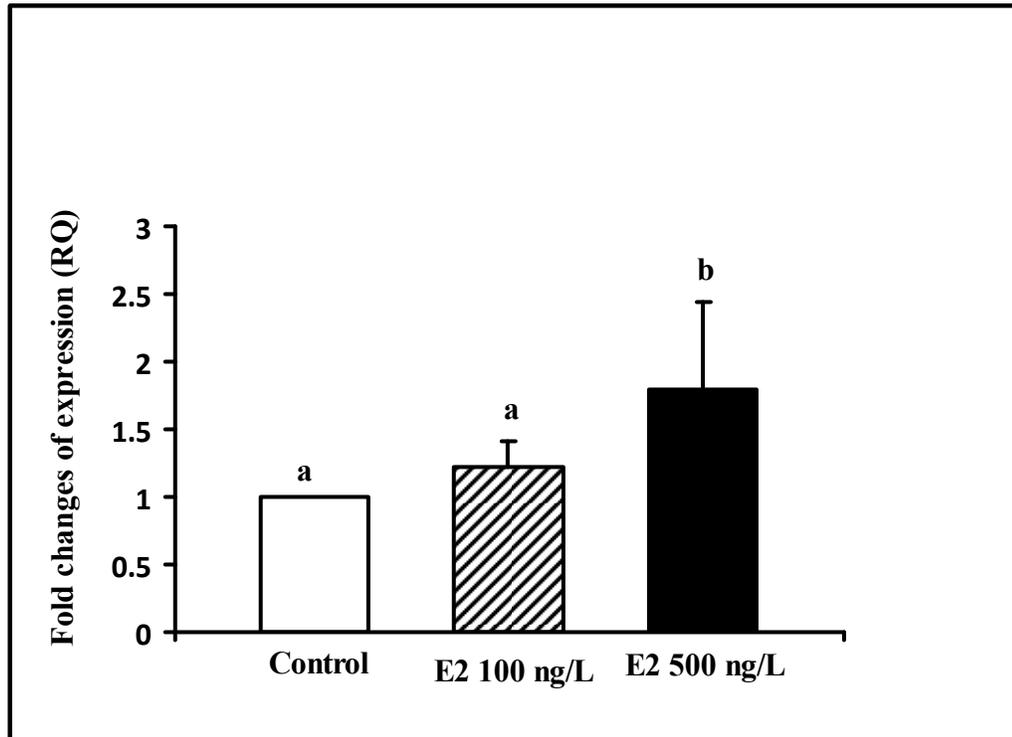
A 40-day exposure to 100 and 500 ng/L of E2 resulted in elevation of *PcEcR* and *PcRXR* mRNA levels in crayfish hepatopancreas compared to control. The *PcEcR* expression levels were not significantly different between control and 100 ng/L E2 treatment (Figure 19; *t*-test,  $p=0.353$ ) with a ~1.2 fold increase when compared to control, whereas mRNA expression levels were significantly elevated ~1.8-fold (*t*-test,  $p=0.029$ ) in the crayfish exposed to 500 ng/L E2 (Figure 19).

The *PcRXR* expression levels increased by ~1.3-fold when exposed to 100 ng/L of E2 compared to control crayfish. However, this increase was not significantly different (Figure 20; *t*-test,  $p=0.234$ ). Whereas the expression levels were significantly elevated by ~1.7-fold (*t*-test,  $p=0.004$ ) in the crayfish exposed to 500 ng/L E2 (Figure 20).

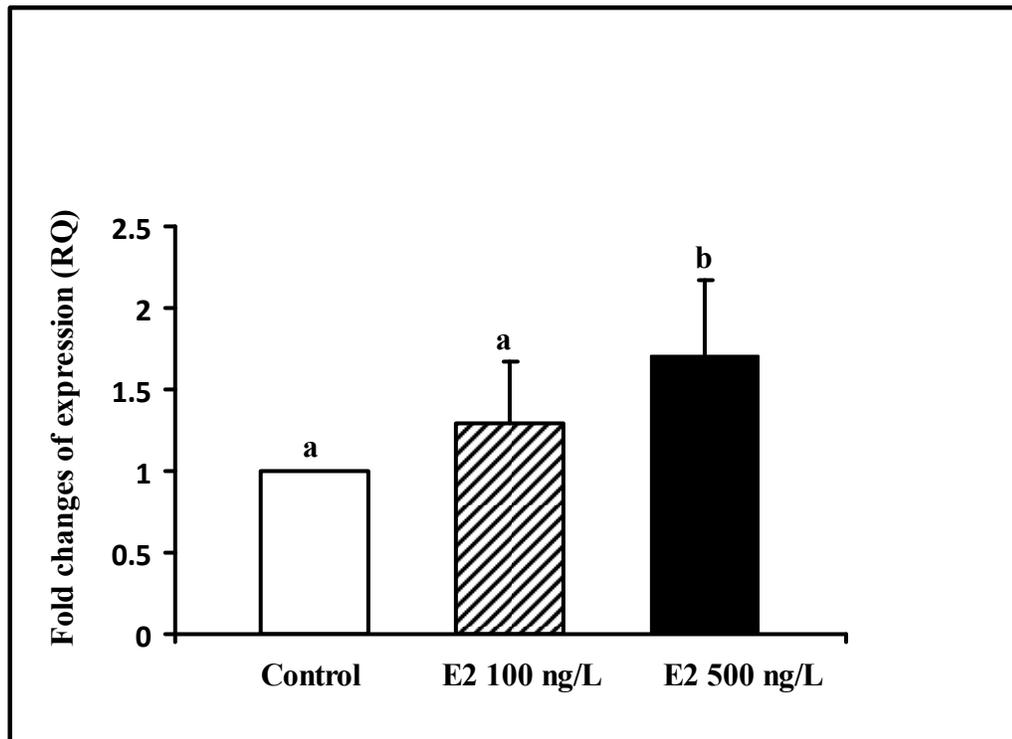
### **C. 2. b. EE2 exposure**

There was an observed increase in *PcEcR* expression levels, with ~1.2-fold increase in crayfish exposed to 100 ng/L of EE2 and ~1.6-fold increase in the ones exposed to 500 ng/L of EE2 (Figure 21). However, these elevations were not statistically significant (*t*-test; EE2 100 ng/L:  $p=0.946$ , EE2 500 ng/L:  $p=0.216$ ).

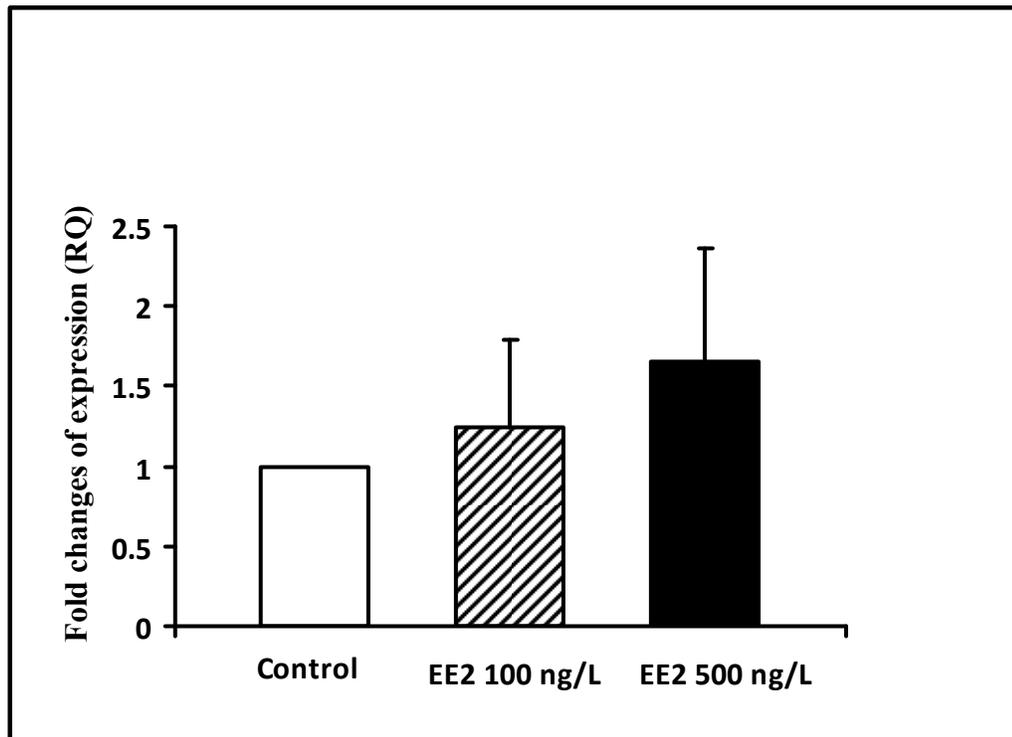
The hepatopancreatic *PcRXR* mRNA levels were significantly elevated in the crayfish exposed to both 100 and 500 ng/L of EE2 (Figure 22). There was an increase in expression by ~1.2-fold (*t*-test,  $p=0.005$ ) when exposed to 100 ng/L, and by ~1.9-fold (*t*-test,  $p=0.008$ ) when exposed to 200 ng/L.



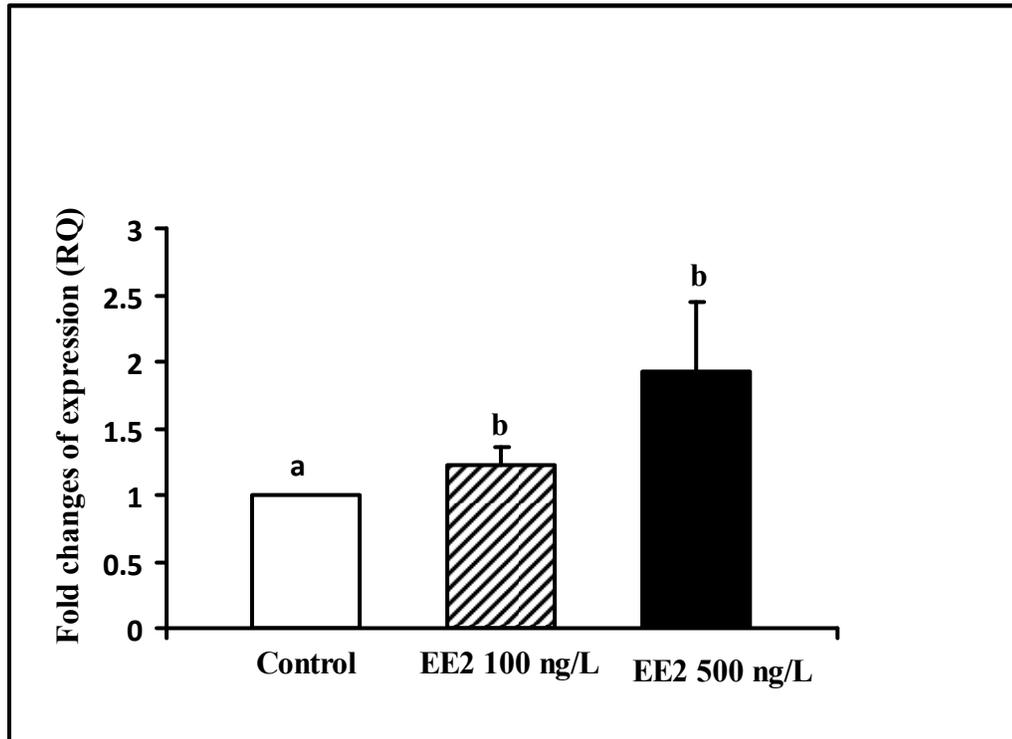
**Figure 19.** Q-RT-PCR assay for the expression of crayfish *Procambarus clarkii* EcR mRNA in hepatopancreas exposed to nominal concentrations E2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M ( $n=3$ ); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant differences from control. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ ).



**Figure 20.** Q-RT-PCR assay for the expression of crayfish *Procambarus clarkii* RXR mRNA in hepatopancreas exposed to nominal concentrations E2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p < 0.05$ ).



**Figure 21.** Q-RT-PCR assay for the expression of crayfish *Procambarus clarkii* EcR mRNA in hepatopancreas exposed to nominal concentrations EE2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. No significant difference was observed between control and treatments (one-way ANOVA,  $p > 0.05$ )



**Figure 22.** *Q-RT-PCR* assay for the expression of crayfish *Procambarus clarkii* RXR mRNA in hepatopancreas exposed to nominal concentrations EE2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M ( $n=3$ ); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ ).

### **C. 3. Measurement of cyclic nucleotides**

Cyclic nucleotides (cAMP and cGMP) extracted from hepatopancreas of *P. clarkii*, exposed for 40 days to sublethal and nominal concentrations (100 and 500 ng/L) of E2 and EE2, were subjected to the respective antibody specific ELISAs to determine the effects of these EDCs on the concentrations of cAMP and cGMP.

#### **C. 3. a. E2 exposure**

When compared to the control crayfish, the intracellular cAMP levels in hepatopancreas increased in crayfish exposed to 100 and 500 ng/L of E2 (Figure 23). The glandular cAMP levels increased by >150-fold in the animals exposed to 100 ng/L of E2 (*t*-test,  $p=0.026$ ), whereas the cAMP levels increased by ~30-fold in the ones exposed to 500 ng/L of E2 (*t*-test,  $p=0.062$ ).

The intracellular cGMP levels did not show any significant difference between the control and E2 treatments (*t*-test; E2 100ng/L:  $p=0.192$ , E2 500 ng/L:  $p=0.482$ ) (Figure 24).

#### **C. 3. b. EE2 exposure**

When exposed to the nominal concentrations of EE2, the intracellular cAMP concentrations in hepatopancreas significantly increased by ~35-fold at 100 ng/L (*t*-test,  $p=0.022$ ), and by ~1000-fold at 500 ng/L (*t*-test,  $p=0.001$ ) (Figure 25).

Similar to the trend observed with E2 exposure, the intracellular cGMP levels were not significantly different between the control and EE2 treatments (*t*-test; EE2 100 ng/L:  $p=0.383$ , EE2 500 ng/L:  $p=0.929$ ) (Figure 26).

#### **C. 4. Expression of calcium transporters**

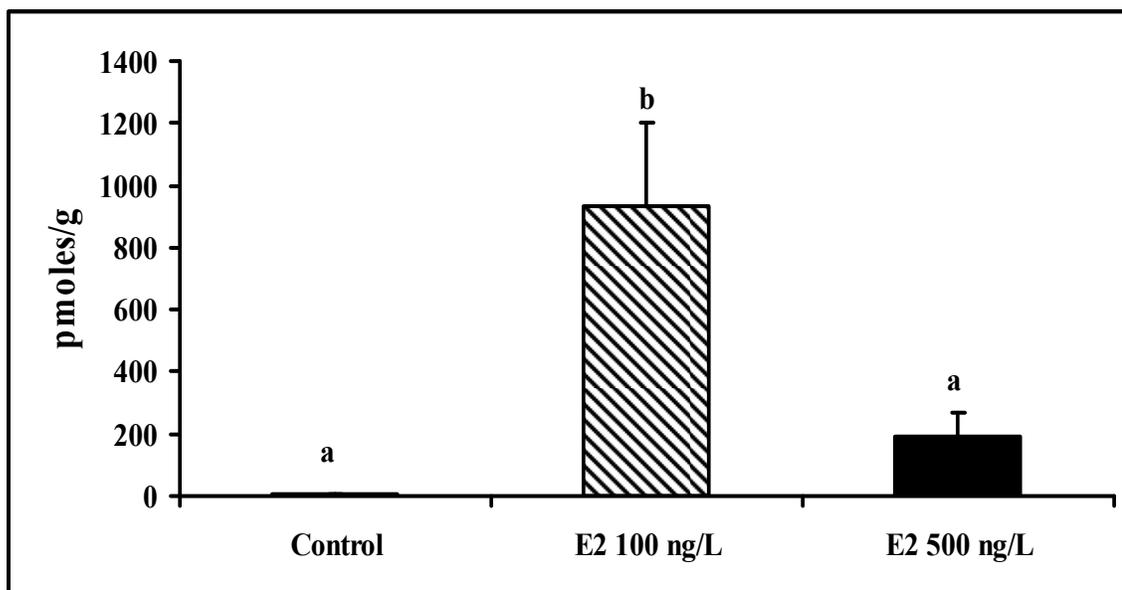
Q-RT-PCR data revealed a general trend towards an increased mRNA expression, when exposed to nominal concentrations (100 and 500 ng/L) of E2 and EE2, for all the three calcium transporters (SERCA, PMCA and CaM) in crayfish hepatopancreas.

##### **C. 4. a. E2 exposure**

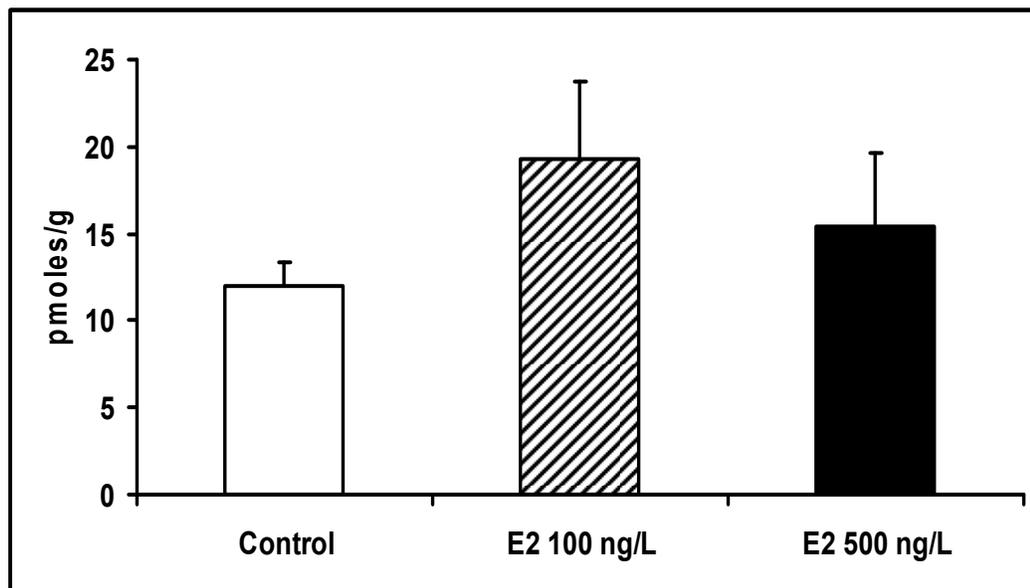
The hepatopancreatic SERCA mRNA showed an increased expression in the crayfish exposed to 100 and 500 ng/L of E2 when compared to control (Figure 27). The ~1.2-fold increase in SERCA expression at 100 ng/L was not statistically significant (*t*-test,  $p=0.343$ ), whereas the ~2.3-fold increase at 500 ng/L was statistically significant (*t*-test,  $p=0.026$ ).

The PMCA expression increased by ~1.5-fold at both E2 concentrations (*t*-test; 100 ng/L:  $p=0.066$ , 500 ng/L:  $p=0.036$ ) (Figure 28).

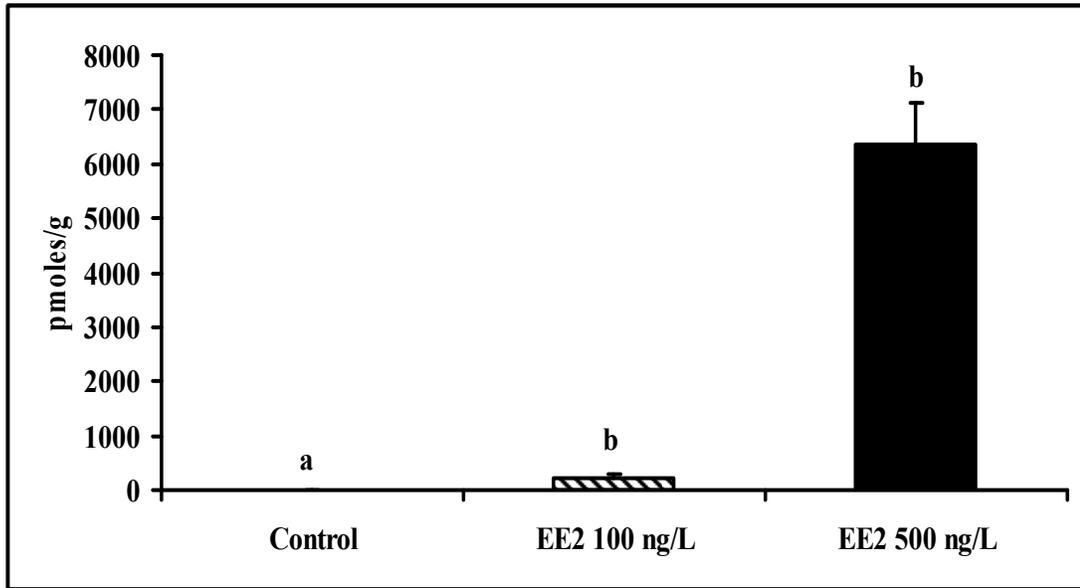
The expression of CaM significantly increased by ~3.4-fold at 100 ng/L (*t*-test,  $p=0.019$ ), and by ~2.2-fold at 500 ng/L (*t*-test,  $p=0.041$ ) (Figure 29).



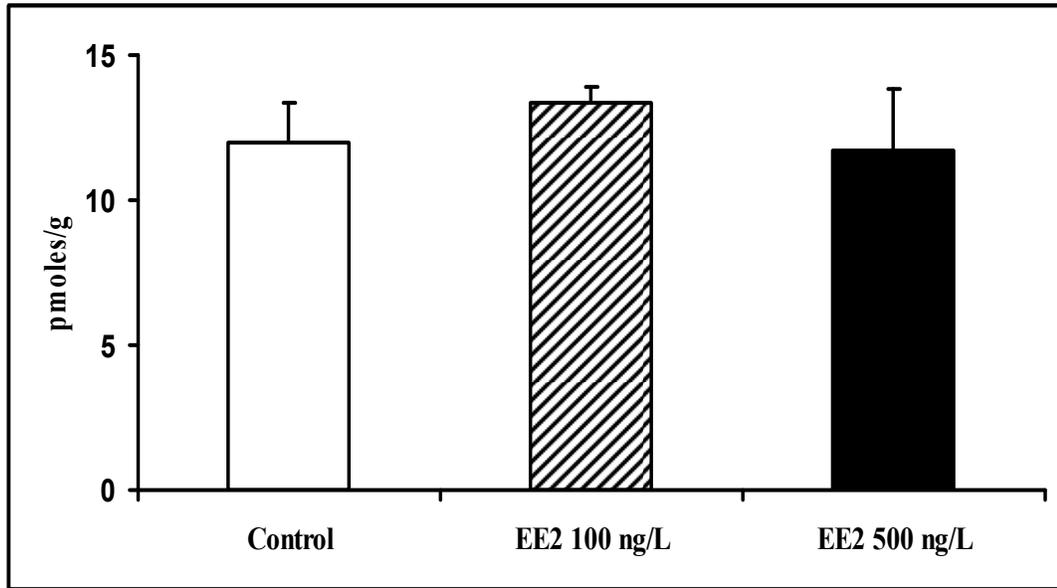
**Figure 23.** Intracellular cAMP levels in hepatopancreas of *Procambarus clarkii* exposed to nominal concentrations of 100 and 500 ng/L of E2. Results are expressed as mean $\pm$ SEM ( $n=3$ ). Different letters above the error bars represent significant differences from control. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ ).



**Figure 24.** Intracellular cGMP levels in hepatopancreas of *Procambarus clarkii* exposed to nominal concentrations of 100 and 500 ng/L of E2. Results are expressed as mean $\pm$ SEM ( $n=3$ ). No significant difference was observed between the control and treatments (one-way ANOVA,  $p>0.05$ ).



**Figure 25.** Intracellular cAMP levels in hepatopancreas of *Procambarus clarkii* exposed to nominal concentrations of 100 and 500 ng/L of EE2. Results are expressed as mean $\pm$ SEM ( $n=3$ ). Different letters above the error bars represent significant differences control. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ ).



**Figure 26.** Intracellular cGMP levels in hepatopancreas of *Procambarus clarkii* exposed to nominal concentrations of 100 and 500 ng/L of EE2. Results are expressed as mean $\pm$ SEM ( $n=3$ ). No significant difference was observed between the control and treatments one-way ANOVA,  $p>0.05$ ).

#### **C. 4. b. EE2 exposure**

When exposed to EE2, the SERCA expression in crayfish hepatopancreas increased by ~1.9-fold at 100 ng/L, and by ~1.6-fold at 500 ng/L (Figure 30). The increase at 100 ng/L was found statistically not significant (*t*-test,  $p=0.093$ ), whereas at 500 ng/L it was significant (*t*-test,  $p<0.0001$ ).

The PMCA expression increased by ~1.1-fold at 100 ng/L, and ~1.8-fold at 500 ng/L of EE2 (*t*-test; 100 ng/L:  $p=0.883$ , 500 ng/L:  $p=0.072$ ) (Figure 31).

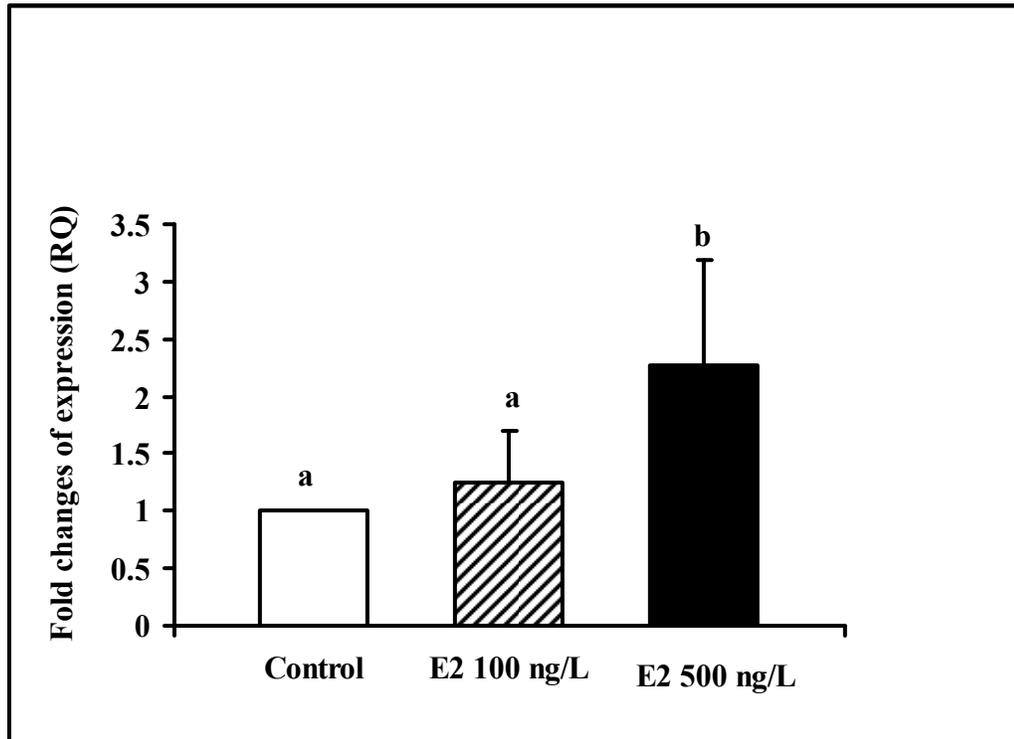
The expression of CaM significantly increased by ~5.9-fold at 100 ng/L (*t*-test,  $p=0.049$ ), and by ~3.5-fold at 500 ng/L (*t*-test,  $p=0.005$ ) (Figure 32).

#### **D. Chitinase gene expression**

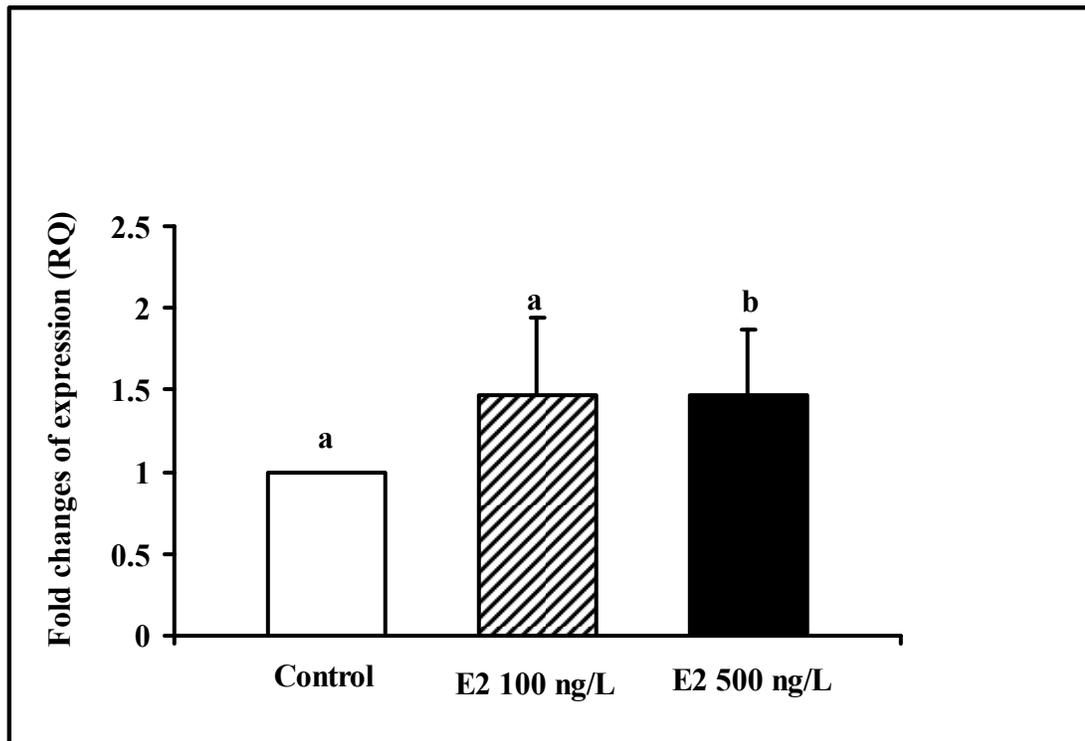
In this study, chitinase is used as a “target gene” for the assessment of molting hormone during natural molting cycle, and the disruption of molting cycle by the anti-molting hormone activities associated with EDCS. The relative expression of chitinase gene in crayfish hepatopancreas during molting cycle, and when exposed to E2 and EE2 was determined by Q-RT-PCR.

##### **D. 1. Molt cycle studies**

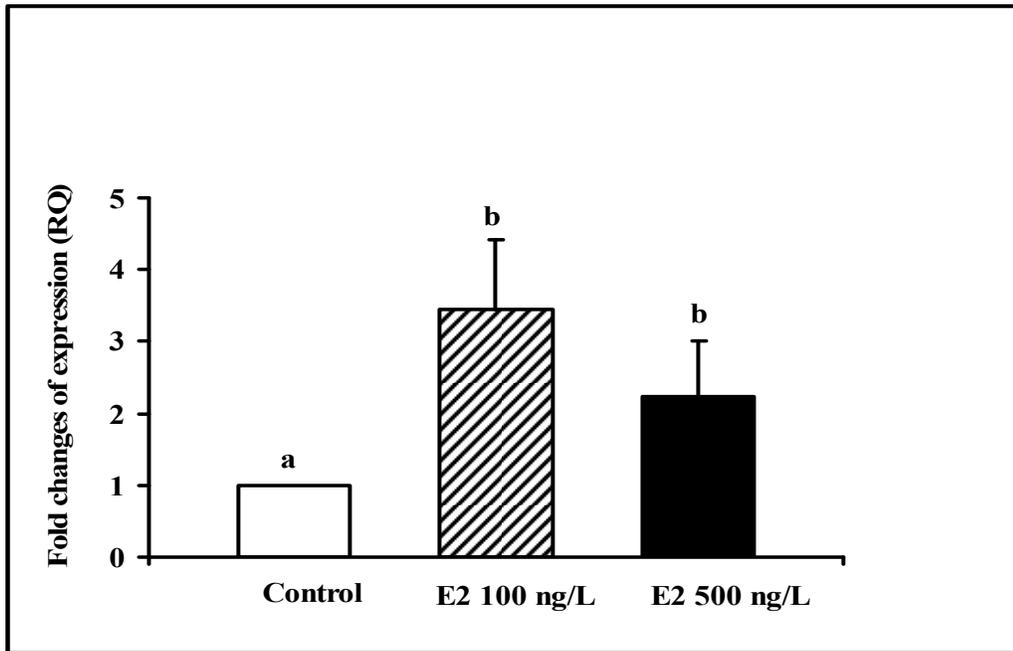
The hepatopancreatic *PcChi* expression was significantly higher during the postmolt when compared to inter and premolt stages of crayfish (*t*-test,  $p=0.008$ ) (Figure 33).



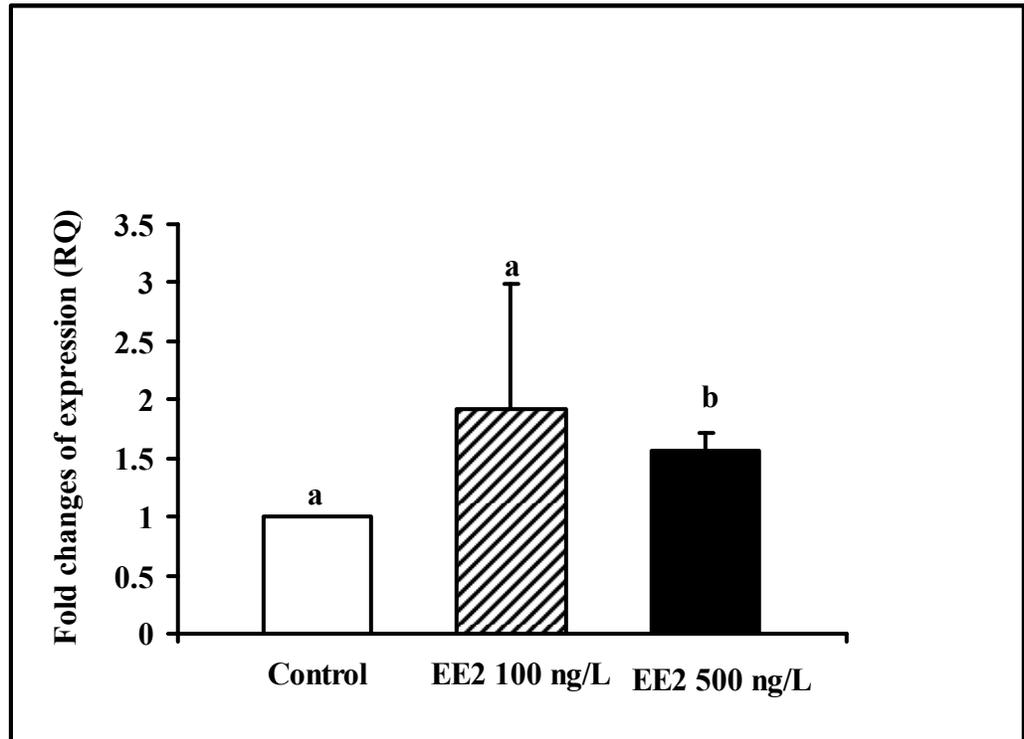
**Figure 27.** Q-RT-PCR assay for the expression of crayfish *Procambarus clarkii* SERCA mRNA in hepatopancreas exposed to nominal concentrations E2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M ( $n=3$ ); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ ).



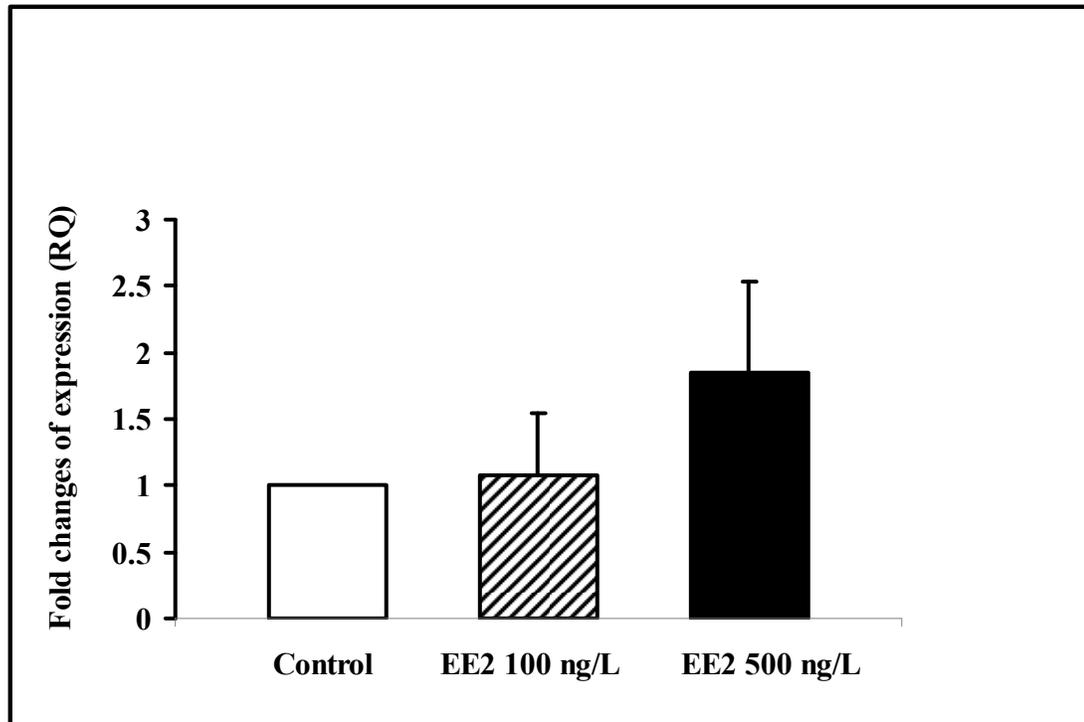
**Figure 28.** Q-RT-PCR assay for the expression of crayfish *Procambarus clarkii* PMCA mRNA in hepatopancreas exposed to nominal concentrations E2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p < 0.05$ ).



**Figure 29.** *Q-RT-PCR* assay for the expression of crayfish *Procambarus clarkii* CaM mRNA in hepatopancreas exposed to nominal concentrations E2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M ( $n=3$ ); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ ).



**Figure 30.** Q-RT-PCR assay for the expression of crayfish *Procamburus clarkii* SERCA mRNA in hepatopancreas exposed to nominal concentrations EE2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p < 0.05$ ).

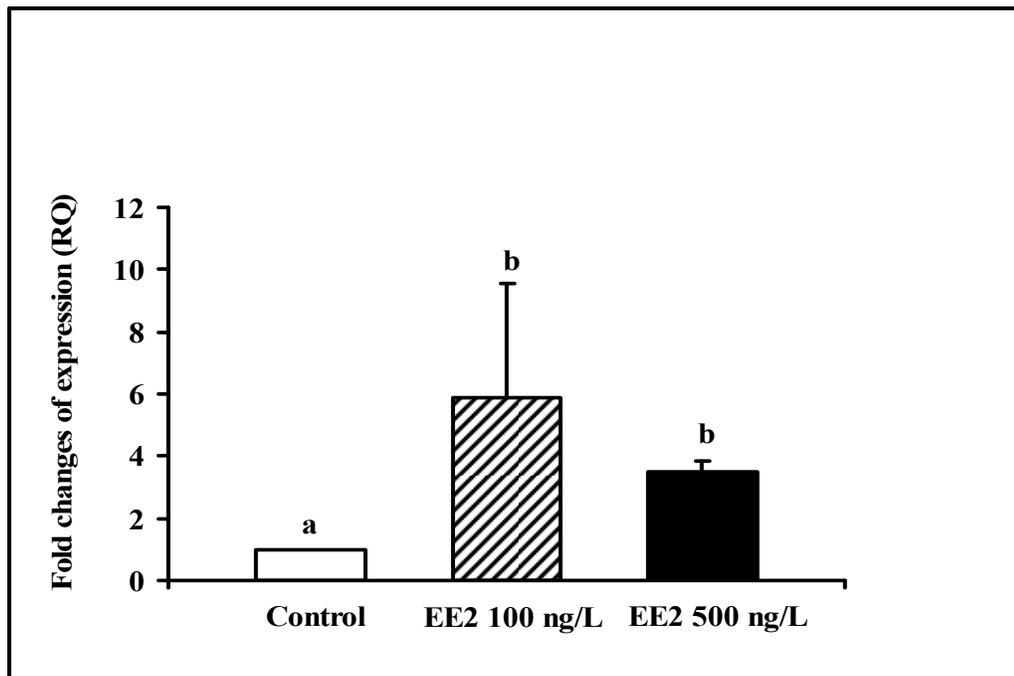


**Figure 31.** Q-RT-PCR assay for the expression of crayfish *Procamburus clarkii* PMCA mRNA in hepatopancreas exposed to nominal concentrations EE2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M (n=3); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. There was no significant difference between treatments and control (one-way ANOVA ,  $p>0.05$ ).

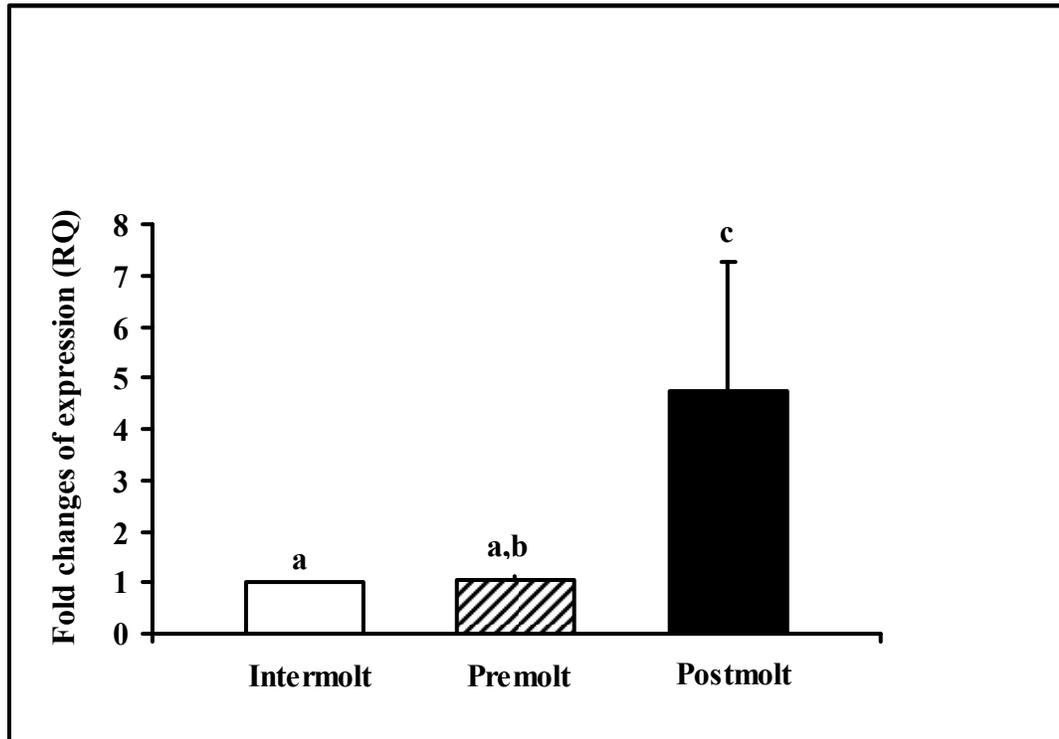
## D. 2. Exposure studies

The hepatopancreatic *PcChi* expression was significantly higher (~2.3-fold) in crayfish exposed to 100 and 500 ng/L of E2 when compared to control crayfish (*t*-test; 100 ng/L E2:  $p=0.047$ , 500 ng/L E2:  $p=0.029$ ) (Figure 34).

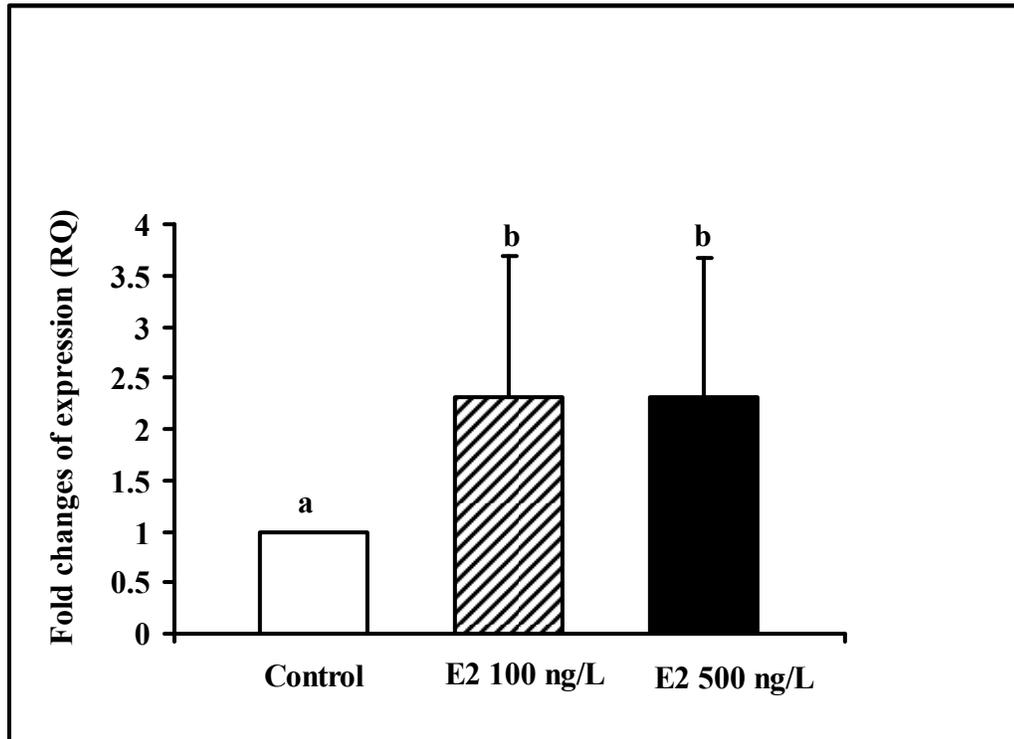
When compared to control, the hepatopancreatic *PcChi* expression was not significantly different in crayfish exposed to 100 ng/L of EE2 (*t*-test,  $p=0.468$ ), whereas the expression was significantly higher (~3.8-fold) in the ones exposed to 500 ng/L of EE2 (*t*-test,  $p<0.0001$ ) (Figure 35).



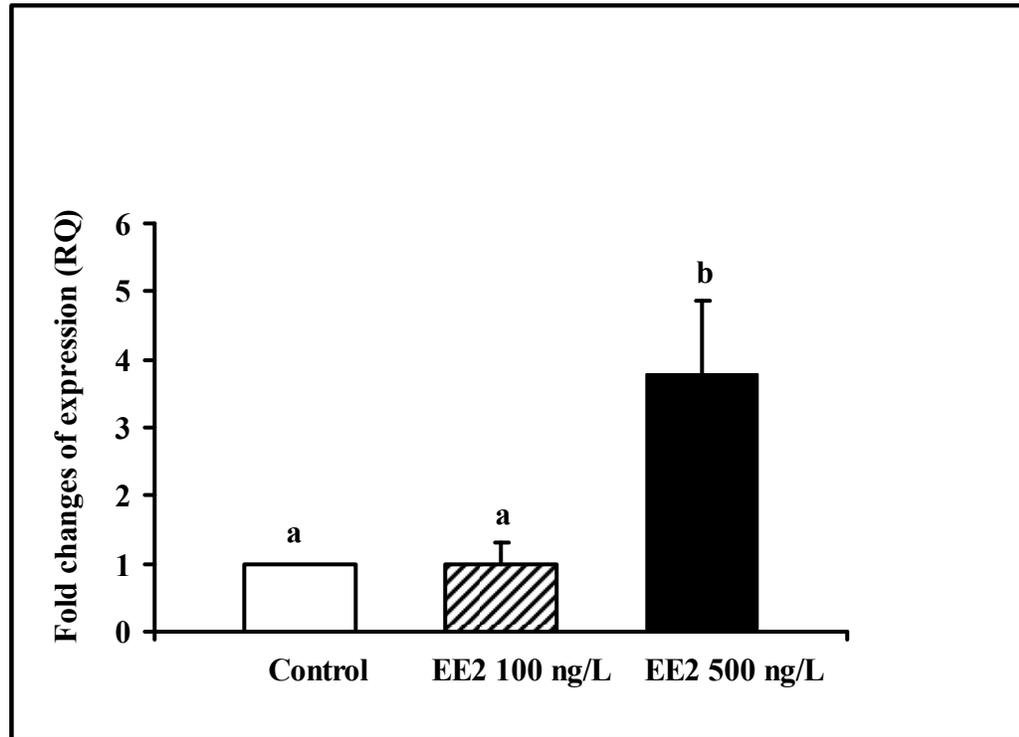
**Figure 32.** *Q-RT-PCR* assay for the expression of crayfish *Procambarus clarkii* CaM mRNA in hepatopancreas exposed to nominal concentrations EE2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M ( $n=3$ ); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ ).



**Figure 33.** *Q*-RT-PCR assay for the expression of *Procambarus clarkii* Chi mRNA in hepatopancreas as a function of molting stage. The calibrator stage was intermolt. Values are represented as means $\pm$ SEM ( $n=3$ ). Different letters above the error bars represent significant differences. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ ).



**Figure 34.** *Q-RT-PCR* assay for the expression of crayfish *Procambarus clarkii* *Chi* mRNA in hepatopancreas exposed to nominal concentrations E2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M ( $n=3$ ); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ ).



**Figure 35.** *Q-RT-PCR* assay for the expression of crayfish *Procamburus clarkii* *Chi* mRNA in hepatopancreas exposed to nominal concentrations EE2 for 40 days. Relative quantification (RQ) expressed as mean  $\pm$ S.E.M ( $n=3$ ); it was performed by normalizing the Ct value of each sample with the Ct value of the endogenous control (18s rRNA gene,  $\Delta$ Ct) and finally calculated using  $\Delta$ Ct of control as calibrator. Different letters above the error bars represent significant difference from control. Statistical analysis was performed using one-way ANOVA ( $p<0.05$ ).

### ***E. E2 and EE2 sample analysis***

Measured concentrations of test solutions for both chemicals were either in close agreement with the nominal values, or slightly divergent (Table. 3 and 4). The concentrations measured were slightly higher for both nominal concentrations (100 and 500 ng/L) with the exception of EE2 at 500 ng/L, where the concentrations were found slightly lower. The effective concentrations were calculated by taking the geometric mean from all measured concentrations.

Nominal (ng/L)	Measured (ng/L)			Effective (ng/L)
	Day 1	Day 20	Day 40	
100	111.3 ± 15.1 (+11.3%)	134.5 ± 13.7 (+34.5%)	131.7 ± 24.7 (+31.7%)	125.4 (+25.4%)
500	532.7 ± 54.2 (+6.5%)	705.9 ± 120.0 (+41.2%)	772.3 ± 329.7 (+54.5%)	662.2 (+32.4%)

**Table 3.** Nominal, measured and effective concentrations of E2 in crayfish-held glass bowls during the 40-day exposure experiment. Concentrations (ng/L) were presented as mean values (n=3) with standard error. Percentage differences relative to nominal concentration are presented in brackets.

Nominal (ng/L)	Measured (ng/L)			Effective (ng/L)
	Day 1	Day 20	Day 40	
100	110.9 ± 43.5 (+10.9%)	146.6 ± 1.5 (+46.6%)	148.2 ± 13.1 (+48.2%)	134.1 (+34.1%)
500	394.1 ± 4.3 (-21.2%)	388.7 ± 27.1 (-22.3%)	428.8 ± 28.3 (-14.2%)	403.5 (- 19.3%)

**Table 4.** Nominal, measured and effective concentrations of EE2 in crayfish-held glass bowls during the 40-day exposure experiment. Concentrations (ng/L) were presented as mean values (n=3) with standard error. Percentage differences relative to nominal concentration are presented in brackets.

## IV. DISCUSSION

### **A. Molt cycle studies**

As expected, all three proposed components of crustacean endocrine pathway, i) nuclear receptors (EcR/RXR), ii) cyclic nucleotides (cGMP/cAMP), and iii) calcium transporters (SERCA, PMCA, and CaM), demonstrated differential temporal expression pattern during molt cycle.

#### **A. 1. Expression of nuclear receptors**

In this study, we first partially cloned two cDNAs encoding PcEcR and PcRXR from the freshwater crayfish, *P. clarkii* (Figure 7 and Figure 8). Since these sequences, which belong to the ligand binding domain, were found to be highly similar to known EcR and RXR sequences of other crustaceans, they appeared to be components of an ecdysteroid receptor heterodimer complex (EcR/RXR) of *P. clarkii*. Following partial cloning, Q-RT-PCR primers were designed (Table. 2) to document the temporal expression of EcR and RXR through the molting cycle.

Changes in mRNA expression levels of PcEcR and PcRXR through different molting stages suggested the hormonal control of these genes at the transcription level. The PcEcR expression increased at the premolt when ecdysone synthesis is upregulated (Figure 10). This finding agrees with an earlier study which documented similar (increased) expression of EcR in the hepatopancreas of other crustacean species, *M. japonicus* during the premolt stage (Asazuma et al. 2007). As Hepatopancreas serves as a major metabolic and storage organ, changes in proteins, lipids, and carbohydrates have

been observed during crustacean molt cycle (Chang 1995). Along with these metabolites changes in activity of ecdysteroid-responsive enzymes, chitinase and chitobiase, have been documented in crustacean hepatopancreas; both of these enzymes manifested higher activity during premolt stage (Zou and Fingerman 1999; Tan et al. 2000). This suggests that various enzymes and genes of physiological importance in crustacean hepatopancreas may be regulated by molting hormones (ecdysteroids).

The mRNA expression pattern of PcEcR during molt cycle did not coincide with that of PcRXR, where the expression increased progressively during the molting period (Figure 11). This implies that the expression of PcRXR is not controlled by ecdysteroid only. For example, Asazuma et al. (2007) found that the hepatopancreatic RXR mRNA expression remained constant throughout the molt cycle of kuruma prawn (*M. japonicus*). The general consensus from previous studies involving whole organism and tissue-specific expression of EcR and RXR (and its ortholog ultraspiracle, USP) suggest that while EcR is responsive to molt cycle with higher expression during premolt stage, its heterodimer partner RXR (or USP) expression did not show any consistent pattern (Durica et al., 1999; Asazuma et al., 2007; Kato et al., 2007; Priya et al., 2009). While cellular responses to ecdysteroids in target cells are mediated by EcRs (Vafopoulou et al., 2005), the same is not true with RXRs which are associated with other nuclear receptors as heterodimeric partners in several other hormonal response systems (Sukov et al., 1994). The role of RXR may be largely allosteric, i.e. it helps in stabilizing EcR in confirmation of binding hormone (ecdysone).

## A. 2. Measurement of cyclic nucleotides

In this study I found a significant increase in cAMP levels in crayfish hepatopancreas during premolt stage (Figure 12), as well as a significant decrease in cGMP levels in the postmolt stage (Figure 13). These findings are in contrast to those from earlier studies which suggested a decrease in the concentrations of cAMP (and in some cases cGMP) during the premolt stage of several crustacean species (Mattson and Spaziani, 1985; Spaziani et al., 1999; Lee and Mykles, 2006; Nakatsuji et al., 2006; Nagai et al., 2009). Those studies have shown that MIH negatively regulates ecdysteroidogenesis by cAMP or/and cGMP mediated signaling pathways. However, those studies involved identification of second messenger pathways that regulate ecdysteroidogenesis in the molting gland, the Y-organ. The studies suggest that MIH regulates molting via either cAMP or cGMP, or both, where the increase in MIH and the MIH-receptor occupancy leads to increase in intracellular cyclic nucleotide levels, leading to the suppression of ecdysteroidogenesis. Although most of the above-mentioned studies implicated cGMP in the regulation of ecdysteroidogenesis, a study by Mattson and Spaziani (1995) implicated cAMP. Thus, there is no general consensus regarding the primary second messenger involved in the signaling pathway associated with ecdysteroidogenesis. Interpretation of the results from these studies in very few species may be misleading. Also, it should be noted that these studies were conducted *in vitro* and involved controlled conditions such as synchronization of molting by eye-stalk ablation, and the cyclic nucleotides were measured over a shorter time periods (within hours). Whereas my study was conducted *in vivo* and the cyclic nucleotides were quantified over the more extended time period associated with the natural molt cycle.

The cGMP and cAMP are found to exhibit species and tissue specificity in their regulation of ecdysteroidogenesis (Nagai et al., 2009). The distribution of MIH receptors varies among tissues, which in turn may influence cAMP/cGMP production, and thereby ecdysteroid levels in an organ system (Watson et al., 2001). The responsiveness of an organ system can be dynamic and the variation in responsiveness may be due to specific levels of cyclic nucleotides rather than a simple increase or decrease in concentrations. In the Y-organ, which is a primary organ for ecdysteroidogenesis, the MIH and its receptor occupancy play a central role in regulation of cyclic nucleotide levels, whereas in a target and a metabolic organ such as hepatopancreas, the cyclic nucleotide levels may be determined by the occupancy of other receptors. The above-mentioned uncertainties may explain the contrary findings of our study. To our knowledge, for the first time our study quantified cyclic nucleotides *in vivo* involving the natural molt cycle of *P.clarkii*.

### **A. 3. Expression of calcium transporters**

One of the major objectives of this study was to determine the relative expression of calcium transporters (SERCA, PMCA, and CaM) during the crayfish molt stages when whole organism  $\text{Ca}^{2+}$  flux was elevated (pre- and postmolt) compared to a baseline condition of relative organismal  $\text{Ca}^{2+}$  balance (intermolt: control). Using Q-RT-PCR technique it is possible to examine the mRNA expression of proteins involved in  $\text{Ca}^{2+}$  balance, and eventually understand the hormonal regulation of these genes. Prior studies have documented upregulation of these calcium transporters during pre- and postmolt compared with intermolt in both epithelial (antennal gland) and non-epithelial (axial abdominal muscle) tissues (Gao and Wheatly, 2004; Wheatly et al., 2004; Gao et al.,

2009). In this study we documented for the first time the relative expression of SERCA and PMCA in another major tissue associated with trans-epithelial  $\text{Ca}^{2+}$  flux during molting, the hepatopancreas; CaM expression in hepatopancreas was earlier documented by Gao et al. (2009). It is quite evident that all three transporters showed uniform increase in expression levels in pre- and postmolt when compared with intermolt. In fact we observed excellent numerical correspondence in the increase of expression for all the calcium transporters in hepatopancreas during the molt cycle (Figures 14, 15, 16). This suggests that calcium transporters chosen for this study (SERCA, PMCA, CaM) play an important role in the crustacean molt cycle, and the common increase in their expression in pre- and postmolt stages suggests that the upregulation is hormonally mediated.

## **B. Exposure studies**

Many anthropogenic compounds, including EDCs are known to disrupt endocrine homeostasis and associated physiological systems, by mimicking and blocking the endogenous hormones (Lyons 2006). Both E2 and EE2 have been implicated as the primary contaminants contributing to the estrogenic activity in surface waters from both the United Kingdom and the United States (Skillman et al., 2006). Most studies attempting to understand endocrine disruption were focused on vertebrate systems, and relatively few focused on the tremendous array of invertebrates that inhabit fresh and marine water environments (Lye et al., 2008). Of the existing studies employing invertebrates, there have been very few studies addressing the mechanisms of action associated with endocrine disruption (Iguchi et al., 2006). Among invertebrates, crustaceans are very vulnerable to EDCs, however only a few studies have assessed a truly endocrine disrupting effect (Hutchinson 2007). In this study we used *P. clarkii* as a

model organism to understand the mechanistic molecular pathways associated with endocrine disruption. Hepatosomatic Index (HSI) provides a crude indication of the potential effects of EDCs and therefore useful in identifying any specific target areas (e.g., hepatopancreas) worthy of further investigation, and is so far there appear to be no comparative studies on HSI in crustaceans following exposure to EDCs (Lye et al., 2008). In vertebrates, particularly fish species, increase in HSI is associated with the induction of vitellogenin (vtg), a major precursor of egg yolk proteins vitellin (vt), in the liver of mature females, but also can be induced in males by exogenous treatment (Johnson et al., 1991; Pait and Nelson, 2002). The observed increases in HSI of crayfish exposed to nominal concentrations of E2 and EE2 in this study (Figures 17 and 18) are unlikely to be attributed to a shift in metabolism toward vtg production, as vt is not found in hepatopancreas. HSI is also a measure of energetic reserves of the liver and metabolic activity, and has been shown to increase in response to chemical contamination and the associated enhanced detoxification process (Slooff et al., 1983). Enlargement of the liver is due to either an increase in cell size (hypertrophy), or an increase in cell number (hyperplasia) (van der Oost et al., 2003).

## **B. 1. Expression of sex-steroid nuclear receptors**

Much of the literature to date on the EDC issue focuses on steroid-hormone receptor-mediated toxicity (Iguchi et al., 2006). E2 and EE2 are strong estrogen agonists (Hutchinson 2002). These agonists compete with their endogenous counterparts for the binding site on the receptor. Recently, EcRs were discovered to be biochemical targets of xenobiotics in crustaceans (Mazurova et al., 2008). Earlier, it has been suggested that EDCs act on the EcR, and raises the threshold of ecdysone required to trigger molting in

crustaceans (Andersen et al., 2001). Studies on the specific mechanisms by which the endocrine system can be disrupted are scarce, and understanding receptor-mediated toxicity is an initial step toward understanding mechanistic molecular pathways associated with endocrine disruption. Thus to better understand molecular mechanisms of endocrine disruption in *P.clarkii*, this study attempted to integrate genomics into ecotoxicology. This study partially cloned hepatopancreatic EcR and RXR transcripts from crayfish, and designed primers to investigate the expression of these genes at the transcriptional (mRNA) level during the natural molt cycle and in the presence of E2 and EE2. As expected, exposure of crayfish to environmental estrogens (E2 and EE2) caused induction of hepatopancreatic EcR and RXR (Figures 19-22). It was important for this study to clarify the expression of EcR/RXR in order to address the mechanistic pathways associated with EDC-induced endocrine disruption in crustaceans. Despite the fact that EcR/USP has been characterized in insects and in some crustaceans (Yao et al., 1992; Durica and Hopkins, 1996; Asazuma et al., 2007; Kato et al., 2007; Wang et al., 2007) little is known about their complex functions in crustaceans. To our knowledge, this is the first study which quantified EcR and RXR mRNA expression levels in a crustacean exposed to environmental estrogens. Previous studies involving vertebrates (Filby et al., 2006) and invertebrates (Hirano et al., 2008) have found similar induction of EcR counterparts upon exposure to EDCs. As mentioned earlier, EcR regulates gene transcription in association with RXR. The induction of RXR mRNA upon exposure to E2 and EE2 may be due to the fact that these EDCs mimic retinoids, the endogenous ligands of RXR. For example Harmon et al. (1995) found methoprene, an insect growth regulator and estrogen (juvenile hormone) agonist, activated mammalian RXR. Also,

there may be a cross-link between ecdysteroid signaling and retinoic-acid signaling pathway. For example, Munetsuna et al. (2009) found RXR-mediated signaling stimulated the endogenous E2 and testosterone synthesis in male rat hippocampal slices; however this pattern is yet to be determined in crustacean cells. Among EDCs, organotins such as TBT and triphenyltin (TPT) have been shown to disrupt RXR-mediated signaling and were thus considered RXR agonists (Grün and Blumberg 2006). At the present time, there has been no documentation of E2 and EE2 being RXR agonists. For the first time, my study documented increased expression of crustacean RXR following exposure to these two chemicals. This effect may be due to the interference by these estrogens at the estrogen response elements (ERE) level. Also, multiple modes of action may exist for sex steroid receptors, and there is probably signaling cross-talk between EcR and RXR through competition for ERE binding.

## **B. 2. Measurement of cyclic nucleotides**

As mentioned earlier, previous studies focusing on the regulatory role of cyclic nucleotide (cGMP/cAMP) on crustacean molting, documented the role of MIH-induced suppression of ecdysteroidogenesis in the YO (Zheng et al., 2008). It is suggested that MIH binds to independent receptors thereby inducing an increase in intracellular cAMP (or cGMP). The increase in the cyclic nucleotide level leads to inhibition of ecdysteroidogenesis. However, there is inconclusive evidence about the MIH receptor and its link to cyclic nucleotide production (Covi et al., 2009). Zheng et al. (2009) cloned receptor guanylyl cyclase (rGC) from Y-organs of blue crab (*Callinectes sapidus*), and hypothesized that this putative receptor is an MIH receptor. The study proposed that rGC activation leads to an increase in cGMP (and not cAMP) which thereby suppresses the

ecdysteroidogenesis by the inhibition of receptor-mediated uptake of cholesterol. However, there is no documentation of ecdysteroidogenesis in other tissues such as hepatopancreas, antennal gland, or cardiac muscle etc. Previous studies focused on understanding the mechanisms associated with molting in YO, as it is a crustacean neuroendocrine gland that produces ecdysone. Our study has chosen hepatopancreas, as it plays a vital role in transcellular  $\text{Ca}^{2+}$  transport associated with molt cycle, and also is a target organ for EDCs. One of the hypotheses of this study is that, in the presence of E2 and EE2, there will be an increase in the induction of cyclic nucleotides in the crayfish hepatopancreas. Our study attempted to determine whether this increase follows genomic or non-genomic mechanisms. In this study, I found a significant increase in cAMP when exposed to these estrogens, whereas, no significant change in cGMP concentrations were observed (Figures 23-26). I found a similar pattern with regard to cyclic nucleotide response in the molt cycle studies, where cAMP was determined to be more responsive than cGMP. To our knowledge, this is the first time my study quantified cyclic nucleotides in hepatopancreas of crayfish exposed to estrogens. Previous studies which looked into the effects of estrogens on cyclic nucleotide levels were limited to *in vitro* studies involving vertebral cell lines. For example, Aronica et al. (1994) observed that the increase in cAMP concentrations in uterine and human breast cancer MCF-7 cells upon the treatment with E2, is due to the increase in the adenylate cyclase activity. The study attributed this increase to the possible binding of estrogen to its high-affinity estrogen receptors located in the membrane and cytoplasmic portion of the cell that do not appear to translocate into the nuclear compartment. Sirotkin et al. (1995) found an increase in the cAMP as well as cGMP concentration in E2-exposed human granulosa

cells. That study suggested that the increase in cyclic nucleotide concentrations was associated with the formation of new steroid receptors. Earlier, there was a common notion where the steroid hormones follow genomic pathways with ligands directly binding to their receptors, which in turn bind to nuclear DNA without the involvement of intracellular cyclic nucleotide-protein kinase system (Catt and Dufau, 1982; Richards and Hedin, 1988). However, some of the above-mentioned previous studies which found the estrogen induced increase in cyclic nucleotide concentrations have broke this notion. Thus there can be two cross-linking pathways associated with cyclic nucleotide production: one, the genomic-pathway which may lead to transcription of estrogen-regulated genes; two, the non-genomic pathway where the estrogens regulate cAMP-mediated gene expression via cAMP-signaling. In order to better understand whether the observed increase in cAMP levels in our study, is associated with a genomic or non-genomic pathway, it will be necessary to determine further downstream elements such as kinases, phosphatases, and phosphodiesterases.

### **B. 3. Expression of calcium transporters**

As described previously, the effects of an estrogen may be mediated by regulation of intracellular signaling mechanisms. The central role of the  $\text{Ca}^{2+}$ -messenger system in various aspects of cell function, makes it a logical focus for examination of possible disturbance in  $\text{Ca}^{2+}$  homeostasis and  $\text{Ca}^{2+}$ -mediated functions as underlying mechanisms of toxicant action (Pounds 1990). In this study I proposed that two intracellular second messenger systems, namely  $\text{Ca}^{2+}$  and the cyclic nucleotides, might play a crucial role in crustacean molt cycle and in its disruption by EDCs.

In general, my study observed an increase in the mRNA expression of the selected  $\text{Ca}^{2+}$  transporters (SERCA, PMCA, and CaM) when exposed to E2 and EE2 (Figures 27-32). Prior to my study, there has been no documentation on the effects of EDCs on crustacean  $\text{Ca}^{2+}$  homeostasis, studies involving vertebral cell lines have implicated various EDCs in affecting the activities of  $\text{Ca}^{2+}$  transporters. Most of these studies observed increase in the intracellular  $\text{Ca}^{2+}$  concentrations. For example, Liu et al. (2009) found E2 replacement in the ovariectomised rats under chronic intermittent hypoxia, increased the SERCA activity and mRNA expression. The increase in mRNA content may be an important molecular mechanism responsible for the increase in SR/ER calcium pump function. Other studies have shown that EDCs such as alkylphenols inhibit SERCA pumps in porcine cerebellar and rat testis microsomes (Khan et al., 2003). He et al. (2004) found an EDC fenvalerate inhibited follicle-stimulating hormone (FSH)-stimulated progesterone (P4) production in human ovarian luteinizing –granulosa cells (hGLCs). The study suggested that inhibition of steroidogenesis is partly mediated through calcium signal. This assumption is based on the fact that the study found decrease in FSH-stimulated cAMP, an increase in intracellular  $\text{Ca}^{2+}$  concentration, and increase in CaM concentration. PMCA activity was increased in renal distal tubular cell line when incubated in E2 (Dick et al., 2003). However, the study found no increase in PMCA expression. The above-mentioned examples of regulation of  $\text{Ca}^{2+}$  transporters by various EDCs may have implications in transcellular  $\text{Ca}^{2+}$  transport and regulation of cellular  $\text{Ca}^{2+}$  concentration. My study for the first time documented the effect of E2 and EE2 on the expression of  $\text{Ca}^{2+}$  transporters in crayfish hepatopancreas. Upregulation of  $\text{Ca}^{2+}$  transporters found in this study is in general agreement with the observed increase in

the intracellular  $\text{Ca}^{2+}$  concentrations manifested in the studies involving vertebral cell lines.

However, it should be noted that one of the studies mentioned earlier (He et al., 2004) found a decrease in cAMP concentration and increase in intracellular  $\text{Ca}^{2+}$  concentration in the presence of an EDC fenvalerate. This is in contrast to the observed increase in cAMP concentrations in our study. It should be noted that unlike our study which involved *in vivo* observations, previous findings involved quantification of cyclic nucleotides *in vitro*. Also, the regulatory systems such as endocrine and hepatopancreatic systems can be dynamic, and their responsiveness can be to specific levels of cyclic nucleotides rather than a simple increase and decrease in concentrations (Covi et al., 2009). This contradiction can also be attributed to several factors such as the type of EDCs, cell types, duration and method of exposure, and the model system chosen for these studies. Cyclic nucleotides and  $\text{Ca}^{2+}$  signaling pathways interact at multiple levels, and defining the specific loci where these two pathways interface will provide better understanding of these signaling components and their effect on steroidogenesis and other metabolic events. Thus understanding mechanisms and events downstream from the production of cAMP needs to be further explored in future studies.

### **C. Chitinase gene expression**

Chitinase (Chi) plays a crucial role in the molt cycle of arthropods, by dissolving chitin in the exoskeleton into more soluble forms so it can be reabsorbed into the body and reutilized for the new exoskeleton (Dall et al., 1990; Tan et al., 2000). In crustaceans, this enzyme is present in integument as well as hepatopancreas, where it is

believed to be involved in chitin degradation in exoskeleton and digestion of chitin-containing food respectively (Watanabe et al., 1998).

### **C.1. Molt cycle studies**

Chi was detected in significant levels in hepatopancreas throughout the crustacean molt cycle (Watanabe et al., 1996). Also, chitin synthesis has been shown to be controlled by the ecdysteroid receptor pathway (Watanabe et al., 1996; Gagou et al., 2002). As the hepatopancreas is the major crustacean storage and metabolic organ, I hypothesized that patterns of Chi expression in this organ would change during the molt cycle. The fact that the Chi expression represents a near terminal event in ecdysteroid signaling, our study assumed hepatopancreatic Chi mRNA an effective molecular biomarker for molt-disrupting effects of EDCs. Thus, in order to understand the crustacean molt cycle and its EDC-mediated disruption, my study looked into changes in the chitinase gene expression in crayfish hepatopancreas during the natural molt cycle, and in the intermolt hepatopancreas extracted from the crayfish exposed to E2 and EE2. I observed a significant increase in the *PcChi* expression in the postmolt stage of the crayfish (Figure 33). This observation did not concur with the findings from previous studies involving other crustacean species, where higher expression was noticed during premolt stage, a stage that is characterized by the chitin degradation by chitinolytic enzymes. For example, hepatopancreatic Chi gene expression was highest during the premolt stage of tiger shrimp *P. monodon*, suggesting Chi regulation by ecdysteroids (Tan et al., 2000).

In *Fenneropenaeus chinensis*, the expression of chitinase (*FcChi*) and chitinase 1 (*FcChi-1*) was detected in all molt stages, but was highest during the premolt stage (Priya

et al., 2009); however the expression fluctuated rather widely within premolt stages D0-D4. However, earlier study by Kono et al. (1995) observed that Chi activity in hepatopancreas did not vary significantly during the molt cycle of kuruma prawn *P. japonicus*. Proespraiwong et al. (2010) in their work on chitinases in *P. monodon* found that Chi 1 and 3 which is expressed specifically in the hepatopancreas, was relatively unchanged in expression during the molt cycle. The same study found Chi 2, which is expressed specifically in the gill, responsive to molt cycle. The results suggested the function of hepatopancreatic chitinases might be related to the digestion of chitinous food and degradation of endogenous chitin prior to molting. In hepatopancreas, although Chi along with other genes such as trypsin and cathepsin, belong to ecdysteroid-responsive group, these genes also function as typical metabolic genes controlling physiological processes such as transport and carbohydrate metabolism, which might also play a significant role during crustacean molt cycle (Shechter et al., 2007). Thus, the above-mentioned species- and -tissue specific differences in chitinase functionality, may explain the contrasting Chi expression found during molt cycle in our study. Most of the above-mentioned studies involved Chi expression in penaeid shrimp. Shetcher et al. (2007) in their search for hepatopancreatic ecdysteroid-responsive genes during the crayfish molt cycle, found all three chi genes (1, 2, and 3) downregulated during premolt stages D1-D2. These dissimilarities in expression patterns during the molt cycle may be explained by differences in hepatopancreas metabolism between penaeid shrimp and crayfish.

## C. 2. Exposure studies

Apolysis is a process where the old exoskeleton detaches from the underlying epidermis as the animal enters the premolt stage of the molt cycle, is triggered by ecdysteroids (Zou and Fingerman, 1999). The process also involves synthesis of chitinolytic enzymes chitinase and chitobiase, which are again under the control of ecdysteroids. Thus, in our study we chose to employ Chi as a biomarker to study the actions of steroid molting hormones of this study, E2 and EE2. Our study observed significant upregulation of *PcChi* mRNA expression in hepatopancreas of crayfish exposed to both estrogens (Figures 34 and 35). As of today, there has been no documentation of the effects of E2 and EE2 on the Chi gene expression in crustaceans. Previous studies involving environmental pollutants and organochlorine compounds (OC) such as Arochlor 1242, endosulfan, 2,4,5-trichlorobiphenyl (PCB29), and kepone upregulated the mRNA expression of another chitinolytic enzyme, N-acetyl- $\beta$ -glucosaminidase (NAG) in the epidermal tissues of the fiddler crab, *Uca pugilator* (Meng and Zou, 2009). The study observed these effects *in vitro*, and the treatment scheme consisted of singular exposure of OC, and a binary treatment involving 20-hydroxyecdysone (20-HE) and OC. Binary exposure led to downregulation of NAG mRNA compared to positive control (20-HE), and it was concluded that OC had an antagonizing effect on epidermal ecdysteroid signaling; however the study was unable to explain the NAG mRNA upregulation associated with singular OC exposure. The estrogens used in our study, E2 and EE2 are strong estrogen receptor agonists (Logie et al., 1998; Snyder et al., 2001). Thus, the upregulation of *PcChi* mRNA in this study suggests agonizing effect of these two estrogens on hepatopancreatic ecdysteroid

signaling in the crayfish, *in vitro*. Earlier we determined this agonizing effect with receptor-binding studies, where E2 and EE2 significantly upregulated EcR/RXR mRNA levels, and the *PcChi* expression confirmed this effect.

The findings of this study are summarized in the Table 5.

	Gene	Molt Cycle Studies			Exposure Studies				
		Inter	Pre	Post	Control	E2 100	E2 500	EE2 100	EE2 500
Nuclear Receptors	EcR	↔	↑	↔	↔	/	↑	/	/
	RXR	↔	↑	↑	↔	/	↑	↑	↑
Cyclic Nucleotides	cAMP	↓	↑	↓	↓	↑	↓	↑	↑
	cGMP	↓	↑	↓	↔	↔	↔	↔	↔
Calcium Transporters	SERCA	↔	↑	↑	↔	/	↑	/	↑
	PMCA	↔	↑	↑	↔	/	↑	/	/
	CaM	↔	↑	↑	↔	↑	↑	↑	↑
Chitinase	Chi	↔	↔	↑	↔	↑	↑	↔	↑

**Table 5.** Summary of findings of the specific aims of this study. “↑” indicates significant increase; “↓” significant decrease; “↔” indicates control or no effect; “/” indicates non-significant increase.

## V. CONCLUSION

There has been a rising interest in the impacts of EDCs on human and environmental health systems in the last two decades. However, despite being the largest subphylum that constitutes 95% of all animal species on the Earth, invertebrates have received lesser attention on the impacts of EDCs. Ecdysozoans which include crustaceans constitute 75% of all animal species, and their developmental and reproductive physiology is regulated by a multi-hormonal system. Their growth which includes periodic shedding (molting) and replacement of cuticle comes directly under the control of steroidal molting hormones, ecdysteroids. As such the EDCs can effect crustacean growth, which can be invisible, but can have profound impact at ecosystem level. Understanding EDC-induced disruption of crustacean ecdysteroid signaling requires functional characterization and expression pattern of genes involved in ecdysteroid signaling (EcR/RXR), and subsequent events that define mechanistic pathway(s). These mechanistic pathways are complex and cross-link with pathways specific to other processes. So far little is known about these complex pathways associated with crustacean ecdysteroidogenesis. This dissertation attempted to delineate a pathway involving ecdysteroid receptors, second messengers (cAMP/cGMP), and  $Ca^{2+}$  transporters, and systematically addressed the EDC-induced effects on these three components. My study hypothesized that these three components play a crucial role in ecdysteroidogenesis. The expression of ecdysteroid receptor (EcR) and heterodimer partner (RXR) showed a typical signaling pattern, i.e. upregulation during premolt, as well as when exposed to E2 and EE2. This suggests that both these estrogens mimic or

block their endogenous hormones. The concentration of cyclic nucleotides did not concur with earlier *in vitro* studies involving YO. However, our *in vivo* assays substantiated the hypothesis that the activation of ecdysteroid receptors leads to an increase in the concentrations of intracellular cyclic nucleotides, the cAMP being more responsive. Upregulation of Ca<sup>2+</sup> transporters suggest an increase in the intracellular Ca<sup>2+</sup> concentration that is typically witnessed in the presence of EDCs. These findings confirm the hypothesis of this study (Table. 5). The expression pattern of Chi gene suggested the agonistic action of E2 and EE2, although its nonconcurring expression pattern during natural molt cycle is probably due to differences in the hepatopancreas metabolism in our model species (crayfish). Taken together, the findings of our study suggest the hypothesized mechanistic pathway –involving estrogen nuclear receptors, second messengers and calcium transporters –play a crucial role in the ecdysteroidogenesis, its disruption, and on the Ca<sup>2+</sup> homeostasis in crayfish. Future progress in further understanding this mechanistic pathway relies on determining the impacts of EDCs on the components further downstream of this pathway.

## REFERENCES

- Ahearn, G. A., Mandal, P. K., & Mandal, A. (2004). Calcium regulation in crustaceans during the molt cycle: A review and update. *Comparative Biochemistry and Physiology, Part A*, 137, 247-257.
- Alcorlo, P., Otero, M., Crehuet, M., Baltanas, A., & Montes, C. (2006). The use of the red swamp crayfish (*Procambarus clarkii, girard*) as indicator of the bioavailability of heavy metals in environmental monitoring in the river guadiamar (SW, Spain). *Science of the Total Environment*, 366, 380-390.
- Alvarez, J., Montero, M., & Garcia-Sancho, J. (1999). Subcellular Ca<sup>2+</sup> dynamics. *Neuronal Information Processing Systems*, 14, 161-168.
- Anderson, H. R., Wollenberger, L., Halling-Sorensen, B., & Kusk, K. O. (2001). Development of copepod nauplii to copepodites — a parameter for chronic toxicity including endocrine disruption. *Environmental Toxicology and Chemistry*, 20, 2821-2829.
- Ankley, G. T., & Giesy, J. P. (1998). Endocrine disruptors in wildlife: A weight of evidence perspective. In R. Kendall, R. Dickerson, W. Suk & J. Giesy (Eds.), (). Pensacola, FL: SETAC.
- Ankley, G. T., Mihaich, E., Stahl, R., Tillitt, D., Colborn, T., McMaster, S., et al. (1998). Overview of a workshop on screening methods for detecting potential anti-estrogenic/androgenic chemicals in wildlife. *Environmental Toxicology and Chemistry*, 17, 68-87.
- Asazuma, H., Nagata, S., Kono, M., & Nagasawa, H. (2007). Molecular cloning and expression analysis of ecdysone receptor and retinoid X receptor from the kuruma prawn, *marsupenaeus japonicus*. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, 148(2), 139-150.
- Azoitei, A., & Spindler-Barth, M. (2009). DNA affects ligand binding of the ecdysone receptor of *drosophila melanogaster*. *Molecular and Cellular Endocrinology*, 303(1-2), 91-99.
- Baskin, R. J. (1971). Ultrastructure and calcium transport in crustacean muscle microsomes. *The Journal of Cell Biology*, 48, 49-60.
- Beckmann, M., Hardege, J. D., & Zeeck, E. (1995). Effects of the volatile fraction of crude-oil on spawning behavior of nereids (annelida, polychaeta). *Marine Environmental Research*, 40, 267-276.

Berridge, M. J., Bootman, M. D., & Lipp, P. (1998). Calcium--a life and death signal. *Nature*, 395(6703), 645-648.

Bondgaard, M., & Bjerregaard, P. (2005). Association between cadmium and calcium uptake and distribution during the moult cycle of females shore crabs, *carcinus maenas*: An in vivo study. *Aquatic Toxicology*, 72, 17-28.

Brion, F., Tyler, C. R., Palazzi, X., Laillet, B., Porcher, J. M., Garric, J., et al. (2004). Impacts of 17 $\beta$ -estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (*Danio rerio*). *Aquatic Toxicology*, 68, 193-217.

Brittsan, A. G., & Kranias, E. G. (2000). Phospholamban and cardiac contractile function. *Journal of Molecular and Cellular Cardiology*, 32(12), 2131-2139.

Bruce, J. I., Straub, S. V., & Yule, D. I. (2003). Crosstalk between cAMP and Ca<sup>2+</sup> signaling in non-excitabile cells. *Cell Calcium*, 34(6), 431-444.

Bruce, J. I., Yule, D. I., & Shuttleworth, T. J. (2002). Ca<sup>2+</sup>-dependent protein kinase--a modulation of the plasma membrane Ca<sup>2+</sup>-ATPase in parotid acinar cells. *The Journal of Biological Chemistry*, 277(50), 48172-48181.

Caldwell, D. J., Mastrocco, F., Hutchinson, T. H., Lange, R., Heijerick, D., Janssen, C., et al. (2008). Derivation of an aquatic predicted no-effect concentration for the synthetic hormone, 17 alpha-ethinyl estradiol. *Environmental Science & Technology*, 42(19), 7046-7054.

Cameron, J. N., & Wood, C. M. (1985). Apparent H<sup>+</sup> excretion of CO<sub>2</sub> dynamics accompanying carapace mineralization in the blue crab (*callinectes sapidus*) following moulting. *Journal of Experimental Biology*, 114, 181-196.

Catt, K. J., & Dufau, M. L. (1982). Hormones action: The regulation of target cell function by peptide, thyroid and steroid hormones. In P. H. Feliy (Ed.), *Endocrinology and metabolism* (pp. 113-199). New York: McGraw Hill.

Chan, S. M., Gu, P. L., Chu, K. H., & Tobe, S. S. (2003). Crustacean neuropeptide genes of the CHH/MIH/GIH family: Implications from molecular studies. *General and Comparative Endocrinology*, 134, 214-219.

Chang, E. S. (1995). Physiological and biochemical changes during the molt cycle in decapod crustaceans: An overview. *Journal of Experimental Marine Biology and Ecology*, 193, 1-14.

Chang, E. S. (1995). Physiological and biochemical changes during the molt cycle in decapod crustaceans: An overview. *Journal of Experimental Marine Biology and Ecology*, 193, 1-14.

- Chang, E. S., & Bruce, M. J. (1993). Regulation of crustacean moulting: A multi-hormonal system. *American Zoologist*, 33, 324-329.
- Chang, E. S., Bruce, M. J., & Tamone, S. L. (1993). Regulation of crustacean moulting: A multi-hormonal system. *American Zoologist*, 33, 324-329.
- Chang, E. S., & O'Connor, J. D. (1988). Crustacea: Molting. In H. Laufer, & R. G. Downer (Eds.), *Endocrinology of selected invertebrate types* (pp. 259-278). New York: Alan R Liss.
- Cheek, O. A., Vonier, P. M., Oberdorster, E., Burow, C. B., & McLachlan, A. J. (1998). Environmental signaling: A biological context for endocrine disruption *Environmental Health Perspectives*, 106(S1), 5-10.
- Chen, D., Zhang, Z., Wheatly, M. G., & Gao, Y. (2002). Cloning and characterization of the heart muscle isoform of Sarco/Endoplasmic reticulum Ca<sup>2+</sup> ATPase (SERCA) from crayfish. *Journal of Experimental Biology*, 205, 2677-2686.
- Claeys, I., Simonet, G., Poels, J., Van Loy, T., Vercammen, L., De Loof, A., et al. (2002). Insulin-related peptides and their conserved signal transduction pathway. *Peptides*, 23(4), 807-816.
- Clotfelter, E. D., Bell, A. M., & Levering, K. R. (2004). The role of animal behavior in the study of endocrine-disrupting chemicals. *Animal Behavior*, 68, 465-476.
- Colborn, T., vom Saal, F. S., & Soto, A. M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives*, 101, 378-384.
- Damstra, T. (2003). Endocrine disrupters: The need for a refocused vision. *Toxicological Sciences : An Official Journal of the Society of Toxicology*, 74(2), 231-232.
- Deamer, D. W. (1973). Isolation and characterization of a lysolecithin-adenosine triphosphatase complex from lobster muscle microsomes. *The Journal of Cell Biology*, 248, 5477-5485.
- Desbrow, C., Routledge, E. J., Brighty, G. C., Sumpter, J. P., & Waldock, M. (1998). Identification of estrogenic chemicals in STW effluent, 1. chemical fractionation and *in vitro* biological screening. *Environmental Science and Technology*, 32, 1549-1558.
- Dick, I. M., Liu, J., Glendenning, P., & Prince, R. L. (2003). Estrogen and androgen regulation of plasma membrane calcium pump activity in immortalized distal tubule kidney cells. *Molecular and Cellular Endocrinology*, 213, 11-18.

- Dick, I. M., Liu, J., Glendenning, P., & Prince, R. L. (2003). Estrogen and androgen regulation of plasma membrane calcium pump activity in immortalized distal tubule kidney cells. *Molecular and Cellular Endocrinology*, 212(1-2), 11-18.
- Durica, D. S., Chung, A. C., & Hopkins, P. M. (1999). Characterization of *EcR* and *RXR* gene homologs and receptor expression during the molt cycle in the crab, *uca pugnator*. *American Zoologist*, 39, 758-773.
- Durica, D. S., Wu, X., Anil kumar, G., Hopkins, P. M., & Chung, A. C. (2002). Characterization of crab *EcR* and *RXR* homologs and expression during limb regeneration and oocyte maturation. *Molecular and Cellular Endocrinology*, 189(1-2), 59-76.
- Dussault, E. B., Balakrishnan, V. k., Borgmann, U., Solomon, K. R., & Sibley, P. K. (2009). Bioaccumulation of the synthetic hormone 17alpha-ethinylestradiol in the benthic invertebrates *chironomus tentans* and *Hyaella azteca*. *Ecotoxicology and Environmental Safety*, 72, 1635-1641.
- Filby, A. L., Thorpe, K. L., & Tyler, C. R. (2006). Multiple molecular effect pathways of an environmental oestrogen in fish. *Journal of Molecular Endocrinology*, 37(1), 121-134.
- Folmar, L. C., Hemmer, M., Hemmer, R., Bowman, C., Kroll, K., & Denslow, N. D. (2000). Comparative estrogenicity of estradiol, ethynyl estradiol and diethylstilbestrol in an in vivo, male sheepshead minnow (*cyprinodon variegatus*), vitellogenin bioassay. *Aquatic Toxicology (Amsterdam, Netherlands)*, 49(1-2), 77-88.
- Gagou, M. E., Kapsetaki, M., Turberg, A., & Kafetzopoulos, D. (2002). Stage-specific expression of the chitin synthase *DmeChSA* and *DmeChSB* genes during the onset of drosophila metamorphosis. *Insect Biochemistry and Molecular Biology*, 32, 141-146.
- Gao, Y., Gillen, C. M., & Wheatly, M. G. (2009). Cloning and characterization of a calmodulin gene (*CaM*) in crayfish *Procambarus clarkii* and expression during molting. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, 152(3), 216-225.
- Gao, Y., & Wheatly, M. G. (2004). Characterization and expression of plasma membrane  $Ca^{2+}$  ATPase (*PMCA3*) in the crayfish *Procambarus clarkii* antennal gland during molting. *The Journal of Experimental Biology*, 207(Pt 17), 2991-3002.
- Grun, F., & Blumberg, B. (2006). Environmental obesogens: Organotins and endocrine disruption via nuclear receptor signaling. *Endocrinology*, 147(6), S50-55.
- Gunnarsson, L., Jauhianen, A., Kristiansson, E., Nerman, O., & Larsson, J. D. G. (2008). Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. *Environmental Science & Technology*, 42(15), 5807-5813.

- Hagedorn, M., Weihrauch, D., Towle, D. W., & Ziegler, A. (2003). Molecular characterization of the SER Ca<sup>2+</sup>-ATPase of *Porcellio scaber* and its expression in sternal epithelia during the moult cycle. *Journal of Experimental Biology*, 206, 2167-2175.
- Hagedorn, M., & Ziegler, A. (2002). Analysis of Ca<sup>2+</sup> uptake into the smooth endoplasmic reticulum of permeabilized sternal epithelial cells during the moulting cycle of the terrestrial isopod *Porcellio scaber*. *Journal of Experimental Biology*, 1935-1942.
- Han, D. W., Patel, N., & Watson, R. D. (2006). Regulation of protein synthesis in Y-organs of the blue crab (*Callinectes sapidus*): Involvement of cyclic AMP. *Journal of Experimental Zoology*, 305, 328-334.
- Harmon, M. A., Mangelsdorf, D. J., Heyman, R. A., & Boehm, M. F. (1995). Activation of mammalian retinoid X receptors by the insect growth regulator methoprene. *Proceedings of the National Academy of Sciences*, 92, 6157-6160.
- Hatakeyama, M., Tessier, D. M., Dunlap, D. Y., Zou, E., & Matsumura, F. (2002). Estrogenic action of b-HCH through activation of c-neu in MCF-7 breast carcinoma cells. *Environment Toxicology and Pharmacology*, 11, 27-38.
- He, J., Chen, J., Liu, R., Wang, S., Song, L., Chang, H. C., et al. (2004). Alterations of FSH-stimulated progesterone production and calcium homeostasis in primarily cultured human luteinizing-granulosa cells induced by fenvalerate. *Toxicology*, 203(1-3), 61-68.
- Hirano, M., Ishibashi, H., Yamauchi, R., Kim, J. -, & Arizono, K. (2008). Expression analysis of ecdysone receptor and ultraspiracle through molting period in mysid crustacean, *Americamysis bahia*. In Y. Murakami, K. Nakayam, S. -. Kitamura, H. Iwata & S. Tanabe (Eds.), *Interdisciplinary studies on environmental chemistry-biological responses to chemical pollutants* (pp. 303-310). Tokyo: Terra Scientific Publishing Company.
- Hofmann, F., James, P., Vorherr, T., & Carafoli, E. (1993). The C-terminal domain of the plasma membrane Ca<sup>2+</sup> pump contains three high affinity Ca<sup>2+</sup> binding sites. *The Journal of Biological Chemistry*, 268(14), 10252-10259.
- Hutchinson, H. T., Pounds, A. N., Hampel, M., & Williams, D. T. (1999). Impact of natural and synthetic steroids on the survival, development and reproduction of marine copepods *tisbe battagliai*. *The Science of the Total Environment*, 233, 167-179.
- Hutchinson, T. H. (2002). Reproductive and development effects of endocrine disrupters in invertebrates: In vitro and in vivo approaches. *Toxicology Letters*, 131, 75-81.
- Hutchinson, T. H. (2002). Reproductive and developmental effects of endocrine disrupters in invertebrates: In vitro and in vivo approaches. *Toxicology Letters*, 131, 75-81.

- Hutchinson, T. H. (2007). Small is useful in endocrine disrupter assessment-four key recommendations for aquatic invertebrate research. *Ecotoxicology*, *16*, 231-238.
- Hutchinson, T. H., Brown, R., Brugger, K. E., Campbell, P. M., Holt, M., Lage, R., et al. (2000). Risk assessment of endocrine disruptors. *Environmental Health Perspectives*, *108*, 1007-1014.
- Iguchi, T., Watanabe, H., & Katsu, Y. (2006). Application of ecotoxicogenomics for studying endocrine disruption in vertebrates and invertebrates. *Environmental Health Perspectives*, *114*(1), 101-105.
- Inglefield, J. R., & Shafer, T. J. (2000). Polychlorinated biphenyl-stimulation of  $Ca^{2+}$  oscillations in developing neocortical cells: A role for excitatory transmitters and L-type voltage-sensitive  $Ca^{2+}$  channels. *The Journal of Pharmacology and Experimental Therapeutics*, *295*(1), 105-113.
- Iwema, T., Chaumot, A., Studer, R. A., Robinson-Rechavi, M., Billas, I. M., Moras, D., et al. (2009). Structural and evolutionary innovation of the heterodimerization interface between USP and the ecdysone receptor ECR in insects. *Molecular Biology and Evolution*, *26*(4), 753-768.
- Johnson, L. L., Casillas, E., Myers, M. S., Rhodes, L. D., & Olson, O. P. (1991). Patterns of oocyte development and related changes in plasma of 17 $\beta$ -estradiol, vitellogenin and plasma chemistry in English sole *parophrys vetulus girard*. *Journal of Experimental Marine Biology and Ecology*, *152*, 161-185.
- Jukosky, J. A., Watzin, M. C., & Leiter, J. C. (2008). Elevated concentrations of ethinylestradiol, 17 $\beta$ -estradiol, and medroxyprogesterone have little effect on reproduction and survival of *ceriodaphnia dubia*. *Bulletin of Environmental Contamination and Toxicology*, *81*, 230-235.
- Kang, J., Park, I. S., Oh, W. Y., Lim, H., Wang, S., Lee, S. Y., et al. (2004). Inhibition of aroclor 1254-induced depletion of stored calcium prevents the cell death in catecholaminergic cells. *Toxicology*, *200*, 93-101.
- Kato, Y., Kobayashi, K., Oda, S., Tatarazako, N., Watanabe, H., & Iguchi, T. (2007). Cloning and characterization of the ecdysone receptor and ultraspiracle protein from the water flea *daphnia magna*. *Journal of Endocrinology*, *193*, 183-194.
- Kavlock, R. J., Daston, G. P., DeRosa, C., Fenner-Crisp, P., Gray, L. E., Kaattari, S., et al. (1996). Research needs for risk assessment of health and environmental effects of endocrine disruptors: A report of the U.S. EPA-sponsored workshop. *Environmental Health Perspectives*, *104*, 715-740.

- Kawecka, I., Lachowicz, L., & Ylinska, L. (2003). *Non-genomic effect of estradiol on plasma membrane calcium pump activity in vitro*. *Polish Journal of Pharmacology*, *55*, 887-893.
- Keller, R. (1992). Crustacean neuropeptides: Structures, functions, and comparative aspects. *Experientia*, *48*, 439-448.
- Khan, S. Z., Kirk, C. J., & Michelangeli, F. (2003). Alkylphenol endocrine disrupters inhibit IP3-sensitive Ca<sup>2+</sup> channels. *Biochemical and Biophysical Research Communications*, *310*, 261-266.
- Khan, S. Z., Kirk, C. J., & Michelangeli, F. (2003). Alkylphenol endocrine disrupters inhibit IP3-sensitive Ca<sup>2+</sup> channels. *Biochemical and Biophysical Research Communications*, *310*(2), 261-266.
- Kluytmans, J. H., Brands, F., & Zandee, D. (1988). Interactions of cadmium with the reproductive-cycle of *mytilus edulis*. *Marine Environmental Research*, *24*, 189-192.
- Kono, M., Wilder, M. N., Matsui, T., Furukawa, K., Koga, D., & Aida, K. (1995). Chitinolytic enzyme activities in the hepatopancreas, tail fan and hemolymph of kuruma prawn *marsupenaeus japonicus* during molt cycle. *Fisheries Science*, *61*, 727-728.
- Korsgaard, B., Andreassen, T. K., & Rasmussen, T. H. (2002). Effects of an environmental estrogen, 17alpha-ethinyl-estradiol, on the maternal-fetal trophic relationship in the eelpout *zoarces viviparus* (L). *Marine Environmental Research*, *54*(3-5), 735-739.
- Kowalska, I., & Ferenc, B. (2009). Calmodulin effects on steroids-regulated plasma membrane calcium pump activity. *Cell Biochemistry and Function*, *27*, 111-117.
- Kuo, T. H., Liu, B. F., Diglio, C., & Tsang, W. (1993). Regulation of the plasma membrane calcium pump gene expression by two signal transduction pathways. *Archives of Biochemistry and Biophysics*, *305*(2), 428-433.
- Lange, R., Hutchinson, T. H., Croudace, C. P., Siegmund, F., Schweinfurth, H., Hampe, P., et al. (2001). Effects of the synthetic estrogen 17 alpha-ethinylestradiol on the life-cycle of the fathead minnow (*pimephales promelas*). *Environmental Toxicology and Chemistry / SETAC*, *20*(6), 1216-1227.
- Langston, W. J., Burton, G. R., Chesman, B. S., & Vane, C. H. (2005). Partitioning, bioavailability and effects of estrogens and xeno-estrogens in the aquatic environment. *Journal of the Marine Biological Association of the United Kingdom*, *85*, 1-31.
- Larsson, D. G. J., Adolfsson-Erici, M., Parkkonen, J., Pettersson, M., Berg, A. H., Olsson, P. E., et al. (1999). Ethinyloestradiol—an undesired fish contraceptive? *Aquatic Toxicology*, *45*, 91-97.

- Laudet, V., & Gronemeyer, H. (2002). *The nuclear receptor facts book*. London: Academic Press.
- Lee, S. G., & Mykles, D. L. (2006). Proteomics and signal transduction in the crustacean molting gland. *Integrative and Comparative Biology*, 46(6), 965-977.
- Liu, J., Glendenning, P., & Prince, R. L. (2003). Estrogen and androgen regulation of plasma membrane calcium pump activity in immortalized distal tubule kidney cells *Molecular and Cellular Endocrinology*, 212, 11-18.
- Liu, Y., Li, W., & Song, W. (2009). Effects of oestrogen on sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase activity and gene expression in genioglossus in chronic intermittent hypoxia rat. *Archives of Oral Biology*, 54, 322-328.
- Logie, C., Nichols, M., Myles, K., Funder, J. W., & Stewart, A. F. (1998). Positive and negative discrimination estrogen receptor agonists and antagonists using site-specific DNA recombinase fusion proteins. *Molecular Endocrinology*, 12, 1120-1132.
- Lye, C. M., Bentley, M. G., & Galloway, T. (2008). Effects of 4-nonylphenol on the endocrine system of the shore crab, *carcinus maenas*. *Environmental Toxicology*, 23, 309-318.
- Lye, C. M., Bentley, M. G., Clare, A. S., & Sefton, E. M. (2005). Endocrine disruption in the shore crab *carcinus maenas*—a biomarker for benthic marine invertebrates? *Marine Ecology Progress Series*, 288, 221-232.
- Lyons, G. (2006). Viewpoint: Policy requirements for protecting wildlife from endocrine disruptors. *Environmental Health Perspectives*, 114(S-1), 142-146.
- MacLennan, D. H., Rice, W. J., & Green, N. M. (1997). The mechanism of Ca<sup>2+</sup> transport by sarco(endo)plasmic reticulum Ca<sup>2+</sup>-ATPases. *The Journal of Biological Chemistry*, 272(46), 28815-28818.
- Maestro, O., Cruz, J., Pascual, N., Martin, D., & Belles, X. (2005). Differential expression of two RXR/ultraspiracle isoforms during the life cycle of the hemimetabolous insect *blattella germanica* (dictyoptera, blattellidae). *Molecular and Cellular Endocrinology*, 238(1-2), 27-37.
- Mattson, M. P., & Spaziani, E. (1986). Regulation of crab Y-organ steroidogenesis in vitro: Evidence that ecdysteroid production increases through activation of cAMP-phosphodiesterase by ca-calmodulin. *Molecular and Cellular Endocrinology*, 48, 135-151.
- Mattson, P. M., & Spaziani, E. (1985). Cyclic AMP mediates the negative regulation of Y-organ ecdysteroid production. *Molecular and Cellular Endocrinology*, 42, 185-189.

- Mazurova, E., Hilscherova, K., Triebekom, R., Heinz-R.K., Marsalek, B., & Blaha, L. (2008). Endocrine regulation of the reproduction in crustaceans: Identification of potential targets for toxicants and environmental contaminants. *Biologia*, 63(2), 139-150.
- Mazurová, E., Hilscherová, K., Triebekom, R., Kohler, H. R., Marsalek, B., & Blaha, L. (2008). Endocrine regulation of the reproduction in crustaceans: Identification of potential targets for toxicants and environmental contaminants. *Biologia*, 63(2), 139-150.
- McCormack, J. G., & Cobbold, P. H. (1991). *Cellular calcium: A practical approach*. Oxford: Oxford University Press.
- McWhinnie, M. (1962). Gastrolith growth and calcium shifts in the freshwater crayfish *orconectes virilis*. *Comparative Biochemistry and Physiology*, 7, 1-14.
- Meng, Y., & Zou, E. (2009). Impacts of molt-inhibiting organochlorine compounds on epidermal ecdysteroid signaling in the fiddler crab, *uca pugilator*, in vitro. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology : CBP*, 150(4), 436-441.
- Meng, Y., & Zou, E. (2009). A molecular biomarker for disruption of crustacean molting: The N-acetyl-beta-glucosaminidase mRNA in the epidermis of the fiddler crab. *Bulletin of Environmental Contamination and Toxicology*, 82(5), 554-558.
- Mu, X., & Leblanc, G. A. (2004). Cross communication between signaling pathways: Juvenoid hormones modulate ecdysteroid activity in a crustacean. *Journal of Experimental Zoology. Part A, Comparative Experimental Biology*, 301(10), 793-801.
- Munetsuna, E., Hojo, Y., Hattori, M., Ishii, H., Kawato, S., Ishida, A., et al. (2009). Retinoic acid stimulates 17 $\beta$ -estradiol and testosterone synthesis in rat hippocampal slice cultures. *Endocrinology*, 150(9), 4260-4269.
- Nagai, C., Asazuma, H., Nagata, S., & Nagasawa, H. (2009). Identification of a second messenger of crustacean hyperglycemic hormone signaling pathway in the kuruma prawn *marsupenaeus japonicus*. *Trends in Comparative Endocrinology and Neurobiology*, 1163, 478-480.
- Nakagawa, Y., Nishimura, K., Oikawa, N., Kurihara, N., & Ueno, T. (1995). Activity of ecdysone analogs in enhancing N-acetylglucosamine incorporation into the cultured integument of *chilo suppressalis*. *Steroids*, 60, 401-405.
- Nakatsuji, T., Sonobe, H., & Watson, R. D. (2006). Molt-inhibiting hormone-mediated regulation of ecdysteroid synthesis in Y-organs of the crayfish (*Procambarus clarkii*): Involvement of cyclic GMP and cyclic nucleotide phosphodiesterase. *Molecular and Cellular Endocrinology*, 253, 76-82.

- Nakatsuji, T., Sonobe, H., & Watson, R. D. (2006). Molt-inhibiting hormone-mediated regulation of ecdysteroid synthesis in Y-organs of the crayfish (*Procambarus clarkii*): Involvement of cyclic GMP and cyclic nucleotide phosphodiesterase. *Molecular and Cellular Endocrinology*, 253(1-2), 76-82.
- Nguyen, H. N., Wang, C., & Perry, D. C. (2002). Depletion of intracellular calcium stores is toxic to SH-SY5Y neuronal cells. *Brain Research*, 924, 159-166.
- Nishikawa, J., Mamiya, S., Kanayama, T., Nishikawa, T., Shiraishi, F., & Horiguchi, T. (2004). Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. *Environmental Science & Technology*, 38(23), 6271-6276.
- Norum, U., Bondgaard, M., Pedersen, T. V., & Bjerregaard, P. (2005). In vivo and in vitro cadmium accumulation during the moult cycle of the male shoe crab *carcinus maenas*—interaction with calcium metabolism. *Aquatic Toxicology*, 72, 29-44.
- Oetken, M., Bachmann, J., Schulte-Oehlmann, U., & Oehlmann, J. (2004). Evidence for endocrine disruption in invertebrates. *International Review of Cytology*, (236), 1-43.
- Ogunbayo, O. A., & Michelangeli, F. (2007). The widely utilized brominated flame retardant tetrabromobisphenol A (TBBPA) is a potent inhibitor of the SERCA Ca<sup>2+</sup> pump. *Biochemical Journal*, 408, 407-415.
- Pait, A. S., & Nelson, J. O. (2002). *Endocrine disruption in fish: An assessment of recent research and results* (NOAA Tech Memo No. 149) National Oceanic Service, Center for Coastal Monitoring and Assessment.
- Parrott, J. L., & Blunt, B. R. (2005). Life-cycle exposure of fathead minnows (*pimephales promelas*) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. *Environmental Toxicology*, 20, 131-141.
- Paschen, W., & Frandsen, A. (2001). Endoplasmic reticulum dysfunction—a common denominator for cell injury in acute and degenerative diseases of the brain? *Journal of Neurochemistry*, (79), 719-725.
- Pawlowski, S., Ternes, T. A., Bonerz, M., Rastall, A. C., Erdinger, L., & Braunbeck, T. (2004). Estrogenicity of solid phase-extracted water samples from two municipal sewage treatment plant effluents and riverine water using the yeast estrogen screen. *Toxicology in Vitro*, 18, 129-138.
- Pottorf, W. J., & Thayer, S. A. (2002). Transient rise in intracellular calcium produces a long-lasting increase in plasma membrane calcium pump activity in rat sensory neurons. *Journal of Neurochemistry*, 83(4), 1002-1008.
- Pounds, J. G. (1990). The role of cell calcium in current approaches to toxicology. *Environmental Health Perspectives*, 84, 7-15.

- Priya, T. A. J., Li, F., Zhang, J., Wang, B., Zhao, C., & Xiang, J. (2009). Molecular characterization and effect of RNA interference of retinoid X receptor (RXR) on E75 and chitinase gene expression in Chinese shrimp *fenneropenaeus chinensis*. *Comparative Biochemistry and Physiology*, *153*, 121-129.
- Proespraiwong, P., Tassanakajon, A., & Rimphanitchayakit, V. (2010). Chitinases from the black tiger shrimp *penaeus monodon*: Phylogenetics, expression and activities. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, *156*(2), 86-96.
- Quackenbush, L. S. (1986). Crustacean endocrinology, a review. *Canadian Journal of Fisheries and Aquatic Sciences*, *43*, 2271-2282.
- Richards, J. S., & Hedin, L. (1988). Molecular aspects of hormone action in ovarian follicular development, ovulation, and lutenization. *Annual Review of Physiology*, *50*, 441-463.
- Rodríguez, E. M., Medesani, D. A., & Fingerman, M. (2007). Endocrine disruption in crustaceans due to pollutants: A review. *Comparative Biochemistry and Physiology, Part A*, *146*, 661-671.
- Safe, S. H. (1995). Do Environmental Estrogens Play a Role in Development of Breast Cancer in Women and Male Reproductive Problems. *Human and Ecological Risk Assessment*. *1*, 17-24.
- Sánchez-López, F. J., Gill, M. D., Martínez, J. L., Aquilera, P. A., & Garrido, P. A. (2004). Assessment of metal contamination in doñana national park (spain) using crayfish (*Procambarus clarkii*). *Environmental Monitoring and Assessment*, *93*, 17-29.
- Shechter, A., Tom, M., Yudkovski, Y., Weil, S., Chang, S. A., Chang, E. S., et al. (2007). Search for hepatopancreatic ecdysteroid-responsive genes during the crayfish molt cycle: From a single gene to multigenicity. *The Journal of Experimental Biology*, *210*(Pt 20), 3525-3537.
- Sirotkin, A. V., Mlyncek, J., Lavrincik, J., Bulla, J., & Hetenyi, L. (1995). The ability of steroid hormones to control cAMP and cGMP production by human granulosa cells in culture. *Cellular Signalling*, *7*(1), 61-65.
- Skillman, A. D., Nagler, J. J., Hook, S. E., Small, J. A., & Schultz, I. R. (2006). Dynamics of 17alpha-ethynylestradiol exposure in rainbow trout (*oncorhynchus mykiss*): Absorption, tissue distribution, and hepatic gene expression pattern. *Environmental Toxicology and Chemistry / SETAC*, *25*(11), 2997-3005.

- Sloof, W., van Kreijl, C. F., & Baars, A. J. (1983). Relative liver weights and xenobiotic-metabolizing enzymes of fish from polluted surface waters in the Netherlands. *Aquatic Toxicology*, 4, 1-14.
- Snyder, M. J., & Mulder, E. P. (2001). Environmental endocrine disruption in decapod crustacean larvae: Hormone titers, cytochrome P450, and stress protein responses to heptachlor exposure. *Aquatic Toxicology*, 55, 177-190.
- Snyder, S. A., Villeneuve, D. L., Snyder, E. M., & Giesy, J. P. (2001). Identification and quantification of estrogen receptor agonists in wastewater effluents. *Environmental Science & Technology*, 35(18), 3620-3625.
- Spaziani, E., Mattson, M. P., Wang, W. L., & McDougall, H. E. (1999). Signaling pathways for ecdysteroid hormone synthesis in crustacean Y-organs. *American Zoologist*, 39, 496-512.
- Spaziani, E., Mattson, M. P., Wang, W. N. L., & McDougall, H. E. (1999). Signaling pathways for ecdysteroid hormone synthesis in crustacean Y-organs. *American Zoologist*, 39(3), 496-512.
- Sternberg, R. M., Hotchkiss, A. K., & Leblanc, G. A. (2008). Synchronized expression of retinoid X receptor mRNA with reproductive tract recrudescence in an imposex-susceptible mollusc. *Environmental Science & Technology*, 42(4), 1345-1351.
- Sucov, H. M., Dyson, E., Gumeringer, C. L., Price, J., Chien, K. R., & Evans, R. M. (1994). RXR alpha mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. *Genes & Development*, 8(9), 1007-1018.
- Szemraj, J., Kawecka, I., Lachowicz, L., & Zylinska, L. (2003). Non-genomic effect of estradiol on plasma membrane calcium pump activity in vitro. *Polish Journal of Pharmacology*, 55(5), 887-893.
- Tamone, S. L., & Chang, E. S. (1993). Methyl farnesoate stimulates ecdysteroid secretion from crab Y-organs in vitro. *General and Comparative Endocrinology*, 89(3), 425-432.
- Tan, S. H., Degnan, B. M., & Lehnert, S. A. (2000). The penaeus monodon chitinase 1 gene is differentially expressed in the hepatopancreas during the molt cycle. *Marine Biotechnology (New York, N.Y.)*, 2(2), 126-135.
- U.S. EPA. (1998). Endocrine disruptor screening and testing advisory committee (EDSTAC) final report. *Environmental Protection Agency*.
- Van den Belt, K., Berckmans, P., Vangenechten, C., Verheyen, R., & Witters, H. (2004). Comparative study on the *in vitro/in vivo* estrogenic potencies of 17beta-estradiol, estrone, 17alpha-ethynyl estradiol and nonylphenol. *Aquatic Toxicology*, 66, 183-195.

Van der Kloot, W. (1969). Calcium uptake by isolated sarcoplasmic reticulum treated with dithiothreitol. *Science*, *164*, 1294-1295.

van der Oost, R., Vindiamian, E., van den Brink, P. J., Satumalay, K., Heida, H., & Vermeulen, N. P. E. (1997). Biomonitoring aquatic pollution with feral eel (*anguilla anguilla*). III. statistical analyses of relationships between contaminant exposure and biomarkers. *Aquatic Toxicology*, *39*, 45-75.

Vom Saal, F. S. (1995). Environmental Estrogenic Chemicals: Their Impact on Embryonic Development. *Human Ecological Risk Assessment*. *1*, 3-16.

Wang, Y. H., Wang, G., & LeBlanc, G. A. (2007). Cloning and characterization of the retinoid X receptor from a primitive crustacean daphnia magna. *General and Comparative Endocrinology*, *150*(2), 309-318.

Watanabe, T., & Kono, M. (1997). Isolation of cDNA encoding a chitinase family protein from cuticular tissues of the kuruma prawn *penaeus japonicus*. *Zoological Science*, *14*, 65-68.

Watanabe, T., Kono, M., Aida, K., & Nagasawa, H. (1996). Isolation of cDNA encoding a putative chitinase precursor in the kuruma prawn *penaeus japonicus*. *Molecular Marine Biology and Biotechnology*, *5*(4), 299-303.

Watanabe, T., Kono, M., Aida, K., & Nagasawa, H. (1998). Purification and molecular cloning of a chitinase expressed in the hepatopancreas of the penaeid prawn *penaeus japonicus*. *Biochimica Et Biophysica Acta*, *1382*(2), 181-185.

Watson, R. D., Lee, K. J., Qiu, S., Luo, M., Umphrey, H. R., Roer, R. D., et al. (2001). Molecular cloning, expression and tissue distribution of crustacean molt-inhibiting hormone. *American Zoologist*, *41*(3), 407-417.

Webster, S. G. (1998). Neuropeptides inhibiting growth and reproduction in crustaceans . In G. M. Coast, & S. G. Webster (Eds.), *Recent advances in arthropod endocrinology* (pp. 33-52). Cambridge: Cambridge University Press.

Wheatly, M. G., Hubbard, M. G., & Corbett, A. M. (2002). Physiological characterization of the Na<sup>+</sup> Ca<sup>2+</sup> exchanger (NCX) in hepatopancreatic and antennal gland basolateral membrane vesicles isolated from the freshwater crayfish *Procambarus clarkii* . *Comparative Biochemistry and Physiology, Part A*, *131*, 343-361.

Wheatly, M. G., Zanotto, F. P., & Hubbard, M. G. (2002). Calcium homeostasis in crustaceans: Subcellular ca dynamics. *Comparative Biochemistry and Physiology, Part B*, 163-178.

- Wheatly, M. G., Zhang, Z., Weil, J. R., Rogers, J. V., & Stiner, L. M. (2001). Novel subcellular and molecular tools to study  $\text{Ca}^{2+}$  transport mechanisms during the elusive moulting stages of crustaceans: Flow cytometry and polyclonal antibodies. *The Journal of Experimental Biology*, 204, 959-966.
- Woin, P., & Bronmark, C. (1992). Effects of DDT and MCPA (4-chloro-2-methylphenoxyacetic acid) on reproduction of the pond snail *lynaea stagnalis*. *Bulletin of Environmental Contamination and Toxicology*, 48, 7-13.
- Wu, X., Hopkins, P. M., Palli, S. R., & Durica, D. S. (2004). Crustacean retinoid-X receptor isoforms: Distinctive DNA binding and receptor-receptor interaction with a cognate ecdysteroid receptor. *Molecular and Cellular Endocrinology*, 218(1-2), 21-38.
- Yao, T. P., Segraves, W. A., Oro, A. E., McKeown, M., & Evans, R. M. (1992). Drosophila ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell*, 71(1), 63-72.
- Yudkovski, Y., Shechter, A., Chalifa-Caspi, V., Auslander, M., Ophir, R., Dauphin-Villemant, C., et al. (2007). Hepatopancreatic multi-transcript expression patterns in the crayfish *cherax quadricarinatus* during the moult cycle. *Insect Molecular Biology*, 16(6), 661-674.
- Zhang, Z., Chen, D., & Wheatly, M. G. (2000). Cloning and characterization of sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) from crayfish axial muscle. *Journal of Experimental Biology*, 203, 3411-3423.
- Zhang, X., Yu, R. M., Jones, P. D., Lam, G. K., Newsted, J. L., Gracia, T., et al. (2005). Quantitative RT-PCR methods for evaluating toxicant-induced effects on steroidogenesis using the H295R cell line. *Environmental Science & Technology*, 39(8), 2777-2785.
- Zheng, J., Nakatsuji, T., Roer, R. D., & Watson, R. D. (2008). Studies of a receptor guanylyl cyclase cloned from Y-organs of the blue crab (*callinectes sapidus*), and its possible functional link to ecdysteroidogenesis. *General and Comparative Endocrinology*, 155(3), 780-788.
- Zou, E. (2005). Impacts of xenobiotics on crustacean molting: The invisible endocrine disruption. *Integrative and Comparative Biology*, 45, 33-38.
- Zou, E., & Fingerman, M. (1997). Effects of estrogenic agents on molting of the water flea, *daphnia magna*. *Ecotoxicology and Environmental Safety*, 38, 281-285.
- Zou, E., & Fingerman, M. (1997). Synthetic estrogenic agents do not interfere with sex determination but do inhibit molting of a cladoceran, *daphnia magna*. *Bulletin of Environmental Contamination and Toxicology*, 58, 596-602.

- Zou, E., & Fingerman, M. (1999). Chitobiase activities in the epidermis and hepatopancreas of the fiddler crab, *uca pugilator*, during the molting cycle. *Marine Biology*, 133, 97-101.
- Zou, E., & Fingerman, M. (1999). Effects of estrogenic agents on chitobiase activity in the epidermis and hepatopancreas of the fiddler crab, *uca pugilator*. *Ecotoxicology and Environmental Safety*, 42, 185-190.
- Zou, E., & Fingerman, M. (1999). Effects of exposure to diethyl phthalate, 4-(tert)-octylphenol, and 2,4,5-trichlorobiphenyl on activity of chitobiase in the epidermis and hepatopancreas of the fiddler crab, *uca pugilator*. *Comparative Biochemistry and Physiology, Part C*, 122, 115-120.
- Zou, E., & Bonvillain, R. (2004). Chitinase activity in the epidermis of the fiddler crab, *uca pugilator*, as an in vivo screen for molt-interfering xenobiotics. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology : CBP*, 139(4), 225-230.
- Zylinska, L., Kowalska, I., & Ferenc, B. (2009). Calmodulin effects on steroids-regulated plasma membrane calcium pump activity. *Cell Biochemistry and Function*, 27(2), 111-117.