CHARACTERIZATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASES IN THE
TRANSCRIPTOME OF THE CRUSTACEAN MOLTING GLAND

Submitted by

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Molting in crustaceans is a complex physiological process that has to occur in order for the animal to grow. The old exoskeleton must be discarded and a new one to be formed from the inside out. Molting is coordinated and regulated mainly by two hormones; steroid hormones named ecdysteroids, which are synthesized and secreted from a pair of Y- organs (YOs) that are located in the cephalothorax and a neuropeptide hormone, the molt inhibiting hormone (MIH), which is secreted from the X-organ/sinus gland complex located in the eyestalks. Molting is induced when MIH is decreased in the blood (hemolymph) which in turn stimulates the YOs to produce and secrete ecdysteroids (molting hormones). There are four distinctive physiological states that the YO can be in throughout the molt cycle; the transition of the YO from the “basal” to the “activated” state happens when the animal enters premolt. During mid-premolt, the YO transitions to the “committed” state, in which the YO becomes insensitive to MIH. In this state, the circulating hemolymph contains high levels of ecdysteroids, which increase to a peak before the actual molt (ecdysis) happens. The YO transitions from the committed to the repressed state in late premolt. Finally, the YO returns back to the basal state in the postmolt stage. MIH binds to membrane receptors, activating a signal transduction pathway divided into “triggering” and “summation” phases. A transient increase in cAMP during the triggering phase leads to prolonged cGMP-dependent suppression of ecdysteroidogenesis during the summation phase. This allows for sustained inhibition of the YO between MIH pulses in the intermolt animal. Cyclic nucleotide phosphodiesterases (PDEs) play an important role by controlling cAMP and cGMP levels. PDEs
hydrolyze the phosphodiester bond in cAMP and cGMP to AMP and GMP, respectively. Mammals have 21 PDE genes that are categorized into 11 families, designated PDE1 to PDE11. Each PDE family has specific catalytic and biochemical properties and tissue distributions. Eight contigs encoding full-length PDE sequences were identified in the *G. lateralis* Y-organ transcriptome. Seven contigs encoding four full-length PDE sequences and three contigs encoding partial-length PDE were identified in the *Carcinus maenas* transcriptome. Multiple sequence alignments showed high sequence identities with orthologs from other species in catalytic (PDEase) and other conserved functional domains. Sequence analysis assigned the Gl-PDE sequences and Cm-PDE sequences to PDE1, PDE2, PDE3, PDE4, PDE5, PDE7, PDE8, PDE9, and PDE11 classes, indicating a high diversity of PDE genes in decapod crustaceans. The reduced sensitivity to MIH by the committed YO is associated with a large increase in PDE activity, which suggests that PDEs modulate the response to neuropeptide during the molt cycle. Non-hydrolyzable analogs of cAMP and cGMP inhibit YO ecdysteroid secretion *in-vitro*. Moreover, *C. maenas* YO ecdysteroidogenesis is inhibited by IBMX, a general PDE inhibitor, and Zaprinast, a specific PDE5 inhibitor. Rolipram, a specific PDE4 inhibitor, has no effect. These data suggest that PDE5 activity modulates the effect of MIH on YO ecdysteroidogenesis. RNA-seq data from MLA showed different mRNA levels for the different PDEs; PDE1 and PDE2 showed a similar pattern as they both increased in intermolt (IM) then decreased dramatically in early premolt (EP), mid premolt (MP), late premolt (LP), and post molt (PM). PDE4 increased in IM followed by a slight decrease and increase in EP and MP then a sharp decline in both LP and PM. Both PDE5 and PDE9 were similar in terms they increased in IM followed by a sharp decrease in EP, MP, LP and they differed as PDE5 increased slightly in PM whereas PDE9 remained decreased. PDE7 began with an increase in IM then a decline with a constant expression level in both EP and MP
followed by dramatic decline in LP and PM. PDE11 showed a typical pattern consistent with the ecdysteroid expression level as it began with a slight increase in IM followed by an increased in EP and reached a peak in MP then declined in a dramatic way in LP and continued decreasing in PM. Taken together, the data suggest that PDE5 and PDE11 play a role in regulating cyclic nucleotide levels in the YO.
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First and foremost, Alhamdullah (means Thanks GOD in Arabic) for giving me everything I’ve dreamt of, and the strength and power required to finish an important milestone in my life. I could have never done this without the faith I have in Allah, the Almighty.

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DEDICATION

In loving memory of my brother “Ahmad” (1983-2013), may Allah gather us together in heaven.
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CHAPTER ONE

Introduction

Background and Rationale:

Although a crustacean grows, its exoskeleton (made from chitin and calcium) does not, so the animal must molt its old exoskeleton to accommodate its expanding body, which is essential for growth, metamorphosis, and reproduction (Skinner, 1962). Depending on the rigidness of the exoskeleton, the crustacean molt cycle is divided into five distinctive stages designated from A to E (Drach, 1939). The molt cycle duration varies depending on the species. The process of molting is hormonally controlled. In preparation for molting, the tissue layer under the exoskeleton detaches and secretes a new exoskeleton. At this stage, the animal has two skeletons – the outer and the inner exoskeleton. After the two outermost layers of the new skeleton are formed, the old skeleton detaches along specific weak points and the animal pulls out, leaving its old skeleton intact except for the split. Mobility is limited immediately after a molt because the exoskeleton is not rigid enough to keep the limbs stiff. Although crustaceans molt throughout their entire life, they molt less frequently with age (Chang et al., 1993).

The crustacean molt cycle is divided into five stages (A-E), based on changes in the exoskeleton. The actual shedding of the exoskeleton occurs at ecdysis (Stage E) and is followed by postmolt stages A and B, which are both marked by thickening and hardening of the new exoskeleton. Anecdysis, or Stage C₄, or intermolt stage, is the interval characterized by a hard exoskeleton that is tightly adhered to the epidermis and is the stage during which the animal feeds and reproduces. Stages D₁-₄, known as premolt or proecdysis, is when the animal prepares for
molting by synthesizing the outermost layers of a new exoskeleton and regenerates lost appendages (Chang and Mykles, 2011).

Molting in crustaceans might be affected by various environmental factors, such as reproduction, nourishment, and migration. Moreover, the frequency and timing of molting in crustaceans can be impacted by some conditions such as salinity and temperature (Skinner, 1985).

**The molting cycle in decapod crustaceans is controlled via two endocrine glands:**

Molting can be stimulated or manipulated by eyestalk ablation (ESA), an acute method to induce molting in the lab, or autotomy of 5 or more walking legs (Multiple Leg Autotomy, MLA), which resembles the natural way. Molting is a very complicated process in which two glands contribute to complete this vital mission. The Y-organs (YOs) are a pair of molting glands located in the anterior body. The YOs secrete steroid molting hormones or ecdysteroids, which stimulate molting processes. The YOs are suppressed by molt-inhibiting hormone (MIH) and the crustacean hyperglycemic hormone (CHH), which are neuropeptides secreted by the X-organ -sinus gland complex found in the eyestalks (Lachaise et al., 1993, Covi et al., 2010).

MIH is a member of a novel neuropeptide family, which has been found only in arthropods. This neuropeptide family regulates a variety of functions including growth, molting, reproduction, and metabolism. MIH binds to hormone receptors on the membranes of YO cells and likely mediates its action via cyclic nucleotide second messengers (Covi et al., 2009). As shown in Fig. 1.1., MIH signaling usually involves an increase in cAMP, followed by a larger increase in cGMP. The delayed increase in cGMP suggests that MIH activates a soluble NO-sensitive guanylyl cyclase (GC-I), as activation of a membrane GC would result in an immediate increase in cGMP. Both cAMP and cGMP inhibit YO ecdysteroidogenesis (Covi et al., 2012). Phosphodiesterases
(PDEs) such as PDE1 and PDE5 hydrolyze cAMP and cGMP and thus control the responsiveness of YOs to MIH (Covi et al. 2012).

Molting is induced when MIH is decreased in the blood, which, in turn, stimulates the YO to produce and secrete ecdysteroids. The YO transitions from the “basal” to the “activated” state and the animal enters premolt. During mid-premol, the YO transitions to the “committed” state, in which the YO becomes insensitive to MIH and CHH (Chang and Mykles 2011). The reduced sensitivity to the neuropeptide MIH is associated with a large increase in PDE activity (Fig1.2.) (Chang and Mykles 2011). This suggests that PDE activity controls the response to MIH and CHH during the molt cycle and may also explain the difference in the effect of IBMX, a universal PDE inhibitor, on the YOs of the two species.

**Cyclic nucleotide phosphodiesterase’s (PDEs) and their contribution in the MIH signaling pathway in the crustacean YO:**

Cyclic nucleotide phosphodiesterases (PDEs) are prevalent enzymes that have been significant and valuable targets in medical and pharmacological fields due to their critical function in regulating the second messengers adenosine 3’5’ cyclic monophosphate (cAMP) and/or guanosine 3’5’ cyclic monophosphate (cGMP) in signal transduction pathways (Murthy and Mangot 2015). Upon binding of the ligand to its specific receptor on the cell membrane, the second messenger’s concentrations will be changed, that alternatively will lead to the signal transmission within the cell. These second messengers are controlled by both the rate of synthesis, with the action of adenylyl/guanylyl cyclase on ATP/GTP and the rate of cAMP/cGMP degradation by the action of PDEs (Fajardo et al., 2014). So, PDEs are enzymes that hydrolyze the 3’ cyclic phosphodiester bond in cAMP and cGMP to AMP and GMP, respectively. Mammals have 21 PDE genes that are categorized into 11 families, designated PDE1 to PDE11. Each PDE
family has specific catalytic and biochemical properties, protein sequences, inhibition tendency, and tissue distributions (Table 1.1) (Sandeep et al., 2008; Francis et al., 2011). Mammalian class I PDEs have an HD domain in the C-terminal half and show high affinity for cAMP and/or cGMP. Protein domains involved in regulation of PDE enzymatic activity and sub-cellular localization are mainly present in the N-terminal half. Some PDEs have phosphorylation sites targeted by protein kinases and lipid modification sites. Approximately 270 aa in the C-terminal catalytic domain are conserved, with a sequence identity of 35% to 50% among different PDE families. Some PDE families are composed of 2 to 4 subfamily genes showing sequence identity of more than 70% and having identical protein domain organization. Multiple transcriptional products, which are generated from most PDE genes by alternative splicing or transcription from distinct promoters, have been identified or predicted in human genome databases (Francis et al., 2011).

The number of PDE genes in crustaceans is unknown. YOs have PDE activity, which is inhibited by IBMX, 8MM-IBMX, and zaprinast, but not EHNA or rolipram (Nakatsuji et al., 2006). These data suggest that the YO has PDE1 and PDE5 activity, but not PDE2 or PDE4 activity. Interestingly, IBMX inhibits ecdysteroidogenesis in the green crab *Carcinus maenas* YO, but not the blackback land crab *Gecarcinus lateralis* YO, which suggests that there is a difference in cyclic nucleotide metabolism between the two species (Nakatsuji et al., 2009; Covi et al., 2008, 2009, 2012).

The purpose of this research project is to identify and characterize the different types of PDEs in the YO of the blackback land crab *Gecarcinus lateralis* and the green crab (*Carcinus maenas*) by using the power of transcriptomics, as well as conventional lab techniques, such as qPCR and *in-vitro* experiments. The hypothesis is based on the premise that the reduced sensitivity to MIH by the committed YO is associated with a large increase in PDE activity, which suggests that PDEs
modulate the response to the neuropeptide, MIH, during the molt cycle. Thus, increasing ecdysteroidogenesis.

This thesis addresses the identification and characterization of the PDEs in the land crab and green crab YO from the transcriptomics data, reports results from YO assays in both land crab and green crab, compares PDE gene expression in different tissues from both the land crab and green crab using qPCR, and reports the effects of MLA and ESA ± rapamycin on PDE gene expression using RNA-seq and qPCR. The thesis concludes with a chapter summarizing the results and their significance and recommending future directions.
Figure 1.1. Proposed MIH signaling pathway regulating ecdysteroidogenesis in decapod crustacean molting gland. The “triggering” phase is initiated by binding of MIH to a G protein-coupled receptor (MIH-R) and activation of adenylyl cyclase (AC); cAMP increases intracellular Ca\(^{2+}\) via cAMP-dependent protein kinase (PKA) phosphorylation of Ca\(^{2+}\) channels. Sensitivity to MIH is determined by phosphodiesterase 1 (PDE1) activity, which varies during the molting cycle. The “summation” phase is mediated by NO and cGMP. Calmodulin (CaM) links the two phases by activating NO synthase (NOS) directly and indirectly via calcineurin (CaN). Dephosphorylation of NOS by CaM can potentially prolong the response to MIH. CaM can also activate PDE1 to inhibit the triggering phase (PDE1 can also hydrolyze cGMP, thus inhibiting the summation phase). cGMP-dependent protein kinase (PKG) inhibits ecdysteroidogenesis. Chronic activation of PKA may directly inhibit ecdysteroidogenesis. Our assumption is that YOs from all decapods are regulated by the same pathway but may differ in the sensitivity of the triggering and summation phases. Other abbreviations: G, G protein; GC-I, NO-sensitive guanylyl cyclase; PDE5, cGMP PDE. From Covi et al., (2012).
Figure 1.2. Hormonal regulation of molting in the blackback land crab, *Gecarcinus lateralis*. Diagram shows the relationship between molt stage, YO state, YO sensitivity to MIH, limb regeneration (R index), YO ecdysteroid synthetic capacity, and hemolymph ecdysteroid titer. During postmolt (A, B, C_{1-3}), intermolt (C_{4}), early premolt (D_{0}), and mid premolt (D_{1,2}), hemolymph ecdysteroid titers are correlated with YO synthetic capacity; during late premolt (D_{3,4}), high ecdysteroid represses YO ecdysteroidogenesis and ecdysteroid titer falls. The YO transitions through four physiological states during the molt cycle: basal (B), activated (A), committed (C), and repressed (R). The B to A transition is triggered by a reduction in MIH; the YOs hypertrophy, but remain sensitive to MIH, as premolt is suspended by MIH injection or by limb bud autotomy (LBA). At the A to C transition, the animal becomes committed to molt, as the YO is less sensitive to MIH and premolt is not suspended by LBA; this transition may be triggered by an increase in MIH or an unidentified tropic factor. At the C to R transition, YO ecdysteroid synthetic capacity remains high, but high hemolymph ecdysteroid titer inhibits ecdysteroid secretion. Molting, or ecdysis (E), marks the R to B transition, during which the YO atrophies and becomes sensitive to MIH. From Chang and Mykles (2011).
**Table 1.1.** Human PDE isozymes are divided into 11 families and differ according to substrate specificity, mechanisms of regulation, and sensitivity to inhibitors. ↑ represents an increase in catalytic activity, whereas ↓ represents a decrease in catalytic activity. * Number of isozymes refers to the number of distinct protein products derived from all genes within a given family that have been identified to date. From Fajardo et al., (2014).

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<th>Isoenzyme Family</th>
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<th>Inhibitors</th>
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<td>cGMP: ↑ PKG: ↑</td>
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CHAPTER TWO

IDENTIFICATION AND CHARACTERIZATION OF THE PHOSPHODIESTERASES (PDES) IN THE DECAPOD CRUSTACEAN’S Y-ORGAN USING TRANSCRIPTOMICS AND qPCR

Summary

Cyclic nucleotide signaling mediates the suppression of the crustacean molting gland (Y-organ or YO) by molt-inhibiting hormone (MIH). When MIH level drops the YO transitions from the basal to the activated state and the animal enters premolt. During mid-premolt, the YO transitions to the committed state, in which the YO becomes insensitive to MIH. Phosphodiesterases (PDEs) hydrolyze the phosphodiester bond in cAMP and cGMP to AMP and GMP, respectively, and thus can modulate the response of the YO to MIH. In some species, PDE inhibitors decrease molting hormone (ecdysteroid) biosynthesis by the YO in-vitro, indicating that PDE activity can keep cyclic nucleotide levels low. Increased PDE activity in the YO is correlated with a reduced sensitivity to MIH when the animal becomes committed to molt. In mammals, 21 PDE genes are organized into 11 families, designated PDE1 to PDE11. Each PDE family has specific catalytic and biochemical properties and tissue distributions. The number and types of PDE genes in crustaceans is unknown. A reference YO transcriptome from the blackback land crab (Gecarcinus lateralis), consisting of 3 biological replicates of intermolt animals, was analyzed for PDE sequences. Nine different contigs encoding seven full-length PDE sequences two partials were identified in G. lateralis. Seven contigs encoding four full-length PDE sequences
and three contigs encoding partial-length PDE were identified in the green shore crab (*Carcinus maenas*) transcriptome. Protein alignments and ClustalX analysis of the Gl-PDE sequences with orthogs from other species in the GenBank database showed that the sequences corresponded to PDE1, 2, 3, 4, 5, 7, 8, 9, and 11. General and selective inhibitors were used to characterize the PDEs regulating ecdysteroid secretion in the green crab, *Carcinus maenas*, YO. IBMX, vinpocetine, EHNA and zaprinast ± rMIH significantly inhibited ecdysteroid secretion, while rolipram, dipyridamole, and BC11-38 did not. This suggests that PDE1, PDE2 and PDE5/11 are primarily responsible for regulating cAMP and cGMP levels. No effect on ecdysteroidogenesis was seen on the blackback land crab, *Gecarcinus lateralis*, YOs when exposed to the same PDE inhibitors *in-vitro*, indicating different regulatory metabolic machineries between the two species.

**Introduction**

Cyclic nucleotide phosphodiesterases (PDEs) are enzymes involved in the regulation of the intracellular concentrations of the second messengers cAMP and/or cGMP (Thompson and Appleman, 1971; Conti, 2000; Soderling and Beavo 2000). PDEs belong to a highly conserved family among all the phyla, and due to their importance in the clinical field, they have received high attention and interest (Levy et al., 2011; Ahmad et al., 2015). Because of the biochemical properties, as well as distinguishing features and complexity of the PDE system, many sophisticated and advanced approaches have been established to understand their role in regulating the cyclic nucleotides cAMP/cGMP in several signaling pathways. This has been linked to the basic pharmacological fact that regulating the degradation of any ligand or second messenger provides greater efficiency, in terms of a prompt and a greater alteration in their percentage concentration, than the regulation and modulation of their synthesis rate (Bender and Beavo, 2006; Halpin, 2008).
In mammalian systems, the PDE I superfamily consists of 11 distinct PDE families where each family is unique in having different isoforms and splice variants. PDEs are the only known enzymes that are capable of hydrolyzing or breaking down the phosphodiester bond in the second messengers cAMP/cGMP to their 5’ inactive monophosphates, so their concentrations remain regulated throughout the cell at all times. PDEs are also effective in controlling the amplitude, spatial, and temporal duration of these cyclic nucleotides as well, so they are not located in unnecessary parts of a cell (Puzzo et al., 2008; Demirbas et al., 2013; Mittal et al., 2017).

Beside the crucial role of PDEs in mammals, PDEs have also been found to be pivotal in other species. For instance, the dunce gene, a cAMP PDE, in Drosophila melanogaster, fruit fly, is important in learning, memory and female fertility (Bellen et al., 1987; Yh et al., 1991; Day et al., 2005). Moreover, these PDEs, as the main players in the cAMP signaling pathway and the cGMP/NO signaling pathway, contribute indirectly in a variety of physiological processes; such as muscle relaxation, visual transduction, endocrine, neuronal, immune, and cardiovascular functions (Yan et al., 2016). Moreover, some PDEs will degrade only cAMP (PDE4, PDE7, PDE8), while others will only hydrolyze cGMP (PDE5, PDE6, PDE9). The rest of the PDE families, PDE1, PDE2, PDE3, PDE10, PDE11, are capable of catalyzing both second messengers as they have a dual specificity (Beavo and Brunton 2000; Mehats et al., 2002). It is thought that an invariant glutamine residue located at the catalytic core is responsible for the PDE cyclic nucleotide selectivity (Xu et al., 2000; Xu et al., 2004; Ke et al., 2011).

All eleven PDE families share a conserved catalytic domain on the carboxyl terminus and a variable regulatory domain on the amino terminus (Francis et al., 2011). About 270 conserved amino acids span the catalytic domain, and the sequence identity can reach high percentages of 35-50% between different PDE families (Houslay and Adams, 2003). This identity can be up to
70% within the same family. The PDE catalytic domain is an alpha helical region that is composed of 16 α-helices which in turn can be divided into three subdomains. These three subdomains form a deep hydrophobic pocket where a Zn\(^{2+}\) binding site is located. Within the PDE catalytic domain, a glutamine switch (Q-switch) is made up of an invariant glutamine residue, which is important to control the selectivity of PDEs toward cAMP or cGMP or both cyclic nucleotides (Xu et al., 2000). On the other hand, regulatory domains differ among the PDE families and that is what makes each PDE family unique and special in terms of their mode of regulation and sensitivity to specific inhibitors. For instance, PDE1 was among the first discovered PDE families; it is particularly regulated by a Ca\(^{2+}\)/Calmodulin (CaM) binding site. PDE3 contains a transmembrane domain and PDE4 is modified by upstream conserved regions (UCRs). PDE8 has two distinct regulatory domains: a response regulatory receiver (REC) and PAS. PDE7 and PDE9 lack specific domains on their amino termini (Omori and Kotera, 2007). On the other hand, about half of the PDE families (PDE2, PDE5, PDE 6, PDE10, PDE11) have tandem GAF domains that function in the dimerization of these PDEs, in addition to the cGMP binding region (Ho et al., 2000; Yausa et al., 2000). Moreover, human PDEs databases show that 21 genes produce transcriptional variants that arose from alternative splicing or transcription from various promoters (Omori and Kotera, 2007; Francis et al., 2011).

The number and types of PDE genes in crustaceans is unknown. Studies by Nakatsuji et al (2008) addressed the hypothesis that the responsiveness of crayfish (*Procambarus clarkii*) YOs to MIH may be caused by the alteration of the PDE activity throughout the molt cycle. Furthermore, YOs have PDE activity, which is inhibited by IBMX, 8MM-IBMX, and zaprinast, but not EHNA or Ro-20-1724 (Nakatsuji, 2006). These data suggest that the YO has PDE1 and PDE5 activity, but not PDE2 or PDE4 activity. Interestingly, IBMX inhibits ecdysteroidogenesis
in the green shore crab *Carcinus maenas* YO, but not the *G. lateralis* YO, which suggests that there is a difference in cyclic nucleotide metabolism between the two species (Nakatsuji, 2009; Covi et al., 2008, 2009, 2012)

This study was conducted to identify and characterize the different PDEs in the crustacean YO. For this purpose, a reference YO transcriptome from the blackback land crab (*Gecarcinus lateralis*), consisting of 3 biological replicates of intermolt animals, was analyzed for PDE sequences that are essential elements in the cAMP/cGMP signaling pathways. Two different transcriptome databases of the green shore crab (*Carcinus maenas*) were used to extract different PDE families. Multiple alignments, phylogeny of PDEs from a variety of orthologs and a comparison between PDEs in both *G. lateralis* and *H. sapiens* are exhibited in this chapter. *In-vitro* YO assays from both *G. lateralis* and *C. maenas* were performed to determine the effects of the different PDE inhibitors on ecdysteriodogenesis. Real-time PCR was conducted to compare the PDE gene expression from different tissues from the both studied decapod species.

**Materials and Methods**

**Animals:**

Adult male *Gecarcinus lateralis* (blackback land crabs) were collected from the Dominican Republic and shipped to Colorado, USA by commercial air cargo. The animals were adapted and acclimated to the new conditions by maintaining them at 27 °C and a relative humidity of ~80%. Intermolt crabs were kept in plastic cages with aspen bedding moistened with 5 p.p.t. Instant Ocean (Aquarium Systems, Mentor, OH). Crabs were maintained in an environmental chamber in a 12 hrs light:12 hrs dark cycle and were fed iceberg lettuce, carrots, and raisins twice a week (Covi et al., 2010). Blackback land crabs molt about once a year. Our other model species, *Carcinus maenas* (green shore crab) was collected from Bodega Bay Harbor in California. Animals were
kept in their optimal conditions either in Bodega Marine Laboratory or when they were shipped to Colorado. At CSU, green crabs were fed chicken liver once a week and were maintained in aerated tanks less than half filled with 30 p.p.t Instant Ocean at 20 °C. The water was changed twice a week unless it became cloudy or a death happened in a tank (Lee et al., 2007).

**Transcriptomics:**

*Gecarcinus lateralis* and *Carcinus maenas* lack a fully sequenced genome. Therefore, a reference YO transcriptome from the blackback land crab (*G. lateralis*), consisting of 3 biological replicates of intermolt animals, was assembled from RNA-seq data. For this purpose, the fiddler crab (*Uca pugnas*) limb bud transcriptome was used as a query to extract the different PDEs in the land crab’s YO transcriptome (Das et al., 2016). *G lateralis* PDEs served as queries to extract different PDEs from two *C. maenas* transcriptome databases (Tepolt & Palumbi 2015; Verbruggen et al., 2015). By using the software perfectBlast, the PDE nucleotide sequences were extracted. Upon extraction, each nucleotide sequence was translated by using the translate tool, EXPASY (https://web.expasy.org/translate/) and the appropriate Open Reading Frame (OPR) was chosen. Then, a standard protein BLAST, blastp, (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), as well as SMARTBLAST (https://blast.ncbi.nlm.nih.gov/smartblast/smartBlast.cgi) was used to find out the corresponded PDE and the possible similar orthologs. Also, a conserved domain search from the Conserved Domain Database (CDD) was used to locate the different PDE domains, (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). InterPro was used for protein sequence analysis and classification (https://www.ebi.ac.uk/interpro)
**In-vitro YO assays:**

Hemolymph was withdrawn from each animal prior to dissecting. 100 μl of hemolymph was combined with 300 μl methanol (MeOH 100%). Green crabs were dissected directly on the assigned date, but the case was a bit different for the land crabs as they were dissected on day 3 post ESA to allow the YOs to be stimulated and activated to secrete ecdysteroids. For both species, one YO of each pair was incubated in 500 μl of crab saline with the appropriate vehicle (as the control), whereas the other pair was incubated in 500 μl of crab saline with the experimental chemical agent, a PDE inhibitor (as the experimental). The incubation time for both control and experimental samples was 4.5 hours, then 200 μl of media was added to 600 μl Methanol (MeOH 100%). Hemolymph and media samples were sent to Bodega Marine Laboratory for ELISA to evaluate the ecdysteroid levels.

**RNA isolation, cDNA synthesis and PCR:**

Different tissues (B, CM, ESG, G, H, HG, HP, MG, T, TG, YO) were harvested from intermolt land crabs and green crabs. All tissues were placed immediately in RNA-later after cleaning the tissues in crab saline. Tissues were kept overnight in 4°C, then transferred to -20°C until the time of RNA purification. Total RNA was isolated from crab tissues using TRIzol reagent (Life Technologies, Carlsbad, CA) as described by (Covi et al., 2010). YO tissues (50-200 mg) were homogenized by using a micro-tube homogenizer system, while all the other tissues were homogenized by a Qiagen TissueLyser II for two minutes at a frequency of 30 revolutions per second. One ml TRIzol was added to the samples, then centrifuged at 12,000 g for 15 min at 4°C. Supernatants were phenol-chloroform extracted and RNA in the aqueous phase was precipitated using isopropanol (0.75 ml per 1 ml TRIzol reagent). RNA was treated with DNase I (Life Technologies), extracted twice with phenol: chloroform:isoamyl alcohol (25:24:1), precipitated
with isopropanol, washed twice with 70% ethanol in DEPC water, and resuspended in nuclease-
free water. A nanodrop spectrophotometer was used to verify the purity of RNA. cDNA was
synthesized using 2 μl total RNA in a 20 μl total reaction with SuperScript III reverse transcriptase
(Life Technologies) and oligo-dT (20) VN primer (50 μmol/l; IDT, Coralville, IA) as described
(Covi et al., 2010). RNA was treated with RNase H (Fisher Scientific, Pittsburgh, PA) and stored
at -80 °C.

End-point PCR was used to amplify the desired product and to increase the yield of each
PDE gene as well as making external standards of the different genes to be used later in qPCR.
Sequence-specific primers (Table 2.2.) were utilized to detect the different PDE products in both
land crab and green crab. Each PCR reaction contained 3 μl DI H₂O, 5 μl Master Mix, 1 μl cDNA
template, and 0.5 μl of each forward and reverse primers. The concentration of the primers was 20
μM. cDNA was amplified in a thermocycler where denaturation occurred at 94 °C for 3 minutes
to initiate the process, then followed by 30-35 cycles of 30 seconds at 94 °C, 30 seconds at the
lowest annealing temperature (see Table 2.1.), 30 seconds at 72 °C. Final elongation was set for 7
minutes at 72 °C. PCR products were then separated on 1% agarose gel that contained TAE buffer
(composed of 40 mM Tris acetate and 2 mM EDTA with an 8.5 pH). Ethidium bromide was
applied to stain the gel and a UV light was used to visualize the gel.

Tissue expression of G. lateralis and C. maenas PDEs:

Real-time PCR (RT-PCR) was used to quantify the expression of the different PDEs in the
following tissue; Brain, Claw Muscle, Eyestalk Ganglia, Gill, Heart, Hind Gut, Hepatopancreas,
Mid Gut, Testis, Thoracic Ganglion, and Y-organ to display a panel comparison between two
crustacean models; the blackback land crab G. lateralis and the green shore crab C. maenas.
Animals from both species were adult intermolt male crabs.
cDNA was synthesized as indicated previously, and a LightCycler 480 thermocycler (Roche Applied Science, Indianapolis, IN) was used to quantify the mRNA transcripts of PDE1, PDE 2, PDE4, PDE7, PDE9, and PDE11 for *G. lateralis* and PDE4, PDE5, PDE9, and PDE11 for *C. maenas*. Each reaction consisted of 1 μl cDNA or standard, 5 μl SYBR Green I Master mix (Roche Applied Science), 3 μl nuclease-free water, and 0.5 μl each of 10 mM forward and reverse primers (Table 2.3). PCR conditions were as follows: an initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 62 °C for 20 s, and extensions at 72 °C for 20 s, followed by melting curve analysis of the PCR product. Concentrations of mRNA transcripts were determined by the LightCycler 480 software (Roche, version 1.5) using a serial dilution of standards of the PCR product for each gene of interest. The amounts of mRNA transcript in copy numbers per μg of total RNA in the cDNA synthesis reaction were calculated based on the standard curve and the calculated molecular weight of dsDNA products.

**Statistical Analysis and software:**

Multiple sequence alignments were generated by utilizing ClustalX version 2.0.12 (Thompson et al., 1997) using the amino acid sequences. A phylogenetic tree was constructed by using PhyML 3.0 (Dereeper et al., 2008; Guindon et al., 2010; Anisimova and Gascuel 2010) and iTOL (Letunic and Bork 2016). A schematic diagram compared the domain organization between the *G. lateralis* and *Homo sapiens* PDEs. For *in-vitro* YO assays, a paired t-test was used to compare the means of ecdyseroid levels secreted from both control and experimental YOs. Primers were designed by IDT. Means for mRNA transcript abundance were compared using an analysis of variance (ANOVA) for tissue distribution versus log copy number. Sigma plot 12.5 software (Systat Software, Inc., Chicago, IL, USA) was used to produce and build up the graphs and figures. The Tukey test was used to determine significance among the means.
Results

Characterization and identification of PDEs from the G. lateralis YO transcriptome and C. maenas cardiac transcriptome:

A de novo transcriptome was used to characterize and identify the different PDE families located in the YO. The extraction resulted in a total of nine PDE contigs, including seven full length sequences and two partial sequences (Table 2.1). A de novo cardiac/assembly of C. maenas transcriptome was utilized to characterize and identify the different PDE families. The extraction resulted in a total of seven PDE contigs, four with full length sequences and three with partial sequences (Table 2.2.). ClustalX analysis assigned the G. lateralis PDEs to PDE1, PDE2, PDE3, PDE4, PDE5, PDE7, PDE8, PDE9, and PDE11. Multiple sequence alignments showed high sequence identities with orthologs from other species in the catalytic and other conserved functional domains. The conserved catalytic domain of all PDEs contained the initiating YHN (or FHN in PDE9) motif, as well as the metal binding motif with a specific sequence signature (HDX2HX4N).

Characterization of Ca²⁺/CaM PDE Gl-PDE1:

A full sequence length, ~ 3037 bp, of PDE1 contig was found in the YO transcriptome through RNA sequencing (RNA-seq). Gl-PDE1 corresponds to the Ca²⁺/ calmodulin PDE in other orthologs, as it contains the following domains: a conserved catalytic domain at the carboxyl terminus and a regulatory domain specifically coding to a Ca²⁺/ calmodulin domain proximal to the amino terminus. Both the DNA and the deduced amino acid sequences are shown in Fig 2.1. Multiple sequence alignments of the G. lateralis PDE1 and other orthologs from different species
showed high levels of sequence identity/similarity (Fig 2.2). Identity to the fruit fly PDE1C was 55%, whereas identity to the human PDE1C was 44%.

Characterization of the GAF-PDEs: Gl-PDE2, Gl-PDE5, Cm-PDE5, Gl-PDE11, Cm-PDE11 and the specific cGMP Gl-PDE9 and Cm-PDE9:

Three GAF-PDEs with full length sequences were established in the G. lateralis YO transcriptome, as they corresponded to PDE2, PDE5, and PDE11 orthologs. All these genes were composed of the conserved catalytic domain and either one or two GAF regulatory domains. Whereas Gl-PDE2, a PDE dual substrate (4729 bp) contained only one GAF domain, namely GAF B (Fig. 2.3), Gl-PDE5, a cGMP binding PDE (6761 bp), and Gl-PDE11, a dual specificity PDE (5752 bp) had two tandem GAF domains (GAF A and GAF B) (Fig. 2.8 & Fig. 2.18). Cm-PDE5 (2996 bp) and Cm-PDE11 (5839 bp) were demonstrated in Figs. 2.9 & 2.19. Multiple sequence alignments showed high sequence identity/similarity. For instance, Gl-PDE2 shared 56% identity with human PDE2A3 and 38% identity with C. elegans PDE2 (Fig. 2.4). Gl-PDE5 shared 89% identity with Cm-PDE5 and 42% identity with H. sapiens PDE5A (Fig. 2.10). The dual PDE, Gl-PDE11, shared 97% identity with Cm-PDE11 and about 72% with D. melanogaster PDE11C, whereas it shared 50% identity with the human PDE11A (Fig. 2.20). Moreover, Gl-PDE2, 5, & 11 and Cm-PDE5 & 11 contained the conserved motif sequence, NKFDE, in their GAF domains. Although Gl-PDE9 (4111 bp) and Cm-PDE9 (4356 bp) are cGMP-specific PDEs, they both lack the GAF domains. The DNA and amino acid sequences are presented in Fig. 2.15 and Fig. 2.16. Multiple alignments showed that Gl-PDE9 has an identity of 93% with Cm-PDE9, 52% with Dm PDE9B and 55% with Hs-PDE9A (Fig. 2.17).
Characterization of cAMP-specific PDEs: Gl-PDE4, Cm-PDE4, Gl-PDE7 and Cm-PDE8:

cAMP-specific PDE, Gl-PDE4, and high affinity cAMP-specific PDE, Gl-PDE7, were found in the YO transcriptome as full length contigs (6130 bp and 7531 bp, respectively). Both PDEs had the conserved catalytic domain near the carboxyl terminus. Two upstream conserved regions (UCRs) have been detected in the regulatory domain in the Gl-PDE4 gene (Fig. 2.5). Cm-PDE4 was found in the cardiac transcriptome as a full length contig (6122 bp) with a conserved catalytic domain proximal to the COOH terminus. One UCR was observed in the regulatory domain region of Cm-PDE4 (Fig. 2.6). Gl-PDE4 has an identity of 91% with Cm-PDE4, 64% with the D. melanogaster dunce gene PDE4D and 60% with H. sapiens PDE4D3 (Fig. 2.7). The DNA and deduced amino acid sequences of Gl-PDE7 are shown in Fig. 2.11. Moreover, Gl-PDE7 has an identity of 46% to both PDE7A in H. sapiens and D. rerio (Fig. 2.12). High affinity cAMP specific and IBMX insensitive PDE, Cm-PDE8, has been revealed in the cardiac transcriptome of the green shore crab as a full length contig (3007 bp). Cm-PDE8 contains a conserved catalytic domain near the carboxy termini and the PAS regulatory domain in the amino terminus (Fig. 2.13). Multiple alignment of Cm-PDE8 with ortholog species showed identity of 44% with Dm-PDE8O and 36% with Hs-PDE8B (Fig. 2.14).

Phylogeny of G. lateralis and C. maenas PDEs with different orthologs:

A phylogenetic tree was constructed using the software iTOL (Interactive Tree Of Life), (https://itol.embl.de/itol.cgi). 102 PDE orthologs from different invertebrate and vertebrate species were compared to the nine G. lateralis PDE contigs from the YO transcriptome and seven C. maenas PDE contigs from the green shore crab cardiac transcriptome. All PDEs from G. lateralis and C. maenas were closely related. Moreover, all sixteen PDEs clustered with their corresponding orthologs (Letunic and Bork 2016) (Fig. 2.21).
Comparison between G. lateralis and H. sapiens PDEs:

As illustrated in Fig. 2.22, all nine PDEs from the blackback land crab were compared with the corresponding human PDE. The domain organization with their interval lengths are shown as well. Catalytic domains are located near the carboxyl terminus in both species. This was the case for all PDEs except for Gl-PDE7 and Gl-PDE9, as their catalytic domains seemed distal to the carboxyl terminus and were not organized with Hs-PDE7 and Hs-PDE9. Moreover, Gl-PDE2 contained only one GAF domain, which contrasted with Hs-PDE2, which has two GAF domains. Moreover, Gl-PDE3 and Gl-PDE8 were found as partial contigs, so no regulatory domains were present to match them to the corresponding human PDEs. PDE1, PDE4, PDE5, and PDE11 were similar in both species regarding their lengths and domain localization.

In-vitro Y-Organ assays in G. lateralis and C. maenas:

Y-organs in both studied species had undergone in-vitro assays to observe the effects of different PDE inhibitors on ecdysteroid synthesis and synthesis. In the green shore crab, C. maenas, PDE inhibitors IBMX, vinpocetine, EHNA, and zaprinast inhibited YO ecdysteroid secretion (Table 2.4), which suggests that PDE1, PDE2, and PDE5/11 play major roles in controlling cyclic nucleotide levels in the YO of C. maenas. Ecdysteroidogenesis was not affected by either rolipram or dipyridamole, which selectively inhibit PDE4 and PDE7 (Table 2.4). In contrast, YOs from the blackback land crab, G. lateralis, showed no effect when exposed to the same PDE inhibitors (Table 2.5).
**PDE gene expression in different tissues in both G. lateralis and C. maenas:**

Quantitative-PCR (qPCR) was used to quantify the mRNA of the nine PDEs in eleven tissues: Brain (B), Claw Muscle (CM), Eyestalk Ganglia (ESG), Gill (G), Heart (H), Hindgut (HG), Hepatopancreas (HP), Midgut (MG), Testis (T), Thoracic Ganglion (TG), and Y-Organ (YO) in the blackback land crab and the green shore crab.

*G. lateralis PDE1* was expressed in low levels in all tissues, but still there was a significant difference between the heart and YO, as well as the claw muscle, to the YO and midgut. *G. lateralis PDE2* and *PDE5* showed relatively high expression in all the tissues with the highest mRNA level in the heart, which contrasted with *PDE11*, which was expressed at lower levels in the same tissues. *Gl-PDE2* showed a significant increase of ~ 100-fold in the heart compared to hepatopancreas and hindgut. Also, the YO demonstrated considerable increase difference (~15-fold change) when compared to the hepatopancreas. *Gl-PDE5* displayed slight significant increases between the YO and midgut (6.4-fold change), and among the heart versus hepatopancreas and midgut (~ 10-fold change). The testes showed the highest *Gl-PDE11* expression and this tissue was different from the claw muscle and hindgut (~8-fold change). An inconsiderable difference was exhibited when the YO was compared to the hindgut (Figs. 2.23 a, b, c, d). Gl-PDE 4, 7, and 9 were expressed in such extremely low levels that they could be barely detected. There was no statistical significance among the tested tissues.

In contrast, *C maenas PDE11* was expressed at high levels in all the tissues except in the HG. A significant difference of mRNA expression of *Cm-PDE11* was seen in the thoracic ganglion versus each of midgut, ~15*10^4-fold increase, and claw muscle, ~5*10^3 fold increase. Also, the eyestalk ganglia were significantly different from the midgut by ~15*10^3-fold increase. *Cm-PDE4*, *Cm-PDE8*, and *Cm-PDE9* were expressed in very low levels in all tissues. *Cm-PDE4*
displayed a minor difference when comparing the claw muscle to both thoracic ganglion and the YO. Moreover, a small statistical significance was noted between the testis and the YO. Cm-PDE8 demonstrated a slight statistical significance observed in the brain versus both the midgut and the thoracic ganglion, also there was a small change between the eyestalk ganglion and thoracic ganglion. Finally, Cm-PDE9 was expressed with minor differences in the thoracic ganglion versus both midgut and hindgut, and between the YO and hindgut (Figs. 2.24 a, b, c, d).

**Discussion**

PDEs are important and crucial players in both cAMP and cGMP signaling pathways. They act as negative regulators that breakdown/hydrolyze these second messengers (Thompson and Appleman, 1971; Conti, 2000; Soderling and Beavo 2000). Since the numbers and types of PDEs are still unknown in crustaceans, a de novo transcriptome (Das et al., 2016) was used to identify and characterize these enzymes. In this novel study, nine PDE contigs were extracted from the YO transcriptome. Sequence analysis assigned the Gl-PDE sequences to seven full length PDEs: PDE1, PDE2, PDE4, PDE5, PDE7, PDE9, and PDE11 and two partial PDEs: PDE3 and PDE8, thus indicating a high diversity of PDE genes found in decapod crustaceans. By similarity and specificity, this group comprises three cAMP-PDEs, two cGMP-PDEs, and four dual-specific PDEs. Moreover, seven PDE contigs were extracted from a de novo assembly of the C. maenas cardiac transcriptome (Tepolt & Palumbi 2015; Verbruggen et al., 2015). Sequence analysis assigned Cm-PDEs to four full-length PDEs: PDE4, PDE8, PDE9, and PDE11 and three partial PDEs: PDE1, PDE5 and PDE3. This prevalent expression of the assorted PDE genes in the YO of the blackback land crab and green shore crab might suggest a vital function in controlling the physiological differences that occur in the molting gland throughout the molt stages.
As a comparison to other arthropod models, the fruit fly, *Drosophila melanogaster*, genome encodes six different PDEs (Day et al., 2005), including the famous *dunce* gene that has been connected to several psychological issues because of its potential role in memory and learning (Walter & Kiger 1984; Bolger et al., 1993).

By examining the deduced DNA and amino acid sequences for Gl-PDEs, as well as the pairwise comparison between Gl-PDEs and Hs-PDEs, the following features were noted: the conserved catalytic domain in all PDE families (which is located in the carboxyl region of the protein and accounts for about 270 amino acids) shares high identity and is composed of a dense alpha-helical structure composed of 16 α-helices which in turn is divided into three subdomains. These α-helices form the active site, which is highly conserved among all PDE families (Charbonne et al., 1986; Francis et al., 2011). Different affinities toward different cyclic nucleotide substrates are assumed to occur because of the slight variation in the catalytic domain for each PDE family (Manallack et al., 2005). Moreover, the active site is organized in two regions: the hydrolysis center and the hydrophobic recognition pocket (Wang et al., 2003; Liu et al., 2007). All PDEs have a similar structure regarding the hydrolysis center as they follow the general hydrolysis mechanism in breaking down and hydrolyzing cAMP/cGMP (Liu et al., 2007). The deep hydrophobic pocket, which can bind to either the substrate or the inhibitor, contains the glutamine switch (Q-switch). The Q-switch has a very important role in PDE nucleotide selectivity, as well as two metal binding sites important in catalytic activity, mainly divalent cations such as Zn$^{2+}$ and possibly Mg$^{2+}$. Histidine residues are essential in chelating such metal ions (Xu et al., 2000; Houslay 2001; Richter et al., 2001; Liu et al., 2007; Ke et al., 2011). The basis of the glutamine switch is to either orient to hydrolyze cAMP or cGMP and, in that case, it will be constrained tightly with hydrogen bonds with the selective cyclic nucleotide. But in the
case of a dual PDE, the Q-switch rotates freely in either direction, depending on which substrate (cAMP/cGMP) needs to be hydrolyzed (Ke et al., 2011).

Interestingly, G. lateralis PDEs contained the conserved catalytic domain proximate to their C-terminus resembling other species. The length of the conserved catalytic domain in all studied Gl-PDEs ranged between 200-270 amino acids, which was comparable with previously characterized PDEs. Furthermore, the catalytic domain was initiated by the conserved signature sequence motif (YHN) in all PDEs, except in PDE9 which started with (FHN) and this was consistent with other orthologs from different invertebrate and vertebrate species (Broderick et al., 2003 and Wang et al., 2003). The metal binding motif with the specific sequence signature (HDX₂HX₄N), which has about 11 invariant amino acids (Manallack et al., 2005), aligned nicely and was identical among G. lateralis PDEs and PDEs from other species.

In contrast, the N-terminal regulatory domain, which borders the catalytic domain, is unique and distinct for each PDE family. N-terminal regulatory domains regulate the enzymatic activity of PDEs. Also, regions that autoinhibit the catalytic domains or regulate PDE subcellular localization are found in the N-terminal region (Omori & Kotera 2007; Azevedo et al., 2014).

The PDE1 family, a dual PDE, is the only family that depends exclusively on the influx of calcium ions to stimulate enzyme activity. This is an example of cross-talk between the cAMP and Ca²⁺ signal transduction pathways. Mammalian PDE1 has two Ca²⁺/calmodulin (Ca²⁺/CaM) regulatory domains that contain binding sites for the Ca²⁺/CaM complex (Gross & Clark 1977; Meeker & Harden 1983; Gooraya et al., 2004; Gooraya & Cooper 2005). In contrast to the human PDE1C, Gl-PDE1 contained only one Ca²⁺/CaM domain, which is similar to the Drosophila PDE1, suggesting that arthropods might not require both binding sites (Sonnenburg et al., 1995; Day et al., 2005).
The GAF domain represents a highly conserved sequence that has been conserved throughout more than 2 billion years of evolution. GAF got its name from the first three proteins in which the sequence was reported: cGMP-specific cyclic nucleotide PDEs, cyanobacterial *Anabaena* adenyl cyclase, and *E. coli* transcription factor PhLA. Although PDE GAF domains bind cGMP, other proteins that contain these sequences do not bind cGMP (Martinez et al., 2002). Mammalian GAF-PDEs contain two tandem GAF domains, GAF-A and GAF-B, in the N-terminal region. Catalytic activation requires at least one of the GAF domains to bind a cGMP. For instance, PDE2 in mammalian systems contain two GAF domains, and GAF-B binds cGMP, whereas in PDE5, GAF-A binds this cyclic nucleotide (Lin et al., 2002; Francis 2005). Such binding causes a conformational change, allowing PKG to phosphorylate a nearby serine, which increases the catalytic activity. PDE11, the most recent PDE discovered and the one most related to PDE5, is still poorly understood, but it contains homologous regulatory domains as in PDE2 and PDE5. All GAF PDEs produce multiple variants via alternative splicing and different initiation sites.

Three GAF-PDEs, PDE2, 5, and 11 have been characterized in this study and they shared features and characteristics with other analogs, such as the conserved signature motif (N(K/R)X₉FX₃DE) specific to the GAF domains. Gl-PDE2 aligned nicely with *C. elegans* and *H. sapiens* with identical regions in the catalytic and regulatory domain. In contrast to Hs-PDE2, which contained two complete GAF domains, Gl-PDE2 contained only the GAF-B domain similar to the protozoan parasite *Trypanosoma brucei* Tb PDE2A and Tb PDE2B (Zoraghi & Seebeck, 2002). Only one GAF domain binds to cGMP or cAMP (Martins et al., 1982; Heikaus et al., 2009). The presence of a single GAF domain in Gl-PDE2 might be enough to bind small molecules such as cAMP/cGMP in an allosteric manner to stimulate the catalytic core domain or might bind a different small molecule, since it is originated from a different ancestor than PDE5 and PDE11.
Due to the fact that Gl-PDE2 is a dual enzyme, it might serve and mediate a cross-talk between cAMP and cGMP signaling pathways (Houslay 2001; Yuasa et al., 2001). Gl-PDE5, Cm-PDE5, Gl-PDE11, and Cm-PDE11 contained two complete tandem GAF domains homologous to the *Homo sapiens* PDEs; Hs-PDE5A and Hs-PDE11A4, respectively. The two GAF domains are linked by a variable region of amino acids which was consistent with another GAF PDEs (Yuasa et al., 2001; Makhlouf et al., 2006).

Gl-PDE9 and Cm-PDE9, cGMP-specific PDEs, contained the conserved catalytic domain which aligned with other ortholog PDEs; however, no other regulatory domains were detected. Mammalian PDE9 has a high affinity for cGMP, and it is 20-100 fold higher than PDE5 and PDE6, respectively. This might indicate that PDE9 will be distributed in cells with low titers of cGMP. Moreover, PDE9 contains the most variant catalytic domain when compared to the other PDE family members. It is likely that cGMP binds to the catalytic domain since it lacks the GAF domain (Fisher et al., 1998; Omori & Cotori 2007; Liu et al., 2008).

Gl-PDE4, Cm-PDE4 and Gl-PDE7 both hydrolyze cAMP and are highly similar to orthologs from other species (Figs. 2.6 & 2.10). In addition to the catalytic domain, Gl-PDE4 contains two Upstream Conserved Regions (UCRs) in its N-terminal region (Fig. 2.5) in a similar location to where GAF domains are found in PDE2, PDE5, and PDE11. This arrangement of domains is equivalent to *dunce* PDE, Dm-PDE4 and the human PDE, Hs-PDE4D. The regulatory domains, UCR1 and UCR2, are separated by sequences with less homology. Although the specific function of PDE4 is still open to assessment, studies on *Drosophila* revealed that a mutated *dunce* locus leads to impaired learning and memory in the fly (Bolger et al., 1993). In contrast, Cm-PDE4 contained only one UCR regulatory domain (UCR2) in its N-terminal region (Fig. 2.6). Human PDE4 isoforms exhibit both long forms (two UCR domains) and short forms (lack UCR1). This
complexity in PDE4 variants defines subcellular localization differences between the short and long forms (Xie et al., 2014). Gl-PDE7 is a PDE that hydrolyzes the second messenger, cAMP; it contained the conserved catalytic core domain and shared high similarities with its orthologs. Interestingly, the ORF of Gl-PDE7 displayed an extremely long sequence and ran to ~1039 AA when compared to either other G. lateralis PDEs or orthologs from different species. The 3’ untranslated region showed a similar pattern and had an extended sequence ~ 7531 bp (Fig. 2.11). Such an observation might be the first of a kind to be seen in any PDE. Thus, it might have an importance in post-transcriptional regulation that is essential in mammalian cells (Matoulkova et al., 2012). Moreover, comparable to other studied PDEs to date, no specific regulatory domains were identified. Cm-PDE8 has a high affinity and specificity to hydrolyze cAMP. But is the only cAMP PDE that is insensitive to IBMX (Omori and Kotera, 2007). Cm-PDE8 displayed similar features compared to orthologs from other species in terms of the conserved catalytic domain and PAS regulatory domain. PAS is an acronym from the first three proteins in which in which the sequence was reported: Periodic circadian protein, Aryl hydrocarbon receptor nuclear translocator protein, and Single-minded protein. The PAS domain is a molecular Velcro that binds small proteins and molecules (Tsai & Beavo, 2012). Unlike other PAS proteins, the regulation of PDE8 through PAS is still unknown (Demirbas et al., 2013). Previous studies on PDE8 family stated that it is a regulator in steroidogenesis in Leydig cells, as well as in adrenal steroidogenesis (Tsai & Beavo, 2012). Such findings might indicate the role of Cm-PDE8 on regulating the molting gland’s ecdysteroidogenesis.

As shown in Figure 2. 21, phylogenetic analysis showed that Gl-PDEs and Cm-PDEs clustered with orthologs from invertebrate and vertebrate species indicating high homology and identity. The only exception observed was with PDE5. Invertebrate and vertebrate PDE5s
clustered in two divergent groups, which might indicate these PDE genes have two different ancestral origins. PDE1, 3, 4, 5, 8, 9, and 11 in both *G. lateralis* and *C. maenas* were closely related suggesting similar physiological systems.

YO *in-vitro* assays for both of our model species, green crab and backblack land crab, investigated the sensitivity to PDE inhibitors. Green crab YOs were sensitive to IBMX (3-isobutyl-l-methylxanthine), a potent non-selective PDE inhibitor (Table 2.4). Similar results have been reported in previous studies; IBMX significantly inhibits PDE in the crayfish *P. clarkii* (Nakatsuji et al., 2006). Moreover, in experiments done on *Manduca* larvae, Smith (1993) reported that the incubation of prothoracic glands (which are counterpart to crustacean YOs) with the molting hormone (PTTH) and IBMX *in-vitro*, blocked the degradation of cAMP but not cGMP. Also, PDE activity was seen in the absence of IBMX in the previous study (Smith 1993). Inhibition of PDEs, except PDE8 and PDE9, by IBMX caused an elevation in cAMP activity in the human adrenal gland (Beavo et al., 1970; Tomes et al., 1993). Vinpocetine, a selective PDE1 inhibitor extensively used on rats in studies on neurodegenerative diseases as in the case of Alzheimer’s showed inhibition of PDE1 (Ahn et al., 1989; Deshmuch et al., 2010). Likewise, memory is enhanced in rodents and humans by vinpocetine, which increases cAMP levels (Deshmukh et al., 2011; Medina et al., 2011). Our results showed that green crab YOs were inhibited by vinpocetine, which might indicate a potential role of PDE1 in controlling ecdysteroid synthesis. EHNA, a specific PDE2 inhibitor, inhibited ecdysteroid secretion by the green crab YOs. Similar effects were also observed in mammalian nervous tissues but was not effective in pharmacological tests (Bessodes et al., 1982; Gomez & Breitenbucher 2013). Zaprinast, a selective PDE5/PDE11 inhibitor, inhibited the green crab YOs. Zaprinast, the precursor of sildenafil (Viagra), inhibits PDE5 and PDE11 with different affinities. Zaprinast inhibited ecdysteroidogenesis in YOs of green crabs especially at
higher concentrations. Similar observations were seen in previous studies: *In-vitro* YO assays in crayfish, *P. clarkia*, demonstrated that Zaprinast partially inhibited PDE activity (Nakatsuji et al., 2006). Experiments on *Drosophila* showed that Zaprinast suppressed PDE6, the closest morphologically to PDE5, in Malpighian (renal) tubules (Broderick et al., 2004; Day et al., 2005).

Interestingly, rolipram (selective-PDE4 inhibitor), dipyridamole (selective-PDE7 inhibitor), and BC11-38 (potent PDE11 inhibitor) had no effect on the ecdysteroid secretion in all *in-vitro* YO assays of the green crab. These results differ from previously published studies on other species: *in-vivo* administration of rolipram showed that there were effective neurodegenerative and neuroprotective impacts on the spinal cord of rat embryos (Richer et al., 2001). Moreover, rolipram inhibited PDE4 in *Drosophila* and mice in therapeutic experiments which were done to find a cure for Fragile-X syndrome (Nikulina et al., 2004; Choi et al., 2015). Dipyridamole was used as an anti-inflammatory agent to inhibit PDE7. This chemical was used in specific locations where PDE7 is normally distributed, such as T-cells, B-cells, skeletal and cardiac muscles (Gresele et al., 2011). This might be the reason for the lack of inhibition of Gl-PDE7 in the YO. The novel PDE11 inhibitor, BC 11-38, was used in mice to inhibit PDE11A4 isoform (homologous to Gl-PDE11) in the brain, particularly the hippocampus, and showed tremendous effects as a therapeutic target for mood and depressive disorders (Kelly 2017). Even though this drug is known to have a high membrane permeability, it is still under investigation because of lack of information concerning whether it can cross the blood-brain barrier. Thus, might be the reason that YO ecdysenoidogenesis was not affected when exposed to the potent PDE11 inhibitor, BC11-38. (Ceyhan et al., 2012; Kelly 2017). Another reason might be, the shortage of adequate information of the nature of PDE11 and how it selects specific inhibitors (Weeks et al., 2009).
lateralis YOs were insensitive to all tested PDE inhibitors in this work and that might point to the difference of the YOs regulatory systems between the two species (Table 2.5).

As illustrated in figure 2.23, a tissue distribution panel was constructed to compare the expression of the different PDEs in the YO with other tissues. Gl-PDE2 and Gl-PDE5 were expressed at their highest levels in heart tissue. Mammalian cardiomyocytes express PDE2, but not PDE5 (Weber et al., 2017). PDE2 was expressed at reasonably high levels compared to its expression in other tissues. Remarkably, Gl-PDE11 was highly expressed in the testis compared to other examined tissues. That pattern is parallel to the mammalian system in which PDE11 is expressed in the prostate and testis, as it is thought to function in spermatogenesis (Franscis 2005). Moreover, low expression of Gl-PDEs in the YO was not surprising as these molting glands were harvested from intermolt animals in which results were anticipated. Fig 2.24 shows the tissue expression of Cm-PDEs; Cm-PDE4, 8, and 9 were expressed in very low levels. Cm-PDE11 was expressed in high levels which might indicate a powerful function of this dual PDE in a variety of tissues, especially in the thoracic ganglion, eyestalk ganglia, and YO. Once again, the different gene expression patterns observed in the two studied species might be due of different metabolic systems and variable modes of regulation.

Conclusions

Nine contigs encoding seven full-length PDE sequences and two contigs encoding partial-length PDE were identified in the Gecarcinus lateralis Y-organ transcriptome. Seven contigs encoding four full-length PDE sequences and three contigs encoding partial-length PDE were identified in the Carcinus maenas transcriptome. Multiple sequence alignments showed high sequence identities with orthologs from other species mainly in the catalytic (PDEase) domain and in the regulatory domains. Sequence analysis assigned the Gl-PDE and Cm-PDEs sequences to
PDE1, PDE2, PDE3, PDE4, PDE5, PDE7, PDE8, PDE9, and PDE11 classes, indicating a high diversity of PDE genes are found in decapod crustaceans. PDE inhibitors IBMX, vinpocetine, EHNA, and Zaprinast inhibited YO ecdysteroid secretion, which suggests that PDE1, PDE2, and PDE5/11 play major roles in controlling cyclic nucleotide levels in the YO of green crab, *C. maenas*. The backblack land crab, *G. lateralis*, YOs showed no response when incubated in the same PDE inhibitors. Cm-PDE11, a dual PDE, was expressed at high levels in different tissues. This might indicate a crucial role of PDE11 in cAMP/cGMP signaling pathways; especially the MIH signaling pathway in the molting gland.
Table 2.1: Shows the nine PDE contigs extracted from the *G. lateralis* YO transcriptome. PDE1, PDE2, PDE4, PDE5, PDE7, PDE9, and PDE11 are full lengths, PDE3 and PDE8 are partial lengths. Abbreviations; AA: Amino Acid; bp: base pair; Ca²⁺/CaM: calcium/calmodulin; cAMP: cyclic adenosine 3’,5’-monophosphate; cGMP: cyclic guanosine 3’,5’-monophosphate; PKA: protein kinase A; PKG: protein kinase G.

<table>
<thead>
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<th>PDE Family</th>
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<th>Contig length</th>
<th>ORF length</th>
<th>Descriptive Name</th>
<th>Substrate Specificity</th>
<th>Regulator</th>
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Table 2.2: Shows the seven PDE contigs extracted from the *C. maenas* transcriptomes. PDE4, PDE8, PDE9, and PDE11 are full lengths, PDE1, PDE3 and PDE5 are partial lengths. Abbreviations; AA: Amino Acid; bp: base pair; Ca^{2+}/CaM: calcium/calmodulin; cAMP: cyclic adenosine 3',5'-monophosphate; cGMP: cyclic guanosine 3',5'-monophosphate; PKA: protein kinase A; PKG: protein kinase G.

<table>
<thead>
<tr>
<th>PDE Family</th>
<th>Contig #</th>
<th>Contig length</th>
<th>ORF length</th>
<th>Descriptive Name</th>
<th>Substrate Specificity</th>
<th>Regulator</th>
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Table 2.3. Oligonucleotide primers used in qPCR to identify gene expression of G1-PDEs and Cm-PDEs in different tissues. Abbreviations: G1, Gecarcinus lateralis; Cm, Carcinus maenas; F, Forward; R, Reverse, PDE, cyclic nucleotide phosphodiesterase; Numbers (1,2,4,5,7,8,9,11), PDE family.

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Figure 2.1. Nucleotide and amino acid sequence of cDNA encoding Gl-PDE1. A full-length open reading frame (ORF) was expressed by the cDNA, the start codon highlighted in yellow and the stop codon highlighted in green. Calcium/calmodulin regulatory domain is indicated by a red box and contains the PDEase_I_N found only in the N-terminus. Ca\(^{2+}\)/calmodulin binding site is indicated in bold red. The conserved catalytic domain is located within the blue boxes started with the initiating (YHN) motif as well as the metal binding motif with a specific sequence signature (HDX\(_2\)HX\(_4\)N) underlined and in blue.
**Figure 2.2.** Multiple alignment of deduced amino acid sequences of PDE1 proteins in one crustacean species, one insect species, one nematode species and one mammalian species. Abbreviations: Gl: *G. lateralis*; Dm: *D. melanogaster*; Ce: *C. elegans*; Hs: *H. sapiens*. Black shading indicates that amino acid residues that are identical or similar in all sequences; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate highly conserved domains. Blue asterisks indicate the signature sequences in the catalytic domain. The colors of the boxes correspond to the colors of the domains in Fig. 2.1.
GLPDE2 4729 nucleotides, structure:  sequence

TACGTTTATAGTTGACACTTTTCTGTTGGTGCGTGACGCTGCGTGGTAACGT

G  S  K  L  T  C  A  G  S  G  T  E

床上

M  G  S  K  L  T  C  A  G  S  G  T  E

床上

421
ATAGCGGCGCATGACCTCCCTCGGGGACCTGTTGGAACACATACGGGAGCCCTACGCTAC 1861
IAGHVASTGTLNNIRDAYAHH 493
CCCTCCTCTTATAGCCGTCGTTAAAGGATCAGCGGGCCTAGCAAGCAGAAGAATACCTTTGC 1921
PLFYRGFRDCTGFKTRNILC 513
TTCCCGATCGACGAGGCAGGGGGCTGATGAGATGGGTGCAGGAGCTCGTAACAAGACTT 1981
FKPIQGQGEVIGVIAELCNKT 533
GGCTCCACTCTCACCTGCGATGAGGAGATTCGCCAGCCTTCTAGTATCTACTCGGCGC 2041
GLHFTFREDEEIATAFSLICYGG 553
ATCTCATACGAGCAACTGGCTCCCTCTCATATAAGAAGATCTCGAGGAGCCTCGCTCACC 2101
ISISNNLLYKKS Ves QVR S 573
CTGTCAGGCGGAGGCTTCCTCAATATCAACATTTCCGCTCGAGTCTCGTGTCACGTAG 2161
LSNEMLFHMKVTKEEVERLR 593
GGTCAGGGCGGAGGTTCTCCGACATTACCAACTCCGCCTGACGCTTGTTCTGTCAGTAC 2221
VQAEVPLTFQFRDFCSFRY 613
TTCCGGGCGGCGAGCTCGTGACGCCCTCTGCACATCCCGGCAGCTCTCGAGGATG 2281
FPRQLADPCSTSPAILSMVES 633
CGTGCCCATGATCAGTAGGATCGAAGCTGCGCTCCTGCCAATCTGACCTTATAG 2341
LGMIKTFRSLRSLAREFTLM 653
GTGCCGAAAGGGTTAATGCGGAGTCACCGGGCTGCTCCCTCTTGCGCTACCC 2401
TACCAAAACATTGTGAGCAGCGCCTCTCCTGTCACCC 2461
YHYNWLHAFSVT 673
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HLALVMWSSMCHDLHGRG 713
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SVMERHLAQAAMCILNNTDC 753
AATCTTGCGAGAAACTCTGAGCCGCGGAGGAGATACACAAAGTTCTCGACCTTATGAGAAGAC 2701
MFLENLSREBYMTFLDLMD 773
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ILALTDLAKHNLRIVESLRQV 793
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ANTGYDPAANQRHHELLICL 813
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ELIKKEFTQGDLEKAMGNM 853
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PELEMBREKAFIPELQFLQFL 873
GATGATGTGCGAAACTCTTGGTTGATAGATGTGCTGCGAGCTGAGA 3061
DDVAIPvYVEIVALKZEPEAB 893
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PYSIKARRNRSRLRVDYK 913
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RRKPKESTSSLEVFEDDSLEE 933
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ELERDES- 940
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CATTACATGTAGGCCAGCCAGAACAGAGGGAGTGGAGTGGAGTGGAGTGGAGTGGAG 3601
TGGGCTAGTCTGCTCCTTGACTCTTGCTAATCGTCTATGTCTAGGGCTGCT 3661
Figure 2.3. Nucleotide and amino acid sequence of cDNA encoding Gl-PDE2. A full-length open reading frame (ORF) was expressed by the cDNA, the start codon highlighted in yellow and the stop codon highlighted in green. The GAF-B regulatory domain is indicated by a green box and contains the \((NK_{xx}FD_{xx}E)\) signature sequence found in all mammalian GAF domains; the sequence is underlined and in green. The conserved catalytic domain is located within the blue boxes started with the initiating (YHN) motif as well as the metal binding motif with a specific sequence signature \((HD_{x3}HX_{i4}N)\) underlined and in blue.
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<tr>
<th>Species</th>
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| GLPDE2   | GDFTQTQVCTVTRCERVQTVGQVIFVAP-C  | 383    |
| Ce.PDE2  | --QPDACSVASNESDRVLPLPMSTI      | 166    |
| Hs.PDE2A3| DLTTIDEFHVQCHYTSVLSTIAFOKEQLGCEQ  | 395    |

GAF-B

| GLPDE2   | LTIVQNLFFHDDVSLRITMAEARQLTDAABCGL  | 423    |
| Ce.PDE2  | -----FDQFLCLNNLSALSICIIIEAKKNTEABDYAVFLH | 203    |
| Hs.PDE2A3| DLQTKNLFFHDDVSLRITIMAERNSALSIFV  | 435    |
|          |                                 | 46     |

| GLPDE2   | DREHGSYLVAVFEDERKEESIEEV LLPQLFPMFYVTS  | 463    |
| Ce.PDE2  | D-ELNKQMVFNN-------------------------ETMLM | 220    |
| Hs.PDE2A3| D-ONELVAVFDE---------------------------GVDDE  | 454    |

| GLPDE2   | GEVRPATQGINGVASTCHLLNNIYAYAPFIFRYGEQ  | 503    |
| Ce.PDE2  | TGGKFDGMYGICVASTMRTMNIDVSRCPFENEEIQ  | 260    |
| Hs.PDE2A3| YEIRPADQGINGVATTQLNNIDYAYPARFYGVDS  | 494    |

| GLPDE2   | TFKARNLPPQDQG-EVGVAELCNGTCHFRTRDE  | 542    |
| Ce.PDE2  | FSIKARNLAPPISSCSLGIGVVLKNBNG--FSHDE  | 298    |
| Hs.PDE2A3| TGFRKARNLPPKQENQEVGVAVLNYLINGAPWSFED  | 534    |

| GLPDE2   | EIATFSLGCGTSINNEVLLKLK/SDELVRLKSEKMF  | 581    |
| Ce.PDE2  | KYIKESYIFVANSIAHIATIAKQEFVVRTRHMEBFKQOG  | 338    |
| Hs.PDE2A3| DLAATFSLGCGSTAHLELKYNKASYRSHLAKKMT  | 573    |

| GLPDE2   | -HMVKKEVERINVQAPLTQHRDFCFAQPPPPIAD  | 620    |
| Ce.PDE2  | EDAVFEVDMRWNDPRDWRYSQNFDEQPPLPVGD  | 378    |
| Hs.PDE2A3| -HMVKSEDDYTKLHGQTPVAAIDSNFEFTYDPSLPE  | 612    |

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Figure 2.4. Multiple alignment of deduced amino acid sequences of PDE2 proteins in one crustacean species, one nematode species and one mammal species. Abbreviations: Gl: G lateralis; Ce: C elegans; Hs: H sapiens. Black shading indicates that amino acid residues that are identical or similar in all sequences; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate highly conserved domains. Blue asterisks indicate the signature sequences in the catalytic domain. Green plus signs indicate the motif sequence in the GAF domain. The colors of the boxes correspond to the colors of the domains in Fig. 2.3.
Figure 2.5. Nucleotide and amino acid sequence of cDNA encoding Gl-PDE4. A full-length open reading frame (ORF) was expressed by the cDNA, the start codon highlighted in yellow and the stop codon highlighted in green. The conserved catalytic domain is located within the blue boxes started with the initiating (YHN) motif as well as the metal binding motif with a specific sequence signature (HDX2HX4N) underlined and in blue. Two Upstream Conserved Regions; UCR1 and UCR2, are found in purple boxes.
Figure 2.6. Nucleotide and amino acid sequence of cDNA encoding Cm-PDE4. A full-length open reading frame (ORF) was expressed by the cDNA, the start codon highlighted in yellow and the stop codon highlighted in green. The conserved catalytic domain is located within the blue boxes started with the initiating (YHN) motif as well as the metal binding motif with a specific sequence signature (HDX$_2$HX$_2$N) underlined and in blue. One Upstream Conserved Region; UCR2, is found in a purple box.
GlPDE4: VETKKVAGSGVLLLDNYTDRIQVLQNMVHCADLSNPTKPL: 657
CmPDE4: VETKKVAGSGVLLLDNYTDRIQVLQNMVHCADLSNPTKPL: 640
Hs.PDE4D: VETKKV
Dm.PDE4D: VETKKVAGSGVLLLDNYTDRIQVLENLVHCADLSNPTKPL: 1037
Ce.PDE4: VETKKVAGSGVLLLDNYTDRIQVLQNMVHCADLSNPTKPL: 573

GlPDE4: ELYKENWVSSIMEEFFQGDRERDQGISPMCDRTATIE: 697
CmPDE4: EMYKENWVSSIMEEFFQGDRERDQGISPMCDRTATIE: 680
Hs.PDE4D: ELYQRTDRIMEEQRQGDRERQGISPMCDRTATIE: 532
Dm.PDE4D: ELYQRTDRIMEEQRQGDRERQGISPMCDRTATIE: 1077
Ce.PDE4: ELYQMNQREMEYQRQGDRERQGISPMCDRTATIE: 613

GlPDE4: KSQVGFIDYIVHPLWETWADLVHPDAQDILDLEENR: 737
CmPDE4: KSQVGFIDYIVHPLWETWADLVHPDAQDILDLEENR: 720
Hs.PDE4D: KSQVGFIDYIVHPLWETWADLVHPDAQDILDLEENR: 572
Dm.PDE4D: KSQVGFIDYIVHPLWETWADLVHPDAQDILDLEENR: 1117
Ce.PDE4: KSQVGFIDYIVHPLWETWADLVHPDAQDILDLEENR: 653

GlPDE4: NRMIPSPSS--SNLKD--------------------------EYPG: 759
CmPDE4: NRMIPSPSS--SNLKD--------------------------EYPG: 742
Hs.PDE4D: QSTIPSPA--PDPEGRQGQTEKFQFELTEDGES: 610
Dm.PDE4D: QSMIPSPPSGVDEPCDRIR--------FQVTLENEDQ: 1150
Ce.PDE4: QSTIPSPA--PDPEGRQGQTEKFQFELTEDGES: 665

GlPDE4: ENSQD-----VPREEELCAAADRIQIQFLEDGSGNRPGP: 795
CmPDE4: ENSQD-----APEEELCAAADRIQIQFLEDGSGNRPGP: 778
Hs.PDE4D: DTEKDSGQSVEEDTCSSDKLCTQDESTIPLDEQVEE: 650
Dm.PDE4D: ENLAELE---EGDESGGESTTGTGTTAASALSGAGGGG: 1188
Ce.PDE4: ------------------------TVEDDHK--------: 674
Figure 2.7. Multiple alignment of deduced amino acid sequences of PDE4 proteins in two crustacean species, one insect species, one nematode species and one mammal species. Abbreviations: Gl: *G. lateralis*; Cm: *C. maenas*; Dm: *D. melanogaster*; Ce: *C. elegans*; Hs: *H. sapiens*. Black shading indicates that amino acid residues that are identical or similar in all sequences; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate highly conserved domains. Blue asterisks indicate the signature sequences in the catalytic domain. The colors of the boxes correspond to the colors of the domains in Fig. 2.5.

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Figure 2.8. Nucleotide and amino acid sequence of cDNA encoding Gl-PDE5. A full-length open reading frame (ORF) was expressed by the cDNA, the start codon highlighted in yellow and the stop codon highlighted in green. Two GAF regulatory domains; GAF-A & GAF-B are indicated by green boxes and contains the (NKxxFDxxE) signature sequence found in all mammalian GAF domains, the sequence is underlined and in green. The conserved catalytic domain is located within the blue boxes started with the initiating (YHN) motif as well as the metal binding motif with a specific sequence signature (HDX2Hx4N) underlined and in blue.
Figure 2.9. Nucleotide and amino acid sequence of cDNA encoding Cm-PDE5. A full-length open reading frame (ORF) was expressed by the cDNA, the start codon highlighted in yellow and the stop codon highlighted in green. Two GAF regulatory domains; GAF-A & GAF-B are indicated by green boxes and contains the (NKxxFDxxE) signature sequence found in all mammalian GAF domains, the sequence is underlined and in green. The conserved catalytic domain is located within the blue boxes started with the initiating (YHN) motif as well as the metal binding motif with a specific sequence signature (HDx2HX4N) underlined and in blue.
### GAF-A

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GlpDE5 : KNYRR-VFTHNKTIGEVSANSMAFK---NPKSFRPLC : 596
CmPDE5 : ---------------------------------------- : -
Dm.PDE5/6 : KNYP--VKNHNR-TALVQAQMFAM--KTKMERFMTLEI : 748
Ce.PDE5 : KNYRR--VAKHWAIGWSVHAMFA--MN--SPDAFTLEA : 493
Hs.PDE5A : KNYRKNNAHNWR--AEQNTGCMFA--KAGKIQNKLTDLEI : 643

********
GlpDE5 : TAFLIGSCHDLDHRCKNNMLETESPLAIYTTSTLEH : 636
CmPDE5 : ---------------------------------------- : -
Dm.PDE5/6 : GlIVACCKHLDHRCTNNAQTKESPAILYTTSTMEH : 788
Ce.PDE5 : ALYRSCCHLDHRCKNNA--MKMTSTPLSIVSTQMER : 533
Hs.PDE5A : ALLAAASCCLDKHGVNSLQREHPLQCLCHIMEH : 683

GlpDE5 : HHFNC-TITLQQEHNFGKTSTIKQVIGNKHPLAT : 676
CmPDE5 : ---------------------------------------- : -
Dm.PDE5/6 : HHFDCGMTISENNKFAASPEDHRSVKTESAIST : 828
Ce.PDE5 : HHFNCVTLQQEHNILKSSAEKRTSLKHPLAT : 573
Hs.PDE5A : HHFDCGMTISQOILGSLSEEKTTIKQPLAT : 723

GlpDE5 : DLALFEPNVARLQLVEDNLCLWDNSDHMLIEAMTFA : 716
CmPDE5 : ---------------------------------------- : -
Dm.PDE5/6 : DLAMYKKNAFLELVEGENKWQGEEXDLGCMMTAC : 868
Ce.PDE5 : DLALFSDKAKLNVILDNNTDEINQEHRLTOAMMGCG : 613
Hs.PDE5A : DLALYKRGEFFELIRKNSNLEDPHQKFLAVMTAC : 763

GlpDE5 : DLCSAKPEMOAETVKVIFEFYEDOGAEK--AAGKNIP : 755
CmPDE5 : ---------------------------------------- : -
Dm.PDE5/6 : DVSLIAPWEVCHKVAKLVAEDFDGSEGELKLQNLQV : 908
Ce.PDE5 : DLVSASKWNCTETVKVIFEFYDAGAE--LSGKTP : 652
Hs.PDE5A : DLSTRKTPROICAIENVATFEDGGERKELNIEDTD : 803

Q-switch
GlpDE5 : VMDRTKVEQAESVGFSGCICPCMELIKLPNTEPI : 795
CmPDE5 : ---------------------------------------- : -
Dm.PDE5/6 : VMDRERKDELFKQVGFIDVCLPLRVLCDTFWIPTL : 948
Ce.PDE5 : VMDRQAHMLPCMVGFGRICPCDLILAFPKNDKAL : 692
Hs.PDE5A : LMNREKKKNKIPSMVGFIDACICQLEAITHVESEDCP : 843

68
Figure 2.10. Multiple alignment of deduced amino acid sequences of PDE5 proteins in two crustacean species, one insect species, one nematode species and one mammal species. Abbreviations: Gl: G lateralis; Cm: C maenas; Dm: D melanogaster; Ce: C elegans; Hs: H sapiens. Black shading indicates that amino acid residues that are identical or similar in all sequences; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate highly conserved domains. Blue asterisks indicate the signature sequences in the catalytic domain. The colors of the boxes correspond to the colors of the domains in Fig. 2.7.

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GIPDE7 7531 nucleotides, structure: TC

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LGGAPRRYSFRFLTLHRRR83

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70
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d h q s v g s e n v r s r l q g 483
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R R H S V P L N L P R L L P R T I R R 603
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G R K C V S N Q G A T T F L H A D C L 703
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S T S I S S K R E F L D R L H E S S K 743
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A L T Y R E Q Q Q Q Q S S W K T R A W 783
GAGAGTCGGCCGGAGGATTTGGGAGGGCTTGGTGCGCCGTGGCTCCTCAGAGAC 3181
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S I P F D H P L C R Q G S G G V E V L 923
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AGTACATATTATTATTATTATTATTATTATA 7531

**Figure 2.11. Nucleotide and amino acid sequence of cDNA encoding Gl-PDE7.**

A full-length open reading frame (ORF) was expressed by the cDNA, the start codon highlighted in yellow and the stop codon highlighted in green. The conserved catalytic domain is located within the blue boxes started with the initiating (YHN) motif as well as the metal binding motif with a specific sequence signature (HDX$_2$HX$_4$N) underlined and in blue.
G1PDE7 : GGSGCDRVKGGGECDPVEPIHHPIRSDTPLTAQDV : 680
Dr.PDE7A : ---------------------------------------- : -
Hs.PDE7A : ---------------------------------------- : -

G1PDE7 : GVHGRKCVSNHQGATTFLHADCLDNHPYRPGRLVRRA : 720
Dr.PDE7A : ---------------------------------------- : -
Hs.PDE7A : ---------------------------------------- : -

G1PDE7 : SLDISTSISKREFLDDRLHESSFPRVDRTNSLEDKVLV : 760
Dr.PDE7A : ---------------------------------------- : -
Hs.PDE7A : ---------------------------------------- : -

G1PDE7 : PREALTYREQQQSSWKTRAWSLNCDDENVCDPREKL : 800
Dr.PDE7A : ---------------------------------------- : -
Hs.PDE7A : ---------------------------------------- : -

G1PDE7 : MKPGMYKLNQGSGYAHGRGSAPLRPEELLGLRGGA : 840
Dr.PDE7A : ---------------------------------------- : -
Hs.PDE7A : ---------------------------------------- : -

G1PDE7 : ERPDYNYLSLRRGAPSQANQGCDVETGSEVGRHTPLT : 880
Dr.PDE7A : ---------------------------------------- : -
Hs.PDE7A : ---------------------------------------- : -

G1PDE7 : STENLPFSEYVSURGRRGSIPFDHGLCRQGSGG : 920
Dr.PDE7A : ---------------------------------------- : -
Hs.PDE7A : ---------------------------------------- : -

G1PDE7 : EVLPSRTGVGNLAVSGGSGAAGFPSVLPHYHEYHRGS : 960
Dr.PDE7A : ---------------------------------------- : -
Hs.PDE7A : ---------------------------------------- : -
**Figure 2.12. Multiple alignment of deduced amino acid sequences of PDE7 proteins in one crustacean species, one fish species, and one mammal species.** Abbreviations: Gl: *G. lateralis*; Dr: *D. rerio*; Hs: *H. sapiens*. Black shading indicates that amino acid residues that are identical or similar in all sequences; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate highly conserved domains. Blue asterisks indicate the signature sequences in the catalytic domain. The colors of the boxes correspond to the colors of the domains in Fig. 2.9.
Figure 2.13. Nucleotide and amino acid sequence of cDNA encoding Cm-PDE8.

A full-length open reading frame (ORF) was expressed by the cDNA, the start codon highlighted in yellow and the stop codon highlighted in green. The conserved catalytic domain is located within the blue boxes started with the initiating (YHN) motif as well as the metal binding motif with a specific sequence signature $(\text{HDX}_2\text{HX}_4\text{N})$ underlined and in blue. The regulatory region, the PAS domain, is shown in an orange box.
Figure 2.14. Multiple alignment of deduced amino acid sequences of PDE7 proteins in one crustacean species, two insect species, and one mammal species. Abbreviations: Cm: *C. maenas*; Dm: *D. melanogaster*; Bm: *B. mori*; Hs: *H. sapiens*. Black shading indicates that amino acid residues that are identical or similar in all sequences; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate highly conserved domains. Blue asterisks indicate the signature sequences in the catalytic domain. The colors of the boxes correspond to the colors of the domains in Fig. 2.13.
Figure 2.15. Nucleotide and amino acid sequence of cDNA encoding Gl-PDE9. A full-length open reading frame (ORF) was expressed by the cDNA, the start codon highlighted in yellow and the stop codon highlighted in green. The conserved catalytic domain is located within the blue boxes started with the initiating (FHN) motif as well as the metal binding motif with a specific sequence signature (HDX2HX4N) underlined and in blue.
Figure 2.16. **Nucleotide and amino acid sequence of cDNA encoding Cm-PDE9.** A full-length open reading frame (ORF) was expressed by the cDNA, the start codon highlighted in yellow and the stop codon highlighted in green. The conserved catalytic domain is located within the blue boxes started with the initiating (FHN) motif as well as the metal binding motif with a specific sequence signature (HDX$_2$HX$_4$N) underlined and in blue.
Figure 2.17. Multiple alignment of deduced amino acid sequences of PDE9 proteins in two crustacean species, two insect species, and one mammal species. Abbreviations: Gl: *G. lateralis*; Cm: *C. maenas*; Dm: *D. melanogaster*; Tc: *T. castaneum*; Hs: *H. sapiens*. Black shading indicates that amino acid residues that are identical or similar in all sequences; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate highly conserved domains. The colors of the boxes correspond to the colors of the domains in Fig. 2.15 & 2.16.
GlPDE11 5752 nucleotides, structure: sequence

GGAGGCGAAGGCTGCTGGCCTGGTGTGCTGGAGGCTAGCTGGACGAGGAGAAA
GAATAGAAGAAATGGTCTAGACGCTGGGCAGCCCAGAGGAGTGTGGTGTAGCTGGACG
TTACACCAGCACAGCTACTGGTGTGCTGGACGGGTCGAT
GGCGGCTGTACGTACGCGTGGCAGACAAACTTGACTTCACGCAGCATCGAGGCAGAGAAA
GAATAGAAGAAAATGTCTCAACAGGTTCGTCTTCCCTTCAAAGGAATGATGGCGCCCACC
TTTACGCCATTCAAGGCTGATGGGTCCCTCAATCTGGAACTGGTGAAGCCGTACGCAGCA
CACCTGAAGGCTTCTGGTGTGAAGGGGGTGTGGGTGAACGGCACGGCCGGCGAGGGCATG
TCGCAGACGGTGCTGGAGCGCAAGGCGGTGGCGGAAGCGTGGCTGGCGTGCCGCGGTGAC
GTGCCGACAGTGATCGTGCATTGCGGCGCGGGATGCCTCAAGGATACACAGGACCTGGCT
CGTCACGCGGAGGAGAAAGGGGCTGATGGAGTGGCCGTGTTGCCCCTCCTCTTCGACCCC
CCCAAAACCCCCGACGATCTGGTGGACTACATGGTGGAGGTGGCTAAGGCGTGCCCCTC
AGCCCCCTCTTCTACTACACATACCTACATACATACAGGACGGCGTAAAG
GAGTGAAGGGCGGAAGGCGAACGCTTGGTACGAGGATGGTACGGCTGACGAGGCGGCGAC
AAGGGCGAAGGCGGCTGACGAGGCGGCGACGAGGGAGGCGACGAGGGCGACGACGACGAC
ATGGAGGCGTGGCTGGACGACCATCAAGATTTCGTGTACGACTACTTCATCAGGAAGGCG
MEAWLDDHHQDVFYDYDFIRKA
TCTAGGCCACATGGTGGGACTCTTGGTTGTCTCCATCGCCTCCGCAGCTGCTGGTAGTA
SRHMVDSWLLSHPQSLGM
GGCCGCGCGGTTACTGGGCGGGCCGGGGGCTAGGGCGGGGGGCGGCTCTGCCACC
GAAGYCGAGPEAGAGAGLAT
ACCCCCCAGAAGCATACAAATCAGGCTCGTTCGTGGCTGACGCCCAATTCGCAAG
TPGQHQNASSGAATPVRK
ATTTCAGCCACAGGATTTGCGAAAGGGGCGCTCCTCAAGGCCATGTTGACCACTATGCAG
ISAHEDFKEKGLLKPIVTID
GGAGACCCACCTTCTATATCCGCTGCCGGCTGCGGAGAATGCGGATCTGGGCAAG
GTPFTISPAAGAANENVAILK
GTTGTCGCAAGTGCCCCCGAGGCGACTCAAGAGGCGCCCTGACGACGACGCAATTAATCTCGAA
VRRKSRTEKLGLDERQLIFE
CCTGTAAGGACGATTTGCAAGACGGCTGATGTCGCCCGCCTGTGGCACAAGATCTCTGCAAG
LVKDICNELDVRPLCHKIQL
AACGTGCTATCCTACAGGACGCTGACAGATGCTGGCTTTTCCTNTAGTAACAGGGGCAAG
NVISILTSADRCSLFLVQGDK
GAGACGGAAGACCCTGCCTCTGCCCAGCTCTCCAGCFTGACGTAACAGACTTGACAGGTG
ETENRCVLSTLFDVNPDSTV
GAGGAGATTTGGAGGAAGAAAGAGATACAGATACGGGGTGATGCTGGCATTGGGGCTAC
EEMEEKEIRIAWGIGTVY
ACGGCGCAGATGGAAGAATGGTTAAACATCCTCTACGCTACGATGTCGACAGGTCTTCAAC
TAQSGAMVNIPDAYADDRFN
TCGTGATCGTCTGTACGCTGGACGGATACGGGCTGCTGTGGTGTAGCTGGACG
SEIDCMYTGKTGSKMLCMPIK
GACAGTACGCTTGATAGGGAGGGGATGCGGGCCTTACAGCTGATGTCGACAGGTCTTCAAC
DSNEGIGVQAVINKHQGPS
TTACACGAGGCGCTGAGAGAAGGTTGGTTAGCTTCCACCTCGCTTCTCGCCAGTTCTTC
FTTADDEKVFESYLQFCIGL

92
Figure 2.18. Nucleotide and amino acid sequence of cDNA encoding Gl-PDE11. A full-length open reading frame (ORF) was expressed by the cDNA, the start codon highlighted in yellow, the stop codon highlighted in green. Two GAF regulatory domains; GAF-A &GAF-B are indicated by green boxes and contains the \((\text{NKxxFDxxE})\) signature sequence found in all mammalian GAF domains, the sequence is underlined and in green. The conserved catalytic domain is located within the blue boxes started with the initiating \((\text{YHN})\) motif as well as the metal binding motif with a specific sequence signature \((\text{HDx}_2\text{HX}_4\text{N})\) underlined and in blue.
CmPDE11  5839 nucleotides

1  GCTGGGGGACCCAGGGCATGAGACCAAGAACCCGGGGTGAGATGAGATCACAGTGGAG
2  GGGGCGGTGGGGAGGATCCAGACCCTTCAGAAGTGCGTGGGGGGGCGCCTCTCCACTCCC
3  GGCAGCGCGAGGCGGGCCAGGAGATCTGGGACTGCAGATGAC
4  A S S G A A T P V R K I S A H E F E K G
5  AGCTTCTTCCAGGAGCCCACTACTACATGAGCMCGGACAGTCTCTCCCCCG
6  G A A N V A I L A K V R K S R T E L
7  CTGGCCGCGTGAAGAGTTGCTATTTCTGCAAGTTGCGCAAGCAGGGGACGAC
8  K G L D E R Q L I F E L V K D I C N D L
9  TCAAGGGATTAGACGAGCCGCAACTTATCTTCTGaAGCTGGAAGACACTGCAATGACC
10  D V R S L C H K I L Q N S I L T N A D
11  TGGATGTCGCTCCCTGTGGCCACACAGATCTGGAGATCCTACCTTTCAATGCCGC
12  R C S L F L V Q G D K E S D N R C L V S
13  ACCGGTGCATCTCCTCCTCTGGAGATTGAAAAGCAGGATGACAGCGGGCCTCAGCTGT
14  T L F D V N P D S T V E E M E E K E E I
15  CACAGCTTTGGACGTGAACACTAGCGGCAAGTTGAGGAGATGAGAGATGGAGAGGA
16  R I A W G S G I V G Y T A Q S G A M L N
17  TCGGATACGCGTGCAGAGTTGCTATTTCTGCAAGTTGCGCAAGCAGGGGACGAC
18  I P D A Y E D D R F N S E I D C M T G Y
19  ATATCTCTGAGTTTATAGAGATGATCGCTTCAATCTCGAGATTGACTGCATAGCGC
20  K T R S M L C M P I K D S C G E V I G V
21  ACAAGACGCCGCTCTATGCTGTGGATCATGCGGCATGACAGGCT
22  A Q V I N K H Q G Q S F T N A D E K V F
23  TGGCCACAGGTTATCAACAGCCTAGGTCAGTCTCTTCTACTGCGATGAGAGGCT
24  E S Y L Q F C G I G L R N A Q L Y E R S
25  TTAAGATCTCCTCCAGTTTGTTGCGCATTTGCCCTCCGCAATGCTGATGAC
26  Q L E V K R N Q V L D L A R I I F E E
27  CCAGGGTACATTGAGGAAGAAATACTACCTTCTCTGAGCTTGCCGGAGCATACTTTC
28  Q S T I E Q V I Y R I M T H T Q S L L Q
29  AAGCAGACACTATAGACGAAAAATGGTGACCCATATTGACAGACACCCAGCCAGCCTTGGC
30  C E R V Q I L V H E A S R G T F S R V
31  AATGTGACAGCCGATGTTTATGACTAGATCCTCGGAAACATCTCGAGAG
32  F D L E V K D L Q G D D A E S R T S P F
33  TGGTATAGTTGGAAGTGATCTGCAAGGAGGATGATGCGAGAAGCCAGCAAGCTCCTGGC
34  E S R F P I N V G I T G H A A T T G E T
35  TCTGTCGCCGTTCCCACTACAGCTAGGGATGATGCGAGAAGCCAGCAAGCTCCTGGC
36  T C I A D Y Q D S R F D Q S V D E N T
37  CTGTGTCGATTTGCTGTATCATACAGATCAGGCTTGGGAAAATCTCTCCAGCT
38  G F R H K S I L C M P I K N T A R K I V
39  CAGGGTTCGCCCAACTTGATCATTCCCTATGCTGAAATCAAGAAGACAGCGCAAGCCAAATAG
40  G V V Q L V N K F D N L P F T S N D E N
41  TGGGATCGTGACATACAAATATTGTAACCTTCTCCCTTTACAAGCAGATGAAAA
42  F L E A F I C G M G I H N T N M Y E

95
Figure 2.19. Nucleotide and amino acid sequence of cDNA encoding Cm-PDE11. A full-length open reading frame (ORF) was expressed by the cDNA, the start codon highlighted in yellow, the stop codon highlighted in green, and the promoter element (CAT box) is highlighted in turquoise. Two GAF regulatory domains; GAF-A & GAF-B are indicated by green boxes and contains the (NKxxFDxxE) signature sequence found in all mammalian GAF domains, the sequence is underlined and in green. The conserved catalytic domain is located within the blue boxes started with the initiating (YHN) motif as well as the metal binding motif with a specific sequence signature (HDX₂HₓNx) underlined and in blue.
Figure 2.20. Multiple alignment of deduced amino acid sequences of PDE11 proteins in two crustacean species, two insect species, and one mammal species. Abbreviations: Gl: G. lateralis; Cm: C. maenas; Dm: D. melanogaster; Tc: T. castaneum; Hs: H. sapiens. Black shading indicates that amino acid residues that are identical or similar in all sequences; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate highly conserved domains. Blue and green asterisks indicate the signature sequences in the catalytic domain and GAF domain, respectively. The colors of the boxes correspond to the colors of the domains in Fig. 2.13.
Figure 2.21. Phylogenetic relationships among the different PDEs of *G. lateralis*, *C. maenas* and orthologs of other species. The 102 deduced amino acid sequences used to generate this tree included the entire open reading frame, catalytic domains, and regulatory domains. *G. lateralis* and *C. maenas* are shown in red font. Letunic and Bork (2016) *Nucleic Acids Res* doi: 10.1093/nar/gkw290
Figure 2.22. A schematic figure showing a comparison of the *G. lateralis* PDEs with the *Homo sapiens* PDEs. Regarding the conserved domains; catalytic domains (PDEase_1) are highly conserved throughout all the PDE families are in yellow boxes. The regulatory domains specific for each family, PDEase_1_N, are shown in bright green boxes, GAF A, or GAF B domains are shown in light green and the PAS domain is shown in purple. The length of each protein in amino acids is shown on the left side of each PDE.
Table 2.4. Effects of PDE inhibitors on the ecdysteroid secretion in the YO of the green shore crab, *Carcinus maenas*. IBMX, a potent and general inhibitor for PDEs, and zaprinast (1 mM) show a very significant effect on ecdysteroidogenesis. Vinpocetine (1mM), EHNA (0.5 mM & 1mM), zaprinast (0.5 mM & 1mM) and zaprinast/rMIH were significant as well. Other inhibitors had no effect on ecdysteroid secretion.

<table>
<thead>
<tr>
<th>PDE Inhibitor (conc.)</th>
<th>PDE</th>
<th>Ecdysteroid Secretion Mean ± SE</th>
<th>N</th>
<th>% Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Inhibitor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBMX (0.5 mM)</td>
<td>General Potent Inhibitor</td>
<td>12.5 ±3.7</td>
<td>7.1±3.0</td>
<td>12</td>
<td>57.2</td>
</tr>
<tr>
<td>Vinpocetine (0.1mM)</td>
<td>PDE1</td>
<td>4.6 ± 1.1</td>
<td>1.8±0.4</td>
<td>13</td>
<td>39.1</td>
</tr>
<tr>
<td>Vinpocetine (0.5 mM)</td>
<td>PDE1</td>
<td>3.5±0.7</td>
<td>1.1±0.5</td>
<td>7</td>
<td>31.4</td>
</tr>
<tr>
<td>Vinpocetine (1.0 mM)</td>
<td>PDE1</td>
<td>2.5±0.5</td>
<td>0.4±0.1</td>
<td>8</td>
<td>16.0</td>
</tr>
<tr>
<td>EHNA (0.1mM)</td>
<td>PDE2</td>
<td>3.2± 2.3</td>
<td>1.3± 0.9</td>
<td>12</td>
<td>40.6</td>
</tr>
<tr>
<td>EHNA (0.5 mM)</td>
<td>PDE2</td>
<td>6.1±2.0</td>
<td>0.5±0.2</td>
<td>8</td>
<td>83</td>
</tr>
<tr>
<td>EHNA (1.0 mM)</td>
<td>PDE2</td>
<td>2.1±0.6</td>
<td>0.8±0.2</td>
<td>8</td>
<td>8.2</td>
</tr>
<tr>
<td>Rolipram (1 mM)</td>
<td>PDE4</td>
<td>16.1±5.5</td>
<td>13.5±3.3</td>
<td>8</td>
<td>84.4</td>
</tr>
<tr>
<td>Zaprinast (0.1 mM)</td>
<td>PDE5/PDE11</td>
<td>5.8±2.0</td>
<td>2.5±1.1</td>
<td>5</td>
<td>43.1</td>
</tr>
<tr>
<td>Zaprinast (0.5 mM)</td>
<td>PDE5/PDE11</td>
<td>3.9±1.2</td>
<td>0.9±0.1</td>
<td>6</td>
<td>23.1</td>
</tr>
<tr>
<td>Zaprinast (1 mM)</td>
<td>PDE5/PDE11</td>
<td>14.5±4.6</td>
<td>4.4±2.8</td>
<td>12</td>
<td>30.2</td>
</tr>
<tr>
<td>Zaprinast (0.5 mM/rMIH)</td>
<td>PDE5</td>
<td>1.5±0.5</td>
<td>0.5±0.5</td>
<td>8</td>
<td>33.3</td>
</tr>
<tr>
<td>Dipyridamole (0.1mM)</td>
<td>PDE7</td>
<td>8.3±2.2</td>
<td>5.2±1.8</td>
<td>12</td>
<td>62.7</td>
</tr>
<tr>
<td>Dipyridamole (0.5mM)</td>
<td>PDE7</td>
<td>4.6±2.2</td>
<td>3.4±2.0</td>
<td>8</td>
<td>74</td>
</tr>
<tr>
<td>Dipyridamole (1.0mM)</td>
<td>PDE7</td>
<td>7.9±1.8</td>
<td>6.7±1.9</td>
<td>8</td>
<td>84.8</td>
</tr>
<tr>
<td>BC 11-38 (0.1 mM)</td>
<td>PDE1</td>
<td>2.1±0.5</td>
<td>3.1±0.6</td>
<td>8</td>
<td>70.0</td>
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<tr>
<td>BC 11-38 (0.5 mM)</td>
<td>PDE1</td>
<td>3.7±0.5</td>
<td>4.3±0.5</td>
<td>8</td>
<td>86.0</td>
</tr>
<tr>
<td>BC 11-38 (1.0 mM)</td>
<td>PDE1</td>
<td>2.9±0.4</td>
<td>2.8±0.3</td>
<td>8</td>
<td>96.0</td>
</tr>
</tbody>
</table>
Table 2.5. Effects of PDE inhibitors on the ecdysteroid secretion in the YO of the blackback land crab, *Gecarcinus lateralis*. All inhibitors had no effect on ecdysteroid secretion as the p-value was higher than 0.05.

<table>
<thead>
<tr>
<th>PDE Inhibitor (conc.)</th>
<th>PDE</th>
<th>Ecdysteroid Secretion Mean ± SE Control Inhibitor</th>
<th>N</th>
<th>% Control</th>
<th>P- value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBMX (0.5 mM)</td>
<td>General Potent Inhibitor</td>
<td>3.4 ±1.0                  3.1±1.3</td>
<td>8</td>
<td>-6.2</td>
<td>P=0.9</td>
</tr>
<tr>
<td>Vinpocetine (0.5 mM)</td>
<td>PDE1</td>
<td>4.5±1.5                      3.3±1.1</td>
<td>8</td>
<td>-26.1</td>
<td>P= 0.6</td>
</tr>
<tr>
<td>EHNA (0.5 mM)</td>
<td>PDE2</td>
<td>5.1±2.3                      4.3±1.6</td>
<td>8</td>
<td>-14.4</td>
<td>P=0.7</td>
</tr>
<tr>
<td>Zaprinast (0.5mM)</td>
<td>PDE5/PDE11</td>
<td>1.5±0.8                     2.2±0.6</td>
<td>8</td>
<td>46.8</td>
<td>P=0.5</td>
</tr>
<tr>
<td>BC 11-38 (0.5 mM)</td>
<td>PDE11</td>
<td>7.4± 2.1                    4.1± 1.5</td>
<td>8</td>
<td>-44.4</td>
<td>P=0.3</td>
</tr>
</tbody>
</table>
Figure 2.23. Tissue distribution panels show the expression of PDE1 (a), PDE2 (b), PDE5 (c), PDE11(d) in eleven tissues in *Gecarcinus lateralis*. qPCR was used to detect the expression of the different PDEs in eleven tissues; Y-Organ (YO), Thoracic Ganglion (TG), Mid Gut (MG), Hind Gut (HG), Eye Stalk Ganglion (ESG), Testis (T), Gill (G), Heart (H), Hepatopancrease (HP), Claw Muscle (CM), Brain (B). Means that were significantly different are represented with a bracket. Data proposed as mean ±1 SE. (n=6)
Figure 2.24. Tissue distribution panels show the expression of PDE4 (a), PDE8 (b), PDE9 (c), PDE11(d) in eleven tissues in *Carcinus maenas*. qPCR was used to detect the expression of the different PDEs in eleven tissues; Y-Orgin (YO), Thoracic Ganglion (TG), Mid Gut (MG), Hind Gut (HG), Eye Stalk Ganglion (ESG), Testis (T), Gill (G), Heart (H), Hepatopancreas (HP), Claw Muscle (CM), Brain (B). Means that were significantly different are represented with a bracket. Data proposed as mean ±1 SE. (n=6)
CHAPTER THREE

EFFECTS OF MOLT INDUCTION METHODS ON CYCLIC NUCLEOTIDE PHOSPHODIESTERASE EXPRESSION IN THE DECAPOD CRUSTACEAN MOLTING GLAND

Summary

cAMP and cGMP, as second messengers, mediate the suppression of the crustacean molting gland (Y-organ or YO) by molt-inhibiting hormone (MIH). When MIH levels decrease, the YO transitions from the basal to the activated state and the animal enters premolt; such a transition requires mTOR. During mid-premolt, the YO transitions to the committed state, in which the YO becomes insensitive to MIH. Cyclic nucleotide phosphodiesterases (PDEs) convert cAMP and cGMP to AMP and GMP, respectively, and therefore can modify the response of the YO to MIH. Seven PDE contigs were extracted from the YO transcriptome. qPCR was used to quantify the effects of molt induction by multiple limb autotomy (MLA) or eyestalk ablation (ESA) ± mTOR inhibitor rapamycin on the mRNA levels of PDE 1, 2, 4, 5, 7, 9, and 11 in Gecarcinus lateralis YO. In response to MLA, all PDEs, except for Gl-PDE5 and Gl-PDE11, were expressed at their highest levels in the intermolt YO. mRNA levels declined during premolt and reached their lowest levels in postmolt. qPCR results from the MLA experiment showed that both Gl-PDE5 and Gl-PDE11 reached high expression levels in mid premolt and late premolt, respectively. MLA transcriptomics revealed that only PDE11 expression maximized at mid premolt. In response to ESA, the mRNA levels of PDE4, 5, 7, 9, and 11 showed no significant change by 7- and 14-days post-ESA. Rapamycin had no significant effect, as PDE mRNA levels were comparable to those
of controls at all time points, indicating that PDE expression is not regulated by mTOR. The qPCR results were consistent with RNA-Seq data, showing similar trends of PDE expression in both MLA and ESA ± rapamycin. The data suggest that transcriptional regulation does not contribute the reduced sensitivity of the committed YO to MIH; the increased PDE activity during mid and late premolt is likely regulated post-transcriptionally in most PDEs. Our data suggest that Gl-
PDE11 is the controlling PDE in the YO and shows mRNA level changes depending on the molt stage. This is consistent with the responsiveness of YO cells to MIH during mid/late premolt.

**Introduction**

Decapod crustaceans must shed their exoskeleton periodically for order to them to grow and increase in size and this mainly happens in terms of molting, ecdysis. Molting in crustaceans represents a significant event in life, and this complex physiological process requires precise coordination and regulation between the action of two hormones. Ecdysteroids are steroid hormones which are synthesized and secreted from a pair of Y-organs (molting glands) located in the anterior cephalothorax of the animal. The molt inhibiting hormone, (MIH) is a neuropeptide hormone, which is produced and secreted from the X-organ/complex gland found in the eyestalks. Molting is induced when the titers of MIH in the blood (hemolymph) decline, leading to the stimulation of the YOs to produce and secrete molting hormones, ecdysteroids. The molt cycle can be divided into the following stages: intermolt, the longest stage in the molt cycle during which the animal practices routine activities such as foraging and mating. Premolt (proecdysis) the stage preceding molting, is subdivided into 3 substages (early premolt, mid premolt, late premolt). During premolt the hemolymph ecdysteroid titers increase until they reach a peak then drop dramatically just before the actual shedding of the exoskeleton (ecdysis). The last stage is postmolt.
(postecdysis); the animal is vulnerable in this stage as it is recovering from this energy consuming process. It also hardens the soft new shell, an essential hallmark observed during this stage.

The molting gland (Y-organ) is a dynamic organ that proceeds through four distinctive physiological phases throughout the molt cycle: basal phase (during intermolt) when hemolymph ecdysteroid levels are inhibited by pulses of MIH. Activated phase (early premolt), the YO shows signs of hypertrophy to elevate ecdysteroidogenesis in response to the declined circulating MIH. The activated YO is still sensitive to MIH since the animal can postpone molting if eyestalk extracts are injected at this period. During mid/late premolt, the YO transitions to the committed phase and it becomes insensitive to MIH and CHH, as ecdysteroid titers reach their maximum. MIH and CHH levels drop dramatically to initiate the actual molting before ecdysis. A repressed YO is observed in postmolt animals, during which calcification and hardening the exoskeleton, as well as claw muscle growth, occur (Nakatsuji et al., 2009; Chung et al., 2010; Chang & Mykles 2011; Covi et al., 2012; Webster et al., 2012; Shyamal et al., 2014).

Exogenous and endogenous cues contribute to the precise timing for the animal to undergo ecdysis; external factors, such as photoperiod, temperature, stress, and crowding (Skinner & Graham 1972; Weis 1976) Internal factors, such as the urge to provide extra space for the growing organs and tissues, as well as the action of the two opposing predominant hormones, MIH and ecdysteroids (Skinner 1985). In crustaceans, several physiological events take place upon reaching molting (ecdysis), including new exoskeleton synthesis, old exoskeleton degradation, claw muscle atrophy, and lost limb regeneration (Skinner 1985; Mykles 1997; Chang & Mykles 2011).

Upon binding of MIH to its receptor on the YO plasma membrane, a transient increase in cAMP, followed by a larger increase in cGMP, is involved. The delayed increase in cGMP suggests that MIH activates a soluble NO-sensitive guanylyl cyclase (GC-I), since activation of a
membrane GC would result in an immediate increase in cGMP. Both cAMP and cGMP inhibit YO ecdysteroidogenesis. Phosphodiesterases (PDEs), such as PDE1 and PDE5, hydrolyze cAMP and cGMP and thus control the responsiveness of YO organs to MIH (Covi et al. 2012).

_G. lateralis_ can be easily manipulated to trigger the process of molting and as in other decapod crustaceans it can be induced by two methods: multiple leg autotomy (MLA) or eyestalk ablation (ESA) (Skinner 1985; Mykles 2001; Chang & Mykles 2011). The autotomy or voluntary loss of 5 or more of the walking legs mimics the natural way of releasing (autotomizing) an appendage when encountering a predator; this will stimulate molting, so the animal will grow a full set of limbs at the next molt. Extirpation of the eyestalks will remove the main source of MIH, which in turn will induce shedding of the old exoskeleton. ESA is a more intense and precise method to induce molting as one can monitor the different molt stages by the increase in hemolymph titers which occurs upon the activation of the YO organs (Lee and Mykles, 2006; Lee et al., 2007b; Covi et al., 2010; Knope & Larson 2014).

mTOR (The mechanistic Target of Rapamycin) is a protein kinase that mediates a variety of cellular functions from cell growth, metabolism, protein and lipid synthesis, to autophagy and cell survival. The mTOR signaling pathway is highly conserved across metazoans (Laplante & Sabatini 2009; Zonko et al., 2010). mTOR is important to increase ecdysteroid synthesis in the insect prothoracic gland (Layalle et al., 2008; Hietakangas and Cohen, 2009). mTOR is crucial for tissue growth in _G. lateralis_ and _C. maenas_ (Abuhagr et al., 2014). mTOR is believed to be upregulated in the activated YO, so injecting the mTOR inhibitor, rapamycin, in-vivo or incubating YOs with this inhibitor in-vitro inhibits YO ecdysteroidogenesis, thus molting (Abuhagr et al., 2016).
The committed YO becomes insensitive to MIH in mid/late premolt, a phenomenon that putatively is due to the large increase in the glandular activity of PDEs. Our hypothesis is that increased PDE activity contributes to reduced sensitivity in mid/ and late premolt YO organs. Moreover, PDE expression requires mTOR activity. The effects of MLA and ESA± rapamycin was determined on the expression of PDEs using both transcriptomics and qPCR.

**Materials and Methods**

*Animals and experimental treatments:*

*Gecarcinus lateralis* Adult male (blackback land crabs) were collected from their natural habitat in the Dominican Republic and then shipped to Colorado, USA by commercial air cargo. The animals were acclimated to the new conditions by maintaining them at 27 °C and a humidity of 75-90%. Intermolt crabs were kept in aerated plastic cages lined with moistened aspen bedding by using 5 p.p.t. Instant Ocean (Aquarium Systems, Mentor, OH). Crabs were maintained in an environmental chamber in a 12 hrs light:12 hrs dark cycle and were fed iceberg lettuce, carrots, and raisins twice a week (Covi et al., 2010). Blackback land crabs molt about once a year and larger crabs molt less frequently.

Two molting induction methods were used in these experiments: multiple leg autotomy (MLA) and eyestalk ablation (ESA). MLA mimics the timing of a natural progression toward molting: limb bud regenerate formation takes about 3-6 weeks before the crab enters premolt and eventually molts (Skinner 1985). ESA, however, is an intense and precise method to induce molting. ESA is effective because the X-Organ/Sinus Gland (XO/SG) (located in the eyestalks) are the primary source of MIH. As a result, ESA has an advantage over MLA in providing a precise time point of YO activation; activation can be verified since ecdysteroid hemolymph titers increase
by day-1 post-ESA (Lee et al., 2007). Limb bud regenerates were used to estimate what molt stage MLA animals are in using a measurement called the Regeneration-index (R-index). Such a measurement is calculated as the length of limb regenerate/100 * carapace width. This value ranges from 0 to ~ 24 upon reaching molting (Skinner and Graham 1972; Yu et al., 2002). Limb bud regeneration can be divided into 2 stages: (1) basal growth involves the formation of a small differentiated limb bud (R-value 8-10). This happens throughout intermolt and low ecdysteroid titers are necessary; (2) proecdysial growth takes place during premolt and high ecdysteroid titers are required (Yu et al., 2002). Three factors can determine the molt stage: ecdysteroid titers, R-value, and the presence/absence of the membranous layer (Moriyasu and Mallet, 1986). A digital caliper was utilized to measure both the limb buds and carapace widths of the MLA animals.

_G. lateralis_ crabs were induced to molt by MLA via autotomizing the eight walking legs (Fig. 3.1). Hemolymph was withdrawn prior to harvesting the YOs from the experimental animals at 5 different molt stages; intermolt (IM), early premolt (EP), mid premolt (MP), late premolt (LP), postmolt (PM). 100 µl of hemolymph was added to 300 µl of methanol (MeOH 100%) then stored at -20C°. Competitive ELISA (Abuhagr et al., 2014b) was used to quantify the hemolymph ecdysteroid titers to determine the accurate molt stage for each animal in addition to the R-value. Harvested YOs were reserved in RNA-later (Ambion®, California) and stored at 20C° until the time of RNA isolation. MLA animals were kept in separate moistened sand cages to provide privacy and mimic their natural habitat (Skinner 1985).

Intemolt animals were ES-ablated using a pair of sharp scissors and then cauterized immediately to minimize the amount of bleeding (Fig. 3.2) To determine the effects of rapamycin (mTOR inhibitor) on YO ecdysteroidogenesis, _in-vivo_ injections were performed. The control group (ESA-rapamycin) crabs were injected by the vehicle (~1% DMSO final concentration) on
Day 0. The experimental group (ESA+ rapamycin) crabs were injected by rapamycin (~10 μM final concentration) on Day 0 (mass of the animal ×0.3μl= amount to inject). Hemolymph samples were collected and YOs were dissected at Day 0 for intermolt (intact) crabs and Days 1, 3, 5, 7, 14 post-injection for ESA ± rapamycin. Competitive ELISA (Abuhagr et al., 2014b) was used to quantify the hemolymph ecdysteroid titers. Harvested YOs were reserved in RNA-later (Ambion®, California) and stored at -20°C until the time of RNA isolation.

**RNA isolation, cDNA synthesis and quantitative real-time PCR:**

Y-organs from both MLA and ESA± rapamycin animals were harvested from land crabs. YOs were placed immediately in RNA-later after cleaning the tissues with crab saline. YOs were kept overnight in 4°C, then transferred to -20°C until the time of RNA purification. Total RNA was isolated from crab YOs using TRIzol reagent (Life Technologies, Carlsbad, CA) as described by (Covi et al., 2010). YO tissues (50-200 mg) were homogenized by using a micro-tube homogenizer system. 1 ml of TRIzol was added to samples, then centrifuged at 12,000 g for 15 min at 4 ºC. Supernatants were phenol-chloroform extracted and RNA in the aqueous phase was precipitated using isopropanol (0.75 ml per 1 ml TRIzol reagent). RNA was treated with DNase I (Life Technologies), extracted twice with phenol: chloroform: isoamyl alcohol (25:24:1), precipitated with isopropanol, washed twice with 70% ethanol in DEPC water, and resuspended in nuclease-free water. cDNA was synthesized using 2 μl total RNA in a 20 μl total reaction with SuperScript III reverse transcriptase (Life Technologies) and oligo-dT (20) VN primer (50 μmol/l; IDT, Coralville, IA) as described (Covi et al., 2010). RNA was treated with RNase H (Fisher Scientific, Pittsburgh, PA) and stored at -80 ºC.

End-point PCR was used to amplify the desired product and to increase the yield of each PDE gene, as well as for making external standards of the different genes to be used later in qPCR.
Primers (Table 3.1) were utilized to detect the different PDE products in land crab. Each PCR reaction contained; 3 μl of DI H₂O, 5 μl Master Mix, 1 μl cDNA template, and 0.5 μl of each forward and reverse primers. The concentration of the primers was 20 μM. cDNA was amplified in a thermocycler where denaturation occurred at 94°C for 3 minutes to initiate the process, followed by 30-35 cycles of 30 seconds at 94°C, and 30 seconds at the lowest annealing temperature (see table 3.1.) 30 seconds at 72 °C. PCR products were then separated on 1% agarose gel that contained TAE buffer (composed of 40 mM Tris acetate and 2 mM EDTA with an 8.5 pH). Ethidium bromide was applied to stain the gel and a UV light was used to visualize the gel.

Real-time PCR (RT-PCR) was used to quantify the expression of Gl-PDEs 1,2,4,5,7,9,11 in each point molt stage of the MLA animals, and Gl-PDE4,5,7,11 in ESA ± rapamycin animals. cDNA was synthesized as indicated previously, and a LightCycler 480 thermocycler (Roche Applied Science, Indianapolis, IN) was used to quantify the mRNA transcripts of Gl-PDE1, PDE2, PDE4, PDE5, PDE7, PDE9, and PDE11 for MLA G. lateralis animals and PDE4, PDE5, PDE7, and PDE11 for ESA ± rapamycin G. lateralis animals. Each reaction consisted of 1 μl cDNA or standard, 5 μl SYBR Green I Master mix (Roche Applied Science), 3 μl nuclease-free water, and 0.5 μl each of 10 mM forward and reverse primers (Table 3.1). PCR conditions were as follows: an initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 62 °C for 20 s, and extensions at 72 °C for 20 s, followed by melting curve analysis of the PCR product. Concentrations of mRNA transcripts were determined by the LightCycler 480 software (Roche, version 1.5) using a serial dilution of standards of the PCR product for each gene of interest. The amounts of mRNA transcript in copy numbers per μg of total RNA in the cDNA synthesis reaction were calculated based on the standard curve and the calculated molecular weight of dsDNA products.
**Bioinformatics:**

The expression of different *Gl-PDEs* in the *G. lateralis* YO was assessed using two databases: the MLA transcriptome and the ESA± rapamycin transcriptome (Das et al., 2016; Shyamal et al., 2018). Differential expression (DE) of *Gl-PDEs* 1,2,4,5,7,9, and 11 were determined at each molt stage (IM, EP, MP, LP, and PM) in MLA animals. Moreover, DE of *Gl-PDEs* 4,5,7, and 11 were assessed at Day 0 for intermolt (intact) crabs; and Days 1, 3, and 7 postmolt for the ESA ± rapamycin animals.

**Statistical analysis and software:**

([https://www.idtdna.com/Primerquest/Home/Index](https://www.idtdna.com/Primerquest/Home/Index)). Primers were designed by IDT software. Means for mRNA transcript abundance were compared using an analysis of variance (ANOVA) for molt stages versus log copy number in the MLA experiment or days post molt versus log copy number in the ESA ± rapamycin experiment. Sigma plot 12.5 software (Systat Software, Inc., Chicago, IL, USA) was used to produce and build up the graphs and figures. Tukey test was used to determine significance among the means.

**Results**

**Effects of Multiple Leg Autotomy (MLA) on Gl-PDEs expression in the molting gland:**

*G. lateralis* animals were induced to molt by automatizing all 8 walking legs. Several weeks later, these crabs entered early premolt. R-values were measured weekly to estimate the molt stage of each crab. To further ensure the accuracy of the crab’s molt stage, hemolymph samples were collected just before dissection, so a competitive ELISA could be performed. Ecdysteroid levels showed that the molting hormone was low in early premolt (EP), elevated during mid premolt (MP), then increased to reach its maximum at late premolt (LP) (Fig. 3.3a).
qPCR results showed that *Gl-PDE4, Gl-PDE7,* and *Gl-PDE9* were expressed in very low levels at all molt stages, especially *Gl-PDE9* (Fig. 3.3 b). Unexpectedly, *Gl-PDE1* was also expressed in low levels, scoring its minimum at early premolt (Fig. 3.3 c) *Gl-PDE2* mRNA level was high in intermolt and decreased gradually through to postmolt. Significant differences were observed between IM/LP and IM/PM, and between EP and PM (Fig. 3.3 c). *Gl-PDE5* displayed a different pattern, as a slight increase was seen from IM to EP and was at its highest expression level in MP, then declined to its lowest level in PM. Statistical differences were noticed between IM/LP and EP/PM in *Gl-PDE5* (Fig. 3.3 c). *Gl-PDE11* demonstrated a similar pattern to ecdysteroid titers in different molt stages. *Gl-PDE11* showed the most robust expression among the rest of *Gl-PDEs*; as it increased progressively to reach its maximum expression in LP, then decreased in PM. Expression levels of *Gl-PDE11* were not identical to the hypothesized trend but was the closest to our expectations. Significance differences were detected between IM/PM and IM/MP (Fig. 3.3 c).

MLA transcriptomics exhibited different expression patterns of *Gl-PDEs.* *Gl-PDE2, Gl-PDE7, Gl-PDE8* and *Gl-PDE9* were expressed at their highest levels at IM and lowest levels at PM (Fig. 3.4 a, b). *Gl-PDE4* showed levels of similar expression levels in IM, EP, MP; then dropped upon reaching PM (Fig. 3.4 b). *Gl-PDE5* expression was high in IM, gradually decreased until reaching LP, then slightly increased in PM (Fig. 3.4 b). Statistical significance was seen between IM/PM in *Gl-PDE2, 4, 7, 8,* and *9; whereas there was a significant difference between IM/LP in *Gl-PDE5.* Once again, *Gl-PDE11* displayed a unique pattern; it was the highest gene to be expressed to reach a peak in MP, then drop dramatically when approaching PM and a significant decrease was seen between these two molt stages (Fig. 3.4 c).
**Effects of Eyestalk Ablation (ESA)± rapamycin on Gl-PDEs expression in the molting gland:**

*G. lateralis* crabs were induced to molt ESA. For the qPCR results, YOs were harvested from 3 different groups and intact animals were dissected on Day 0, control (DMSO) group (ESA-rapamycin) animals were dissected on Days 1,3,5,7, and 14, and experimental (rapamycin) group (ESA+ rapamycin) animals were also dissected on Days 1,3,5,7, and 14. ESA ±rapamycin animals were either injected with DMSO (control group) or rapamycin (experimental group) on Day 0.

A significant increase from Day 0 to Day 1 was seen in the expression of Gl-PDE4, Gl-PDE7, Gl-PDE11 in both control and experimental YOs. Expression then levels off with no difference between the two groups (Fig. 3.5 a, c, d). Gl-PDE5 showed no noticeable elevation in its expression from Day 0 to Day1 and displayed a similar trend with the other PDEs. But there was a slight increase in Gl-PDE5 expression from Day 5 to Day 7 in the control group. Also, there was a significant decrease from Day 3 to day 5 in the experimental group (Fig. 3.5 b). Expression of Gl-PDE4 mRNA increased significantly at day 1 post ESA + rapamycin and Day 3 post ESA – rapamycin when compared to intact animals on Day 0 (Fig. 3.5 a). There was a slight, but a significant, increase in Gl-PDE5 expression between day 5 and day 7 in the control group (Fig. 3.5 b). Gl-PDE7 mRNA levels increased drastically from Day 0 to Day 1 and a statistical significance was observed in Day 1 in the control group when compared to Day 0 (Fig.3.5 c). Gl-PDE11 expression levels illustrated a significant increase in Day 1 post-ESA in experimental animals and 3 days post-ESA in control animals when compared to Day 0 (Fig. 3.5 d).

Comparable to the ESA ± rapamycin qPCR experiment, ESA ± rapamycin transcriptomics showed similar trends and patterns of Gl-PDEs expression in both control and experimental animals; an increase was seen from Day 0 to Day 1 as the YO is activated upon the ablation of the eyestalks.
Gl-PDE4 expression levels increased significantly at 1-day post-ESA in control and experimental animals compared to Day 0 (Fig. 3.6 a). Gl-PDE5 mRNA levels showed a statistical difference between Day 0 and Day 1 post-ESA in control and experimental crabs (Fig. 3.6 b). Expression levels of Gl-PDE7 exhibited a significant increase between Day 0 and Day 3 post-ESA in control animals. Gl-PDE11 expression levels increased extremely from Day 0 to Day 1 which resulted in a significant increase at 1-day post-ESA in both control and experimental animals (Fig. 3.6 d).

Discussion

cAMP and cGMP signaling pathways control a plethora of intracellular proteins that vary depending on the cell type and function. Such signaling pathways are widespread from prokaryotes to eukaryotes and are involved in activating protein kinases, including PKA and PKG. The only known negative regulators of cAMP and cGMP are cyclic nucleotide phosphodiesterases (cN-PDEs), which fall under the Class I PDE superfamily. The PDE superfamily includes eleven genes designated PDE1 to PDE11. Each family shares common biochemical features, substrate specificity, cellular/sub-cellular localization, pharmacological characteristics, and regulatory mode. Although all eleven genes include a well-conserved catalytic domain located in the carboxyl terminus, each gene has its unique regulatory domains. Our hypothesis is that an increase in PDE expression will occur in G. lateralis Y-organs at mid/late premolt stages, thus decreasing the responsiveness of YOs to (MIH). Such a finding parallel results from studies done on crayfish, in which glandular PDE activity was detected in YOs at specific molt stages (Nakatsuji et al., 2006a; Nakatsuji et al., 2009), as well as in green crab YOs (Mattson & Spaziani, 1985b). Prior to the current study, it was unknown which of the PDE genes might be expressed in the YOs and contribute to the most critical time of the crustacean’s life.
Hemolymph ecdysteroid titers increased when animals were induced to molt by MLA (Fig. 3.3 a). qPCR showed that \textit{Gl-PDE}4, \textit{Gl-PDE} 7, and \textit{Gl-PDE} 9 mRNA transcripts were expressed in low levels. Moreover, \textit{Gl-PDE}1 and \textit{Gl-PDE}2 showed different patterns (Fig. 3.3 c). None of the above \textit{Gl-PDE} expression profiles met our hypothesis since we did not see increased PDE expression in mid/late premolt YOs. We conclude that these enzymes might be regulated post-transcriptionally to stabilize the mRNA and to further enhance and modify the structure of the final protein product. Such observations were symmetrical to the beta-subunit in \textit{HsPDE5} (Lerner et al., 2006). Moreover, studies on \textit{HsPDE3B} adipocytes revealed both transcriptional and post-transcriptional modifications (Yan et al., 2007). Another interpretation might be that MLA has no effect on the expression of \textit{Gl-PDE}1,2,4,7, and 9. It is also possible that target cells interpret nuanced changes in PDE activity and even slight changes present a potent impact on the target cell response (Sette & Conti 1996). Conversely, \textit{Gl-PDE5} showed its highest expression in MP, then dropped upon reaching PM. \textit{Gl-PDE11} reached its maximum expression in LM and decreased in PM (Fig. 3.3 c). These findings do support our first hypothesis, that PDE activity in mid/late premolt is responsible for the YO insensitivity to MIH, which in turn triggers the animal to be committed to molt (Nakatsuji et al., 2009). Consequently, \textit{Gl-PDE5} (a c-GMP specific PDE) and \textit{Gl-PDE11} (a dual PDE) might play a remarkable role in the YO’s MIH signaling pathway.

Results of the MLA transcriptomics studies demonstrated the relative expression of \textit{Gl-PDEs} (Fig. 3.4 a, b). \textit{Gl-PDE} 2,4,7, and 9 were relatively higher in IM and then decreased gradually while reaching their lowest expression in PM. \textit{Gl-PDE5} was high in intermolt then dropped to its minimum expression in LM and slightly increased in PM (Fig. 3.4 b). \textit{Gl-PDE11} expression reached a peak in MP then declined in PM accompanied with a significant difference between these two stages (Fig. 3.4 c). We conclude that MLA had no impact on \textit{Gl-PDE} 2,4,7, and
9 expression since these results were consistent with the qPCR results seen in the MLA experiment. Notably, \textit{Gl-PDE5} and \textit{Gl-PDE11} depicted different trends as was observed in the qPCR results from the MLA experiment. Our data strongly suggest that both \textit{Gl-PDE5} and \textit{Gl-PDE11} act on the intracellular levels of cAMP/cGMP and both converge to stimulate ecdysteroid synthesis in the molting gland. This finding is consistent with recent studies in humans, in which \textit{Hs-PDE11A} exerted a regulatory effect on cortisol excretion and synthesis (Ceyhan et al., 2012; Vezzosi et al., 2012). In contrast to \textit{Gl-PDE5}, inhibition of \textit{PDE5A} in rat and mouse Leydig cells by sildenafil (Viagra) \textit{in-vivo}, illustrated an activation of the NO/cGMP pathway, thus increasing testosterone synthesis (Saraiva et al., 2009; Andric et al., 2010).

mTOR is a conserved serine/threonine kinase and found from yeast to humans. It represents a central node for a variety of cellular processes, such as gene transcription, protein synthesis, cell growth, and cell metabolism (Cornu et al., 2013). mTOR is important during molting in the activated YO in crustaceans (Abuhagr et al., 2014), as such an activation in early premolt YOs upregulates ecdysteroid synthesis (Mykles 2010). In the fruit fly, \textit{Drosophila melanogaster}, metamorphosis depends on mTOR signaling pathway in the molting gland (prothoracic gland or PG) (Layalle et al., 2008).

Our second goal was to determine whether \textit{Gl-PDE} expression requires mTOR activity or not. For this purpose, ESA was performed to induce molting. ESA ± rapamycin qPCR/transcriptomics results revealed an activation from Day 0 to Day 1 in the expression of \textit{Gl-PDE4,5,7, and 11}, which then leveled off on all other days post-ESA (Fig. 3.5 a, b, c, d) and (Fig. 3.6 a, b, c, d). Furthermore, no differences were observed between control and experimental groups. In contrast, previous studies showed that PDE4D5 binds Rheb (upstream regulator of mTOR) in a noncatalytic novel fashion to inhibit mTOR, and that this binding will only be
dissociated if intracellular cAMP levels are elevated (Kim et al., 2010). Furthermore, previous studies provide evidence of cross talk between mTOR and cAMP signaling pathways; an increase in cAMP concentration inhibits mTOR complexes, thus suppressing the catalytic activity of mTOR (Xie et al., 2011). So, cAMP can either activate or inhibit mTOR depending on the tissue type and cAMP distribution (Kwon et al., 2004; Rocha et al., 2008). PDEs might have an indirect regulation mechanism, but this is not fully understood. Taken together, mTOR does not contribute to PDE expression in the crustacean’s *G. lateralis* molting gland, indicating a different mechanism of regulation might be controlling Gl-PDEs, which is distinct from mammalian PDEs.

Conclusions

Molting in the blackback land crab, *Gecarcinus lateralis*, can be induced by two methods: Multiple Leg Autotomy (MLA) and Eyestalk Ablation (ESA). In mid/late premolt, molting glands (YOs) become insensitive to MIH and the animal is committed to molt. Second messengers cAMP and cGMP play a crucial role in the MIH signaling pathway and their intracellular concentrations are regulated with cyclic nucleotide phosphodiesterases (PDEs). MLA and ESA ± rapamycin transcriptomes were screened for PDE expression. qPCR for the two molt induction experiments was used to validate the transcriptomics results. MLA showed no effect on *Gl-PDE1, 2, 4, 5, 7*, and 9, suggesting these genes might be regulated in a post-transcriptional manner. Conversely, *Gl-PDE11* (a dual PDE) is the dominant PDE in the YO and shows molt-dependent changes in the mRNA levels that is consistent with a role in reducing sensitivity in mid/late premolt. ESA increased mRNA levels of *Gl-PDE4, 5, 7* and 11, and was associated with YO activation. Such increases do not require mTOR activity. These data were not consistent with our hypothesis that mTOR regulates PDE expression as there was no significant difference between the control and experimental groups based on both transcriptomics and qPCR results.
Table 3.1. Oligonucleotide primers used in qPCR to identify gene expression in GI-PDEs in MLA and ESA ± rapamycin experiments. Abbreviations: Gl, Gecarcinus lateralis; F, Forward; R, Reverse, PDE, cyclic nucleotide phosphodiesterase; Numbers (1,2,4,5,7,9,11), PDE family.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’-3’)</th>
<th>Amplicon Product (bp)</th>
<th>Annealing Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gl-PDE1 -F2</td>
<td>GGTGGCAAGTGGAAAGATAAAG</td>
<td>226</td>
<td>62 C°</td>
</tr>
<tr>
<td>Gl-PDE1 -R2</td>
<td>CCTCCTCGTCTCTCTCTTAGT</td>
<td></td>
<td>62 C°</td>
</tr>
<tr>
<td>Gl-PDE2 -F2</td>
<td>GGTGGTAGTGACGTATT</td>
<td>301</td>
<td>62 C°</td>
</tr>
<tr>
<td>Gl-PDE2 -R2</td>
<td>TCCCTCTTCTCTCTCTCTCT</td>
<td></td>
<td>62 C°</td>
</tr>
<tr>
<td>Gl-PDE4 -F1</td>
<td>AGGCTTCTGTGTGGTACATATC</td>
<td></td>
<td>62 C°</td>
</tr>
<tr>
<td>Gl-PDE4 -R2</td>
<td>CACAAACTTGATCCCTCAATC</td>
<td>260</td>
<td>62 C°</td>
</tr>
<tr>
<td>Gl-PDE5 -F2</td>
<td>CAGACCACCGATCTTTATT</td>
<td></td>
<td>62 C°</td>
</tr>
<tr>
<td>Gl-PDE5 -R1</td>
<td>TCCTCGACCCGATTTCTATG</td>
<td></td>
<td>62 C°</td>
</tr>
<tr>
<td>Gl-PDE7 -F1</td>
<td>CATGGAAGGCAATTTGGCTAAG</td>
<td>283</td>
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<tr>
<td>Gl-PDE7 -R1</td>
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</tr>
<tr>
<td>Gl-PDE9-F1</td>
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<td>Gl-PDE11-R2</td>
<td>CGACTGATGTCATTCATATC</td>
<td></td>
<td>62 C°</td>
</tr>
</tbody>
</table>
Figure 3.1. Molting can be induced by Multiple Limb Autotomy (MLA). In this picture, the crab reached ecdysis and is pulling itself out of the old exoskeleton upon losing all the walking legs. Molting in this case is important, so the animal can grow with a full set of limbs.
Figure 3.2. Molting can be induced by Eyestalk Ablation (ESA). This picture depicts the removal of both eye stalks, thus eliminating the main source of MIH (molt-inhibiting hormone).
Figure 3.3. Effects of MLA on the YO expression of *Gl-PDE*4, *Gl-PDE* 7, *Gl-PDE* 9 (a) and YO expression of *Gl-PDE* 1, *Gl-PDE* 2 *Gl-PDE* 5, *Gl-PDE* 11(b). *Gl-PDE* mRNA expression for the different families was quantified by qPCR at each molt stage point; intermolt (IM), early premolt (EP), mid premolt (MP), late premolt (LP), post molt (PM). n= 10-12 for each molt stage. Data presented as mean ±1 S.E. No significant differences were observed in the means of *Gl-PDE*4,7 and 9. mRNA expression of *Gl-PDE* 1,2,5,11 appeared with different trends. Means within the same gene that were significantly different are represented with a bracket.
Figure 3.4. Relative expression of *Gl-PDE*2,9 (a), *Gl-PDE*4,5,7,8 (b) and *Gl-PDE*11 (c). Transcriptomics was used to assess the effects of molt stage on PDE expression. There was a significant difference between intermolt (IM) and postmolt (PM) in all *Gl*-PDEs, except *Gl-PDE*11 which elucidated a statistical significance between mid premolt (MP) and PM. *Gl-PDE*11 showed the highest expression among all PDEs (scales are different for each set of PDEs). Means within the same gene that were significantly different are represented with a bracket.
Figure 3.5. Effect of ESA± rapamycin on the expression of *Gl-PDE4* (a), *Gl-PDE5* (b), *Gl-PDE7* (c), and *Gl-PDE11* (d) in *G. lateralis* YO. qPCR was used to evaluate the expression of *Gl-PDE4,5,7,11* at point times 1,3,5,7,14 days post ESA in control (DMSO) and experimental (rapamycin) injected animals. mRNA PDEs increased from Day 0 to Day 1, then leveled off throughout the rest time points. Means within the same gene that were significantly different are represented with a bracket. Data presented as mean± S.E (n=8-10).
Figure 3.6. Effect of ESA± rapamycin on the expression of Gl-PDE4 (a), Gl-PDE5 (b), Gl-PDE7 (c), and Gl-PDE11 (d) in G. lateralis YO. Transcriptomics was used to evaluate the expression of Gl-PDE4,5,7,11 at point times 1,3,7 days post ESA in control (DMSO) and experimental (rapamycin) injected animals. mRNA PDEs increased from Day 0 to Day 1, then leveled off throughout the rest time points. Means within the same gene that were significantly different are represented with a bracket. Data presented as mean± S.E.
CHAPTER FOUR

SUMMARY AND FUTURE DIRECTIONS

Cyclic nucleotide signaling mediates the suppression of the crustacean molting gland (Y-organ or YO) by molt-inhibiting hormone (MIH). When MIH level drops the YO transitions from the basal to the activated state and the animal enters premolt. During mid-premolt, the YO transitions to the committed state, in which the YO becomes insensitive to MIH. Phosphodiesterases (PDEs) hydrolyze the phosphodiester bond in cAMP and cGMP to AMP and GMP, respectively, and thus can modulate the response of the YO to MIH. In some species, PDE inhibitors decrease molting hormone (ecdysteroid) biosynthesis by the YO in-vitro, indicating that PDE activity can keep cyclic nucleotide levels low. Increased PDE activity in the YO is correlated with a reduced sensitivity to MIH when the animal becomes committed to molt. In mammals, 21 PDE genes are organized into 11 genes, designated PDE1 to PDE11. Each PDE family has specific catalytic and biochemical properties and tissue distributions. A reference YO transcriptome from the blackback land crab (Gecarcinus lateralis), consisting of 3 biological replicates of intermolt animals, was analyzed for PDE sequences. Nine different contigs encoding seven full-length PDE sequences two partials were identified. Moreover, seven different contigs encoding five full-length PDE sequences two partials were identified in the C. maenas transcriptome. Protein alignments and ClustalX analysis of the Gl-PDE and Cm-PDE sequences with orthologs from other species in the GenBank database showed that the sequences corresponded to PDE1, 2, 3 4, 5, 7, 8, 9, and 11. General and selective inhibitors were used to characterize the PDEs regulating ecdysteroid secretion in the green crab, Carcinus maenas YO. IBMX, vinpocetine, EHNA and zaprinast ± rMIH significantly inhibited ecdysteroid secretion, while rolipram, dipyridamole, and BC11-38
did not. This suggests that PDE1, PDE2 and PDE5/11 are primarily responsible for regulating cAMP and cGMP levels. No effect on ecdysteroidogenesis was seen on the blackback land crab, *Gecarcinus lateralis*, YOs when exposed to the same PDE inhibitors *in-vitro*, indicating different regulatory metabolic machineries between the two species.

PDE gene expression was examined in different tissues of *G. lateralis* and *C. maenas* by qPCR. Gl-PDE2 and Gl-PDE5 showed mild levels of mRNA expression. Cm-PDE11 had the highest expression among all examined PDEs which might indicate a pivotal role in cAMP/cGMP signaling pathways.

qPCR was used to quantify the effects of molt induction by multiple limb autotomy (MLA) or eyestalk ablation (ESA) ± mTOR inhibitor rapamycin on expression of PDE 1, 2, 4, 5,7,9,11 in *Gecarcinus lateralis* YO. In response to MLA, all PDEs, except for PDE5 and PDE11, were expressed at their highest levels in the intermolt YO. mRNA levels declined during premolt and reached their lowest levels in postmolt. qPCR results from the MLA experiment showed that both Gl-PDE5 and Gl-PDE11 reached high expression levels in mid premolt and late premolt, respectively. MLA transcriptomics revealed that only PDE11 expression maximized at mid premolt. In response to ESA, the mRNA levels of PDE4, 5, 7, 9, and 11 showed no significant change by 7- and 14-days post-ESA. Rapamycin had no significant effect, as PDE mRNA levels were comparable to those of controls at all time points, indicating that PDE expression is not regulated by mTOR. The qPCR results were consistent with RNA-Seq data, showing similar trends of PDE expression in both MLA and ESA ± rapamycin. The data suggest that transcriptional regulation does not contribute to the reduced sensitivity of the committed YO to MIH; the increased PDE activity during mid and late premolt is likely regulated post-transcriptionally in most PDEs. Our data suggest that PDE11 is the controlling PDE in the YO and shows mRNA level
changes depending on the molt stage. This finding is consistent with the responsiveness of YO cells to MIH during mid/late premolt.

The number of PDE families were unknown before this project, and it was proposed that two PDE families might contribute in the MIH signaling pathway. The outcome of this project was surprising, as nine different PDEs, each with their unique properties and characteristics were found in the YO transcriptome of *G. lateralis* and the transcriptome of *C. maenas*. Moreover, our data suggest that PDE11 might be the prominent enzyme in the YO.

Future studies should investigate the protein levels of these PDEs in the YO and other tissues. Rapamycin has no effect on PDE mRNA levels, indicating mTOR activity does not control PDE gene expression in the activated YO. However, Activin/TGFβ signaling, which is required for YO commitment, may control PDE gene expression in mid and late premolt. Transcriptomics and qPCR can be used to determine whether SB431542, an inhibitor on Activin/TGFβ signaling, affects PDE mRNA levels.
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