CHARACTERIZATION OF THE ADIPOKINETIC HORMONE/CORAZONIN-RELATED PEPTIDE SIGNALLING SYSTEM IN THE MOSQUITO, *AEDES AEGYPTI*

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Abstract

The adipokinetic hormone/corazonin-related peptide (ACP) is an insect neuropeptide structurally intermediate between corazonin (CRZ) and adipokinetic (AKH) hormones, which all demonstrate homology to the vertebrate gonadotropin-releasing hormone (GnRH). AKH and CRZ are best known to function in energy mobilization and cardioacceleration, respectively, however, the function of the ACP signalling system remains unclear. Here the gene encoding the ACP receptor in *Aedes aegypti* has been identified. Functional deorphanization of *Aedae*ACPR-I revealed a highly specific response for its native ligand, ACP. Analysis of spatial and developmental expression profiles reveals enrichment of *ACP* and *ACPR* in the central nervous system and post-eclosion, respectively. The cell-specific distribution of the *ACP* and *ACPR* within the central nervous system was examined revealing expression within distinct regions of the brain, thoracic ganglia, and abdominal ganglia. The findings of this thesis point to a role of ACP within the nervous system functioning either as a neuromodulator or neurotransmitter.

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Table of Contents

List of Tables

List of Figures

Chapter 1: General Introduction

Chapter 2: Molecular identification, transcript expression, and functional deorphanization of the adipokinetic hormone/corazonin-related peptide receptor in the disease vector, *Aedes aegypti*.

Chapter 3: Revealing the distribution and cell-specific expression of the adipokinetic hormone/corazonin-related peptide and its receptor and utilizing RNA interference to knockdown *ACP* and *ACPR* transcript levels

Chapter 1: General Introduction

Aedes aegypti

Aedes aegypti are anthropophilic mosquitoes prevalent in the tropics and subtropics, where they transmit several diseases including, Dengue fever, Yellow fever, and Chikungunya (Price et al., 2015). Recently, incidences of these diseases have increased at an alarming rate. According to the World Health Organization, Dengue virus affects approximately 50-100 million people per year and 3.6 billion people live in Dengue endemic areas and are constantly at risk of infection (Price et al., 2015). Yellow fever affects approximately 200,000 people per year, and the Chikungunya virus, which was responsible for infecting 1.6 million people during an outbreak in India and Reunion island (Africa), has spread to the Caribbean and Florida putting more individuals at risk (Mavalankar et al., 2008). Recently, there has been tremendous emphasis on *A. aegypti*'s role in the transmission of the ssRNA Zika flavivirus, a virus that is the causative agent for microcephaly, characterized as an abnormally small head due to incomplete brain development, and other neurological complications (Huang et al., 2016). The Zika virus is well established in Brazil and in 2015, spread alarmingly northwards to the United States (Saiz et al., 2016). As of January 2017, active circulation of the Zika virus has been reported in almost all Latin American and Caribbean countries (Baud et al., 2017).

The incidence of diseases transmitted by *A. aegypti* has increased over the years with a decline in the development of insecticides (Vézilier et al., 2013). Moreover, traditional insecticides utilized for control of insect pest populations are becoming increasingly ineffective due to increased resistance on the part of the mosquitoes (Ranson et al., 2011). Thus, studying and understanding these organisms may be invaluable in developing novel vector control strategies that limit pathogen transmission in nature. These control strategies include but are not

limited to, the development of pesticides to prevent mosquito population growth, as well as the potential development of vaccines against viruses transmitted by *A. aegypti*.

Neuropeptides

Organisms employ a wide variety of signalling molecules, which function to modulate activities of their individual systems to maintain homeostasis (Nässel, 2002) . An especially important class of signalling molecules are the neuropeptides, which are oligopeptides involved in the processing and transmission of information from the nervous system to effector systems (Mercier et al., 2007). Characterized as 5-50 residue long peptides, they are biosynthesized in neural tissue, and may act as neurotransmitters, neurohormones, and neuromodulators (Hoyer and Bartfai, 2012). Their neurohormonal function involves secretion into the haemolymph to initiate a compensatory or regulatory response elsewhere in the organism (Nässel, 2002). Neuropeptides can also act as neurotransmitters, demonstrated by their ability to transmit electrical signals from one axon to another (Mercier et al., 2007). Additionally, neuropeptides can also enhance signals or decrease them illustrating their modulatory function. Initially, neuropeptides are produced as larger precursors in peptidergic neurons, which are enzymatically processed into active peptides (Hökfelt et al., 2003; Nässel and Larhammar, 2013). Neuropeptide precursors typically contain a signal peptide and propeptide (inactive) region. Signal peptide sequences are N-terminal extensions of nascent polypeptide chains that mediate protein targeting to the membrane of the endoplasmic reticulum (Blobel, 1980). After removal of the signal peptide via signal peptidase, propeptides are processed through enzymatic cleavage and post translational modification(s) to become active peptides that are stored within vesicles prior to secretion (Hökfelt et al., 2003; Nässel and Larhammar, 2013) .

To elicit a response, mature neuropeptides bind to their cognate receptors triggering a diversity of intracellular effects including transcriptional and post transcriptional alterations of target genes and proteins ultimately bringing about a physiological change in the organism (Hoyer and Bartfai, 2012). With the exception of a few neuropeptides, for example the insulin-like peptides that act on a tyrosine kinase receptor (RTK), the majority of receptors for neuropeptides are guanosine protein-coupled receptors (GPCRs) (Nässel and Winther, 2010). GPCRs contain a signature seven trans-membrane domain with an extracellular N-terminus and intracellular C-terminus (Vanden Broeck, 1996). The heterotrimeric G-protein, bound to the C-terminus of the GPCR, is composed of three subunits (α, β, γ) (Pierce et al., 2002). Upon activation of the receptor, a conformational change occurs and the GDP bound to the G protein is replaced with a GTP molecule (Mercier et al., 2007). Additionally, the $\beta\gamma$ subunits dissociate as a dimer from the α subunit, both of which can target other signalling proteins and intracellular cascades.

Neuropeptides are structurally and functionally the most diverse class of signalling molecules in the nervous system (Nässel, 2000). The earliest trace of neuropeptides dates back to some of the most primitive metazoans, the cnidarians, where nervous systems are thought to have emerged (Grimmelikhuijzen et al., 1996; Nässel, 2000). The primordial nervous system found in early metazoans is characterized as a loose network of neurons. A great number of neuropeptides can be found in more basal organisms, such as *Caenorhabditis elegans*, which possess 119 neuropeptide precursor genes, which can be subdivided into three major families according to the sequence and structural similarities of their derived peptides (Frooninckx et al., 2012). Of these genes, 40 encode insulin-like peptides, 31 belong to the FMRF-amide-like (Phe-Met-Arg-Phe-amide) family, and the remaining 48 are termed neuropeptide-like proteins

(Frooninckx et al., 2012). Insect neuropeptides are known to function in a vast array of processes including osmoregulation, diapause, reproduction, metabolism, development, pigment synthesis, control of skeletal, heart, and visceral muscle, and colour change (Nässel, 1993).

Most metazoans belong to one of two evolutionary lineages, the protostomes and deuterostomes, which are thought to have diverged about 700 million years ago (Douzery et al., 2004). Neuropeptide signalling is ubiquitous throughout both lineages but it is often unclear whether there exists evolutionary relationships between the neuropeptides used by both the protostomes and the deuterostomes. One exception is the widely known Gonadotropin releasing hormone (GnRH) signalling pathway, which has been found in deuterostomia as GnRHs and in protostomia as GnRH-like peptides, red pigment concentrating hormone (RPCH), corazonin, adipokinetic hormone (AKH), and adipokinetic hormone/corazonin-related peptide (ACP) (Roch et al., 2011).

Gonadotropin releasing hormone in deuterosomes

Gonadotropin releasing hormones (GnRH) belong to a group of neuropeptides originally discovered and isolated from the hypothalamus of vertebrates that control secretions of the anterior pituitary gland. First discovered in pigs (Matsuo et al., 1971) and sheep (Amoss et al., 1971), GnRH controls reproductive maturation and function in humans and other vertebrates through its regulation of synthesis and release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Kah et al., 2007). Its mammalian structure was thought to be unique until GnRH was identified in non-mammalian vertebrates, where it became apparent that diverse forms of this peptide hormone exist in vertebrates. At present, 16 variants have been identified in vertebrates (Kavanaugh et al., 2008; Kim et al., 2011) and 12 in urochordates (Sakai et al., 2017). Basic characteristics of vertebrate GnRH's include an N-terminal pyroglutamate, a

high degree of conservation in the first four amino acids on the N-terminus and last two residues on the C-terminus, as well as an amidated C-terminus (Roch et al., 2011). In addition to possessing a signal peptide, GnRH prepropeptides possess a GnRH-associated peptide (GAP) region following the GnRH sequence (Schneider et al., 2006). Within this prohormone, the GnRH sequence is the only region that appears highly conserved (Tsai, 2006).

Two types of GnRHs, GnRH-I and GnRH-II, have been characterized in most vertebrates, whereas a third subtype, GnRH-3/GnRH-III, has been identified in teleost fish (where all three types are found in some teleosts) and lampreys (Decatur et al., 2013; Kavanaugh et al., 2008; Millar et al., 2004). In addition to vertebrates, the GnRH family of peptides have been identified in urochordates, where 12 GnRH peptides have been identified in ascidians (Sakai et al., 2017). In contrast to vertebrate and non-ascidian invertebrate GnRH genes where a single GnRH is encoded by a gene, multiple ascidian GnRHs arise from a single gene (Kawada et al., 2013; Matsubara et al., 2016). A GnRH-like peptide has also been identified in the cephalochordate *Branchiostoma floridae* (Roch et al., 2014), and in the echinoderms, *Strongylocentrotus purpuratus* (Rowe and Elphick, 2012) and *Asterias rubens* (Tian et al., 2016). GnRH receptors (GnRHRs) belong to the Class A GPCR family where in most vertebrates two to three forms of GnRHRs are present (Millar et al., 2004). Invertebrate deuterostomian GnRHRs in ascidians, amphioxus, and echinoderms have also been isolated, ranging from one to four receptors in each organism (Sakai et al., 2017).

As previously mentioned, vertebrate GnRH-I triggers the release of LH and FSH from the pituitary gland, thus regulating the hypothalamic-pituitary-gonadal (HPG) axis. Stimulation of the HPG axis leads to steroid production and gametogenesis in the vertebrate gonads. This endocrine function of GnRH-I is highly conserved across organisms possessing a pituitary gland

(Tsai and Zhang, 2008). Additionally, GnRH serves as a peripheral bioactive peptide inducing the release of sex steroids in vertebrate reproductive tissues (Millar et al., 2004). Although the functions of GnRH-II/III are poorly understood, expression in the midbrain and telencephalon, respectively, are suggestive of neuromodulatory roles (Roch et al., 2011).

Gonadotropin releasing hormone in protostomes

Discovery of the vertebrate GnRH peptides as a hypothalamic releasing factor for reproductive hormones, LH and FSH, paved the way for investigation of endocrine control of reproductive systems in more basal organisms. The first lophotrochozoan GnRH-like peptide was isolated and sequenced from the octopus, *Octopus vulgaris* (Iwakoshi et al., 2002). This molecule was termed oct-GnRH, and is a dodecapeptide, deviating from the 10 residue peptides found in most chordates. The receptor for oct-GnRH has been identified and, like the chordate GnRHRs, is a rhodopsin-like GPCR (Kanda et al., 2006). Since then GnRH-like peptides have been identified in a cuttlefish (*Sepia officinalis*), a pacific oyster (*Crassostrea gigas*), a sea hare (*Aplysia californica*), a marine worm (*Capitella teleta*), a leech (*Helobdella robusta*), and a scallop *(Patinopecten yessoensis*) (Sakai et al., 2017). The receptor for *A. californica* GnRH-like peptide has also been cloned and is similarly a rhodopsin-like GPCR (Kavanaugh and Tsai, 2016).

The reproductive role of GnRH extends to mollusks as oct-GnRH has been shown to induce contraction of the oviduct and promote the release of sex steroids, including testosterone, progesterone, and 17β-estradiol-like steroids from the spermatozoa and follicle in octopus (Iwakoshi et al., 2002; Kanda et al., 2006) . In contrast, GnRH-like peptide in *A. californica* exhibited no effects on reproduction, instead regulating substrate attachment, feeding, and opening of parapodia (Tsai et al., 2010).

The first GnRH-type peptide to be isolated from ecdysozoans, and invertebrates as a whole, was the crustacean hormone red pigment-concentrating hormone (RPCH), from the shrimp *Pandalus borealis* (Fernlund and Josefsson, 1972). RPCH peptides in crustaceans are pleiotropic and function in modulating rhythms of the stomatogastric and swimmeret system, concentration of pigment granules in the integumental cells, and movement of pigment in the cells of the eye (Gäde and Marco, 2006; Rao, 2001). Receptors for RPCH are localized in the epidermal chromatophores where they concentrate the pigment granules resulting in an intense red colour characteristic of crustaceans, such as crabs (Rao, 2001).

Soon after the discovery of RPCH, a related peptide was isolated from the corpora cardiaca of locusts *Schistocerca gergaria* and *Locusta migratoria*, as a result of its lipid mobilizing function and was named adipokinetic hormone (AKH) (Mayer and Candy, 1969; Stone et al., 1976). Identification of an AKH precursor revealed a similar architecture to GnRH whereby the AKH peptide is located immediately after the N-terminal signal peptide (Schulz-Aellen et al., 1989). In 1989, an AKH-like peptide was isolated from the cockroach *Periplaneta americana*, and on account of its cardioccelerator properties, was termed corazonin (CRZ) (Veenstra, 1989). A structural intermediate between AKH and corazonin peptides was identified in 2010, and was termed adipokinetic hormone/corazonin-related peptide (ACP). The discussion that follows will focus on the AKH, ACP, and CRZ signalling systems within arthropods.

Adipokinetic hormone

Adipokinetic hormone (AKH) is an insect neuropeptide, produced by intrinsic neurosecretory cells of the corpora cardiaca (CC), a neuroendocrine gland situated behind the insect brain (Diederen et al., 2002). Initially, AKHs were reported as hyperglycemic and hyperlipemic factors in CC extracts of *P. americana* (Steele, 1961) and *S. gregaria* (Mayer and

Candy, 1969), respectively. It wasn't until 1976, where AKH was first isolated, purified, and sequenced from the CC of *L. migratoria* (Stone et al., 1976). Although AKH sequences demonstrate variability across insect species, some hallmarks have been deduced which consist of: (a) 8-10 residue peptides, (b) an N-terminus blocked with pyroglutamate (pQ), (c) an amidated C-terminus, (d) an aliphatic amino acid in position 2, (e) a Trp residue at position 8, and (f) an aromatic residue at position 4 (either Phe or Tyr) (Gäde et al., 1997). To date, over 60 different forms of AKHs have either been elucidated or predicted from genomic databases (Gäde and Marco, 2013).

AKH primarily functions to mobilize lipids (thus "adipokinetic") from the insect fat body, an organ analogous to the liver and adipocytes of vertebrates, essential during energy requiring processes such as flight and locomotion (Mercier et al., 2007). The fat body of insects is characterized as loosely assembled tissue located underneath the epidermis, and primarily functions to store lipids and carbohydrates (Martins et al., 2008). The nervous system, upon reception of stress signals, releases AKH neuropeptides from the CC which interact with their receptors on the fat body and stimulate the release of energy-rich compounds including diacylglycerols (DAG), trehalose, and in some cases proline, into the haemolymph to fuel the high physical activity of the insect (Gäde and Auerswald, 2003). Gäde and Auerswald illustrate the mechanisms by which trehalose and DAGs are released from adipocytes upon AKH binding to its receptor, the schematic of which is illustrated in Figure 1 (Gäde and Auerswald, 2003). In this case, the mechanism is dependent on which type of Gα subunit is activated (G_q or G_s). A G_q protein-coupled receptor is activated by insects that oxidize only carbohydrates for flight (eg. cockroaches), or those that oxidize carbohydrates in conjuction with proline (eg. beetles, (Auerswald and Gade, 1999; Auerswald et al., 1998) or lipids (eg. locusts) (Van Der Horst et al.,

1999) . Upon AKH binding, G_q activates a membrane bound enzyme, phospholipase C, which breaks down phosphatidylinositol-4,5-bisphosphate (PIP_2) to synthesize inositol trisphosphate (IP_3) and diacylglycerol (DAG). The DAGs subsequently activate protein kinase C (PKC) and phospholipase A (PLA) to form free fatty acids (FFAs) utilized by the organisms as an immediate energy source. Simultaneously, IP_3 acts upon the endoplasmic reticulum to release Ca^{2+} that activates phosphorylase *b* kinase, which in turn activates glycogen phosphorylase; this enzyme is responsible for liberating glucose-1-phosphate, which through a series of reactions, is then converted into trehalose. In addition to carbohydrate release, some insects, such as locusts and the tobacco hornworm moth, also liberate lipids (Gäde and Auerswald, 2003). To liberate lipids, the AKH peptide activates a G_s protein-coupled receptor whereby the enzyme adenylyl cyclase is activated resulting in increased cyclic-AMP levels (cAMP). cAMP and Ca^{2+} work together to activate a triacylglycerol lipase that produces DAGs in locusts and moths and FFAs in beetles. In the tsetse fly and a number of beetles, the FFAs undergo β-oxidation to produce acetyl-CoA, which is combined with alanine to form proline (Auerswald and Gade, 1999; Weeda et al., 1980).

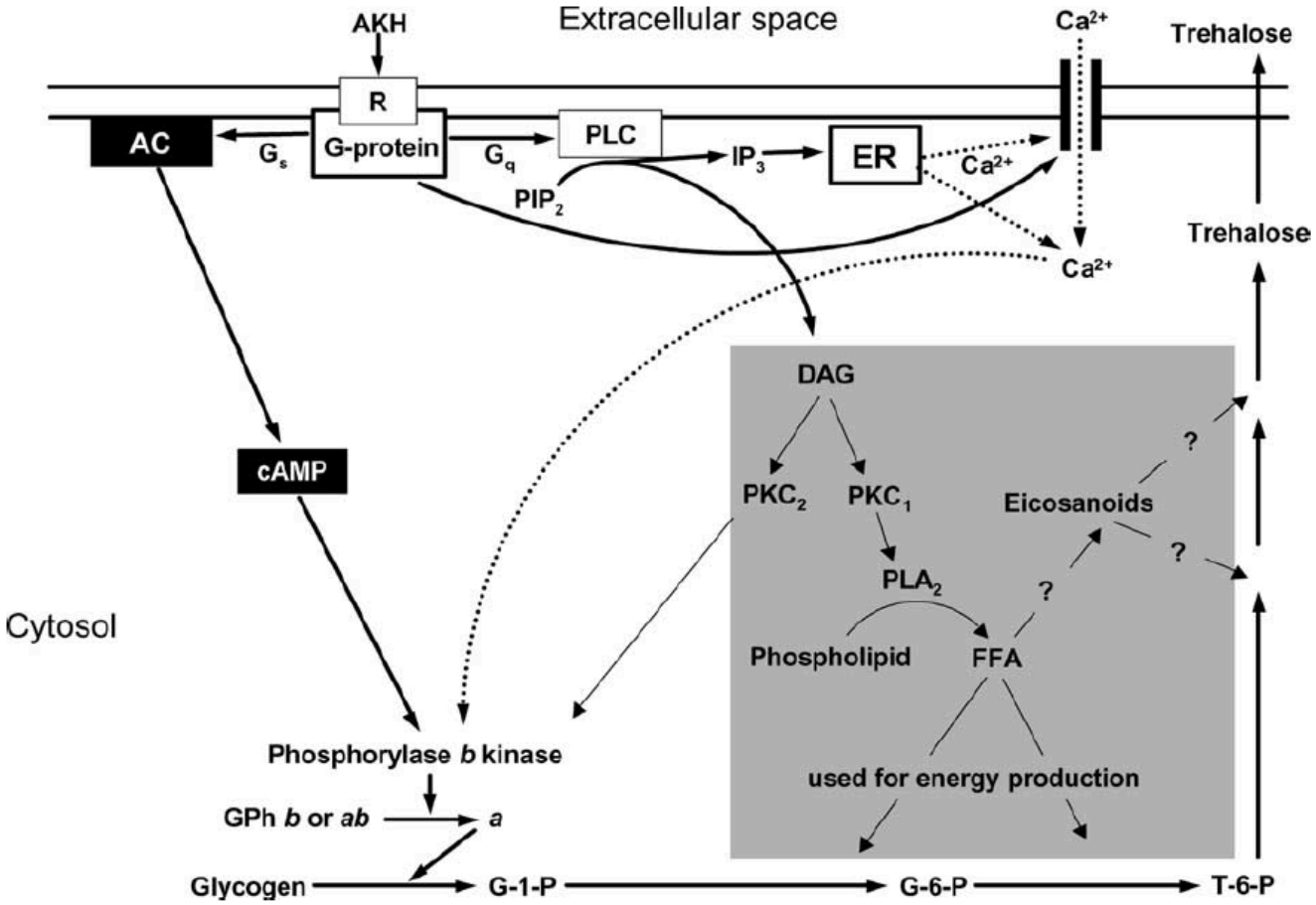

Figure 1. Proposed intracellular mechanism of the AKH peptides role in lipid and carbohydrate mobilization in locusts, beetles, and cockroaches. Abbreviations are as follows, AC = adenylyl cyclase, cAMP = cyclic AMP, GPh = Glycogen phosphorylase, G-1/6-P = glucose-1/6-phosphate, T-6-P = trehalose-6-phosphate, PIP_2 = phosphatidyl inositol bisphosphate, PLC = phospholipase C, DAG = diacylglycerol; IP_3 = inositol trisphosphate, ER = endoplasmic reticulum, FFA = free fatty acids, PKA = protein kinase A, PKC = protein kinase C. This figure was adapted from Gäde and Auerswald, 2003, with the permission of Elsevier.

The role of AKH in metabolism was first discovered as a lipid/carbohydrate mobilizing hormone in cockroaches and migratory locusts, however the purification and identification of the peptide occurred several years later in 1976 by the Stone group (Mayer and Candy, 1969; Steele, 1961; Stone et al., 1976). Later on, Ward and colleagues conducted studies on the kissing bugs *Rhodnius prolixus* and *Triatoma infestans*, where they observed that after 60 minutes of flight activity the bugs began demonstrating diminished lipid concentration in the flight muscle and increased FFAs in the haemolymph (Ward et al., 1982). This finding was supported further by research conducted by Marco and colleagues who found that conspecific injections of 10pmol synthetic AKH into *R. prolixus* and *T. infestans* resulted in a significant increase in haemolymph lipid levels (Marco et al., 2013). Similarly, injections of species-specific AKH in *L. migratoria* and *P. americana* resulted in lipid mobilization and increased levels of circulating trehalose, respectively (Oguri and Steele, 2003; Scarborough et al., 1984; Van Der Horst, 2003). Proline mobilization was observed in dung beetles, *Scarabaeus* spp. and the fruit beetle *Pachnoda sinuata* (Gäde and Auerswald, 2002). RNA interference (RNAi) mediated knockdown of the AKHR receptor in 5th instar female *R. prolixus* resulted in significantly elevated triacylglycerol (TAG) levels in the fat body and flight muscle tissues 10 days post knockdown (Alves-Bezerra et al., 2016). In *D. melanogaster*, genetic ablation of AKH producing cells via the driving of apoptosis transgenes has been shown to reduce haemolymph trehalose levels in larvae and starved adults (Isabel et al., 2005). In addition to their fat mobilizing function, AKHs have been shown to stimulate heart beat rate in prepupae of *Drosophila melanogaster* (Noyes et al., 1995). In locusts, AKHs have also been shown to inhibit the synthesis of haemolymph and tissue proteins (Carlisle and Loughton, 1986).

To determine AKH distribution in insects, Schooneveld *et al*. conducted immunohistochemical studies and found that AKH was predominantly localized to the neurosecretory cells intrinsic to the CC of the locust, *L. migratoria*, with some immunoreactivity observed in the sub-esophageal ganglion (Schooneveld et al., 1983; Schooneveld et al., 1985). Subsequent studies, in *D. melanogaster* (Noyes et al., 1995) and *L. migratoria* (Bogerd et al., 1995) utilizing *in situ* hybridization, localized AKH transcript solely in neurosecretory cells of the CC. Similar AKH-like immunoreactivity was observed in the CC of *D. melanogaster* (Isabel et al., 2005), *L. migratoria* and *S. gregaria* (Diederen et al., 1987), *Glossina morsitans* (Attardo et al., 2012), *R. prolixus* (Patel et al., 2014), *Anopheles gambiae* (Kaufmann and Brown, 2006), and *A. aegypti* (Kaufmann et al., 2009).

In 2002 Staubli and colleagues made a significant breakthrough in insect molecular neuroendocrinology as they identified the first insect AKH receptors (Staubli et al., 2002). Primers were designed based on known sequences of the *D. melanogaster* gonadotropin hormone releasing hormone receptor (GnRHR). A 1347bp length PCR product was obtained, and identified as the AKH receptor. They then designed degenerate primers based on conserved regions of the *D. melanogaster* AKH receptor and subsequently identified the *Bombyx mori* AKH receptor (Staubli et al., 2002). The receptors isolated from the fruit fly and the silkworms were found to be GPCRs and demonstrated homology with the vertebrate GnRH receptors (Staubli et al., 2002). Since then, AKHRs have been identified in *Manduca sexta* (Ziegler et al., 2011), *P. americana* (Hansen et al., 2006), *Blatella germanica* (Huang et al., 2012), *A. gambiae* (Belmont et al., 2006; Kaufmann and Brown, 2006), *R. prolixus* (Zandawala et al., 2015a) and a great number have also been predicted in other insect species based on available genomic sequences (Grimmelikhuijzen and Hauser, 2012; Hauser and Grimmelikhuijzen, 2014). The

AKHR gene in *A. aegypti* was identified and alternative splicing results in three different transcript variants *AedaeAKHR-IA/B*, *AedaeAKHR-II* (Kaufmann et al., 2009; Oryan et al., 2018). Functional characterization of AKHRs using heterologous receptor assays in *P. americana* (Hansen et al., 2006), *B. mori* (Shi et al., 2011; Zhu et al., 2009), *A. gambiae* (Belmont et al., 2006; Hansen et al., 2010), and *A. aegypti* (Oryan et al., 2018) revealed receptor activation only by AKH, with the exception of the *B. mori* AKHR which was activated by ACP at a 100-fold higher dose (Zhu et al., 2009).

Using endpoint reverse transcription PCR (RT-PCR) analyses, AKHR transcript expression was shown to be present in the abdomen and ovaries of adult *A. gambiae* (Kaufmann and Brown, 2006), *A. aegypti* and *Culex pipens* (Kaufmann et al., 2009). Recently, quantitative expression analysis of AKHRs I and II in *A. aegypti* revealed enrichment in the nervous tissue, reproductive accessory organs, and the carcass of adult mosquitoes (Oryan et al., 2018). Furthermore, AKHRs IA and II were found to be enriched in adult *A. aegypti* with greater expression observed in early adults (Oryan et al., 2018). In *R. prolixus*, using quantitative PCR (qPCR), AKHR transcript levels are the greatest in the dorsal vessel, fat body, diaphragm and abdominal nerves, and lastly the prothoracic glands with any associated fat body (Zandawala et al., 2015a). In female 5th instar *R. prolixus* AKHR mRNA levels are the highest in the flight muscle and fat body. Since there is fat body associated with reproductive tissue, a role for AKH in *R. prolixus* reproduction is possible. Indeed, a reproductive function has been demonstrated before as injections of AKH into female *Gryllus bimaculatus* resulted in a significant reduction in ovary mass and number of terminal oocytes produced (Lorenz, 2003). Furthermore, knocking down the AKHR receptor in the tsetse fly, *Glossina morsitans*, resulted in impaired "milk" production during pregnancy (Attardo et al., 2012). AKH has also been shown to inhibit

vitellogenesis of oocytes in *L. migratoria* (Moshitzky and Applebaum, 1990). Finally, AKH has been identified as a component of the seminal fluid proteins in adult male *A. aegypti* and *Aedes albopitus* (Boes et al., 2014) and, as already suggested (Kaufmann et al., 2009), future studies should examine if AKH peptides have a direct or indirect influence on insect reproduction.

Corazonin

Corazonin (CRZ) is an insect undeca-neuropeptide first discovered in *P. americana* due to its significant cardioexcitatory activity on the isolated cockroach heart (Veenstra, 1989). It is synthesized by neurosecretory cells of the pars lateralis, a cluster of neurons within the protocerebrum that project their axons towards the corpora cardiaca, where it is stored and subsequently released into the haemocoel upon the arrival of a stimulus (de Velasco et al., 2007). Despite the sequence of CRZ (Table 1) remaining largely conserved across insect species, to date no universal function has been described for this neuropeptide. Multiple physiological roles have been assigned to CRZ, including the induction of melanization in crowding and pre-swarming *L. migratoria* and *S. gregaria* (Tawfik et al., 1999) populations, initiation of ecdysis in *M. sexta* (Kim et al., 2004), and the reduction in silk spinning rates in *B. mori* (Tanaka et al., 2002). The role of corazonin in development extends to the oriental fruit fly *Bactrocerca dorsalis*, where knockdown of the *BacdoCRZR* resulted in a delay in larval to pupal transition (Hou et al., 2017). In *D. melanogaster,* CRZ has been shown to regulate nutritional and oxidative stress as well as feeding (Kubrak et al., 2016), nutrient sensing (Miyamoto and Amrein, 2014), and fecundity (Bergland et al., 2012). In male flies, CRZ has been shown to act on its receptor in a small cluster of serotonergic neurons on the accessory glands and promote the transfer of sperm and seminal fluids (Tayler et al., 2012).

Designated as a cardio-acceleratory peptide, CRZ has recently been shown to also increase heart beat rate in *R. prolixus* (Patel et al., 2014). However, a study on *A. gambiae*, utilizing RNA interference, demonstrated that knockdowns of CRZ and its receptor did not significantly alter heart rate (Hillyer et al., 2012). The aforementioned physiological functions of CRZ are mediated by its receptor (CRZR), first discovered in *D. melanogaster*, and like AKHR and GnRHR, it too belongs to the rhodopsin-like family of GPCRs (Cazzamali et al., 2002). Thus far, in addition to *D. melanogaster*, CRZR has been cloned in *M. sexta* (Kim et al., 2004), *B. mori* (Kim et al., 2004), *A. gambiae* (Hillyer et al., 2012), *A. aegypti* (Oryan et al., 2018), *R. prolixus* (Hamoudi et al., 2016), *B. dorsalis* (Hou et al., 2017), and *Musca domestica* (Sha et al., 2012). Functional characterization of CRZRs from *R. prolixus* (Hamoudi et al., 2016), *B. dorsalis* (Hou et al., 2017), *A. aegypti* (Oryan et al., 2018), *D. melanogaster* (Park et al., 2002), and *A. gambiae* (Belmont et al., 2006; Hansen et al., 2010) revealed strict specificity for CRZ.

Table 1. Amino acid sequences of *Aedes aegypti* neuropeptides AKH, ACP, CRZ, and the mammalian GnRH. Similarities between peptides are denoted by similar coloured residues. Blue residues mark conserved, but not identical, residues.

Neuropeptide	Amino acid Sequence
AKH	pQLTF--TPSW--amide
ACP	pQVTF--SRDWNAamide
CRZ	pQ-TFQYSRGWTNamide
GnRH-I	pQ--HWSYGLRPGamide

In *A. gambiae* utilizing qPCR, Hillyer *et al*. showed that CRZ displays bimodal developmental expression profile with greatest transcript abundance observed in 2nd instar larvae and 1 day old adults (Hillyer et al., 2012). Similarly, CRZR expression is bimodal, with greatest transcript levels in the 2nd instar larva and pupal stages. Thus, these findings support the induction of ecdysis, a role previously ascribed to CRZ in the lepidopteran insect *M. sexta* (Kim et al., 2004). Hillyer *et al*. found two transcript variants of CRZ, both of which result in the same biologically active peptide (Hillyer et al., 2012). However, in their qPCR analyses, the CRZ levels measured represent both transcript variants, thus whether these two transcripts are differentially expressed during certain developmental stages remains unknown. Developmental expression analysis of CRZR in *A. aegypti* revealed enrichment in the pupal and adult stages, suggestive of an ecdysis-related role (Oryan et al., 2018). CRZR transcript was also localized to nervous tissue and the primary reproductive organs (i.e. ovaries and testes) of adult *A. aegypti* (Oryan et al., 2018). Recently, Hamoudi and colleagues conducted a qPCR analysis of CRZR in different tissues of 5th instar *R. prolixus*. The spatial expression demonstrated greatest enrichment of the CRZR transcript in the CNS and dorsal vessel of the bug (Hamoudi et al., 2016). Expression in the dorsal vessel is consistent with the previous described cardiostimulatory role. However, a temporal expression profile of CRZR in 5th instar *R. prolixus* demonstrated that transcript levels were not up-regulated around the time of ecdysis. Moreover, dsRNA knockdown of the CRZR receptor prior to ecdysis failed to produce bugs with impaired or irregular ecdysis (Hamoudi et al., 2016). Due to its sequence similarity with AKH, Patel *et al*. investigated CRZs ability to mobilize lipids. Synthetic CRZ (2µg) was injected into adult male *R. prolixus*, however it had no effect on haemolymph lipid levels (Patel et al., 2014). Similarly, subjecting the dorsal vessel of 5th instar *R. prolixus* to increasing doses of AKH *in vitro*, failed to

alter heart rate, illustrating the presence of two structurally-related yet functionally-unrelated signalling systems.

Adipokinetic Hormone/Corazonin-Related Peptide

In 1999, Siegert and colleagues discovered a novel neuropeptide in *L. migarotria*, they termed "peptide A", to describe a novel member of the AKH family of peptides due to its sequence similarity with well-known AKHs (Siegert, 1999). The peptide was classified as an AKH with unknown function. Similarly, in 2006 Belmont and colleagues isolated an orphan GPCR from *A. gambiae* that was similar in sequence to AKH and corazonin (Belmont et al., 2006). Kaufmann and colleagues in 2006 identified a peptide, which they labelled as AKH-II in *A. gambiae* (Kaufmann and Brown, 2006). Also in 2006, Li *et al*. discovered a peptide and its receptor in the flour beetle, *Tribolium castaneum* of similar sequence to previously known AKHs/AKHRs (Li et al., 2008). All of these peptides and receptors at the time of discovery were labelled as inactive AKH peptides, but interestingly, were later identified as the adipokinetic hormone/corazonin-related peptide (ACP) or its receptor (ACPR). Hansen and colleagues in 2010, further examined the *A. gambiae* orphan GPCR identified by Belmont and colleagues (Belmont et al., 2006) and successfully identified the ligand for the receptor (Hansen et al., 2010). The peptide ligand was found to be structurally intermediate between AKH and CRZ, and thus the Hansen group termed this novel peptide the adipokinetic hormone/corazonin-related peptide (ACP). Subsequently, they went on to functionally characterize the ACP receptor in *A. gambiae* and *T. castaneum*, both of which were found to express two transcript variants. Utilizing *in vitro* heterologous bioassays, the Hansen group demonstrated that the AKH, CRZ, and ACP receptors were only activated by their corresponding ligand, and hence are independent signalling systems (Hansen et al., 2010). Recently, Zandawala *et al*. characterized the ACP

signalling system in *R. prolixus*, where they discovered one peptide, and three receptor variants (Zandawala et al., 2015b). In terms of receptor functional deorphanization, a similar result to the Hansen *et al*. study was obtained by the Zandawala group in *R. prolixus* where the AKH, ACP, and CRZ peptides did not cross react as only ACP stimulated ACPR (Zandawala et al., 2015b). However, *in vitro* studies characterizing *B. mori* AKHR have shown that high concentrations of ACP can activate the AKH receptor (Zhu et al., 2009). It was also shown that CRZ cannot activate either ACPR (*R. prolixus*; Zandawala et al. 2015b, *A. gambiae*, *T. castaneum*; Hansen et al., 2010) or AKHR (*A. aegypti*; Oryan et al., 2018 and *A. gambiae*; Hansen et al., 2010*)* and neither AKH nor ACP can activate CRZR (*A. aegypti*; Oryan et al., 2018, *A. gambiae*; Hansen et al., 2010, and *R. prolixus*; Zandwala et al., 2015b), thus a possibility for cross reactivity occurs only between AKH and ACP, but never with CRZ, which indicates that the AKH/AKHR and ACP/ACPR signalling systems are more closely related to one another.

Hauser and Grimmelikhuijzen using comprehensive *in silico* analyses proposed a model (Fig. 2) whereby an ancestral form of GnRH and GnRHR duplicated prior to the emergence of bilaterians, resulting in two branches, one producing an AKH-like peptide/receptor system and the other producing a CRZ-like peptide/receptor signalling system (Hauser and Grimmelikhuijzen, 2014). The ACP system arose through further duplication of the AKH/AHKR hormonal system, which evolved into its own signalling system and over time has been lost in some insects such as *Apis mellifera* and *D. melanogaster*. Also, after the establishment of these three independent signalling systems, the CRZ/CRZR system was lost in several arthropods and in vertebrates completely. Thus, this scenario is a prototypical illustration of the co-evolution of receptors and ligands (Hauser and Grimmelikhuijzen, 2014)

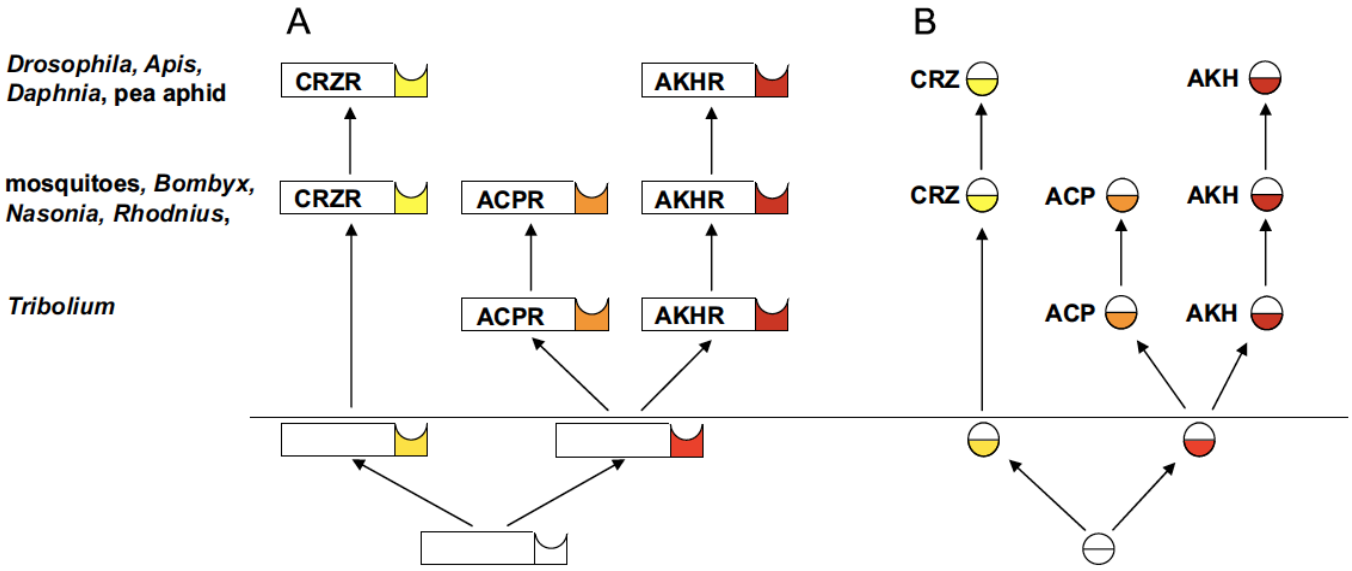

Figure 2. Proposed evolutionary mechanism giving rise to the AKH, ACP, and CRZ signalling systems. The proposition provides a potential scenario for the co-evolution of the neuropeptide receptors (A) and their corresponding ligands (B). Receptors or peptides below the horizontal line is ancestral and thus not identifiable. Duplication of the ancestral receptor/peptide resulted in an ancestral CRZ/CRZR, and AKH/AKHR. Further duplication of AKH/AKHR resulted in the ACP/ACPR signalling system. This figure was adapted from Hansen *et al.* 2010 with the permission of the American Society for Biochemistry and Molecular Biology.

To date, no physiological role has been ascribed to ACP, however qPCR analyses attempt to elucidate a potential function. In *A. gambiae* a recent microarray analysis of gene expression profiles revealed highest expression of ACP (*AnogaAKH-II*) in males in comparison to larvae and females (Marinotti et al., 2005). Expression profiles of *ACP* and *ACPR* show that both peptide and receptor are expressed the greatest in the head of *T. castaneum* (Hansen et al., 2010). During development in *T. castaneum*, both the ACP peptide and its receptor transcript are prominently expressed in 24-48 hr old eggs, and larvae 96 hrs -15 days old. Recently, it has been shown that 5th instar *R. prolixus* express both *ACP* and *APCR* in the corpora cardiaca/corpora allata complex in addition to the rest of the CNS (lacking the CC/CA) (Zandawala et al., 2015b). Additionally, a developmental expression profile in *R. prolixus* showed that expression of both ACP and ACPR transcripts increased significantly after ecdysis (Zandawala et al., 2015b). Since ACP is structurally related to both AKH and CRZ, Patel *et al*. investigated whether any functional similarity existed between the three peptides (Patel et al., 2014). Injections of *Rhopr*ACP into adult male *R. prolixus* failed to affect haemolymph lipid levels, in contrast to AKH, which was found to increase haemolymph lipid levels. In addition, exposing the dorsal vessel of 5th instar *R. prolixus* to increasing doses of ACP *in vitro* failed to alter heartbeat frequency (Patel et al., 2014). Similarly, injections of ACP into *A. gambiae* resulted in no significant difference in haemolymph lipid or trehalose levels (Kaufmann and Brown, 2008). Additionally, *L. migratoria* ACP did not display any metabolic function as it was not able to mobilize lipids or carbohydrates, nor alter heart beat frequency (Siegert, 1999). Thus, in *R. prolixus* and *L. migratoria*, ACP does not seem to act as a cardio-excitatory agent, nor does it function to liberate lipids. Furthermore, RNAi techniques to knockdown the ACP receptor in *T. castaneum* did not result in any differences in physical appearance, egg number, or mortality

between ds*Trica-ACPR* knockdown beetles and control EGFP injected beetles (Hansen et al., 2010). Immunocytochemistry localizing ACP in first instar *T. castaneum* larvae showed 3-4 strongly stained cells in each hemisphere of the brain with axons projecting towards the brain neuropil, suboesophaeal ganglion (SOG), thoracic ganglia, and abdominal ganglia (Hansen et al., 2010). Based on the staining pattern a neurosecretory role for ACP was suggested in *T. castaneum*.

Although, ACP is a structural intermediate of AKH and ACP, there seems to be no functional overlap in *R. prolixus*. Expression profiles of the transcripts encoding ACP along with its receptor in *R. prolixus* and *T. castaneum*, suggest potential neuronal functions for this signalling system, thus leaving the door wide open for future studies to better understand this relatively novel discovered neuropeptide signalling system. Whether similar expression patterns, receptor variants, and exclusivity of the ACP receptor for its ligand is observed in *A. aegypti* remains unknown.

Research objectives and hypotheses

Insects constitute the largest and most diverse animal group, and are of ecological importance as many insects play key roles in the pollination of flowering plants (Gäde et al., 1997). Despite the importance of insects, our knowledge of their neuroendocrinology remains incomplete, as studies focusing on their neuropeptides have lagged far behind those of vertebrates. *A. aegypti* mosquitoes are the principal vectors for the Dengue fever, Yellow fever, Chikungunya, and Zika viruses, all of which have a significant impact on human morbidity and mortality (Barón et al., 2010). A thorough understanding of mosquito biology is required to devise novel methods to reduce and prevent mosquito-borne diseases. My research is focused on the identification and functional characterization of the ACP neuropeptide receptor (ACPR) in *A.*

aegypti. ACPR along with its corresponding peptidergic ligand, ACP, have been shown to be present in many arthropod species; however their function remains unknown (Hansen et al., 2010; Patel et al., 2014; Siegert, 1999; Zandawala et al., 2015b). Despite ACP/ACPR being structurally related to AKH and CRZ and their receptors, studies in *R. prolixus* have demonstrated a lack of functional overlap between these signalling systems (Patel et al., 2014). Whether the functional exclusivity extends to *A. aegypti* ACP is one of the goals I will address with my proposed research. Thus, to functionally characterize ACPR in the mosquito, firstly, I will identify the expressed transcripts encoding ACPR in adult *A. aegypti*. Identifying functional forms of the receptors are important in determining the function of this signalling system. Previous investigations have identified an ACP neuropeptide within *A. aegypti*, albeit it was mischaracterized as a non-functional AKH family member (Kaufmann et al., 2009), which indicates the presence of an ACP receptor within *A. aegypti* is highly likely considering the above mentioned reports of ligand receptor co-evolution (Hauser and Grimmelikhuijzen, 2014). **Therefore, I hypothesize that an ACP receptor is present (and its transcript is expressed) in the adult mosquito, *A. aegypti*.**

Secondly, I will examine functional activation of *A. aegypti* ACPR following stimulation with ACP and structurally-related peptides including AKH and corazonin. ACP receptors in *T. castaneum*, *A. gambiae*, and *R. prolixus* have demonstrated specificity to their respective cognate ligands (Hansen et al., 2010; Zandawala et al., 2015b). **Thus, I hypothesize that the *A. aegypti* ACPR will similarly demonstrate strict specificity for *Aedae*ACP**.

Thirdly, the spatial and temporal transcript expression patterns, as well as localization of ACPR in *A. aegypti* will be examined allowing for the discovery of potential physiological roles of this neuropeptide signalling pathway. Both ACP and/or ACPR transcripts have been shown to

be expressed throughout the nervous system of *R. prolixus* (Zandawala et al., 2015b), *T. castaneum* (Hansen et al., 2010), *A. gambiae* (Kaufmann and Brown, 2006) and *A. aegypti* (Kaufmann et al., 2009); **therefore, I hypothesize that *AedaeACP* and *AedaeACPR* transcripts will be enriched in the nervous system of *A. aegypti.***

Additionally, to further characterize ACP/ACPR, fluorescent *in situ* hybridization (FISH) will be conducted to localize both the peptide as well as the receptor transcript, while immunohistochemistry will be used to determine the distribution of the ACP peptide. **I hypothesize that FISH and immunohistochemistry would localize the ACP peptide and transcript to the central nervous system, more specifically within neurosecretory cells of the brain and thoracic ganglia of *A. aegypti*.** Previous literature has localized *Aedae*ACP (Kaufmann et al., 2009) and *Anoga*ACP (Kaufmann and Brown, 2006) to the corpora cardiaca, as is expected. However, immunoreactivity was also observed in the brain and thoracic ganglia, which was attributed to "AKH-II". We now know that AKH-II is ACP, and thus to confirm this localization pattern, I will be utilizing FISH. This hypothesis is further supported with previous reports of ACP immunoreactivity throughout the CNS of *T. castaneum* (Hansen et al., 2010), and *R. prolixus* (Patel et al., 2014), with no immunoreactivity observed outside the nervous system. Moreover, localization of the *ACP* and *APCR* transcript has not been examined previously. As mentioned above, ACPR transcript expression has been observed in the CNS of *R. prolixus* (Zandawala et al., 2015b) and *T. castaneum* (Hansen et al., 2010), **thus it was hypothesized that FISH will localize *ACPR* mRNA transcript to the central nervous system of *A. aegypti***.

Lastly, to uncover a potential function for this neuropeptide signalling pathway, knockdown of the transcripts encoding the ACP peptide and its receptor utilizing RNAi may result in phenotypic differences in adult *A. aegypti*. Knockdown of both *Aedae*ACP peptide and

receptor transcript was mediated via dsRNA feeding of larval *A. aegypti*. In the beetle *T. castaneum*, knockdown of the ACP receptor did not result in any changes to physical appearance, egg number, or mortality between knockdown and control beetles (Hansen et al., 2010). **Therefore, it was hypothesized that RNAi will knockdown expression of *A. aegypti ACP* and *ACPR*.**

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Chapter 2:

Molecular identification, transcript expression and functional deorphanization of the adipokinetic hormone/corazonin-related peptide receptor in the disease vector, *Aedes aegypti*

Prefix

The recently discovered adipokinetic hormone/corazonin-related peptide (ACP) is an insect neuropeptide structurally intermediate between corazonin (CRZ) and adipokinetic (AKH) hormones, which all demonstrate homology to the vertebrate gonadotropin-releasing hormone (GnRH). To date, the function of the ACP signaling system remains unclear. In the present study, we molecularly identified the complete open reading frame encoding the *Aedes aegypti* ACP receptor (ACPR), which spans nine exons and undergoes alternative splicing giving rise to three transcript variants. Only a single variant, *Aedae*ACPR-I, yielding a deduced 577-residue protein, contains all seven transmembrane domains characteristic of rhodopsin-like G protein-coupled receptors. Functional deorphanization of *Aedae*ACPR-I using a heterologous cell culture-based system revealed highly-selective and dose-dependent receptor activation by *Aedae*ACP (EC_{50} of 10.25nM). Analysis of the *Aedae*ACPR-I and *Aedae*ACP transcript levels in post-embryonic developmental stages using quantitative RT-PCR identified enrichment of both transcripts after adult eclosion. Tissue-specific expression profiling in adult mosquitoes reveals enrichment of the *Aedae*ACPR-I receptor transcript in the central nervous system, including significant abundance within the abdominal ganglia. Further, the *Aedae*ACP transcript is prominently detected within the brain and thoracic ganglia. Collectively, these results indicate a neuromodulator or neurotransmitter role for ACP and suggest this neuropeptide may function in regulation of post-ecdysis activities.

Introduction

Neuropeptides are structurally and functionally the most diverse class of signaling molecules that function in intercellular communication in multicellular organisms (Klavdieva, 1995). In insects, neuropeptides play a fundamental role in the regulation of various physiological processes including development, reproduction, osmoregulation, as well as behavior and feeding (Nässel, 1993). One of the first neuropeptides to be isolated and purified from insects was the adipokinetic hormone (AKH), which is produced by neurosecretory cells of the corpora cardiaca (CC), and are one of the most extensively studied family of neuropeptides (Diederen et al., 2002). Insect AKH neuropeptides have been functionally well characterized to stimulate the release of energy-rich compounds including diacylglycerols, trehalose, and in some cases proline, into the haemolymph to fuel the activity of the insect (Lorenz and Gäde, 2009). In addition to these catabolic actions, AKH peptides have been shown to stimulate heart beat and inhibit the synthesis of haemolymph and tissue proteins (Gäde and Marco, 2006). The AKH receptors (AKHR), first isolated from *Drosophila melanogaster* and *Bombyx mori*, are G protein-coupled receptors (GPCR) (Park et al., 2002; Staubli et al., 2002). Both AKH and AKHR demonstrate homology with the vertebrate gonadotropin releasing hormone (GnRH) and its receptor (GnRHR), respectively (Hauser et al., 1998; Staubli et al., 2002). Another insect neuropeptide family that is structurally similar to the vertebrate GnRH signaling system includes the corazonin peptides. Corazonin (CRZ) is an insect undeca-neuropeptide first discovered in *Periplaneta americana* due to its significant cardioexcitatory activity on the isolated cockroach heart (Veenstra, 1989). Since then, CRZ has been shown to also increase heart rate in the kissing bug, *Rhodnius prolixus* (Patel et al., 2014). Despite the CRZ sequence remaining largely conserved across insect species (primarily pQTFQYSRGWTNamide), to date no universal function has been described for this neuropeptide (Predel et al., 2007). Multiple physiological

roles have been described for CRZ, including the induction of melanization in swarming populations of *Locusta migratoria* and *Schistocerca gregaria*, initiation of ecdysis in *Manduca sexta*, the reduction in silk spinning rates in *B. mori*, and in social insects, has been proposed as a central regulator of behaviour and caste identity (Gospocic et al., 2017; Kim et al., 2004; Tanaka et al., 2002; Tawfik et al., 1999).

Over the last decade, a third signaling system evolutionarily- and structurally-related to the AKHRs and CRZRs and their neuropeptides was identified and named adipokinetic hormone/corazonin-related peptide (ACP) (Hansen et al., 2010). ACP and its receptor (ACPR), like the AKH/AKHR and CRZ/CRZR signaling systems, also demonstrate homology to the vertebrate GnRH/GnRHR system. Upon analysis of this structural intermediate it became evident that ACP and its receptor ACPR were in fact already described in a number of insects, however, were at the time characterized as AKHs and AKHRs (Belmont et al., 2006; Kaufmann and Brown, 2006; Kaufmann et al., 2009; Li et al., 2008; Shi et al., 2011; Siegert, 1999; Zhu et al., 2009). Unlike AKH, but similar to CRZ, the ACP amino acid sequence at the N-terminus (pQVTFSRDW) demonstrates conservation across most arthropods (Hansen et al., 2010). Characterization of the ACP signaling system in *R. prolixus*, *Tribolium castaneum*, and *Anopheles gambiae* revealed that the AKH, CRZ, and ACP receptors were only activated by their corresponding ligand, and thus are suggested to be independent signaling systems (Hansen et al., 2010; Zandawala et al., 2015). However, *in vitro* studies characterizing *B. mori* AKHR and ACPR have shown that high concentrations of ACP can activate the AKH receptor, and *vice versa* (Shi et al., 2011; Zhu et al., 2009). In both *R. prolixus* and *A. gambiae*, it was also shown that CRZ cannot activate either ACPR or AKHR and furthermore, neither AKH nor ACP can activate CRZR, which indicates that the AKH/AKHR and ACP/ACPR signaling systems are

more closely related to one another (Hansen et al., 2010; Zandawala et al., 2015). To date, no functions have been assigned to the ACP signaling system, but expression profiles of ACP and ACPR in *R. prolixus*, and *T. castaneum*, show that both peptide and receptor are primarily expressed in nervous system and, to a lesser extent, in the reproductive tissue of *R. prolixus* (Hansen et al., 2010; Zandawala et al., 2015). Furthermore, ACP transcript was detected in the head and thorax of adult *Aedes aegypti* with a similar transcript distribution observed in 4th instar larvae, pupae, and adult *A. gambiae* (Kaufmann and Brown, 2006; Kaufmann et al., 2009). Developmental expression profiles have revealed high expression of both ACP and its receptor before and after hatching of eggs in *T. castaneum* and after ecdysis in *R. prolixus*, and thus a role in early larval development and post-ecdysis, respectively, has been proposed (Hansen et al., 2010; Zandawala et al., 2015). Additionally, assays testing the potential for cardio-excitatory or lipid mobilization roles of ACP in *R. prolixus* yielded negative results, which indicates that ACP, AKH, and CRZ are independent signaling systems with distinct functions (Patel et al., 2014). Further investigations are clearly necessary in order to assign a physiological role for the ACP/ACPR neuropeptide system in insects.

Aedes aegypti mosquitoes are principal vectors for a variety of pathogens including dengue fever, yellow fever, chikungunya, and Zika viruses, all of which have a significant impact on human morbidity and mortality (Barón et al., 2010). A thorough understanding of mosquito biology is required to devise novel methods to reduce and prevent mosquito-borne diseases. In an attempt to advance our understanding of the ACP signaling system in *A. aegypti*, we have identified and functionally deorphanized the *A. aegypti* ACPR (*Aedae*ACPR). Furthermore, to begin identification of prospective target tissues and physiological roles, we have determined the post-embryonic developmental expression profile and the spatial expression

pattern in the adult mosquito of the transcripts encoding the ACP precursor peptide as well as a functional ACP receptor in *A. aegypti*.

Materials & Methods

Animals

Aedes aegypti (Liverpool strain) eggs were hatched in plastic containers half-filled with deionized water at an initial density of approximately 100 larvae/litre of water. Larvae were fed a 2% brewers yeast, 2% liver powder solution daily, and adults were provided with a 10% sucrose solution through a microcentrifuge tube fitted with a cotton ball wick allowing feeding *ad libitum*. Larvae and pupae were maintained in an incubator at 26°C on a 12:12 hour light: dark cycle. Colony upkeep involved adult females being fed sheep's blood in Alsever's solution weekly (Cedarlane Laboratories Ltd., Burlington, ON) using an artificial feeding system described previously (Rocco et al., 2017). All experiments on adults were performed on either one or four-day old male and female mosquitoes that were sucrose-fed only and had been isolated during the pupal stage and transferred *en masse* into small glass microchambers.

Isolation and cloning of cDNA encoding A. aegypti ACPR

Gene specific forward and reverse primers were designed using Primer 3 in Geneious Software (Biomatters Ltd, Auckland, New Zealand) based on a predicted incomplete *A. aegypti* ACPR sequence (XM_001653870) described previously (Hansen et al., 2010) to amplify a 975bp partial fragment using Q5 High Fidelity DNA Polymerase (New England Biolabs, Whitby, On) and whole adult female *A. aegypti* cDNA as template. The PCR product was purified, A-tailed, cloned into pGEM-T vector (Promega, Madison, WI, USA) and nucleotide sequence was confirmed by Sanger sequencing (Center for Applied Genomics, Hospital for Sick Children, Toronto, ON). After successful validation of the cloned partial sequence, primers were designed (as mentioned above) to perform 5' and 3' rapid amplification of cDNA ends (RACE)-PCR utilizing the Clontech SMARTer 5'/3' RACE Kit (Takara BIO USA Inc, CA, USA). To facilitate cloning of amplicons, the linker sequence GATTACGCCAAGCTT, which overlaps

with the pRACE vector provided in the kit, was added to the 5' ends of the gene specific primers (Table 1). First-strand cDNA synthesis was prepared using 1μg total RNA from adult female head using the 3' CDS primer (provided in the kit) and a gene-specific reverse primer to generate template cDNA for 3' and 5' RACE, respectively. Initial attempts at 5'RACE using the 5'CDS primer (provided in the RACE kit) for first-strand cDNA synthesis and subsequent PCR with the SeqAmp DNA Polymerase (Takara BIO USA Inc, CA, USA) was not successful for this target. Thus, the protocol was modified to generate first-strand cDNA using a gene-specific reverse primer (*Aedae*ACPR-R1, Table 1) and subsequent nested RACE-PCR reactions utilized Q5 High Fidelity DNA Polymerase *in lieu* of SeqAmp DNA Polymerase. Nested PCR reactions utilized gene specific forward (3' RACE) and reverse (5' RACE) primers and a universal primer mix (UPM) to amplify the complete cDNA encoding *A. aegypti* ACPR. Optimal PCR cycling parameters for subsequent amplification of ACPR were determined empirically. Specifically, for 3' RACE this included an initial denaturation at 94°C for 1 min, followed by 40 cycles of 30 s at 94°C, 30s at 68°C, and 3 min at 72°C to amplify PCR products using SeqAmp DNA Polymerase. For 5' RACE, the Q5 High Fidelity DNA Polymerase was utilized with the following cycling parameters, 30 s at 98°C, followed by 30 cycles of 5 s at 98°C, 15 s at 65-68°C, 1 min 10 s at 72°C, with a final extension step of 2 min at 72°C. Following two rounds of PCR using nested gene-specific primers, amplicons were gel extracted and cloned into the linearized pRACE vector and miniprep samples were then sent for sequencing. Finally, primers were designed at the 5' and 3' ends of the complete cDNA sequence (including UTRs) and at the start and stop codons of the sequence (region including only the open reading frame, excluding UTRs), and were used for subsequent PCR amplification of the receptor with Q5 High Fidelity DNA polymerase to confirm base pair accuracy.

Gene Structure and Phylogenetic Analyses

Mapping of exon-intron boundaries of *A. aegypti ACPR* gene was determined using the cloned complete cDNA sequence as a query against the *A. aegypti* genome scaffolds database available locally on a lab computer running Geneious Pro Bioinformatics Software (Biomatters Ltd, Auckland, New Zealand). Positions of introns and exons were further confirmed using the BDGP splice site prediction server using the standardized data set of *D. melanogaster* genes (Reese et al., 1997). Membrane topology of ACPR-I, II, and III were predicted using the Constrained Consensus TOPology prediction server (CCTOP) (Dobson et al., 2015). The deduced *Aedae*ACPR-I, II and III protein sequences were aligned to the human gonadotropin-releasing hormone receptor 1 along with ACP, AKH, and CRZR receptors from other species (see Appendix; Table S1) using ClustalW in MEGA 6.06 (Tamura et al., 2013). Relationships between the various receptor sequences were determined through neighbour-joining (Saitou and Nei, 1987) and maximum-likelihood phylogenetic analysis methods (Jones et al., 1992). Bootstrap values are based on 1000 replicates.

Preparation of mammalian expression constructs

Amplicons encoding just the open reading frame (start ATG to stop codon) were used as template for re-amplification using a modified forward primer possessing the consensus Kozak translation initiation sequence (Kozak, 1984; Kozak, 1986) at the 5' end of the start codon. The resulting product was cloned into pGEM-T Easy vector and then subcloned into the mammalian expression vector, pcDNA 3.1^+ (Life Technologies, Burlington, ON). Construct directionality was confirmed by Sanger sequencing and plasmid DNA was purified from overnight bacterial cultures using a PureLink MidiPrep Kit (Invitrogen, Burlington, ON) and subsequently used for transfection of mammalian cells for the receptor functional assay. Analysis of multiple independent sequences obtained from RACE-PCR revealed a number of single nucleotide

polymorphisms (SNPs) that localized to various sites along the full cDNA sequence. Upon further analysis, it was determined that only a single SNP at nucleotide position 1924 within the open reading frame resulted in an amino acid change that differed from that predicted in the *A. aegypti* genome. To determine whether this difference in the resulting residue, in comparison to the *A. aegypti* genome database, confers any difference to the functional activity of the receptor, site directed mutagenesis was performed. Specifically, 5' phosphorylated primers were designed (Table 1) with the forward primer possessing an adenine (position 1924) consistent with the *A. aegypti* genome sequence whereas our consensus sequence contained a thymine in this nucleotide position. Using these modified primers, asymmetric PCR was performed using a pGEM-T Easy plasmid construct as template to replace the Ile_{472} in the cloned receptor with an Asn_{472} matching the *A. aegypti* genome. Mutation of the coding sequence was verified by sequencing and sub-cloned into the mammalian expression plasmid, pcDNA3.1$^+$ (as described above).

Cell culture, transfections, and bioluminescence assay

Functional activation of *Aedae*ACPR-I was assayed using a previously established cell culture system involving a recombinant Chinese hamster ovary (CHO)-K1 cell line stably expressing aequorin (Paluzzi et al., 2012). Cells were grown in Dulbecco's modified eagles medium: nutrient F12 (DMEM:F12; 1:1) media containing 10% heat-inactivated fetal bovine serum (FBS; Wisent, St. Bruno, QC), 200μg/mL Geneticin, 1x antimycotic-antibiotic to approximately 90% confluency, and were transiently transfected with pcDNA3.1$^+$ expression vector possessing *Aedae*ACPR-I using Lipofectamine LTX and Plus Reagent transfection system (Invitrogen, Burlington, ON) following a 3:1:1 transfection reagent (μL): Plus reagent (μL): plasmid DNA (μg) ratio. At 48 hours post-transfection, cells were detached from the culture flasks using Dulbecco's phosphate buffered saline (DPBS) containing 5mM EDTA and

resuspended in BSA medium (DMEM-F12 media containing 0.1% bovine serum albumin, 1X antimycotic-antibiotic) to a concentration of 10^6-10^7 cells/mL. Coelenterazine h (Promega, Madison, WI, USA) was added to the cells to a final concentration of 5μM, and incubated for 3 hours in the dark at room temperature on a stirrer set at 200 rpm. The cell suspension was then diluted 10-fold and incubated at room temperature for an additional hour. Serial dilutions of peptide ligands (Table 1) were prepared in BSA medium (10^{-5} to 10^{-12} M), and loaded in quadruplicates into 96-well luminescence plates (Greiner Bio-One, Germany). All peptides were commercially synthesized (Genscript, Piscataway, NJ) at a purity >90% and were prepared in dimethyl sulfoxide at a stock concentration of 1mM. Cells were loaded into each well with an automatic injector unit and luminescence was measured for 20 seconds using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). BSA medium alone was utilized as a negative control and 5 x 10^{-5} M ATP was used as a positive control, which acts on endogenously expressed purinoceptors (Iredale and Hill, 1993; Michel et al., 1998). EC_{50} values were calculated in GraphPad Prism 7.02 (GraphPad Software, San Diego, USA) from dose-dependent curves from four independent transfections.

Tissue dissections, RNA extraction, and cDNA synthesis

Lightly CO_2-immobilized four-day old adult male (n=30) and female (n=20) *A. aegypti* were submerged in Dulbecco's phosphate buffered saline (DPBS; Wisent Corporation, St. Bruno, QC), and the following body segments and/or tissues were dissected and isolated: head, midgut, Malpighian tubules, hindgut, ovaries, testes, accessory reproductive tissues, and carcass (remaining fat body, musculature, and cuticle). Tissue/organ samples were lysed in RNA lysis buffer containing 1% 2-mercaptoethanol. Whole adult RNA was obtained by submerging several males and females in RNA lysis buffer containing 1% 2-mercaptoethanol and using a sterile plastic pestle to disrupt the tissue. To measure the developmental expression profile for

*Aedae*ACPR, first to fourth instar larvae, pupae, as well as one- and four-day old adult mosquitoes were collected and submerged in RNA lysis buffer and flash frozen in liquid nitrogen. Total RNA was isolated from whole animal and individual adult tissues samples mentioned above using the PureLink™ RNA mini kit following manufacturer protocol with an on-column DNase treatment to remove genomic DNA (Invitrogen, Burlington, ON). Purified total RNA samples were quantified with a Take3 micro-volume plate and measured on a Synergy Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). To assess *ACP* and *ACPR* transcript levels, cDNA was synthesized from 20ng total RNA using the iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Mississauga, ON) following manufacturers protocol, including a ten-fold dilution of cDNA following synthesis.

Quantitative PCR

ACP and ACPR transcript abundance was quantified on a StepOnePlus™ Real Time PCR system (Applied Biosystems, Carlsband, CA) using PowerUP™ SYBR® Green Master Mix (Applied Biosystems, Carlsband, CA). Cycling conditions were as follows: 1) UDG activation 50°C for 2 min, 2) 95°C for 2 min, and 3) 40 cycles of i) 95°C for 15 seconds and ii) 60°C for 1 minute. Gene-specific primers designed over different exons were used to amplify *ACPR*, with the forward primer designed over exon 5 (nucleotides 1458-1476) to ensure specificity for ACPR-I, and the reverse primer over exon 6 (nucleotides 1585-1603). Gene specific primers amplifying *Aedae*ACP were designed over multiple exons (Table 1; forward: nucleotides 89-112, reverse: nucleotides 403-421) based on a previously published mRNA sequence (Genbank Accession Number: FN391984) (Kaufmann et al., 2009). Relative expression levels were determined using the $\Delta\Delta C_T$ method and were normalized to the geometric mean of *rp49*, *rpL8*, and *rps18* reference genes, which were previously characterized and determined as optimal

endogenous controls (Paluzzi et al., 2014). The *AedaeACPR* spatial expression profile was determined using 7-9 biological replicates, all of which included three technical replicates per reaction and a no-template negative control. The *AedaeACPR* developmental expression is an average of 3-5 biological replicates that each included duplicate technical replicates for each target gene and a no-template negative control. The *AedaeACP* spatial expression profile consisted of 3-4 biological replicates and the developmental expression is an average of 3-5 biological replicates. Specificity of primers for target mRNA were assessed by conducting no reverse-transcriptase controls, analysis of dissociation curves, and Sanger sequencing of amplicons. Data were analyzed using a one-way ANOVA with Dunnett's multiple comparisons test where $p < 0.05$ was considered significant.

Table 1. Primer information for oligonucleotides used for initial amplification of partial cDNA, 5' and 3' RACE and RT-qPCR analysis to determine temporal and spatial transcript expression.

Oligo Name	Sequence (5'-> 3')	Function
AedaeACPR-F1[a]	GGTCACACCGAAACGACAGTGG	Amplification and cloning of partial AedaeACPR
AedaeACPR-R1[a]	TGGACCTCCTCTGGGCTGC	Amplification and cloning of partial AedaeACPR and 1st strand cDNA synthesis for 5' RACE
AedaeACPR-R2	GACCGATTGGAGATTTCACAC	5'RACE
AedaeACPR-R3	CAGAACAGGTTGTAAGCCGTCT	5'RACE
AedaeACPR-R4	TCCACCGAGCATTATTTTGC	5'RACE
AedaeACPR-R5	TCCAGTGGGATCATGATGAAG	5'RACE
AedaeACPR-LR5	GATTACGCCAAGCTTCCTCCAGTGGGATCATGATGAAG	5'RACE and cloning of 5' RACE amplicon
AedaeACPR-LF1	GATTACGCCAAGCTTGGTCACACCGAAACGACAGTGG	3' RACE
AedaeACPR-LF2	GATTACGCCAAGCTTGTTGGATCGGTGCTTTGCTGTGAT	3' RACE
AedaeACPR-LF3	GATTACGCCAAGCTT GGCTTACAACCTGTTCTGCGTGG	3' RACE and cloning of 3' RACE amplicon
AedaeACPR-ORFkozak-F	GCCACCATGTATCTTTTCGGCAGGATTGCG	ORF cloning for functional receptor assay
AedaeACPR-ORF-R	TGATTTATCATCGCCAGCCACC	ORF cloning for functional receptor assay
AedaeACPR-N4721-F	AGGAATGGCAGCACCG	Generation of ACPR-I-N472I variant
AedaeACPR-N4721-R	GGCGAAGATCCCGTTG	Generation of ACPR-I-N472I variant
AedaeACPR-qPCR-F	GGGATGCGACTTCGTTGTA	qPCR amplification of AedaeACPR-I
AedaeACPR-qPCR-R	TCGCGGTCAAACATGTACC	qPCR amplification of AedaeACPR-I
AedaeACP-qPCR-F[b]	ATGTGTTCTCTAAGGCGAAATAGC	qPCR amplification of AedaeACP
AedaeACP-qPCR-R[b]	TTACAGGTGCCCATTCGAA	qPCR amplification of AedaeACP

[a] Primers based on partial ACPR mRNA sequence (Accession number: XM_001653870.2; Hansen *et al*. 2010) identified through homology based *in silico* analysis of the *A. aegypti* genome.
[b] Primers based on ACP mRNA sequence (Accession number: FN391984.1; Kaufmann *et al*. 2009)

Results

AedaeACPR receptor variants

Herein, the complete cDNA sequence encoding the *A. aegypti* adipokinetic hormone/corazonin-related peptide (ACP) receptor (Fig. 1a) was identified. Following initial cloning and sequence analysis, three transcript variants were identified, *Aedae*ACPR-I, ACPR-II, and ACPR-III (Fig. 1b). *Aedae*ACPR-I is 2596 bp (GenBank Accession number: MF461644), which includes a 1734 bp open reading frame (ORF) encoding a 577 residue receptor (Fig. 1a). The cloned 5' untranslated region (UTR) is 509 bp in length and the 3'UTR is 346 bp and contains a predicted polyadenylation sequence (nucleotide position 2547-2552). *Aedae*ACPRs II and III are transcript variants of 2442 bp (GenBank: MF461645) and 2240 bp (GenBank: MF461646) in length, which yield deduced proteins comprised of 328 and 243 amino acids, respectively. Only *Aedae*ACPR-I has the seven hydrophobic transmembrane (TM) domains characteristic of GPCRs, whereas *Aedae*ACPR-II has five TM domains and *Aedae*ACPR-III has only three TM domains (Fig. 2). A number of single nucleotide polymorphisms (SNP) were observed across the entire cDNA sequence. Of those occurring within the ORF only one SNP (nucleotide 1924, found within the seventh exon, which corresponds to the C-terminus of the receptor) results in a different amino acid at residue Ile_{472}, compared to the Asn_{472} in the *A. aegypti* genome.

The gene structure was modeled by comparing the cloned cDNA sequence to the *A. aegypti* genome database using Geneious Bioinformatics software (Biomatters Ltd., Auckland, New Zealand). Analysis of the *A. aegypti* ACPR-I receptor shows that it maps over nine exons located on supercontig 1.417 spanning a total genomic region of 266.881 kb. The exons are 810 bp, 191 bp, 228 bp, 202 bp, 154 bp, 197 bp, 177 bp, 83 bp, and 549 bp long. Alternative splicing of the *A. aegypti* ACPR gene results in the absence of either exon 5 only or absence of both

exons 4 and 5, which yields *Aedae*ACPR-II and *Aedae*ACPR-III, respectively, both resulting in-frame translation shifts and premature stop codons, and consequently, truncated ORFs (Fig. 1b).

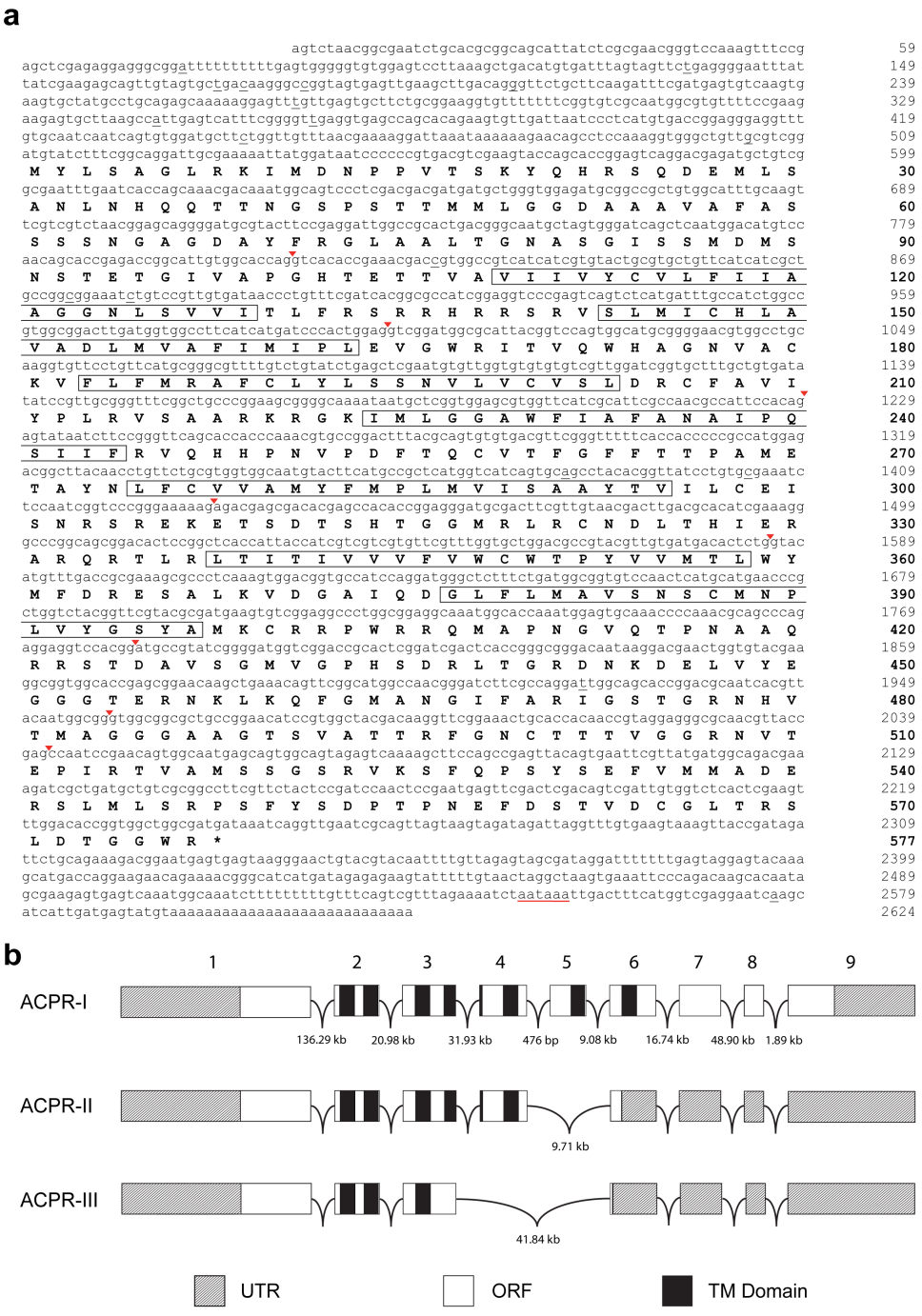

Figure 1. ***Aedes aegypti*** **adipokinetic hormone/corazonin-related peptide receptor variant I (ACPR-I) cDNA, amino acid sequence, and predicted gene structure. (a)** Lower case letters denote cDNA, and uppercase letters represent amino acid residues with positions denoted by the numbers on the right of the sequences with bolded numbers indicating amino acid positions. Exon-exon boundaries within the cDNA are denoted by inverted red arrowheads. Underlined nucleotides indicate single nucleotide polymorphisms that differed from the *A. aegypti* genome. A putative polyadenylation signal is underlined in red in the 3'UTR. The predicted hydrophobic alpha-helices that form the transmembrane domains are outlined by black rectangular boxes. **(b)** The splicing pattern of the *Aedae*ACPR gene based on BLAST analyses of cloned cDNA and splice site prediction analysis. Alternative splicing gives rise to three receptor mRNA variants where *ACPR-I* possesses all exons, *ACPR-II* lacks exon 5, and *ACPR-III* lacks both exons 4 and 5. Boxes representing exons are drawn to scale whereas intervening introns are not drawn to scale.

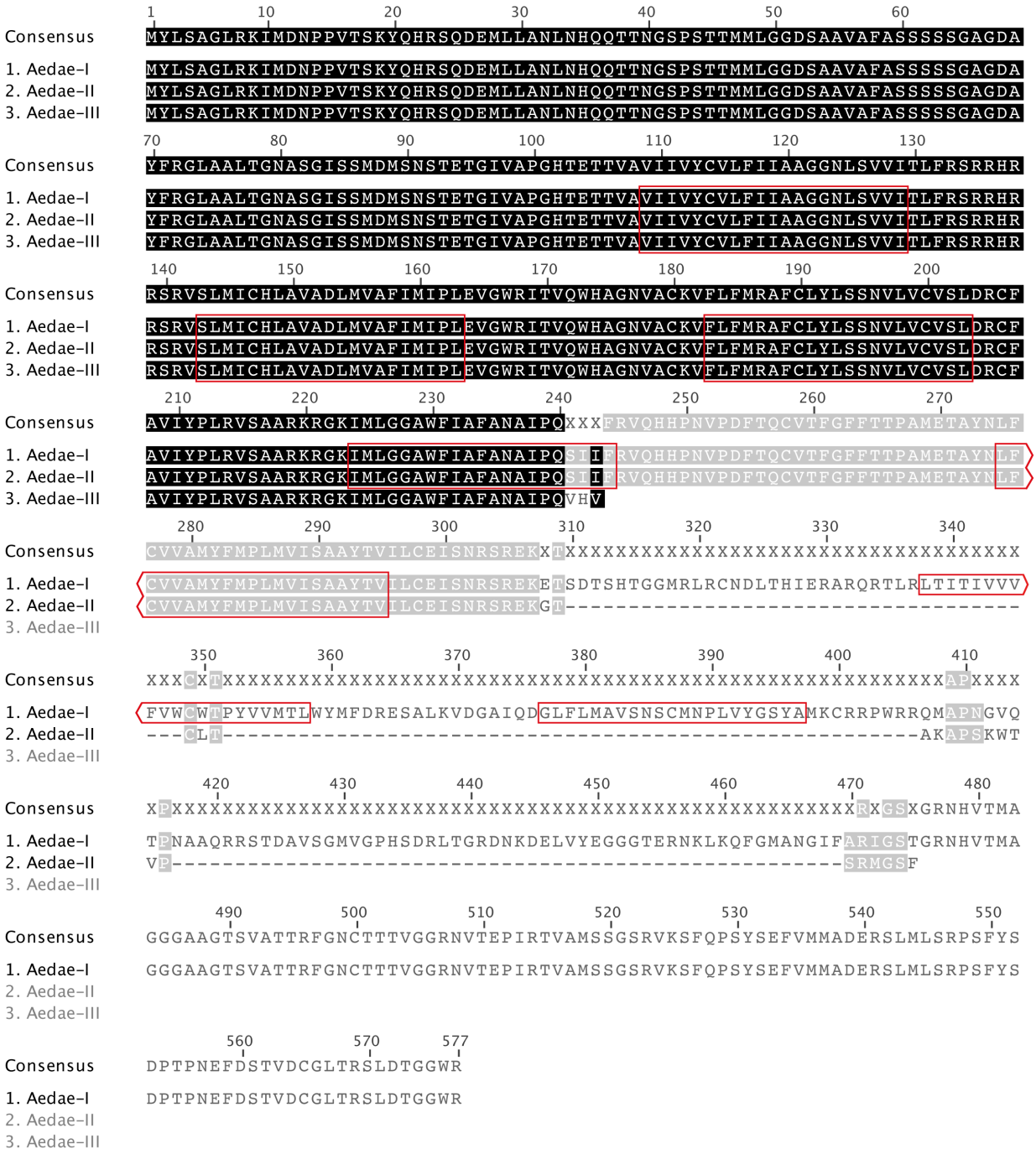

Figure 2. Sequence alignment *Aedes aegypti* ACP receptor variants I, II, and III. Aligned amino acid sequences of ACPRs from *A. aegypti*, transcript variant I (GenBank: MF461644), transcript variant II (GenBank: MF461645), and transcript variant III (GenBank: MF461646). Residues outlined in red indicate predicted transmembrane domains based on the *A. aegypti* sequences. Highlighting of residues indicates % identity with black denoting 100% sequence identity, dark grey denotes 80-100% identity, and light grey represents amino acid positions with 60-80% identity.

Sequence and Phylogenetic Analysis

Alignment of the *A. aegypti* ACPR-I deduced protein sequence, which is the only receptor isoform we identified that contains all seven predicted transmembrane domains, with selected receptors from *A. gambiae*, *T. castaneum*, *R. prolixus*, and *B. mori*, reveals conservation of the ACP receptor across insect species (Fig. 3). Specifically, *Aedae*ACPR-I shares 59.4% sequence identity with the *A. gambiae* ACP receptor, 42.4% identity with the *R. prolixus* ACPR-C, 41.5% identity with the *T. castaneum* ACPR and 33.8% identity with the *B. mori* ACP receptor. Overall, there is a high degree of conservation over the seven predicted transmembrane (TM) domains, particularly over TM regions one, two, three, five and seven. Strong sequence identity is also observed in the first and second intracellular loops, as well as the first extracellular loops. All of the receptor sequences, except for *Bommo*ACPR, which harbors an Asp in place of Asn, possess the conserved NPXXY motif in the seventh TM domain characteristic of rhodopsin-like (family A) GPCRs (Mirzadegan et al., 2003; Rosenbaum et al., 2014). Another conserved motif found in rhodopsin-like GPCRs is the E/DRY motif adjacent to the second intracellular loop (Mirzadegan et al., 2003). In particular, the ACP receptors possess a DRF motif in the silkworm *B. mori* and DRC motifs are found in the mosquitoes *A. gambiae* and *A. aegypti*, in place of the characteristic DRY motif found in hemipteran *R. prolixus* and coloepteran *T. castaneum*, which have more conserved features of rhodopsin-like GPCRs (Mirzadegan et al., 2003; Palczewski, 2006).

Phylogenetic analysis using the neighbor-joining and maximum-likelihood methods (not shown) yielded trees with highly similar topologies (Fig. 4). All the ACP receptors analyzed are positioned within a single clade that is sister group to the clade comprised of the AKH receptors. Together, the AKH and ACP receptor clades form a monophyletic group which is a sister group to the clade comprised of CRZ receptors. The *Aedae*ACPR-I identified herein clusters closely

with the other insect ACPRs that have previously been identified and functionally characterized. Predicted ACPRs from other mosquito species including, *Anopheles darlingi*, *Culex pipiens*, and the Asian tiger mosquito *Aedes albopictus* also cluster close to the *A. gambiae* and *A. aegypti* ACP receptors. In the tick, *Ixodes scapularis*, five putative ACPRs are predicted based on available EST and genome data (Gulia-Nuss et al., 2016), and these are found to be basal relative to the insect AKHRs and ACPRs.

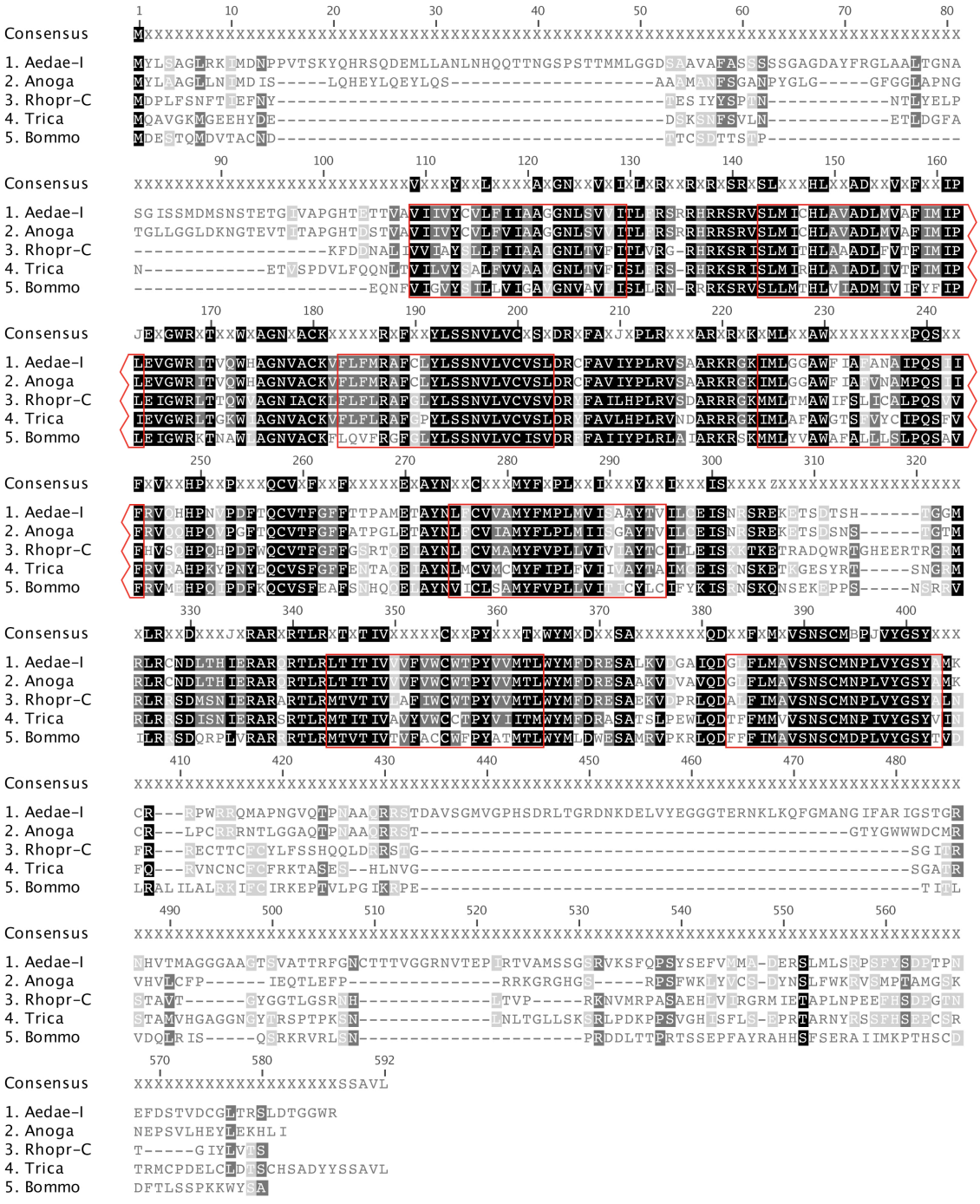

Figure 3. Sequence alignment of select insect adipokinetic hormone/corazonin-related peptide receptors (ACPR). Aligned amino acid sequences of ACPRs from *A. aegypti* (Variant I, GenBank: MF461644) *R. prolixus* (GenBank: AKO62858), *B. mori* (GenBank: NP_001127726), *T. castaneum* (GenBank: ABX52400), and *A. gambiae* (GenBank: ABX52399). Residues outlined in red indicate predicted transmembrane domains based on the *A. aegypti* ACPR sequence. Highlighting of residues indicates % identity with black denoting 100% sequence identity, dark grey denotes 80-100% identity, and light grey represents amino acid positions with 60-80% sequence identity.

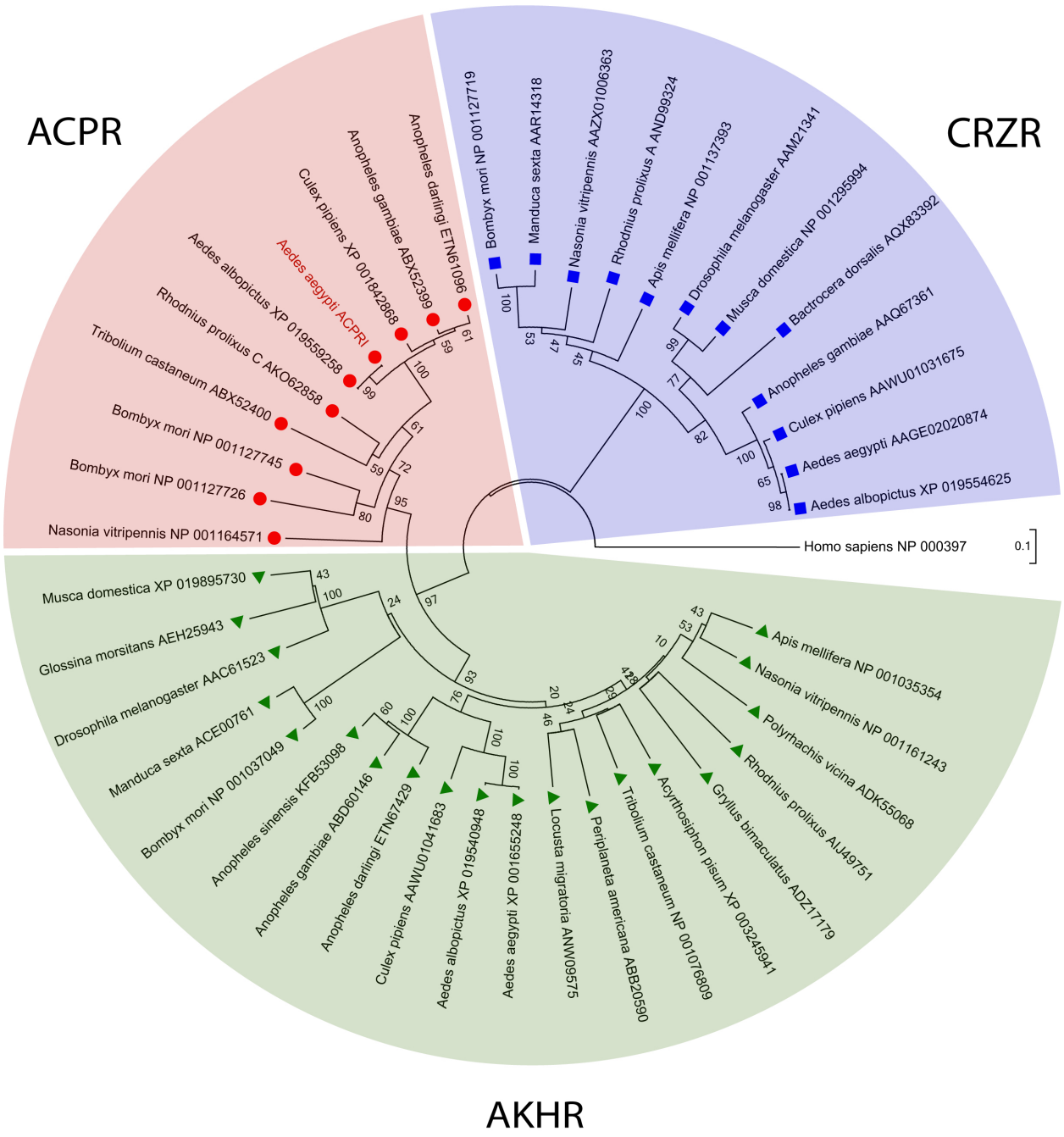

Figure 4. Phylogenetic relationship of adipokinetic hormone receptors (AKHR, △), corazonin receptors (CRZR, □) and adipokinetic hormone/corazonin-related peptide receptors (ACPR, ○) from insects. Tree was constructed using the neighbour-joining analysis (with 1000 bootstrap replicates). Branch lengths indicate the number of amino acid substitutions per site and numbers adjacent to nodes denotes the percentage support for the clustering of the related sequences in that particular clade. Receptor protein sequences are labelled by species name and identified with their GenBank accession number. The *Aedae*ACPR-I receptor cloned in this study is denoted in red text. The human gonadotropin releasing hormone receptor isoform 1 (GenBank: NP_000397) was included in the analysis and designated as the outgroup. References for sequences are included in Table 2.

Table 2. GenBank accession numbers and references for receptor sequences in Fig. 4.

Receptor	Putative Ligand(s)	Accession Number	Reference(s)
Acyrthosiphon pisum AKHR	AKH	XP_003245941	(Li et al., 2016)
Aedes aegypti AKHR1	AKH	XP_001655248	(Nene et al., 2007)
Aedes albopictus AKHR	AKH	XP_019540948	
Anopheles gambiae AKHR	AKH	ABD60146	(Kaufmann and Brown, 2006)
Anopheles darlingi	AKH	ETN67429	(Mendes et al., 2010)
Anopheles sinensis	AKH	KFB53098	(Zhou et al., 2014)
Culex pipiens AKHR	AKH	AAWU01041683	(Li et al., 2016)
Apis mellifera AKHR	AKH	NP_001035354	(Elsik et al., 2014)
Bombyx mori AKHR	AKH, ACP	NP_001037049	(Huang et al., 2010; Ou et al., 2014; Staubli et al., 2002; Suetsugu et al., 2013; Zhu et al., 2009)
Musca domestica AKHR		XP_019895730	
Manduca sexta AKHR	AKH	ACE00761	(Ziegler et al., 2011)
Drosophila melanogaster AKHR	AKH	AAC61523	(Hauser et al., 1998; Staubli et al., 2002)
Locusta migratoria AKHR		ANW09575	
Polyrhachis vicina AKHR		ADK55068	
Nasonia vitripennis AKHR	AKH	NP_001161243	(Hansen et al., 2010)
Tribolium castaneum AKHR	AKH	NP_001076809	(Hansen et al., 2010)
Periplaneta Americana AKHR	AKH	ABB20590	(Hansen et al., 2010)
Rhodnius prolixus AKHR	AKH	AIJ49751	(Zandawala et al., 2015b)
Gryllus bimaculatus AKHR	AKH	ADZ17179	(Konuma et al., 2012)
Glossina morsitans AKHR	AKH	AEH25943	
Aedes aegypti CRZR	CRZ	AAGE02020874	(Nene et al., 2007)
Aedes albopictus CRZR		XP_019554625	
Anopheles gambiae CRZR	CRZ	AAQ67361	(Belmont et al., 2006)
Apis mellifera CRZR	CRZ	NP_001137393	(Elsik et al., 2014)
Culex pipiens CRZR	CRZ	AAWU01031675	(Li et al., 2016)
Bombyx mori CRZR	CRZ	NP_001127719	(Hansen et al., 2010; Ou et al., 2014; Yamanaka et al., 2008)
Drosophila melanogaster CRZR	CRZ	AAM21341	(Cazzamali et al., 2002)
Nasonia vitripennis CRZR	CRZ	AAZX01006363	(Werren et al., 2010)
Bactrocera dorsalis CRZR	CRZ	AQX83392	(Hou et al., 2017)
Rhodnius prolixus CRZR-A	CRZ	AND99324	(Hamoudi et al., 2016)
Manduca sexta CRZR	CRZ	AAR14318	(Kim et al., 2004)
Musca domestica CRZR	CRZ	NP_001295994	
Anopheles gambiae ACPR	ACP	ABX52399	(Hansen et al., 2010)
Anopheles darlingi ACPR	ACP	ETN61096	(Marinotti et al., 2013)
Aedes albopictus ACPR	ACP	XP_019559258	
Culex pipiens ACPR	ACP	XP_001842868	(Li et al., 2016)
Tribolium castaneum ACPR	ACP	ABX52400	(Hansen et al., 2010)
Nasonia vitripennis ACPR	ACP	NP_001164571	(Hansen et al., 2010)
Bombyx mori ACPR1	ACP, AKH	NP_001127726	(Hansen et al., 2010; Kim et al., 2004)
Bombyx mori ACPR2	ACP, AKH	NP_001127745	(Hansen et al., 2010; Kim et al., 2004)
Rhodnius prolixus ACPR-C	ACP, AKH	AKO62858	(Zandawala et al., 2015a)
Ixodes scapularis ACPR-1	ACP	ABJB010361713	(Ayllón et al., 2015)
Ixodes scapularis ACPR-2	ACP	ABJB010171383	(Ayllón et al., 2015)
Ixodes scapularis ACPR-3	ACP	ABJB010083490	(Ayllón et al., 2015)
Ixodes scapularis ACPR-4	ACP	ABJB010179456	(Ayllón et al., 2015)
Ixodes scapularis ACPR-5	ACP	ABJB010141465	(Ayllón et al., 2015)

Functional characterization of AedaeACPR-I

A heterologous receptor functional assay involving CHO-K1 cells was used to validate the cloned receptor as a bona fide ACP receptor. Indeed, the *Aedae*ACPR-I was dose-dependently activated by *Aedae*ACP (EC_{50} =1.025x10^{-8} M) (Fig. 5a), confirming the proposed identity of the receptor based on phylogenetic analysis. Kinetic analysis of receptor activation demonstrated maximal luminescence response was evident over the first five seconds following application of the ACP peptide, indicative of an immediate and transient elevation of intracellular calcium levels elicited through activation of the ACP receptor (Fig. 5b). Our results also confirm the specificity of the ACP receptor for the ACP peptide alone (Table 3), since no luminescence response indicative of receptor activation was observed in response to the closely related peptides, *Aedae*AKH and *Aedae*CRZ, or other tested peptides, specifically *Aedae*CAPA-1 and pyrokinin-1 (*Aedae*PK1), which share no structural similarity to *Aedae*ACP. Modification of the isoleucine residue (Ile_{472}) obtained in our cDNA to the genome consistent asparagine residue (Asn_{472}) resulted in no change to receptor activation by its endogenous ACP ligand, as determined by equal luminescent response by both the cloned ACPR (containing Ile_{472}) or the isoform that was generated (containing Asn_{472}) that matches the *A. aegypti* genome (*Aedae*ACPR-I-N472I; Fig 6a). No luminescence signals were obtained in untransfected cells, or those transfected with empty pcDNA 3.1^{+} vector (Fig. 6b), indicating that the luminescent response observed when *Aedae*ACPR-I was exposed to *Aedae*ACP was a result of binding and activation of the receptor and the subsequent mobilization of Ca^{2+}, which is required for the luminescent response detected using this heterologous system.

Table 3. Structure of peptides tested in the heterologous receptor functional assay and summary of activity in eliciting a luminescent response.

Peptide Name	Peptide Sequence	EC_{50} (nM)[a]
*Aedae*ACP	pQVTFSRDWNAa	10.25
*Aedae*AKH	pQLTFTPSWa	NA[b]
*Aedae*CRZ	pQTFQYSRGWTNa	NA[b]
*Aedae*CAPA1	GPTVGLFAFPRVa	NA[b]
*Aedae*CAPA-PK1	AGNSGANSGMWFGPRLa	NA[b]

[a] EC_{50} values are the averages of multiple independent biological replicates involving CHOK1-aeq cells transiently expressing *Aedae*ACPR-I
[b] No activity (NA) detected when tested with peptide titres up to a maximum of 10μM

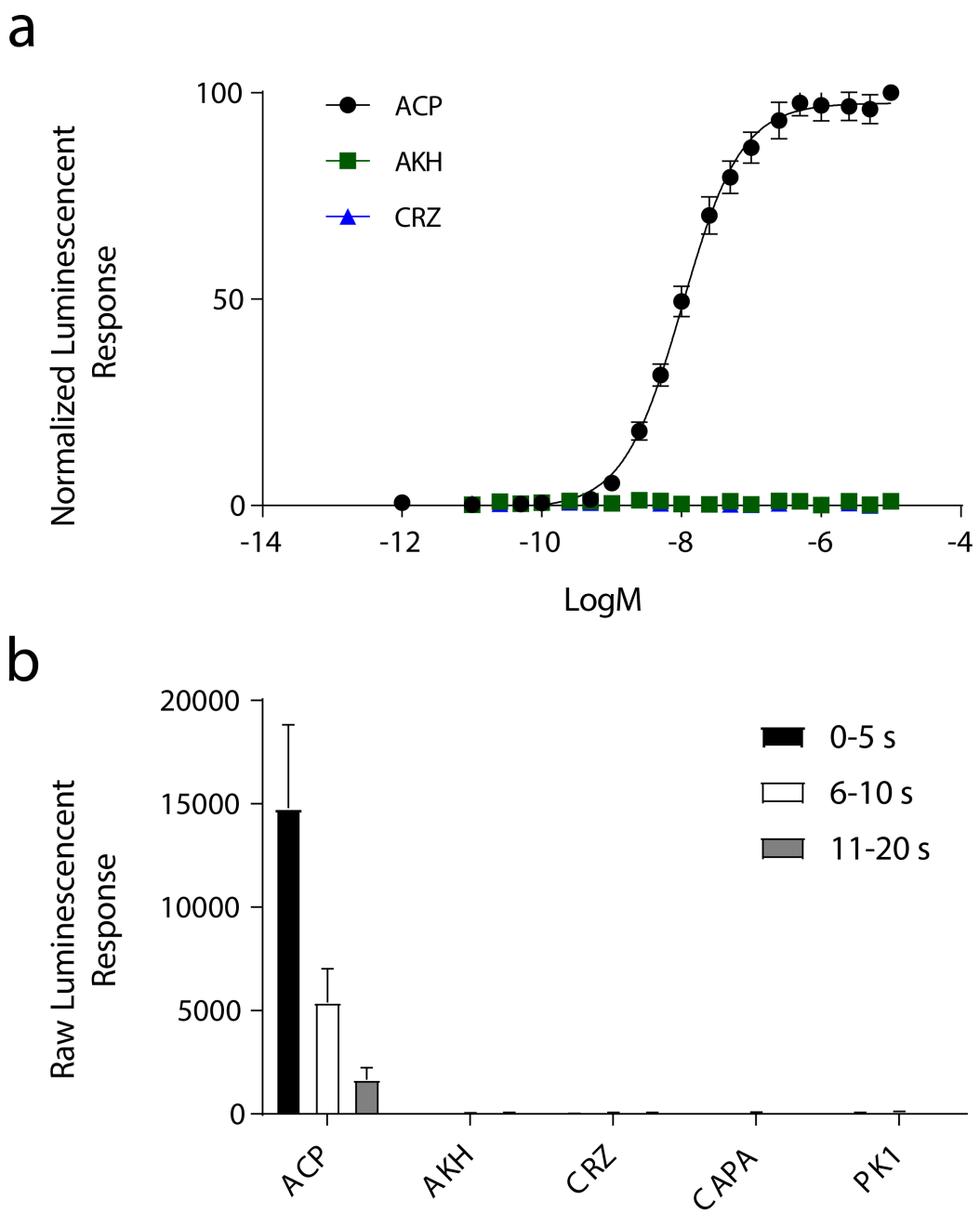

Figure 5. Functional heterologous receptor assay of CHO-K1 aequorin cells transiently expressing *Aedae*ACPR-I. (***a***) Dose dependent effect on the bioluminescence response (mean 0-15s) after the addition of between 10^{-12} – 10^{-5} M doses of *A. aegypti* ACP, AKH and CRZ peptides. Luminescence is normalized to the BSA control and plotted relative to the maximal response (10^{-5} M). The EC_{50} for *Aedae*ACP is 1.025×10^{-8} M. No receptor activation was detected when challenged with *Aedae*AKH and *Aedae*CRZ or *Aedae*CAPA1 and *Aedae*PK1 (not shown). (***b***) Kinetics of the bioluminescence response measured between 0-5s, 6-10s, and 11-20s time intervals, following the addition of 10^{-6} M of the above peptides normalized to vehicle control (BSA media). Data represent the mean ± standard error (n=4).

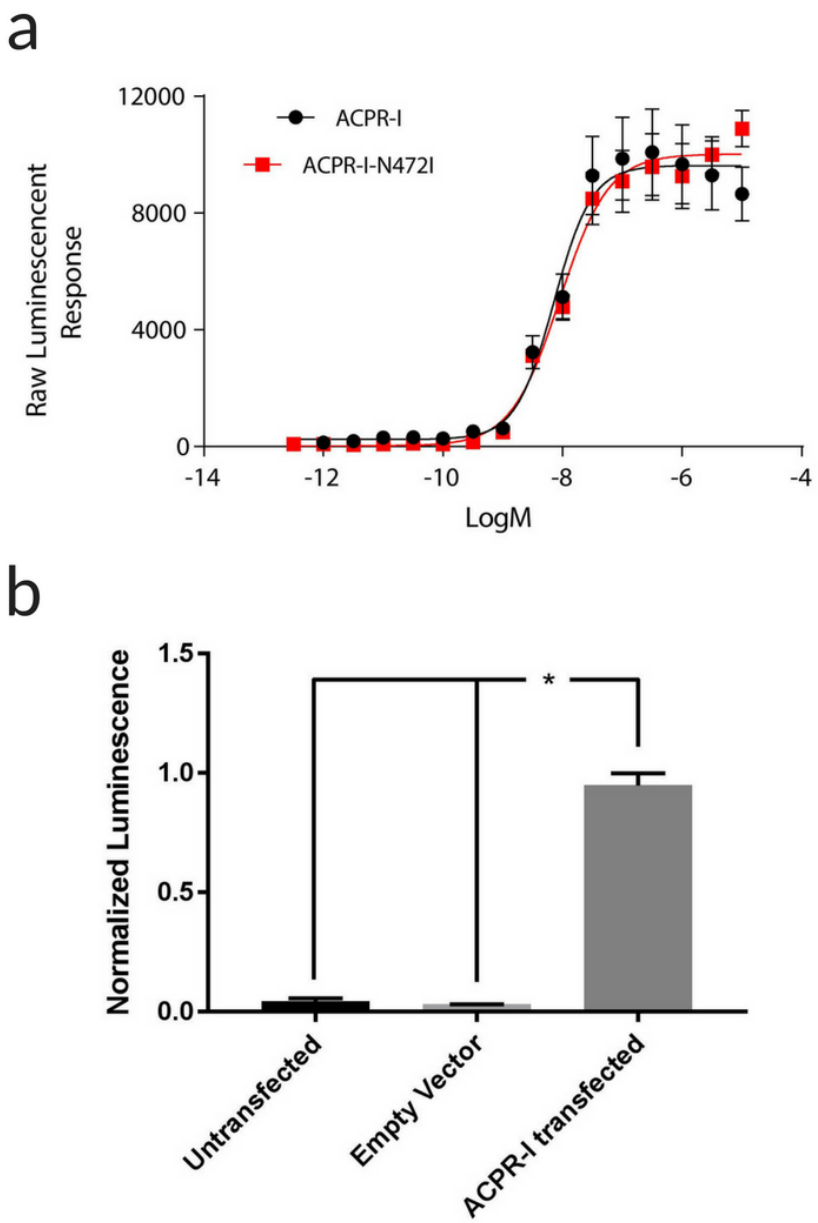

Figure 6. Functional heterologous receptor assay of CHO-K1 aequorin cells transiently expressing the *Aedae*ACPR-I and *Aedae*ACPR-I-N472I, and validation of CHOK1-aequorin expression of ACPR-I. (***a***) Dose dependent effect on the bioluminescence response (mean 0-15s) after the addition of between 10-12 – 10-5 M doses of AedaeACP to AedaeACPR-I and AedaeACPR-I-N472I. (***b)*** Effect of 10^{-6} M *Aedae*ACP on untransfected CHOK1-aeq cells or cells transfected with either the empty pcDNA 3.1^{+} vector or pcDNA expressing *ACPR-I*. Luminescence is normalized to the BSA control. * denontes significance ($p<0.05$) as determined by a one way ANOVA (Dunnett's multiple comparisons test). Data represent the mean ± standard error (n=4).

ACPR and ACP transcript expression profile

We utilized RT-qPCR to measure levels of the *ACPR* transcript in individual tissues/organs of adult *A. aegypti* and across development stages of the mosquito from larvae to adult in order to reveal potential physiological functions for ACP signalling. Transcript expression profiles are solely indicative of *ACPR-I* transcript levels (see methods), since ACPR-II and ACPR-III are truncated variants that encode receptor isoforms that lack the seven TM domains typical of GPCRs and thus are not expected to be functionally responsive to the ACP peptide. Developmental expression profiling of *Aedae*ACPR revealed enrichment of the ACP receptor transcript following the transition from pupal to adult stages (Fig. 7a). One-day and four-day old male *A. aegypti* had the highest levels of *ACPR* transcript abundance. A similar expression pattern was observed for *Aedae*ACP with greatest enrichment in one-day old and four-day old male mosquitoes (Fig. 7b).

Examination of the spatial expression profiles in both male and female *A. aegypti* revealed significant enrichment of *ACPR* in the abdominal ganglia of both sexes when compared relative to expression in the whole adult (males, $p=0.0025$ and for females, $p=0.0016$) (Fig. 7c,e). ACPR transcript was also observed in the carcass, accessory reproductive tissue, testes, head and thoracic ganglia of adult male mosquitoes, although not significantly enriched as found in the abdominal ganglia (Fig. 7c). Similarly, ACPR transcript was also found in the head, thoracic ganglia, hindgut, and accessory reproductive tissues of adult female *A. aegypti* (Fig. 7e). *AedaeACP* transcript was detected in the central nervous system, where it was enriched in the brain and thoracic ganglia of male (Fig. 7d) and significantly enriched in the head ($p=0.0127$) and thoracic ganglia ($p=0.004$) of female mosquitoes (Fig. 7f).

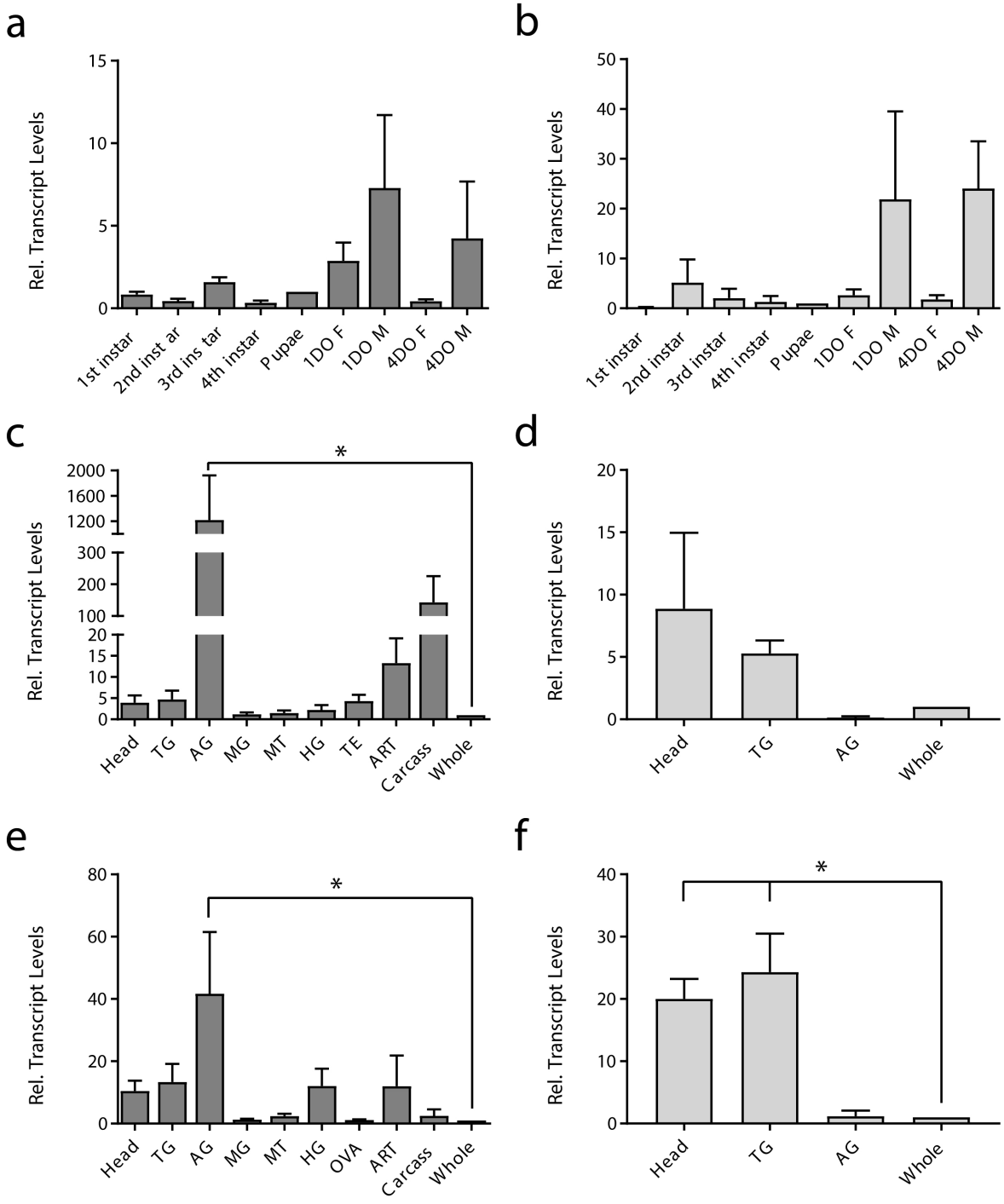

Figure 7. Transcript expression pattern of *ACPR-I* and *ACP* during post-embryonic development and in specific tissues of four-day old adult *A. aegypti*. Developmental expression of *ACPR-I* (***a***) and *ACP* (**b**) transcript is analyzed across post-embryonic stages of the mosquito with expression shown relative to transcript levels in pupa. Spatial expression is analyzed in various tissues from four-day old adult males, *ACPR-I* (***c***) and *ACP* (**d**), and females, *ACPR-I* (***e***) and *ACP* (**f**) with transcript abundance shown relative to levels in whole body adult mosquitoes. Abbreviations: thoracic ganglia (TG), abdominal ganglia (AG), midgut (MG), Malpighian tubules (MT), hindgut (HG), ovaries (OVA), testes (TE), accessory reproductive tissue (ART). Data represent mean ± standard error, * denotes significance ($p<0.05$) as determined by a one-way ANOVA, Dunnett's multiple comparisons test.

Discussion

In the present study, we have identified and functionally characterized the *A. aegypti* adipokinetic hormone/corazonin-related peptide receptor (ACPR). ACPR and its ligand (ACP), are structurally intermediate to the adipokinetic hormone (AKH) and the corazonin (CRZ) systems, which collectively are insect neuropeptides that demonstrate homology to the vertebrate gonadotropin-releasing hormone (GnRH). The AKH/AKHR and CRZ/CRZR neuropeptide signalling systems have been described in many insect species. Both AKH and CRZ are pleiotropic, with the former primarily functioning in the mobilization of energy-rich compounds and the latter serving as a cardio-excitatory factor (Gäde and Auerswald, 2003; Veenstra, 1989). To date, an ACPR has been identified in *R. prolixus*, *T. castaneum*, *A. gambiae*, and a number of other candidates are predicted based on genomic data mining; however, a physiological function has yet to be described for the ACP/ACPR system (Hansen et al., 2010; Zandawala et al., 2015).

We have identified three transcript variants, one of which (*Aedae*ACPR-I) yields a complete rhodopsin-like GPCR comprising 577 residues and seven transmembrane (TM) domains. Transcript variants II and III differ in their splicing of exons 4 and 5, which results in truncated receptors with either five or three TM domains, respectively, which are unlikely to be functional. Similarly, previous studies in *R. prolixus* (Zandawala et al., 2015) and *A. gambiae* (Hansen et al., 2010) revealed multiple receptor variants, whereas only a single variant was identified in *T. castaneum* (Hansen et al., 2010). Specifically, in *R. prolixus*, three ACPR variants have been identified, two of which contain the typical seven TM domains (*Rhopr*ACPR-B and *Rhopr*ACPR-C), and one which contains five TM domains (Zandawala et al., 2015). Additionally, two ACPR isoforms have been identified in *A. gambiae*, *Anoga*ACPR-A and *Anoga*ACPR-B, both of which result in receptors possessing seven TM domains (Hansen et al., 2010). Through phylogenetic analyses, we confirmed that the receptor isolated in this study is an

ortholog of other insect ACP receptors (Hansen et al., 2010; Shi et al., 2011; Siegert, 1999; Zandawala et al., 2015).

Using a heterologous receptor assay, we determined the functional activation of *Aedae*ACPR-I testing various peptidergic ligands. Our findings indicate that the receptor is activated solely by its native ligand *Aedae*ACP, whereas other tested peptides, even closely-related peptides sharing structural similarity, namely *Aedeae*AKH and *Aedae*CRZ, failed to activate *Aedae*ACPR-I. This confirms that the putative *Aedae*ACPR isolated herein is in fact an ACPR. Similar binding specificity of ACP receptors has been observed previously in *T. castaneum*, *A. gambiae*, and *R. prolixus* with EC_{50} values reported in the low nanomolar range (Hansen et al., 2010; Zandawala et al., 2015). Additionally, previous research in the aforementioned insects, and recently in *A. aegypti*, have also observed that the AKH receptors are not activated by ACP or corazonin peptides, and similarly, the corazonin receptors are not activated by ACP or AKH peptides (Hamoudi et al., 2016; Hansen et al., 2010; Oryan et al., 2018). Thus, consistent with these previous observations, we determined that although these neuropeptide systems are structurally and evolutionarily related, they are indeed independent of one another and do not exhibit any cross talk in *A. aegpyti*. Notably, however, the *R. prolixus* AKH was able to activate *Rhopr*ACPR-C expressed in CHOK1/Gα16 cells (Zandawala et al., 2015). Although the receptor activation was considerably lower and latent compared to ACP, there is potential for cross-talk between the AKH and ACP neuropeptide signaling systems in *R. prolixus* (Zandawala et al., 2015). Furthermore, previous studies in *B. mori* have revealed that high concentrations of *Bommo*-ACP (previously referred to as AKH3) resulted in the activation of *Bommo*-AKHR with an EC_{50} of 1.07×10^{-6} M, whereas sensitivity to its natural AKH ligand was approximately 100-fold higher with an EC_{50} of 1.17×10^{-8} M (Zhu et al., 2009). Similarly,

high concentrations of the AKH peptides in *B. mori*, *Bommo*-AKH1 and *Bommo*-AKH2, were also found to activate putative *B. mori* ACPRs (A28, and A29), albeit at significantly higher concentrations than ACP peptide (Shi et al., 2011).

Transcript expression profiles of *A. aegypti ACPR* were measured to reveal potential functional roles for ACP by determining prospective target tissues. Examination of the tissue-specific expression profile of four-day old adult male and female mosquitoes revealed expression of *ACPR* in the CNS, with significant enrichment in the abdominal ganglia, and enrichment of the ACP transcript in the brain and thoracic ganglia. This is not surprising, as similar expression of the ACP receptor and peptide in nervous tissue has been observed previously in other insects. Consistent with the quantified ACP expression profile data determined herein, ACP transcripts in *A. aegypti* and *A. gambiae* were detected solely in the head and thorax segments of adult mosquitoes (Kaufmann and Brown, 2006; Kaufmann et al., 2009). In fifth instar and adult *R. prolixus*, ACPR transcript was found to be enriched in the CNS and the corpus cardiacum /corpora allata (CC/CA) complex (Zandawala et al., 2015). *ACPR* expression in *T. castaneum* was revealed to be greatest in the brain in comparison to the torso (i.e. body minus the head) (Hansen et al., 2010). Expression of *Aedae*ACPR within the CNS suggests that ACP may be functioning as a neuromodulator and/or a neurotransmitter. This possibility is supported by the extensive varicose immunoreactive staining of ACP in the central nervous system of *T. castaneum* first instar larvae, where a neurosecretory role was suggested (Hansen et al., 2010). Specifically, immunoreactivity (IR) was observed in three to four neurons in the brain, the central brain neuropil with projections from the brain descending into the suboesophageal ganglion (SOG), thoracic ganglia, and abdominal ganglia (Hansen et al., 2010). These projections within the brain neuropil and ganglia were strongly varicose with no projections

observed exiting the CNS. Immunocytochemistry using an antiserum against *D. melanogaster* AKH, which recognizes both AKH and ACP in *A. gambiae* and *A. aegypti* (Kaufmann and Brown, 2006; Kaufmann et al., 2009), revealed immunoreactivity throughout the mosquito nervous system. In *A. gambiae* AKH-like immunoreactivity was observed in two pairs of lateral neurosecretory cells in the brain, but was indicated to likely represent staining of ACP-producing neurons, since AKH synthesis and storage is restricted to the corpus cardiacum (Kaufmann and Brown, 2006). Similarly, AKH-like immunoreactivity was observed in two pairs of lateral neurosecretory cells in the brain of *A. aegypti*, again likely indicative of ACP-producing neurons (Kaufmann et al., 2009). Within the fused thoracic ganglia of adult *A. aegypti* and *A. gambiae*, AKH-like immunoreactivity was observed within at least one to a few cells within the ventral region of the pro- and mesothoracic ganglia (Kaufmann and Brown, 2006; Kaufmann et al., 2009). Thoracic extracts were negative for ACP-like activity in *A. gambiae* (Kaufmann and Brown, 2006; Kaufmann et al., 2009), however, expression of the AKH transcript in adult male *A. aegypti* is absent in the head and thorax region (Kaufmann and Brown, 2006; Kaufmann et al., 2009); thus, it is unclear if the cells within the thoracic ganglia are ACP- or AKH-producing neurons in mosquitoes although the current findings indicate that ACP transcript is prominently expressed in both brain and thoracic regions of the nervous system in *A. aegypti*. In *R. prolixus*, ACP-like immunoreactivity is observed in two bilaterally-paired cell bodies at the anterior portion of the protocerebrum near the optic lobes, and one bilateral pair of cell bodies medially positioned in the protocerebrum (Patel et al., 2014). The extensive, yet confined, immunohistochemical distribution of ACP throughout the nervous system of insects (Hansen et al., 2010; Kaufmann and Brown, 2006; Kaufmann et al., 2009), taken together with the prominent but restricted neuronal expression of *Aedae*ACP transcript along with the significant

enrichment of the ACP receptor within the abdominal ganglia of adult *A. aegypti* strongly supports that ACP may be functioning centrally within the nervous system as a neurotransmitter or neuromodulator. In *L. migratoria* ACP was identified in the storage lobe of the CC, in contrast to the glandular lobe where AKH is found, suggesting synthesis of this neuropeptide within neurosecretory cells of the brain (Siegert, 1999). Furthermore, given that *ACPR* was found in the CC/CA complex, Zandawala *et al*. postulated that ACP in *R. prolixus* may be involved in the regulation of other hormones in a manner similar to its distant vertebrate homolog, GnRH (Zandawala et al., 2015). We observed *Aedae*ACPR transcript expression in other tissues as well including the female hindgut and male carcass in *A. aegypti*. ACPR expression in the hindgut, the primary site of reabsorption of ions and metabolites (Coast et al., 2002), was unexpected since neither AKH nor CRZ have been found to regulate hydromineral balance. Thus, this possible function for ACP on the hindgut will require further investigation. Detection of the ACPR transcript in the carcass, which includes fat body and body wall musculature, suggests that ACP and AKH may share a lipid mobilizing function, although this is unlikely since ACP was shown to have no influence on lipid or carbohydrate metabolism in female *A. gambiae* nor did it influence energy stores in male insects of *L. migratoria* or *P. americana* (Kaufmann and Brown, 2008). Interestingly, contrary to our predictions, males on a whole tended to have higher ACPR transcript abundance relative to females and enrichment in the female carcass was not observed. Transcript expression was also observed in the accessory reproductive tissues of both males and females. Expression of ACPR in reproductive tissue has also been observed in *R. prolixus* (Zandawala et al., 2015). Perhaps, in addition to structural homology between ACPR and GnRHR, there is a yet undiscovered functional conservation between the two signaling systems. Furthermore, AKHR, another homolog of GnRHR, has been

shown to play a role in egg development in *Gryllus bimaculatus* and *Caeborhabditis elegans*, and influences lipid metabolism essential for lactation in *Glossina morsitans* (Attardo et al., 2012; Lindemans et al., 2009; Lorenz, 2003). Additionally, analysis of seminal fluid protein content of *A. albopictus* revealed the AKH peptide as one of the proteins transferred from male to female mosquitoes during mating (Boes et al., 2014). Recently, CRZR transcript expression in *R. prolixus* was also observed in male and female reproductive tissues, which suggests some potentially overlap with regard to reproductive targets in insects (Hamoudi et al., 2016).

We also investigated the molecular expression of the ACP signaling system during development and found that both ACP and ACPR transcript levels are enriched after adult eclosion albeit more so in males. Similar findings were observed in *R. prolixus* (Zandawala et al., 2015); however, in contrast, *ACPR* transcript levels in *T. castaenum* were highest in late embryonic and early larval stages and decreased thereafter as the beetle progressed in development (Hansen et al., 2010). In *A. aegypti*, *Aedae*ACPR expression could be indicative of a post-eclosion function for the ACP system. Interestingly, analysis of both spatial and temporal expression profiles reveals greater expression of *ACP* and *ACPR* in adult males compared to females, which agrees to an earlier microarray analysis in *A. gambiae* that determined higher ACP expression in adult males, compared to adult female and last instar **A**larvae (Marinotti et al., 2005). There is no clear explanation for such a sex-specific difference in ACP and ACPR transcript expression, however similar to our findings, male *D. melanogaster* express greater levels of the AKH receptor than their female counterparts (Hauser et al., 1998).

Currently, no definitive function for ACP has been determined and functional studies in other insects have revealed that ACP does not influence energy mobilization and so does not duplicate the actions of AKH (Kaufmann and Brown, 2008; Patel et al., 2014). Additionally,

ACP failed to increase heart-beat frequency, suggesting that the physiological actions of ACP does not mirror the most established function of CRZ (Patel et al., 2014). Here we have functionally identified the ACP receptor and quantitatively examined its transcript expression profile, along with that of its peptidergic ligand, in the blood feeding arthropod, *A. aegypti*. Based on receptor transcript expression profiles, a number of targets for ACP have now been identified including notably the abdominal ganglia, accessory reproductive tissues and carcass. Taken together, enrichment of the ACP receptor in the abdominal ganglia and relatedly, the ACP transcript in the brain and thoracic ganglia, suggests a neurotransmitter/neuromodulatory role in the mosquito *A. aegypti*.

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Chapter 3:

Revealing the cell-specific expression of the adipokinetic hormone/corazonin-related peptide along with its receptor and utilizing RNA interference to infer the physiological function of this signalling system

Prefix

The adipokinetic hormone/corazonin-related peptide (ACP) is an insect neuropeptide structurally intermediate between corazonin (CRZ) and adipokinetic (AKH) hormones, which all demonstrate homology to the vertebrate gonadotropin-releasing hormone (GnRH). Various studies have characterized the CRZ and AKH signalling systems within many insect species, and most notable are the cardioacceleratory and energy mobilization functions, respectively. In contrast, the function of ACP and its receptor, ACPR, which have now been identified in a number of insect species, remains unknown. Despite ACP/ACPR being structurally related to AKH and CRZ and their receptors, studies have shown it to be functionally unrelated to the two later signalling pathways. Here, we aim to examine the cell-specific distribution of the ACP/ACPR signalling system and use RNA interference to knockdown expression of the ACP peptide and receptor in the disease vector mosquito, *Aedes aegypti*. Immunohistochemical analysis and fluorescence *in situ* hybridization reveals expression of the ACP peptide and transcript to a number of cells in the central nervous system, including putative lateral neurosecretory cells in the protocerebrum of the brain and ventrally localized cells within the first two regions of the fused thoracic ganglia. Notably, no ACP-immunoreactive cells or ACP transcript expressing cells were observed in the six abdominal ganglia. Interestingly, ACP

receptor transcript was localized solely within the abdominal ganglia of the central nervous system, with no cells expressing the transcript detected in the brain or thoracic ganglia. Based on the localization of the ACP peptide and its receptor within distinct regions of the nervous system, a neurotransmitter or neurmodulatory role is suggested.

Introduction

Adipokinetic hormones (AKHs) are a large family of insect neuropeptides synthesized exclusively in the corpora cardiaca (CC), a small neurohaemal organ closely associated with the insect brain (Diederen et al., 1987; Diederen et al., 2002). AKHs are released into the haemolymph during physical activity to stimulate the release of energy substrates from the fat body (an organ analogous to adipose tissue and the mammalian liver) (Gäde and Auerswald, 2003). In addition to functioning comparably to glucagon and adrenaline, AKHs are pleiotropic and function as neurohormones, neurotransmitters, and neuromodulators. Additional functions attributed to AKHs include the stimulation of heart beat rate, oxidative stress, the inhibition of protein synthesis, and extension of life span (Gäde and Marco, 2006; Zandawala et al., 2017).

The insect corazonins, another pleiotropic group of neuropeptides, are structurally related to the AKHs but their actions are somewhat different. Corazonin (CRZ), first discovered and named due to its cardioacceleratory effect in *P. americana* has also been shown to function in ecdysis and melanization in other insects (Kim et al., 2004; Tawfik et al., 1999; Veenstra, 1989). Immunohistochemical studies in *P. americana* (Veenstra and Davis, 1993) and *D. melanogaster* (Choi et al., 2005) have localized CRZ to a number of dorsolateral neurons in the pars lateralis of the protocerebrum as well as neurons in each ganglia of the ventral nerve cord. The sequence for corazonin is highly conserved across insect species, thus it is interesting that apart from stimulating heart rates of *P. americana* and *R. prolixus*, there is no clear common denominator for the various activities of CRZ (Patel et al., 2014; Veenstra, 1989). Besides AKHs cardioexcitatory role in *D. melanogaster* prepupae (Noyes et al., 1995), another area of overlap between the biological actions of AKH and CRZ is the implication of both neuropeptides in nutritional and oxidative stress (Zandawala et al., 2017). Whether there exists any functional

overlap between these two signaling pathways remains unclear; however, it has been suggested that AKHs and corazonin have indeed specialized in that they control different aspects of stress (Hansen et al., 2010).

The recently discovered adipokinetic hormone/corazonin-related peptide (ACP), as its name implies, is structurally similar to AKH and CRZ. Studies in a number of insects have demonstrated that the three neuropeptide pathways, namely AKH, CRZ and ACP, remain independent as all three receptors demonstrate highly-selective specificity for their respective ligands (Hamoudi et al., 2016; Hansen et al., 2010; Oryan et al., 2018; Wahedi and Paluzzi, 2018; Zandawala et al., 2015). So far the functional role(s) of ACP remains unknown, as biological assays have revealed that ACP does not mobilize lipids in *A. gambiae* (Kaufmann and Brown, 2008), *R. prolixus* (Patel et al., 2014), *L. migratoria* (Siegert, 1999), nor does it alter heart beat rate in *R. prolixus* (Patel et al., 2014) and *A. gambiae* (Hillyer et al., 2012). Expression analyses of the peptide and receptor in *R. prolixus*, and ACPR in *T. castaneum*, had revealed enrichment in nervous tissue (Hansen et al., 2010; Zandawala et al., 2015). In *T. castaneum*, ACP was localized to a number of neurons in each hemisphere of the brain, with projections throughout the CNS (Hansen et al., 2010). ACP-like immunoreactivity was also observed throughout the nervous system of *R. prolixus*, and like *T. castaneum*, no projections were observed exiting the CNS (Hansen et al., 2010; Patel et al., 2014). Furthermore, knockdown of the ACP receptor using RNAi in *T. castaneum* did not result in any clear effect (Hansen et al., 2010).

AKH, ACP, and CRZ along with their respective receptors do not always occur together in all arthropods. The ACP signalling system is not present in *D. melanogaster*, *P. humanus*, *A. pisum*, *D. pulex*, and the honey bee *Apis mellifera*, whereas the corazonin signalling system is

missing from *T. castaneum* and *A. pisum* (Hansen et al., 2010; Hauser and Grimmelikhuijzen, 2014). It is believed that the ACP signalling system arose through gene duplication of an AKH-like ancestor prior to the emergence of arthropods (Hauser and Grimmelikhuijzen, 2014). The AKH ancestor itself arose through gene duplication of a GnRH-like ancestor into the CRZ and AKH signalling systems (Hauser and Grimmelikhuijzen, 2014).

Mosquitoes are the world's most important disease vectors, as they transmit parasites and viruses that infect millions annually. The physiology and behaviour of these mosquitoes, and all organisms, are regulated by neuropeptides. The diverse functions of neuropeptides make them prime targets for the development of novel insect control agents (Boonen et al., 2009). However, before designing such control agents targeting neuropeptide signaling, the *in vivo* physiological roles of specific neuropeptide systems need to be elucidated. Given the endogenous *A. aegpyti* ACP receptor (ACPR) was recently deorphanized and found to be enriched in the early adult stage (Wahedi and Paluzzi, 2018), the present study sought to map the distribution of the ACP peptide and its receptor in order to reveal a potential function for this neuropeptide system within the mosquito *A. aegypti*. Immunohistochemistry was used to determine the distribution of the ACP peptide within the nervous system. Utilizing fluorescence *in situ* hybridization, the cell-specific expression of *ACP* and *ACPR* in the CNS of *A. aegypti* was examined. Lastly, I sought to deduce the functional importance and physiological role of the ACP signaling system via targeted RNAi of the *AedaeACPR* and *AedaeACP* gene.

Materials and Methods

Animals

Aedes aegypti (Liverpool strain) eggs were hatched in plastic containers half-filled with deionized water at an initial density of approximately 100 larvae/litre of water. Larvae were fed a solution of 2% brewer's yeast, 2% liver powder daily, and adults were provided with a 10% sucrose solution through a microcentrifuge tube fitted with a cotton ball wick allowing feeding *ad libitum*. Larvae and pupae were maintained in an incubator at 26°C on a 12:12 hour light: dark cycle. Colony upkeep involved adult females being fed sheep's blood in Alsever's solution weekly (Cedarlane Laboratories Ltd., Burlington, ON) using an artificial feeding system described previously (Rocco et al., 2017). All experiments on adults were performed on either one or four-day old male and female mosquitoes that were sucrose-fed only and had been isolated during the pupal stage and transferred *en masse* into small glass microchambers.

Immunohistochemistry

Adult male and female *A. aegypti* tissues were dissected in 1x nuclease-free Dulbecco's phosphate-buffered saline (DPBS) and subsequently treated with 4% paraformaldehyde fixative for one hour at room temperature. Tissues were then washed once with DPBS and then incubated with 4% Triton X-100 at room temperature for one hour, followed by three 10 minute washes with DPBS. The polyclonal rabbit antiserum to *Drome*AKH (generously provided by Dr. Mark Brown, University of Georgia) diluted 1:500 was preincubated in a 0.4% Triton X-100, 5% normal sheep serum (NSS, v/v) in DPBS at 4ºC overnight prior to use. The nervous tissue was then incubated in the antiserum for 48 hours on a flatbed rocker at 4ºC. Following this, tissues were washed with DPBS four times over the course of an hour and were subsequently incubated overnight (16-18 h) with Goat anti-rabbit Alexa Fluor® 488 IgG (H+L) secondary antibody (Molecular Probes, Life Technologies, Eugene, OR) diluted 1:500 with 10% NSS in DPBS at

4ºC, and then washed with DPBS four times over the course of an hour. As a negative control, the preincubated *Drome*AKH antiserum was preincubated with 50 μM *Aedae*ACP peptide overnight prior to its use in immunohistochemistry. Tissues were mounted on cover slips with mounting media comprised of DPBS with 50% glycerol containing 4 µg/mL 4′,6- Diamidino-2-phenylindole dihydrochloride (DAPI) and were visualized on a Nikon Eclipse T*i* fluorescence microscope (Neville, NY).

Preparation of Digoxigenin-labeled RNA probes

The distribution of cells expressing the *A. aegypti ACP* and *ACPR* mRNA within the CNS was determined using fluorescent in situ hybridization (FISH) following a similar protocol as previously described (Paluzzi et al., 2008). To synthesize the sense and antisense probes, *A. aegypti ACP* was amplified via PCR (ACP F2/ACP R2, for primers see Table 5) using whole adult cDNA as template. Primer sequences for *AedaeACP* are based on a previously published sequence (Kaufmann et al., 2009). The open reading frame (ORF) was chosen as the target sequence and was amplified and ligated to pGEM-T vector (Promega, Madison, WI). The T7 promoter sequence (5'-AATTGTAATACGACTCACTATAGGGCG-3') at the 5' end of the sense strand and the 5' end of the anti-sense strand was added to the PCR products via directional screening and subsequent amplification of *ACP* from the pGEM-T vector using a combination of a T7 promoter sequence primer and either a gene-specific forward primer for anti-sense probe template or a gene-specific reverse primer for sense probe template. To localize receptor transcript, two target probes were designed for *A. aegypti ACPR* encoded by the primers ACPR-3'F2/ACPR-RNAi-R and a more downstream target was amplified using the primers ACPR-qPCR-F/ACPR-R (see Table 5). ACPR gene-specific primers with the addition of the T7

promoter sequence were utilized to add the T7 sequence to 5' end of the target for sense probe (control) and 3'end of the target for anti-sense probe templates.

Subsequently, digoxigenin (dig) labeled antisense and sense ACP and ACPR probes were generated by *in vitro* transcription, using the T7 RNA Polymerase Mix and 10X Reaction Buffer from the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, Whitby, ON) and the DIG RNA Labeling Mix, 10X conc. (Roche Applied Science, Mannheim, Germany), following the manufacturer's protocol. Once DIG-labeled RNA synthesis was complete, template DNA was removed with DNase I (New England Biolabs, Whitby, ON) and run on a non-denaturing 1% agarose gel to confirm RNA probe integrity. RNA probes were quantified by UV spectroscopy using a Take3 micro-volume plate and measured on a Synergy Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

Fluorescence in situ hybridization

One and four-day old adult *A. aegypti* (for *ACPR* and *ACP*, respectively) tissues dissected in 1x nuclease-free DPBS were immediately placed 0.2mL PCR tubes containing fixation solution (4% paraformadehyde) where they were incubated at room temperature for 60 minutes on a rocker. Tissues were subsequently washed 5 times with 0.1% Tween-20 in DPBS (PBT) and treated with 1% H_2O_2 (diluted in DPBS) for 10 minutes at room temperature to quench endogenous peroxidase activity. Tissues were then treated with 4% Triton X-100 (Sigma Aldrich, Oakville, Ontario, Canada) prepared in PBT and incubated for one hour at room temperature to digest the tissues. Tissues were then washed three times with PBT to stop the digestion. A secondary fixation was performed for 20 minutes in fresh 4% paraformaldehyde fixation solution described above and later washed three times with PBT to remove all traces of fixative. Tissues were then rinsed in a 1:1 mixture of PBT-RNA hybridization solution (50%

formamide, 5x SSC, 100 μg/mL heparin, 100 μg/mL sonicated salmon sperm DNA and 0.1% Tween-20), which was subsequently removed and replaced with 100% hybridization solution and tissues held at RT. Aliquots (100 μL/tube of samples) of RNA hybridization solution were boiled at 100°C for five minutes and then cooled on ice for five minutes, giving rise to the prehybridization solution. The tissues were incubated in the prehybridization solution at 56°C for 60 minutes. Following prehybridization, 200/300ng of anti-sense RNA probe for *ACP*/*ACPR* (or sense RNA probe for controls) was added per 100μL of hybridization solution and this solution was heated to denature at 80°C for 3 minutes and then cooled on ice for 5 minutes. The prehybridization solution was then removed and tissues were incubated in the hybridization solution containing probe for approximately 16 hours at 56°C in the thermocycler block.

The next day, tissues were washed twice with hybridization solution and subsequently with 3:1, 1:1 and 1:3 (vol/vol) mixtures of hybridization solution-PBT (all pre-warmed to 56°C). The samples were then washed with PBT pre-warmed to 56°C and cooled to room temperature. To reduce non-specific staining, tissues were blocked with PBTB (1x DPBS, 0.1% Tween-20, 1% Molecular Probes block reagent; Invitrogen, Carlsbad, CA) for one hour. The tissues were then incubated in a solution containing PBTB and a 1:400/1:300 (*ACP*/*ACPR*) dilution of a mouse anti-DIG biotin-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 1.5 hours on a rocker and protected from light. The antibody incubation was followed by several washes in PBTB over the course of 1 hour. Tissues were then incubated in a 1:100 dilution of horseradish peroxidase-streptavidin solution (Molecular Probes, Life Technologies, Eugene, OR) in PBTB for 1 hour and the tissues were washed with PBTB several times over an hour. Then, the tissues were washed twice with PBT and once with DPBS. Afterwards, a tyramide solution was prepared consisting of Alexa Fluor 568 tyramide dye

(1:200) in amplification buffer containing 0.0015% H_2O_2 (Life Technologies, Eugene, OR). After the last DPBS wash was completely removed from the tissues, the tyramide solution was added to the tissues and incubated in the dark for 1 hour on a rocker at room temperature. The tyramide solution was then removed and the tissues were washed with DPBS ten times over the course of an hour. Tissues were mounted on cover slips with mounting media comprised of DPBS with 50% glycerol containing 4 μg/mL 4′,6- Diamidino-2-phenylindole dihydrochloride (DAPI) and were visualized on a Nikon Eclipse T*i* fluorescence microscope (Neville, NY).

Preparation of ACP and ACPR dsRNA targets

Primers were designed to amplify a region of the *ACP* and ACPR genes as a target for dsRNA synthesis (Table 5). Two methods were utilized for generation of dsRNA, a) dsRNA producing bacteria (HT115(DE3) *Escherichia coli* cells expressing the L4440 double-T7 vector) and b) *in vitro* transcription of target dsRNA. Initial attempts at RNA interference focused on knockdown of *ACP* with dsRNA produced by the HT115 (DE3) *E. coli*. The *ACP* target regions were amplified (target 1: ACP-F1/ACP-R3 and target 2: ACP-F3/R1) and cloned into pGEM-T-Easy and then subcloned into the L4440 vector (Addgene, Plasmid #1654) which possesses two T7 promoters, one flanking either side of the multiple cloning site. The first target encoded by primers ACP-F1/ACP-R3 spans the 1st and part of the 2nd exon (expected product size: 357bp), including the 5'UTR and the second target encoded by primers ACP-F3/ACPR-R1 spans the 2nd and 3rd exons (expected product size: 325bp) (Fig. 1). The L4440 plasmid containing the ds*ACP* target sites was then transformed into HT115 (DE3) *E. coli* cells purchased from the University of Minnesota *Caenorhabditis* Genetics Center (http://biosci.cbc.umn.edu/CGC/CGChomepage.htm) for the purpose of bacterial feeding as described below. As a control, HT115 (DE3) cells were transformed with the empty L4440

vector. Successful transformants were screened for presence of the ACP inserts and sequenced to confirm construct identity. The HT115(DE3) *E. coli* cells are genetically modified to be deficient in RNase III enzymes that degrade dsRNA and are IPTG inducible to express T7 polymerase, thus allowing for the abundant expression of dsRNA. The primers used for amplification of the target region for *ACP* were designed to prevent complete overlap with the region amplified by our primers used for qPCR detection in order to avoid amplification of bacterial produced dsRNA and false detection of mRNA transcripts. The qPCR primers (ACP –F2/ACP-R2) span nucleotides 89-421 on the *ACP* gene (Accession number: FN391984.1; (Kaufmann et al., 2009), dsACP target 1 is more upstream of the region targeted by the qPCR primers, spanning nucleotides 1-357, offset by 88bp on the 5' end and 64bp on the 3' end. The second dsACP target spans nucleotides 205-529 and is more downstream than the region targeted by the qPCR primers, offset by 116bp on the 5' end and 108bp on the 3' end.

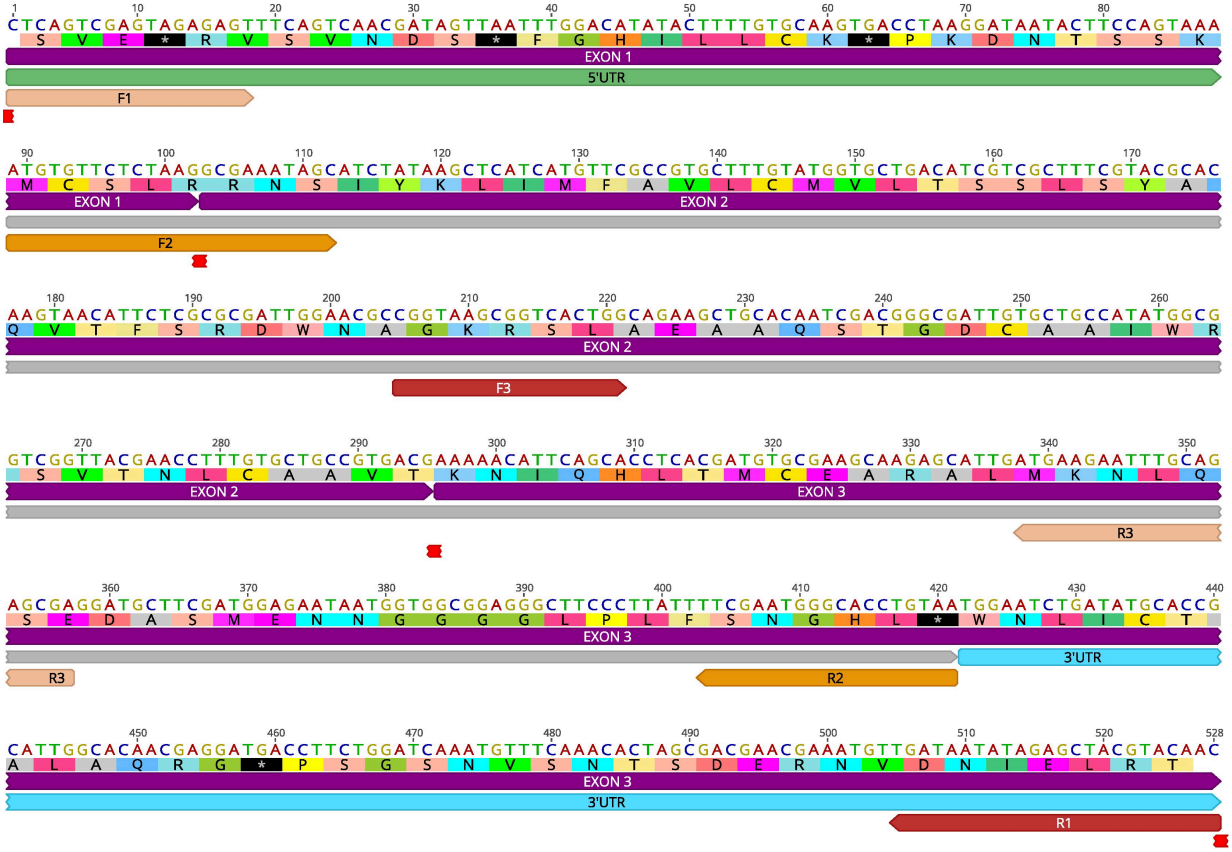

Figure 1. Primer sets mapped to *A. aegypti ACP* gene. The ACP gene spans three exons, denoted in purple. Targets for dsRNA synthesis included regions encoded by primers F1/R3 (beige arrows) and F3/R1 (red arrows). Primers F2/R2 (spanning the open reading frame, denoted in grey) were utilized for generation of the FISH probe template and qPCR analysis of *ACP* gene expression. Top letters indicate nucleotides (positions of which are denoted by numbers above). Second panel of letter indicates the translated sequence. 5'UTR = 5' untranslated region (green), 3'UTR = 3' untranslated region (blue). ACP encoding region of cDNA spans nucleotide positions 176-205.

The second method of dsRNA synthesis involved amplification of target regions for *ACP, ACPR*, and the ampicillin resistance gene (ARG) as a non-native control target with a target region of 658bp plus 48 bp from the two T7 sequences added to the 5' and 3' ends, thus 706bp. *ACP* (F1/R3) and *ACP* (F3/R1) were amplified from the L4440 vector described above using T7 primers, and thus the expected product size is 222bp larger (the distance between the two T7 promoters in the L4440 vector in the empty vector). As discussed below, the ACP (F1/R3) target was pursued further as a target for *ACP* knockdown with *in vitro* dsRNA feeding. The *ACPR* target was amplified via PCR (ACPR-F RNAi/ACPR-R-RNAi) and subsequently reamplified with the same forward and reverse primers modified to possess the T7 promoter sequence at the 5' end, with an expected product size of 633bp. The region targeting ACPR includes exon 5, the exon specific for the functional isoform of the ACP receptor in *A. aegypti* (Wahedi and Paluzzi, 2018). The *ARG* target was initially amplified from the pGEM-T vector and was then reamplified with modified forward and reverse primers possessing the T7 promoter sequence at the 5' end. Double stranded RNA was synthesized by *in vitro* transcription using the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, Whitby, ON) following manufacturers recommendations. The double stranded RNA was subsequently purified by adding one volume of 3M Na^+ CH_3COO^- and 8 volumes of nuclease free water. An equal volume of phenol chloroform was then added to the reaction, and the aqueous phase was collected. Two volumes of 100% ethanol were added to the collected fraction and incubated at -80°C for one hour to precipitate the RNA. The RNA was then pelleted by centrifugation at 13,000 rpm 4°C for one hour. The supernatant was removed and the pellet was then rinsed with ice cold 70% ethanol. The reaction was centrifuged again at 13,000 rpm for 5 minutes, the supernatant was then removed and the pellet was then air dried for 15 minutes and resuspended in 100uL of

nuclease-free water, and an aliquot was diluted and run on a non-denaturing 1% agarose gel to confirm dsRNA integrity. Purified dsRNA samples were quantified by UV spectroscopy using a Take3 micro-volume plate and measured on a Synergy Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

Introduction of dsRNA to A. aegypti larvae

a) *Feeding of dsRNA producing bacteria*

HT115 (DE3) cells containing the L4440 plasmid with the *ACP* target region were inoculated overnight with shaking at 37ºC in liquid LB media supplemented with 10µg/mL of tetracycline and 100µg/mL of ampicillin. The next day the cultures were diluted 10-fold in flasks containing LB media supplemented with antibiotics mentioned above, and were incubated at 37ºC with shaking at 300rpm until they reached an OD_{595} of 0.4 determined by using a Synergy 2 Microplate Reader. After reaching the desired optical density, the cells were induced to produce dsRNA by adding IPTG to a final concentration of 0.4mM, and incubated at 37ºC with shaking at 300rpm for an additional 4 hours. After induction with IPTG, the bacterial cultures were centrifuged at 5000rpm for 10 minutes, and the bacterial pellet was resuspended in 5mL of LB media (not supplemented with antibiotics) which was stored in 500µL aliquots at -80 ºC. There were four treatments, i) control larvae not fed any bacteria, ii) larvae fed bacteria expressing the empty L4440 vector, iii) larvae fed bacteria expressing the *ACP* target region encoded with primers F1/R3, and iv) larvae fed bacteria expressing the *ACP* target region encoded with primers F3/R1 (see Table 5). Each treatment included 40, 3^{rd}-4^{th} instar larvae, in 100mL of deionized water that were starved 24 hours prior to bacteria treatment. The larvae were fed 500µL of bacteria daily which was supplemented with 250µL of larval feed approximately 8 hours post bacteria exposure. Pupae were isolated and total RNA was collected from one day old

adult mosquitoes using the Monarch® Total RNA Miniprep Kit (New England Biolabs, Whitby, ON) following manufacturers protocol.

b) dsRNA soaking method

Following a protocol similar to that described previously (Chasiotis et al., 2016), fourth instar mosquito larvae were soaked in 500ng/μL of ds*ACP*, ds*ARG*, and ds*ACPR* with approximately 50 larvae in 300μL of water for two hours. Larvae were then transferred into 100mL of deionized water and 24 hours later they were fed larval feed. Pupae were collected and total RNA from one day old adult mosquitoes was isolated using the Monarch® Total RNA Miniprep Kit (New England Biolabs, Whitby, ON) following manufacturers protocol.

RT-PCR and qPCR

RNA extracted from larvae samples was used as template for cDNA synthesis using iScript™ reverse transcriptase (BioRad, Mississauga, ON). RT-PCR was used to determine *ACP* expression post bacteria feeding using primers described previously (Table 2, Wahedi and Paluzzi, 2018). Conditions for PCR using OneTaq DNA Polymerase were as follows: initial denaturation 94ºC for 3s, 32 cycles of 1) 94ºC for 20s, 2) 60ºC for 30s, and 3) 68ºC for 30s, and a final extension at 68ºC for 5 minutes. Samples were run on a 1% agarose gel to qualitatively examine expression levels. For the *in vitro* dsRNA feeding method, knockdown efficiency was determined in one day old adult mosquitoes using qPCR. Methods and primers to measure *ACP* and *ACPR* expression for qPCR were the same as those used in Chapter 2 and published recently (Wahedi and Paluzzi, 2018). A Grubbs's test (α=0.05) was utilized to identify outliers in the data.

Table 1. Primers used to amplify *A. aegypti ACP* and *ACPR* gene fragments for dsRNA synthesis, FISH probe synthesis, and RT-qPCR analysis.

Oligo Name	Sequence (5'-> 3')	Function
ACPR-RNAi F	GTCCGTCGTGATAACCCTG	Initial amplification of dsACPR target
ACPR-RNAi F T7	AATTGTAATACGACTCACTATAGGGCG/ GTCCGTCGTGATAACCCTG	Addition of T7 promoter sequence to dsACPR target
ACPR-RNAi-R	ATCCCTCCGGTGTGGCT	Initial amplification of dsACPR target and ACPR FISH target
ACPR-RNAi-R-T7	AATTGTAATACGACTCACTATAGGGCG/ ATCCCTCCGGTGTGGCT	Addition of T7 promoter sequence to dsACPR target and ACPR FISH target
ACP-F1	CTCAGTCGAGTAGAGAGTTTCAGTC	Amplification of dsACP target 1
ACP-F3	CGGTAAGCGGTCACTGG	Amplification of dsACP target 2
ACP-R1	CGTTGTACGTAGCTCTATATTTTCA	Amplification of dsACP target 1
ACP-R3	TCGCTCTGCAAATTCTTCAT	Amplification of dsACP target 2
ARG-RNAi F	TATAGGGAGACATCGAACTGGATCTCAACAG	Initial amplification of dsARG target
ARG-RNAi-F-T7	TTTAATACGACTCACTATAGGGAGACATCGAAC	Addition of T7 promoter sequence to dsARG target
ARG-RNAi-R	TATAGGGAGTAGATAACTACGATACGGGAGGG	Initial amplification of dsARG target
ARG-RNAi RT7	TTTAATACGACTCACTATAGGGAGTAGATAAC	Addition of T7 promoter sequence to dsARG target
ACPR-3'F2	GTTGGATCGGTGCTTTGCTGTGAT	Initial amplification of ACPR target for FISH
ACPR-3'F2-T7	AATTGTAATACGACTCACTATAGGGCGGTTGGATCGGTGCTTTGCTGTGAT	Addition of T7 promoter sequence to ACPR target for FISH
ACPR-R	TGGACCTCCTCTGGGCTGCGTT	Initial amplification of 2nd ACPR target for FISH
ACPR-R-T7	AATTGTAATACGACTCACTATAGGGCGTGGACCTCCTCTGGGCTGCGTT	Addition of T7 promoter sequence to 2nd ACPR target for FISH
ACP-F2	ATGTGTTCTCTAAGGCGAAATAGC	Amplification of ACP target for FISH
ACP-R2	TTACAGGTGCCCATTCGAA	Amplification of ACP target for FISH
AedaeACPR-qPCR-F-T7	AGGAATGGCAGCACCGS	Addition of T7 promoter sequence to 2nd ACPR target for FISH
AedaeACPR-qPCR-F	GGGATGCGACTTCGTTGTA	qPCR amplification of AedaeACPR-I and initial amplification of 2nd ACPR target for FISH
AedaeACPR-qPCR-R	TCGCGGTCAAACATGTACC	qPCR amplification of AedaeACPR-I
AedaeACP-qPCR-F[b]	ATGTGTTCTCTAAGGCGAAATAGC	qPCR amplification of AedaeACP
AedaeACP-qPCR-R[b]	TTACAGGTGCCCATTCGAA	qPCR amplification of AedaeACP

Results

ACP-like immunoreactivity

Wholemounts of central nervous systems from four day old adult male and female mosquitoes revealed ACP-like immunostaining in two pairs of lateral neurosecretory cells and their axons in the anterior protocerebrum (Fig. 2A). Staining in these cells was abolished in the ACP preabsorbed antiserum control treatments (Fig. 2B). Staining of two to three cells was also observed in the thoracic ganglia on the ventral side of the prothoracic segment just anterior to the junction between the pro and meso segments of the ganglia (Fig. 2C, E) which was abolished in the ACP preabsorbed controls (Fig. 2D). No ACP-like immunostaining was observed in the abdominal ganglia (Fig. 2F).

A. aegypti ACP and ACPR transcript localization

Assessment of cell specific expression of *A. aegypti ACP* and *ACPR* was accomplished using fluorescence *in situ* hybridization (FISH). Sense and antisense probes were generated for *ACP* and *ACPR* (Fig. 3) The probe for *ACP* targeted the ORF with an expected product size of 333bp plus 74bp, the latter distance representing the region between the T7 promoter and the multiple cloning site of the pGEM-T-Vector. Consistent with the expectations, there is a band under the 500bp marker for both the antisense (Fig. 3A) and sense (Fig. 3B) probes targeting *ACP*. Two probes were designed to target *ACPR*, the first probe encoded by primers 3'F2 + ACPR R RNAi and the second probe is encoded by ACPR-qPCR-F and ACPR-R (see Table 5). The expected product size for the first probe is 343bp and the expected product size for the second probe is 322 bp. Consistent with the expectations, there are bands around the 350bp range for the antisense probe 1 (Fig. 3C) and antisense probe 2 (Fig. 3D). To confirm the band was RNA rather than the template DNA, an equal dilution of template DNA was run alongside the

RNA probe, and the DNA band is visibly less intense than the lanes loaded with the RNA probe synthesized by *in vitro* transcription. Similar findings were observed for both sense probes targeting ACPR (Fig. 3E).

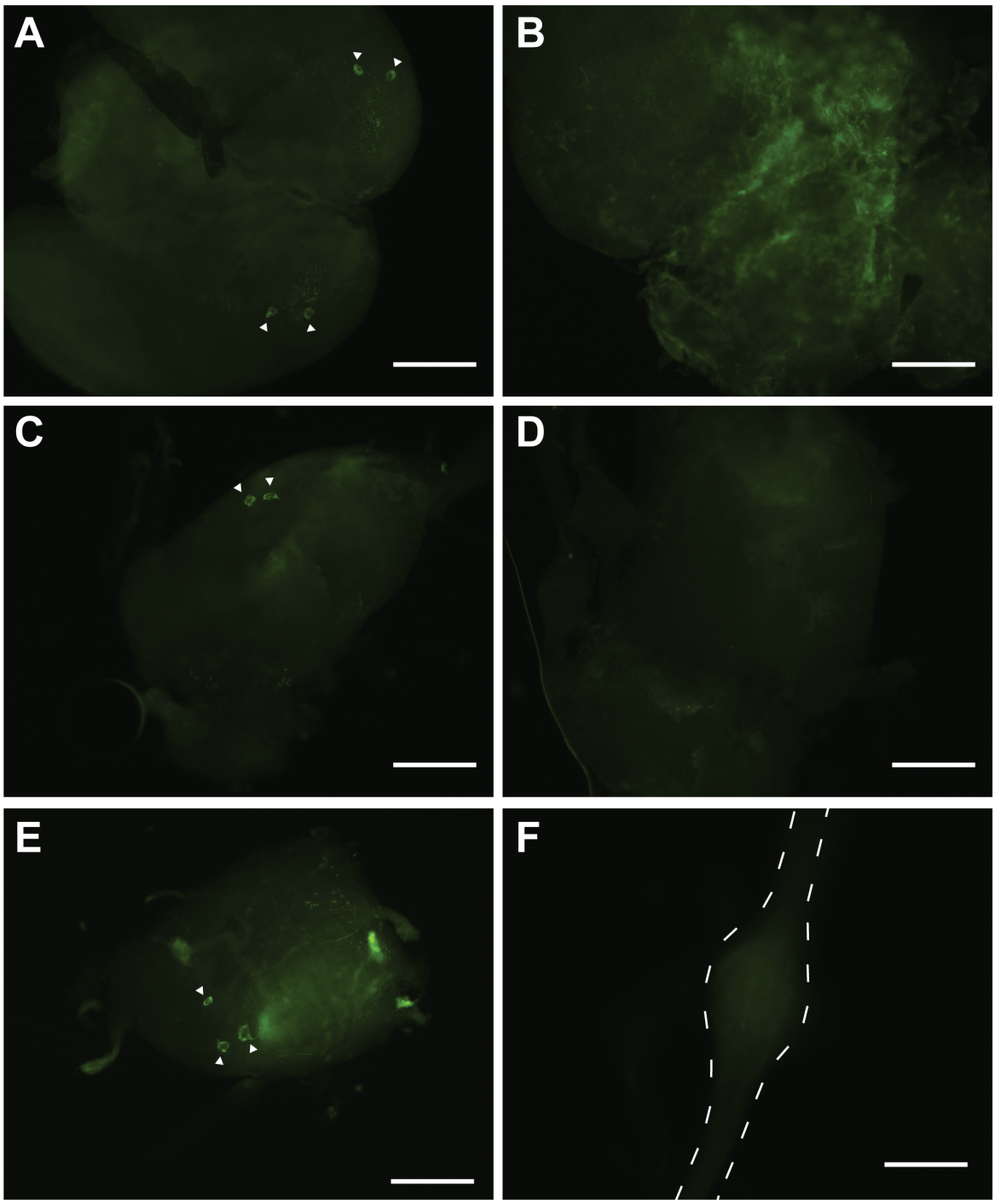

Figure 2. Immunolocalization of *Aedae*ACP in nervous tissue of adult *A. aegypti* (sucrose fed) with the *Drome*AKH antiserum. ACP-like immunoreactivity (indicated by white arrowheads) was observed in two pairs of lateral neurosecretory cells in the brain (A) and two to three cells in the thoracic ganglia (C, E). Antiserum preabsorbed with *Aedae*ACP showed no staining in the brain (B) or thoracic ganglia (D). No immunostaining was observed in the abdominal ganglia (F). All images were taken at the same exposure. Scale bars A-E, 100µm, F, 50 µm.

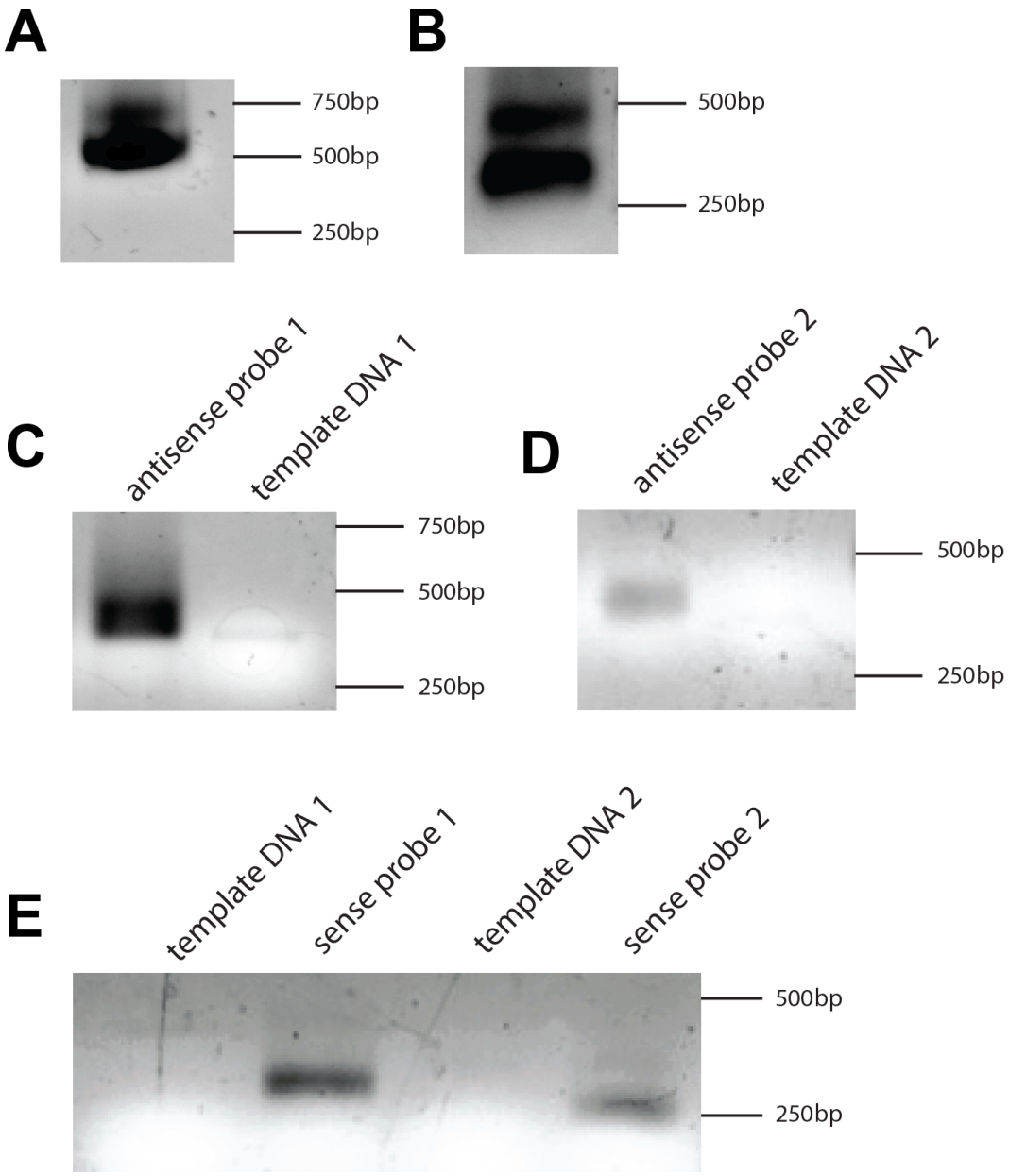

Figure 3. *AedaeACP* and *AedaeACPR* RNA probes. ACP antisense (A) and sense (B) probes produced by primers ACPF2/ACPR2. Antisense probes 1 produced by primers ACPR-3'F2/ACPR-R RNAi (C) and 2 produced by primers ACPR-qPCR-F/ACPR-R (D) targeting *ACPR*. An equal dilution of template DNA was run beside the RNA probe to confirm ssRNA probe synthesis. Complementary sense probes 1 and 2 targeting ACPR are shown in panel E. Samples were run on a 1% agarose gel.

The CNS, including the brain, thoracic ganglia, and six abdominal ganglia of the ventral nerve cord was surveyed for *AedaeACP* and *ACPR* transcript. Similar to the *Aedae*ACP localization pattern, wholemounts of nervous tissues from four day old adult male and female mosquitoes revealed fluorescence signals corresponding to *ACP* mRNA transcript in two pairs of lateral neurosecretory cells in the anterior protocerebrum and more specifically the supraesophageal ganglion (Fig. 4A). Fluorescence in these cells were not observed in the sense probe control (Fig. 4B). Similar to *Aedae*ACP immunolocalization, fluorescence signals were observed in two to three cells were also observed in the thoracic ganglia on the ventral side of the prothoracic segment just anterior to the junction between the pro and meso segments of the ganglia (Fig. 4C, E) which was not observed in the *ACP* sense probe controls (Fig. 4D). No *ACP* transcript fluorescence was observed in the abdominal ganglia (Fig. 4F).

AedaeACPR transcript was localized to the abdominal ganglia of one day old adult male and female mosquitoes. Similar staining was observed in both male and female mosquitoes. Findings indicate *ACPR* transcript in two pairs of cells positioned laterally on either side of the abdominal ganglia (Fig. 5, 6A). Interestingly, these cells appeared to be on two different focal planes of the ganglia with one pair positioned more dorsally while the other positioned more ventrally (Fig 5). Both pairs of neurosecretory cells were observed for both antisense probes (Probe 1 Fig. 4AB, Probe 2 Fig. 5CD). Interestingly, no staining was observed in the terminal (6^{th}) abdominal ganglia. No specific fluorescence was observed in the sense probe (2) (Fig.65B) or no probe controls (Fig. 6C). Similar findings were observed for the sense probe (1) control (data not shown). No fluorescence signals were observed in the thoracic ganglia, nor any specific signals in the brain of adult mosquitoes with either antisense probe (data not shown).

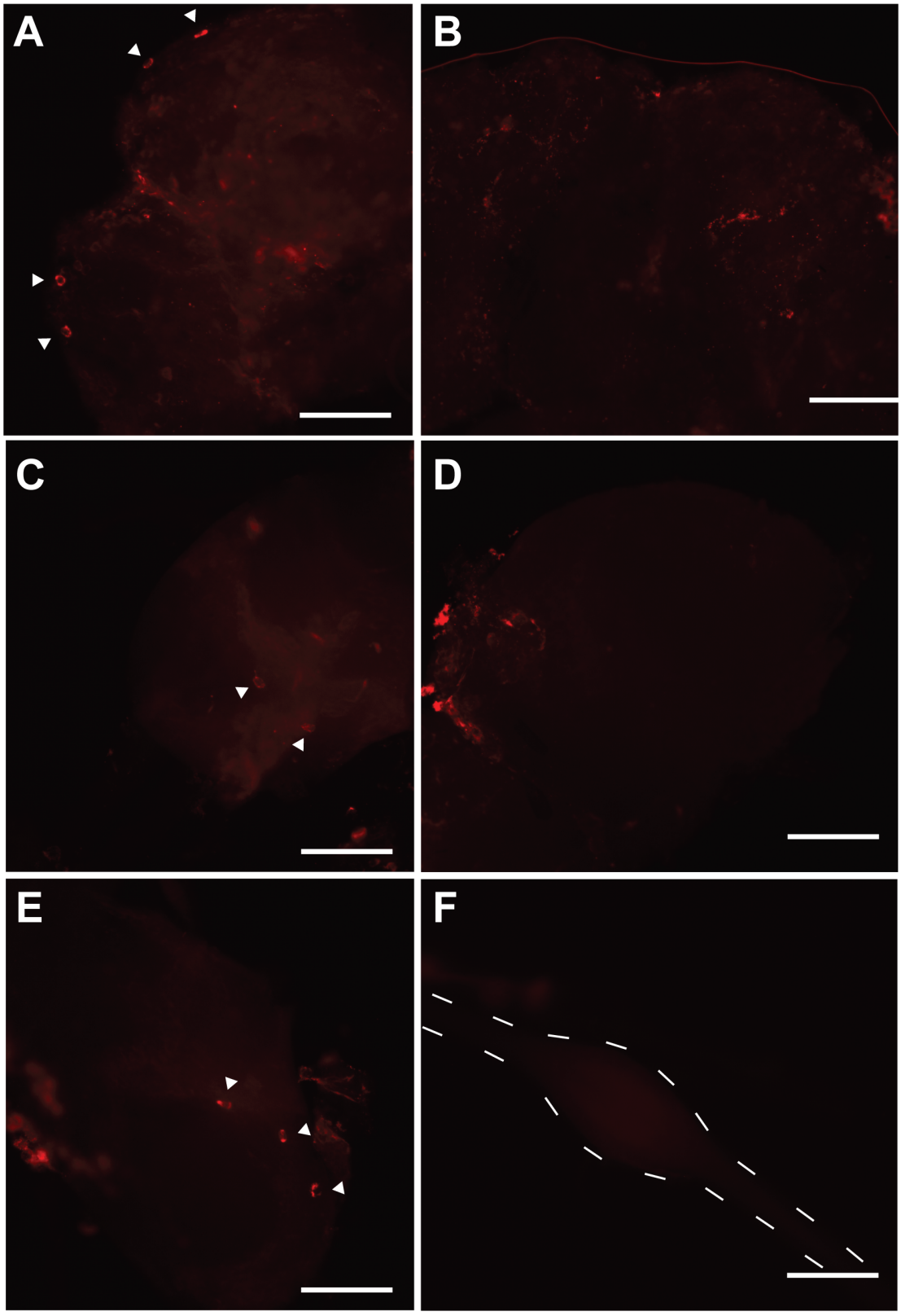

Figure 4. Distribution of *ACP* mRNA transcript in nervous tissue of adult *A. aegypti* (sucrose fed). *ACP* transcript (indicated by white arrowheads) was observed in two pairs of lateral neurosecretory cells in the brain (A) and two to three cells in the thoracic ganglia (C, E). *ACP* sense probe showed no staining in the brain (B) or thoracic ganglia (D). No fluorescence was observed in the abdominal ganglia (F). All images were taken at the same exposure. Scale bars A-E, 100µm, F 50 µm.

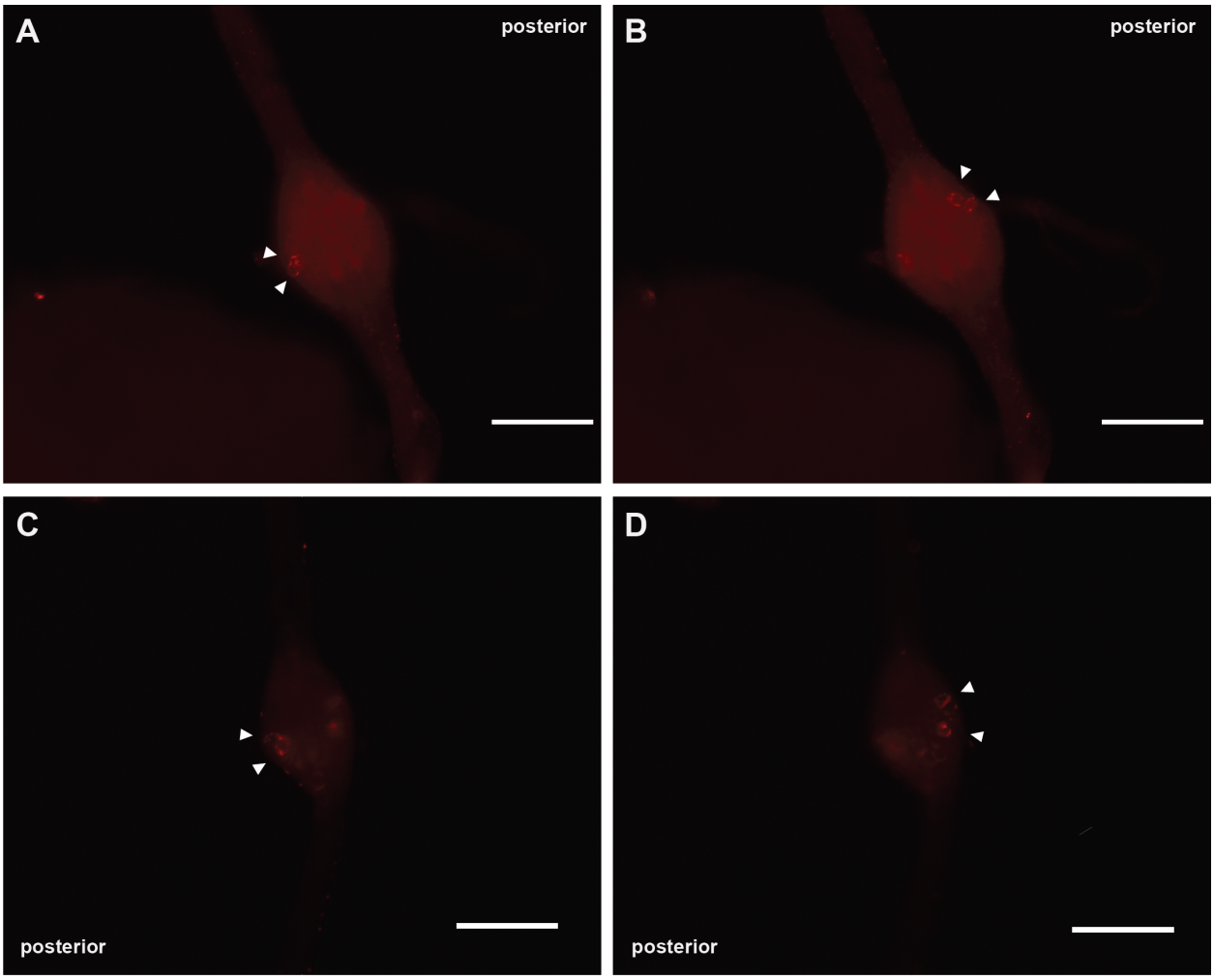

Figure 5. Distribution of *ACPR* mRNA transcript in nervous tissue of adult *A. aegypti* (sucrose fed). *ACPR* transcript (indicated by white arrowheads) was observed in two pairs of lateral neurosecretory cells in the abdominal ganglia. Panels (A) and (B) demonstrate the same ganglia in different focal planes utilizing antisense probe 1. Panels (C) and (D) demonstrate the same ganglia in different focal planes utilizing antisense probe 2. All images were taken at the same exposure. Scale bars are 50 μm.

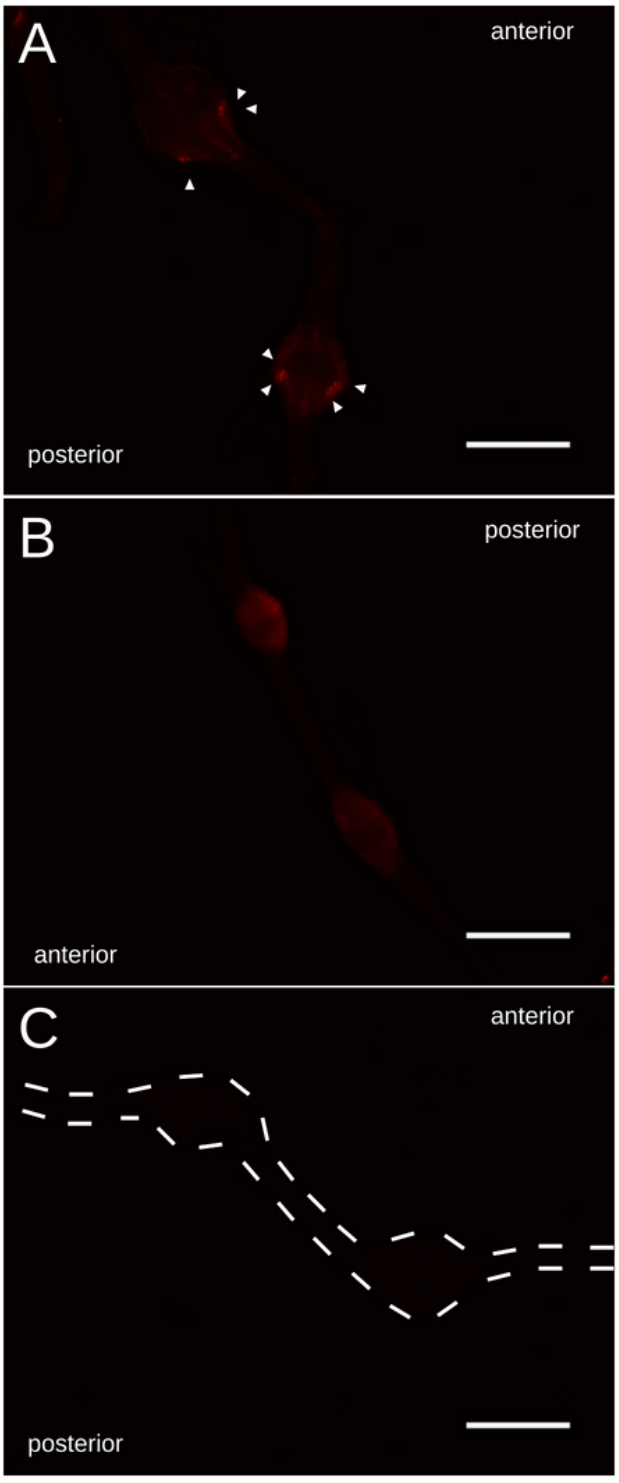

Figure 6. Validation of *ACPR* mRNA transcript distribution in nervous tissue of adult *A. aegypti* (sucrose fed). *ACPR* transcript (indicated by white arrowheads) was observed in two pairs of lateral neurosecretory cells in the abdominal ganglia. (A) antisense probe 2. (B) sense probe 2. (C) no probe control. All images were taken at the same exposure. Scale bars are 100 µm.

ACP and ACPR dsRNA knockdown

HT115 (DE3) dsACP feeding induced RNAi

To find a physiological role for the ACP system, *A. aegypti* larvae were fed dsRNA either in the form of bacteria or *in vitro* synthesized dsRNA to knockdown the ACP peptide and receptor. Initial attempts at RNAi focused on the knocking down the ACP peptide using the HT115 (DE3) cells, an RNase III deficient strain of *E. coli* as well as IPTG inducible T7 polymerase expression. ACP target 1 (product of F1/R3 primers), and ACP target 2 (product of F3/R1 primers) for dsRNA synthesis was cloned into the L4440 vector which possesses two T7 promoters in an inverted orientation flanking the MCS. Exposure of 3rd instar larvae to HT115(DE3) expressing either dsACP (1) or dsACP (2) did not result in knockdown of the ACP transcript levels in one day old adult male and female *A. aegypti* (Fig. 7A). There was no apparent reduction in band intensity in the two dsACP fed larval treatments when compared to the non-bacteria and empty L4440 vector fed controls.

Endogenous controls *rp49*, *rpL8*, and *rpS18* expression appeared equivalent as indicated by similar band intensities (Fig. 7A). Similar findings were observed even under chronic exposure of 3rd instar larvae to 4th instar larvae to ds*ACP* producing bacteria, whereby no reduction in *ACP* expression was evident in one day old adult *A. aegypti* when the experiment was repeated for three additional biological replicates (data not shown).

In vitro dsACP feeding-induced RNAi

The second method employed to knockdown *ACP* and *ACPR* transcripts utilized *in vitro* synthesized ds*ACP*, ds*ACPR*, along with ds*ARG* as a control. Synthesis of dsRNA corresponding to dsACP F1/R3 and F3/R1 resulted in expected bands at approximately 579bp and 547bp, with no bands observed in the no T7 polymerase controls as well as no template control (Fig. 8A).

dsACP F1R3 treated larvae resulted in a less intense band in adult male and females suggestive of *ACP* knockdown, whereas band intensity of ds*ACP* F3R1 adults mirrored those of control non-dsRNA exposed larvae (Fig. 7B). Endogenous control *rp49* expression in all treatments remained stable as indicated by similar band intensities. Based on this initial partially successful RNAi approach, future attempts at knocking down *ACP* transcript abundance utilized target 1 encoded by primers F1/R3.

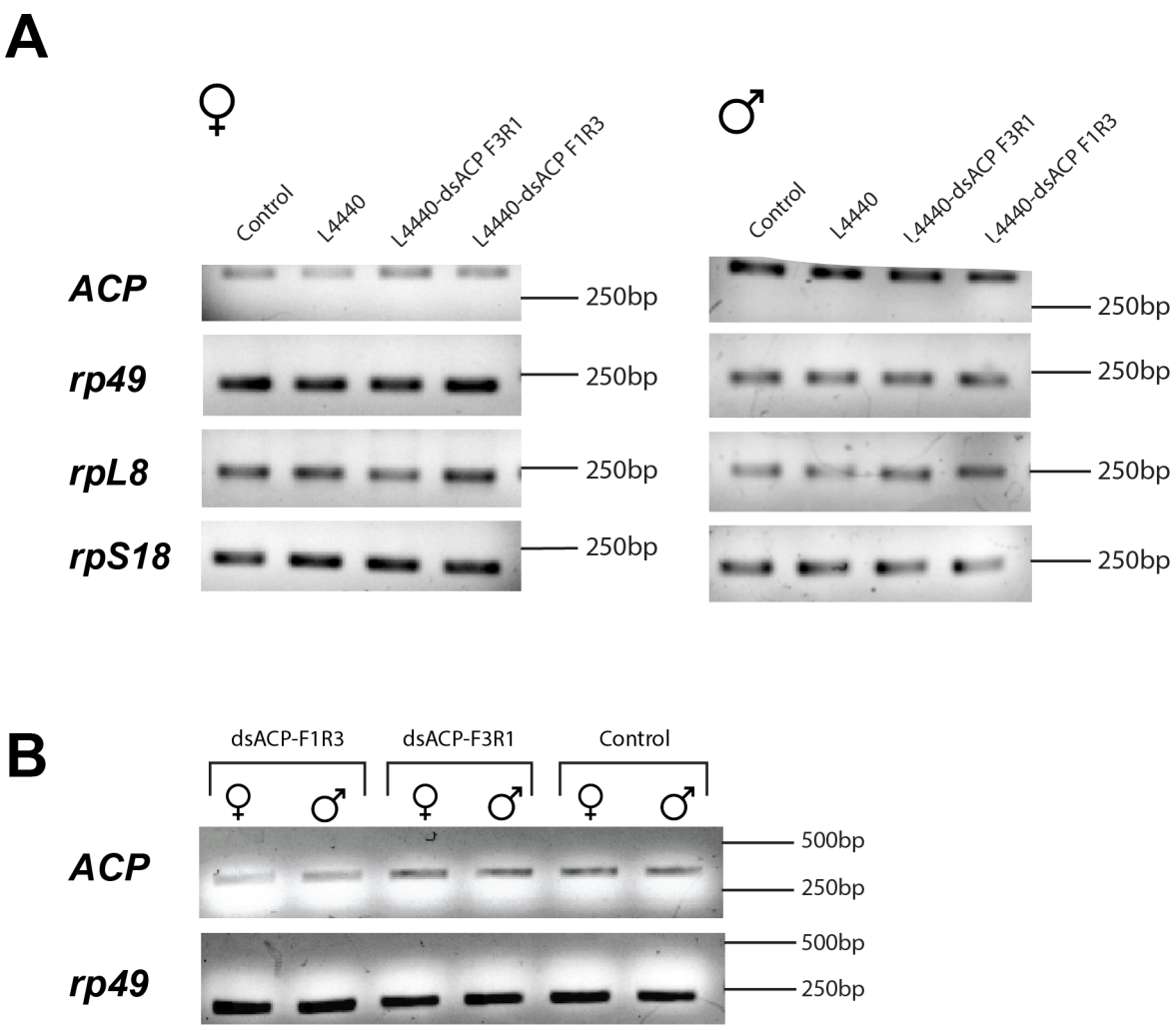

Figure 7. RT-PCR analysis of *ACP* expression shown in representative gels of ds*ACP* RNAi one day old adult male and female *A. aegypti*. (A) Top panel: 3rd instar larvae fed ds*ACP* (F1/R3 and F3/R1) expressing HT115(DE3) *E. coli*. Control treatments included bacteria expressing the empty L4440 vector, and non bacteria fed larvae. (B) Top panel: 4th instar larvae fed *in vitro* synthesized ds*ACP* (F1/R3 and F3/R1). Control treatment included larvae not exposed to dsRNA. Bottom panels are representative gels for endogenous controls *rp49* (A, B), *rpL8* (A), and *rpS18* (A) is included to demonstrate cDNA integrity of the samples. Samples were run on a 1% agarose gel.

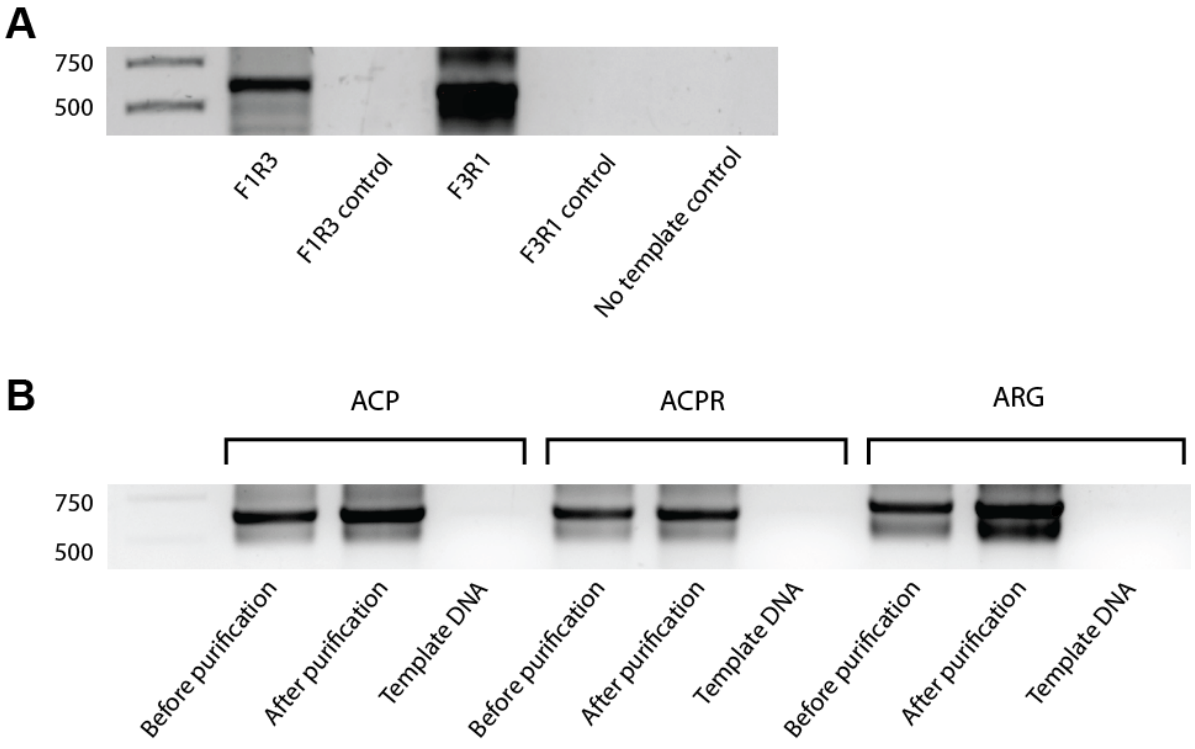

Figure 8. *AedaeACP* and *AedaeACPR* dsRNA products. (A) Double stranded ACP targets 1 (ACP-F1/ACP-R3) and 2 (ACP-F3/ACP-R1). No polymerase and no template controls were run to demonstrate synthesis of RNA. (B) Double stranded RNA targeting *ACP*, *ACPR*, and a control gene not expressed by *A. aegypti*, *ARG*. Samples before and after RNA purification were run showing presence of dsRNA target of interest. An equal dilution of template DNA was run to demonstrate successful synthesis of dsRNA. Samples were run on a 1% agarose gel.

Double stranded RNA targeting *ACP*, *ACPR*, and a control non-native gene *ARG* (ampicillin resistance gene) were synthesized and bands of expected sizes were 579 bp, 633bp, and 706bp, respectively (Fig. 8B). Although the first RNAi treatment resulted in an apparent knockdown of *ACP* in one day old adult mosquitos, subsequent feeding of 4th instar larvae with ds*ACP*, ds*ACPR*, and ds*ARG* did not consistently result in any change in *ACP* or *ACPR* expression levels in one day old adult female mosquitoes (Fig. 9A, B, respectively). Similarly, there was no significant change in *ACP* or *ACPR* expression levels in ds*ACP* and *ds*ACPR fed larvae compared to ds*ARG* and non-dsRNA fed control male *A. aegypti* (Fig 9C, D, respectively).

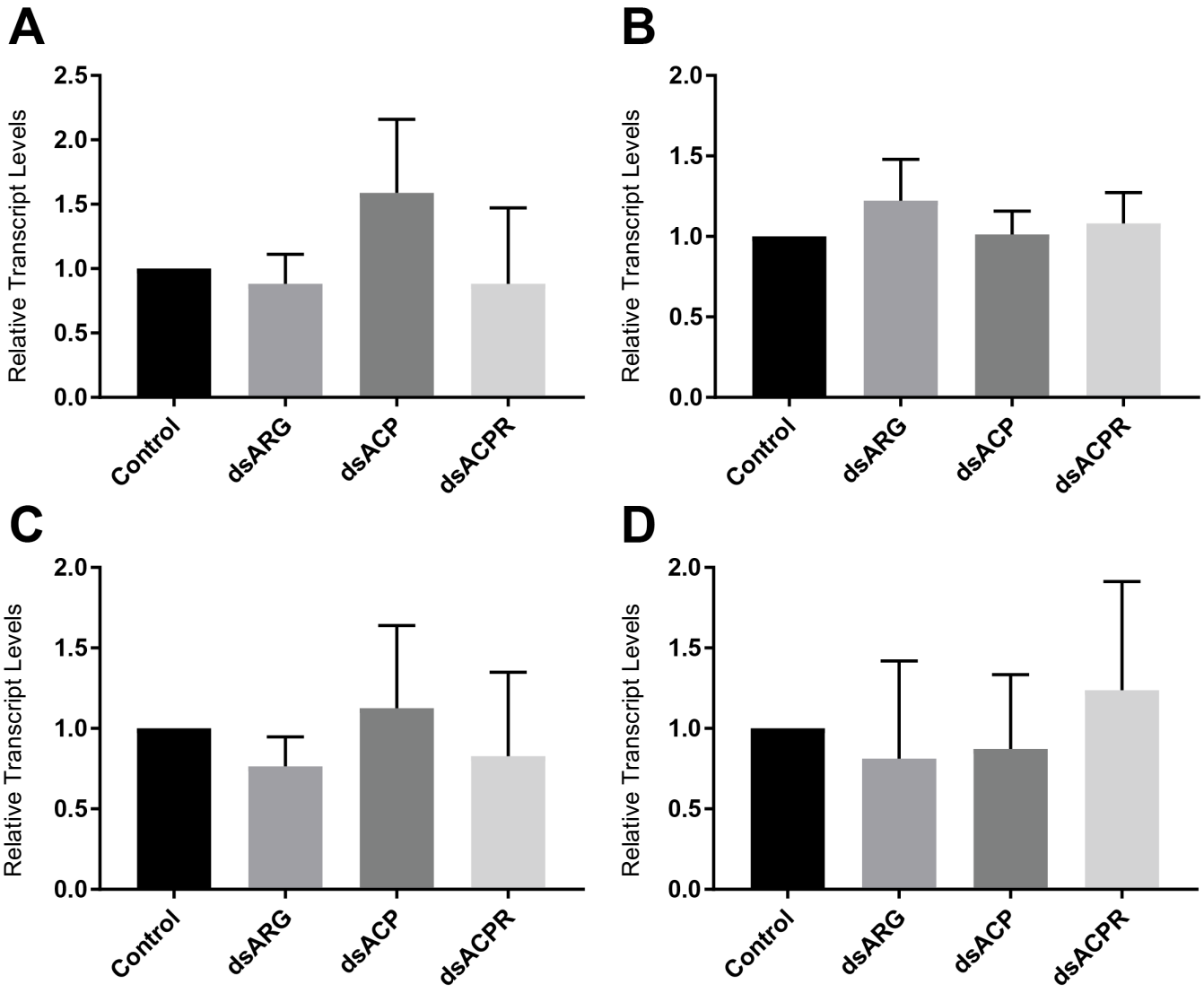

Figure 9. *AedaeACP* and *AedaeACPR* expression of dsRNA fed RNAi in one day old adult male and female *A. aegypti*. Female *ACP* (A) and *ACPR* (B) transcript expression and male *ACP* (C) and *ACPR* (D) transcript expression levels in control, dsARG, dsACP, and dsACPR exposed 4th instar larvae. Transcript expression levels are relative to control non-dsRNA fed larvae expression in one day old adults. Data is an average of 3-4 biological replicates and represent mean ± standard error.

Discussion

The adipokinetic hormone/corazonin-related peptide (ACP) signalling system is widespread throughout insects. Although the ACP peptide and receptor sequence demonstrates similarity to AKH/AKHR and CRZ/CRZR, a functional relationship between the three neuropeptide signalling systems has yet to be established. In the previous chapter the ACP receptor in *A. aegypti* was identified as well as its selectivity for its ligand, ACP. Enrichment of *ACP* and *ACPR* expression levels were identified in the CNS of adult mosquitoes. In the current study, cellular localization of the ACP peptide and transcript, along with the ACPR transcript was examined throughout the central nervous system of *A. aegypti*. This is the first report of the cell specific distribution of the ACP receptor in any insect. To attempt to elucidate the function of the ACP/ACPR signalling system within *A. aegypti*, RNAi was utilized to knockdown the peptide and receptor in *A. aegypti*. To induce RNAi targetting *ACP* and *ACPR* two methods were employed that included larval feeding of dsRNA producing HT115(DE3) *E. coli* or *in vitro* synthesized dsRNA.

Distribution pattern of ACP and ACPR in the CNS

Previous research localized *Aedae*AKHs I-II to the brain, corpora cardiaca, and thoracic ganglia of *A. gambiae* and *A. aegypti* (Kaufmann and Brown, 2006; Kaufmann et al., 2009). Indeed, *Aedae*AKH-II was later identified as *Aedae*-ACP, thus, the immunoreactivity observed in the brain and thoracic ganglia was attributed to ACP since previous studies has revealed synthesis and storage of AKH is restricted to the corpus cardiacum (Bogerd et al., 1995; Diederen et al., 1987; Diederen et al., 2002; Hansen et al., 2010; Kaufmann and Brown, 2006; Kaufmann et al., 2009; Noyes et al., 1995). Here, ACP-like immunoreactivity was observed in two pairs of lateral neurosecretory cells in the anterior region of the protocerebrum, consistent

with previous findings in *A. aegypti* and *A. gambiae* (Kaufmann and Brown, 2006; Kaufmann et al., 2009). In the present study, two to three cell bodies were identified in the pro segment of thoracic ganglia, consistent with previous literature where ACP-like immunoreactivity was reported in one cell in the prothoracic segment of ten-day old female *A. aegypti* (Kaufmann et al., 2009) and three clusters of cells within the pro and meso segments of the *A. gambiae* (Kaufmann and Brown, 2006) thoracic ganglia. In *T. castaneum*, ACP-like immunoreactivity was observed in 3- 4 neurons within the anterior region of each hemisphere of the brain with their axons projecting to the brain neuropil, thoracic ganglia, and abdominal ganglia (Hansen et al., 2010). *R. prolixus* ACP immunoreactivity was also observed in two bilaterally paired cell bodies located in the anterior protocerebrum (Patel et al., 2014). Axons from these cells project towards one pair of cells in the posterior region of the brain, which project ventrally and posteriorly out of the brain and throughout the CNS (Patel et al., 2014). No ACP-like immunoreactivity was observed outside of the CNS in either *T. castaneum* or *R. prolixus* (Hansen et al., 2010; Patel et al., 2014). The current study established that cell-specific ACP transcript localization paralleled ACP-like immunoreactivity in *A. aegypti*, with two bilateral pairs of neurosecretory cells in the anterior protocerebrum, thus resolving previous uncertainties in labelling of AKH/ACP-like immunoreactivity (Kaufmann and Brown, 2006; Kaufmann et al., 2009).

It is well established that AKH is found in the corpora cardiaca, and in line with this view, AKH has been previously immunolocalized to the CC of *A. aegypti* (Kaufmann et al., 2009), *L. migratoria* (Diederen et al., 1987; Diederen et al., 2002; Schooneveld et al., 1983; Schooneveld et al., 1985), *S. gregaria* (Diederen et al., 1987; Diederen et al., 2002), *A. gambiae* (Kaufmann and Brown, 2006), *R. prolixus* (Patel et al., 2014), and *D. melanogaster* (Isabel et al.,

2005; Nässel and Winther, 2010). AKH transcript localization utilizing *in situ* hybridization has been restricted to the CC in *L. migratoria* (Bogerd et al., 1995) and *D. melanogaster* (Noyes et al., 1995). In contrast, CRZ has been localized to neurosecretory cells within the brain that project towards the CC in *P. americana* (Veenstra and Davis, 1993), *R. prolixus* (Patel et al., 2014), *Protophormia terranovae* (Cantera et al., 1994) as well as a number of insects from six different orders (Roller et al., 2003). Expression of corazonin mRNA in the insect brain was confirmed by *in situ* hybridization in the CNS of the waxmoth *Galleria mellonella* (Hansen et al., 2001). The neurosecretory cells identified in the brain are situated in the pars lateralis and pars intercerebralis, a dorso-medial region of the protocerebrum rich in neurosecretory cells (Hartenstein, 2006). Given that ACP shares greater similarity to AKH, it is interesting that its distribution within the CNS is more closely related to previous reports of CRZ distribution.

Examining the distribution of the ACP receptor within the adult mosquito, and particularly in the CNS where it was previously found to be enriched (Wahedi and Paluzzi, 2018), may provide insight into the role of this neuropeptide signalling pathway. Within *A. aegypti* ACPR transcript was localized to two pairs of laterally positioned cells within first 5 abdominal ganglia. The distribution of the ACP receptor at the protein or transcript level has not been previously examined in any insect species. The AKH receptor has been localized to the fat body of *D. melanogaster* (Grönke et al., 2007), as is expected due to its metabolic function on energy compounds. The CRZ receptor has been localized to seven dorso-medially positioned cells on either side of the *R. prolixus* brain (Hamoudi et al., 2016). Localization of the *A. aegypti* ACP peptide within the brain and thoracic ganglia, and the receptor in the abdominal ganglia, which is a region more downstream in the CNS, is suggestive of a role for ACP within the CNS, perhaps functioning as a neuromodulator or neurotransmitter. Based on the ACP-like

immunoreactivity in *T. castaneum* and *R. prolixus* it was proposed that ACP is released within the CNS and a neurosecretory role for ACP was also suggested (Hansen et al., 2010; Patel et al., 2014).

The ACP-like immunoreactivity and ACP transcript expression observed in the thoracic ganglia suggests the presence of neurosecretory cells or interneurons. There are a variety of interneuronal cell types, such as local amacrine neurons, wide-ranging projection neurons, intersegmental ascending and descending neurons (Nässel and Homberg, 2006). Interneurons can be located within a single ganglion and synapse locally or they can send axonal processes throughout different ganglia and synapse with a far-reaching neuron. They are often implicated in the regulation of nervous system derived factors. In contrast, neurosecretory cells are generally monopolar with their axonal processes often projecting directly to peripheral tissues where their products are released (Nation, 2002). Limited literature exists on the presence of neurosecretory cells in the thoracic ganglia, thus the nature of the cells identified in the thoracic ganglia remains unclear. A representative schematic summarizes immunohistochemical and fluorescence *in situ* hybridization findings for ACP and ACPR (Fig. 10).

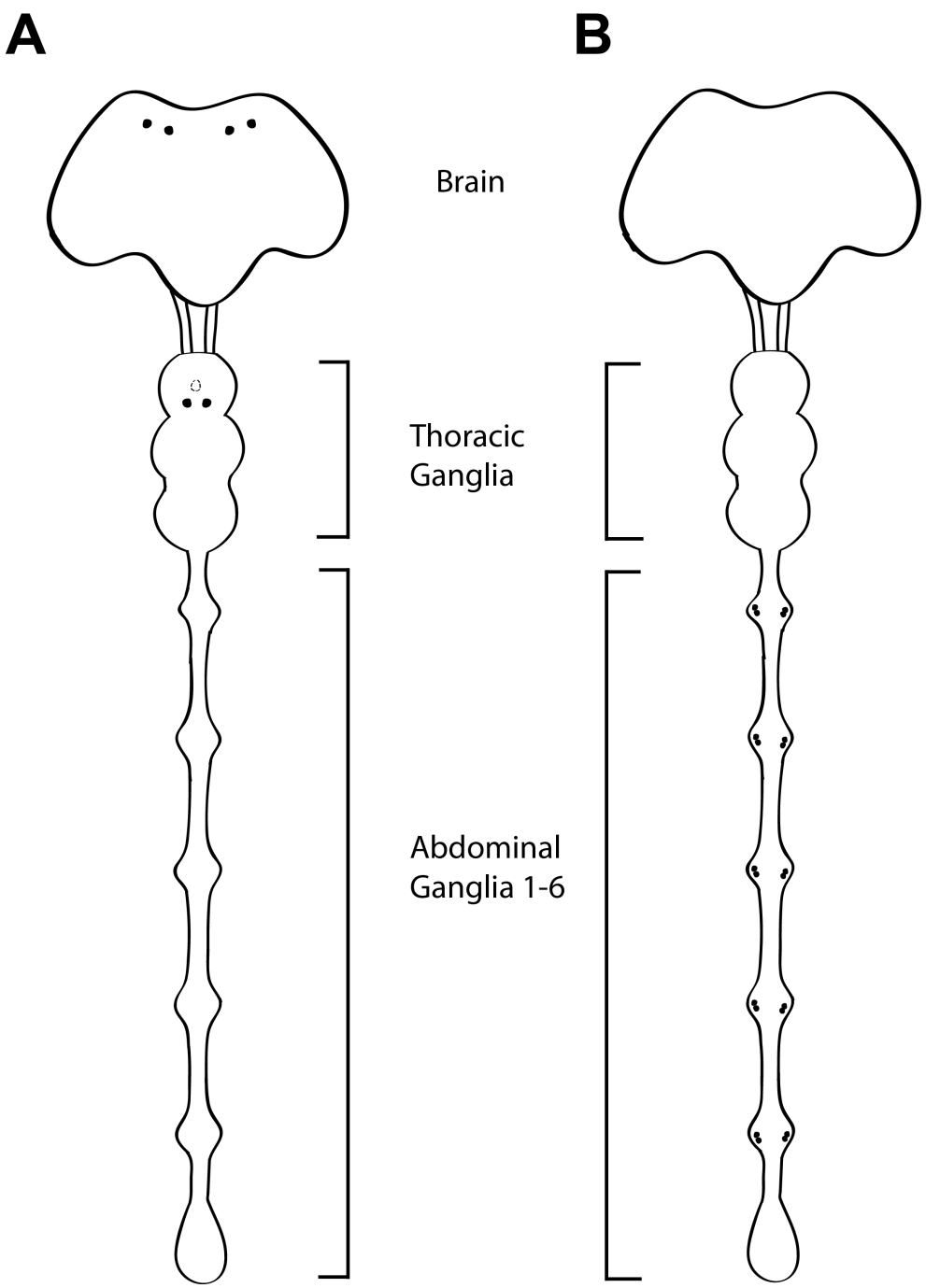

Figure 10. Schematic representation of ACP peptide and transcript (A) and ACPR transcript (B) distribution in the CNS of adult *A. aegypti*. Solid circles indicate somata with 100% frequency of detection.

dsRNA knockdown of ACP and ACPR

In an attempt to elucidate the physiological role of the ACP signalling system through observation of phenotypic differences, RNA interference was utilized in an attempt to knockdown expression of *ACP* and *ACPR* in adult *A. aegypti*. Mosquito larvae fed *E. coli* expressing *ACP* dsRNA failed to knockdown *ACP* expression levels in adult male and female *A. aegypti*. Lack of success with the first method prompted employment of the second, more traditional *in vitro* dsRNA feeding method. Although initial qualitative assessment of RT-PCR products showed evidence of *ACP* knockdown mediated by *in vitro* dsRNA feeding, further attempts which were monitored by qPCR, failed to knockdown *ACP* expression. Furthermore, *ACPR* expression was also unchanged in one day old adult male and female mosquitoes relative to controls. Thus, a physiological function could not be deduced since silencing of either *ACP* or *ACPR* was not successful. Feeding of larvae with either dsRNA directly or transgenic bacteria producing dsRNA was utilized to knockdown expression of either *ACP* or *ACPR* in early adult stage mosquitoes as previous temporal expression analyses revealed elevated *ACP* and *ACPR* transcript abundance in one-day old mosquitoes, particularly in males (Wahedi and Paluzzi, 2018). The dsRNA feeding conditions utilized here were modified from protocols used to feed *Caenorhabditis elegans* nematodes (Kamath et al., 2001; Timmons et al., 2001). A recent study conducted by Whyard and colleagues using the HT115(DE3) cells to suppress select testis genes in adult male *A. aegypti* resulted in over 60% and up to 92% sterility of males (Whyard et al., 2015) . Although knockdown efficiency was not mentioned, percentage sterility post dsRNA feeding of testes-specific genes is representative of knockdown efficiency. Thus, the lack of *ACP* knockdown observed here, may not be due to the methodological approach but the target gene of interest. More specifically in this case, accessibility of the dsRNA to the neurosecretory cells

expressing *ACP* in the brain and thoracic ganglia. Similarly, feeding of larvae with *in vitro* synthesized dsRNA failed to alter *ACP* or *ACPR* expression levels, whereas Whyard and colleagues observed reduction in target genes in adult mosquitoes that emerged from larvae fed dsRNA (Whyard et al., 2015). Therefore, it is unclear whether the unsuccessful RNA interference is a result of the inability of the dsRNA to reach the nervous tissue or the target region of the *ACP* and *ACPR* genes were not suitable to mediate knockdown. Only one target for *ACPR* was chosen for RNAi thus future efforts should aim to target different regions of the gene. It must be noted that this was the only available target region that included exon 5, which is an area of the transcript that is exclusive to the functional *ACPR-I* variant and is localized upstream of the qPCR primers.

Studies involving RNAi, regardless of mode of dsRNA synthesis, have shown that dsRNA systemically spreads and can function across cell boundaries (Fire et al., 1998). Thus, the means by which the RNA is introduced to the organism (injection vs feeding) is not critical for gene inactivation (Tabara et al., 1998; Timmons and Fire, 1998). Therefore, initiation of RNAi is possible either by soaking larvae in *E. coli* expressing the target gene dsRNA or directly in a solution of dsRNA, as RNA can be absorbed through the gut by epithelial cells and be distributed to somatic tissues and the germ line (Tabara et al., 1998).

Knockdown of the ACP receptor in *T. castaneum* (55-65%) did not result in any differences in physical appearance, egg number, or mortality between the ACP receptor knockdown and control dsEGFP injected beetles (Hansen et al., 2010). The lack of significant phenotypic difference could be a result of insufficient silencing of the receptor, increased *ACP* expression to rescue the dsACPR effect, the redundancy of the ACP system when either AKH or/and CRZ are also present, or simply an inability to see small behavioural changes.

Summary: integrating the whole

The *A. aegypti* ACP receptor identified in this study demonstrates homology with previously identified ACP receptors and, like the bulk of neuropeptide receptors, is a canonical seven transmembrane domain GPCR. The receptor isolated and deorphanized herein, in common with orthologs identified in other insects such as *R. prolixus* (Zandawala et al., 2015), *T. castaneum*, *A. gambiae* (Hansen et al., 2010), demonstrates strong specificity for its ligand, ACP, and is not activated by structurally-related peptides, AKH and CRZ. Correspondingly, the AKHRs and CRZRs in *A. aegypti* (Oryan et al., 2018), *A. gambiae* (Hansen et al., 2010), *R. prolixus* (Hamoudi et al., 2016) are only activated by their native ligands, therefore illustrating the presence of these three similar yet independent signalling pathways. Furthermore, phylogenetic analyses suggests co-evolution of the AKH, ACP, and CRZ peptides and their receptors, all three of which derive from a common GnRH-like ancestor (Hauser and Grimmelikhuijzen, 2014). Receptor/ligand co-evolution is not unique to invertebrates, indeed, structurally similar oxytocin and vasopressin like peptides are present in all vertebrates (Stafflinger et al., 2008). The receptors for these neuropeptides are also structurally related, however, like ACP, AKH, and CRZ, oxytocin and vasopressin do not activate one anothers receptor (Birnbaumer et al., 1992; Kimura et al., 1992). Oxytocin and vasopressin, now possessing different actions, are both implicated in the control of social behaviour highlighting some aspects of the original function of the common ancestor from which they evolved (Donaldson and Young, 2008). This is what is suggested for the AKH and CRZ, and potentially ACP. Given that these neuropeptides co-evolved with their receptors, giving rise to coexisting systems that each have a specialized function, all three may harbor some aspects of the original function of their GnRH-like ancestor (Hansen et al., 2010). The absence of the ACP system in

some organisms as well as the loss of CRZ/CRZR in other organisms supports this idea, as the three systems may serve on another as backups whereby the presence of two is enough to maintain overall function.

Consistent with spatial expression profiles in *R. prolixus* (Zandawala et al., 2015) and *T. castaneum* (Hansen et al., 2010), ACP and ACPR transcript expression was primarily enriched in the CNS, and receptor expression was also observed in reproductive tissue of the kissing bug and mosquito. Findings from immunohistochemistry and FISH support spatial expression analyses of *ACP*, with *ACP* localization within the brain and thoracic ganglia of adult mosquitoes and no immunoreactivity detected in the abdominal ganglia. Further, *ACPR* distribution was identified solely in the abdominal ganglia (1^{st}-5^{th}) consistent with significant enrichment of the transcript in the ventral nerve cord measured by the earlier spatial expression analysis (Wahedi and Paluzzi, 2018). Immunohistochemical and FISH staining for ACP was consistent with previous literature and FISH localization of the ACPR transcript to neurosecretory cells in the abdominal ganglia is very suggestive of a neuromodulatory for ACP. Many neuropeptides have been identified in the abdominal ganglia of insects such as allatostatins, allatotropins, CAPA (or periviscerokinins), crustacean cardioactive peptide, leucokinins, bursicon, FMRF/FLRF amides, corticotropin-releasing factor diuretic hormones, pigment dispersing factor, pheromone biosysnthesis activating neuropeptides, pyrokinins, tachkinin-related peptides, insulin like peptides (Nässel, 2002). Thus, there is still work to be done to determine which particular neurosecretory cells in the abdominal ganglia, and particularly what product they synthesize, may be under the upstream control of ACP derived from either the brain or thoracic ganglia and mediated via the abdominal ganglia-localized ACPR.

Future directions

Isolation, functional characterization, and transcript expression and distribution analyses have identified the ACP system within *A. aegypti*, however no physiological role could yet be confirmed. Future research localizing the ACP receptor in the abdominal ganglia as well as reproductive tissue of adult mosquitoes will aid in uncovering the function of this neuropeptide pathway. Determining the intracellular signalling pathway and other downstream effects at the cellular level may uncover a neurotransmitter role. Additionally, further research should focus on identifying neuropeptides expressed in close proximity to the *ACPR* expressing neurosecretory cells in the abdominal ganglia that may associate with this pathway in a well-characterized downstream signalling system. Furthermore, research should focus on elucidating the potentially shared function (if any) between AKH, ACP, and CRZ. Previous literature has implicated both AKH and CRZ in nutritional and oxidative stress responses (Bednářová et al., 2015; Bharucha et al., 2008; Kubrak et al., 2016). Thus, perhaps ACP may share a stress-related function in insects, as it doesn't function in cardioacceleration or lipid mobilization, the most established functions to date for CRZ and AKH, respectively (Kaufmann and Brown, 2008; Patel et al., 2014; Siegert, 1999).

Currently, I have commenced RNAi injections into adult stage *A. aegypti* to silence *ACPR* and *ACP* expression and potentially uncover a role for ACP. I am also carrying out a lipid/carbohydrate assay following closely a protocol previously described for mosquitoes (Kaufmann and Brown, 2008), which will determine whether ACPs functional independence from AKH extends to *A. aegypti*. As previously mentioned, neuropeptides are prime targets for pest control strategies, and given the fact that ACP is missing in the honeybee *Apis mellifera* and *D. melanogaster* (Hansen et al., 2010), it may prove as an ideal target for insecticides.

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