FUNCTIONAL ELUCIDATION OF THE ADIPOKINETIC HORMONE/CORAZONIN RELATED PEPTIDE AND INSIGHTS INTO ITS RECEPTOR SPECIFICITY USING RECEPTOR CHIMERA IN *AEDES AEGYPTI* MOSQUITO

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Abstract

Mosquitoes *Aedes aegypti* are an essential organism to study as they are significant vectors responsible for transmitting arboviral diseases such as Yellow fever, Dengue and Zika virus. The adipokinetic hormone/corazonin-related peptide (ACP) is an insect neuropeptide that is structurally intermediate between corazonin (CRZ) and adipokinetic hormone (AKH). A well-known function of the AKH signaling system is the mobilization of energy while CRZ was initially described as a cardioacceleratory factor. Contrastingly, the role of ACP and its receptor (ACPr) remains unclear in many insects. Despite the structural similarity and the close evolutionary relationship between ACP and AKH, their signaling systems function independently due to the high selectivity of their corresponding receptors. In light of this knowledge, this thesis sought to advance our understanding of the role of the ACP in *A. aegypti* by mapping the distribution of ACP in the nervous system, examining the ACPr transcript expression in the abdominal ganglia, assessing the possible involvement of ACP in controlling energy substrate levels, and determining adult survival rate after injection with ACP. Moreover, this study aimed to determine the most critical regions of the ACPr necessary for ligand-binding specificity and discern specific residues critical for functional ligand-binding by singly replacing the three ACPr extracellular loops or critical highly conserved residues and incorporating those from the AKH receptor and testing these ACPr chimeras using a heterologous functional assay. Together, this study gives insight to advance our mechanistic understanding of the broad and critical functions of the ACP signaling system in *A. aegypti*.

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List of Abbreviations

ACP	adipokinetic hormone/corazonin-related peptide
ACPr	adipokinetic hormone/corazonin-related peptide receptor
AG	abdominal ganglia
AKH	adipokinetic hormone
AKHr	adipokinetic hormone receptor
ANOVA	analysis of variance
BSA	bovine serum albumin
CA	corpora allata
CC	corpora cardiaca
CCTOP	Consensus constrained topology prediction
cDNA	complimentary DNA
CHO	chinese hamster ovary
CNS	central nervous system
CRZ	corazonin
CRZr	corazonin receptor
DAG	diacylglycerol
DAPI	4’,6-diamidino-2-phenylindole
DH	diapause hormone
dH_2O	distilled water
DMEM	Dulbecco’s modified eagles medium
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
dsRNA	double-stranded RNA
ECL	extracellular loop
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FSH	follicle-stimulating hormone
GDP	guanosine diphosphate

GnRH	Gonadotropin-releasing hormone
GnRHr	Gonadotropin-releasing hormone receptor
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
LB media	lysogeny broth media
LH	luteinizing hormone
MIP	myoinhibitory peptide
mRNA	messenger RNA
NMR	nuclear magnetic resonance
NSC	neurosecretory cell
NSS	normal sheep serum
OD	optical density
OL	optic lobe
ORF	open reading frame
PBAN	pheromone biosynthesis-activating neuropeptide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PI	pars intercerebralis
PK1	pyrokinin-1
PK2	pyrokinin-2
PL	pars lateralis
qPCR	quantitative PCR
RNA	ribonucleic acid
RNAi	RNA interference
RPCH	red pigment-concentrating hormone
Rp49	ribosomal protein 49
Rpl8	60S Ribosomal Protein S18
RpS18	40S Ribosomal Protein L8
RT	room temperature
RT-PCR	reverse transcription PCR

RT-qPCR	reverse transcription qPCR
SAR	structure-activity relationship
SEG	subesophageal ganglion
SP	Sex peptide
TAG	triacylglycerol
TMDs	transmembrane domains
VNC	ventral nerve cord

Statement of Contributions

Chapter One

This chapter was written by S. Afifi with valuable guidance and editorial support from Prof. J.P. Paluzzi.

Chapter Two

This chapter was written by S. Afifi with valuable guidance and editorial support from Prof. J.P. Paluzzi. All experiments, data collection, and data analysis were performed by S. Afifi.

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Chapter Four

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Chapter 1:

General Introduction

1.1 Mosquitoes *Aedes aegypti*

Insects are one of the largest groups of animals on earth that play significant roles in ecosystems (Huang et al., 2010). Insects are essential as biomedical models due to the many discoveries in muscle contraction, digestion, as well as crucial developmental and metabolic pathways that could be applied to vertebrate systems (Law and Wells, 1989). Mosquitoes are considered one of the deadliest creatures on the earth since they are responsible for a considerable number of human deaths and illnesses every year (Kotsakiozi et al., 2017; Omodior et al., 2018). Diseases are transmitted when the mosquitoes ingest pathogens from an infected host during a blood meal and transfer these into their following host (Kuno and Chang, 2005). Generally, the body size of male mosquitoes is smaller than females (Christophers, 1960; Shettima et al., 2021). The lifespan of male adult mosquitoes is three to seven days on average, while the female mosquito with a sufficient food supply could live up to five months or longer, with an average lifespan of ~ six weeks (Christophers, 1960; Clements, 2000; Goindin et al., 2015).

Male *Aedes aegypti* adult mosquito behavior is different from the female mosquito as they feed only on the plant nectar and do not transmit disease or bite. On the other hand, the female mosquitoes, in addition to feeding on plant nectar, must bite to get a blood meal (Briegel, 1985, 1986; Clements, 2000; Shettima et al., 2021). The blood meal provides a source of protein which is essential to mature their eggs (Briegel, 1986, 1985; Shettima et al., 2021). To find the female *A. aegypti* mosquitoes their preferred host, they are attracted to chemical compounds released by

mammals, including carbon dioxide (CO_2) and ammonia (Bohbot et al., 2013; Ghaninia et al., 2019). Therefore, the females *A. aegypti* are typically excessively studied because of their significant contribution to transmitting many arboviruses through their blood-feeding (Shettima et al., 2021). The global world population growth, urbanization, and the escalating risk of importation through increased travel and international trade aided these vectors' worldwide spread and persistence (Neiderud, 2015). Since *A. aegypti* mosquitoes are extremely anthropophilic and thrive close to humans, they have become tremendously efficient in spreading and transferring arthropod-borne viruses (Powell and Tabachnick, 2013).

The *A. aegypti* mosquito is an infamous disease vector for pathogens such as Dengue, Yellow fever, Chikungunya and Zika virus (Saiz et al., 2016; Kotsakiozi et al., 2017). Dengue is one of the most prominent of these diseases, which is responsible for affecting ~50 -100 million people per year worldwide (Price et al., 2015). Additionally, the Chikungunya virus affected about 1.6 million individuals during an epidemic outbreak in Reunion Island and India, placing even more people at risk (Mavalankar et al., 2008). Moreover, an enormous emphasis on *A. aegypti* function in the transmission of the Zika flavivirus, a virus that causes microcephaly in newborn babies characterized as a small head abnormality due to the incomplete development of the brain and other neurological problems (Huang et al., 2016). The Zika virus was also notably widespread northwards to the United States, and it became well-established in Brazil as well (Saiz et al., 2016). Furthermore, active Zika virus circulation was reported in most of the Caribbean and Latin American populations (Baud et al., 2017).

The incidence of diseases vectored by the *A. aegypti* mosquito has risen over decades, with a decline in the development of insecticides (Vézilier et al., 2013). Furthermore, as a consequence of mosquitoes becoming more resistant, the traditional pesticides and insecticides utilized to

control insect pest populations gradually became ineffective (Ranson et al., 2011). Therefore, enriching our understanding and studying these organisms might be invaluable in developing and creating novel vector control strategies to limit pathogen transmission in nature and their population growth. On top of that, it might help develop vaccines against viruses transmitted by the *A. aegypti* mosquito as well as reduce the incidence of these vector-borne diseases.

1.2 Neuropeptides

Living organisms use a wide variety of signaling molecules which play a role in modulating the activities of their systems to maintain homeostasis (Nässel, 2002). A fundamental class of signaling molecules are the neuropeptides, which are implicated in transmitting and processing the information from the nervous system to the effectors (e.g. muscles, glands) (Mercier et al., 2007). Neuropeptides are small signaling molecules in multicellular organisms synthesized as precursors in neuroendocrine cells and neurons (Llopis-Giménez et al., 2019; Russo, 2017). These precursors are modified and processed into mature peptides that bind to their receptors in membranes of target cells and tissues where they may perform as neurotransmitters, neuromodulators, and neurohormones (Altstein and Nässel, 2010). Their neurohormonal role begins the following secretion into the haemolymph to initiate a regulatory or compensatory response in a different site inside the organism (Nässel, 2002). Moreover, neuropeptides may act as neurotransmitters due to their capability to transfer electrical signals from one neuron to another (Fieber, 2019; Mercier et al., 2007). Further, neuropeptides could also act as neuromodulators that decrease or enhance electrical signals (Mercier et al., 2007). Thus, neuropeptides play a vital part in the chemical cell-cell communication systems between the different cells (Barón et al., 2010; Llopis-Giménez et al., 2019; Nässel and Winther, 2010).

Mature neuropeptides bind to their corresponding receptors to stimulate a response that activates intracellular effectors, eventually causing physiological changes inside the organism (Hoyer and Bartfai, 2012). Most of the receptors for neuropeptides are G protein-coupled receptors (GPCRs), whose structure has been well-identified over time (Gilman, 1987; Nässel and Winther, 2010). Specifically, GPCRs display a typical architecture consisting of seven-transmembrane domains (TMDs), each comprising 20 to 30 hydrophobic amino acids and characterized by an intracellular C-terminus and an extracellular N-terminus (Gilman, 1987; Vanden Broeck, 1996). Moreover, it has been demonstrated that the extracellular loops (ECLs) play a vital role in ligand-binding recognition and activation of the GPCRs (Wheatley et al., 2012). The heterotrimeric G-protein that binds to the C-terminal domain of the GPCR consists of three subunits, including alpha (α), beta (β), and gamma (γ) subunits (Pierce et al., 2002). A conformational change occurs during receptor activation as the guanosine diphosphate (GDP) bound to the G protein is substituted with a guanosine triphosphate (GTP) molecule (Mercier et al., 2007).

Neuropeptides are considered functionally and structurally the most diverse group of signaling molecules in the nervous system (Nässel, 2002). The earliest traces of neuropeptides are thought to date back to the cnidarians, which are one of the most primitive metazoans (Grimmelikhuijzen et al., 1996; Nässel, 2002). The importance of neuropeptides in insects resides in regulating various aspects of behavior and physiology including reproduction, osmoregulation, locomotion, metabolism, pigment synthesis, color change, feeding, diapause, control of the heart, visceral and skeletal muscle and development (Barón et al., 2010; Llopis-Giménez et al., 2019; Nässel, 1993; Nässel and Winther, 2010). The neurosecretory system of insects consists of neurosecretory cells (NSCs) located within the brain, subesophageal ganglion (SEG), and the ventral nerve cord (VNC) that houses the ganglia of the stomatogastric nervous system and the

abdominal ganglia (AG) (Hartenstein, 2006). The brain and the SEG are fused together in the mosquitoes; the NSCs send their axons to stimulate neurohaemal organs, such as the corpora allata (CA) and corpora cardiaca (CC), in order to release neuroactive products into the haemolymph (Bräunig, 1987; Hartenstein, 2006; Nässel, 1996).

Insects are great models for studying and understanding neuropeptidergic systems as they are easy to use for laboratory experiments due to their versatility and simplicity (Predel and Eckert, 2000). Numerous studies of the neuropeptidergic system have utilized several insect orders (e.g. Coleoptera, Hymenoptera, Blattodea, Lepidoptera, and Hemiptera), with particular attention to those insect species that cause phytosanitary crises in human environments or crops (Riehle et al., 2002; Roller et al., 2008). These studies reinforce our understanding of the environmental plasticity of insects and their adaptation to adverse circumstances and different environments (Hawthorne, 1997). Moreover, these studies might provide new targets for developing novel insecticide agents (Geary and Maule, 2010; Van Hiel et al., 2010). Thus, the characterization of these neuropeptides and their cognate GPCRs could be utilized to design insecticides and pest control agents with high specificity.

1.3 Gonadotropin-releasing hormone

The protostomes and deuterostomes are believed to have diverged back to ~700 million years ago, and the majority of metazoans belong to one of these two evolutionary lineages (Douzery et al., 2004). Although neuropeptide signaling is abundant throughout both lineages, it is usually not obvious if evolutionary relationships exist between the neuropeptides used by the deuterostomes and protostomes. One exception is the well-known Gonadotropin-releasing hormone (GnRH) signaling pathway (Roch et al., 2011). The GnRH was initially discovered in

sheep (Amoss et al., 1971) and pigs (Matsuo et al., 1971) and isolated from the vertebrate hypothalamus that controls the anterior pituitary gland secretions. In humans and other vertebrates, GnRH regulates the reproductive function and maturation via its control of synthesis and release of the gonadotropins, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Kah et al., 2007). The GnRH signaling pathway has been identified as GnRHs in deuterostomes and as GnRH-like peptides in protostomes, red pigment concentrating hormone (RPCH), adipokinetic hormone (AKH), corazonin (CRZ), and adipokinetic hormone/corazonin-related peptide (ACP) (Roch et al., 2011).

The RPCH, a crustacean hormone isolated from the *Pandalus borealis* shrimp, was the first GnRH-like peptide to be isolated and sequenced from invertebrates (Fernlund and Josefsson, 1972). In crustaceans, the RPCH peptides have an essential role in pigment granule concentration in the integumental cells, pigment movement in the eye cells, as well in regulating the stomatogastric system and swimmeret rhythms (Rao, 2001; Gäde and Marco, 2006). Additionally, the RPCH receptors were found concentrated in the pigment granules of the epidermal chromatophores, giving the characteristic intense red color to crustaceans (e.g. crabs) (Rao, 2001). Shortly after the RPCH discovery, a related peptide was isolated and purified from corpora cardiaca (CC) of the locusts *Locusta migratoria* and *Schistocerca gergaria* due to its role in lipid mobilization, known as adipokinetic hormone (AKH) (Mayer and Candy, 1969; Stone et al., 1976). Following that, an AKH-like peptide was identified and isolated from the cockroach *Periplaneta americana* and, as the result of its cardio-acceleration effects, was named corazonin (CRZ) (Veenstra, 1989). Over two decades later, another neuropeptide was discovered, a structural intermediate between AKH and CRZ peptides, that became known as adipokinetic

hormone/corazonin-related peptide (ACP) (Hansen et al., 2010). The following discussion will be focused on the AKH, CRZ, and ACP signaling systems.

1.4 Adipokinetic hormone

Adipokinetic hormones (AKHs) were one of the first neuropeptides to be purified and isolated in insects (Stone et al., 1976; Diederen et al., 1987, 2002). Moreover, AKHs are among one of the most extensively characterized peptide hormones in insects, as they have been described in ~40 family members from the major orders of insects (Schaffer et al., 1990; Oudejans et al., 1991; Gäde et al., 1994, 2006). AKH is produced and released by neurosecretory cells of the corpora cardiaca (CC), a neuroendocrine gland located behind the insect brain (Diederen et al., 2002) that is homologous to the mammalian anterior pituitary gland. Initially, the AKHs were described as hyperlipemic, and hyperglycemic factors in the CC extracts from the cockroach *P. americana* and the locust *S. gregaria*, respectively (Steele, 1961; Mayer and Candy, 1969). The structure of AKH was not clearly identified until 1976, where the AKH was first isolated, sequenced and purified from the CC of the locust, *L. migratoria* (Stone et al., 1976). Despite AKH sequences showing some variability between insect species, some hallmark features were determined, including a peptide consisting of 8-10 amino acids with an amidated C-terminus and pyroglutamate at the N-terminus (Gäde et al., 1997; Zhu et al., 2009).

AKH was termed "adipokinetic" due to its primary function of regulating lipid from the insect fat body (an organ functionally analogous to the mammalian adipocytes and liver due to its role in utilization, detoxification, and energy storage), which is essential during energy-requiring expensive activities such as locomotion and flight (Mercier et al., 2007; Liu et al., 2009). The insect fat body is distinguished as a loosely formed tissue located beneath the epidermis, and its

primary function is to store carbohydrates and lipids (Martins and Pimenta, 2008). During the reception of stress signals, the insect nervous system releases AKHs from the CC, which then bind with their receptors on the fat body and promote the release of several energy-rich compounds (Gäde and Auerswald, 2003). These energy-rich compounds include trehalose, diacylglycerols (DAG), and in some cases proline, that are released into the haemolymph to fuel their activities (Gäde and Auerswald, 2003).

The function of AKH in metabolism was first described as a carbohydrate/lipid mobilizing hormone in migratory locusts and cockroaches; however, only years later did Stone and colleagues succeed in identifying and purifying the AKH peptide (Steele, 1961; Mayer and Candy, 1969; Stone et al., 1976). Afterward, studies were conducted on the kissing bugs (also known as triatomine bugs) *Rhodnius prolixus* and *Triatoma infestans* by Ward and his research group, had noticed that after 60 minutes of the bug's flight activity, the kissing bugs began to show an increase in the level of free fatty acids in their haemolymph and a decrease in the concentration of lipid in their flight muscles (Ward et al., 1982). This finding was confirmed by further research done on *R. prolixus* and *T. infestans* by Marco's group, who demonstrated that after injection with 10 pmol synthetic AKH, a significant increase in lipid levels in the haemolymph was observed (Marco et al., 2013). Similarly, lipid mobilization and elevation in the trehalose levels in circulation were observed after the species-specific AKH injections occurred in the locust *L. migratoria* and the cockroach *P. americana*, respectively (Scarborough et al., 1984; Oguri and Steele, 2003; Van der Horst, 2003). The mobilization of proline was detected in several beetles, such as dung beetles *Scarabaeus* spp. and fruit beetles *Pachnoda sinuata* (Gäde and Auerswald, 2002). In *R. prolixus*, RNA interference (RNAi) mediated knockdown of the AKH receptor (AKHr) resulted in a

significant increase in the level of triacylglycerol (TAG) in both the flight muscle and fat body tissues (Alves-Bezerra et al., 2016).

In addition to the fundamental function of mobilizing metabolites during energy-requiring processes, AKH has been shown to be involved in controlling carbohydrate homeostasis in the haemolymph of the fruit fly *Drosophila melanogaster* and silkworm *Bombyx mori* larvae (Oda et al., 2000; Kim and Rulifson, 2004). Furthermore, many studies observed that AKH plays a crucial role in insect development, behavior, reproduction and diapause (Lorenz, 2003; Hahn and Denlinger, 2007). Despite the structural unrelatedness between the AKH neuropeptides and glucagon, AKH in insects has a similar functional role to adrenaline and glucagon in mammals (Ziegler et al., 1984; Bednářová et al., 2013). Additional roles of the AKHs have been discovered, such as the stimulation of heart-beat rate, regulation of oxidative stress, inhibition of protein synthesis, and life span extension (Gäde and Marco, 2006; Zandawala et al., 2018).

AKH in insects is deemed functionally equivalent to the vertebrate hormone glucagon as it promotes a fast mobilization of lipids and carbohydrates from the fat body during starvation (de Brito Sanchez et al., 2021). A recent study in *Drosophila* demonstrated that AKH signals are involved in two divergent signaling pathways, alternatively competing with regard to rest and locomotor activity (Pauls et al., 2021). During the day, it was revealed that the AKH causes an increase in the activity of the fruit flies through the octopaminergic system; on the other hand, during the night, AKH signaling prevents the increase in their activity levels by sending signals to the fat body (Pauls et al., 2021). Also, they suggested that regulation of this activity involves octopamine feedback signaling from the octopaminergic neurons to AKH-producing cells (Pauls et al., 2021).

Immunohistochemical studies were conducted in *L. migratoria* to determine the distribution of AKH in insects, which has been extensively localized to the neurosecretory cells in the CC, with some immunoreactivity detected in the subesophageal ganglion (SEG) (Schooneveld et al., 1983, 1985). Subsequent studies using *in situ* hybridization in *L. migratoria* and *D. melanogaster* showed that AKH transcript was localized only in the neurosecretory cells of the CC (Bogerd et al., 1995; Noyes et al., 1995). Furthermore, AKH-like immunoreactivity was detected in the CC of various insects, including *L. migratoria* and *S. gregaria* (Diederen et al., 1987), *D. melanogaster* (Isabel et al., 2005), *Anopheles gambiae* (Kaufmann and Brown, 2006), *A. aegypti* (Kaufmann et al., 2009), *Glossina morsitans* (Attardo et al., 2012), and *R. prolixus* (Patel et al., 2014).

In 2002, a substantial breakthrough in insect molecular neuroendocrinology was made by Staubli and colleagues as they succeeded in isolating and determining the first insect AKH receptors from the fruit fly *D. melanogaster* and the silkworms *Bombyx mori* (Staubli et al., 2002). These AKH receptors showed homology with the mammalian GnRH receptors (Staubli et al., 2002). Following that, the AKH receptors have been identified in several insects such as *P. americana* (Hansen et al., 2006), *A. gambiae* (Belmont et al., 2006; Kaufmann and Brown, 2006), *Blatella germanica* (Huang et al., 2011), *Manduca sexta* (Ziegler et al., 2011), and *R. prolixus* (Zandawala et al., 2015b), as well as a significant number in other insect species have been predicted according to the available genomic sequences (Grimmelikhuijzen and Hauser, 2012; Hauser and Grimmelikhuijzen, 2014). The AKHr gene was recognized in the mosquito *A. aegypti*, and alternative splicing led to three different transcript variants: *AedaeAKHr-IA*, *AedaeAKHr-IB*, and *AedaeAKHr-II* (Kaufmann et al., 2009; Oryan et al., 2018). Using heterologous receptor assays to characterize the AKH receptors functionally in several insects, such as in *A. gambiae* (Belmont

et al., 2006; Hansen et al., 2010), *P. americana* (Hansen et al., 2006), *A. aegypti* (Oryan et al., 2018), and *B. mori* (Zhu et al., 2009; Shi et al., 2011) demonstrated that the AKH receptor-activated only by its specific AKH peptide, except for the *B. mori* because AKH receptor at a 100-fold higher dose resulted in activation by the ACP peptide (Zhu et al., 2009).

The AKH receptor transcript expression was revealed using reverse transcription PCR (RT-PCR) analyses in the ovaries and the abdomen of adult *Culex pipens*, *A. aegypti*, and *A. gambiae* (Kaufmann and Brown, 2006; Kaufmann et al., 2009). Lately, quantitative expression analyses in adult mosquito *A. aegypti* showed enrichment of the AKH receptor transcripts I and II in the nervous tissue, the carcass, and the accessory reproductive organs (Oryan et al., 2018). Furthermore, the transcript levels of AKH receptor using quantitative PCR (qPCR) in *R. prolixus* were highly enriched in the fat body, abdominal nerves, dorsal vessel, and diaphragm (Zandawala et al., 2015b). Thus, there is a high possibility for the AKH to have a role in reproduction in insects because the fat body is usually associated with the reproductive tissue. In *L. migratoria*, AKH showed inhibition of oocyte vitellogenesis (Moshitzky and Applebaum, 1990). Moreover, injection of AKH in the female *Gryllus bimaculatus* led to a significant decrease in the ovary mass observed, and several terminal oocytes were produced (Lorenz, 2003). Additionally, AKH receptor knockdown caused impaired milk production in the tsetse flies *Glossina morsitans* during pregnancy (Attardo et al., 2012). Also, in adult males mosquitoes *Aedes albopitus* and *A. aegypti*, AKH has been determined as components of seminal fluid proteins (Kaufmann et al., 2009; Boes et al., 2014). Therefore, further studies should be done to further investigate if AKH has a direct and/or indirect influence on the reproduction of insects.

1.5 Corazonin

Corazonin (CRZ), derived from the Spanish word for heart, corazón, is another closely related insect neuropeptide that was first discovered and isolated from the heart of the American cockroach, *P. americana* (Veenstra, 1989). CRZ is produced chiefly in neuroendocrine cells of the pars lateralis of the protocerebrum and released via the CC (de Velasco et al., 2007; Predel et al., 2007). CRZ is widespread in several insects species and shares some structural homology with AKH (see **Table 1-1**) but differs in its functions (Hansen et al., 2010; Hauser and Grimmelikhuijzen, 2014; Wahedi, 2018). CRZ was named because of its significant cardio-excitatory effect in many insects, including the kissing bug *R. prolixus* and cockroach *P. americana* (Veenstra, 1989; Patel et al., 2014). However, a study that utilized RNAi on the mosquito *A. gambiae* revealed that CRZ and its receptor knockdowns did not significantly alter heart-beat rate (Hillyer et al., 2012). Thus, the cardio-stimulatory activity of CRZ is absent in adult mosquitoes of *A. gambiae* (Hillyer et al., 2012).

To date, a universal role of CRZ remains poorly established, as it has been associated with multiple physiological roles, including ecdysis initiation in *M. sexta*, a decrease in the rate of silk-spinning in *B. mori*, and melanization induction in *S. gregaria* and *L. migratoria* (Veenstra, 1989; Tawfik et al., 1999; Tanaka et al., 2002; Kim et al., 2004). Moreover, the function of CRZ has extended to an involvement in larval development in the oriental fruit fly, *Bactrocerca dorsalis*, where the *BacdoCRZ* receptor knockdown led to a delay in the transition of the larval to pupal stage (Hou et al., 2017). CRZ has also been observed to regulate fecundity (Bergland et al., 2012), sensing of nutrients (Miyamoto and Amrein, 2014), as well as oxidative stress and feeding (Kubrak et al., 2016) in *D. melanogaster*. Furthermore, CRZ was observed to be involved in promoting the transmission of sperm and seminal fluids in male flies (Tayler et al., 2012).

Table 1-1. Comparison between the amino acid sequences of the mosquito *A. aegypti* neuropeptides ACP, AKH and CRZ. Similarities between peptides are represented by similar colored of the amino acids. Residues are highlighted in blue are conserved but not identical. These alignments demonstrate that ACP is structurally related and intermediate between AKH and CRZ (Hansen et al., 2010; Hauser and Grimmelikhuijzen, 2014; Marchal et al., 2018; Wahedi, 2018).

Neuropeptides	Amino acid Sequences
ACP	**pQVTF--SRDWNAamide**
AKH	**pQLTF--TPSW--amide**
CRZ	**pQ-TFQYSRGWTNamide**

The CRZ receptor (CRZr) has been cloned in numerous insect species, including *D. melanogaster* (Cazzamali et al., 2002), *B. mori* (Kim et al., 2004), *Musca domestica* (Sha et al., 2012), *M. sexta* (Kim et al., 2004), *B. dorsalis* (Hou et al., 2017), *A. gambiae* (Hillyer et al., 2012), *R. prolixus* (Hamoudi et al., 2016) and *A. aegypti* (Oryan et al., 2018). The CRZ receptor's functional characterization studies in many insects such as *D. melanogaster* (Park et al., 2002), *B. dorsalis* (Hou et al., 2017), *R. prolixus* (Hamoudi et al., 2016), *A. gambiae* (Belmont et al., 2006; Hansen et al., 2010) and *A. aegypti* (Oryan et al., 2018) demonstrated strict specificity to their CRZ peptide. Using RT-qPCR, the developmental expression profile study on the CRZ transcript showed the highest enrichment in the 2nd instar larval stage and one-day-old adult *A. gambiae* mosquito (Hillyer et al., 2012). Similarly, the expression of CRZr observed the highest transcript abundance in the 2nd instar larvae and pupa stages. Furthermore, Oryan and colleagues conducted a developmental expression analysis in the mosquito *A. aegypti* showed enrichment of the CRZ receptor in the pupa and adult stages, suggesting that CRZ has an ecdysis-related function (Oryan et al., 2018). Therefore, these findings support the role of CRZ in the ecdysis induction in mosquitoes, similar to what was previously determined in *M. sexta* (Kim et al., 2004).

Spatial expression analysis using RT-qPCR was performed in different tissues of 5th instar *R. prolixus* that revealed a high enrichment of CRZr transcript in the dorsal vessel and central nervous system (CNS) of the kissing bug (Hamoudi et al., 2016). Thus, the CRZr transcript in the dorsal vessel shows consistency with the previously recognized cardio-stimulatory function. Additionally, a study conducted on the adult stage *A. aegypti* mosquito also detected the localization of the CRZr transcript in the nervous tissue and the reproductive organs (Oryan et al., 2018). Given the structural similarity between CRZ and the AKH sequence (**Table 1-1**), Patel and colleagues aimed to examine if CRZ affected lipid mobilization. By injecting the synthetic CRZ

into adult males *R. prolixus*, they found no effect on the lipid levels in the haemolymph (Patel et al., 2014). Similarly, the dorsal vessel of 5th instar *R. prolixus* was isolated *in vitro* and subjected to high doses of AKH that did not change the heart-beat rate. Thus, these findings provide evidence of the existence of two structurally-related signaling systems that are unrelated functionally.

1.6 Adipokinetic Hormone/Corazonin-Related Peptide

In 1999, a novel neuropeptide called “peptide A" was discovered in the locust *L. migarotria*. Siegert and colleagues classified this peptide as a new novel member of the peptides AKH family due to its similarity in sequence with the AKHs, but its function was unknown (Siegert, 1999). Interestingly, unlike the classic AKHs which are found in the glandular lobe of the CC, this new peptide was present in the secretory lobe of the CC indicating it is synthesized by neurons in the brain (Siegert, 1999). Several years after this discovery, an orphan GPCR was isolated from the mosquito *A. gambiae,* which also showed sequence similarity to both the AKH and CRZ receptors (Belmont et al., 2006). That same year, a peptide was identified in *A. gambiae* and termed "AKH-II " (Kaufmann and Brown, 2006). In the flour beetle, *Tribolium castaneum*, Li and colleagues succeeded in identifying a peptide and its receptor that displayed sequence similarity to previously well-known AKHs and AKH receptors (Li et al., 2008). All of these neuropeptides and receptors that were discovered and mentioned previously were referred to as inactive peptides of the AKH family due to their sequence similarities to the AKH but inability to mobilize energy substrates. Interestingly, Hansen and colleagues later determined these AKH-like peptides (and the cognate receptors) were part of an independent signaling system (Hansen et al., 2010).

Furthermore, Hansen and his research group examined the orphan GPCR of the mosquito *A. gambiae* identified previously by Belmont and colleagues; and they succeeded in determining the ligand for this receptor (Belmont et al., 2006; Hansen et al., 2010). This neuropeptide ligand was identified as being structurally intermediate between CRZ and AKH; hence, Hansen and colleagues decided to name this novel neuropeptide the adipokinetic hormone/corazonin-related peptide (ACP) (Hansen et al., 2010). Further, Hansen *et al.* characterized the functionality of ACP receptors in *T. castaneum* and *A. gambiae*, where each has two transcript variants (Hansen et al., 2010). Therefore, in the same study, they used an *in vitro* heterologous functional bioassay and found that the three receptors ACP, AKH, and CRZ were activated by their respective ligand only. Therefore, they were considered as three independent signaling systems (Hansen et al., 2010).

In 2015, Zandawala and colleagues succeeded in characterizing the ACP signaling system in the kissing bug *R. prolixus* where they found one ACP peptide transcript and three transcript variants of the ACP receptor (Zandawala et al., 2015a). Moreover, in *R. prolixus*, similar results were observed to the Hansen group regarding functional deorphanization of the ACP receptor as the CRZ and AKH peptides did not have activity; instead, the ACP was the only peptide that stimulated the ACP receptor (ACPr) (Zandawala et al., 2015a). On the other hand, *in vitro* studies done in 2009 on *B. mori* observed that ACP under a high dose concentration was able to activate the AKH receptor (Zhu et al., 2009). Moreover, studies performed on several insect species such as *T. castaneum*, *A. gambiae*, *A. aegypti*, and *R. prolixus* revealed that neither AKH nor ACP receptors could be activated by CRZ. Similarly, neither AKH nor ACP peptides were able to activate CRZ receptors (Hansen et al., 2010; Zandawala et al., 2015a; Oryan et al., 2018). Therefore, according to these studies, the cross-reactivity might occur only between ACP and AKH while never observed with CRZ, indicating that the AKH and ACP signaling systems are

more closely related than the CRZ signaling system. Many studies utilizing the comprehensive *in silico* analyses proposed a model where an ancestral form of GnRH and GnRH receptor (a-GnRHr) was duplicated before the emergence of bilaterians, leading to two branches, one producing a CRZ-like peptide/receptor (CRZr) signaling system and the other giving an AKH-like peptide/receptor (AKHr) signaling system (**Fig. 1-1**) (Hansen et al., 2010; Hauser and Grimmelikhuijzen, 2014; Marchal et al., 2018; Zhou et al., 2018). Furthermore, the AKH hormonal system was duplicated prior to the emergence of the phylum Arthropoda, resulting in the ACP and AKH signaling systems. Thus, AKH and ACP signaling systems were suggested to be paralogous due to gene duplication in the arthropod lineage (Hansen et al., 2010; Hauser and Grimmelikhuijzen, 2014; Marchal et al., 2018; Zhou et al., 2018). Since the ACP/ACPr signaling system arose through further AKH hormonal system duplication and then evolved into its own hormonal system, and over time, the ACP (and its receptor) has been lost in some insects (e.g. *D. melanogaster* and *Apis mellifera*) (Hansen et al., 2010). Additionally, the CRZ system was lost in many vertebrates and arthropods. Thus, the proposed scenario (**Fig. 1-1**) illustrates the coevolution of the three ligands and their receptors confirming that ACP, AKH and CRZ are three independent signaling systems (Hansen et al., 2010; Hauser and Grimmelikhuijzen, 2014; Marchal et al., 2018; Zhou et al., 2018).

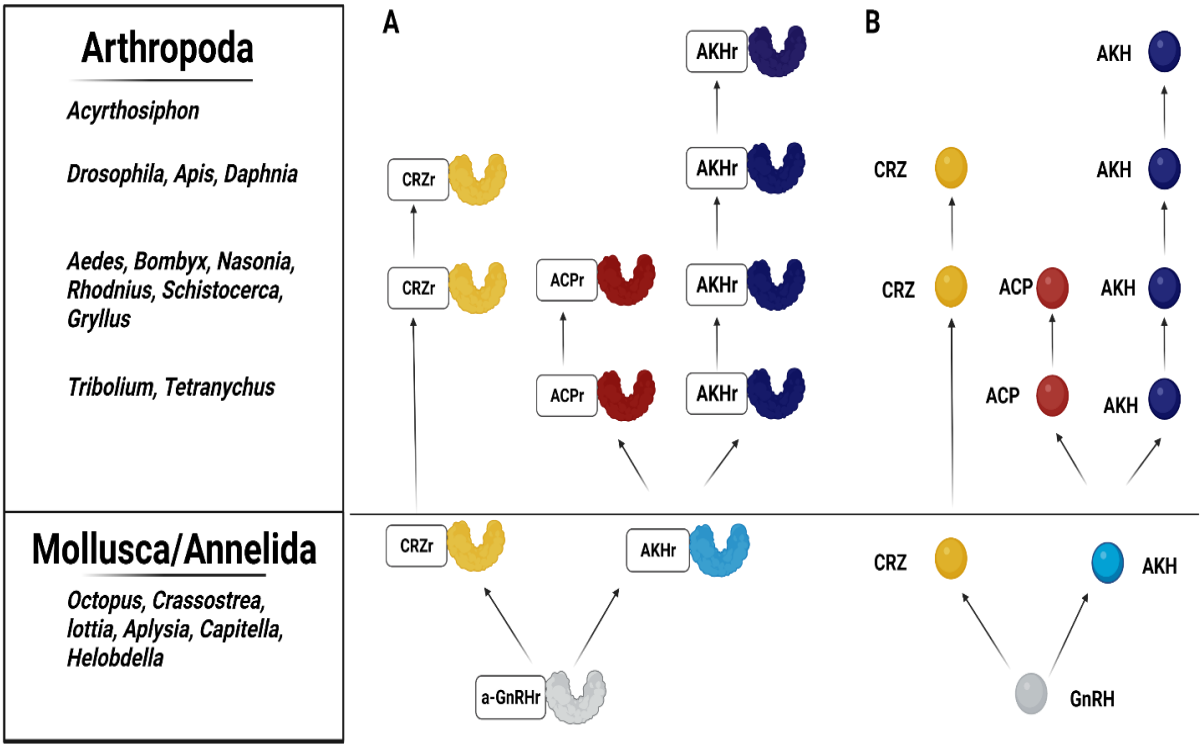

Figure 1-1. A proposed scenario of the evolutionary mechanism giving rise to the CRZ/ACP/AKH signaling systems in different species. The scheme presents a suggested scenario for the coevolution of the neuropeptide (CRZr/ACPr/AKHr) receptors (**A**) and their corresponding ligands (CRZ/ACP/AKH) (**B**). Receptors or peptides below the horizontal line are ancestral GnRH-like receptors (a-GnRHr), and ligands (a-GnRH) were duplicated before the Mollusca and Annelida emergence and diverged, leading to AKH- and CRZ-like ligands and AKHr-like receptors and CRZr-like receptors. Ancestral receptor/peptide duplication resulted in an ancestral CRZ/CRZr and AKH/AKHr. Later the AKH/AKHr duplication resulted in the ACP/ACPr signaling system. This figure was created by BioRender (BioRender.com) and adapted based on several studies and literature data (Hansen et al., 2010; Hauser and Grimmelikhuijzen, 2014; Marchal et al., 2018; Zhou et al., 2018).

To date, the physiological function of the ACP signaling system remains unclear, while transcript enrichment analyses have attempted to find a potential role. In *T. castaneum*, expression profiles showed the highest expression of both the ACP peptide and its receptor in the head of the flour beetle (Hansen et al., 2010). Also, the ACP peptide and its receptor were found prominently expressed during early developmental stages include in eggs and larvae of *T. castaneum* (Hansen et al., 2010). In 2015, Zandawala and colleagues revealed that both ACP and ACPr were expressed mainly in the CNS, more specifically in the corpora cardiac (CC)/corpora allata (CA) complex in 5th instar *R. prolixus* (Zandawala et al., 2015a). Further, a developmental expression examination revealed that after ecdysis in *R. prolixus*, a significant increase was observed in the expression of both the ACP peptide and its receptor (Zandawala et al., 2015a). Similar findings were detected in the mosquito *A. aegypti*, where RT-qPCR was used to determine the developmental expression profile that identified the enrichment of both the ACP and its receptor transcripts in the adult mosquito post-eclosion, specifically in the one and four-day-old adult mosquito (Wahedi and Paluzzi, 2018). Furthermore, the tissue-specific expression pattern revealed the highest expression of the ACP peptide in the nervous system of the adult mosquito *A. aegypti*, particularly in the brain and the thoracic ganglia, while the ACP receptor showed high enrichment in the abdominal ganglia (Wahedi and Paluzzi, 2018).

Since the ACP signaling system shares structural similarities to AKH and CRZ peptide systems, Patel and colleagues examined if any similarity in function has existed between the ACP, AKH, and CRZ in kissing bugs *R. prolixus* (Patel et al., 2014). They observed that ACP did not affect the haemolymph lipid levels as well as failed to change the heart rate frequency (Patel et al., 2014). Similarly, an earlier study showed that the locust ACP (referred then to as 'peptide A') did not change the heart-beat frequency nor display any metabolic role as it could not mobilize

carbohydrates or lipids in *L. migratoria* (Siegert, 1999). Therefore, in both *L. migratoria* and *R. prolixus*, ACP does not seem to have a role in liberation of lipids, nor does it act as a cardio-acceleratory agent. In addition, in 2008, ACP (known as AKH-II previously) injections into adult *A. gambiae* mosquitoes showed no significant difference in lipid levels in the haemolymph (Kaufmann and Brown, 2008). Notably, unlike the previous studies, a recent study on the male cricket *G. bimaculatus* showed that ACP regulates the concentration of lipids and carbohydrates in the haemolymph (Zhou et al., 2018). Additionally, a more recent study observed that ACP regulates the utilization of muscle lipids during a long-term flight in *L. migratoria* (Hou et al., 2021).

In the first instar larvae of the *T. castaneum*, ACP showed localization into three to four intensely stained cells in each brain hemisphere sending axonal projections to the brain neuropil, thoracic ganglia, and abdominal ganglia (Hansen et al., 2010). Furthermore, a recent study performed on Orthoptera showed that in some insect species (e.g., *L. migratoria*), the ACP is expressed by brain neurons in the pars lateralis (PL) and pars intercerebralis (PI) regions; whereas in other insect species (e.g. *P. americana*), the expression of ACP is produced only by neuroendocrine cells of the PL of the brain (Veenstra, 2021).

Based on the transcript expression profile and immunolocalization of ACP in the CNS, it suggested a potential neurotransmitter, neuromodulatory, or neurosecretory functions for the ACP signaling system in insects. To date, the functional role of the ACP remains unclear in most arthropods. Therefore, this knowledge gap leaves the door wide open for further future investigations to understand better the specific role of this relatively understudied ACP signaling system in insects.

1.7 Research Objectives and Hypotheses

Insects represent the most diverse and largest animal group. Many insects have ecological importance as they play vital roles in pollinating flowering plants (Gäde et al., 1997). Furthermore, they also have biomedical significance as they participated in many discoveries that occurred firstly in insects and then were applied to (or studied in) the mammalian systems, such as metabolic, muscle contractions, and digestion pathways (Law and Wells, 1989). Despite all of these important benefits of insects, our knowledge of their neuroendocrinology remains limited and requires further research, as most studies focus on the neuropeptides of the vertebrates more than the invertebrates (Corbière et al., 2019; Fieber, 2019). The mosquito *A. aegypti* is one of the primary vectors responsible for many diseases such as Yellow fever, Dengue fever, Zika virus, and Chikungunya, which significantly impact human health and mortality, making them an essential organism for research (Barón et al., 2010). Thus, understanding mosquito biology demands researchers create new strategies as well as develop novel methods to avoid and/or reduce outbreaks of mosquito-borne diseases.

As mentioned above, the ACP signaling system was discovered in insects, which is evolutionarily and structurally related to CRZ and AKH signaling systems (Hansen et al., 2010). Despite the ACP and its corresponding peptidergic receptor (ACPr) were identified in several arthropod species, unfortunately, our knowledge of their main functional role in most arthropods remains unclear (Hansen et al., 2010; Patel et al., 2014; Zandawala et al., 2015a). Therefore, the primary goal of this thesis sought to advance our understanding and bridge the knowledge gap of the ACP/ACPr signaling system by mapping its immunological distribution, determining functions in adults *A. aegypti*, and looking at the key structural features of the ACP receptor. Experiments

in this current thesis were conducted on the adult mosquito species *A. aegypti* as a model to investigate the localization and specific physiological role of ACP and its receptor. The knowledge gained from the interaction between the ligands with their respective receptor is thought to be very helpful in using specific receptors as targets for the development of selective biorational insecticides (Hill et al., 2018).

In the first data chapter of this thesis (Chapter 2), the first objective was to localize the ACP distribution in the adult *A. aegypti* nervous system using immunohistochemistry in order to provide insight that could allow for the discovery of a potential function of this neuropeptide signaling system. Both the ACP and/or its receptor transcripts have been observed to be expressed throughout the CNS of *T. castaneum* (Hansen et al., 2010), *A. gambiae* (Kaufmann and Brown, 2006), and *R. prolixus* (Zandawala et al., 2015a). Furthermore, a previous report from our research group has succeeded in confirming the enrichment of ACP and its receptor in the nervous tissue of *A. aegypti*, where the ACP peptide was expressed in the brain and thoracic ganglia while the ACP receptor was highly expressed in the abdominal ganglia (Wahedi and Paluzzi, 2018). Moreover, based on the evidence from a study done by our group showing AKH-like immunoreactivity, two pairs of cells were revealed in the anterior protocerebrum of the brain, more specifically in the supraesophageal ganglion, while two to three cells in the pro-thoracic segment of the thoracic ganglia in adult *A. aegypti* (Wahedi, 2018). Therefore, by using an antiserum against the ACP peptide specifically, it was hypothesized that the ACP-expressing cells are confined to the brain and thoracic ganglia but project axons towards the abdominal ganglia of adult *A. aegypti*.

The second objective was to determine the ACP receptor transcript expression profile in the abdominal and terminal ganglia of the male and female adult *A. aegypti* by utilizing RT- qPCR.

According to a prior study that demonstrated the ACP receptor was expressed outside the nervous tissues of the adult mosquito, such as the hindgut and the accessory reproductive tissues (Wahedi and Paluzzi, 2018), this suggested that the ACPr might have a functional role in the metabolite and ion reabsorption and/or reproductive processes. Similarly, the ACPr was also expressed in reproductive tissue in *R. prolixus* (Zandawala et al., 2015a). A study in the *L. migratoria* also showed that the projection from the terminal abdominal ganglia regulates the contractile activity of the hindgut (Donini et al., 2002). Based on these findings, the working hypothesis was that the ACPr is more expressed in the terminal ganglia than all of the pre-abdominal ganglia in adult mosquitoes, suggesting it could have an effect on the reabsorption and/or reproductive physiology of the adult mosquitoes.

Next, numerous studies mentioned earlier have characterized the most common role of CRZ and AKH systems within many insect species, which is cardio-excitatory function and regulation of the lipid homeostasis, respectively (Steele, 1961; Mayer and Candy, 1969; Stone et al., 1976; Veenstra, 1989; Kaufmann et al., 2009; Marco et al., 2013; Patel et al., 2014). In contrast, the central functional role of the ACP and its receptor is not well-established in most insects. From a previous study, we know that the ACP receptor was expressed in other tissues outside the nervous tissue, such as the carcass, which includes the fat body of male adult mosquitoes (Wahedi and Paluzzi, 2018). Consequently, to uncover a potential peripheral role for this neuropeptide signaling pathway, the third objective was to examine one of the potential targets of ACP function in terms of energy substrate mobilization. A biochemical assay was utilized by injecting *Aedae*-ACP and *Aedae*-AKH synthetic peptides into female and male adult *A. aegypti* to assess whether the ACP contributes toward energy substrate mobilization in this insect. Therefore, it was hypothesized that ACP modulates energy substrates levels and energy metabolism in adult *A. aegypti* mosquitoes.

The rationale for this hypothesis is further supported by previous reports suggesting that ACP regulates carbohydrate and lipid concentration in the haemolymph of the adult male cricket *G. bimaculatus* as well as the regulation of the lipid utilization in the locust *L. migratoria* (Zhou et al., 2018; Hou et al., 2021).

As mentioned in the preceding section, using CRISPR/Cas9 system-mediated gene knockout revealed that, although the ACP mutant affects the utilization and oxidation of lipid in the locust *L. migratoria*, no impairment was observed in the adult longevity compared to the wild-type locusts (Hou et al., 2021). Hence, the fourth objective of the current research aimed to determine the survival rate after injection with *Aedae*-ACP synthetic peptide in both female and male adult *A. aegypti* mosquitoes. Specifically, I hypothesized that injection of ACP would impact the survival rate of the adult mosquito.

Lastly, using the heterologous system, a series of analogues based on the *A. aegypti* original ACP sequence were recently designed and screened against the ACP receptor to identify the key residues vital for receptor activation (Wahedi et al., 2019). This structure-activity relationship (SAR) of the ligand can provide insight and motivation to this current research by following similar SAR analysis but focusing on the ACP receptor by creating chimeras. Therefore, to further characterize the ACP receptor of *A. aegypti*, in the second data chapter (Chapter 3), the fifth objective of this thesis was to determine how the ACP receptor (ACPr) elicits strong specificity for its native ligand in the *A. aegypti* mosquito by mutagenizing specific regions of the ACP receptor to identify features most critical for ligand-mediated activation and specificity using a heterologous functional assay. Hence, ACP receptor chimeras were generated by swapping out a single complete extracellular loop region (ECL1, ECL2, and ECL3) from the native *Aedes* ACP

receptor or selecting highly conserved residues within the three ACPr extracellular loops and replacing them with corresponding sequences from the *A. aegypti* AKH receptor (AKHr).

As mentioned before, previous investigations have demonstrated that AKH and ACP, and their receptors, are more closely related to each other compared to CRZ (Hansen et al., 2010; Zandawala et al., 2015a; Oryan et al., 2018). Additionally, previous literature showed that although the close evolutionary relationship and structural similarity between the AKH and the ACP, their signaling systems function independently due to the strict selectivity of their corresponding ligands with their respective cognate receptors (Hansen et al., 2010; Patel et al., 2014; Zandawala et al., 2015b). Further, the extracellular loops (ECLs) play an essential function in activating and recognizing ligand-binding of the GPCRs (Wheatley et al., 2012). Identifying the functional form of the ACP receptor is necessary for demarcating the function of this signaling system. As a result, it was hypothesized that the complete replacement of each ACP receptor extracellular loop is detrimental to the ligand-binding recognition sensitivity. Moreover, the working hypothesis was that the modification of extracellular loops of the ACP receptor to the ECLs from the AKH receptor decreases sensitivity to binding of its native ligand, ACP, while increasing its promiscuous activation by AKH, its most structurally- and evolutionarily-related ligand.

1.8 References

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Chapter 2:

Functional elucidation and cell-specific expression of the adipokinetic hormone/corazonin-related peptide in the disease vector mosquito, *Aedes aegypti*

2.1 Summary

The adipokinetic hormone/corazonin-related peptide (ACP) is an insect neuropeptide structurally intermediate between corazonin (CRZ) and adipokinetic hormone (AKH). Several studies have characterized the AKH and CRZ signaling systems within diverse insect species, and the most notable functions include energy mobilization and cardio-acceleratory activity, respectively. In contrast, the function of the ACP signaling system in insects remains unclear. The current study aimed to elucidate the function of ACP in the adult mosquito *Aedes aegypti* by localizing the distribution of ACP in the nervous system using immunohistochemistry and examining transcript abundance of the ACP receptor in the abdominal ganglia, determining the potential involvement of ACP in metabolism controlling energy substrate levels, and finally, assessing adult longevity after injection with ACP. In adult mosquitoes, ACP immunoreactivity is localized in two pairs of lateral interneurons in the pars lateralis of the brain and 2-3 cells in the thoracic ganglia. Further, extensive ACP-immunoreactive axonal projections with prominent blebs and varicosities were detected passing through each abdominal ganglia. Hence, the immunolocalization of ACP spanning the anterior and posterior regions of the central nervous system in the adult *A. aegypti* mosquito suggests that ACP may function as a neurotransmitter and/or neuromodulator. Notably, there is no significant difference in the transcript abundance of the ACP receptor between the abdominal ganglia and the terminal abdominal ganglion. After ACP injection of both the male and female mosquitoes, the results revealed a similar survival rate between the ACP-injected and sham-injected mosquitoes. Biochemical analyses of energy substrates revealed a reduction in abdomen glycogen stores in response to ACP and AKH, but a corresponding hypertrehalosaemic effect only in response to AKH in female mosquitoes. Interestingly, both ACP and AKH led to a significant increase in haemolymph carbohydrate levels

while having no influence on glycogen stores in male mosquitoes. Finally, no significant changes in the abdomen and haemolymph lipid levels were observed in both sexes.

2.2 Introduction

Neuropeptides play a crucial role in regulating several physiological and behavioral processes such as feeding, development, water balance and reproduction in multicellular animals, including arthropods (Barón et al., 2010; Nässel and Winther, 2010). Three neuropeptide signaling systems that exist in invertebrates show homology to the mammalian gonadotropin-releasing hormone (GnRH) system (Hansen et al., 2010; Gäde et al., 2011; Roch et al., 2011; Li et al., 2016). These three neuropeptide systems are well known as the adipokinetic hormone (AKH) family, the corazonin (CRZ) family and the adipokinetic hormone/corazonin-related peptide (ACP) family.

One of the first insect neuropeptide families to be purified and isolated, AKHs, are synthesized exclusively by neurosecretory cells (NSC) of the corpora cardiaca (CC), a small neurohaemal organ that is associated closely with the brain in insects (Stone et al., 1976; Diederen et al., 1987; Diederen et al., 2002). AKHs are an extensively studied family of neuropeptides that play a fundamental function in regulating energy homeostasis in adult insects by mobilizing lipids and/or carbohydrates from the fat body (an organ equivalent to the vertebrate liver and adipose tissue) during high physical activities, including locomotion and flight (Gäde et al., 1997). Additionally, AKHs are recognized to be involved in the maintenance of carbohydrate homeostasis in insect larvae (Ziegler et al., 1984, 1990; Kim and Rulifson, 2004). Although the insect AKH neuropeptides and vertebrate glucagon are not structurally related, AKH has a similar functional role in insects that adrenaline and glucagon control in vertebrates (Ziegler et al., 1984; Bednářová et al., 2013). Additional roles of the AKHs include the regulation of oxidative stress, life span extension, heart-beat rate stimulation and protein synthesis inhibition (Gäde and Marco, 2006; Zandawala et al., 2018).

Another closely related insect neuropeptide, CRZ (from corazón, Spanish for 'heart'), shares some structural homology with AKH but differs notably in its functions. CRZ is produced chiefly in the neuroendocrine cells of the pars lateralis (PL) of the protocerebrum and released via the CC (Predel et al., 2007). Immunohistochemical studies in the cockroach *Periplaneta americana* (Veenstra and Davis, 1993) and fruit fly *Drosophila melanogaster* (Choi et al., 2005) showed that CRZ localized to several dorsolateral neurons in the PL and also in each abdominal ganglia of the ventral nerve cord (VNC). CRZ was first discovered in the cockroach, *P. americana* and was named because of its cardio-excitatory effect in many insects, including the kissing bug *Rhodnius prolixus* (Veenstra, 1989; Patel et al., 2014). However, the cardio-stimulatory activity of CRZ is absent in adult *Anopheles gambiae* mosquitoes (Hillyer et al., 2012). To date, the universal function of CRZ remains poorly understood, although it has been associated with additional functions, including melanization, ecdysis as well as responses to metabolic and osmotic stress (Kim et al., 2004; Kubrak et al., 2016; Tawfik et al., 1999; Veenstra, 1989; Zandawala et al., 2021).

Recently, a third structurally-related signaling system was discovered in the *A. gambiae* mosquito and other insects named the adipokinetic hormone/corazonin-related peptide (ACP), which is evolutionarily related to AKH, CRZ and their receptors (Hansen et al., 2010). Although ACP and its receptor (ACPr) are structurally intermediate between AKH and CRZ and their receptors, their functional role in insects is not well established. Nonetheless, several studies have revealed that AKH, CRZ and ACP neuropeptide signaling systems function independently as the three receptors show highly-selective specificity for their respective ligands (Hansen et al., 2010; Zandawala et al., 2015; Hamoudi et al., 2016; Oryan et al., 2018; Wahedi and Paluzzi, 2018). Several biological assay studies in many insects, including kissing bugs and mosquitoes, have shown that ACP does not regulate the mobilization of energy and thus does not replicate the actions

of AKH (Kaufmann and Brown, 2008; Patel et al., 2014). Furthermore, ACP does not affect the rate of the heart-beat, indicating that the physiological actions of ACP do not overlap with the most recognized function of CRZ (Patel et al., 2014).

Interestingly, none of the previous studies could determine the precise function of ACP until a study suggested that it regulates the haemolymph concentration of carbohydrates and lipids in the male cricket, *Gryllus bimaculatus* (Zhou et al., 2018). This evidence indicated that ACP in crickets might indeed share a functional role with AKH in the regulation of energy homeostasis (Zhou et al., 2018). Moreover, a very recent study conducted in the locust, *Locusta migratoria,* revealed that ACP regulates muscle lipid utilization during long-term migratory flight (Hou et al., 2021). Contrary to the utilization of lipids as a primary energy substrate in locusts, the primary source of energy used by mosquitoes is carbohydrates in the form of trehalose, but prolonged taxation and movement will also result in the breakdown of lipids (Briegel et al., 2001).

Expression analyses of the ACP peptide and its receptor (ACPr) transcripts in *R. prolixus*, and specifically ACPr in *Tribolium castaneum*, had demonstrated enrichment in the nervous tissue (Hansen et al., 2010; Zandawala et al., 2015). ACP was localized to numerous neurons in each hemisphere of the brain, with projections throughout the central nervous system (CNS) in *T. castaneum* (Hansen et al., 2010). Furthermore, in *R. prolixus*, ACP-like immunoreactivity was revealed throughout the nervous system, and similar to the ACP-like distribution in *T. castaneum*, there are no projections detected exiting the CNS (Hansen et al., 2010; Patel et al., 2014). Moreover, a very recent study conducted in Orthoptera revealed that the ACP expression is surprisingly different from other insect species, as it was interesting to find that ACP is also being produced by neuroendocrine cells of the pars intercerebralis (PI), while in other insect species, ACP appeared to be expressed by neurons in the PL of the brain (Veenstra, 2021).

In addition, a previous report confirmed enrichment of ACP and ACPr transcripts in the CNS of *A. aegypti*, where the ACP transcript was found with increased abundance in the brain and thoracic ganglia while the ACPr transcript was significantly enriched in the abdominal ganglia (Wahedi and Paluzzi, 2018). Furthermore, the *A. aegypti* ACPr transcript was expressed in other tissues outside the nervous system, including the male carcass, suggesting that the ACP signaling system might also have a role in peripheral tissues (Wahedi and Paluzzi, 2018).

Thus, the current study aimed to advance our understanding of the role of the ACP/ACPr signaling system in the adult *A. aegypti* mosquito by firstly mapping the distribution of the ACP peptide throughout the CNS. It was hypothesized that the ACP expressing cells are confined to the brain and thoracic ganglia but send axons into the abdominal ganglia. Secondly, the ACPr transcript expression pattern was examined in the pre-terminal abdominal ganglia and the terminal ganglion in both the male and female mosquitoes using quantitative RT- PCR. Since previous research from our group revealed that ACPr transcript was expressed in other tissues outside the nervous system, including the accessory reproductive tissues and hindgut (Wahedi and Paluzzi, 2018), this suggests a role in reproductive processes and/or reabsorption of metabolites and ions. The insect hindgut is considered a primary site of reabsorption (Coast et al., 2002). Moreover, a study conducted in *L. migratoria* indicated that the neuronal projections from the terminal abdominal ganglia could lead to modulation of hindgut contractile activity (Donini et al., 2002). In *R. prolixus,* ACPr expression has also been detected in reproductive tissue (Zandawala et al., 2015). Therefore, I hypothesized that ACPr is more highly expressed in the terminal ganglion than the pre-terminal abdominal ganglia in adult mosquitoes, suggesting that ACP signaling plays a role in modulating reproductive and/or reabsorption physiology. Thirdly, a biochemical assay was used to quantify energy substrates following injection with synthetic peptides, *Aedae*-ACP and

Aedae-AKH, to determine their effect on mobilization of energy substrates and their depletion from stores in both male and female adult *A. aegypti*. Thus, I hypothesized that ACP impacts energy metabolism and modulates levels of energy substrates in adult mosquitoes. Lastly, adult longevity (or survival rate) was assessed after injection of male and female mosquitoes with *Aedae*-ACP synthetic peptide. In this experiment, I hypothesized that ACP would affect adult longevity of both sexes.

2.3 Materials and Methods

2.3.1 Experimental animals and rearing conditions

Aedes aegypti mosquito rearing was carried out as described previously (Rocco et al., 2017; Wahedi and Paluzzi, 2018). Briefly, adults of *A. aegypti* (Liverpool strain) were obtained from an established laboratory colony in the Department of Biology, York University (Toronto, ON, Canada). Larvae of *A. aegypti* were hatched from semi-desiccated eggs oviposited onto Whatman filter papers in a plastic container filled with double-distilled water and were fed daily with several drops of a feed solution comprised of 2% (w/v) beef liver powder and 2% (w/v) brewer's yeast. Pupae were transferred to beakers containing distilled water. Larvae, pupae and adult mosquitoes were reared in an incubator (26°C, 12:12 hour light: dark cycle). Adult male and female mosquitoes were supplied with a 10% sucrose solution through a cotton ball wick fitted in a microcentrifuge tube. Colony maintenance included adult females that were blood-fed using an artificial membrane feeding system every two days with sheep's blood in Alsever's solution (Cedarlane Laboratories Ltd., Burlington, ON, Canada). All experiments were carried out using one to four-day-old female and male adults mosquitoes (fed *ad libitum* with 10% sucrose) that had been isolated and transferred into glass mesh-covered jars in their pupal stage.

2.3.2 Immunohistochemistry

Male and female one and four-day-old adult *A. aegypti* were briefly anesthetized with CO_2, and nervous system tissues (i.e., brain, thoracic ganglia, and abdominal ganglia) were dissected at room temperature (RT) in 1x nuclease-free Dulbecco's phosphate-buffered saline (1x DPBS) and then transferred immediately to 4% paraformaldehyde (PFA) for fixation overnight at 4 °C. The nervous tissues were then washed three times, 15 minutes each with 1x DPBS and the tissues were

then incubated at RT for one hour with 4% Triton X-100, 2% bovine serum albumin (BSA), and 10% normal sheep serum (NSS) prepared in 1x DPBS. Following this, tissues were washed several times with 1x DPBS, each wash for 15 minutes to remove all traces of permeabilization solution. The nervous tissues were incubated with an anti-ACP mouse polyclonal ACP antiserum (diluted 1:1000), which was a kind gift from Prof. Jan Veenstra (Patel et al., 2014; Veenstra, 2021), prepared in 1x DPBS with 0.4% Triton X-100, 2% BSA(w/v) and 2% NSS(v/v) at 4 °C for 4 days with gentle agitation. As a negative control, the control tissues were incubated under the same conditions and in the same solution [0.4% Triton X-100 containing 2% BSA (w/v) and 2% NSS (v/v) in 1x DPBS] but without adding the ACP primary antiserum. After the four-day ACP primary antiserum incubation, tissues underwent three 15-minute washes with 1x DPBS. The nervous tissues were then incubated overnight at 4°C with Alexa Fluor 594-Goat anti-mouse IgG (H+L) secondary antibody (1:200 dilution; Molecular Probes, Life Technologies, Eugene, OR) made up in 1x DPBS with 10% NSS(v/v). Following overnight incubation at 4°C with gentle agitation, tissues were rinsed several times with 1x DPBS at RT and mounted on glass coverslips with mounting media [1x DPBS with 50% glycerol containing 4 μg/mL 4′,6- Diamidino-2-phenylindole dihydrochloride (DAPI)] and imaged on a Nikon Eclipse T*i* fluorescence microscope (Neville, NY).

2.3.3 Abdominal and terminal ganglia dissections, RNA extraction, cDNA synthesis and RT-qPCR

Lightly CO_2-immobilized one-day-old adult *A. aegypti* male (n = 50-60) and female (n = 40-50) in each biological replicate were submerged in 1x DPBS. In adult mosquitoes, only six abdominal ganglia appear as a result of the fusion of the 1st abdominal ganglion to the metathoracic ganglion and the 7th and 8th ganglia are fused into a terminal ganglion (Brown and Cao, 2001).

Thus, the abdominal ganglia (2^{nd}-6^{th}) and the fused terminal ganglion were dissected and isolated. Tissues were kept in a 1x DNA/RNA protection reagent (New England Biolabs, Whitby, ON). Total RNA was isolated and purified using the Monarch® Total RNA Miniprep Kit following the manufacturer's protocol and guidelines (New England Biolabs, Whitby, 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 ACPr transcript levels, cDNA was synthesized using the iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Mississauga, ON) following manufacturer recommendations, including a ten-fold dilution of cDNA following synthesis. The ACPr transcript abundance was quantified on a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Carlsbad, CA) using PowerUP™ SYBR® Green Master Mix (Applied Biosystems, Carlsbad, CA). The conditions of thermal cycling were as follows: (1) UDG activation 50 °C for 2 minutes, (2) 95 °C for 2 minutes, and (3) 40 cycles of (i) 15 seconds at 95 °C and (ii) 1 minute at 60 °C. Gene-specific primers amplifying *Aedae*ACPr mRNA sequence (see **Table 2-1**) were published previously (Wahedi and Paluzzi, 2018). Expression levels were normalized to the geometric mean of Ribosomal protein 49 (rp49), 60S Ribosomal Protein S18 (rpl8), and 40S Ribosomal Protein L8 (rpS18) housekeeping genes (see **Table 2-1**) that were determined and utilized previously as optimal endogenous controls (Paluzzi et al., 2014). The transcript levels were plotted relative to the abdominal ganglia. *Aedae*ACPr expression profile was determined using 4-5 biological replicates, including three technical replicates per reaction and a no-template negative control. Data were analyzed using a two-tailed t-test ($p < 0.05$) GraphPad Prism 8.02 (GraphPad Software, San Diego, USA).

Table 2-1. Information for oligonucleotides used for RT-qPCR analysis to determine spatial transcript expression. Forward and reverse primers were based on ACPr mRNA sequence (Genbank Accession number: XM_001653870.2) (Wahedi and Paluzzi, 2018). The reference genes forward and reverse primers are rp49 (Genbank Accession number: AY539746), 60S rpl8 (Genbank Accession number: XM_00165766140S) and rpS18 (Genbank Accession number: XM_001660270) (Paluzzi et al., 2014).

Oligo Name	Sequence (5'-> 3')	Function
***Aedae*ACPr-F**	GGGATGCGACTTCGTTGTA	qPCR amplification of *Aedae* ACPr
***Aedae*ACPr-R**	TCGCGGTCAAACATGTACC	qPCR amplification of *Aedae* ACPr
rp49-F	ACAAGCTTGCCCCCAAC	qPCR reference genes
rp49-R	GCGATTTCGGCACAGTAGA	qPCR reference genes
rpl8-F	AACCGTCAAGCAAATCATCC	qPCR reference genes
rpl8-R	GTCACCGGTCTTCTCCTCC	qPCR reference genes
rpS18-F	TAAAAATGTCGCTCGTGATCC	qPCR reference genes
rpS18-R	AATCGGGGATCTTGTACTGG	qPCR reference genes

2.3.4 Intrathoracic peptide injections and biochemical analyses

To assess the influence of ACP on nutrient energy substrate levels in the adult *A. aegypti*, synthetic *Aedae*-ACP and *Aedae*-AKH (see **Table 2-2**, GenScript, Piscataway, NJ, USA) 1mM stocks originally dissolved in Dimethyl sulfoxide (DMSO) were diluted in 1x DPBS. A physiologically-relevant dose of 10 pmol for both peptides was injected into each adult mosquito as this amount of *Aedae*-AKH was observed previously to elicit a hypertrehalosemic effect in adult *A. gambiae* (Kaufmann and Brown, 2008). To perform injections, a Nanojet III Programmable Nanoliter Injector (Drummond Scientific Company, Broomall, PA, USA) was fitted with fine-tipped glass needles made with a micropipette puller. The female and male one to four-day-old sugar-fed adult *A. aegypti* mosquitoes were injected with 10 pmol of synthetic *Aedae*-ACP or *Aedae*-AKH (positive control) into the dorsal lateral thorax near the base of the wing. For sham-injected controls, an equivalent volume of DMSO diluted identically in 1x DPBS was injected. At 90 minutes post-injection, two abdomens from females and three abdomens from males were pooled. Each mosquito was carefully opened at the segmental line between the last two abdominal segments to allow haemolymph to bleed or diffuse into the 1x DPBS. Haemolymph was collected by incubating two females or three males together in 100 µL 1x DPBS. After 10 min, 90 µL of the haemolymph solution was collected for the biochemical assay. The remaining abdomens were pooled and homogenized in 200 µL of 2% w/v sodium sulfate solution (2% w/v $NaSO_4$).

Carbohydrate, lipid, and glycogen levels were measured using a modified version of a previously published protocol (Van Handel and Day, 1988; Kaufmann and Brown, 2008). For the separation of sugar and lipids, 1.6 mL (v:v, 1:1) of chloroform/methanol ($CHCl_3$–MeOH) was added to each centrifuge tube, and then the tubes were centrifuged at 3000 rpm for 1 min. Next, the supernatants were transferred into fresh centrifuge tubes, while the pellets were retained for

glycogen analysis. Then, 0.6 mL of distilled H_2O (dH_2O) was added to the supernatant and then centrifuged at 3000 rpm for 1 min. The top portion (water/methanol) was used for the sugar analysis, while the bottom fraction (chloroform) was kept for the lipid analysis. Serial dilutions (25, 50, 100, 150 and 200 µl) of 0.1% of anhydrous glucose in dH_2O were used for the sugar and glycogen standardization. The glycogen in the precipitate and the standards were measured by adding 2 mL of anthrone reagent per tube immediately, whereas, for the sugar in the aqueous fraction, the tubes were heated on a heating block ~ 90-110 ^oC until the solvent evaporated down into ~ 0.1-0.2 mL then 2 mL of anthrone reagent was added per tube. Next, all tubes were heated for 17 minutes at ~90-110 ^oC and then removed from the heating block and allowed to cool. A series of 50, 100, 200 and 400 µl of 0.1% soybean oil in chloroform was used as a standard for the lipid analysis. The lipid portion of the samples and the soybean oil standards were placed at 90-110 ^oC to evaporate the solvent, and later 200 µL of sulfuric acid (H_2SO_4) was added and heated for 10 minutes in the heating block. Subsequently, 1 mL of vanillin-phosphoric acid reaction/tube was added, and the tubes were removed from the heating block and allowed to cool. A reddish color appeared within ~5 minutes, and the reaction was stable for up to 30 minutes.

Absorbance values for 100 µL/well from processed experimental animals and standard samples were measured in 96-well plates by a microplate reader. The optical density (OD) at λ= 625 nm was determined and converted to microgram per individual female and male mosquito based on the regression equations derived from the standard curves. Biochemical assays were performed over 3-5 independent biological replicates, including at least three technical replicates per reaction on each plate. Statistical analyses were completed using GraphPad Prism 8.02 (GraphPad Software, San Diego, USA) utilizing a one-way ANOVA and Tukey's multiple comparison post-test ($p < 0.05$).

Table 2-2. Summary of the structure of peptides used in the injections for the biochemical assay and survival rate.

Peptide Name	Peptide Sequence
***Aedae*-ACP**	pQVTFSRDWNAa
***Aedae*-AKH**	pQLTFTPSWa

2.3.5 Injection with Aedae-ACP and determination of the survival rate

The preparations and injections of the female and male one to two-day-old adult *A. aegypti* mosquitoes (n = 15-20) with synthetic *Aedae*-ACP (and diluted DMSO as control) were done as described above in the biochemical analyses section. Following injection, mosquitoes were placed into recovery containers. Injected male and female mosquitoes were provided 10% sucrose solution, and their survival was checked daily for a total duration of 30 days. Data presented represents an average of three independent biological replicates. Survival rates for the mosquitoes were monitored daily, and the survival curve was analyzed with Kaplan-Meier survival analysis and Log-rank Mantel-Cox test ($p < 0.05$) using GraphPad Prism 8.02 (GraphPad Software, San Diego, USA) compared with their corresponding sham-injected controls.

2.3.6 Graphical representation

All of the data were compiled using Microsoft Excel and then transferred into GraphPad Prism 8.02 (GraphPad Software, San Diego, USA) to generate all of the figures and conduct the statistical analyses, which are described in detail in each figure caption.

2.4 Results

2.4.1 ACP immunoreactivity

A representative image of the ACP immunoreactivity in the central nervous system (CNS) of adult *A. aegypti* mosquitoes (**Fig.2-1**), the procedure was repeated several times, and the same distribution of ACP immunoreactivity was observed. In the adult mosquito CNS, ACP immunostaining was localized in two pairs of lateral cells in the brain, and their axonal projections were revealed in the anterior protocerebrum (**Fig. 2-1A**). Also, two cells were observed on the ventral side of the prothoracic segment, and one cell was faintly stained just anterior to the junction between the prothoracic segment and mesothoracic segments of the fused thoracic ganglia. Additionally, two cells were observed in the mesothoracic segments of the ganglia (**Fig. 2-1B**). Faintly staining axonal processes were observed on the dorsal surface in the thoracic ganglia that continued into the abdominal ganglia (data not shown). Furthermore, ACP immunoreactive processes were detected with prominent blebs and varicosities passing through each abdominal ganglia, including the terminal ganglion (**Fig. 2-1C, D**). A summary schematic diagram of ACP peptide distribution in the CNS of adult *A. aegypti* is presented in (**Fig. 2-2**).

2.4.2 Transcript abundance of ACPr in the first six abdominal ganglia and terminal ganglion

RT-qPCR was utilized to measure levels of the ACPr transcript in the pre-terminal abdominal (2^{nd} to 6^{th}) ganglia and terminal ganglion in one-day-old male and female adult *A. aegypti* mosquitoes. Notably, the expression profiling of *Aedae*ACPr revealed that there is no significant enrichment between the pre-terminal abdominal ganglia and the terminal ganglion in both sexes (males, $p = 0.7929$ and for females, $p = 0.7856$) (**Fig. 2-3A, B**).

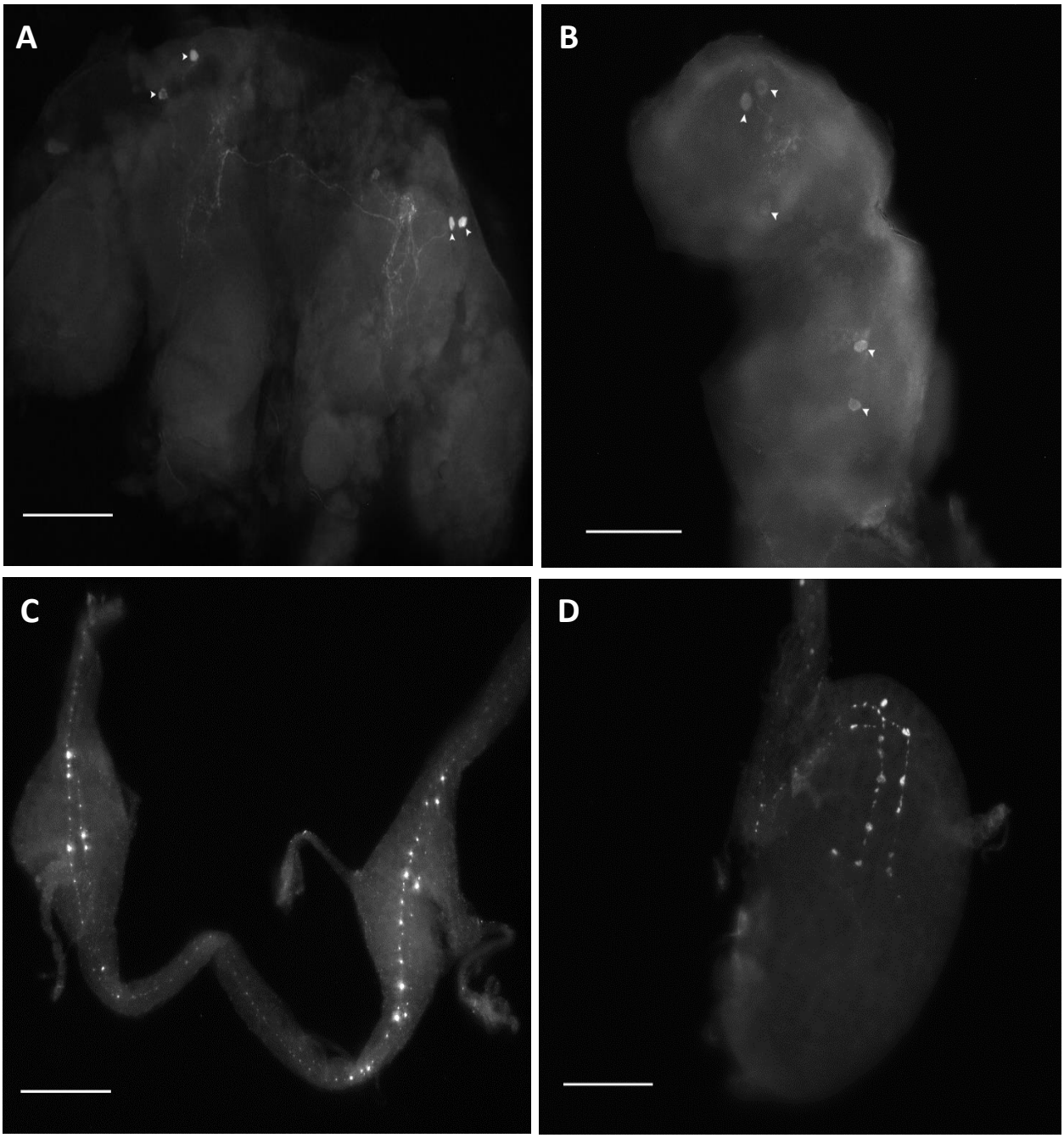

Figure 2-1. Immunolocalization of *Aedae* ACP in the nervous system of adult *A. aegypti*. ACP immunoreactivity (arrowheads) in two pairs of lateral interneurons in the brain **(A)**, a few faintly stained cells in the fused thoracic ganglia **(B)**, and extensive immunoreactive processes in all abdominal ganglia **(C)**, including the terminal abdominal ganglion **(D)**. Scale bars A-B 100μm, C-D 50μm. These images are representative of dozens of preparations that were observed in 4-6 independent experimental replicates.

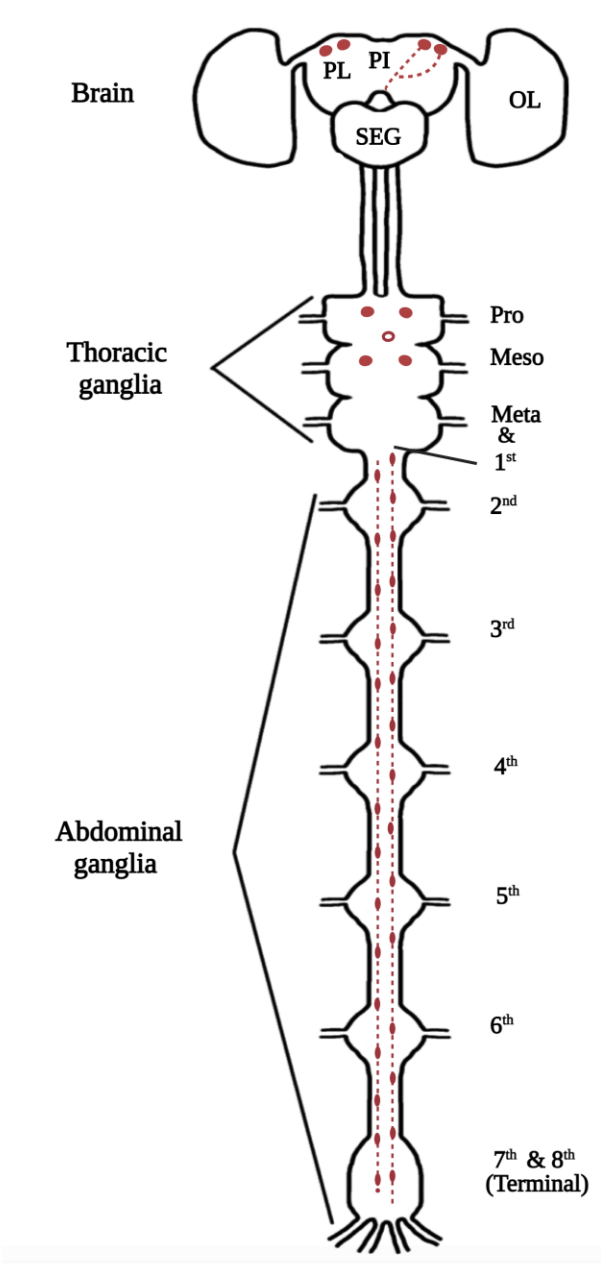

Figure 2-2. Schematic overview illustrating the distribution of immunoreactive staining for the ACP peptide in the CNS of adult *A. aegypti*. The ACP immunoreactivity was strongly detected in two pairs of cells in the pars lateralis of the brain. Two to three cells were detected in the ventral prothoracic ganglia, while two cells were observed in the mesothoracic ganglia. Extensive ACP immunoreactive processes were observed with varicosities and prominent blebs passing through all abdominal ganglia, including the terminal abdominal ganglion. Abbreviations: (PL) pars lateralis, (PI) pars intercerebralis, (SEG) subesophageal ganglion and (OL) optic lobe. Schematic is not drawn to scale. Filled cells (●) represent a 100% detection frequency, while the unfilled cells (O) indicate inconsistent detection frequency.

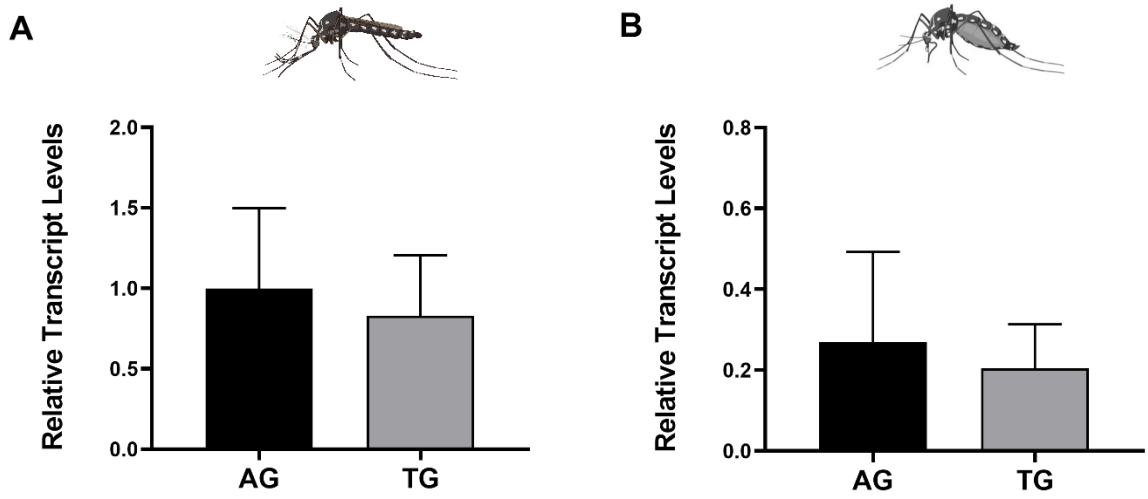

Figure 2-3. Transcript expression pattern of ACPr in abdominal and terminal ganglia of one-day-old adult *A. aegypti.* ACPr transcript abundance in **(A)** male and **(B)** female pre-terminal abdominal ganglia and terminal ganglion**.** Abbreviations: (AG) pre-terminal abdominal ganglia, (TG) terminal abdominal ganglion. Data represent mean ± standard error, as determined using an unpaired two-tailed t-test. Data is an average of 4-5 independent biological replicates. The mosquitoes images were created using BioRender (BioRender.com).

2.4.3 Metabolic effects of Aedae-ACP and Aedae-AKH

As a first step towards discovering physiological roles for the ACP in *A. aegypti,* a potential function related to energy substrate mobilization was examined. The synthetic *Aedae*-ACP and *Aedae*-AKH were separately injected into both females (**Fig. 2-4**) and males (**Fig. 2-5**), one to four-day-old adult *A. aegypti* mosquitoes. Following a 90 min incubation, the glycogen and lipid content of abdomens and lipid and carbohydrate levels in the haemolymph were determined.

At 90 min post-injection, haemolymph carbohydrate content in female mosquitoes was unchanged following *Aedae*-ACP injection but was significantly increased in females injected with *Aedae*-AKH ($p < 0.0001$; **Fig. 2-4A**). For male mosquitoes, injection with both *Aedae*-ACP and *Aedae*-AKH led to significantly elevated carbohydrate levels in the haemolymph ($p = 0.0024$ and $p = 0.0202$, respectively) (**Fig. 2-5A**). Conversely, glycogen level in the abdomen was significantly reduced in both *Aedae*-ACP and *Aedae*-AKH treated female mosquitoes ($p = 0.0279$ and $p = 0.0322$, respectively) (**Fig. 2-4B**), whereas neither peptide influenced glycogen in the abdomen of male mosquitoes (**Fig. 2-5B**). Furthermore, no significant changes in the haemolymph and abdomen lipid levels were observed after the injection with *Aedae*-ACP and AKH in both female (**Fig. 2-4C, D**) and male mosquitoes (**Fig. 2-5C, D**), respectively.

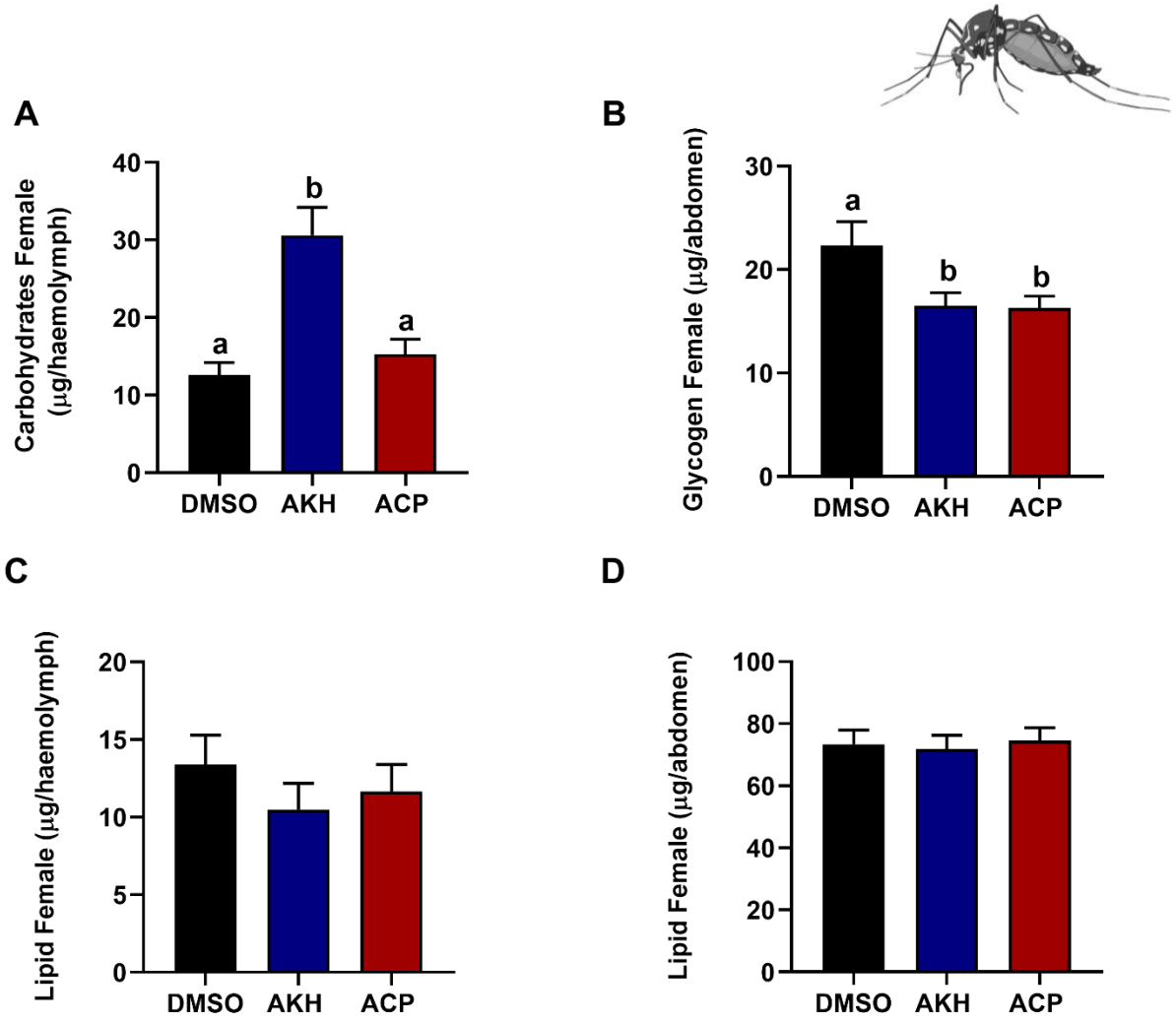

Figure 2-4. Effects of ACP and AKH on carbohydrate, glycogen and lipid mobilization in sugar-fed female adult *A. aegypti*. (A) Carbohydrate in the haemolymph and **(B)** glycogen levels in the abdomen. **(C)** Lipid levels in the haemolymph and **(D)** lipid levels in the abdomen. Different letters denote bars that are significantly different from one another as determined by a one-way ANOVA and Tukey's multiple comparison post-test ($p < 0.05$). Data represent the mean ± standard error. Data is an average of 4-5 independent biological replicates. The mosquito image was created using BioRender (BioRender.com).

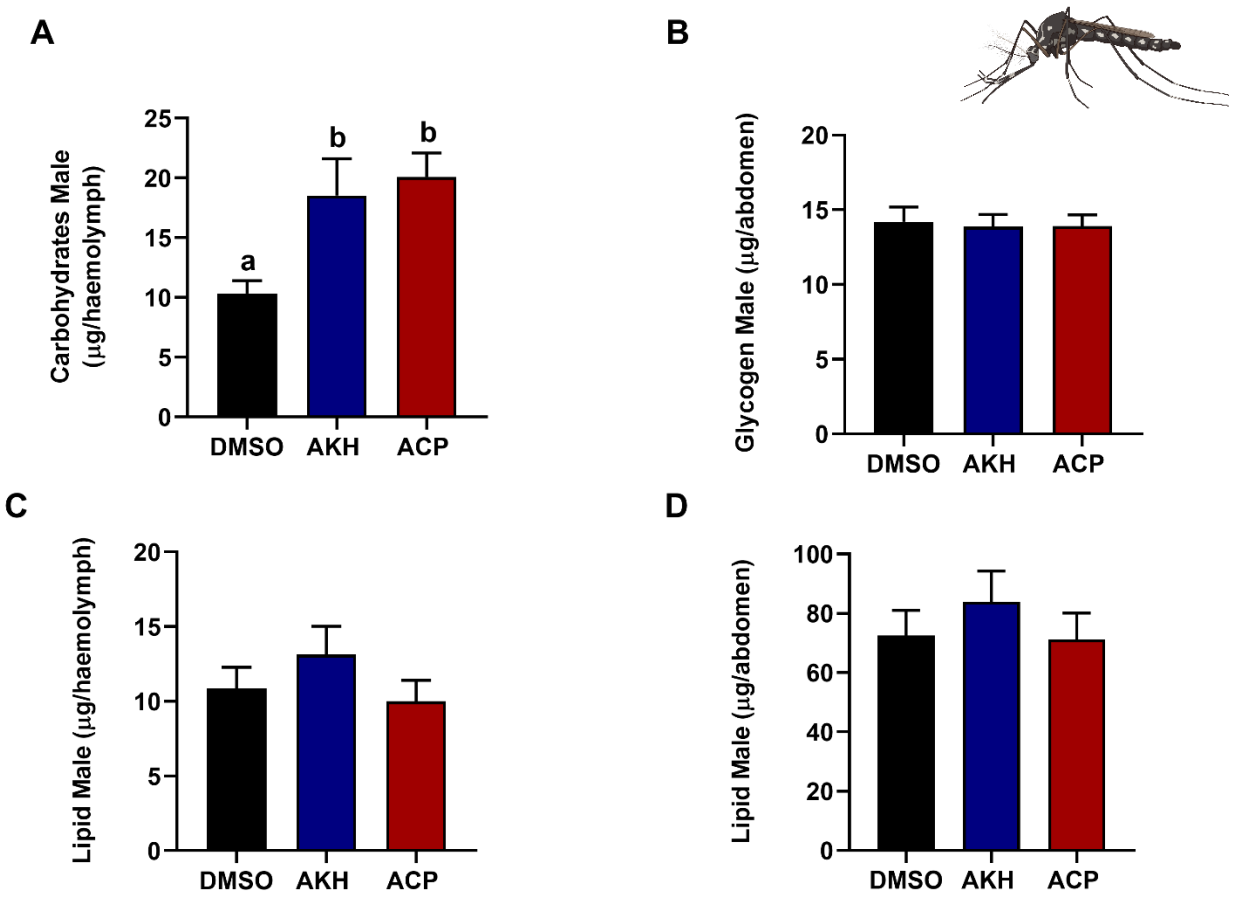

Figure 2-5. Effects of ACP and AKH on carbohydrate, glycogen and lipid mobilization in sugar-fed male adult *A. aegypti.* (A) Carbohydrate in the haemolymph and **(B)** glycogen levels in the abdomen. **(C)** Lipid levels in the haemolymph and **(D)** lipid levels in the abdomen. Different letters denote bars that are significantly different from one another as analyzed by a one-way ANOVA and Tukey's multiple comparison post-test ($p < 0.05$). Data represent the mean ± standard error. Data is an average of 4-5 independent biological replicates. The mosquito image was created using BioRender (BioRender.com).

2.4.4 Survival rate determination

In light of the above findings that indicate ACP contributes towards the regulation of energy substrate levels, I next aimed to determine if ACP signaling influences adult longevity (i.e. the survival rate). To examine this possibility, both male and female one to two-day-old adult *A. aegypti* mosquitoes were injected with *Aedae*-ACP, and their survival was tracked for 30 days. Over the entire 30-day observation period, the statistical analysis showed no significant difference between the survival of ACP-injected mosquitoes compared to the sham-injected control mosquitoes ($p = 0.1462$). Thus, these results demonstrate that ACP-injected mosquitoes exhibit a survival rate similar to the DMSO-treated control mosquitoes (**Fig. 2-6A).** However, reanalyzing a subset of the data, specifically days 10-30 post ACP injection, there was a nearly significant difference between groups ($p = 0.0536$) as determined using Log-rank (Mantel-Cox) test (**Fig. 2-6B**); however, more research is needed to clarify the potential role of ACP on adult *A. aegypti* survival.

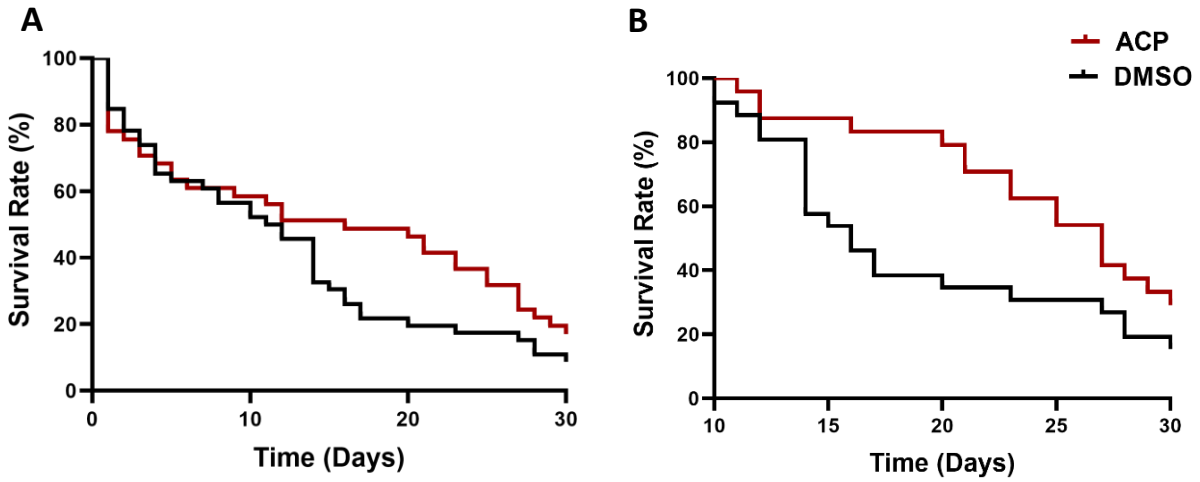

Figure 2-6. Survival rate plots comparing the effect of ACP injection vs. sham-injected adult *A. aegypti* mosquitoes. Survival plot from 0-30 days **(A)** and 10-30 days **(B)**. ACP-injected mosquitoes had a survival rate similar to that of sham-injected (DMSO) control mosquitoes **(A)**. Beginning the analysis following a 10 day recovery (to account for animals that died likely due to injection alone), ACP injected mosquitoes show a nearly significantly higher survival rate compared to sham-injected control mosquitoes ($p = 0.0536$) **(B)**. Sham-injected control (DMSO) mosquitoes are indicated in the black trace, while the experimental mosquitoes injected with ACP are shown in the red trace. Data were analyzed with Kaplan-Meier survival analysis and Log-rank Mantel-Cox test. Data is an average of three independent biological replicates.

2.5 Discussion

The adipokinetic hormone/corazonin-related peptide (ACP) and its receptor (ACPr) are recently discovered and are now known to be extensively widespread throughout many insects (Hansen et al., 2010). Although the ACP neuropeptide and its receptor demonstrate similarity to adipokinetic hormone (AKH) and corazonin (CRZ) along with their cognate receptors, a functional relationship between these three signaling systems has not been determined, but instead, they appear to function independently (Hansen et al., 2010; Wahedi and Paluzzi, 2018; Zandawala et al., 2018). CRZ has many known functions, including cardio-stimulatory activity, melanization, ecdysis regulation and stress physiology, while AKH is well established for its role in regulating energy mobilization in insects (Gäde et al., 1997; Kim et al., 2004; Kubrak et al., 2016; Veenstra, 1989; Zandawala et al., 2021; Ziegler et al., 1990). A previous study demonstrated the presence of the ACP/ACPr signaling system in *A. aegypti* and revealed mRNA enrichment in nervous tissue, particularly in the brain and thoracic ganglia for the ACP peptide (Kaufmann et al., 2009; Wahedi and Paluzzi, 2018) and in the abdominal ganglia for the ACPr (Wahedi and Paluzzi, 2018). In the current study, the cellular localization of the ACP peptide was observed throughout the central nervous system (CNS) of adult *A. aegypti* mosquitoes using immunohistochemistry. Similar enrichment of the ACPr transcript was observed between the pre-terminal abdominal ganglia and the terminal ganglion as determined using quantitative RT-PCR (RT-qPCR). In addition, the effects of ACP on energy substrate mobilization were examined by injecting the synthetic peptide into adult female and male mosquitoes. Lastly, to further elucidate functional roles of the ACP/ACPr signaling system in mosquitoes, the survival rate after ACP injection of adult *A. aegypti* mosquitoes was measured.

2.5.1 Distribution pattern of ACP in the CNS

Previous studies localized *Aedae*AKHs I and II to the brain, thoracic ganglia, and corpora cardiaca (CC) of *A. aegypti* and *A. gambiae* (Kaufmann and Brown, 2006; Kaufmann et al., 2009). *Aedae*AKH-II was later characterized as *Aedae*-ACP (Hansen et al., 2010); therefore, the immunoreactivity which was herein detected in the brain and thoracic ganglia was attributed to ACP as previous findings have observed that the AKH synthesis and storage are confined to the CC (Bogerd et al., 1995; Diederen et al., 1987; Diederen et al., 2002; Hansen et al., 2010; Kaufmann et al., 2009; Kaufmann and Brown, 2006; Noyes et al., 1995).

In this current study, ACP immunoreactivity was localized in two pairs of lateral cells in the anterior protocerebrum of the *A. aegypti* brain. Moreover, two to three cell bodies were identified in the ventral side of the prothoracic segment; two cells were also observed in the mesothoracic segment of the fused thoracic ganglia. In addition, extensive ACP-immunoreactive axonal processes with varicosities and prominent blebs were also detected passing through each abdominal ganglion. A previous study showed that varicosities in the invertebrates were involved in the cell-to-cell interactions and the formation of the presynaptic terminals that induce specific changes in their functions and structures (Giachello et al., 2012). Further, several studies found that the varicosities in the sea hare *Aplysia californica* and the snail *Helix pomatia* play a role in the neurotransmitter release modulation and growing neuron remodelling (Bailey and Chen, 1988; Cibelli et al., 1996; Ghirardi et al., 1996, 2000; Hatada et al., 2000; Angers et al., 2002; Chin et al., 2002; Giachello et al., 2012). Therefore, ACP immunoreactive varicosities and blebs observed in the *A. aegypti* mosquito axonal processes demonstrate evidence of cell-to-cell communication, suggesting this neuropeptide acts within the CNS as a neurotransmitter or neuromodulator in the mosquito. Further, a similar observation to the current study was reported earlier (Wahedi, 2018),

whereby the *Aedae*-ACP transcript was localized by fluorescent *in situ* hybridization in two pairs of lateral cells in the brain, 2-3 cells in the thoracic ganglia, while no neurons expressing the *Aedae*-ACP transcript were detected in the abdominal ganglia. The current results are also consistent with a previous study in the kissing bug, *R. prolixus*, with ACP immunoreactivity detected in the brain, particularly in two bilaterally paired cell bodies located in the protocerebrum (Patel et al., 2014). Additionally, a study published recently in the locust, *L. migratoria,* revealed that ACP peptide was detected in numerous cells in the bilateral forebrain and the par intercerebralis (Hou et al., 2021).

Lately, a study that occurred in the Orthoptera observed that the expression of ACP is notably different from other insect species. By examining the ACP-immunoreactivity in the adult *Schistocerca gregaria, G. bimaculatus* and *L. migratoria,* many neuroendocrine cells were observed in the pars intercerebralis that projected to the CC, while a small number of the interneurons in the pars lateralis of the brain were detected (Veenstra, 2021). Unlike the distribution of ACP in the brain of Orthoptera, the results of the ACP immunoreactivity of the adult American cockroach *P. americana* brain (Veenstra, 2021) show consistency in the distribution of ACP within the adult brain of *A. aegypti* mosquitoes. Specifically, in the adult *P. americana*, ACP immunoreactive interneurons were expressed only in a small number of ACP interneurons in the lateral part of the brain (i.e. pars lateralis), while no ACP neuroendocrine cells in the pars intercerebralis were detected (Veenstra, 2021). Hence, based on the immunoreactive distribution of ACP along with the characteristics of this staining within distinct regions of the CNS in adult *A. aegypti*, this suggests that this neuropeptide may hold a function in the coordination or communication between the anterior and posterior regions of the nervous system, suggesting a role as a neurotransmitter and/or neuromodulator.

Furthermore, previous studies prior to Hansen et al., 2010 discovering the ACP system reported that AKH-like immunoreactivity was detected in 2 pairs of lateral neurosecretory cells in the brain at the anterior region of the protocerebrum in both *A. aegypti* and *A. gambiae*. However, as was suggested later, this AKH-like immunoreactivity associated with the protocerebrum is likely to represent the cross-reactivity of ACP-producing neurons since the storage and synthesis of AKH were determined to be restricted to the CC (Diederen et al., 2002; Hansen et al., 2010; Kaufmann et al., 2009; Kaufmann and Brown, 2006). Moreover, AKH-like immunoreactivity was observed within one cell in the prosegment of thoracic ganglia in ten-day-old female *A. aegypti*, while three clusters of cells in the thoracic ganglia were observed specifically within the prothoracic and mesothoracic segments of *A. gambiae* (Kaufmann and Brown, 2006; Kaufmann et al., 2009). ACP-like immunoreactivity was determined in three to four neurons in the brain within the anterior region of each hemisphere, and their axons project processes to the thoracic and abdominal ganglia in the flour beetle, *T. castaneum* (Hansen et al., 2010). ACP-like immunoreactivity wasn't detected outside of the CNS in either *R. prolixus* or *T. castaneum*, nor was it associated with neurohaemal organs (Hansen et al., 2010; Patel et al., 2014), suggesting its physiological role is constrained within the nervous system. Similarly, the results of this study also indicate that ACP-immunoreactivity is restricted within the CNS, suggesting its functional role may be constrained to the mosquito nervous system.

In a few insect species, ACP has been detected in interneurons, and no neuroendocrine cells of the pars intercerebralis projecting to the CC were observed (Hansen et al., 2010; Patel et al., 2014). Thus, the ACP immunoreactive cells that were detected in the adult *A. aegypti* brain appear to be interneurons signaling to the ventral nerve cord (VNC), whereas the cells in the thoracic ganglia might signal to the periphery. There are several types of interneuronal cells, such

as inter-segmental ascending and descending neurons, local amacrine neurons and wide-ranging projection neurons (Nässel and Homberg, 2006). In addition, neuropeptides in insects are expressed by both neuroendocrine cells and interneurons. The interneurons have axons projecting within the CNS, and neuroendocrine cells have axons that leave the CNS and release their products, such as hormones, into the haemolymph (Veenstra, 2021). Moreover, neurosecretory cells are usually monopolar, which have axonal processes that are mostly projected directly to the peripheral tissues where their products are released. On the other hand, the interneurons are generally involved in the regulation of nervous system-derived factors. As they could send their axonal processes forming synapses in different ganglia with a far-reaching neuron, or they might also be located locally with a synapse within a single ganglion (Nation, 2002; Nässel and Homberg, 2006). In insects, several neuropeptides have been detected in the brain and the abdominal ganglia (Nässel, 2002). In contrast, limited studies exist on the presence of neurosecretory cells in the thoracic ganglia; therefore, the nature of the cells that were observed in the *A. aegypti* mosquito thoracic ganglia remains unclear and further investigations are required.

The action of ACP in the adult *A. aegypti* mosquito could be derived from either interneuron in the brain or neurosecretory cells in the thoracic ganglia (or both) and mediated via the abdominal ganglia. According to a previous study that confirmed ACPr transcript was enriched in the abdominal ganglia in the adult mosquito *A. aegypti* (Wahedi and Paluzzi, 2018), this also supports the notion that the abdominal ganglia might be a likely primary target of ACP action. Consequently, there is still work that needs to be done to determine the function of the ACP immunoreactive interneurons in the brain and the cells observed in the thoracic ganglia, as well as understand what triggers the activation of these neurons producing ACP. Additionally, ACP-immunoreactive axonal processes observed might regulate or have an activity in the abdominal

ganglia. This suggests that ACP might be acting as a modulator and could influence the release of other neuropeptides, such as pyrokinin, insulin-like peptides, leucokinins and CAPA, which are expressed in the abdominal ganglia (Nässel, 2002; Hellmich et al., 2014; Sajadi et al., 2020); however, further studies are required to determine downstream targets of ACP signaling in the CNS.

2.5.2 Transcript expression pattern of ACPr in the abdominal and terminal ganglia

In larval mosquitoes, the ventral nerve cord has eight separate abdominal ganglia. On the other hand, in adults, six distinct abdominal ganglia are present due to the fusion of the 1st abdominal ganglion to the meta-thoracic ganglion, while the 7th and 8th ganglia are fused, forming the terminal ganglion (Brown and Cao, 2001). Therefore, the transcript expression profiles of *A. aegypti* ACPr of the abdominal ganglia and the terminal ganglion were measured to help in the functional interpretation to reveal potential roles for ACP. Examination of the abdominal ganglia (2nd-6th) and the fused terminal ganglion (7th & 8th) in one-day-old *A. aegypti* male and female mosquitoes showed that there is no significant difference in ACPr transcript abundance between the abdominal and terminal ganglia in both sexes. Thus, these results are consistent with the observations of ACP immunoreactivity as we found that there is similar distribution in the form of axonal projections in each of the abdominal ganglia. Moreover, the widespread varicosities and blebs that were revealed indicate sites of communication (i.e. synapse) within each of the ventral nerve cord ganglia. Also, a previous report demonstrated the enrichment of the ACPr transcript in the abdominal ganglia while the ACP transcript was most abundant in the brain, followed by the thoracic ganglia (Wahedi and Paluzzi, 2018). All of these suggest that ACP has a neuromodulator and/or neurotransmitter function in the male and female adult *A. aegypti* mosquito. However,

further studies are necessary to validate this proposed role and downstream targets of ACP/ACPr signaling in the adult *A. aegypti* mosquito.

2.5.3 Metabolic actions of Aedae-ACP

AKH and ACP are more closely related to each other compared to the CRZ system (Hansen et al., 2010). As a result of this closer structurally similarity between AKH and ACP, this study sought to determine whether the metabolic function of AKH extends to ACP in adult *A. aegypti* mosquitoes. Similar to the findings in female *A. gambiae* (Kaufmann and Brown, 2008), our results confirm that injection of synthetic AKH resulted in an increase in haemolymph carbohydrate levels and a reduction in glycogen stores in the female *A. aegypti* mosquitoes. Thus, in *A. aegypti*, AKH can also be designated as a hypertrehalosaemic hormone, and like other AKHs, functions comparably to the vertebrate hormone glucagon in female adult mosquitoes. In contrast, there was only a marginal insignificant increase in the haemolymph carbohydrate levels in response to ACP; however, interestingly, there was a significant reduction in the glycogen stores in females. This is in contrast to previous findings in female *A. gambiae*, where ACP (then referred to as AKH-II) was found to have no effect on glycogen stores (Kaufmann and Brown, 2008). To date, no study has worked to determine the metabolic effect of ACP on the male mosquito. Surprisingly, the current data indicate that both AKH and ACP caused a significant increase in the carbohydrate level in the haemolymph, while having no influence on the glycogen stores in male mosquitoes. A previous study showed that the *A. aegypti* ACPr is highly expressed (over 100-fold) in the carcass, including the fat body in adult male mosquitoes but not in female mosquitoes (Wahedi and Paluzzi, 2018). This difference in the peripheral enrichment of ACPr could explain the difference in the metabolic effects of ACP between male and female adult *A. aegypti* mosquitoes. With regards to

metabolic actions in relation to lipids, the results of this study revealed that there are no changes in haemolymph or abdomen lipid levels in *Aedae*-AKH or *Aedae*-ACP injected mosquitoes, irrespective of sex. Similarly, injection of *Rhopr*-ACP did not result in lipid mobilization in *R. prolixus*; this effect was only observed in response to *Rhopr*-AKH (Patel et al., 2014). The primary metabolic fuel in mosquitoes is not identical to that in *R. prolixus*; specifically, the primary energy substrate used by mosquitoes are carbohydrates in the form of trehalose, but prolonged movement or energy demand could also result in lipid breakdown (Clements, 1992; Briegel et al., 2001).

Recently, a study conducted on the locust *L. migratoria*, demonstrated that ACP facilitates the utilization and oxidation of lipid during their long-term flight. Specifically, using metabolomic and transcriptomic analyses, it was shown that ACP is involved in the oxidation and the transport of fatty acids in the flight muscles (Hou et al., 2021). In mosquitoes, the most abundant free amino acid is proline, which has been suggested as a possible flight metabolite in *A. aegypti* (Scaraffia and Wells, 2003). Thus, ACP may be involved in the conversion of energy substrates, as proline is a key intermediate in metabolism functioning as a transporter of acetyl units from the fat body to the flight muscle, where they re-enter the citric acid cycle and are oxidized to produce ATP (Weeda et al., 1980; Scaraffia and Wells, 2003). Previous studies have implicated CRZ and AKH in nutritional and oxidative stress (Bharucha et al., 2008; Bednářová et al., 2015; Kubrak et al., 2016; Zandawala et al., 2021). Thus, ACP might share a stress-related role in insects.

2.5.4 Determination of the Survival rate

A recent study showed that ACP and other insect neuropeptides such as CRZ and *hugin* encoding genes were differentially expressed during *Plasmodium berghei* infection in the brain of the malaria vector, *Nyssorhynchus albimanus* (Alvarado-Delgado et al., 2021). This study also observed that after *P. berghei* infection, the ACP transcript showed a significant increase in expression in the brain, suggesting that ACP might have a role in the immune or stress response in *Ny. albimanus* (Alvarado-Delgado et al., 2021). In the current study, I aimed to determine if adult longevity (i.e. the survival rate) was altered after the injection of male and female *A. aegypti* with synthetic *Aedae*-ACP. Generally, in comparison with control sham-injected mosquitoes, no significant difference in the survival rate of female and male mosquitoes was observed after the ACP injection over the entire 30 day-long observation period. On the other hand, beyond the first ten days post-injection, which saw large treatment-independent mortality likely as a result of injury following injection, the data reveal a substantial increase in survival in the ACP-injected mosquitoes compared to the control mosquitoes. Therefore, further investigation should be done to confirm and clarify the role of ACP in the survival rate. For instance, injecting different doses of ACP in adult mosquitoes may reveal dose-dependent effects that could influence the survival rate of adult mosquitoes. Additionally, a recent study in *L. migratoria* demonstrated that despite the ACP mutants eliciting differences in lipid utilization and oxidation, there is no impairment on their survival rate with similar longevity to that of wild-type locusts (Hou et al., 2021). Consequently, further investigation is required to explore the mechanism and functioning of ACP in insects.

2.5.5 Concluding remarks

The data presented here provide evidence of the distribution of ACP within the nervous system in the adult mosquito *A. aegypti*. The localization of the *A. aegypti* ACP peptide was found within neurons in the brain and thoracic ganglia, with axonal processes projecting into the abdominal ganglia without evidence of processes leaving the nervous system, suggesting a function for ACP retained in the CNS as a neurotransmitter or neuromodulator. ACP receptor transcript quantification in the abdominal and terminal ganglia revealed that there is no significant difference between the expression of ACPr in the pre-terminal abdominal ganglia and the terminal ganglion in both sexes. Further, to examine a potential functional overlap between AKH and ACP, a biochemical substrate mobilization assay revealed a reduction in glycogen stores in the female adult *A. aegypti,* while an increase in the carbohydrate haemolymph level in the male *A. aegypti* was found in response to ACP injection. Finally, the adult survival rate experiment showed that no significant difference occurred between the control and the ACP-injected adult mosquitoes, but more work is needed as the results suggest an increase in longevity after ACP injection. This current study provides insight to enhance our mechanistic understanding of the crucial roles of the ACP signaling system in the adult *A. aegypti,* which might be paramount for developing novel approaches and improving existing pest control strategies in order to lessen the burden of these medically important disease vectors.

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2.7 References

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Chapter 3:

Insights into adipokinetic hormone/corazonin-related peptide receptor specificity and key residues important for ligand activation in the mosquito, *Aedes aegypti*

3.1 Summary

Neuropeptides have a crucial role in regulating various behavioral and physiological processes in insects. Adipokinetic hormone/corazonin-related peptide (ACP) and adipokinetic hormone (AKH) are two neuropeptides that demonstrate homology to the vertebrate gonadotropin-releasing hormone (GnRH). Despite the structural similarity and the close evolutionary relationship between the ACP and the AKH, their signaling systems function independently. To date, the role of ACP and its receptor (ACPr) remains unclear in the *Aedes aegypti* mosquito. Structure-activity relationships (SARs) are often carried out on peptide ligands to determine critical residues for bioactivity and receptor activation; however, residues and features necessary for ligand binding and specificity in the receptors themselves are less studied. Herein, this study focuses on the ACP and AKH that share the closest evolutionary relationship and examines structural features of their receptors critical for conferring receptor activation and ligand specificity. Firstly, to determine the specific regions of the ACPr that are most critical for ligand fidelity and specificity, ACPr chimeras were created by singly replacing each of the three ACPr extracellular loops (ECLs) in their entirety and incorporating the corresponding ECLs from the AKH receptor (AKHr). Heterologous functional assays determined that the three ACPr ECL chimera receptors with complete replacement of full individual ECLs showed no response to either ACP or AKH. These results suggest that the complete replacement of each extracellular loop singly is crucial to ligand binding and recognition. Secondly, through a more targeted approach, I aimed to determine specific residues critical for functional ligand-binding by substituting select highly conserved residues within the three ECLs of the ACPr with those from the AKHr. Modifications of specific and highly conserved residues in these ACPr ECLs chimeras suggest that the third extracellular loop contains the most critical features necessary for ACP binding and receptor

activation. In addition, the combination of two selectively-modified ACPr ECLs demonstrated a significant decrease in response to *Aedes* ACP compared to the native ACPr response. Interestingly, the combination of the ACPr ECL 2 and ECL3 chimeras showed the same response to *Aedes* AKH as the ACPr, while the combination of the ACPr ECL1 and ECL2 chimeras showed a significantly increased response to *Aedes* AKH compared to the ACPr. Lastly, combining all of the ACPr ECLs chimeras together resulted in a significant decrease in response to *Aedes* ACP compared to the ACPr response. In contrast, a significantly increased response to *Aedes* AKH was observed in the receptor chimera combining selected modifications in all three ECLs compared to native ACPr. Hence, in this current study, the particular residues that are essential for ACP ligand interaction were identified due to the detrimental effect that occurred in ACPr activation after the selective modification of key residues localized within the extracellular domains of the receptor.

3.2 Introduction

Neuropeptides and their G protein-coupled receptors (GPCRs) play a central role in the regulation of a wide variety of physiological, behavioral and developmental processes during the life cycle of arthropods, such as feeding, growth, metabolism, reproduction, locomotion and development. They also play a crucial part in the chemical cell-to-cell communication systems between different types of cells (Nässel, 2002; Nässel and Homberg, 2006; Barón et al., 2010; Nässel and Winther, 2010; Nässel and Zandawala, 2019). Most neuropeptide receptors are G protein-coupled receptors (GPCRs), and they typically contain seven transmembrane domains with an intracellular C-terminus and N-terminus localized to the extracellular environment (Gilman, 1987; Vanden Broeck, 1996). The seven transmembrane domains are connected by three extracellular loops (ECLs) and three intracellular loops, with the former participating in the binding and interaction with a specific ligand (Caers et al., 2012b; Pierce et al., 2002; Vanden Broeck, 1996; Wise, 2012; Zandawala et al., 2015a). In recent years, neuropeptides have been extensively studied in various insect orders such as Blattodea, Diptera, Coleoptera, Hymenoptera, Hemiptera, and Lepidoptera (Riehle et al., 2002; Roller et al., 2008; Traverso et al., 2016; Chang et al., 2018; Llopis-Giménez et al., 2019; Bläser et al., 2020; Ragionieri and Predel, 2020).

Adipokinetic hormone (AKH), corazonin (CRZ) and adipokinetic hormone/corazonin related peptide (ACP) are three neuropeptides that were found in invertebrates and considered homologous to the mammalian gonadotropin-releasing hormone (GnRH) (Hansen et al., 2010; Gäde et al., 2011; Roch et al., 2011; Li et al., 2016). The ACP receptor (ACPr) is activated by a neuropeptide very closely related to both AKH and CRZ and was hence known as ACP (Hansen et al., 2010). Despite the ACP signaling system being structurally intermediate between AKH and CRZ signaling systems, the functional role of ACP and its receptor in most arthropods remains

unclear. Moreover, these three neuropeptides and their receptors are not always found together in all arthropods. For instance, mosquitoes have all the three neuropeptide signaling systems (AKH, ACP, and CRZ) while CRZ is absent in the beetle *Tribolium castaneum*, and ACP is missing in the fruit fly *Drosophila melanogaster* (Hansen et al., 2010). Interestingly, the lack of ACP in *Drosophila* has restricted the potential use of molecular genetic tools that could examine its physiological functions (Hansen et al., 2010; Zandawala et al., 2015a).

AKH system was duplicated before the emergence of the phylum Arthropoda, leading to the ACP and AKH neuropeptide systems (Hansen et al., 2010). Owing to gene duplication in arthropod lineage, ACP and AKH signaling systems were considered to be paralogous (Hauser and Grimmelikhuijzen, 2014). However, various studies showed the high selectivity of the ACP, AKH, CRZ receptors and their response only with their cognate ligands. Therefore, these three neuropeptides and their receptors are indeed considered to work independently (Hamoudi et al., 2016; Hansen et al., 2010; Oryan et al., 2018; Wahedi and Paluzzi, 2018; Zandawala et al., 2015a). *In vitro* studies demonstrated that high concentrations of AKH can activate the ACP receptor and *vice versa* in the silkworm *Bombyx mori* (Zhu et al., 2009; Shi et al., 2011). However, it is important to note that CRZ failed to activate AKH and ACP receptors (and *vice versa*) in *Anopheles gambiae* and *Rhodnius prolixus*, which suggested that the AKH and ACP are more related to each other compared to the CRZ system (Hansen et al., 2010; Zandawala et al., 2015a). Thus, these three signaling systems are a good example of receptor-ligand co-evolution (Caers et al., 2012b; Li et al., 2016).

Many species of mosquitoes are known as vectors of pathogens that cause several diseases in humans and other vertebrates. The mosquito *Aedes aegypti* is such a chief vector for pathogens, including Dengue and Yellow fever, Chikungunya and Zika viruses that are the causative agents

of chronic and acute illnesses in humans globally (Kotsakiozi et al., 2017). As a result of ongoing environmental change and global trade, there has been an increase in the geographic range of these diseases; therefore, a deep understanding of mosquito transcriptomics, genomics, and the regulation of physiological processes by neuropeptides are essential.

The specificity of the ligand for its receptor arises from the recognition of the binding pocket of the GPCR. Hence, to enhance our understanding of how the ACPr is selectively activated by its native ACP ligand, this study implements a structure-activity analysis through the generation of chimera receptors using mutagenesis. Previously, a series of analogues based on the original ACP peptide of *A. aegypti* were designed and screened against the ACPr using a heterologous system to identify the crucial residues of the ligand necessary for receptor activation (Wahedi et al., 2019). This insight gained on the ACP ligand guided this research study focusing instead on the receptor (ACPr) by designing receptor chimera. Thus, in this current study, I used the adult mosquito *A. aegypti* as a model insect to study critical residues and features that facilitate specificity of ACPr for its ligand (ACP) while preventing promiscuous activation by the structurally- and evolutionarily-related peptide, AKH (Hansen et al., 2010; Hauser and Grimmelikhuijzen, 2014; Tian et al., 2016; Zandawala et al., 2018). To achieve this, I first generated ACP receptor chimera using mutagenesis by singly replacing the three ACPr extracellular loops (ECL1, ECL2, and ECL3) in their entirety and then incorporating the corresponding ECL from the AKH receptor (AKHr). These chimeras were then tested using a functional receptor assay to confirm whether ACP was still functional after swapping out the ACPr ECL1, 2 and 3 with the ECL1, 2 and 3 of AKHr. Secondly, following bioinformatic analysis and multiple sequence alignment of multiple insect ACP and AKH receptors, I predicted the specific residues in the ACPr extracellular loops that might be most critical for ligand fidelity and

specificity in the mosquito *A. aegypti*. Three independent ACPr chimeras were generated after selecting highly conserved residues of the three ACPr extracellular loops and replacing them with residues highly conserved in insect AKH receptors. Thus, this study set out to test the following hypotheses: (i) Modification of extracellular loops of the ACPr determines sensitivity to binding of its native ligand, specifically ACP, while altering its sensitivity to other structurally-related ligands, such as AKH; (ii) ACPr chimera containing all three extracellular loops or their highly-conserved residues from insect AKH receptors will elicit improved responsiveness to AKH.

3.3 Materials and Methods

3.3.1 Heterologous functional receptor assay

3.3.1.1 Generation of A. aegypti ACP receptor chimera by site-directed mutagenesis

To create ACP receptor (ACPr) chimera, the native *A. aegypti* ACPr sequence was used to incorporate the three extracellular loops of the *A. aegypti* AKH receptor (AKHr) sequences using the Primer3 module in Geneious® 6.1.8 Software (Biomatters Ltd, Auckland, New Zealand) based on a previously published sequences (Wahedi and Paluzzi, 2018; Wahedi et al., 2019). The open reading frame (ORF) of the cloned *A. aegypti* ACPr was inserted into pBudCE4.1 mammalian expression vector following procedures described previously (Wahedi and Paluzzi, 2018; Wahedi et al., 2019). The ACPr chimeras were created by singly replacing the ACPr extracellular loops (ECLs) and incorporating ECLs of *Aedes* AKHr (see **Fig. 3-1**; schematic overview of the approach). The Consensus Constrained Topology Prediction (CCTOP) Web Server was used as a guide to reveal and determine the positions of extracellular loops in order to design the forward and reverse primers used in PCR-based site-directed mutagenesis (Dobson et al., 2015). The forward and reverse primers were designed using Primer 3 in Geneious Software (**Table 3-1**). The functional validation of the three ACPr chimeras was examined using a heterologous functional assay described previously (Wahedi and Paluzzi, 2018). Also, to identify the specific key residues that are highly conserved in the ACP and the AKH receptors that might be critical for ligand-binding specificity, the sequences of both these receptors in *A. aegypti* were compared with homologous sequences from other insects (see **Table 3-2**), with the analysis focusing on conserved residues within the extracellular loops (ECL1, ECL2, and ECL3) of these two distinct receptor families using the ClustalW online server. The Primer3 module in Geneious® 6.1.8 Software was used to design the forward and reverse primers used to substitute the highly conserved residues for

the three selective ECLs chimeras of *Aedes* ACPr were created using Primer 3 in Geneious Software (**Table 3-3**).

Q5 High Fidelity DNA Polymerase (New England Biolabs, Whitby, ON) was used to amplify the three extracellular loop fragments and the native *Aedae*ACPr construct in pBudCE4.1 Gα15 was used as a template (Wahedi et al., 2019). PCR products (ACPr ECL1, ACPr ECL2 and ACPr ECL3) were assembled into pBudCE 4.1 Gα15 using NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs, Whitby, ON) following the protocol guidelines. Briefly, each PCR product was mixed with HiFi DNA Assembly Master Mix and pBudCE 4.1 Gα15 (using a 2:1 insert:vector ratio) and then incubated in a thermocycler at 50°C for 30 minutes. For the competent cell transformation, each HiFi cloning assembled product was mixed with NEB 5-alpha competent *E. coli* cells and placed on ice for 30 minutes, followed by a 1-minute heat shock at 42°C before finally placing the mixture on ice for at least 10 minutes. Then, SOC media was added into the mixture that was immediately spread onto LB agar media plates containing Zeocin™ antibiotic and incubated overnight at 37°C. The recombinant bacteria containing these constructs were grown overnight in liquid cultures of LB media containing 25 μg/ml of Zeocin™ antibiotic. DNA isolation was then carried out using the Monarch® Plasmid Miniprep Kit (New England Biolabs, Whitby, ON) following recommended guidelines. All of the Miniprep DNA samples were quantified and submitted for Sanger sequencing (Center for Applied, Genomics, Hospital for Sick Children, Toronto, ON) to verify base-pair accuracy and orientation. Endotoxin-free and higher titer plasmid DNA was purified from overnight bacterial cultures using a ZymoPURE™ II Plasmid Midiprep Kit (Zymo Research, Irvine, USA) and used for transfection of mammalian cells utilized in the functional heterologous receptor assay.

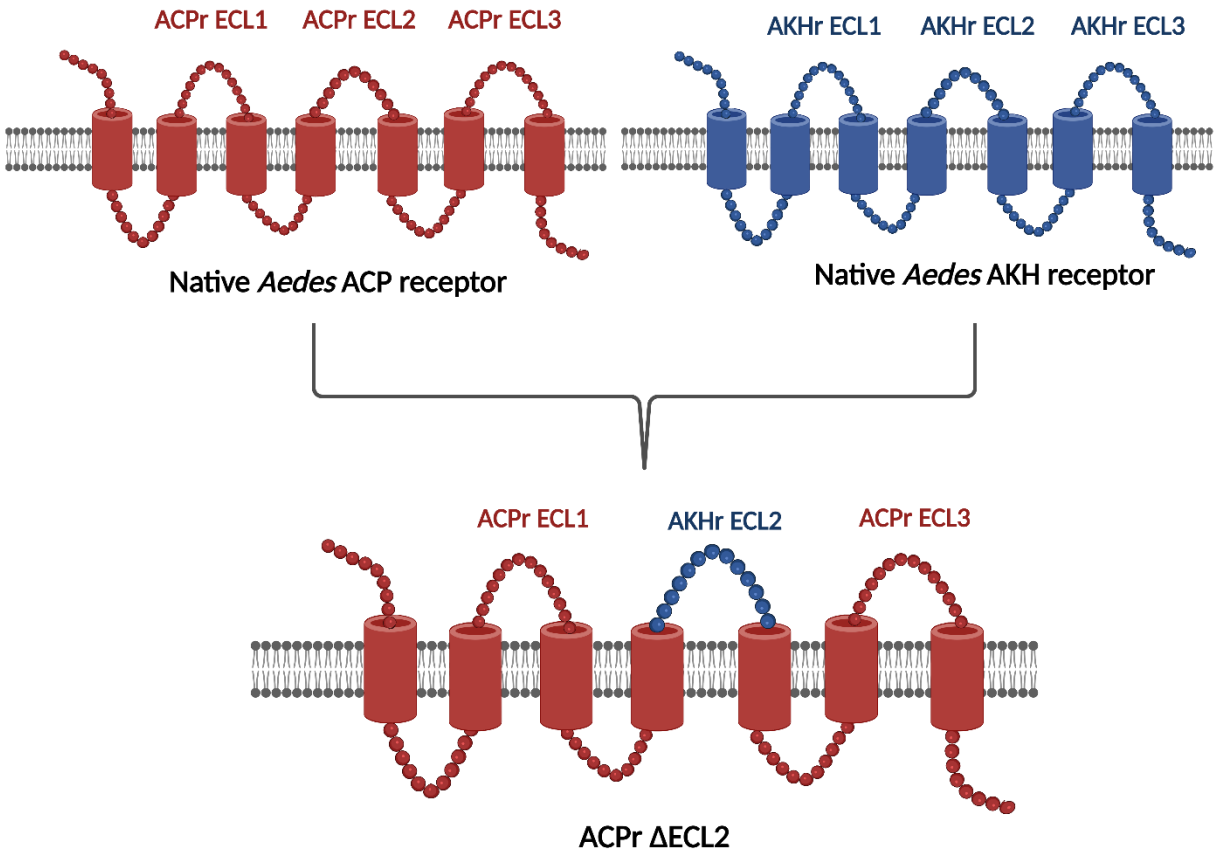

Figure 3-1. Schematic diagram showing the *Aedes* ACPr chimera using the extracellular loop of *Aedes* AKHr and the native sequence of *Aedes* ACPr. The original *Aedes* AKH receptor and the three extracellular loops of AKHr (AKHr ECL1, AKHr ECL2, AKHr ECL3) are presented in blue. The original *Aedes* ACP receptor and the three extracellular loops of ACPr (ACPr ECL1, ACPr ECL2, and ACPr ECL3) are shown in red. For brevity, only the ACPr ECL2 chimera (ACPr ΔECL2) is shown in the above example at the bottom of the figure. This illustration was created using BioRender (BioRender.com).

Table 3-1. Primers used for mutagenesis creating the three ECLs chimera of *Aedes* ACPr.

Primers Name	Sequences (5′- > 3′)
ECL1-F1	GGCGATATCATGTGTCGTTTCCTGTTCATGCGG
ECL1-F2	TGGAGGGCGGGCGATATCATGTGTCGT
ECL1-F3	TGTCCAATGGAGGGCGGGCGATATC
ECL1-R1	CAGTTGCCGCCCACAGTGGGATCATGATG
ECL1-R2	CTCCATTGGACAGTTGCCGCCCACAGTG
ECL1-R3	TCGCCCGCCCTCCATTGGACAGTTGC
ECL2-F2a	TACTTTCTCGAATGACAACTACCATACG
ECL2-F2b	GACGTATAATACTTTCTCGAATGACAAC
ECL2-F2c	GCAATGTGTGACGTATAATACTTTCTC
ECL2-R1	CAGGTGTGGTTCGGGTGTATTTCGAAGATTATACTCTGTG
ECL2-R2	TATTATACGTCACACATTGCTCATACCAGGTGTGGTTCGGG
ECL3-R1	TCCAGCCAGAGTGTCATCACAACGTACG
ECL3-R2	CTGATTTTTGATCCAGCCAGAGTGTCATC
ECL3-R3	CAACGTTTTCCGCTGATTTTTGATCCAGCC
ECL3-F1	ATCAACGTGTGGGGCTCTTTCTGATGGCG
ECL3-F2	CGGAAAACGTTGATCAACGTGTGGGGCTC
ECL3-F3	TCAAAAATCAGCGGAAAACGTTGATCAACG
pBud_632F	CGAAATTAATACGACTCACTATAGGG
pBud_771R	TGATGATGACCGGTATGCAT

Table 3-2. GenBank accession numbers and references for the receptor sequences used in Fig. 3-3. The receptors that lack references were predicted by automated computational analysis.

Receptor	Deorphanized/ Putative Ligand(s)	Accession Number	Reference(s)
Aedes aegypti ACP	ACP	AVA08868	(Wahedi and Paluzzi, 2018)
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)
Acyrthosiphon pisum AKHr	AKH	XP_003245941	(Li et al., 2016)
Acyrthosiphon pisum AKHr1	AKH	XP_001655248	
Aedes aegypti AKHr isoform 1a	AKH	AVI09459	(Oryan et al., 2018)
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)
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	AAN10047	(Park et al., 2002)
Drosophila melanogaster		AAC61523	(Hauser et al., 1998)
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	
Schistocerca gregaria AKHr	AKH	AVG47955	(Marchal et al., 2018)
Daphnia pulex AKH NMR		ACD75498	

Table 3-3. Primers used for mutagenesis to substitute specific residues of ACPr ECLs that are highly conserved and may be important for ligand specificity of *Aedes* ACPr.

Primers Name	Sequences (5′- > 3′)
MODECL#1F1	CGTGATGTGCCGGGTGATGCTGTTCTTCCGGGCG
MODECL#1F2	GGCATGCGGGGGACGTGATGTGCCGGGTGATGC
MODECL#1F3	ATTACGGTCCAGTGGCATGCGGGGGACGTGATG
MODECL#1R1	CGTAATGGCCCATCCGATCTCCAGTGGCATCATG
MODECL#1R2	GCATGCCACTGGACCGTAATGGCCCATCCGATCT
MODECL#2F1	CCATGGAGACGGCTTACAACCTGTTCGGCATGGTGG
MODECL#2F2	TTTTCACCACCCCCGCCATGGAGACGGCTTACAAC
MODECL#2F3	GTGTGACGTTCAACTTTTTCACCACCCCCGCCATG
MODECL#2R1	GTCCGTCACGTTTGGGTGGTGCTCAACGTGGAAGAT
MODECL#2R2	CACACACTGCTCATAGTCCGTCACGTTTGGGTGGT
MODECL#2R3	GAAAAAGTTGAACGTCACACACTGCTCATAGTCCG
MODECL#3F1	ATCCAGAAAGGGCTCTTTCTGTTCGCGGTGTCCA
MODECL#3F2	AAGTGGACCAGCGCATCCAGAAAGGGCTCTTTCT
MODECL#3F3	AAAAGCGCCCTCAAAGTGGACCAGCGCATCCAG
MODECL#3R1	CCAGTACCAGAGTGACATCACATAGTACGGCGTC
MODECL#3R2	GCTTTTGCGGTCAAGCCAGTACCAGAGTGACATCA
MODECL#3R3	TCCACTTTGAGGGCGCTTTTGCGGTCAAGCCAGT
561 F	GGCGTTTTGTCTGTATCTGAG
561 R	CTCAGATACAGACAAAACGCC
885 F	TATCCTGTGCGAAATCTCCAA
885 R	TTGGAGATTTCGCACAGGATA

3.3.1.2 Cell culture, transfections, and calcium bioluminescence receptor assay

Functional activation of the three *Aedae* ACPr chimeras (ACPr ECL1, ACPr ECL2 and ACPr ECL3), as well as the three selectively modified *Aedae* ACPr ECLs chimeras, were carried out following a previously described mammalian cell culture system involving a 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) media containing 10% heat-inactivated fetal bovine serum (FBS; Wisent, St. Bruno, QC), 200μg/mL geneticin, and antimycotic-antibiotic mixture as reported previously (Wahedi and Paluzzi, 2018). Cells were grown to approximately 90% confluency and were transfected with the native ACPr, the full ECL ACPr chimera (ECL1-3) and the selectively modified ACPr ECLs chimera using Lipofectamine 3000 transfection reagent (Invitrogen, Burlington, ON) following manufacturer guidelines for DNA to transfection reagent ratios. A pcDNA mCherry construct was used as a control to assess transfection efficiency and to further validate activation specificity of the ACPr ECLs chimeras. Cells were harvested for the functional assay by detaching cells from the culture flasks at 48-hours post-transfection using 5mM ethylenediaminetetraacetic acid (EDTA) in Dulbecco's PBS. Cells were prepared for the receptor functional assay as described previously (Wahedi and Paluzzi, 2018).

Transfected cells were resuspended at a concentration of 10^6 cells/mL in assay media and incubated with coelenterazine (~ 3 hours) as reported previously (Oryan et al., 2018). Cells prepared for the functional assay were loaded into each well of the plate using an automated injector unit, and the luminescent response was measured with a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Stocks of *Aedae*ACP (pQVTFSRDWNAa) and *Aedae*AKH (pQLTFTPSWa) peptides (see Chapter 2, **Table 2-2**) were commercially synthesized

(purity >90%; Genscript, Piscataway, NJ) and prepared in assay media (0.1% BSA in DMEM: F12). Luminescence response to *Aedae*ACP and *Aedae*AKH peptides applied onto the native ACPr, each of the three full ECL *A. aegypti* ACPr chimera and also the selectively modified *A. aegypti* ACPr were examined. Negative controls were carried out using BSA media alone, whereas 50μM ATP was used as a positive control. Luminescence data was subtracted from BSA control, plotted and normalized relative to the maximal ATP response (10^{-5} M), and analyzed in GraphPad Prism 8.02 (GraphPad Software, San Diego, USA).

3.3.2 Statistical analyses

Data was compiled using Microsoft Excel and transferred to Graphpad Prism software v.7 to create all figures and conduct statistical analyses. Receptor functional assay average luminescence output data were analyzed by performing either a t-test or one-way ANOVA followed by Tukey's multiple comparison post-test to compare between control and different experimental peptide treatments. In all statistical tests, $p<0.05$ was considered significant.

3.4 Results

3.4.1 Ligand-receptor interaction heterologous functional assay

3.4.1.1 Incorporating the three full ECLs of Aedes AKH receptor into the Aedes ACPr chimera

The first step for determining the ACP receptor (ACPr) specificity and understanding the particular regions of the ACPr that are the most critical for ligand fidelity and specificity was creating ACPr chimera based on the native *Aedes* ACPr sequence and incorporating extracellular loops from the *Aedes* AKH receptor. The functional assay aimed to confirm whether ACP was still functional and capable of activating the ACPr chimera after swapping out the ACPr ECL 1, 2 and 3 with the corresponding ECLs of the AKH receptor. Using a heterologous functional assay, the luminescent response of CHO-K1 aequorin cells expressing the *A. aegypti* ACPr or one of the three chimera ACPr, which are the ACPr ECL1 (ACPr ΔECL1), ACPr ECL2 (ACPr ΔECL2), ACPr ECL3 (ACPr ΔECL3), was used to validate their activation. The results confirmed that the ACPr elicited the highest response to *Aedes* ACP ($p < 0.0001$), as this peptide binds with its native receptor (**Fig. 3-2A**). In contrast, it was noticeable that the three full ECL chimera ACPr receptors showed no response with either the *Aedes* ACP (**Fig. 3-2A**) or *Aedes* AKH (**Fig. 3-2B**).

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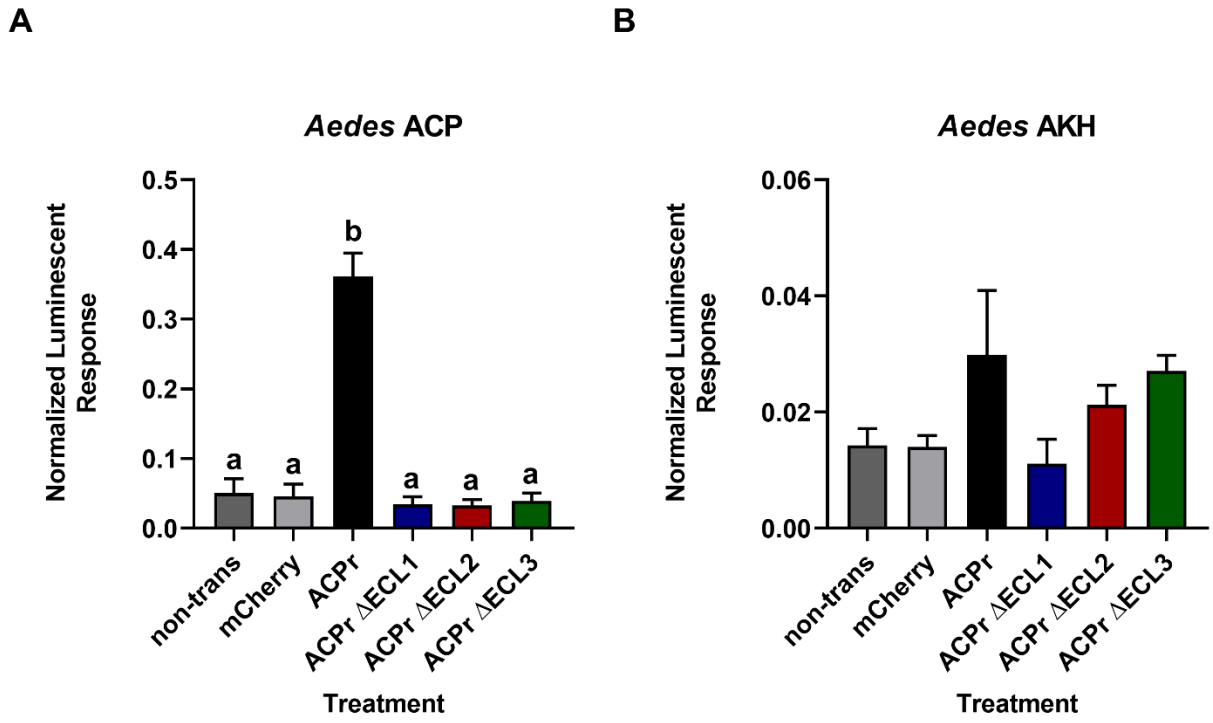


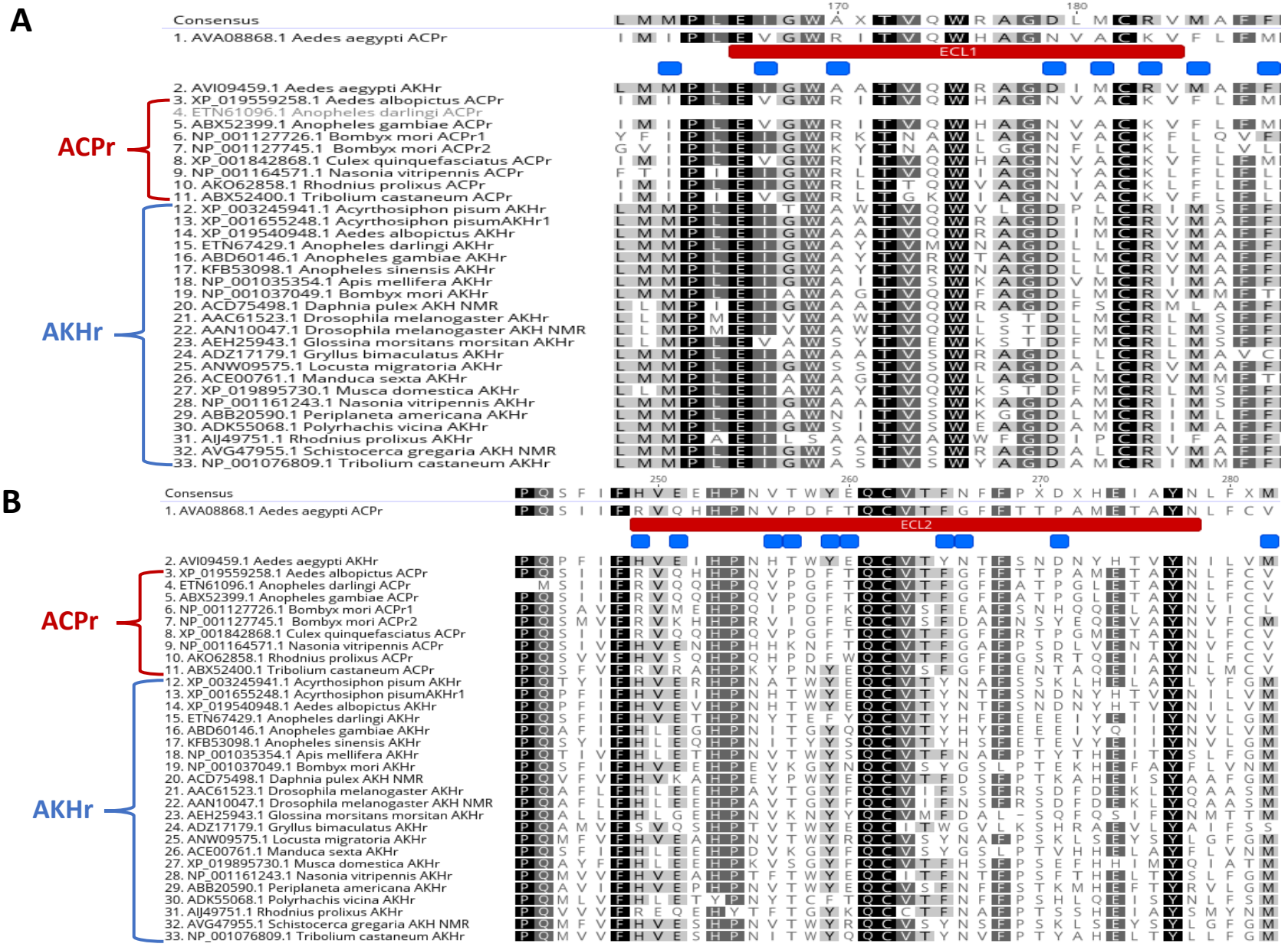
Figure 3-2. Functional heterologous receptor assay expressing either the native *A. aegypti* ACPr or one of three full ECL chimeras of the ACPr. (A) Normalized luminescent response of the native ACPr and three ACPr ECLs chimera after treatment with the *Aedes* ACP. (B) Normalized luminescent response of the native ACPr and the three ACPr ECls chimera after treatment with the *Aedes* AKH. The untransfected control (non-trans), transfected control (mCherry), native ACP receptor (ACPr), ACPr ECL1 chimera (ACPr ΔECL1), ACPr ECL2 chimera (ACPr ΔECL2), and ACPr ECL3 chimera (ACPr ΔECL3), respectively. Background luminescent response (BSA control) was subtracted from experimental samples and normalized relative to the maximal luminescence following treatment of the ATP (10^{-5} M). Different letters denote bars that are significantly different from one another, as determined by a one-way ANOVA and Tukey's multiple comparison post-test ($p < 0.05$). Data represent the mean ± standard error (n=3).

3.4.1.2 Identification of specific key residues in the three ACPr-ECLs

The most extreme and dramatic step was the removal of the full individual extracellular loop domains as described in the previous section. A further step was trying to target specific residues in each of the ECLs 1, 2 or 3, rather than replacing the entire extracellular loop. This might help to identify the highly conserved residues in the ECL 1, 2 and 3 that may confer ligand binding specificity. Thus, I aimed at discerning specific critical residues necessary for functional ligand-binding that are highly conserved and important for ligand specificity. Therefore, multiple sequence alignment of ACP and AKH receptors from different insect species revealed putative residues within the three ECLs that may contribute towards ligand specificity (**Fig. 3-3**). For instance, the valine amino acid (V) that was highly conserved in the ECL1 of the ACP receptors (ACPr) was substituted with isoleucine (I), which is highly conserved in AKH receptors **(Fig. 3-3A)**; and similar substitutions at highly conserved residues were performed along the entire region of the three ECLs. The GenBank accession numbers and any relevant references for insect ACPr or AKHr sequences used for the multiple alignments of the ACP and AKH receptors were gathered in **Table 3-2**.

Subsequently, three new independent ACPr-ECL chimeras were designed to modify these specific residues in the ECLs 1, 2 or 3 rather than replacing the entire ECL. Interestingly, it was noticed that the ACPrΔECL1 selective chimera showed the same response to the *Aedes* ACP as did the native ACPr (**Fig. 3-4A)**, while the selective ACPrΔECL2 chimera showed a significantly higher response ($p < 0.0001$) to the *Aedes* ACP compared to the response observed by native ACPr (**Fig. 3-4A)**. In contrast, the selective ACPrΔECL3 chimera showed a significant decrease in response ($p < 0.0001$) to the *Aedes* ACP compared to the response by the native ACPr (**Fig. 3-4A**). All of the three ACPrΔECLs showed a significantly higher

response to the *Aedes* AKH than the native ACPr response; in particular, the ACPrΔECL2 and ACPrΔECL3 chimeras demonstrated the highest response ($p < 0.0001$) to the *Aedes* AKH, being also significantly higher than the response observed with ACPrΔECL1 ($p = 0.0348$) (**Fig, 3-4B**).


A
Consensus
1. AVA08868.1 Aedes aegypti ACPr
ECL1
2. AVI09459.1 Aedes aegypti AKHr
3. XP_019559258.1 Aedes albopictus ACPr
4. ETN61096.1 Anopheles darlingi ACPr
5. ABX52399.1 Anopheles gambiae ACPr
6. NP_001127726.1 Bombyx mori ACPr1
7. NP_001127745.1 Bombyx mori ACPr2
8. XP_001842868.1 Culex quinquefasciatus ACPr
9. NP_001164571.1 Nasonia vitripennis ACPr
10. AKO62858.1 Rhodnius prolixus ACPr
11. ABX52400.1 Tribolium castaneum ACPr
12. XP_003245941.1 Acyrthosiphon pisum AKHr
13. XP_001655248.1 Acyrthosiphon pisumAKHr1
14. XP_019540948.1 Aedes albopictus AKHr
15. ETN67429.1 Anopheles darlingi AKHr
16. ABD60146.1 Anopheles gambiae AKHr
17. KFB53098.1 Anopheles sinensis AKHr
18. NP_001035354.1 Apis mellifera AKHr
19. NP_001037049.1 Bombyx mori AKHr
20. ACD75498.1 Daphnia pulex AKH NMR
21. AAC61523.1 Drosophila melanogaster AKHr
22. AAN10047.1 Drosophila melanogaster AKH NMR
23. AEH25943.1 Glossina morsitans morsitan AKHr
24. ADZ17179.1 Gryllus bimaculatus AKHr
25. ANW09575.1 Locusta migratoria AKHr
26. ACE00761.1 Manduca sexta AKHr
27. XP_019895730.1 Musca domestica AKHr
28. NP_001161243.1 Nasonia vitripennis AKHr
29. ABB20590.1 Periplaneta americana AKHr
30. ADK55068.1 Polyrhachis vicina AKHr
31. AIJ49751.1 Rhodnius prolixus AKHr
32. AVG47955.1 Schistocerca gregaria AKH NMR
33. NP_001076809.1 Tribolium castaneum AKHr
ACPr
AKHr
B
Consensus
1. AVA08868.1 Aedes aegypti ACPr
ECL2
2. AVI09459.1 Aedes aegypti AKHr
3. XP_019559258.1 Aedes albopictus ACPr
4. ETN61096.1 Anopheles darlingi ACPr
5. ABX52399.1 Anopheles gambiae ACPr
6. NP_001127726.1 Bombyx mori ACPr1
7. NP_001127745.1 Bombyx mori ACPr2
8. XP_001842868.1 Culex quinquefasciatus ACPr
9. NP_001164571.1 Nasonia vitripennis ACPr
10. AKO62858.1 Rhodnius prolixus ACPr
11. ABX52400.1 Tribolium castaneum ACPr
12. XP_003245941.1 Acyrthosiphon pisum AKHr
13. XP_001655248.1 Acyrthosiphon pisumAKHr1
14. XP_019540948.1 Aedes albopictus AKHr
15. ETN67429.1 Anopheles darlingi AKHr
16. ABD60146.1 Anopheles gambiae AKHr
17. KFB53098.1 Anopheles sinensis AKHr
18. NP_001035354.1 Apis mellifera AKHr
19. NP_001037049.1 Bombyx mori AKHr
20. ACD75498.1 Daphnia pulex AKH NMR
21. AAC61523.1 Drosophila melanogaster AKHr
22. AAN10047.1 Drosophila melanogaster AKH NMR
23. AEH25943.1 Glossina morsitans morsitan AKHr
24. ADZ17179.1 Gryllus bimaculatus AKHr
25. ANW09575.1 Locusta migratoria AKHr
26. ACE00761.1 Manduca sexta AKHr
27. XP_019895730.1 Musca domestica AKHr
28. NP_001161243.1 Nasonia vitripennis AKHr
29. ABB20590.1 Periplaneta americana AKHr
30. ADK55068.1 Polyrhachis vicina AKHr
31. AIJ49751.1 Rhodnius prolixus AKHr
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33. NP_001076809.1 Tribolium castaneum AKHr
ACPr
AKHr

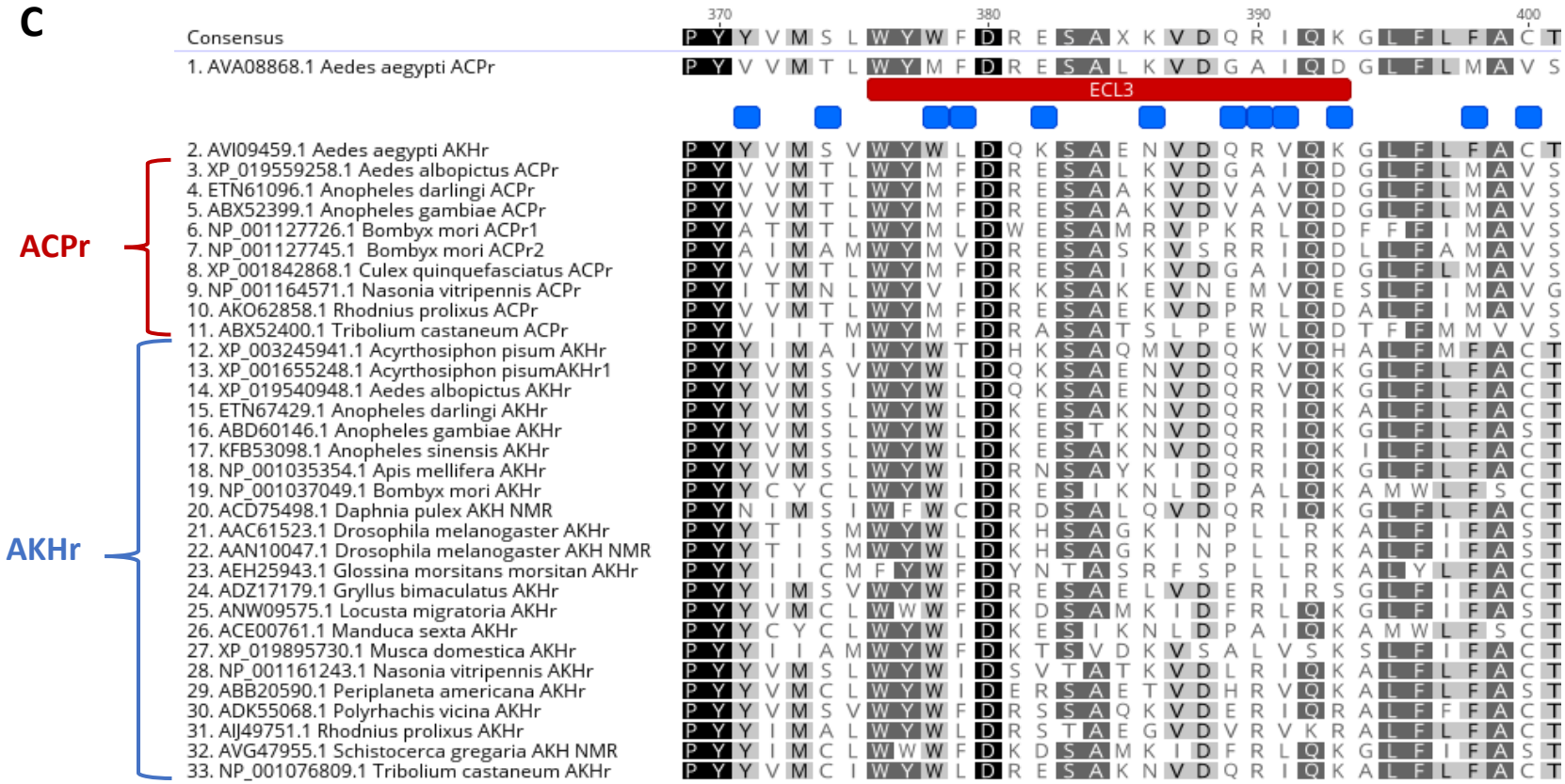

Figure 3-3. Sequence alignment of select insect ACP receptors (ACPr) and AKH receptors (AKHr) to identify specific key residues which are highly conserved and could be important for the ligand specificity conferred by the extracellular loops. (A) ECL1, (B) ECL2 and (C) ECL3. Residues outlined on the top with blue boxes indicate specific key residues which are highly conserved. Highlighting of residues indicates % identity with black denoting 100% sequence identity, dark grey denotes 80 to 100% identity, light grey represents amino acid positions with 60 to 80% sequence identity, and white denotes amino acid positions with less than 60% sequence identity. Receptor protein sequences are labelled by species name and identified with their GenBank accession number. References for sequences are included in **Table 3-2**.

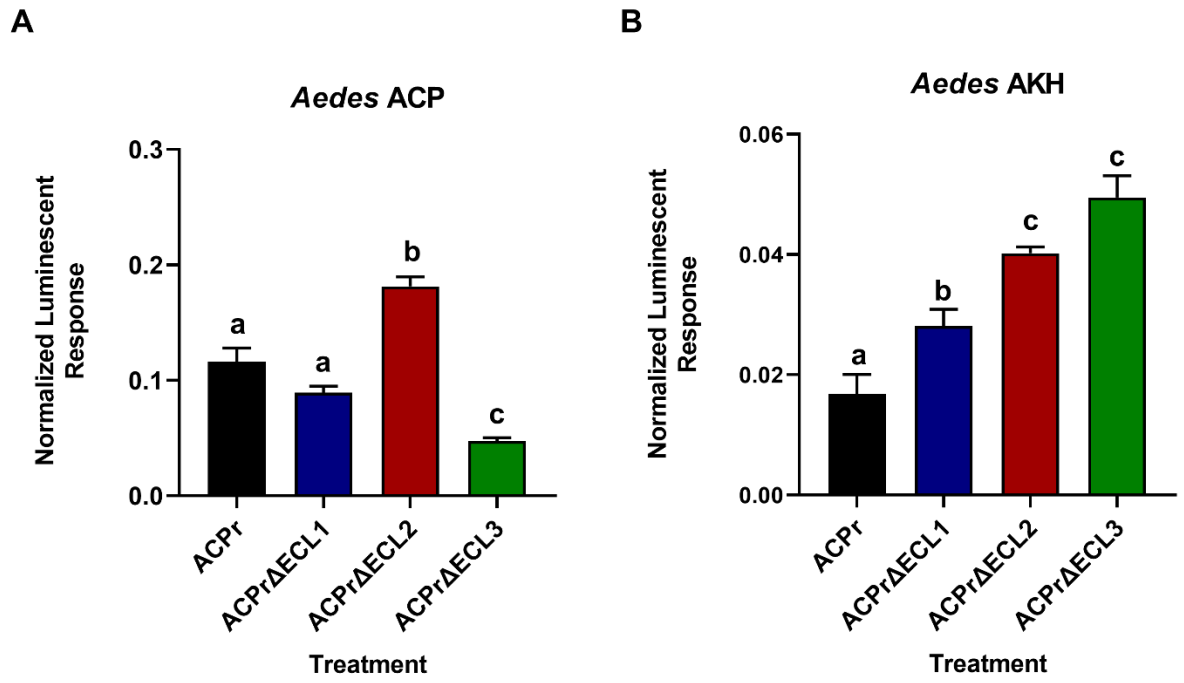

Figure 3-4. Bioluminescence responses of CHO-K1 aequorin cells transiently expressing either the native *A. aegypti* ACPr or the three chimeras with selectively modified ECLs yielding the new ACPr chimeras. (A) Normalized luminescence of the three independent ECLs chimera following treatment with the *Aedes* ACP. (B) Normalized luminescence of the three independent ECLs chimera after treatment with the *Aedes* AKH. Background luminescence (i.e. BSA control) was subtracted from experimental samples and normalized relative to the maximal luminescent response following ATP treatment (10^{-5} M). Different letters denote bars that are significantly different from one another as determined by a one-way ANOVA and Tukey's multiple comparison post-test ($p < 0.05$). Data represent the mean ± standard error (n=3).

3.4.1.3 Combination of two selectively modified ECLs of the ACPr chimeras

After observing the activity of ACPr chimera with selectively modified residues in individual ECLs, the next aim was to determine the effect of the combination of two selectively modified chimera ACPr ECLs chimeras. Thus, a combination of ACPr ECL1 chimera with ACPr ECL2 chimera (ACPrΔECL 1+2), ACPr ECL2 chimera with ACPr ECL3 chimera (ACPrΔECL 2+3) and lastly, ACPr ECL1 chimera with ACPr ECL3 chimera (ACPrΔECL 1+3) was occured. The results demonstrated that all of them showed a significant decrease compared to the native ACPr response to the *Aedes* ACP ($P < 0.0001$) (**Fig. 3-5A**). On the other hand, it was notably observed that the combination of the chimera ACPrΔECL 2+3 or ACPrΔECL 1+3 showed the same response as the native ACPr response to *Aedes* AKH, while the combination of the chimera ACPrΔECL 1+2 showed a significantly higher response ($P = 0.0407$) to the *Aedes* AKH compared to the native ACPr (**Fig. 3-5B**).

3.4.1.4 Combination of all three selectively modified ECLs of the ACPr chimera

A combination of the three selectively modified ACPr ECLs chimeras (ACPrΔECL 1+2+3) was tested to determine the effect of these combined selective substitutions on receptor sensitivity and activation by the two above-mentioned ligands, *Aedes* ACP and AKH. The results revealed a significantly decreased response for the combined chimera ACPrΔECL 1+2+3 compared to the native ACPr response to *Aedes* ACP ($P < 0.0001$) (**Fig. 3-6A**), while in contrast, the combined chimera ACPrΔECL 1+2+3 resulted in a significantly increased response to the *Aedes* AKH ($P = 0.0217$) (**Fig. 3-6B**).

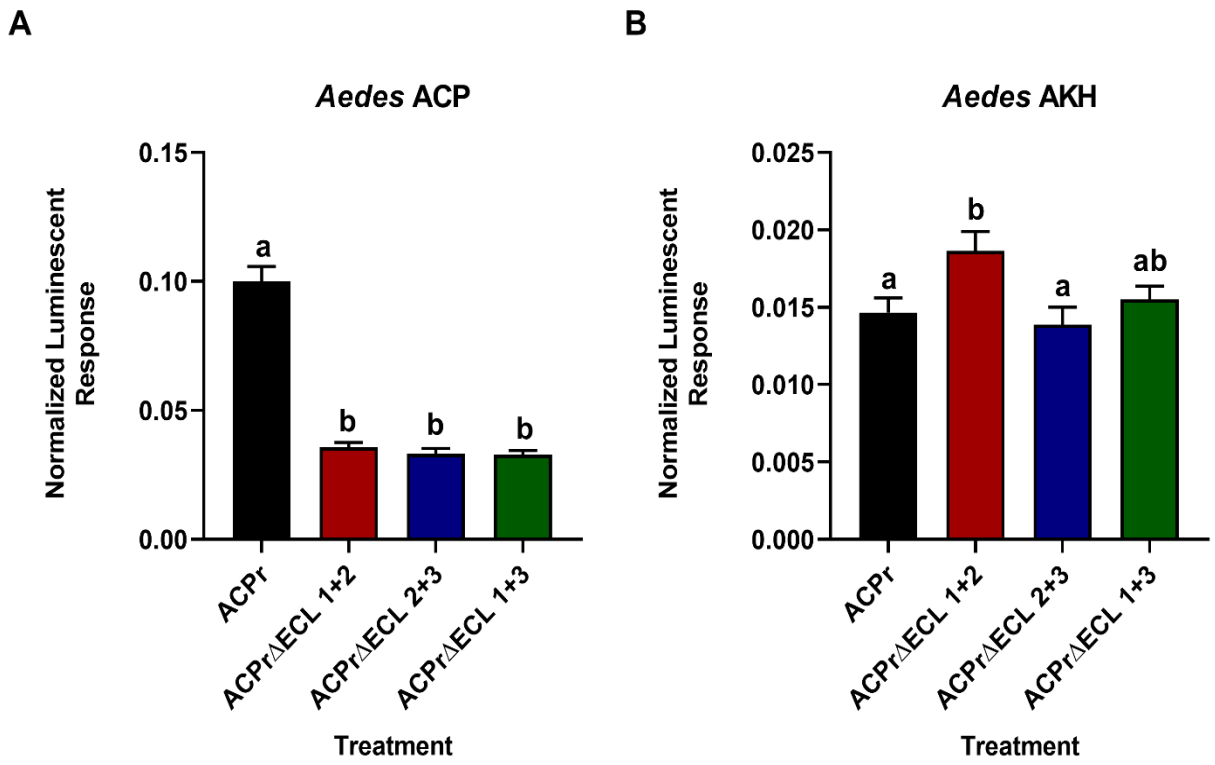

Figure 3-5. Receptor functional assay of CHO-K1 aequorin cells transiently expressing either the native *A. aegypti* ACPr or the combination of two selectively modified ECLs of the ACPr chimeras. (A) Normalized luminescent response of the ACPr chimera containing two selectively combined modified ECLs following treatment with the *Aedes* ACP. (B) Normalized luminescent response of the ACPr chimera containing two selectively combined ECLs following treatment with the *Aedes* AKH. Background luminescent response obtained following the vehicle control (BSA media) was subtracted from the experimental samples and normalized relative to the maximal luminescence following ATP treatment (10^{-5} M). Different letters denote bars that are significantly different from one another as determined by a one-way ANOVA and Tukey's multiple comparison post-test ($p < 0.05$). Data represent the mean ± standard error (n=3).

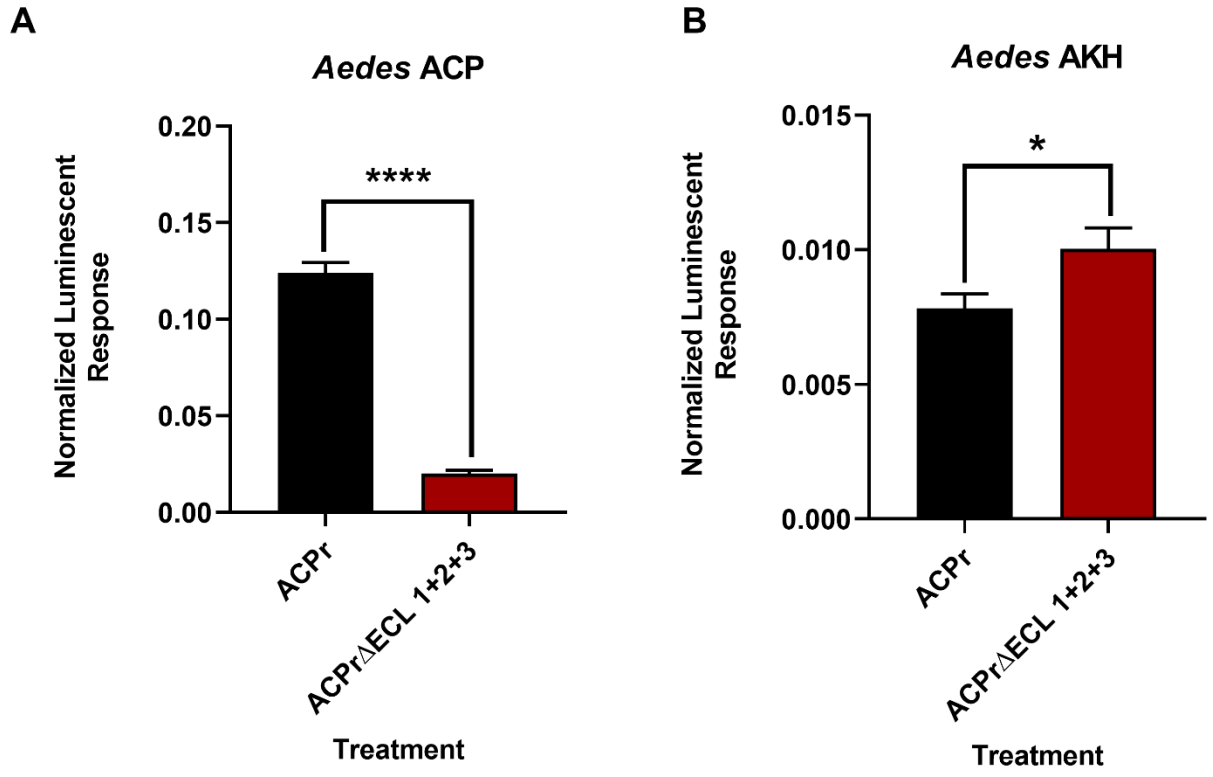

Figure 3-6. Bioluminescence responses of CHO-K1 aequorin cells transiently expressing either the native *A. aegypti* ACPr and the combination of all three selectively modified ECLs of the ACPr chimeras. (A) Normalized luminescence of the combination of the three selectively modified ACPr ECls chimera after treatment with the *Aedes* ACP. (B) Normalized luminescence of the combination of the three selectively modified ACPr ECls chimera following treatment with the *Aedes* AKH. Background luminescent response (BSA control) was subtracted from experimental samples and normalized relative to the maximal luminescent response following treatment of ATP (10^{-5} M). Data represent mean ± standard error (n=3), (*) denotes significance ($p<0.05$) as determined using an unpaired two-tailed t-test.

3.5 Discussion

The adipokinetic hormone/corazonin-related peptide (ACP) is structurally intermediate between AKH and CRZ (Hansen et al., 2010) and was initially identified as adipokinetic hormone II (AKH II) (Siegert, 1999; Kaufmann and Brown, 2006; Kaufmann et al., 2009). This discovery provided new questions and evidence on the role of ACP neuropeptide in insects. Previous *in vitro* studies characterizing AKH receptor (AKHr) in the silkworm *Bombyx mori* have demonstrated that high concentrations of AKH can activate the ACP receptor (ACPr) and *vice versa* (Zhu et al., 2009; Shi et al., 2011), which indicates that ACP and AKH are closely related to each other (Hansen et al., 2010). In arthropods, despite ACP and AKH being two distinct signaling systems, there is a notable similarity between these two peptides (Hansen et al., 2010). This similarity caused ACP to be misclassified previously as a member of the AKH peptide family in several studies prior to the discovery of the ACP receptor (Siegert, 1999; Kaufmann and Brown, 2006; Kaufmann et al., 2009). Moreover, extensive structure-activity studies were carried out in several species on their AKH receptors using natural and synthetic analogs of the AKH neuropeptide family (Ziegler et al., 1991, 1998; Gäde, 1992; Poulos et al., 1994; Lee and Goldsworthy, 1996; Lee et al., 1997; Gäde et al., 2000; Velentza et al., 2000; Caers et al., 2012a; Marco and Gäde, 2015, 2019). On the other hand, very limited data on biological actions or structure-activity relationships is available for the ACP system in insects.

A recent study examined the structure-activity relationship (SAR) of the ACP ligand characteristics in *A. aegypti*, using the ACP analog ligands as well as the natural AKH ligands, to determine the necessity for ACP receptor activity and specificity (Wahedi et al., 2019). However, the SAR of the ACP receptor in *A. aegypti* is yet to be investigated. Consequently, the present study intended to advance our understanding of SAR of two GnRH-related systems in *A. aegypti*

sharing the most recent evolutionary origin that sustain independence of function and signaling despite their relatively high degree of ligand and receptor homology (Hansen et al., 2010; Hauser and Grimmelikhuijzen, 2014; Tian et al., 2016; Zandawala et al., 2018). Specifically, this study set out to examine the SAR of the ACPr in the *A. aegypti* mosquito by substitution of key regions or selected residues of the receptor. In doing so, this provides insight into the specific structural features necessary for ligand fidelity and sustaining independence of the ACP and AKH signaling systems in insects.

As a consequence of the sequence conservation between the ACP and AKH signaling system, specific regions of the ACPr potentially most critical for ligand specificity were examined after creating ACPr chimera by singly replacing the three extracellular loops (ECLs) of the native ACPr and incorporating those from the AKHr of *A. aegypti*. Overall, it was observed that the three ACPr ECL chimera receptors showed no response to either AKH or ACP, indicating these ligands could no longer bind or the chimera were non-functional when a single extracellular loop of ACPr (ECL 1, 2 and 3) was switched with the ECL 1, 2 and 3 of the AKHr. Given these results, it suggests that the complete replacement of each ECL leads to a highly detrimental effect on the functional ligand-binding. Thus, these results reveal that the entire extracellular domains 1, 2 and 3 play a critical role in forming the ACP ligand-binding pocket. In agreement with these results, it is well documented for a variety of G-protein coupled receptors (GPCRs) that the ECLs play a key role in the activation and ligand-binding recognition (Wheatley et al., 2012). For example, one study focused on the modifications of ECLs impacted ligand-binding and activation of GnRH by substituting the ECL2 domain of the human GnRH receptor with the ECL2 of the chicken GnRH receptor (Sun et al., 2001). They determined that the ECL2 domain is crucial for the ligand-binding

and the activation of the GnRH receptors and can confer activity to mammalian antagonists (Sun et al., 2001).

As a subsequent step following the replacement of the whole individual ECLs, highly conserved amino acids within the three ECLs of ACPr and AKHr in several insects were identified, and then these specific residues were modified to generate three new independent ACPr ECLs chimeras. Thus, the most highly conserved residues between ACP and AKH receptors were selected in order to discriminate particular residues critical for ligand-binding and functionality. The results suggest that selectively modified residues with the third extracellular loop appear to be the most critical for ACP activation. Similarly, a previous study showed that the ECL3 of the GPCRs is more than just a connector between two transmembranes, but it is also involved in receptor activation and ligand selectivity (Lawson and Wheatley, 2004). Moreover, another study observed a similar finding to the current study results but in another neuropeptide using the chimera receptors to determine the function of the extracellular loops in the pheromone biosynthesis-activating neuropeptide (PBAN)/pyrokinin GPCRs from insects (Choi et al., 2007). PBAN is a peptide considered to be a pyrokinin-2 (PK2) family member that is known to stimulate the hindgut motility and induce the biosynthesis of the sex pheromone in many insects; but different from the diapause hormone (DH), which is a pyrokinin-1 (PK1) family member (Raina et al., 1989; Rafaeli and Jurenka, 2003; Zhang et al., 2004a, 2004b; Choi et al., 2007; Jurenka, 2015; Ahn and Choi, 2018; Lajevardi and Paluzzi, 2020). After generating chimera receptors by swapping the third extracellular domain between a PBAN-receptor from a moth and pyrokinin-receptors from *Drosophila,* similar observations to the present study results indicated that the third extracellular loop of these receptors is essential and directly shared in peptide ligand recognition (Choi et al., 2007). Separately, a more recent study revealed that the replacement of Pro238 located

in the 3rd ECL in *D. melanogaster* sex peptide receptor (*Drm*SPR) with the corresponding residue L310 from mosquitoes *A. aegypti* sex peptide receptor *(Aea*SPR) led to reducing its sensitivity of the sex peptide (SP) without changing its myoinhibitory peptide (MIP) sensitivity (Lee et al., 2020). Therefore, further research will be needed to identify the specific amino acid residues that are the most critical in the third ECL of ACPr necessary for ligand binding and activation in *A. aegypti*.

A recent *in silico* study done on the stick insect, *Carausius morosus*, using the AKH receptor model, showed that crucial residues in ligand binding were located at the second ECL and the sixth and seventh transmembrane domains (Birgul Iyison et al., 2020). Using the molecular modelling and mutagenesis studies, a study that occurred in the human GnRH receptor suggested that the fourth transmembrane domain /ECL2 junction plays a critical role for the peptide ligand binding and also in the conformational selection of the receptor (Forfar and Lu, 2011). Consequently, I decided to examine the effect of combining two ACPr ECL chimeras. Interestingly, the results revealed that all doubly combined ACPr ECLs chimeras showed a significantly decreased response to *Aedes* ACP compared to the native ACPr response. On the other hand, regarding the *Aedes* AKH response, a few observations can be highlighted. First, the combination of the ACPr ECL2 and ACPr ECL3 chimeras showed the same response as the ACPr, while the combination of the ACPr ECL1 and ACPr ECL2 showed a significant increase response to the *Aedes* AKH compared to the native ACPr response. Thus, the interactions between two modified ACPr ECLs may play a key role in enhancing the response to the AKH ligand. Lastly, in this present study, a combination of the three ACPr ECLs chimeras 1, 2 and 3 were occured. The results demonstrated a significantly reduced response to the *Aedes* ACP, while in contrast, a significantly increased response for the combined three ACPr ECLs to *Aedes* AKH. Therefore,

this study has succeeded to identify the particular residues of ACPr that are fundamental for ACP ligand binding fidelity while simultaneously elucidating the residues which help to prevent promiscuous binding by the closely related peptide, AKH. However, the specific amino acids that are most important for ACP ligand binding within each extracellular domain of ACPr remains to be further elucidated.

3.5.1 Concluding remarks

Taken together, up to now, no sufficient information regarding the cognate receptor of ACP of *A. aegypti* was available. While some extracellular loops might not be directly involved in peptide binding, they could help to maintain the appropriate conformational structure that allows the binding of the peptide with its receptor (Choi et al., 2007). This current study suggests that all extracellular loop domains of the *A. aegypti* ACPr are involved in binding with its corresponding ligand. Notably, however, residues within the third extracellular domain appear to be the most critical for the ACP ligand-binding and recognition. Furthermore, a highly detrimental effect in the ACP binding recognition was observed, after the combination of the three ACPr ECLs chimeras, while contrarily, an increased response occurred toward the *Aedes* AKH. Consequently, this study succeeded in identifying the specific residues within ACPr that are pivotal for the identity of the ACP ligand activation. Collectively, the data obtained in this current study set the framework toward understanding the ACP ligand-binding pocket and also provide fundamental insight regarding the key residues that play a key role in the activation of the ACP and AKH receptors by their corresponding ligands in *A. aegypti* mosquitoes. Moreover, it might give insight into design of some high-affinity agonists or antagonists to block the function of ACP and AKH, which could further facilitate the future design of insecticides against the same receptor of several insects.

3.6 References

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Chapter 4:

General discussion: Summary and future directions

4.1 Summary integrating the whole thesis

The *A. aegypti* adult mosquito transmits pathogens causing numerous harmful human diseases (Saiz et al., 2016; Kotsakiozi et al., 2017); thus, understanding the biology of the mosquito is needed to provide insight to develop new novel compounds geared toward vector control. The ACP is an insect neuropeptide structurally intermediate between the AKH and CRZ signaling systems (Hansen et al., 2010). Numerous studies on insects showed that the CRZ was initially described as having a cardio-stimulatory effect, while the most prominent role of the AKH is the mobilization of energy reserves (i.e., lipid and carbohydrates) (Veenstra, 1989; Gäde et al., 1997). To date, the functional role of the ACP signaling system in most arthropods remains unclear. The current research has provided the groundwork for our understanding of the ACP/ACPr singling system in the adult yellow fever mosquito, *A. aegypti*. The major findings related to the specified goals of this study are summarized below.

In brief, the findings from this research have succeeded in elucidating the ACP distribution within the CNS in the *A. aegypti* adult mosquito using immunohistochemistry, examining transcript expression of the ACP receptor in the pre-terminal abdominal ganglia and the terminal ganglion using the RT-qPCR, and determining the potential involvement of ACP in modulating energy substrate levels and energy metabolism as determined using the biochemical substrate mobilization assay. Furthermore, the current study determined that the entire ECLs of the *A. aegypti* ACP receptor play a key role in facilitating binding with its respective ligand. Lastly, this study also succeeded in identifying the specific residues within the ACP receptor that are

fundamental for the ACP ligand binding and activation in the *A. aegypti*. Specifically, the residues within the 3rd ECL appear to be the most vital for the ACP ligand-binding and activation as determined using a heterologous functional receptor assay.

In chapter two, the results revealed that the ACP peptide was localized within two pairs of lateral cells within the pars lateralis in the anterior region of the brain, as well as a few stained cells in the thoracic ganglia in adult *A. aegypti* mosquitoes. Further, extensive ACP immunoreactive axonal processes with prominent varicosities were detected projecting within each abdominal ganglia, including the terminal ganglia, with no evidence of arborization of these processes outside the CNS. These results herein are consistent with a previous molecular study that confirmed prominent enrichment of the ACP transcript in the brain and the thoracic ganglia, while a significantly abundant enrichment of the ACP receptor transcript was detected within the abdominal ganglia in the adult *A. aegypti* (Wahedi and Paluzzi, 2018). Therefore, these results suggest that the ACP signaling system serves as a neuromodulatory and/or neurotransmitter function in the *A. aegypti* nervous system.

Additionally, no difference between the transcript abundance of the ACP receptor in all of the pre-terminal abdominal ganglia and the terminal ganglion was observed in both male and female adult mosquitoes. These results are consistent with the ACP immunoreactive staining results that found a similar distribution observed within each abdominal ganglia as well as the terminal ganglion in the adult mosquito.

In mosquitoes, trehalose is the carbohydrate used as the primary metabolic fuel; on the other hand, lipid utilization is the primary energy source in the locusts (Clements, 1992; Briegel et al., 2001). Further, recent studies showed that ACP regulates the concertation of lipid and the carbohydrates in the male adult *G. bimaculatus* haemolymph while also modulating the muscle

lipid utilization during the flight in the adult *L. migratoria*. Thus, this studies in orthopteran insects suggests that the ACP might share a physiological functional role with AKH in the energy homeostasis regulation (Zhou et al., 2018; Hou et al., 2021). Moreover, a study showed that the ACP receptor is expressed outside the nervous system of adult mosquitoes in the male carcass that includes the fat body (Wahedi and Paluzzi, 2018).

Hence, as a first step toward discovering the physiological roles of the ACP in the *A. aegypti* adult mosquito, I examined the potential functional overlap between ACP and AKH using a biochemical assay. The results detected an elevation in the carbohydrate level in the male *A. aegypti*, while a decrease in glycogen stores in the female adult *A. aegypti* was revealed in response to ACP injection. On the other hand, no significant changes in the haemolymph and abdomen lipid levels were detected in both adult sexes. Thus, the results from this current study suggest that ACP could be involved in regulating the energy metabolism and energy substrate levels in the adult mosquito. Collectively, the compilation of results in chapter two presented in this thesis provides a more comprehensive understanding of the distribution of ACP in the CNS and suggests a physiological role of the ACP signaling system in adult *A. aegypti*.

Phylogenetic analyses have previously indicated the co-evolution of the AKH, CRZ, and ACP peptides along with their corresponding receptors, and that all three systems arose from a common GnRH-like ancestor (Hauser and Grimmelikhuijzen, 2014). Furthermore, several studies revealed that the AKH and the ACP signaling systems are more closely related than either is to the CRZ signaling system (Hansen et al., 2010; Zandawala et al., 2018). Therefore, the ACP and AKH signaling systems are ideal examples of ligand-receptor co-evolution (Caers et al., 2012b; Li et al., 2016). Importantly, enrichment of our knowledge about these pathways could be harnessed to develop effective mimetic neuropeptide analogs that might disrupt these pathways as approaches

to design a new generation of biorational peptide-based agents for pest control strategies. Also, a previous study showed that the ECLs could assist in maintaining the proper conformational structure that permits the binding of the receptors with their corresponding ligands (Choi et al., 2007). Unfortunately, only a few studies have highlighted the structure-activity relationships of the receptor revealing features and residues that are vital for peptide ligand activation and specificity.

Accordingly, chapter three in this thesis focused on examining the structural features in the ECLs of ACP and AKH receptors that are essential for conferring ligand specificity and receptor activation. First, the importance of the ECLs on ligand-binding activation of the ACPr and the AKHr was tested by creating three ACPr ECL chimeras after swapping out the full ACP receptor ECLs with their respective ECLs from the *A. aegypti* AKH receptor and testing these chimera using functional receptor assays. The findings emphasized that the entire ECLs play a central role in the ACP ligand-binding recognition, as the three ACPr ECL chimeras revealed no response to the ACP or the AKH.

Furthermore, this study also highlighted the highly conserved residues in the three ECls of the ACP with those from the AKH receptors by conducting bioinformatic analysis and multiple alignments of sequences from many insect species. Following three new independent ACP receptor chimeras were executed after selecting highly conserved residues within each of the three ACPr ECLs and substituting them with those residues that are highly conserved in AKHr ECLs. What was surprising was that the third ECL appeared to have the most vital features since residues in this ECL were found to be the most essential for ACP receptor binding recognition and activation.

Moreover, the results have indicated a highly detrimental effect on the ACP binding activation and recognition after combining the three modified ACP receptor ECLs chimeras; conversely, an increased response was observed following challenge with the closely-related peptide, AKH. Consequently, findings from this research demonstrate that modifying ECLs of the ACP receptor to the ECLs from the AKH receptor leads to reduced sensitivity to binding of its native ligand, ACP, while improving responsiveness to AKH. Therefore, these data would be highly important when further analyzing the potential use of ACPr and AKHr as targets for creating novel pest control agents.

4.1 Future directions

This present study has dramatically furthered our knowledge of the ACP/ACPr signaling system in the adult mosquito *A. aegypti;* however, there are many unanswered questions pertaining to the specific role of the ACP in *A. aegypti*; thus, additional studies should be performed. Various neuropeptides have been localized in the insect's abdominal ganglia, such as octopamine, crustacean cardioactive peptides, insulin-like peptides, CAPA, leucokinins, pigment dispersing factor, and pyrokinins (Nässel, 2002; Hellmich et al., 2014; Sajadi et al., 2020; Pauls et al., 2021). For instance, octopamine is produced by octopaminergic neurons located mostly along the midline in the abdominal ganglia, while the AKH is released and produced by the corpora cardiaca of the brain (Stone et al., 1976; Monastirioti, 1999). A recent study suggested that the activity and rest regulation in the fruit fly *Drosophila melanogaster* is involved by octopamine feedback from the octopaminergic neurons to AKH -producing cells (Pauls et al., 2021). This finding begged the question as to whether the ACP may also be released to target other neurons directly or indirectly in the ventral nerve cord. Thus, future studies could include identifying the intracellular ACP

signaling pathway and other downstream effects at the cellular level might aid in further uncovering the function in the CNS as a potential neuromodulator or neurotransmitter.

In addition, further research is necessary to validate whether the ACP signaling pathway has a possible peripheral role in reproduction since a previous finding showed that the ACP receptor transcript is expressed in both male and female adult *A. aegypti* accessory reproductive organs (Wahedi and Paluzzi, 2018). Moreover previous studies revealed that both AKH and CRZ are involved in oxidative stress and nutritional responses (Bednářová et al., 2015; Kubrak et al., 2016; Zandawala et al., 2021). Therefore, more research should be geared towards elucidating if there is any potentially shared role between ACP, AKH, and CRZ in the stress-related functions of arthropods.

Specific amino acid residues in ECLs could play a crucial role in agonist and/or antagonist binding to GPCRs (Hjorth et al., 1994). Thus, point mutations or site-directed mutagenesis of a subset of the highly conserved residues within each extracellular domain will be required to provide more information for modeling the ligand-receptor structural interaction. Exploring the indispensable amino acid residues of these two GnRH-related receptors in *A. aegypti* mosquito that are fundamental for activation by their respective ligands would help to clarify how these two evolutionarily related systems uphold specific signaling networks avoiding their cross-activation onto the structurally related but functionally distinct signaling systems. Hence, future investigations could focus on determining specific amino acids in each extracellular loop individually, with a specific focus on the third ECL that was determined herein to be the most critical. This could confirm the most conserved and indispensable residues that are directly involved in the recognition and binding to the ACP ligand.

Besides, the next steps for future research could include utilizing nuclear magnetic resonance (NMR) to be adopted, as was used for the ortholog of AKH and its receptor in a crustacean (Jackson et al., 2018; Jackson and Gäde, 2021). Thus, this could help us determine the structural similarities and differences between *A. aegypti* ACPr and AKHr, which might shed light on the molecular interaction explaining how these receptors bind with their unique ligands. Lastly, neuropeptides are ideal targets for the development of lead compounds for peptidomimetic control strategies; given that the ACP signaling system is missing in the *D. melanogaster* and honeybee *Apis mellifera* (Hansen et al., 2010), it might prove as a promising target for species-specific pesticides and/or insecticides.

4.3 References

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