Diuretic hormones of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)

by

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Abstract

Neuropeptides are diffusible signal molecules mediating vital physiological processes. We have been interested in a group of neuropeptides and their receptors involved in osmoregulatory neuroendocrine system which has been suggested as a possible target for development of new biopesticides. Since the genome sequence of the *T. castaneum* has recently been completed, we were able to identify the respective genes encoding three peptide hormones from *T. castaneum* that were characterized for their diuretic activities in other insects: one calcitonin-like (CT-like DH31) and two corticotropin releasing factor-like (CRF-like DH37 and DH47, the numbers indicates the number of amino acid residues). This peptide is expressed at all developmental stages and in the central nervous system (CNS), Malpighian tubules (MT) and gut. The synthetic peptide TricaDH31 also has been show to be biologically active, inducing significant excretions in adults beetles. When *Tcdh31* was silenced using RNAi, adults had deformed wings and abnormal body shape. Mortality in adults was high, the number of eggs laid was reduced as well as the hatchability of the eggs. The two biologically active CRF-like peptides in *T. castaneum*, are encoded by one gene which undergoes alternative splicing. When *Tcdh47* was knocked down, high mortality occurred as well as low oviposition and egg hatchability. Similar effects were observed with silencing of both CRF-like genes. However, RNAi of *Tcdh37* transcripts had similar, but less severe effects. Adults also had deformed wings when both CRF-like genes were silenced, but not when just one of them was knocked down. These results indicate that CRF-like genes could

have additional biological functions to their roles in dieresis. We tested the *in vivo* activity of these peptides. TenmoDH47 induced high excretions in adults, whereas TenmoDH37 induces smaller excretions. We identified the respective genes encoding two putative receptors for TricaDH31 as Glean_13321 and Glean_02694 (*Trica-ctr1* and *Trica-ctr2*, respectively) and two receptors for CRF-like peptide as Glean_12799 and Glean_07104 (*Trica-crfr1* and *Trica-crfr2*, respectively). The CT-like receptors are expressed at all developmental stages, in the CNS and MT. RNAi of the receptors revealed that only *Trica-ctr2* silencing caused significant mortality and reduction in the number of eggs laid. The CRF-like receptors are expressed at all developmental stages. Adults also had deformed wings and laid fewer eggs after RNAi of *Trica-crfr1*. RNAi of *Trica-crf2* also caused significant mortality. These peptides and receptors seem to fine tune the beetle physiology and may have functions not yet known.

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Tricadh31	*Tribolium castaneum* diuretic hormone 31 gene
TricaDH31	*T. castaneum* diuretic hormone 31 peptide
Tricadh37	*T. castaneum* diuretic hormone 37 gene
TricaDH37	*T. castaneum* diuretic hormone 37 peptide
Tricadh47	*T. castaneum* diuretic hormone 47 gene
TricaDH47	*T. castaneum* diuretic hormone 47 peptide
Trica-ctr1	*T. castaneum* calcitonin-like receptor 1 gene
TricaCTR1	*T. castaneum* calcitonin-like receptor 1 protein
Trica-ctr2	*T. castaneum* calcitonin-like receptor 2 gene
TricaCTR2	*T. castaneum* calcitonin-like receptor 2 protein
Trica-crfr1	*T. castaneum* corticotropin-like receptor 1 gene
TricaCRFR1	*T. castaneum* corticotropin-like receptor 1 protein
Trica-crfr2	*T. castaneum* corticotropin-like receptor 2 gene
TricaCRFR2	*T. castaneum* corticotropin-like receptor 2 protein
LocmiDH	*Locusta migratoria* diuretic hormone
DippuDH31	*Diploptera punctata* diuretic hormone 31
TenmoDH37	*Tenebrio molitor* diuretic hormone 37
TenmoDH47	*Tenebrio molitor* diuretic hormone 47
RhoprDH31	*Rhodnius prolixus* diuretic hormone 31
ManseDH41	*Manduca sexta* diuretic hormone 41

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Chapter 1

Introduction

Worldwide socio-economic improvements have been possible through agricultural progresses in human history. With the use of synthetic insecticides the yield of several crops were drastically increased, which has impacted our society, health and environment. Several approaches have been made to reduce losses caused by insects, including the development of new insecticides and the improvement of natural biological control. However, the success of these strategies is variable and dependent on crop system, insect-pest ecology and biology, and socio-economic factors.

Recently, a number of review papers discussed the potential use of insect peptides to control agriculturally important pests (Altstein, 2001; Gade and Goldsworthy, 2003; Hoffmann and Lorenz, 1998; Masler et al., 1993). These novel types of insecticides would be safer to the environment and humans, selective to the targeting species or a group of insects, and will not affect beneficial organisms. Diuretic peptides were mentioned as potential tools to develop strategies to break the insect homeostasis and cause death.

Insect osmoregulation

In insects the water and ion balance are tightly controlled to maintain homeostasis (Gade, 2004). As in most of terrestrial organisms, insects conserve their water content rather than expel it, and efficient osmoregulation is critical to their success (Dow and Davies, 2006). Diuretic hormones, any peptide that

increases water loss in the whole insect either by increasing Malpighigian tubules (MT) secretion and/or inhibiting fluid reabsorption from the hind gut (Gade, 2004), are involved in water and ion balance, as are antidiuretic neuropeptides, those peptides that decrease water loss in the insect body either by inhibiting tubule secretion and/or by promoting the reabsorption of ions and water in the hindgut. The definition of diuretic hormone is not clear and the term diuretic peptides is more broad including all peptides, well know or not, which have effects on ion and water balance.

Many diuretic and antidiuretic peptides were recently discovered and described, (Beyenbach, 2003; Coast, 1996, 1998; Gade, 2004; Gade et al., 1997) mainly resulting from the phenomenon of reverse genetics. In the majority of these studies, the peptides or genes have been identified based on homologous sequences without functional implications. In a number of studies, clear diuretic activities of peptides were documented in isolated insect excretion systems, which does not rule out possible additional functions.

Mechanisms keeping water balance and the importance of malpighian tubules

In the insect circulatory system, there is little or no blood pressure and urine formation is by secretion instead of filtration as in the kidney of mammals. It may be due to the presence of the tracheal system that does not require hemolymph to transport gas, making the circulation rate variable (Gade et al., 1997). The MT arise from the junction between midgut and hindgut, and secrete

a solution rich in KCl or NaCl, where water and other solutes move by passive diffusion (Gade, 2004; Ramsay, 1952, 1954). Some active transport is involved in the excretion of toxic substances (Beyenbach, 2003; Dow and Davies, 2006). Active transport is driven by a vacuolar-type proton pump (V-H^+-ATPase) located on the apical membrane of the MT principal cells coupled with antiporters for Na^+/H^+ and K^+/H^+ exchange. Through this mechanism blood-feeding insects secrete NaCl in the tubule lumen and non-blood feeding insects secret KCl. Then urine formed in the tubule is secreted into the hindgut lumen, where there is selective reabsorption of essential metabolites such as water and ions. Reabsorption occurs mainly in the ileum and determines the final composition of the excreta (Gade, 2004). Diuretic peptides have a variety of pathways by which they can regulate fluid transport. Many of these peptides function though second messengers, mainly cyclic adenosine monophosphate (cAMP), which can increase MT secretion, preferentially through increasing Na^+ transport in locusts. Another well known pathway is the nitric oxide/guanosine 3`,5`-cyclic monophosphate (cGMP) in *Drosophila melanogaster*, which can increase secretion through MTs independently of cAMP or Ca^{++} (Dow and Davies, 2006; Gade et al., 1997).

Epithelial cells in the MT secrete a wide range of organic solutes, which leads to the sequesteration of toxic compounds to be excreted (Dow and Davies, 2006). Several enzymes are found in the tubules, such as the alcohol dehydrogenase, cytochrome P450s and glutathione transferases (Dow and Davies, 2006). Over-expression of the later two is related to insecticide

resistance in many insects (Catania et al., 2004; Enayati et al., 2005). This suggests that the MTs maybe be a major site in determining insecticide resistance.

The MT are spread throughout the insect body and display rapid fluid secretion rates. They open into the hindgut exposing them to insect pathogens (Dow and Davies, 2006). Their importance as an autonomous immune system has been characterized in *D. melanogaster*. They are capable of sensing bacterial attack and creating an effective killing response independent of the fat body (McGettigan et al., 2005), which was shown by rapid increases in expression of the genes that were known for immune functions. Once the diuretic peptides have acted directly on the tubule function, any interference in this system may result in several problems to insect maintaining homeostasis, immunity and the integrity of other systems.

Diuretic peptides

In multicellular organisms, communication between cells is achieved by the use of signaling molecules. Some of the important molecules are the peptides and protein hormones which are produced in endocrine and neural cells. Usually these molecules are produced as large precursors and need to be cleaved and modified in order to be active when released to the extracellular environment (Li et al., 2008). These molecules are involved in several physiological processes and can produce different hormonal signals or act as neurotransmitters in insect depending on the developmental stage or tissue. The

neuropeptides are usually named according to their known function in the insect in which they were discovered. However, some neuropeptides, such as the diuretic peptides may perform different functions in addition to the ones their names suggest. Most of the studies regarding insect diuretic peptides rely on the ability of the peptide to induce secretion in the MT, but this does not tell us if these peptides are involved in other processes in insects.

The corticotropin-releasing factor (CFR)-like diuretic hormones are one of the well known families of peptides involved in osmoregualtion in insects. The first identified peptide of this family was isolate from 10,000 trimmed heads of pharate adults of *Manduca sexta*. They are structurally similar to the vertebrate CFR/urotensin/sauvagine family of peptides thus named as CRF-like diuretic hormones (CRF-DH) (Gade, 2004). New peptides in this family were isolated from several insect species, including moths, termites, locusts, crickets, cockroaches, beetles, mosquitoes, and flies. It seems that most species have two of these peptides, one short (30-37 amino acids) and the other, long (41-47 amino acids) (Gade, 2004).

The mode of action of CRF-DH seems to be common across the species with some degrees of interspecific cross-bioactivity (Coast et al., 1992; Gade et al., 1997). CRF-DH activates an adenylate cyclase in the primary MT cells, increasing the levels of cAMP in these cells (Gade et al., 1997). This increases the conductance across the basolateral membrane of the primary cells. Then, Na^+ from the hemolymph enters the cells, increasing the intracellular Na^+ concentration. This presents a mechanism for the coupled transport of Na^+/H^+

add water or others solutes through the basal membrane in opposite directions. Water and solutes flow passively via the osmotic gradient (Beyenbach, 1995, 2003). Structural studies suggests that cAMP may also affect mitochondrial location and action (Gade et al., 1997). Mitochondria move to the brush border and into microvilli, it is presumed that CRF-DH (via cAMP) also increase the activity of the apical V-H^+-ATPase by increasing in ATP concentration. Subsequently the cation movement across the apical membrane to luminal side is stimulated (Gade et al., 1997).

A second family of diuretic hormones are the kinins that were first isolated in the cockroach *Leucophaea maderae* (Gade et al., 1997; Holman et al., 1991) and now there are more than 20 know kinins from several insects. The mature peptides are small (6 – 13 amino acids). Although their main action in the MT is diuresis, they are also involved in the myotropic action and perhaps also in the release of digestive enzymes in the gut (Gade et al., 1997). Until now, kinins have not been found by mining the genome sequence of *T. castaneum.*

Other important families of diuretic hormones are the calcitonin-like diuretic peptides (CT-DH), the arginine vasopressin-like diuretic peptides (AVPL) and the cardioacceleratory peptides. The first AVPL was isolated from the subesophageal ganglion of the *Locusta migratoria* (Proux et al., 1987). Interestingly, searches of the *D. melanogaster* and *A. gambiae* genomes did not uncover AVPL gene homologs, which suggests that this gene had been lost in higher dipteran insects. However, this gene was found in *T. castaneum* and in vivo injection of AVPL strongly increased diuretic activity in this beetle (Aikins et

al., 2008). A calcitonin-like diuretic hormome, CT-DH or DH31, has been isolated from brain and Corpora Cardiaca (CC) of the cockroach *Diploptera punctata*. This peptide was shown to stimulate secretion in MT not only in *Diploptera* but also in *Locusta* (Furuya et al., 2000). Using the sequence of the cockroach DH31 peptide, (Coast et al., 2001) a peptide with 71% identity in the *D. melanogaster* genome was found. Synthetic *D. melanogaster* DH31, also, increased the secretion rate of the MT. The same research group has shown that in *Drosophila* the calcitonin-like peptides stimulate dieresis through a cAMP dependent mechanism located in the principal cells of the MT, where an apical vacuolar V-ATPase is stimulated. The presence and activity of DH31 have been studied in the blood-sucking bug *Rhodnius prolixus*. Immuno reactive cells have been found in the CNS, salivary glands, hind gut and the neurohemal site in last instar of *Rhodnius prolixus* (Brugge et al., 2001). Co-localization of Dippu-DH31 with serotonin-like was found in cells of the mesothoracic ganglionic mass and in neurohemal sites on the abdominal nerves. This DH31 is not co-localized with CRF-like or kinin peptides. Dippu-DH31 stimulated low levels of secretion in 5^{th} intsar MT (14 folds). However, in combination with serotonin-like, Dippu-DH31 increased the rate of secretion from the tubules, in a additive manner. Recently, it has been shown that Dippu-DH31 does not increase the cAMP content of *Rhodnius* tubules (Brugge et al., 2008) similar to what is found in *Manduca sexta* (Furuya et al., 2000) and in *D. punctata* (Tobe et al., 2005). Contrasting with this finding, the cAMP level is increased by DH-like peptides in the Malpighian tubules of *Shistocerca americana*, *D. melanogaster* and *Anopheles gambiae*

(Coast et al., 2001). DH31-like peptide immunoreactivity has been also found in the CNS and gut of the milkweed bug *Oncopeltus fasciatus*. However, it is not known if these peptides control dieresis or which secondary messager pathways are used in these insects (Brugge and Orchard, 2008).

The receptors of diuretic peptides

Insect neuropeptides exert their action by binding to membrane receptor - the G-protein coupled receptors (GPCRs). After being activated by the extracellular ligand the GPCRs initiate an intracellular second messaged cascade, frequently known to involve cAMP or cGMP. These receptors have a typical topology that consists of transmembrane domains with seven hydrophobic α-helices and several conserved motifs (Hauser et al., 2006). The GPCRs are classified into four families: Family A includes rhodopsin-like receptors, Family B consists of secretin-like receptors, Family C includes metabotropic glutamate like receptors, and, Family D is for atypical receptors (Hauser et al., 2006). In *Drosophila* and other insects the diuretic hormone receptors (both receptors for CRF-DH and CT-DH) are members of the secretin family. The receptors of DH44 (CRF-like) and DH31 (CT-like) have been deorphanized in *D. melanogaster* recently and allow homology searches in other species. The first GPCR of the family B was identified in *M. sexta* (Reagan, 1994; Reagan et al., 1994). Since diuretic activity has been show by a large group of peptides, the functional characterization of the receptors is difficult. However, this work became easier due the availability of several insect genomes. Using sequence similarity, diuretic

hormone receptors could be identified in insect genomes, cloned and used in assays to determine their ligands.

Trubolium castaneaum

Besides its economical importance, the red flour beetle, *T. castaneum*, is well recognized as a model organism among eukaryotes. It is a member of Coleoptera, the largest insect Order with a great biological diversity (Lorenzen et al., 2005), 40% of all insect species are in this order. Coleopterans are the most successful group of metazoans, and many species cause economical losses to several crops worldwide. Therefore, studies of *T. castaneum* can provide insights to understand the basis of biological processes in other coleopteran insect species. It has been suggested that studies involving this beetle may help to understand the evolution of higher insects with complex development and can contribute to several areas of biological research. Several genetic and genomic tools have been developed for *T. castaneum*, and now, with its whole genome sequenced, forward and reverse genetic approaches are available to facilitate functional genetic analysis (Brown et al., 2003). This beetle belongs to the group of insects having the ability to absorb atmospheric water in the hindgut (cryptonephrideal complex). The physiology of the cryptonephridial complex (rectal complex) now can be explored using these molecular genetics tools.

The completion of the *T. castaneum* genome sequence provided an excellent opportunity to identify neuropeptides in Coleoptera, for which less is known in comparison to other insect orders such as Diptera. The goal of this

study is to identify, clone and characterize the biological function of diuretic peptides CRF-DH and CT-DH and their receptors involved in the neuroendocrine control of the *T. castaneum* excretion system.

Chapter 2

Introduction

Worldwide socio-economic improvements have been possible through agricultural progresses in human history. With the use of synthetic insecticides the yield of several crops were drastically increased, which has impacted our society, health and environment. Several approaches have been made to reduce losses caused by insects, including the development of new insecticides and the improvement of natural biological control. However, the success of these strategies is variable and dependent on crop system, insect-pest ecology and biology, and socio-economic factors.

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In multicellular organism, communication between cells is achieved by the use of signaling molecules. Among the most important molecules are the peptides and protein hormones that are produced in endocrine and neural cells. Usually these molecules are produced as large precursors and need to be cleaved and modified in order to be active when released to the extracellular environment (Li et al., 2008). These molecules are involved in several

physiological processes and can produce different hormonal signals or act as neurotransmitters in insect depending on the developmental stage or tissue. The neuropeptides are usually named according to their known function in the insect in which they were discovered. However, some neuropeptides, such as the diuretic peptides, may perform different functions other than their name suggest. Most of the studies regarding insect diuretic peptides rely on the ability of the peptide induced secretion on the MTs, but it does not tell us if these peptides are involved in other processes in insects.

A conserved group of peptides similar to vertebrate corticotropin releasing hormone (CRF)-like has been found to have diuretic activity in the beetle *Tenebrio molitor* L. (Furuya et al., 1998), and named the CRF-like diuretic hormone or TenmoDH47 (47 amino acids). Diuretic hormones, like TenmoDH47, were mentioned as potential tools to develop strategies that would break insect homeostasis and provoke death. The completion of the *T. castaneum* genome sequence provides an excellent opportunity to identify neuropeptides in Coleoptera, for which less is known in comparison to other insect orders such as Diptera. The objective of this study is to identify, clone and characterize the biological function of diuretic peptides CRF and CT-like and their receptors involved in the neuroendocrine control of the *T. castaneum* excretion system.

We identified and cloned two putative diuretic hormone genes of *T. castaneum*. We studied their expression patterns and biological function using RNA interference (RNAi). We also identified their putative receptors and studied their biological function as well. There are two receptors of CRF-like peptides

(*Trica-crfr1* and *Trica-crfr2)* and two receptors for CT-like peptides (*Trica-ctr1* and *Trica-ctr2*)

Material and Methods

Identifying and cloning *T. castaneum* diuretic hormone genes

TblastN was used to search the *T. castaneum* genome for sequences similar to the diuretic hormones of *D.melanogaster*. Three putative diuretic hormone genes were initially found: two CRF-like (*Tricadh47* and *Tricadh37*) and one calcitonin-like (*Tricadh31*). *Tricadh31* was also found in our EST database and the clone was recovered from the DNA plate. CRF-like genes, for which clones were not found within EST database, were cloned by nested PCR of the larval cDNA library by using the primers designed on the predicted exons. After sequence analysis, we found that the two CRF-like peptides were encoded by a single gene that undergoes alternative splicing. The primers used are described in the Table 1. PCR reactions were subject to an initial hold at 94^{o}C for 3min followed by 30 to 35 cycles of: 94^{o}C, 30s; 55^{o}C, 1min; 72^{o}C, 1min. A final hold of 72^{o}C for 10min ensured full extension of the PCR products. PCR products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide (0.5µg/ml). The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen). The eluted DNA was TA-cloned into pGEM-T Easy (Promega) and used to transform chemically competent DH5α cells (New England Biolabs).

Identifying *T. castaneum* diuretic hormone receptors genes

TblastN was used to search *T. castaneum* for sequences similar to the diuretic hormones receptors previously identified in other insect species.

The conceptual translations of predicted GPCRs genes were analyzed to confirm the orthologous relationships with the known receptors in other insects. The phylogenetic analyses were using performed PAUP v4b2 (Swofford, 1993) to generate a distance tree for the group of closely related GPCRs from all insects. The range of GPCRs from different species in the analysis were expanded until the tree reached to the clear outgroup (with bootstrapping value higher than 70). Multiple sequence alignment of GPCRs was performed using ClustalW, and Boxshade (http://www.ch.embnet.org/software/BOX_form.html), and the transmembrane domains were predicted using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html).

Cloning of putative *T. castaneum* diuretic hormone receptors genes

The predicted coding regions for each receptor (Hauser et al., 2006) were amplified from a cDNA library (TB, *T. castaneum* adult brain) by the inverse polymerase chain reaction (IPCR) using internal primers (Table 1). Reaction tubes were subject to an initial hold at 98^{o}C for 1min followed by 35 cycles of: 98^{o}C, 5s; 55 to 60^{o}C depending on the melting temperature of the primers, 3min; 72^{o}C, 1min. A final hold of 72^{o}C for 10min ensured full extension of PCR products. A proofreading polymerase was used in IPCR (Phusion High Fidelity – New EnglandBiolabs).

Rapid amplification of cDNA ends (RACE) was used to obtain the 5' end of *Trica-ctr2*. Chimeric PCR was used to obtain the full length sequences of *Trica-crfr2* and *Trica-ctr2*. The primers used are described in Table 2. The PCR conditions and cloning was done following the manufacturer's instructions (GeneRacer® Kit with SuperScript® III RT and TOPO TA Cloning® kit for Sequencing - Invitrogen)

Real-time quantitative RT-PCR and Semi-quantitative RT-PCR

Total RNA (from a pool of three individuals of different stages or tissues) was prepared using TRIzol reagent (Invitrogen). The stages tested were early embryo (EE, <24 hr), late embryo (LE, >24 hr), early larval (EL,<24 h post-hatching), late larval (LL, older than fifth instar including prepupae), early pupal (EP,<24 h post pupation), late pupal (LP, >24 h postpupation), early adult (EA, <24 h post eclosion), and late adult (LA, 1-week old). Tissue-specificity of expression was examined by quantitative PCR using SYBR premix Ex taq (Takara Bio., USA) in the CNS including all the ganglia and brain, MT, hindgut, and the remaining carcass, which excluded the aforementioned tissues. The fold-differences of the target molecule were standardized and compared with the control gene ribosomal protein S3 (RP3, GenBank accession number CB335975), using the $^{\Delta\Delta}$CT method (Livak and Schmittgen, 2001).

Expression patterns of alternatively spliced products *Tricadh37* and *Tricadh47* were examined using exon-specific primers (Table 1). The reaction tubes were subjected to an initial hold at 94^{o}C for 3min followed by 30 cycles of: 94^{o}C, 30s; 55^{o}C, 1min; 72^{o}C, 1min. A final hold of 72^{o}C for 10min ensured the

full extension of the PCR products. PCR products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide (0.5μg/ml).

In situ hybridization

Digoxigenin labeled DNA probes were generated by using PCR DIG Probe Synthesis Kit (Roche). Dissected tissue (gut, MT, CNS) or paraffin sections (whole last instar larvae) were used for in situ hybridization. Tissues were fixed with 4% paraformaldehyde at 40°C, washed 3 times for 15 minutes with PBST (PBS and 0.2% Triton-X-100), treated with 10μg/mL proteinase K, and hybridized at 48°C for 20-30h. After hybridization, tissues were blocked in 1% BSA, incubated with anti-digoxigenin-alkaline phosphatase (Roche, 1:1000 dilution in 1% BSA) overnight at 40°C, and nitroblue tetrazolium salt/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP, Roche, 1:50 dilution in AP buffer) was added to the substrate to develop color, Color development was stopped by repeated washes with PBS and the samples mounted in 100% glycerol. CNS from larva, pupa and adult were dissected, and all ganglia can be seen in Appendix 3 as a reference.

Immunohistochemistry for CRF-like peptides

The CNS was dissected in PBS and fixed overnight in Bouin at 4°C. The tissues were then washed 3 times 15 min in PBST (PBS with 0.1% Triton X-100) and incubated with rabbit anti-TenmoDH37 (1:200) in PBST at 4°C overnight. The sample was washed 3 times 15 min with PBST and incubated overnight with

a goat anti-rabbit antibody conjugated with Cy3 (Jackson Immuno Research, West Glove, Pensylvania) in PBST at 4^{o}C. The tissues were washed with PBST and mounted on a glass slide for observation using a confocal microscope LSM510.

Functional Assay

Transient expression of DH receptors was performed in the CHO-WTA11 (Euroscreen) cell line that exhibits stable expression of the luminescent reporter aequorin and Ga16, which is known to be a promiscuous linker mobilizing intracellular Ca^{++}. Cells were grown in complete Ham's F12 medium at 37 ℃ in 5% CO_2. Transfection with respective *dhr* genes was performed using FuGene6 (Roche Molecular Biochemicals) according to the manufacturer's protocol at a DNA to FuGene6 ratio of 3:1. Before functional assays, cell suspensions were incubated in coelanterazine h (Molecular Probes) according to previously defined protocols (Park et al., 2003).

Luminescence assays were performed in opaque 96-well microplates (Corning) using an Orion Microplate Luminomiter. After the addition of cells to a well, luminescence was recorded for 20 s. Each 96-well microplate contained multiple wells for positive controls (50 μL Triton 0.1%) and negative controls (50 μL BSA). Luminescence values at each ligand concentration was integrated during the 20-s response interval and normalized to the highest ligand response in each plate after the subtraction of background values obtained from negative controls. Luminescence measured in replica wells (2 wells) for one concentration

of ligand was averaged for the analysis. Data collected from at least three replica plates were used for analysis with the Origin analysis program (OriginLab Corp., Northampton, MA). The following peptides were used in this assay: Tenmo DH47 (*Tenebrio molitor*), Tenmo DH37, Trica DH31 (*T. castaneum* Dr. David Schooley from University of Nevada provided our peptides.

In vivo excretion: chamber assay

Excretions from individual insects were detected using a modified system originally developed by Coast (2004). The chamber assay consists of a single beetle placed in a small chamber that is supplied with dry air (10 cm^3 per min with ~3.2% RH). The outlet of the chamber is connected to the humidity analyzer (RH300, Sable system) measuring vaporized excretion from the insect. Calibration with known amount of Ringer's solution in the chamber found that it is possible to detect the secretion of less than 1 nL solution that is instantly vaporized in the system by using a regression equation (Figure 1 – data collected by Jamie Aikins). The saline used in the study was originally developed for *T. molitor* and contained (in mM) 90 NaCl, 50 KCl, 5 $MgCl_2$, 2 $CaCl_2$, 6 $NaHCO_3$, 4 NaH_2PO_4, 10 glycine, 10 proline, 6 histidine, 10 serine, 8 glutamine, 50 glucose, adjusted to pH 7.0 with NaOH (Nicolson, 1992). Continuous 15-min recordings were made at a 60-Hz sampling rate after the treatment. All experiments used 5 to 10 days old adults, and were performed at room temperature, which varied between 22 and 25^oC

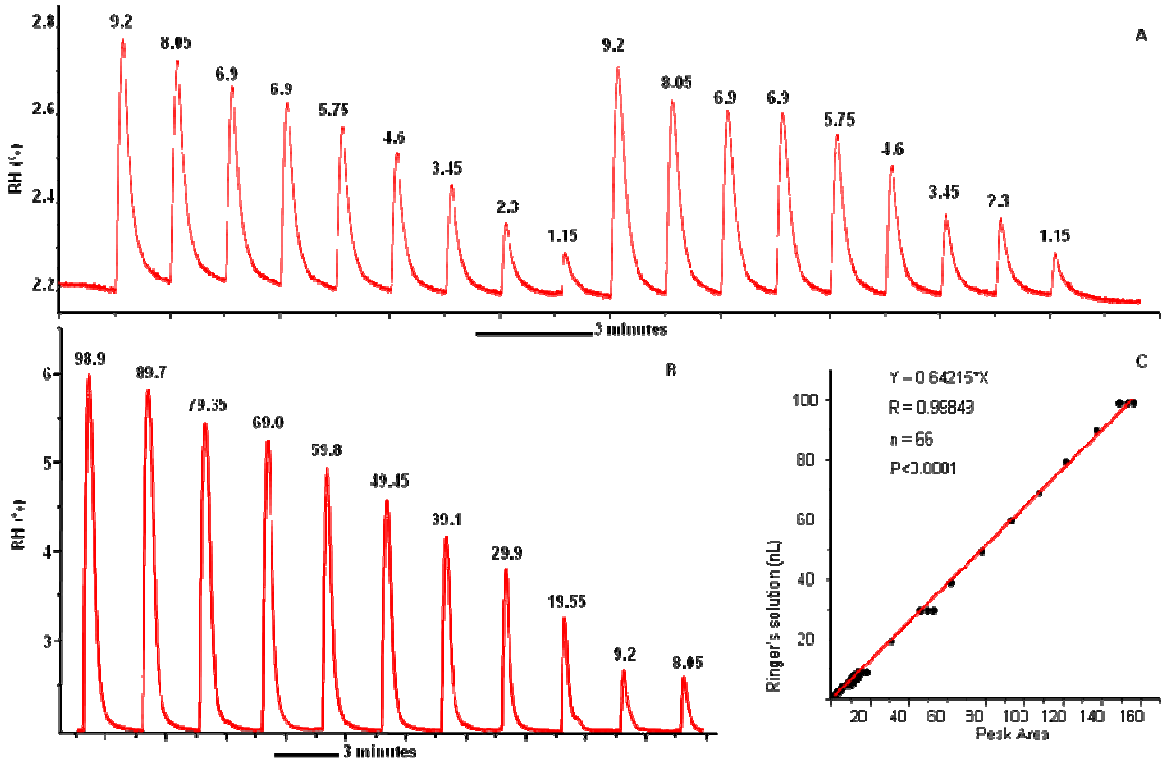


Figure 1. Humidity chamber assay measuring vaporized solution in the airflow. A and B. Different amounts of saline solution were injected in the chamber (I to 100 nl) and the change in relative humidity (RH) were observed. C. Regression between amount of saline injected in the chamber and the area of the graphs from A and B.

Table 1 Primers used to amplify the diuretic hormone genes and the receptorsgenes. All primers used to generate dsRNA had T7 (taatacgactcactataggg) at the 5' end.

Gene	Forward (5'-3')	Reverse (5'-3')	Use	Amplicon Size (bp)
DH31	915 cctggatactattcgccgat	920 ggaactgtttggaaattgaag	RT-PCR	300
DH37 (Exon 1-4)	1001 gacaacatcaccatcggctttctgc	999 tccactctctcatacactgactag	RT-PCR	822
DH37 (Exon 2-4)	1019 gtggcttgtttgttcttggtgcg	999 tccactctctcatacactgactag	RT-PCR	683
DH47 (Exon 1-5)	1001 gacaacatcaccatcggctttctgc	913 gtgattggagaaatgcccttt	RT-PCR	621
DH47 (Exon 2-5)	1019 gtggcttgtttgttcttggtgcg	913 gtgattggagaaatgcccttt	RT-PCR	400
DH37	985 ccttccgagcgaagtatcca	999 tccactctctcatacactgactag	*In situ*	177
DH47	987 aatcccgaagcaaaagagc	914 ttgaagatttcaccgacact	*In situ*	247
DH31	915 cctggatactattcgccgat	920 ggaactgtttggaaattgaag	*In situ*	300
Trica-crfr1	942 ttaccgtattcctcaacgtg	945 ccgtcaacgctgaaacaact	qRT-PCR	240
Trica-ctr1	967 gagatgcggaaacgatgaag	969 gagatgcggaaacgatgaag	qRT-PCR	140
Trica-crfr1	935 cgtttacagtttgcaggttc	938 ggagtccaaggacagctccg	qRT-PCR	297
Trica-crfr2	928 aacgagacgacctatgtcaa	931 tccagtgccccctttggagc	qRT-PCR	351
Trica-ctr2	940 tgaatggaactttaatgaac	945 ccgtcaacgctgaaacaact	*In situ*	838
Trica-ctr1	966 ccacatccagctgtttatc	968 ctccggtgtaatggtagttg	*In situ*	612
Trica-crfr1	935 cgtttacagtttgcaggttc	939 acgaatatctggttcttcgc	*In situ*	986
Trica-crfr2	930 gacccagccacatgcacttg	933 tccccgaacgtagtctggat	*In situ*	680
DH37	985 ccttccgagcgaagtatcca	986 taaatttttgagatattctcga	dsRNA	137
DH47	987 aatcccgaagcaaaagagc	988 attgtcgtaagttccggaagc	dsRNA	207
DH31	1297 ttctcaagccgccaaacatc	1298 cagagagggtgggaaaaggg	dsRNA	448
DH37/47	1479 gttgttgttgtaagaagtga	1480 gttgttgttgtaagaagtga	dsRNA	484
Trica-ctr1	1377 gagcggaatgagaataagtg	1378 catcatcgtcctcatttacagcg	dsRNA	250
Trica-crfr2	1379 ctctataccaacaggaccac	1380 atgtggaagtaatgtagcag	dsRNA	500
Trica-crfr1	1381 ggcggaattagcttgcgaac	1382 tccacataaaatccgcaagg	dsRNA	419
Trica-ctr2	1383 gacggtgaatggaactttaatg	1384 gaggaaggtgaagaggacgacg	dsRNA	342

RNA interference

DNA templates for double-stranded RNA (dsRNA) synthesis were prepared by PCR with gene specific primers based on DH and DHR gene sequences (Table 1). To knock down all four isoforms of the CRF-like gene, specific primers were designed in the exon three which is shared by all isoforms. dsRNA was synthesized using the MEGAscript RNAi Kit (Ambion) according to the manufacturer's protocol. A total of 200nl of dsRNA solution was injected at a concentration of 1.0 mg/ml in 0.1mM sodium phosphate, pH 7, containing 5mM KCl. Last instar larvae were injected through the abdomen. After injection, larvae were allowed to rest in Petri dishes for 1 h, then returned to the incubator in Petri dishes with flour/yeast substrate and monitored daily for mortality, developmental rate, and for abnormal behaviors. After pupation, the insects were sorted by sex and kept in an individual well of a 96-well plate. In each well of the plate, flour/yeast and wheat granules were placed to facilitate the adult emergence. RT-PCR to confirm the suppression of target gene expression was conducted in 3 day old adults (10–15 days after dsRNA injection. Ten days after emergence adults were set for single pair matings for 5 couples in glass vials (2 cm diameter x 6 cm high) containing flour/yeast and granules of wheat to help insect movement. Three days after mating, the flour/yeast was replaced with triple sieved flour/yeast to facilitate egg collection. At 16 and 19 days after adult emergence, eggs and hatching larvae were collected, counted and, placed into flour/yeast in plastic Petri dishes (5cm diameter x 1cm high). Egg hatching was checked several times afterward and the final number of larvae obtained from

each plate was counted 10 days after egg collection. The adult mortality was evaluated until 30 days after emergence. Figure 2 summarizes the protocol used in the RNAi experiments.

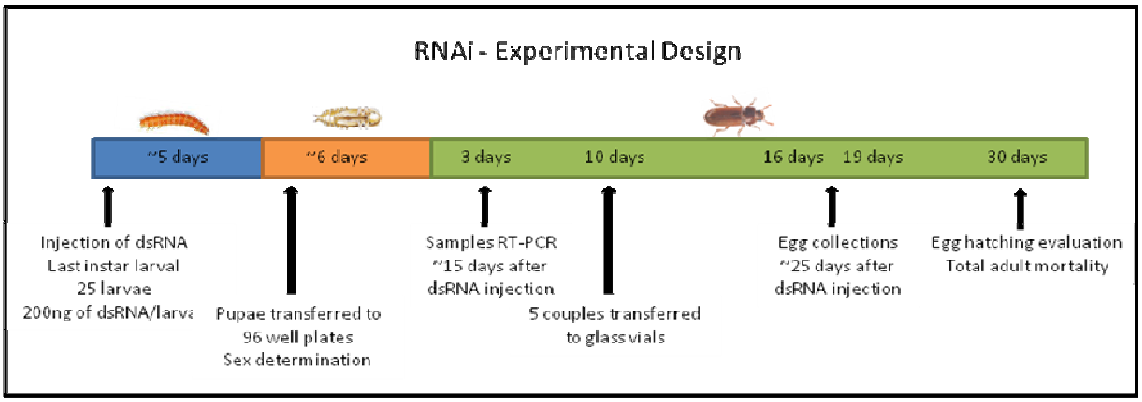


Figure 2. Schematic diagram showing the schedule for examination of RNAi phenotypes. Larvae were injected with 200ng of dsRNA and placed in Petri dishes with food. In the pupal stage, each individual was separated into cells of 96 well plates after the sex was determined. Three days after adult emergence, 3 adults were used for RT-PCR to examine the efficiency of the RNAi. Couples were put in glass vials (5 vials – insects 10 days old) to evaluate effects of RNAi on the reproduction. Eggs were collect twice at 16 and 19 days after adult emergence. The hatchability of eggs was continuously observed several times until 30 days after adult emergence. Insects were observed daily checking mortality and abnormal behavior or morphology. Three biological replications were done. In the first replication 50 to 100 larvae were injected in each treatment. In the second and third replications just 35 to 30 larvae were injected.

Results

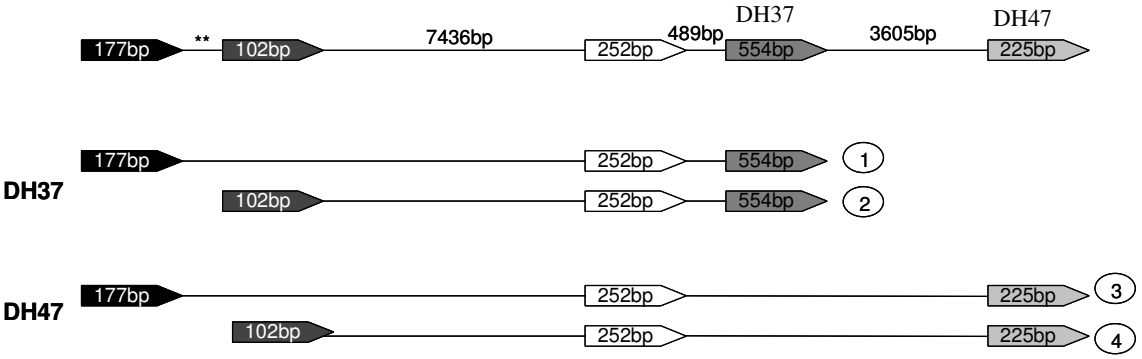
The results section is divided in two parts: in the first section the CRF-like gene/ligands and their receptors are described, and in the second part the calcitonin-like gene/ligand and receptors are described.

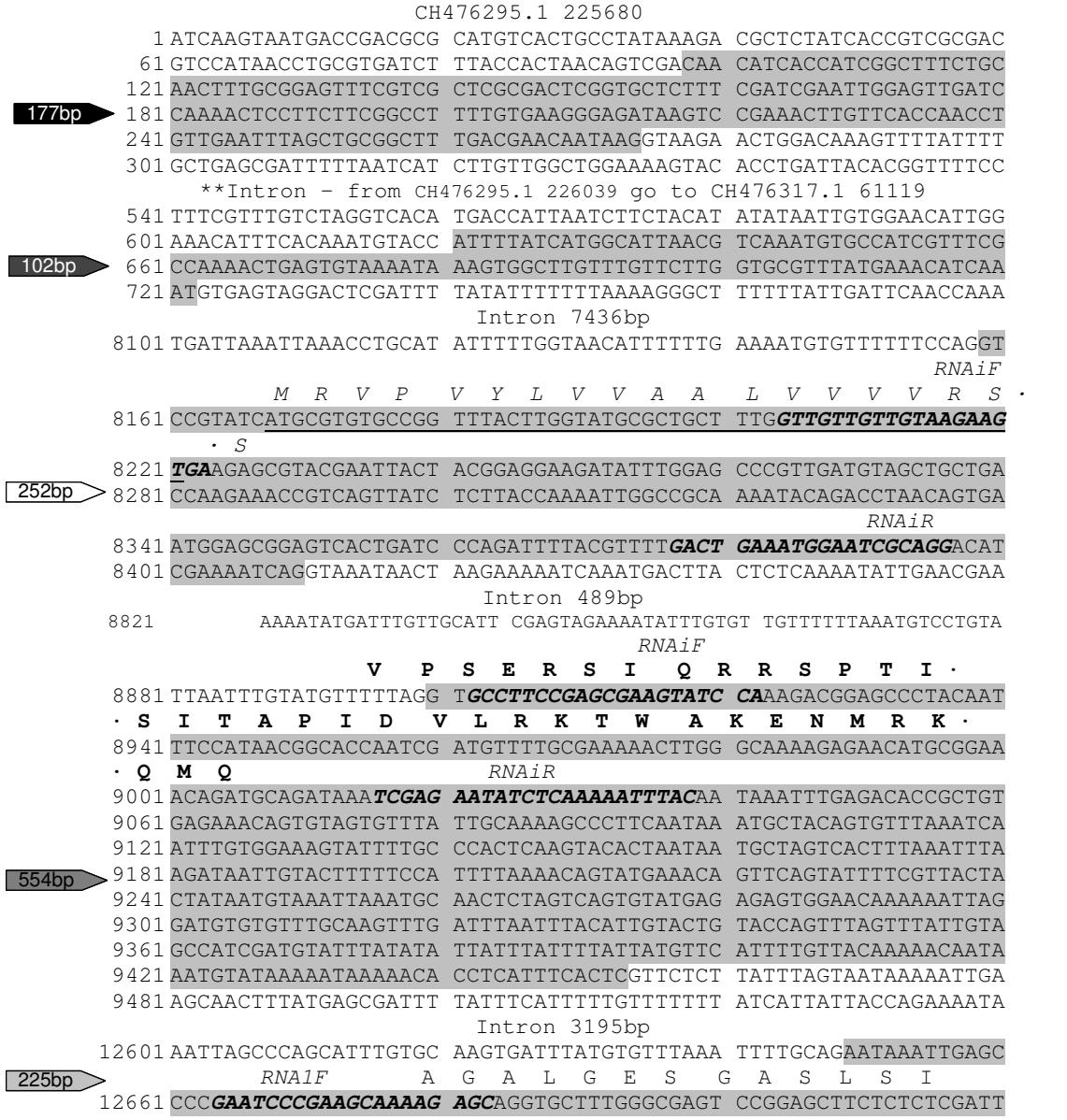
CRF-like DH genes and their receptors

Based on the bioinformatics predictions using *D. melanogaster* DH sequences (done by Dr Bin Li), the initial prediction was that *T. castaneum* had two CRF-like genes: *Tricadh37* and *Tricadh47*. However, cloning the full-length cDNAs for each CRF-like DH revealed that those two peptides are from one gene which undergoes alternative splicing for two different transcripts encoding the CRF-like peptides; TricaDH37 and TricaDH47 where the names indicate the numbers of amino acid residues. The gene has five exons, the translation start point located in the common exon (exon 3). The first two alternatively spliced exons contain 5' untranslated regions (UTR) and the first in-frame methionine starts in the common (exon exon 3) followed by a putative signal peptide. The gene structure and sequence of the CRF-like gene are shown in Figure 3. Using primer 1001, located in the exon 1 or the primer 1019 in exon 2 and primer 999 located in the exon 4, we obtained a fragment of DH37. Similarly, using the same primers from the exon 1 or 2 and primer 913, which is in the last exon, *Tricadh47* is obtained. Thus, all four differently spliced forms were confirmed by RT-PCR.

Multiple sequence alignments with the mature CRF-like peptides from different species were generated to understand the evolutionary relationships

among the CRF-like peptides. *T. castaneum* CRF-like peptides have high similarity with their orthologs from *Tenebrio molitor* (Figure 4).





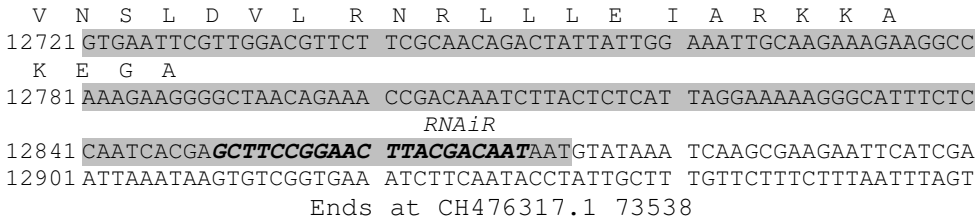


Figure 3. *Tribolium castaneum* CRF-like gene structure and sequence. The gene has two alternative transcripts with mutually alternative exons and transcription start points. The gray boxes indicate exons. The first exon was found in scaffold CH476295.1 and all other exons in the scaffold CH476317.1. The primers used to generate dsRNA are indicated in bold and italic font. The signal peptide is indicated with underlined font in exon 3. DH37 mature peptide is indicated in exon 4 with bold font. DH47 mature peptide is indicated in exon 4.

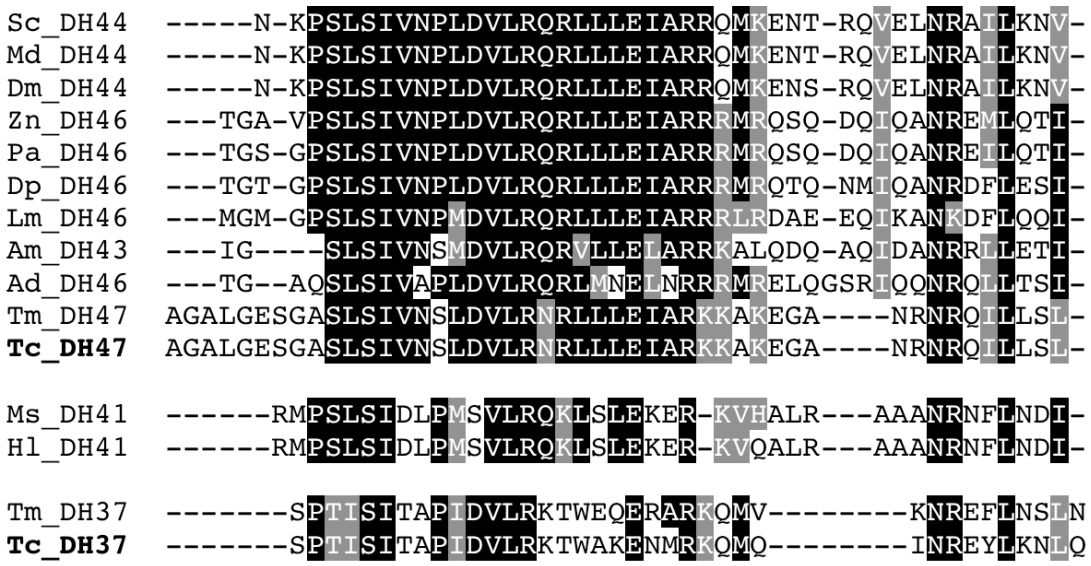
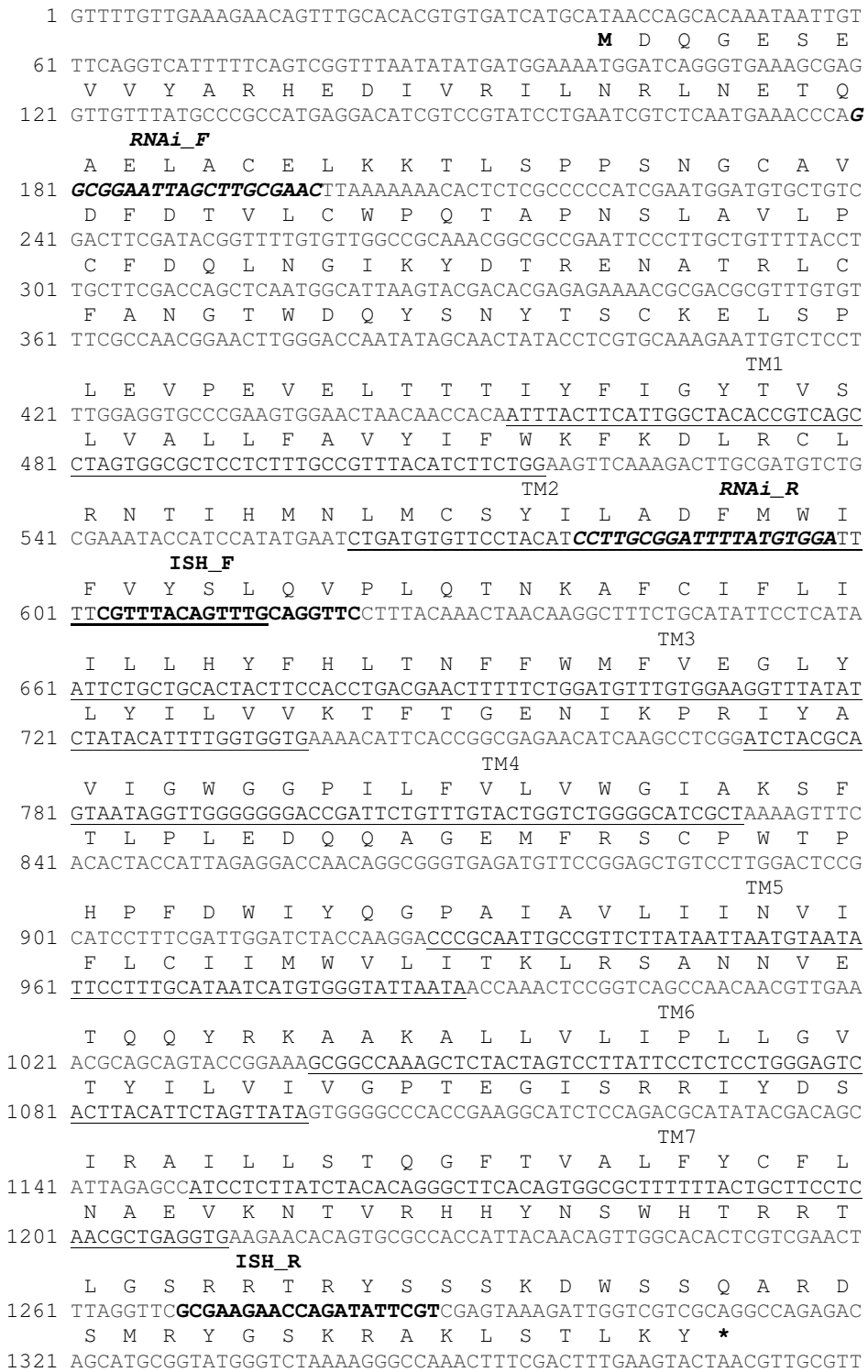


Figure 4. Multiple sequence alignment of DH ligand sequences. Identical (inverted box) and similar amino acids (gray box) are by the 50% majority rule (Li, B. et al., 2008).

A TblastN search was performed using a *D. melanogaster* CRF-like receptor sequence and hits with high scores from *T. castaneum* and mosquitoes were used to build a phylogenic tree (Figure 10). Based on the clustering patterns of the closely related receptor sequences from other insects with *D. melanogaster* and mosquito receptors, which have been functionally characterized, we were able to identify orthologous genes representing *T. castaneum* CRF and calcitonin-like receptors. The gene structure and sequence of the CRF-like receptor genes are shown in Figures 5 and Figure 6. Both genes have 9 exons and similar topology. The open reading frame (ORF) of *Trica-crfr1* is 1272bp, while the ORF of *Trica-crf2* is 1383bp. The locations of the primers used to generate dsRNA and probes for *in situ* hybridization are also shown in

the sequences. The transmembrane domains were predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).



```
1381 TTAGTCTGTATCAAACAAATTCGTACAGAAAAAGGGGGTCCACATGCTCGACCGGTACTA
1441 CTACAACGGTTGTGGTAGTACCGTTCCCGAA
```

Figure 5. *Trica-crfr1* gene structure and sequence (ORF: 1272bp). Transmembrane segments predicted by TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) are indicated by underlined font in the nucleotide sequence. The primers used to generate dsRNA are shown in bold and italic font; whereas the primers used to generate probes for *in situ* hybridization are show in bold font. Sequence shown is from CM000279 10513802 to 10491091. Introns are not shown.

```
   1 CATATATCACCCGTGCAAAC AAACTGATAAAGAAAATCTT CGCTCGAATCCACCTCAATT
                                              M   S  W  S  E  P  L
  61 GTGTTTCGCAGATTCGAGTG TCAAAATGCATCATCAACAA TGAGTTGGTCCGAACCTCTC
     P  Q  E  P  E  P  V   D  A  D  L  W  P  S   S  E  N  V  L  N
 121 CCCCAGGAGCCGGAGCCCGT CGACGCCGACTTGTGGCCCA GTTCCGAGAATGTCCTCAAC
                                                          RNA1F
     E  T  E  D  I  K  I   R  L  N  I  T  L  Q   H  C  T  S  L  Y
 181 GAAACAGAGGACATCAAGAT CCGACTCAACATCACCTTAC AACACTGCACCAGCCTCTAT
     T  N  R  T  T  A  L   A  E  T  H  P  D  G   F  C  P  V  T  T
 241 ACCAACAGGACCACAGCCCT CGCAGAAACCCACCCAGATG GGTTTTGCCCCGTCACCACC
     D  G  L  L  C  W  P   P  T  P  I  N  E  T   T  Y  V  K  C  F
 301 GATGGGCTGCTGTGCTGGCC GCCCACGCCCATCAACGAGA CGACCTATGTCAAGTGCTTC
     A  E  L  M  N  I  R   Y  D  D  T  Q  N  A   T  R  V  C  L  A
 361 GCCGAATTGATGAACATCAG ATACGACGACACACAGAATG CCACAAGAGTTTGCCTCGCT
     N  G  T  W  T  K  A   D  Y  S  K  C  T  E   I  I  L  I  P  D
                            TM1
 421 AATGGGACATGGACGAAAGC CGATTATTCCAAATGCACCG AAATCATTCTTATCCCCGAC
                                                          TM2
     V  E  T  Q  A  T  I   Y  F  V  G  Y  V  L   S  L  I  T  L  S
 481 GTCGAAACCCAAGCCACGAT TTATTTCGTCGGATACGTTT TAAGTTTAATTACACTGTCG
                                                          TM3
     I  A  L  G  I  F  T   Y  F  K  E  L  R  C   L  R  N  R  I  H
 541 ATAGCTTTGGGAATTTTCAC ATATTTCAAAGAGCTGCGCT GCTTGAGAAACCGAATTCAC
     M  N  L  M  W  S  Y   M  L  M  Y  I  M  W   I  L  T  L  T  V
 601 ATGAATCTCATGTGGTCTTA CATGTTAATGTACATAATGT GGATTCTGACCCTGACGGTT
     L  G  S  K  G  G  T   G  A  S  I  A  C  I   F  V  I  T  L  L
 661 CTGGGCTCCAAAGGGGGCAC TGGAGCCTCCATAGCTTGCA TATTCGTCATCACACTGCTA
           RNAiR                                          TM4
     H  Y  F  H  I  S  T   F  F  W  M  F  V  E   G  L  Y  L  Y  I
 721 CATTACTTCCACATTTCCAC GTTTTTTTGGATGTTTGTTG AGGGCCTGTATCTTTATATT
     L  V  V  E  T  L  T   R  E  N  Y  K  L  R   V  Y  Y  V  C  I  G
 781 CTCGTCGTTGAAACACTGAC CAGGGAGAATTATAAATTGA GGGTTTATGTGTGCATCGGG
                                                          TM5
     W  G  L  P  M  I  F   I  L  V  W  V  I  V   K  S  F  I  P  A
 841 TGGGGCTTGCCAATGATTTT CATCCTCGTTTGGGTGATTG TCAAGAGCTTTATCCCGGCA
                ISH_F
     A  G  D  P  A  T  C   T  W  F  N  S  H  D   V  D  W  I  F  Q
 901 GCGGGCGACCCAGCCACATG CACTTGGTTCAACAGTCATG ACGTGGACTGGATTTTCCAA
                                                          TM6
     G  P  T  M  L  V  L   L  L  N  L  A  F  L   L  A  I  M  W  V
 961 GGGCCTACAATGCTGGTTCT CCTGCTGAATTTGGCCTTCC TGCTGGCCATAATGTGGGTC
     L  I  T  K  L  R  S   A  N  T  V  E  T  Q   Q  Y  H  K  A  A
1021 CTCATAACAAAACTGCGATC TGCCAATACGGTGGAAACCC AACAGTATCATAAAGCGGCC
     K  A  L  L  V  L  M   P  L  L  G  I  T  Y   V  I  T  I  Y  A
1081 AAAGCCCTCCTCGTACTTAT GCCCTTGCTGGGCATCACCT ACGTTATCACAATCTACGCC
                               Tm7
     P  T  P  D  K  K  S   E  I  I  F  E  C  V   R  A  V  L  L  S
1141 CCAACCCCTGATAAGAAATC GGAAATCATTTTCGAGTGCG TCCGCGCTGTCCTTCTCTCG
     T  Q  P  L  N  E  H   P  P  I  C  Q  S  Y   S  V  A  I  T  G
1201 ACACAGCCATTGAACGAGCA TCCACCAATTTGCCAAAGCT ATTCGGTTGCAATTACAGGG
     L  H  C  R  S  I  L   L  L  L  K  H  G  G   A  E  H  R  P  P
1261 CTTCACTGTCGCTCTATTCT ACTGCTTCTTAAACACGGAG GTGCAGAACACCGTCCGCCA
                                                          ISR_R
     P  L  R  N  V  E  N   T  T  I  S  G  P  I   Q  T  T  F  G  E
1321 CCACTTCGAAACGTGGAAAA CACGACGATCTCTGGGCCCA TCCAGACTACGTTCGGGGAG
     S  Q  Q  G  L  V  P   Q  V  T  H  G  E  Y   T  V  R  E  K  I
1381 TCGCAGCAAGGACTGGTCCC CCAGGTCACGCACGGAGAGT ATACGGTGAGGGAAAAAATA
     F  N  D  S  L  G  T   I  T  F  G  S  I  *
1441 TTCAATGACTCTTTGGGGAC AATCACGTTTGGCTCGATTT GAGTGATCTCAAGTCGGGAG
1501 GATCCAAGTCAGCAGTTCTT GTAATAACTCACTTTTGACA TTGGCGGTTTTTTGTGAGAG
1561 GGTTTATATCGAGAGCTGGT GA
```

Figure 6. *Trica-crfr2* gene structure and sequence (ORF: 1383bp). Transmembrane segments predicted by TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) are indicated by underlined font in the nucleotide sequence. The primers used to generate dsRNA are shown in bold and italic font; where the primers used to generate probes for *in situ* hybridization are show in bold font. Sequence shown is from CM000284 13821701 to 13837104. Introns are not shown.

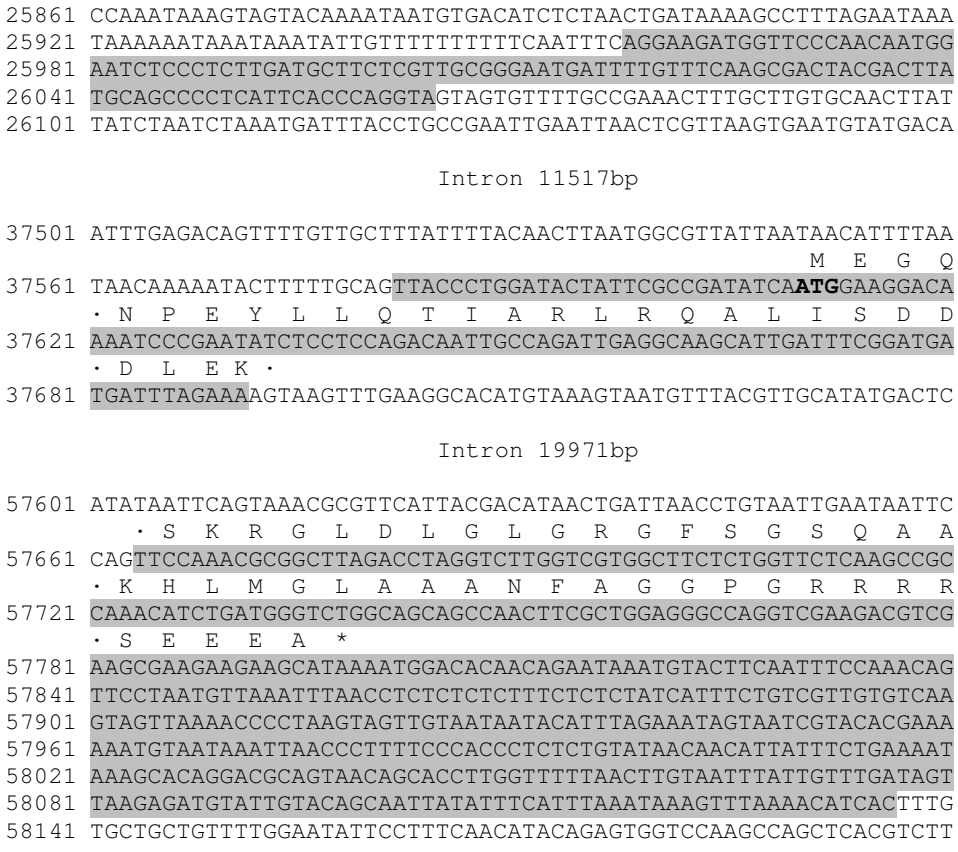
Calcitonin-like genes and their receptors

The *Tricadh31* was found in our EST database and recovered from the DNA plates and its sequence is shown in Figure 7A. A multiple sequence alignment was determined and shown in Figure 7B. The highest sequence identity of the TricaDH31 is with the aphid AcypiDH31, *Acyrthosiphon pisum*, which is 62%. The identity of the *T. castaneum* DH31 with the other species is around 50%. The mature peptide region is highly conserved throughout all the insect orders.

The gene structures and sequences of the two CT-like receptors are shown in Figure 8 and Figure 9. To obtain a full length clone of *Trica-ctr2*, RACE was used as described in the Methods section. The predicted 5' and 3' ends were not corrected in the Glean prediction and the first and last exon was not found in our clone sequences. The predicted 5' end was missing 103 amino acids and the new 3' end was found in a different scaffold, which indicates that

the genome assembly was incomplete. The ORF of *Trica-ctr1* and *Trica-ctr2* is 1218bp and 1230bp, respectively.

A



B

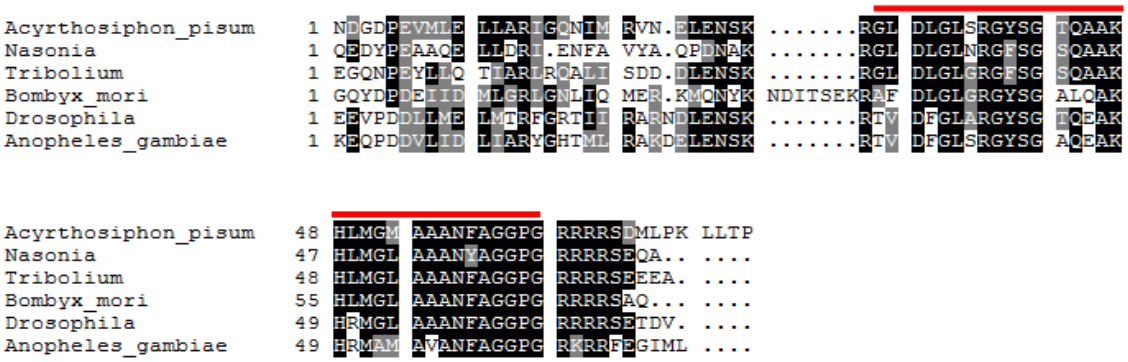


Figure 7. A. Sequence of *Tricadh31*. The exons are indicated by gray boxes. B. Multiple sequence alignment of DH31 peptides from different insect

orders: *Acyrthosiphon pisum*, Hemiptera (XM_001945866.1); *Nasonia*, Hymenoptera (XM_001599898.1); *Bombyx mori*, Lepdoptera (NM_001130907.1), *Drosophila*, Diptera (NM_164825.1), *Anopheles gambiae*, Diptera (XM_321755.4). Identical (inverted box) and similar amino acids (gray box) are by 50% majority rule. The sequence of *Tricadh31* shown is from CH476267 402706 to 370426.

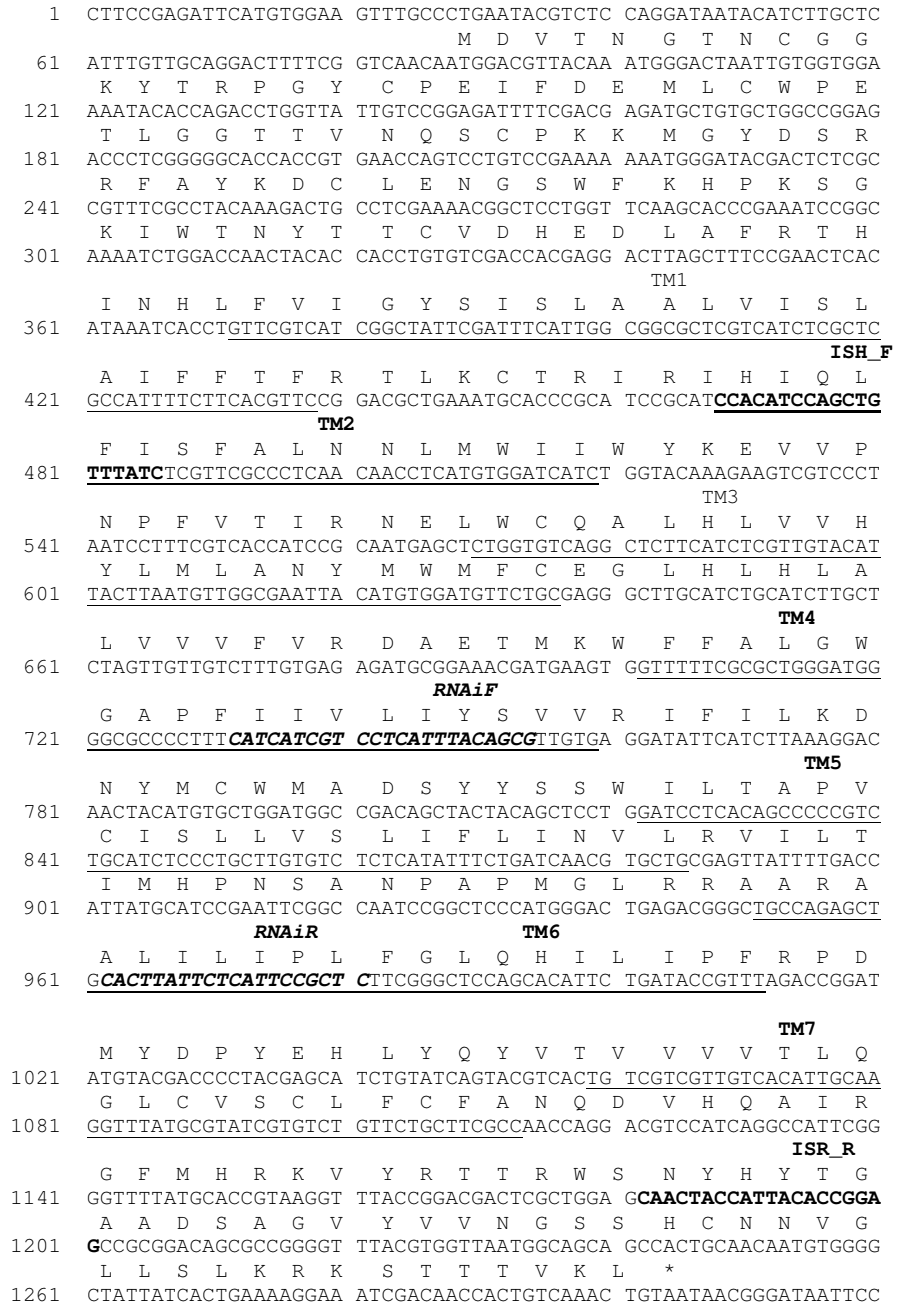


Figure 8. *Trica-ctr1* gene structure and sequence (ORF: 1218bp). Transmembrane segments predicted by TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) are indicated by underlined font in the nucleotide sequence. The primers used to generate dsRNA are shown in bold and italic font; whereas the primers used to

generate probes for *in situ* hybridization are show in bold font. The sequence shown is from CM000280 12763502 to 12751368. Introns are not shown.

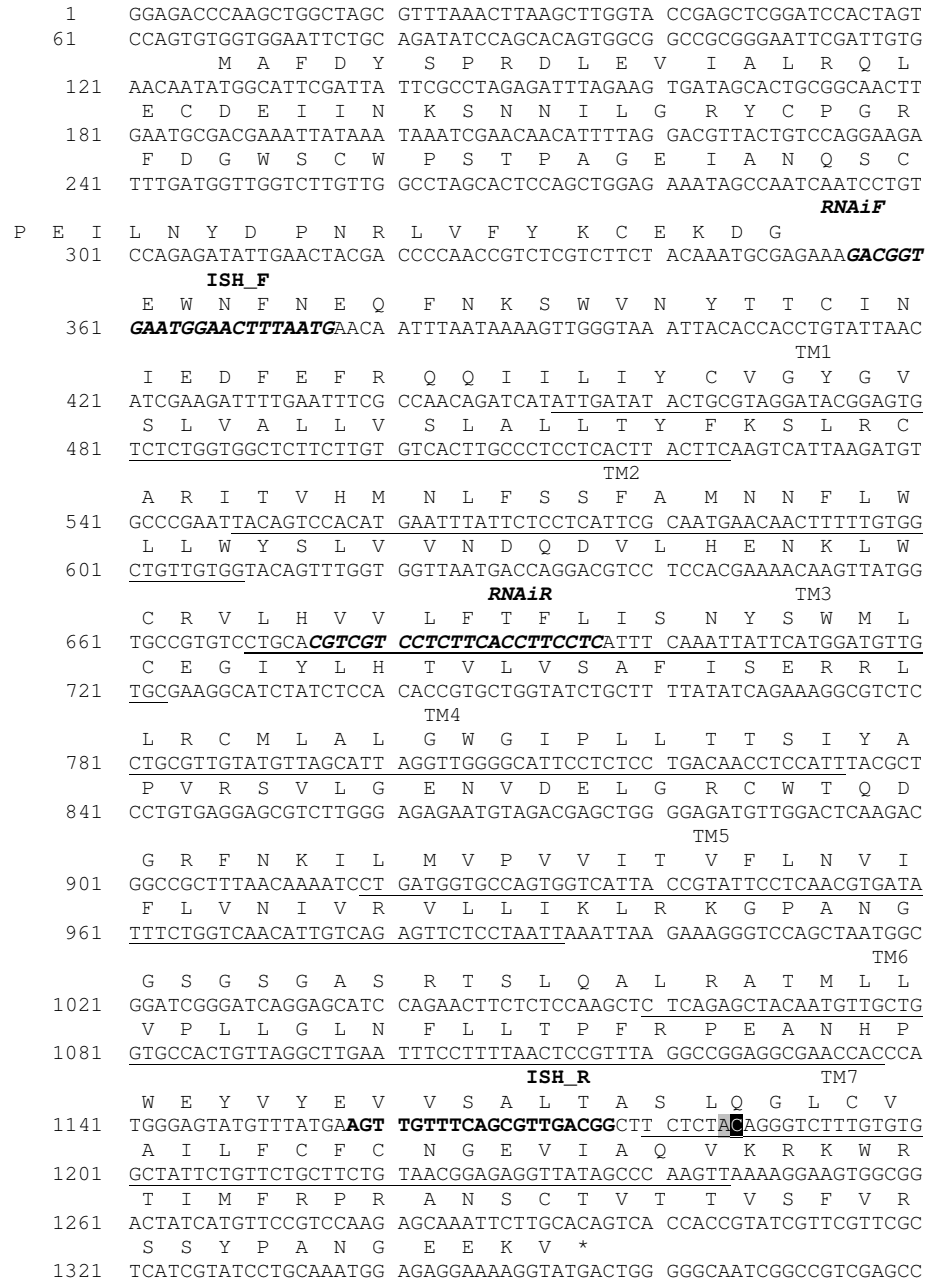


Figure 9. *Trica-ctr2* gene structure and sequence (ORF: 1230bp). Transmembrane segments predicted by TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) are indicated by underlined font in the nucleotide sequence. The primers used to generate dsRNA are shown in bold and italic font; whereas the primers used to

generate probes for *in situ* hybridization are show in bold font. The sequence is from CM000278 15823760 to 15741565(A) and from CH476486.1 11082 (C) to 6326. Introns are not shown.

The phylogenetic analysis of *T. castaneum* diuretic hormone receptors revealed that the calcitonin-like receptors formed two clusters with receptors from Diptera, implying earlier gene duplication. A multiple sequence alignment was also performed and revealed that sequences of calcitonin-like receptors are highly conserved between dipterans and *T. castaneum* (Appendix 1). The relationship among the CRF-like receptors was not clear unlike the case of calcitonin-like receptors (Figure 10) indicating independent gene duplications at multiple intervals. The CRF-like receptors are also conserved among the Diptera and *T. castaneum,* while the sequences of the mosquito prediction for CRF-like receptor contained divergent sequences between transmembrane domains (Appendix 2).

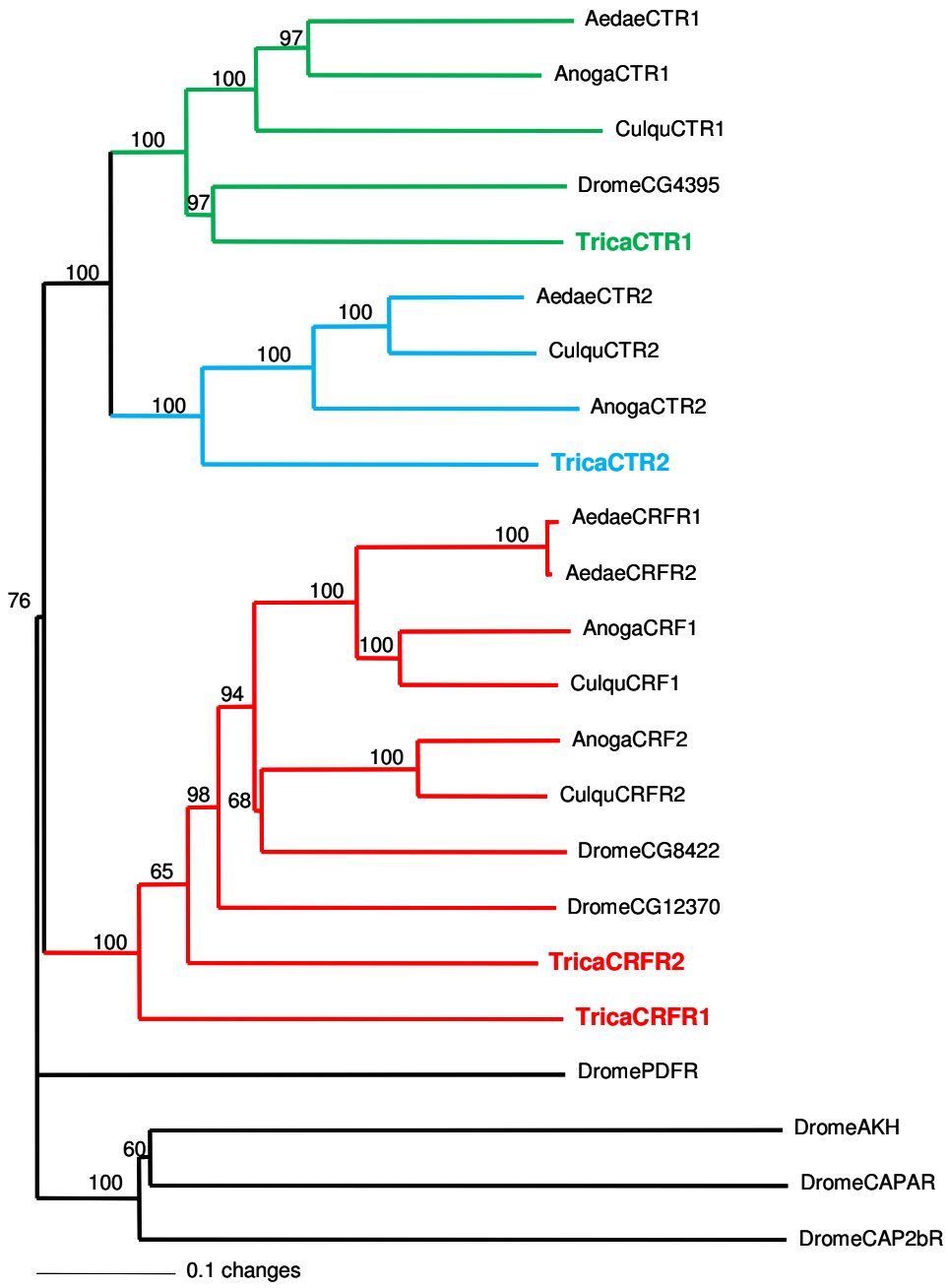


Figure 10. Phylogenetic relationship of *T. castaneum* calcitonin-like receptors and CRF-like receptors with other members of the family B of GPCR (secretin family) from *Aedis aegypti* (Aedae), *Anopheles gambiae* (Anoga), *Culex quinquefacius* (Culqu) and *Drosophila melanogaster* (Drome). The outgroups are Drosophila receptors of AKH, CAP2b and

CAPA. Numbers at the nodes are for percent support in 1000 bootstraps. Accession numbers: AedaeCRFR1, EU273351.1; AedaeCRFR2, XM_001659059.1; AedaeCTR1, XM_001651938.1; AedaeCTR2, XM_001660544.1; CulquCTR1, XM_001862151.1; CulquCTR2, XM_001864861.1; DromeCG12370, NM_165907.3; DromeCG4395, NM_132615.2; DromeCG8422, NM_137116.2; AnogaCTR1, XM_321982.3; AnogaCTR2, XM_318856.3; AnogaCRF1, XM_315466.4; AnogaCRF2, XM_315468.4; TricaCRFR1, XM_001808544.1; TricaCRFR2, XM_970323.2; TricaCTR1, XM_001808544.1; TricaCTR2, XM_963937.1; DromeCAPAR, AF522188.1; DromeCAP2bR, NM_206418.2; DromeAKH, AF522194.1; CulquCRF1, BK006347.1; CulquCRFR2, EU273352.1; DromePDFR, NM_130651.2. The sequences of TricaCTR2 and TricaCRF2 available at NCBI or Beetlebase are listed as reference since they are not annotated correctly.

Real-time quantitative PCR (qRT-PCR)

Stage-specific real-time quantitative PCR revealed that CRF-like receptors mRNA were detectable throughout almost all developmental stages (detailed information about these stages can be seen in Methods). *Trica-ctr1* and *Trica-ctr2* transcripts were detected in Malpighian tubules (MT). *Trica-ctr1* was constitutively expressed during larval stages, with peaks in early larval and early

pupal stages. Expression of this gene was also detected in the gut and in the hindgut (Figure 11).

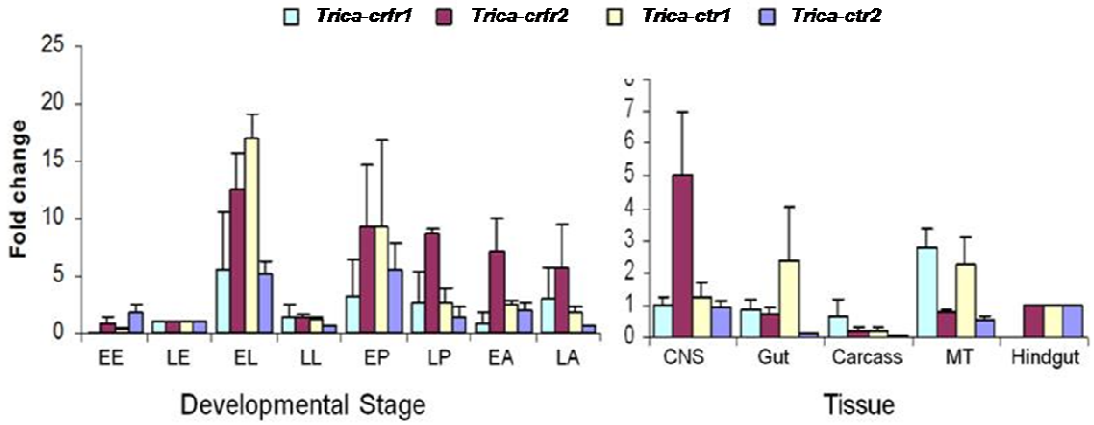


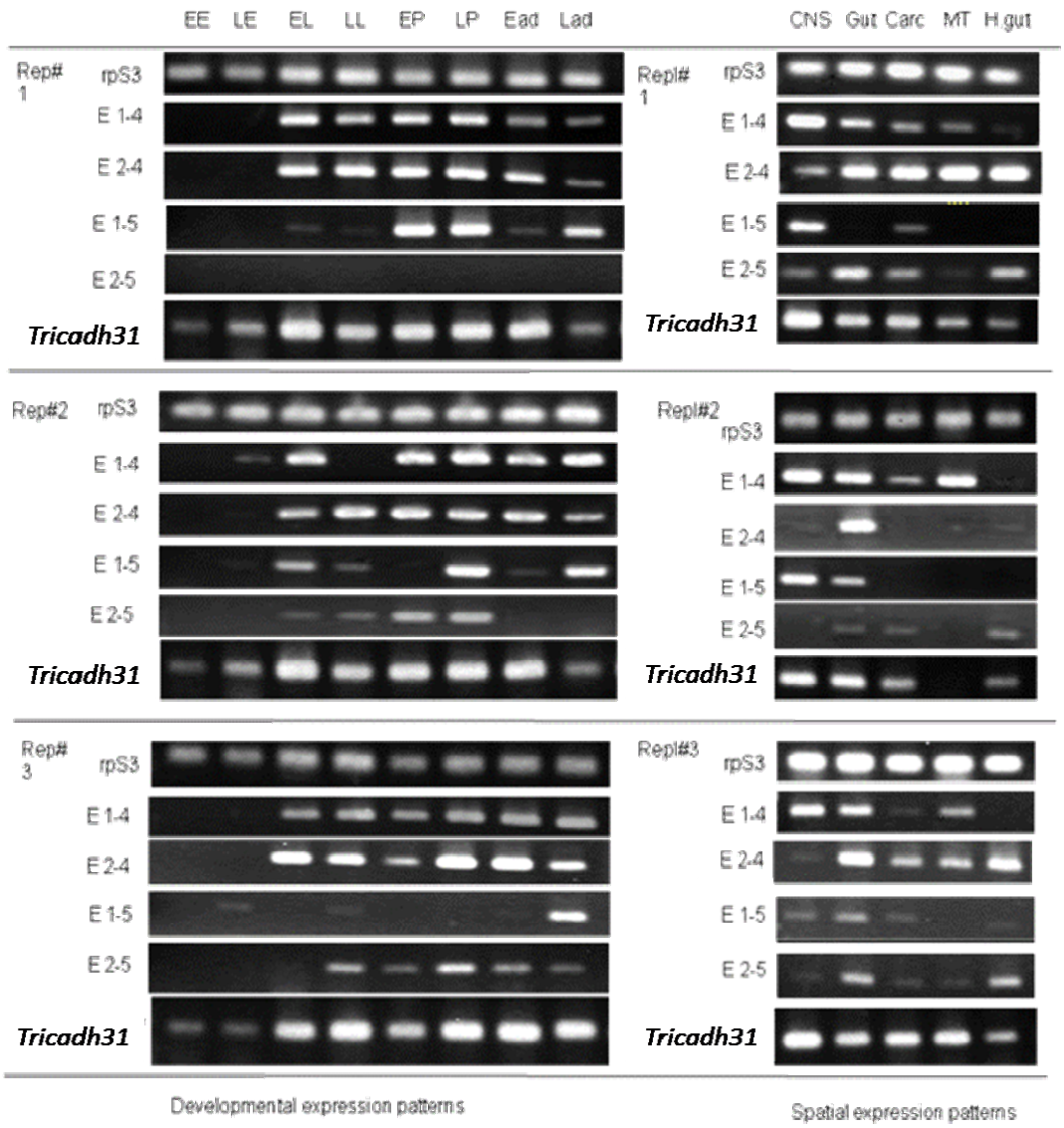
Figure 11. Quantitative RT-PCR in different stages and tissues for diuretic hormone receptors of *T. castaneum*. Pools of tissue dissected from three insects were used for total RNA isolation. The averages and standard deviations of three biological replications are shown. CNS, central nervous system, MT, Malpighian tubules.

Semi-quantitative reverse transcription PCR (RT-PCR)

The *Tcdh31* was expressed throughout the developmental stages with lower expression in embryonic stage (Figure 12). The gene is also highly expressed in the CNS and in the gut, but at a lower level in the hindgut. The CRF-like gene isoforms are not expressed in the early embryo, and just in two replications we found expression in the late embryo stage. This could be due to the sensitivity of this experiment or they may not be expressed. In all other stages or tissue at least one isoform is expressed (Figure 12). This experiment

showed that there is high variability among the insects sampled. Three insects were used in each sample plot.

A



B

Gene	Developmental Stage								Tissue				
	EE	LE	EL	LL	EP	LP	EA	LA	CNS	Gut	Carc	MT	HGut
rpS3	+++	-++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+-+	+++
E1-4		-	+++	++	+++	+++	+++	+++	+++	+++	+++	+-+	
E2-4			+++	+++	+++	+++	+++	+++	+	+++	+++	++	++
E1-5			+	++	+	++	-+	+++	+++	++	++		
E2-5		-	+	++		+		+	++	+++	+++	+	+++
Tricadh31	+++	-++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++

Figure 12. Semi-quantitative RT-PCR of the four different isoforms of the *T. castaneum* CRF-like gene and DH31. EE, early embryo; LE, late embryo; EL, early larva; LL, late larva; EP, early pupa; LP, late pupa; Ead, early adult; Lad, late adult; CNS, central nervous system; Carc, carcass; MT, Malpighian tubules. E 1-4 and E 2-4 are DH37 isoforms; E 1-5 and E 2-5 are DH47 isoforms. A. Raw data; B. Summary of expression.

In situ hybridization of *Tricadh37*

The larval brain revealed strong staining in two clusters of cells on each side (Figure 13). Two pairs of cells were stained in abdominal ganglia 1 through 6, and in the last adnominal ganglion. The positions of the cells in the first six abdominal ganglia were different from the last one. In the last abdominal ganglion the stained cells were located in the center of the ganglion while on the other abdominal ganglia they were located more laterally. On the terminal abdominal ganglia two clusters of cells were stained. One with four cells and the other with two cells stained. The staining on the pupal tissues was similar to the larval staining. However, cells were also stained on the pupal gut (Figure 13). The adult brain also shows staining similar to the pupal and larval brains, with

two clusters of cells (four pairs of cells). In the adults, two pairs of cells in thoracic ganglion 1 and 2 were detected. In adult, the third thoracic ganglia and first abdominal ganglion more cells were stained than in the corresponding larval and pupal ganglia (Figure 13). One bilateral pair of cells was strongly stained in the adult abdominal ganglia 2 to 5. The staining in the adult abdominal ganglion 6 was also different from the earlier stages; where the position of the cells stained was not bilateral, they were located at the center of the ganglion.

Immunohistochemistry of TricaDH37

Four pairs of neurons were immunoreactive to rabbit anti-TenmoDH37 in the brain (Figure 14A and Figure 15). Immunoreactivity was also found on the corpora cardiaca (Figure 14A). In the first six abdominal ganglia bilateral pairs of neurons were immunoreactive with projection to the insect hemocel (Figure 14B). The immunoreaction on the last abdominal ganglion was different than first abdominal ganglia, being the neurons were not bilateral (Figure 14C). The terminal abdominal ganglion did not have neurons with projections to the insect hemocel (Figure 14D). Immunoreactivity was also found in the gut and hindgut (Figure 14E, F and G). Projection of neurons in the cryptonephredial complex was found in the hindgut (Figure 14G). The results of immunohistochemisty and *in situ* hybridization were very similar. The main difference was the cells weakly stained on some ganglia which were not immunoreactive to the antibody (Figure 14).

In situ hybridization of *Tricadh47*

The majority of cells stained were located in the brain, subesophageal ganglion, abdominal ganglia 1 to 3 and in the terminal abdominal ganglia in the larval, pupal and adult stages (Figure 16 and 17). In the brain, several cells were stained, four pairs were strongly stained at the anterior lateral position of the brain, five cells on the posterior part of the brain and, one pair located in the center of the anterior part. The larval and adult brains had similar staining pattern. In the pupal brain just two pairs at the anterior central part and one pair in the posterior part showed strong staining. In subesophageal ganglia at least two pairs of cells were stained in all stages. In the thoracic ganglia of adults eight cells were strongly stained, whereas in the pupal stage just six cells were weakly stained. Six pairs of cells were strongly stained in the terminal abdominal ganglion in all stages (Figures16 and 17).

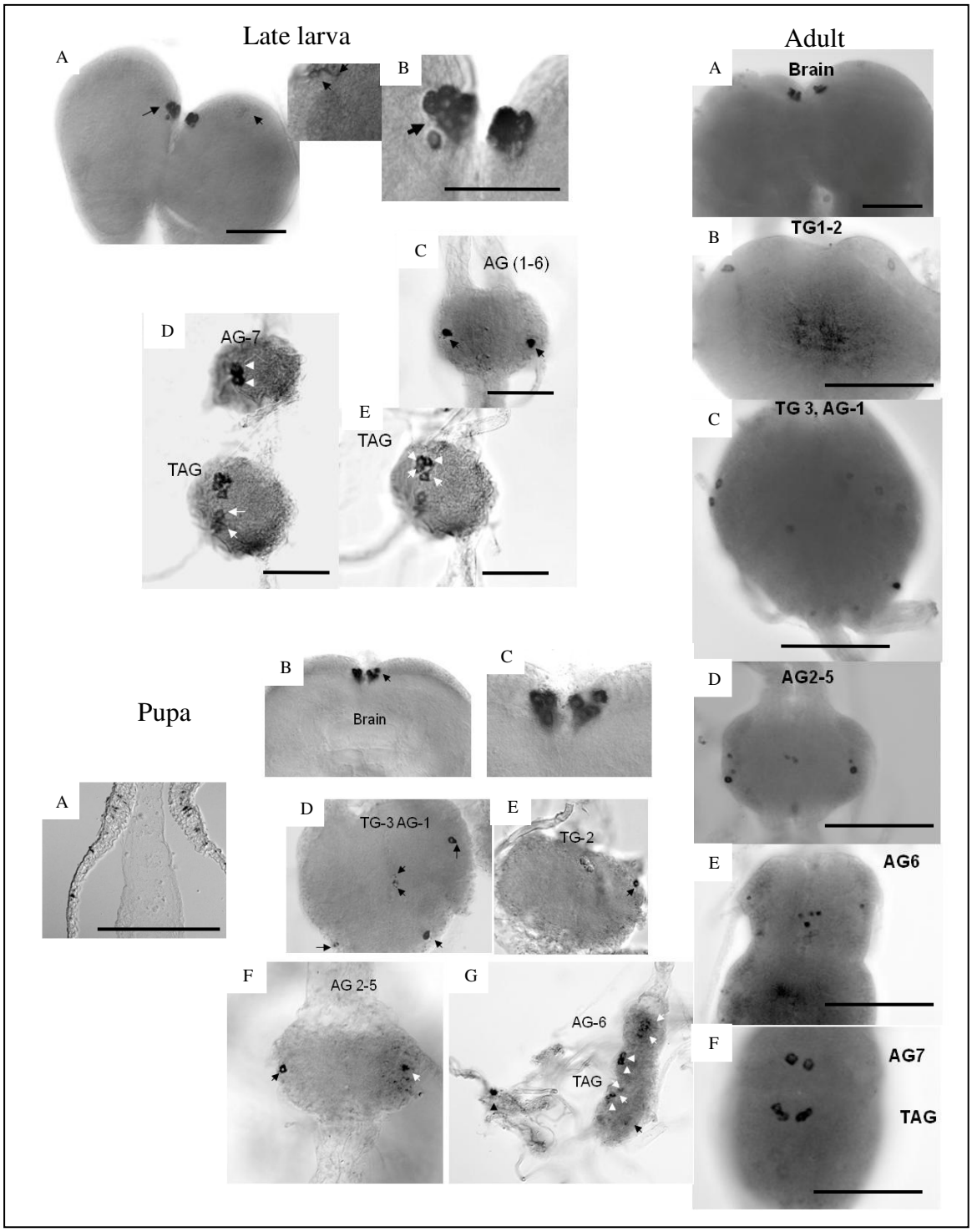


Figure 13. *In situ* hibridazation of *Tricadh37* in larva, pupa and adults of *T. castaneum*. Arrows indicate cells with strong staining. TG, thoracic

glanglion; AG, abdominal ganglion; TAG, terminal abdonominal ganglion. **Larva**: A. Brain with 2 anterior centraly located cluster of cells strongly stained; B. Magnification of the cluster of cells in the anterior central region of the brain show the presence of 4 cells in each cluster; C. AG 1-7 with 1 pair of cells strongly stained; D. AG7 and TAG, AG7 with one pair of strongly stained cells; E. TAG with 3 pairs of cells strongly stained. **Pupa**: A. Staining in the pupal gut; B. Brain with 2 cluster of cells in similar position found in larval brain; C. Magnification of the cluster of cells in the brain revealing the presence of 4 cells in each cluster; D. Represents TG3 and AG1 with one pair of cells strongly stained; E. TG2 with stained cells; F. Represents AG 2-5 with one pair of cells strongly stained; AG7 and TAG, AG7 with one pair of strongly stained cells and TAG with 3 pairs of stronlgy stained cells. **Adult**: A. Brain with cluter of cells at similar position found in larva and pupal brains; B. TG 1-2 with 1 pair of cells stained in the anterior region; C. TG 3 and AG 1 with 1 pair of strongly stained cells; D. AG 2-5 with 1 pair of cells strongly stained and other cells weakly stained; E. AG6 with 2 pairs of cells showing strong staining; F. AG7 and TAG, AG7 showing 2 pairs of strongly stained cells and TAG with 3 pairs of stained cells.

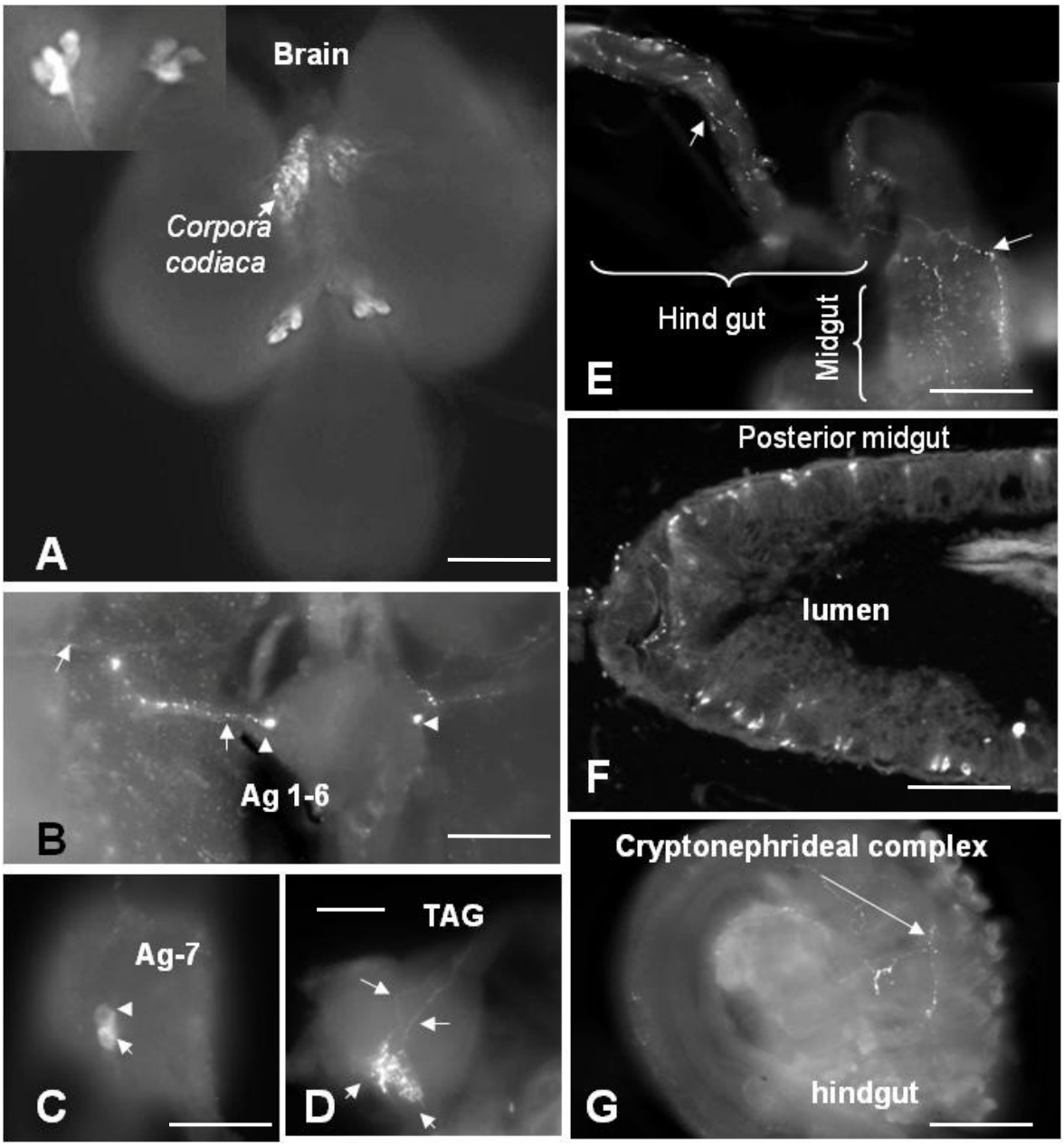


Figure 14. Immunohistochemistry of TricaDH37 with rabbit anti-TenmoDH37. A. Immunoreactivity in four pairs of neurons on brain and on Corpora Cardiaca cells. B. Abonominal ganglia showing the immunoreactivity found in abdominal ganglia 1 to 6: a pair of neurons on each side of the ganglion with projections to the insect body. C. A immunoreactive pair of cells on the last abdominal ganglion. D. Two

immunoreactive neurons on the terminal ganglia with projection not bilateral as seen on the abnominal ganglia 1 to 6. E. Projection of immunoreactive neurons on the gut. F. Immunoreactivity on the peripheral region of the midgut. G. Hindgut with immunoreactions on the cryptonephredial complex. Scale bars: 100µm A, B, C, D and E; 50µm F and G.

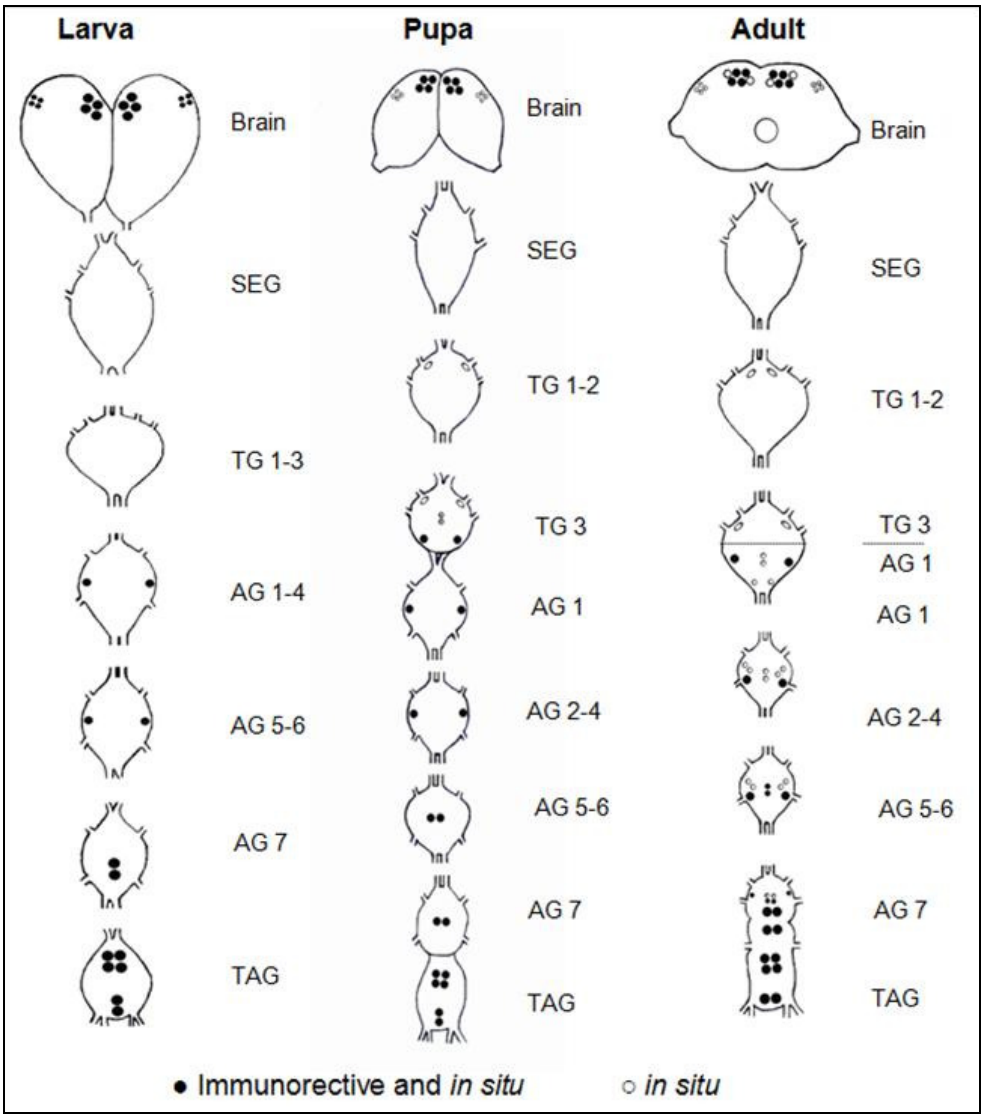


Figure 15. *T. castaneum* immunoreactive cells to rabbit anti-TenmoDH37 and cells stained with specific probes to *Tricadh37* on different stages.

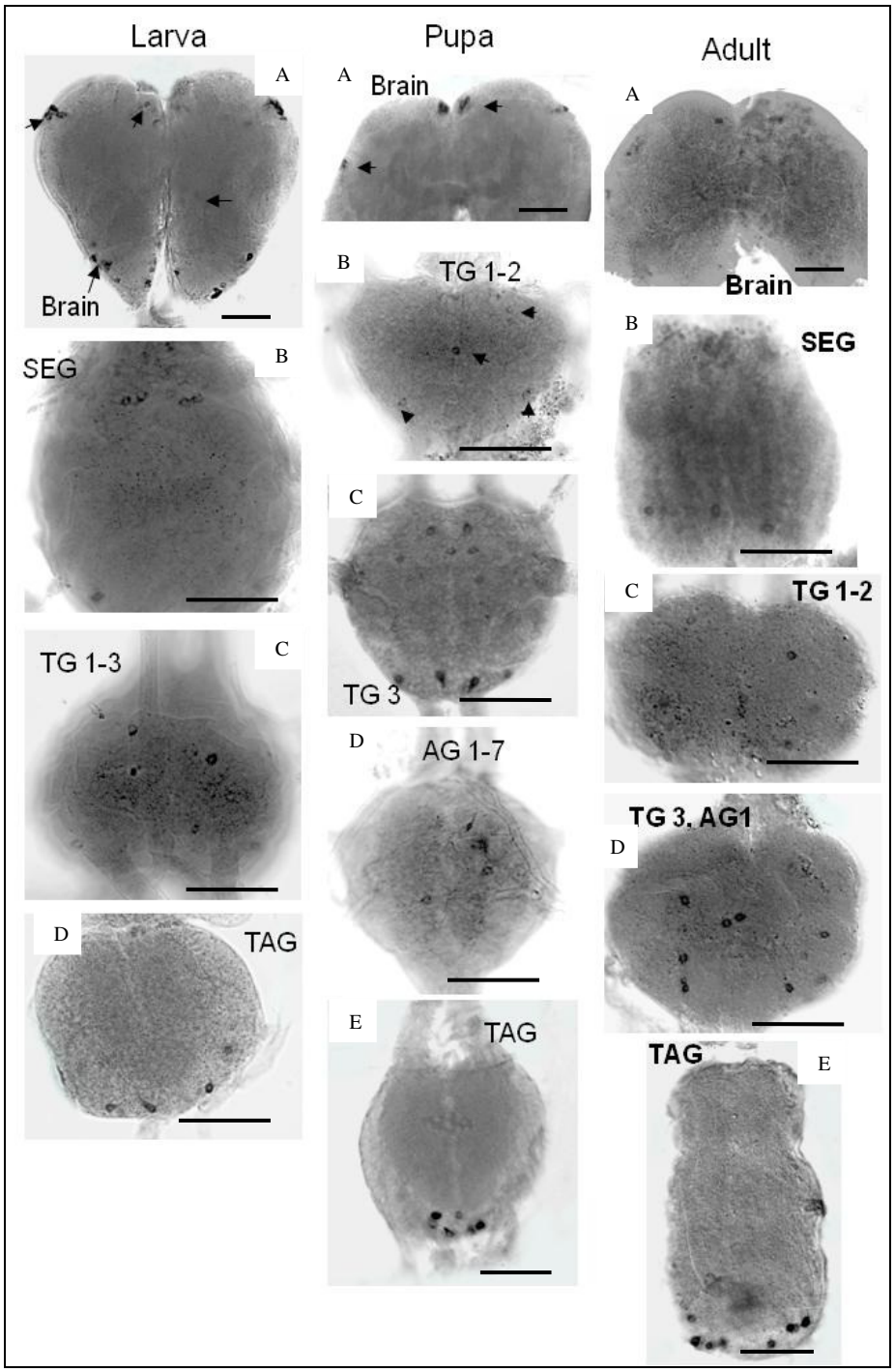


Figure 16. *In situ* hibridazation of *Tricadh47* in larva, pupa and adults of Tribolium. Arrows indicate cells with strong staining. SEG, subesophageal ganglion; TG, thoracic glanglion; AG, abdominal ganglion; TAG, terminal abdonominal ganglion. Scale bars 50µm.

Larva: A. Brain with 2 anterior laterally located cluster of cells strongly stained, 2 pairs centraly located at the anterior and posterior region and, 2 cells in each lobe at the posterior region also strongly stained; B. SEG with 2 pairs of cells strongly stained at anterior region and 1 pair at posterior region. C. TG 1-3 with 4 cells lateraly located showing strong staining; D. TAG with 3 pairs of cells strongly stained at the posterior region. **Pupa**: A. Brain with stained cells in similar position observed in larva; B. TG with 4 cells strongly stained; C. TG3 with 5 pairs of cells stronlgy stained; D. AG 1-7 showing no stained cells; E. TAG with 3 pairs of cells anteriorly located strongly stained. **Adult**: A. Brain with 2 pairs of cells strongly stained anterior centrally located; B. SEG with 2 pairs of cells stained at the posterior region of the ganglion; C. TG 1-2 with 4 pairs of stained cells; D. TG 3 and AG 1 with 5 pairs of cells strongly stained; E. TAG with 3 pairs of cells in the posterior region strongly stained.

In situ hybridization of *Tricadh31*

In addition to the staining in the central nervous system (CNS), *TricaDH31* expression was also found on the midgut and hindgut (Figure 18). The staining seems to be located at the outer layer of cells of the lumen.

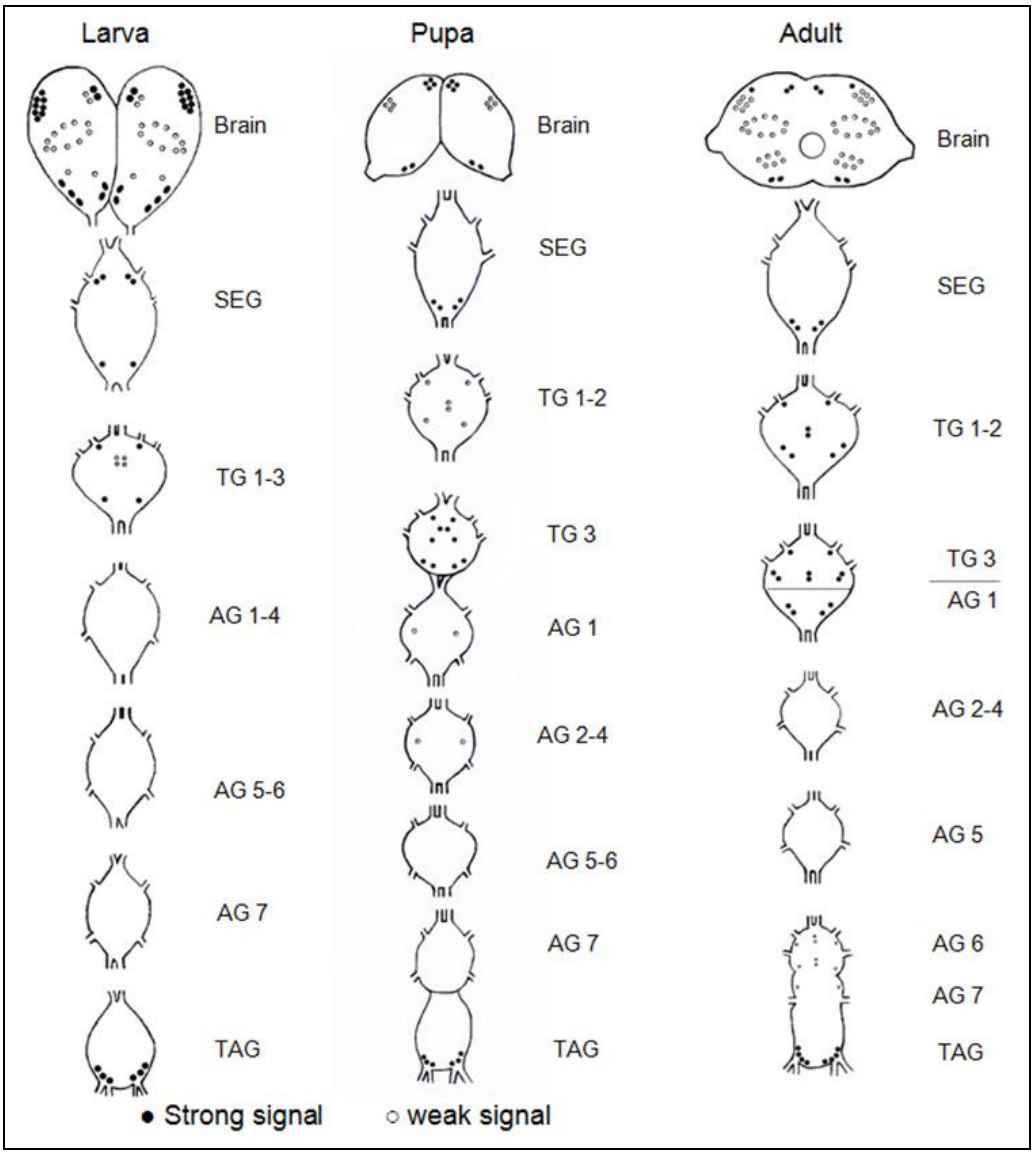


Figure 17. *In situ* hibridazation of *Tricadh47* in larva, pupa and adults of *T. castaneum*.

The adult CNS had the most cells with strong staining among all diuretic hormone genes (Figure 19 and 20). In the adult brain ten pairs of cells at the anterior part were strongly stained, however in the pupa and larval stages, the staining in those cells were not strong. No strong staining was observed on the

larval brain and just one pair of cells laterally located at the posterior part on each lobe of the brain were strongly stained in the pupal brain. In the subesophageal ganglion, abdominal ganglia 5-6 and in the terminal abdominal ganglia of larva, pupa and adults had strongly stained cells. The remaining ganglia did not show strongly stained cells in the developmental stages, staining in adult CNS were the most consistently strong (Figure 19 and 20). Abdominal ganglia 5 and 6 had one pair of cells which were strongly stained in all developmental stages. The terminal abdominal ganglion also had two pairs of cells strongly stained at all developmental stages.

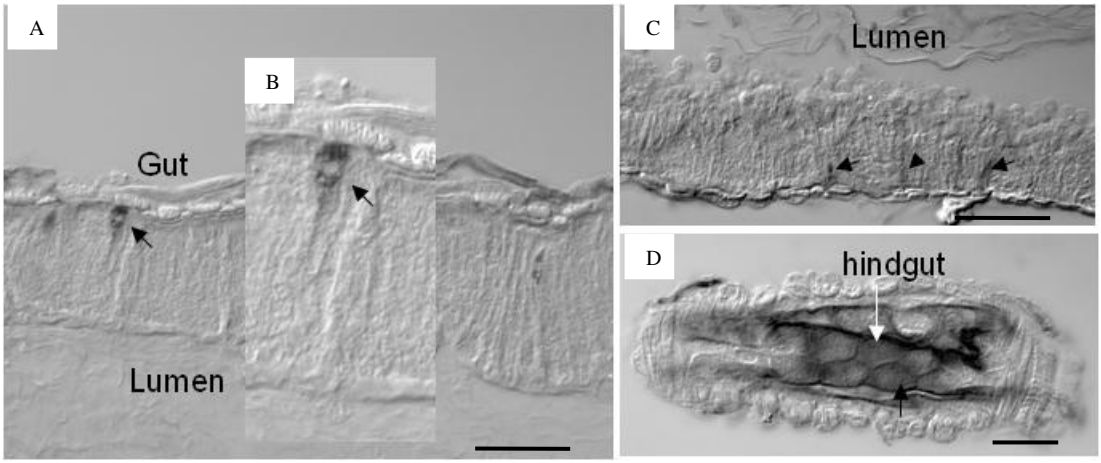


Figure 18. *In situ* hybridization of *Tricadh31* in larval gut. Arrows indicated cells stained. A. Gut; B. Detail showing staning; C. Lumen; D. Hindgut. Scale bars 50µm.

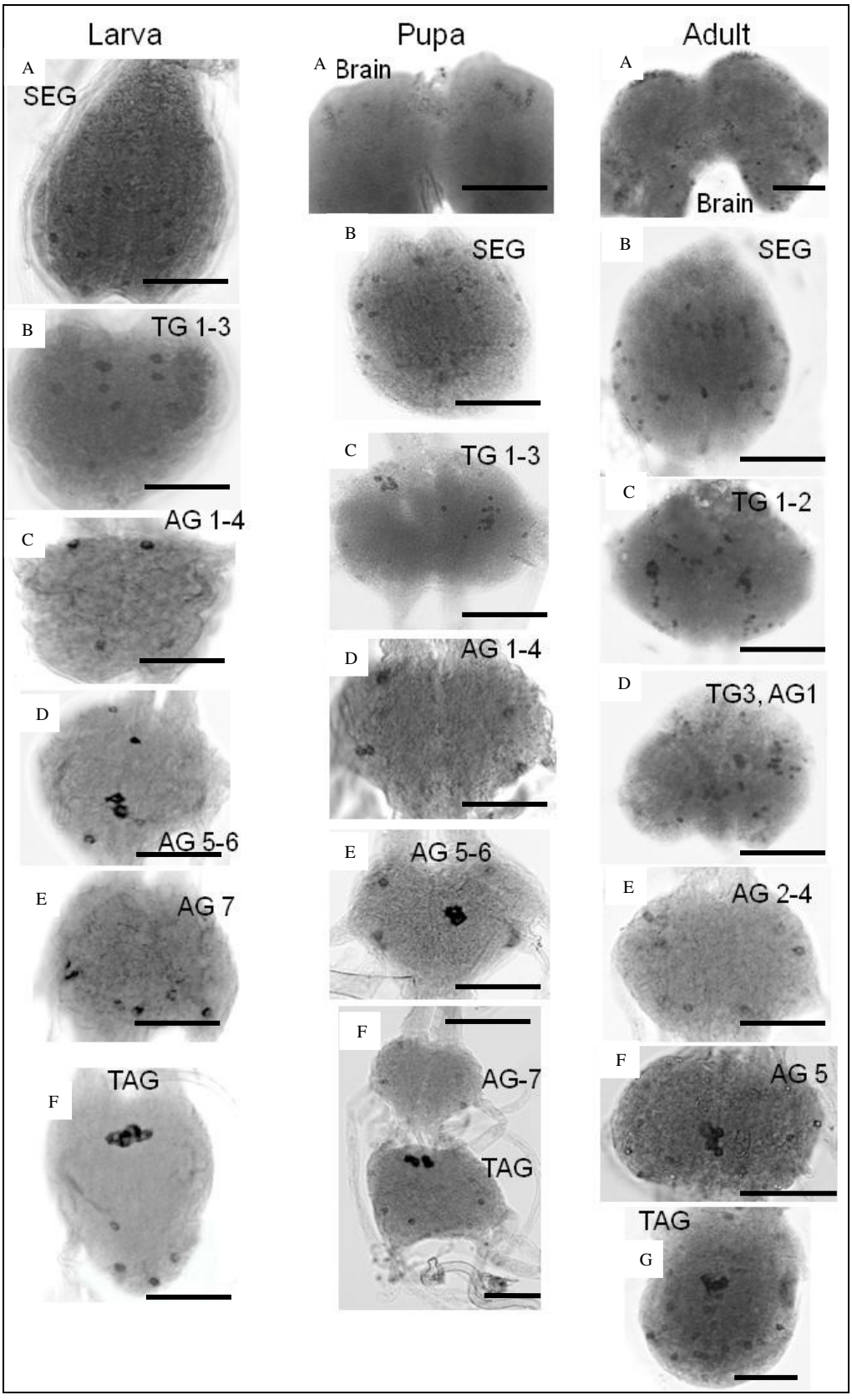

Larva
A
SEG
B
TG 1-3
C
AG 1-4
D
AG 5-6
E
AG 7
F
TAG
Pupa
A
Brain
B
SEG
C
TG 1-3
D
AG 1-4
E
AG 5-6
F
AG-7
TAG
Adult
A
Brain
B
SEG
C
TG 1-2
D
TG3, AG1
E
AG 2-4
F
AG 5
TAG
G

Figure 19. *In situ* hibridazation of *Tricadh31* in larva, pupa and adults of *T. castaneum*. SEG, subesophageal ganglion; TG, thoracic glanglion; AG, abdominal ganglion; TAG, terminal abdonominal ganglion. Scale bars 50µm. **Larva**: A. SEG with 8 pairs of cells stained; B. Represents TG 1-3 showing 5 pairs of cells strongly stained at the anterior part and 4 pairs at the posterior part; C. AG 1-4 showing 1 pair of cells stained at the posterior part; D. AG 5-6 with 1 pair of cells strongly stained at the center of the ganglion; E. AG7 with two pair of cells weakly stained at the lateral part of the ganglion; F. TAG with two pairs of cells strongly stained at the center and two pairs with normal staining at the posterior lateral part. **Pupa**: A. Brain with weakly stained cells; B. SEG with 5 cells strongly stained at both side and 1 pair at the center; C. TG 1-3 with 3 pairs of cells strongly stained; D. AG 1-4 with lateraly stained cells; E. AG 5-6 with 1 pair of cells strongly stained at the center and 4 cells lateraly located with weakly staining; E. AG7 and TAG, where AG7 shows weakly stained cells and TAG 2 pairs strongly stained. **Adult**: A. Brain with several cells strongly stained; B. SEG with 4 pairs of cells located at anterior region strongly stained and 1 cells with similar staining located at posterior region, several cells with weak staining can be seen; C. TEG 1-2 with 2 clusters of cells strongly stained; D. TEG3 and AG1 with 2 clusters of stained cells at each side and two pairs in the center; E. AG 2-4 with 10 stained cells lateraly located; F. AG5 showing 2 cells strongly

stained at the center; G. TAG with 2 pairs of cells strongly stained at the center and weakly stained cells laterally located.

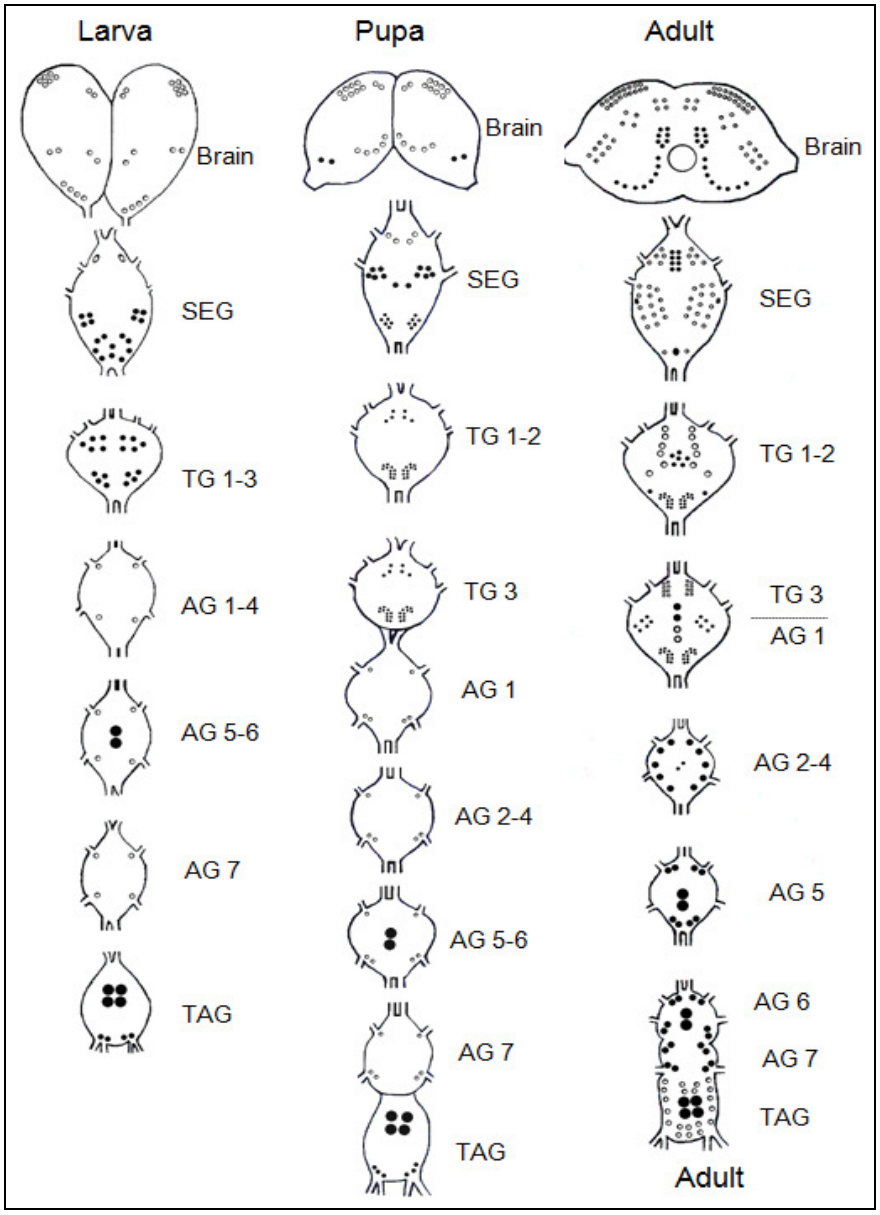


Figure 20. *In situ* hibridazation of *Tricadh31* in larva, pupa and adults of *T. castaneum*.

In situ hybridization of calcitonin-like receptors genes

Expression of both *T. castaneum* calcitonin-like receptors was detected in the Malpighian tubules. Expression of *Trica-ctr1* was detected in CNS but not in the hind gut or midgut (Figure 21) as detected with qRT-PCR. Several cells were found in the brain, but with weak staining. In the subesophageal ganglion two pairs of cells were strongly stained, whereas in the abdominal ganglia just one pair was stained. In the thoracic ganglion four pairs of cells were strongly stained.

In situ hybridization of calcitonin-like receptors genes

The CRF-like receptors were not detected in the CNS although the *Trica-crfr2* gene is highly expressed in CNS according to qRT-PCR results (Figure 21 and 11, respectively). *Trica-crfr1* staining was detectable in the hindgut. *Trica-cfr2* staining was detected in the midgut, hindgut, MT, aorta near the brain, epidermal cells and in epithelial cells of the ileum (Figure 21 and Table 2).

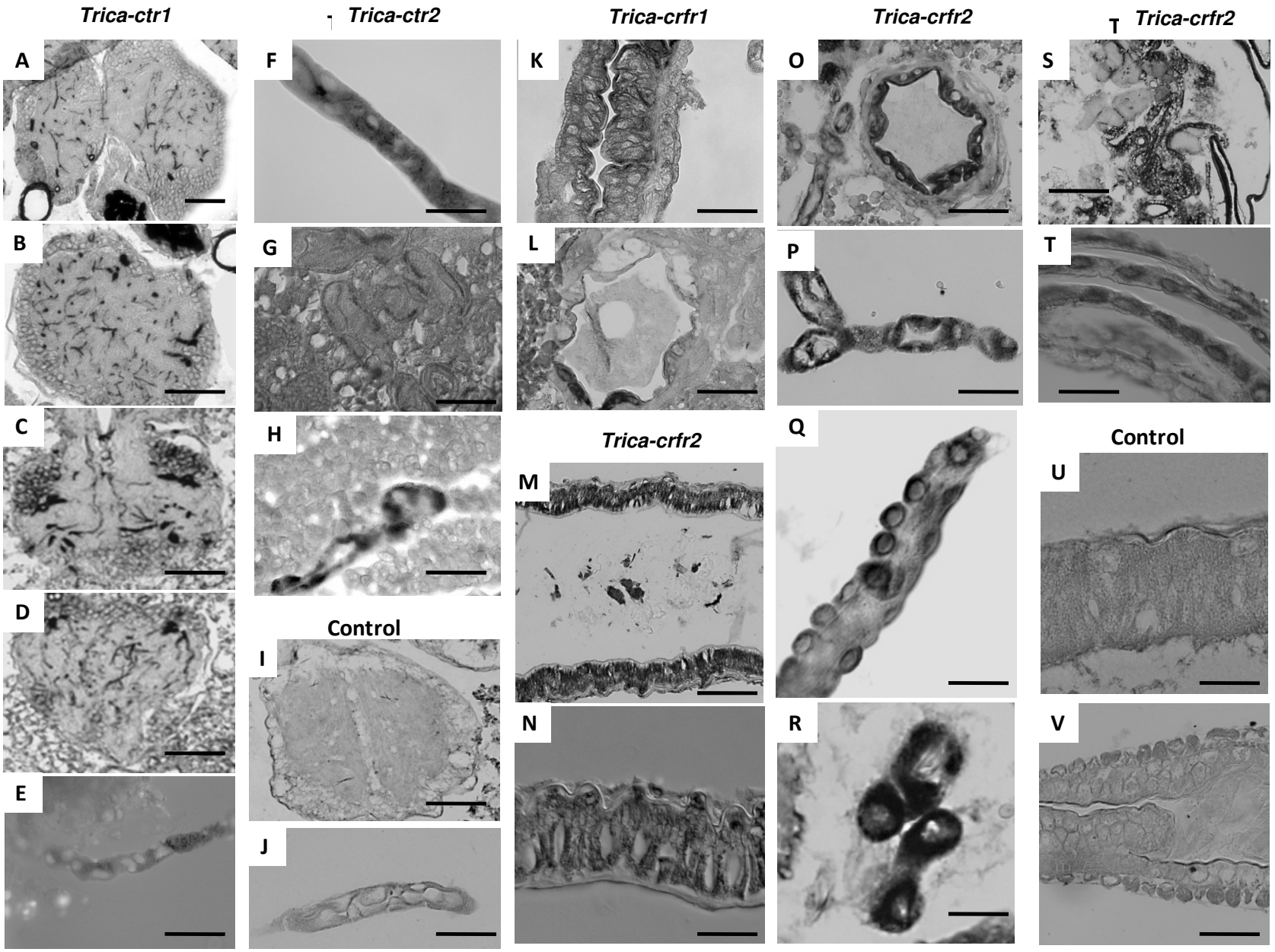

Trica-ctr1
Trica-ctr2
Trica-crfr1
Trica-crfr2
T Trica-crfr2
A
F
K
O
S
B
G
L
P
T
C
H
Trica-crfr2
Q
Control
M
U
D
Control
I
N
R
V
E
J

Figure 21. *In situ* hibridazation of diuretic hormones receptors of *T. castaneum*. A. Larval brain; B. Subesophageal ganglion; C. Terminal abdominal ganglion; D. Abdominal ganglion; E. Malpighian tubules (MT) whole mount; F. MT whole mount; G. MT section; H. MT section; I. Terminal ganglion showing no staining in control; J. MT control; K. Hindgut; L. Hindgut with higher magnification; M. Gut section; N. Midgut; O. Hindgut and MT section; P. MT whole mount; Q. Aorta near to brain whole mount; R. Aorta near brain section; S. Epithelial cells; T. Illeum and epithelial cells; U. Control midgut section; V. Control hindgut section. Scale bars 100µm A, B, C, D and I; 50 µm E, P, Q, F and J; 25 µm G, H, K, L, M, N, O, R, S, T, U, U and V.

Table 2. Expression of diuretic hormone receptors in *T. castaneum* larva.

Receptor	**Tissue**		
	CNS	**Malpighian tubules**	**Gut**
Trica-ctr1	**+**	**+**	
Trica-ctr2		**+**	
Trica-crf1			**+**
Trica-crf2			**+**

Functional Assay of *Trica-ctr1 and Trica-crfr1*

Two receptors were tested in our functional assay as described in the Methods section, however only TricaCRFR1 was successfully deorphanized. Low levels of response of TricaCTR1 to TricaDH31 was also detected and determination of the EC_{50} was not possible. TricaCRFR1 responded to TenmoDH37 ranging from 2nM to 4000nM (Figure 22). The EC_{50} of TenmoDH37 was 163.17nM and was lower than TenmoDH47 (Figure 23B). All the data was normalized to the control BSA. TricaCTR1 was also tested; however no response to TricaDH31 was detected. Interestingly this receptor responded to BommoDH31 and AnogaDH31 but the response was low and the EC_{50} could not be calculated (Figure 23A).

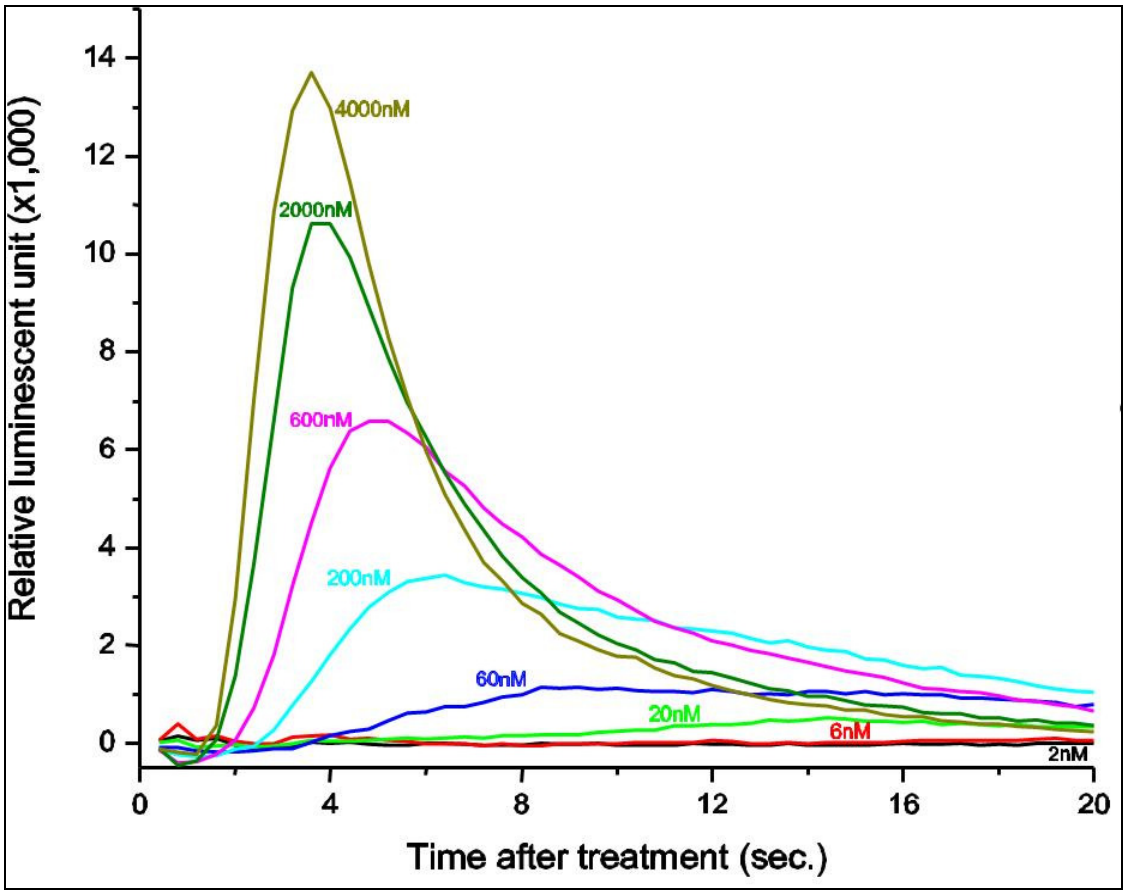


Figure 22. Luminescent reporter assay showing typical responses to varying doses of ligand TenmoDH37.

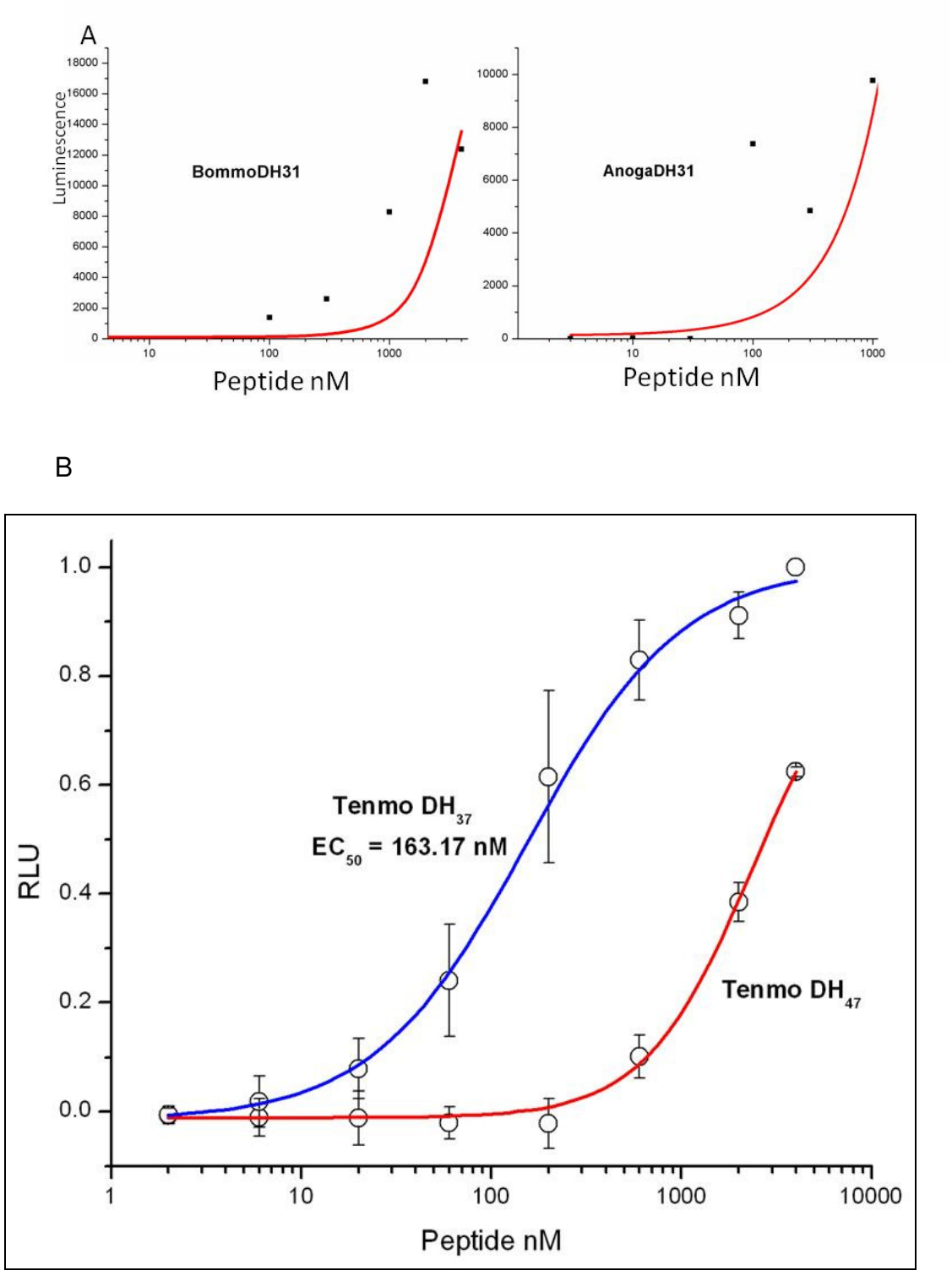


Figure 23. A. *Trica-ctr1* response to BommoDH31 and AnogaDH31, graphs represent average of 3 plates. B. Dose-responses of *Trica-crfr2* to

TenmoDH37 and TenmoDH47. Bars indicate standard error for minimum three replicated plates.

Response of *T. castaneum* to injections of diuretic hormones: chamber assay

Injections of TenmoDH47, TenmoDH37 and TricaDH31 into adult *T. castaneum* induced significant levels of immediate excretions (Figure 24). The responses were in a dose dependent manner in the range of doses that we tested. TenmoDH47 induced the higher excretions, followed by TricaDH31. TenmoDH37 induced significant excretions in concentrations at 1µM (50nL were injected at this concentration). A similar pattern was found in the percentage of response to the injections (excretions higher than the Ringer's injections (Figure 25). In the positive control, 8-Brome cAMP, 56% of the adults showed significant levels of excretion. A higher percentage of significant excretion compared to the positive control was found after injections of TenmDH47, and a lower percentage to TenmoDH37 and TricaDH31.

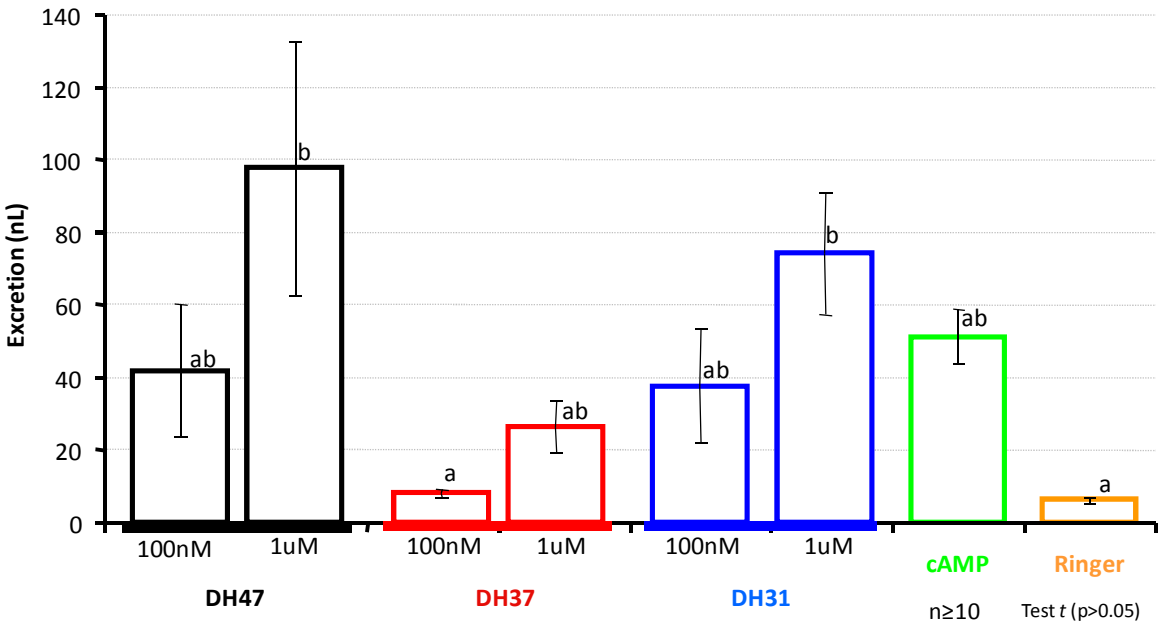


Figure 24. *In vivo* excretions (nL) induced by injections of TenmoDH47, TenmoDH37 and TricaDH31 (50nL) at different concentrations *T. castaneum* adults (10 to 15 days old). The 8-Br cAMP and Ringer's solutions were the positive and negative controls, respectively. The data were obtained for 15 minutes after the injection. Pair-wise student t-test was performed (P>0.05).

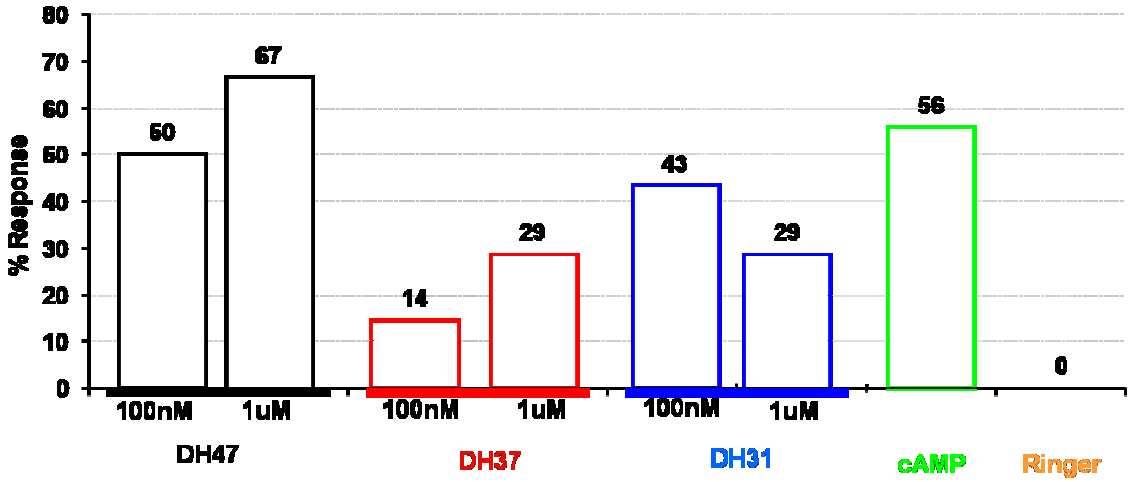


Figure 25. Percentage of response (excretion higher than negative control) of *T. castaneum* (10 to 15 days old) adults to injections of TenmoDH47, TenmoDH37 and TricaDH31 (50nL) at different concentrations. The 8-Br cAMP and Ringer's solutions were the positive and negative controls, respectively. The data were collected for 15 minutes, immediately after the injection.

The excretion patterns among insects and according to neuropeptides injected is variable. TenmDH47 usually induces one or two humidity peaks indicating the excretion (Figure 26 A, B and C). Only one excretion can occur in the first 5 minutes, one between 10 to 14 min after injection or both. The first excretion is always the highest. TricaDH31 and TenmoDH37 induced excretion mainly 5 minutes after injections (Figure 26B). TricaDH37 also induced excretions 10 minutes after injection, but they were very small when compared to excretions induced by TenmoDH47.

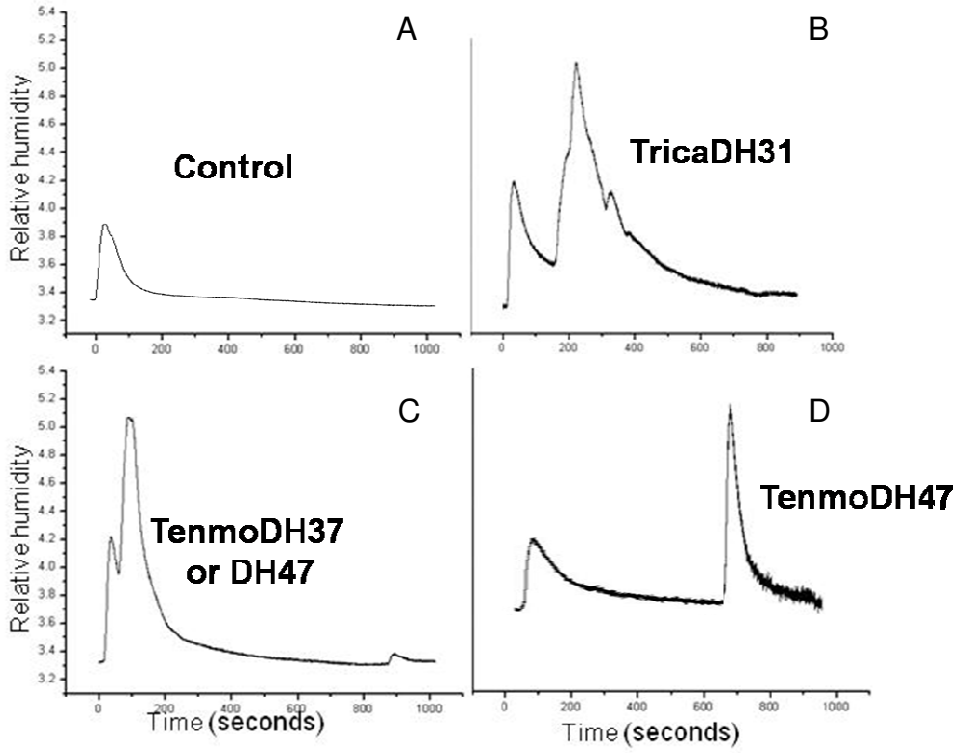


Figure 26. Types of excretions induced by diuretic hormones in *T. castaneum*. A. No response; B. High excretion 5 min after injection of the peptides. C. Two excretions peaks, one before 5 min and one between 10 and 14 min after injection; D. Only one excretion peak between 10 and 14 min after injection.

RNA interference of DH genes and their receptors

Injections of dsRNA of *Trica-crfr1* in the last larval instar increased the duration of the pupal stage (Figure 27A), however *Trica-crfr2* RNAi showed no difference from control. No treatments induced significant mortality during larval and pupal stages, while most of the mortality was observed in the adult stage. When both CRF-like genes, *Tricadh37* and *Tricadh47*, were knocked

downalmost 60% of the insects died 30 days after adult emergence (Figure 27B), which was similar to *Tricadh47* knock down alone. *Trica-crfr1*, *Trica-ctr2*, *Tricadh37* and, *Tricadh31* silencing also induced significant mortalities (~30%) from control treatment.

More than 45% of the adults show morphological defects when *Tcdh31* expression was suppressed (Figure 27C and Figure 29).The adults had deformed elytra, abdomen flatted, and with round shape. These deformed elytra appeared to have more flour attached, which may have interfered with mating as a physical barrier. Their wings would not fold correctly. The adults were also bigger than the wild type. Similar elytral defects were observed when both CRF-like genes and TricaCRFR1 were knocked down, but the body shape and size were normal (Figure 30). The number of eggs and the egg hatchability was extremely reduced when *Tcdh47* was silenced (Figure 27D and Figure 28A). When both CRF-like genes were knocked down the number of eggs oviposited was reduced but the egg hatchability was not reduced as compared to when only *Tcdh47* was knocked down (Figure 27D and Figure 28A). TricaCTR2 and TricaCRFR2 genes silencing also reduced the oviposition (Figure 27D) but not the hatchability of the eggs.

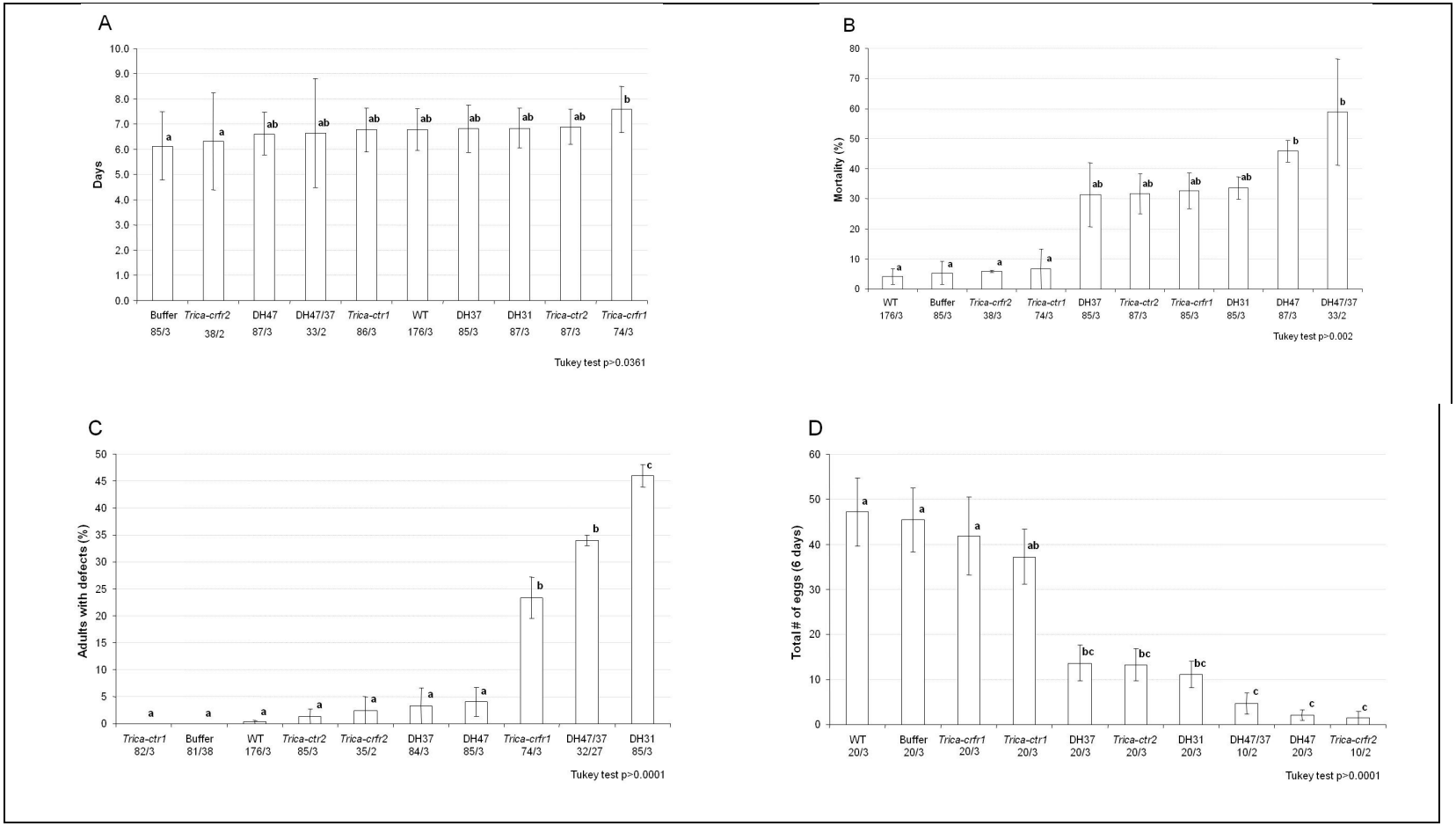


Figure 27. Effects of dsRNA injections of diuretic hormone genes and receptors in *T. castaneum*. A. Duration of pupal stage. B. Total mortality after one month of adult emergence. C. Percentage of adults that emerge with defects. D. Total number of eggs collected 6 days from one couple.

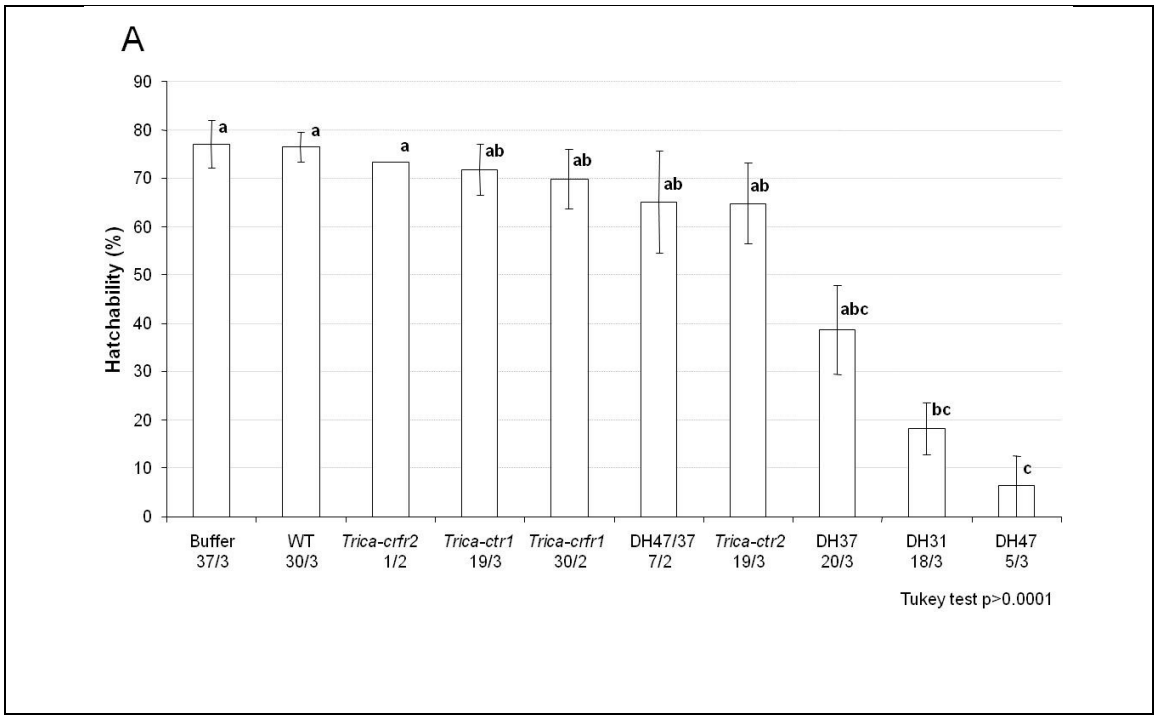


Figure 28. Effects of dsRNA injections of diuretic hormone genes and receptors on the hatchability of eggs of *T. castaneum*.

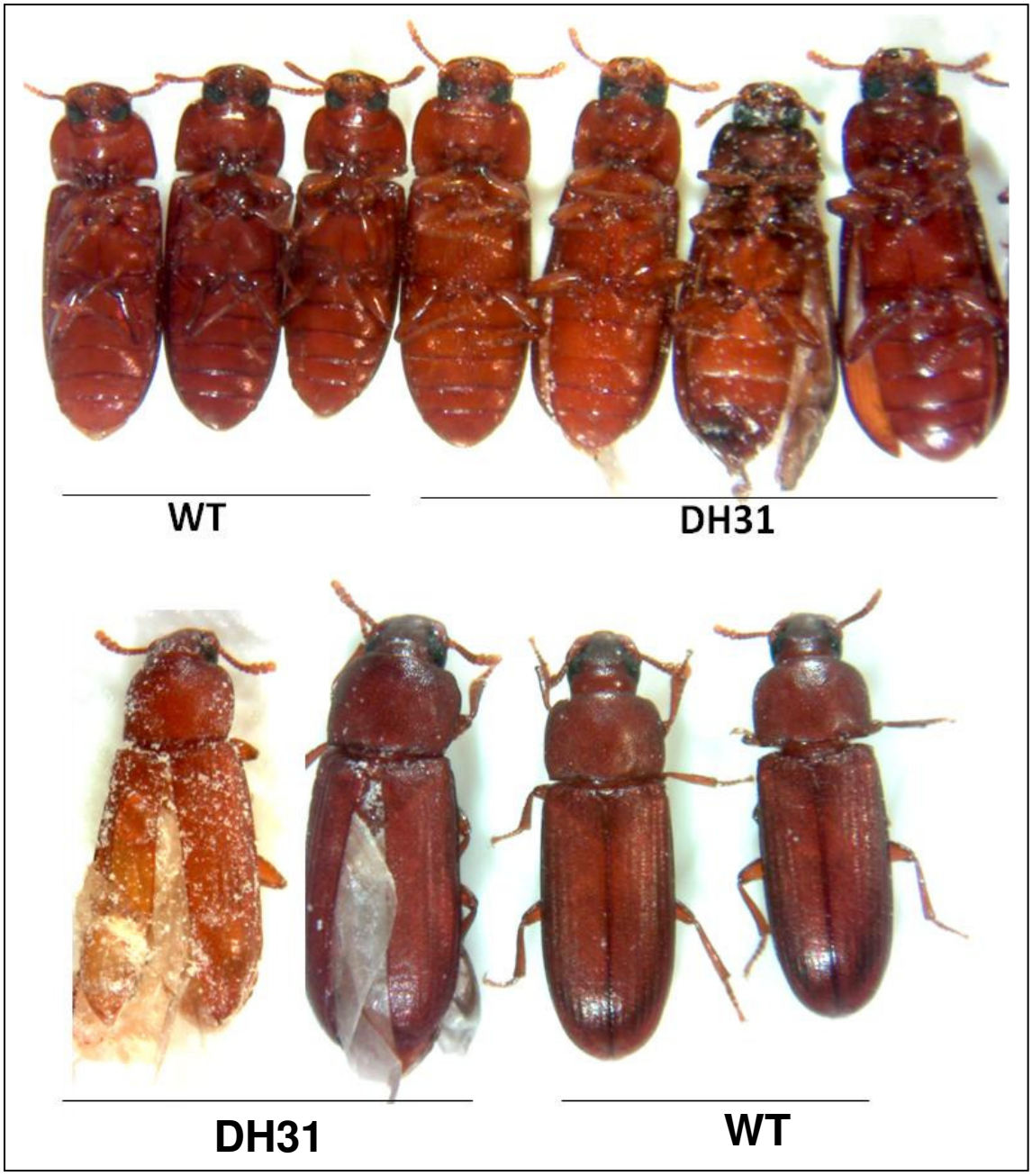


Figure 29. Morphological defects observed in adults when *Tricadh31* dsRNA was injected in the last larval instar of *T. castaneum*.

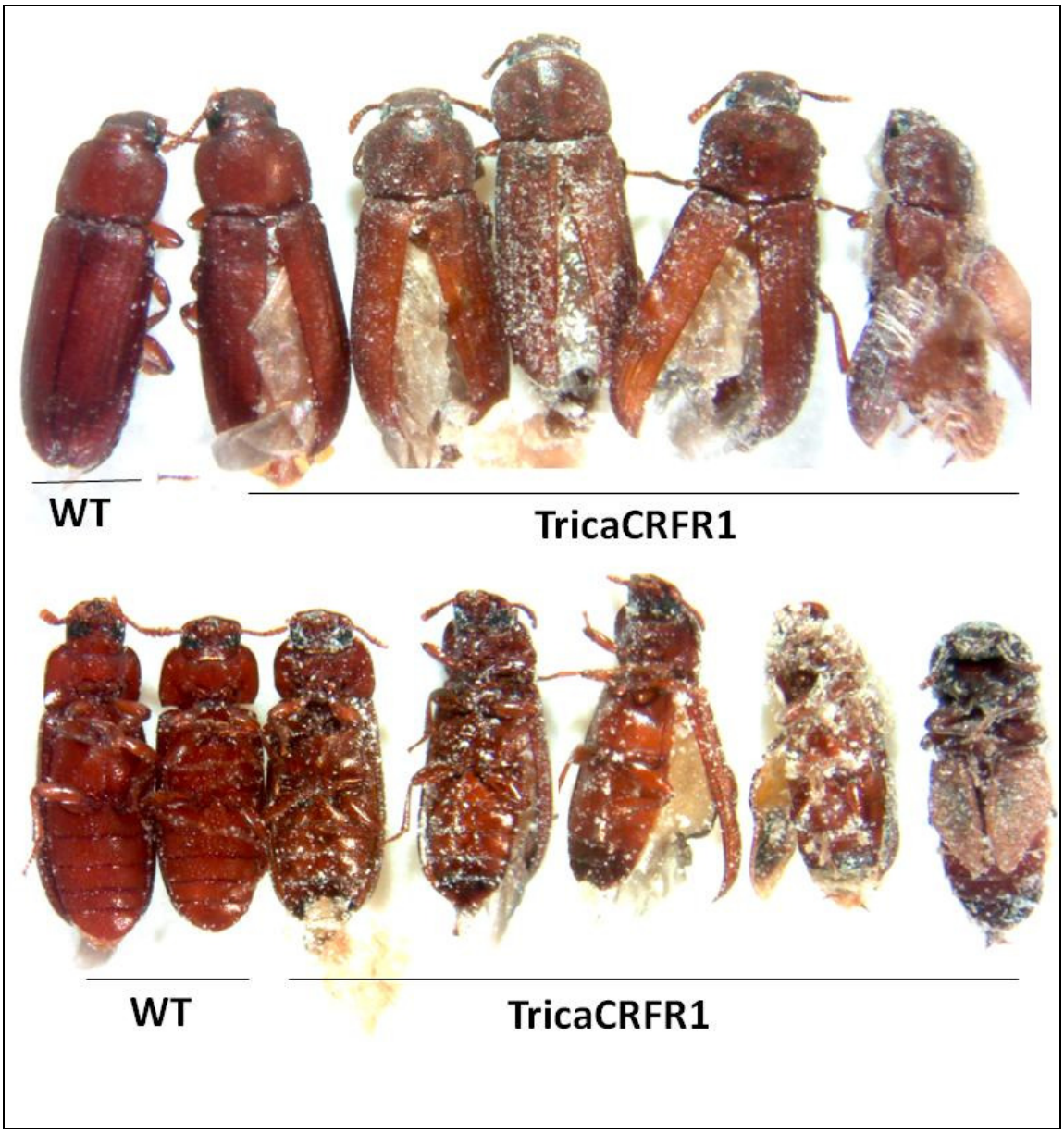


Figure 30. Morphological defects observed in adults when *Trica-crf1* dsRNA was injected in the last larval instar of *T. castaneum*.

RT-PCR was performed to check the efficiency of RNAi experiments, results are shown on the Figure 31.

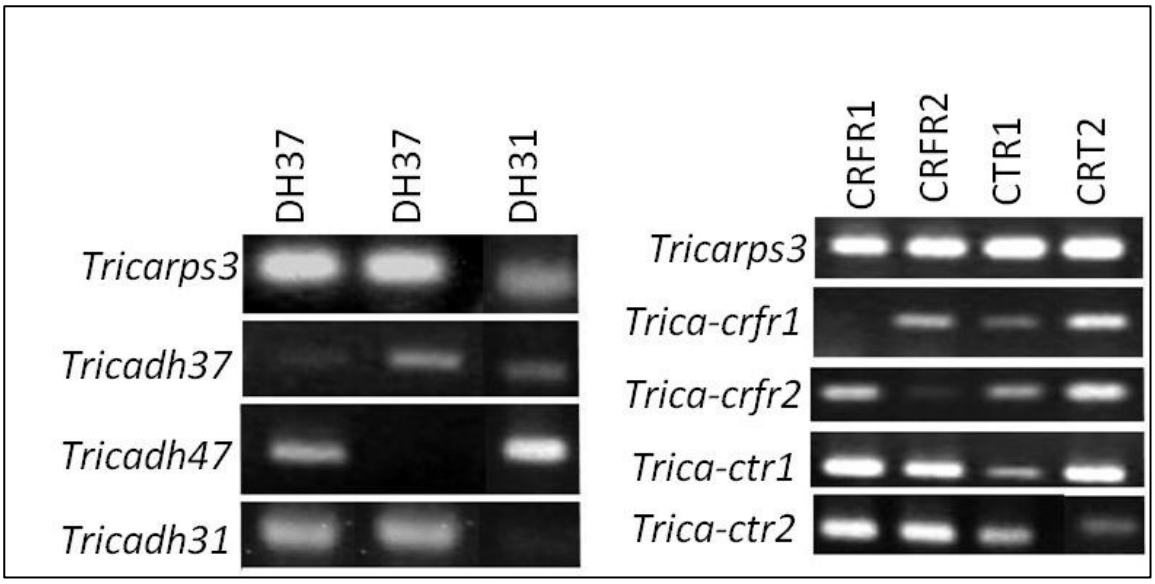


Figure 31. RT-PCR showing the suppression of mRNA levels in dsRNA-injected *T. castaneum*. Each lane contains the RT-PCR product of three individuals treated with dsRNA after 35 PCR cycles. The picture is representative of three biological replications.

Discussion

T. castaneum calcitonin-like peptide

We have shown that *T. castaneum* has three diuretic hormones, one calcitonin-like neuropeptide (TricaDH31) and two CRF-like neuropeptides (TricaDH47 and TricaDH37). TricaDH 31 is encoded by one gene and our *in vivo* assay showed that the peptide induced significant excretions in adults. In addition, semi-quantitative RT-PCR results show the presence of transcripts in all stages and tissues studied. *In situ* hybridization results indicate that *Tricadh31* is present in gut and in the entire CNS during larval, pupal and adult stages. We tried immunohistochemistry with DippuDH31 antibody (*Diploptera puntata*)

(Furuya et al., 2000) with larval, pupal and adult CNS, however we did not find any consistent staining in *T. castaneum*. Since we had superworms (*Zophobas morio*) available in the laboratory we tried immunohistochemistry with the same antibody (data not show). We found two pairs of cells strongly stained in each brain lobe. In *T. castaneum* we found similar staining in just one larval brain and did not find any other staining in different stages in our modified immunohistochemistry protocols. TricaDH31 and DippuDH31 have high sequence identity, having just two amino acid changes. This antibody was used to map DH31-like immunoreactivity in *Rhodinius prolixus* (Te Brugge et al., 2005). Immunoreaction in *R. prolixus* was found in cell bodies and processes throughout the CNS, hindgut, in the salivary inervation of 5th instar nymphs. This was similar to the immunoreactions observed in *D. punctata* (Furuya et al., 2000). Our *in situ* hybridization results show the presence of *Tricadh31* transcripts in CNS, hindgut and midgut. The staining in the gut is present in the outer layer of cells in the lumen at the heamolymph surface. The staining of CNS cells in adult stage was stronger than earlier stages and new cells revealed the presence of *Tricadh31* transcripts in abdominal ganglia. The lack of immunoreactions with anti-DippuDH31 in *T. castaneum* CNS could be influenced by several factors such as different levels of hydration or level of starvation as observed in *Manduca sexta* and *R. prolixus* (Brugge and Orchard, 2002; Chen et al., 1994; Te Brugge et al., 2005)

The calcitonin-like peptides of insects species from different orders are highly conserved, and in some cases they have identical sequences as observed

for DippuDH31 and RhoprDH31 (*R. prolixus* DH31) (Brugge and Orchard, 2008). *T. castaneum* DH31 has only two amino acid changes in comparison with DippuDH31. The peptides are conserved among species with different feeding strategies and these peptides may play a role not only in dieresis but also in feeding (Brugge and Orchard, 2008). In *R. prolixus* the immunoreactivity of DH31 is co-localized with serotonin, which is released from neurohaemal cell termini in response to feeding. It was suggested that DH31 can be released in association with serotonin or on its own, since there are also DH31 immunoreactive cells not co-localized with serotonin staining, and both may be involved in feeding and diuresis at fine levels (Brugge and Orchard, 2008). Our *in vivo* assay (or chamber assay) showed that injections of 50nL of TricaDH31 in a concentration of 100nM were able to stimulate excretions of 40nL (4x bigger than control). Interestingly, the number of beetles responding to injections of this peptide (excretions higher than those observed with Ringer's solution) was low, approximately 40%. We used adults of the same age and the experiments were performed at the same time everyday to avoid diurnal variation. However, low response to diuretic peptides can occur throughout the year and the best results were obtained from July to October, which is similar to vasopressin-like peptide (Aikins et al., 2008). There are also response variations throughout the day and the best responses were obtained between 2AM to 7PM (personal observation). Similar variability of response in *R. prolixus* to DippuDH31 has been reported (Te Brugge, 2005). The type of response is also different from CRF-like peptides. The responses to TricaDH31 were observed during the first five minutes after the injection,

whereas the CRF-peptides can induce two excretions peaks (one around 5 minutes and a small excretion 12-14 minutes after injection).

The presence and activity of the calcitonin-like DH31 have been demonstrated in several insect species (Brugge and Orchard, 2008; Coast, 2001, 2004; Furuya et al., 2000). However, RNA interference has been used to study the biological function of diuretic peptides in a few species (Aikins et al., 2008). We knocked down the expression of *Tricadh31* by the injection of 200nL of dsRNA solution in the last instar larvae. Besides the mortality observed in adult stage (30%), insect had deformed wings and abnormal body shape. The insects were able to mate, however the number of eggs laid was greatly reduced (less than one third of the wild type) and just 20% of the eggs hatched. We do not know the physiological mechanisms behind the effects observed. The morphological defects have not been reported in other species and the effects on reproduction can be related to feeding arresting. More detailed studies are needed to understand the underlying molecular functions of diuretic hormones in insects. It is clear that they are not involved in just water and ion balance.

T. castaneum CRF-like peptides

Based on computational predictions, we first thought that *T. castaneum* had two genes encoding TricaDH37 and TricaDH47. After cloning we conclude that there is just one gene, which undergoes alternative splicing. There are 4 different isoforms that can be transcribed, two for each peptide. They are expressed differently in the CNS, gut and MT and exon 1 or 2 may be promoters.

The mature peptides are also conserved in different orders; however the similarity within coleopteran orders is higher. The calcitonin-like peptides are more conserved and there are cases where peptides from species of different orders have identical sequence (Brugge and Orchard, 2008). We studied the temporal and spatial expression patterns of each isoform. Although in each stage we found at least one isoform being expressed for each CRF-like peptide (except in embryos where just one isoform of *Tricadh37* is present) the *Ttricadh37* seems to be present in more developmental stages than *Tricadh47.* All isoforms were found in the CNS, gut, Mapighian tubules (MT), carcass and hindgut. To further investigate the expression of these genes/peptides we used *in situ* hybridization and immunohistochemisty. Only antibodies raised against TenmoDH37 were available. Similarly to *D. melanogaste*r the expression of this CRF-like peptide appears to be limited to two clusters of four cells in the pars intercerebralis, however the fly has just three cells in each cluster (Cabrero et al., 2002). These cells were also detected with *in situ* hybridization. An additional cluster of four cells located bilaterally in each brain lobe was found. In *D. melanogaster* there are no DH37 neurosecretory cells in the abdominal region. However, we were able to detect neurosecretory cells in abdominal ganglia 1 through 7 and terminal abdominal ganglion. Other species including *M. sexta* (Chen et al., 1994), *Locusta migratoria* (Johnson et al., 2005) and the bug *Rhodnius prolixus* (Brugge et al., 2001) also have abdominal neuroendocrine cells. It seems that only the order Diptera does not have this neurosecrotory cells in the abdomen. In *L. migratoria*, in addition to CRF-like peptides, the abdominal neuroendocrine cells

also express leucokinins and these peptides act synergistically (Thompson et al., 1995). Unfortunately, it is not know where leucokinins are expressed in *T. castaneum.* Similar to *D. melanogaster* (Cabrero et al., 2002) the number of neuroendocrine cells espressing DH37 in *T. castaneum* does not increase from larval, pupal and adult stages, but does increase in *M. sexta* (Veenstra and Hagedorn, 1991). We also found immunoreactive cells inervations of TricaDH37 in the midgut, hindgut and cryptonephridial complex; however we did not found any staining of *Tricadh37* transcripts using *in situ* hybridization. The peptide might be produced elsewhere, secreted, and then transported to these organs where it performs its biological functions.

In situ hybridization results revealed that *Tricadh47* is expressed in several cells in the brain in all developmental stages, however the number of cells stained in each stage is significantly different. During the pupal stage the number of cells expressing *Tricadh47* is dramatically reduced in the brain, but the number of cells expressing this gene in other ganglia is constant during development. Based on the numbers of cells and the intensity of the staining we can speculate that *Tricadh47* is less expressed in brain during pupal stage, whereas *Tricadh37* is continuous expressed during development and *Tricadh31* is more highly expressed in adult brains. However, we do not know the physiological concentrations of each peptide in the insect blood and how active they are. Our chamber assay showed that the same concentrations of TenmoDH37, TenmoDH47 and TricaDH31 induced different patterns of excretions. TenmoDH47 induced higher levels of excretions, followed by

TricaDH31 and TenmoDH37. However, the low response to TenmoDH37 could be due low cross reactivity. TenmoDH37 is the only CRF-like peptide to have the C-terminus non-amidated (Furuya et al., 1998), while in *T. castaneum* it is amidated. In the dung beetle *Onthophagus gazelle*, TenmoDH37 had more than 1000 times lower activity than ManseDH41 in elevating cyclic AMP in Malpighian tubules (Holtzhausen and Nicolson, 2007). However, the complete *L. migratoria* and *D. punctata* CRF-like peptides are able to stimulate similar secretion rates of TenmoDH37 in *Tenebrio molitor* (Wiehart et al., 2002a; Wiehart et al., 2002b). We also observed that CRF-like peptides are able to induce one or two excretions in *T. castaneum.* Usually 5 minutes after injection one excretion peak occurs and another one occurs 12 to 14 minutes after the injection. However, the first excretion is always higher than the second one.

Using exon specific RNAi, we were able to knock down *Tricadh37*, *Tricadh47* or both. The highest mortality occurred when *Tcdh47* or both CRF-like forms were silenced (50 to 60% of the adults died 30 days after adult emergence). *Tricadh37* silencing caused similar mortality to *Tcdh31* RNAi (approximately 35%). Only when both CRF-like isoforms were knock down almost 35% of the adults showed similar morphological defects to those observed when *Tricadh31* was silenced. The adults of *Tricadh31* knock down were bigger than the wild type, showing that this gene is clearly involved in development. The number of eggs produced was also lower when CRF-like genes were knocked down, however, as observed with the mortality rates of CRF-like genes silencing, RNAi of *Tricadh47* or both CRF-like reduced the

oviposition (less than 10 eggs per female in 6 days) more than when *Tricadh37* alone was silenced alone (15 eggs per female in 6 days). The wild type females laid approximately 48 eggs per six days. Interestingly, the hatchability was also more reduced by RNAi of *Tricadh47* or both genes than the RNAi of *Tricadh37.* We do not know if the *T. castaneum* CRF-like peptides act synergistically or additively with each other or with the calcitonin-like peptides, vasopressin-like peptides or kinins. In *L. migratoria* it was show that LocmiDH (a CRF-like peptide) acts synergistically with DippuDH31, whereas DippuDH46 acts additively with DippuDH31 (Furuya et al., 2000). However, most studies focus only on MT excretion or the secondary messenger used by the diuretic peptides. Our RNAi results show that CRF-like genes might be involved in other physiological processes in addition to diuresis, since the larva developed to adult stages without problems where dieresis is highly important. The morphological defects observed only when both CRF-like genes where silenced suggest that they have overlapping functions in the pupa during metamorphosis. However, the effects of *Tricadh37* RNAi are less severe than the combination of either CRF-like genes or *Tricadh47* alone. We do not know if the insect had reduced feeding which resulted in lower oviposition and egg viability or if the CRF-like genes play important roles in reproduction. It has been suggested that diuretic hormones can interfere with insect feeding strategies (Brugge and Orchard, 2008).

T. castaneum diuretic hormone receptors

To further investigate the biological and diuretic hormones in *T. castaneum* we cloned the predicted CRF-like (*Trica-crfr1* and *Trica-crfr2* genes) and calcitonin-like (*Trica-ctr1* and *Trica-ctr2* genes) receptors and studied their expression pattern, ligand-receptor relationships and used RNAi to study their biological function. Our phylogenetic analysis showed that *T. castaneum* calcitonin-like receptors 1 and 2 are well clusted with Diptera receptors 1 and 2 (Figure 10). However, the clustering of CRF-like receptors is not as clear as the calcitonin-like receptors. Using qRT-PCR the diuretic hormone receptors transcripts were detected in all developmental stages, in CNS, MT, gut and hindgut. Only *Trica-crfr1* was not detectable in hindgut. *In situ* hybridization results showed the presence of *Trica-ctr1* gene only in CNS and hindgut and *Trica-ctr2* in MT. CRF-like receptors' staining was only found in hindgut. These differences in expression profiles could be attributed to low sensitivity of *in situ* hybridization, since these receptors have an usually low copy numbers. In *D. melanogaster* both calcitonin and CRF-like receptors are expressed in the larval CNS and their expression is co-localized with corazonin (Johnson et al., 2005). In this insect DH44 and DH31 receptors are suggested to inhibit corazonin release. Since all neurons expressing corazonin in *D. melanogaster* also express both diuretic hormone receptors, it is suggested that there is a closer association between corazonin signaling and upstream regulation by the convergence of DH31 and DH44 signaling pathways. DH receptor signaling in *D. melanogaster* seems to be involved in corazonin release. Unfortunately, in *T. castaneum,* little

is known about the expression or co-expression and localization of neuropetides in the CNS and peripheral nerves. It is not known if corazonin is also involved in diuresis; this is a multi-functional peptide involved in the cardioactivity in *Periplaneta americana* (Veenstra, 1989). It is also involved in ecdysis initiation (Kim et al., 2004). In *Aedes aegypti,* two CRF-like receptors are also expressed in the CNS and MT (Jagge and Pietrantonio, 2008). One of them (named AegeGPRDIH1) has expression changes paralleling mosquito excretions after blood feeding. In *Nilaparvata lugens,* immunoreactivity of one diuretic hormone receptor was found only in MT, on the outer layer of cells in contact with the hemolymph. In *T. castaneum,* we found expression of calcitonin-like peptides and receptors in CNS, MT and gut. We can speculate that the expression of the receptors in CNS indicates unknown functions of these receptors. They may be involved in secretion of other peptides in *T. castaneum* as occur in *D. melanogaster* where corazonin release is controlled by this receptors (Johnson et al., 2005), but more detailed studies are needed. The CRF-receptors may also be involved in other physiological process rather than dieresis. We found staining in the aorta neat to brain, in the MT and gut for *Trica-crfr1* gene. It is not known if diuretic hormone receptors are involved in heart beating or play some role in AKH pathway.

We tried to functionally characterize *T. castaneum* diuretic hormone receptors using heterologous expression of the receptors in CHO cells. TricaCRFR2 responded to concentrations of TenmoDH37 ranging from 2nM to 4000nM. The EC50 of TenmoDH37 was 163.17nM and was lower than

TenmoDH47. TricaCTR1 also responded to BommoDH31 and AnogaDH31, however the doses needed for receptors sensibilization were high and we could not calculate the EC_{50}.

Finally, to study the biological roles of *T. castaneum* diuretic hormone receptors we used RNAi. Primers to synthesize dsRNA were designed from regions of low similarity among the receptors. The injections were done in the last larval instar and evaluations were performed for 30 days after adult emergence. Only *Trica-crfr1* RNAi increase the duration of the pupal stage by 1 day.and approximately 23% of the adults had deformed wings and mortality in the adult stage was significantly higher than in the control (30%). *Trica-crfr2* RNAi did not cause this effect but inhibited the oviposition almost completely. *Trica-ctr2* RNAi also caused similar mortalities and reduction in the number of eggs laid by each female. The effects on the percent egg hatching were small when compared to RNAi of *Tricadh31*, the putative ligand of this receptor. These receptors are expressed differently throughout insect development and tissues and may play unknown roles in the insect physiology. This also occurs with the ligands, which are expressed differently during development and in different tissues. A considerable amount of work is needed to understand these diuretic hormones and its receptors regulatory pathways. This scenario seems to be even more complicated when other diuretic peptides such as vasopressin-like and anti-diuretic factors are taken in consideration.

Conclusions

We have identified and cloned one calcitonin-like peptide and two calcitonin-like receptors in *T. castaneum*. This peptide is expressed in all developmental stages and in CNS, MT and gut. The synthetic peptide TricaDH31 have also shown to be biologically active by inducing significant levels of excretions in adult beetles. When *tricadh31* was silenced using RNAi, adults had deformed wings and abnormal body shape. The mortality in adult stage was high, the number of eggs laid was reduced as well as the hatchability of the eggs. The calcitonin-like receptors are also expressed in all developmental stages, CNS and MT. RNAi with the receptor genes revealed that only *Trica-ctr2* silencing caused significant mortalities and reduction in the number of eggs laid. Therefore, either calcitonin-like ligand or receptors are both vital to normal development in *T. castaneum*.

There are two biologically active CRF-like peptides in *T. castaneum.* These peptides are encoded by one gene which undergoes alternative splicing. The gene has 5 exons and 4 isoforms can be transcribed. We used exon specific RNAi to silence each or both CRF-like genes. When *Tricadh47* was knocked down, high mortality occurred as well as low oviposition and egg hatchability. Similar effects were observed with silencing of both CRF-like genes. However, RNAi of *Tricadh37* transcripts had similar and less severe effects. Adults also had deformed wings when both CRF-like genes were silenced but not when just one of them was knocked down. These results indicate that CRF-like genes could have different biological functions in addiction to their role in dieresis. We

also tested the *in vivo* activity of these peptides. TenmoDH47 induced high excretions in adults, whereas TenmoDH37 induces smaller excretions. The receptors of CRF-like peptides were also identified and cloned in this studied. There are two receptors which are expressed in all developmental stages. Adults also had deformed wings and laid fewer eggs after RNAi of *Trica-crfr1*. RNAi of *Trica-crf2* also caused significant mortalities. These peptides and receptors seem to have a role in fine tuning beetle physiology and may have additional functions not yet known.

References

Aikins MJ, Schooley DA, Begum K, Detheux M, Beeman RW, Park Y. 2008. Vasopressin-like peptide and its receptor function in an indirect diuretic signaling pathway in the red flour beetle. Insect Biochemistry and Molecular Biology 38(7):740-748.

Altstein M. 2001. Insect neuropeptide antagonists. Biopolymers 60(6):460-473.

Beyenbach KW. 1995. Mechanism and Regulation of Electrolyte Transport in Malpighian Tubules. Journal of Insect Physiology 41(3):197-207.

Beyenbach KW. 2003. Transport mechanisms of diuresis in Malpighian tubules of insects. Journal of Experimental Biology 206(21):3845-3856.

Brown SJ, Denell RE, Beeman RW. 2003. Beetling around the genome. Genetical Research 82(3):155-161.

Brugge VAT, Nassel DR, Coast GM, Schooley DA, Orchard I. 2001. The distribution of a kinin-like peptide and its co-localization with a CRF-like

peptide in the blood-feeding bug, Rhodnius prolixus. Peptides 22(2):161-173.

Brugge VAT, Orchard I. 2002. Evidence for CRF-like and kinin-like peptides as neurohormones in the blood-feeding bug, Rhodnius prolixus. Peptides 23(11):1967-1979.

Brugge VAT, Orchard I. 2008. Distribution and activity of a Dippu DH31-like peptide in the large milkweed bug Oncopeltus fasciatus. Peptides 29(2):206-213.

Cabrero P, Radford JC, Broderick KE, Costes L, Veenstra JA, Spana EP, Davies SA, Dow JAT. 2002. The Dh gene of Drosophila melanogaster encodes a diuretic peptide that acts through cyclic AMP. Journal of Experimental Biology 205(24):3799-3807.

Catania F, Kauer MO, Daborn PJ, Yen JL, Ffrench-Constant RH, Schlotterer C. 2004. World-wide survey of an Accord insertion and its association with DDT resistance in Drosophila melanogaster. Molecular Ecology 13(8):2491-2504.

Chen YT, Veenstra JA, Hagedorn H, Davis NT. 1994. Leucokinin and Diuretic Hormone Immunoreactivity of Neurons in the Tobacco Hornworm, Manduca-Sexta, and Colocalization of This Immunoreactivity in Lateral Neurosecretory-Cells of Abdominal Ganglia. Cell and Tissue Research 278(3):493-507.

Coast GM. 1996. Neuropeptides implicated in the control of diuresis in insects. Peptides 17(2):327-336.

Coast GM. 1998. Insect diuretic peptides: Structures, evolution and actions. American Zoologist 38(3):442-449.

Coast GM. 2001. Diuresis in the housefly (Musca domestica) and its control by neuropeptides. Peptides 22(2):153-160.

Coast GM. 2004. Continuous recording of excretory water loss from Musca domestica using a flow-through humidity meter: hormonal control of diuresis. Journal of Insect Physiology 50(5):455-468.

Coast GM, Hayes TK, Kay I, Chung JS. 1992. Effect of Manduca-Sexta Diuretic Hormone and Related Peptides on Isolated Malpighian Tubules of the House Cricket Acheta-Domesticus (L). Journal of Experimental Biology 162:331-338.

Coast GM, Webster SG, Schegg KM, Tobe SS, Schooley DA. 2001. The Drosophila melanogaster homologue of an insect calcitonin-like diuretic peptide stimulates V-ATPase activity in fruit fly Malpighian tubules. Journal of Experimental Biology 204(10):1795-1804.

Dow JAT, Davies SA. 2006. The Malpighian tubule: Rapid insights from post-genomic biology. Journal of Insect Physiology 52(4):365-378.

Enayati AA, Ranson H, Hemingway J. 2005. Insect glutathione transferases and insecticide resistance. Insect Molecular Biology 14(1):3-8.

Furuya K, Harper MA, Schegg KM, Schooley DA. 2000. Isolation and characterization of CRF-related diuretic hormones from the whitelined sphinx moth Hyles lineata. Insect Biochemistry and Molecular Biology 30(2):127-133.

Furuya K, Schegg KM, Schooley DA. 1998. Isolation and identification of a second diuretic hormone from Tenebrio molitor. Peptides 19(4):619-626.

Gade G. 2004. Regulation of intermediary metabolism and water balance of insects by neuropeptides. Annual Review of Entomology 49:93-113.

Gade G, Goldsworthy GJ. 2003. Insect peptide hormones: a selective review of their physiology and potential application for pest control. Pest Management Science 59(10):1063-1075.

Gade G, Hoffmann KH, Spring JH. 1997. Hormonal regulation in insects: Facts, gaps, and future directions. Physiological Reviews 77(4):963-1032.

Hauser F, Cazzamali G, Williamson M, Blenau W, Grimmelikhuijzen CJP. 2006. A review of neurohormone GPCRs present in the fruitfly Drosophila melanogaster and the honey bee Apis mellifera. Progress in Neurobiology 80(1):1-19.

Hoffmann KH, Lorenz MW. 1998. Recent advances in hormones in insect pest control. Phytoparasitica 26(4):323-330.

Holman GM, Nachman RJ, Schoofs L, Hayes TK, Wright MS, Deloof A. 1991. The Leucophaea-Maderae Hindgut Preparation - a Rapid and Sensitive Bioassay Tool for the Isolation of Insect Myotropins of Other Insect Species. Insect Biochemistry 21(1):107-112.

Holtzhausen WD, Nicolson SW. 2007. Beetle diuretic peptides: The response of mealworm (Tenebrio molitor) Malpighian tubules to synthetic peptides, and cross-reactivity studies with a dung beetle (Onthophagus gazella). Journal of Insect Physiology 53(4):361-369.

Jagge CL, Pietrantonio PV. 2008. Diuretic hormone 44 receptor in Malpighian tubules of the mosquito Aedes aegypti: evidence for transcriptional regulation paralleling urination. Insect Molecular Biology 17(4):413-426.

Johnson EC, Shafer OT, Trigg JS, Park J, Schooley DA, Dow JA, Taghert PH. 2005. A novel diuretic hormone receptor in Drosophila: evidence for conservation of CGRP signaling. Journal of Experimental Biology 208(7):1239-1246.

Li B, Predel R, Neupert S, Hauser F, Tanaka Y, Cazzamali G, Williamson M, Arakane Y, Verleyen P, Schoofs L and others. 2008. Genomics, transcriptomics, and peptidomics of neuropeptides and protein hormones in the red flour beetle Tribolium castaneum. Genome Research 18(1):113-122.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25(4):402-408.

Lorenzen MD, Doyungan Z, Savard J, Snow K, Crumly LR, Shippy TD, Stuart JJ, Brown SJ, Beeman RW. 2005. Genetic linkage maps of the red hour beetle, Tribolium castaneum, based on bacterial artificial chromosomes and expressed sequence tags. Genetics 170(2):741-747.

Masler EP, Kelly TJ, Menn JJ. 1993. Insect Neuropeptides - Discovery and Application in Insect Management. Archives of Insect Biochemistry and Physiology 22(1-2):87-111.

McGettigan J, McLennan RKJ, Broderick KE, Kean L, Allan AK, Cabrero P, Regulski MR, Pollock VP, Gould GW, Davies SA and others. 2005. Insect renal tubules constitute a cell-autonomous immune system that protects the organism against bacterial infection. Insect Biochemistry and Molecular Biology 35(7):741-754.

Nicolson S. 1992. Excretory Function in Tenebrio-Molitor - Fast Tubular Secretion in a Vapor-Absorbing Insect. Journal of Insect Physiology 38(2):139-146.

Park Y, Kim YJ, Dupriez V, Adams ME. 2003. Two subtypes of ecdysis-triggering hormone receptor in Drosophila melanogaster. Journal of Biological Chemistry 278(20):17710-17715.

Proux JP, Miller CA, Li JP, Carney RL, Girardie A, Delaage M, Schooley DA. 1987. Identification of an Arginine Vasopressin-Like Diuretic Hormone from Locusta-Migratoria. Biochemical and Biophysical Research Communications 149(1):180-186.

Ramsay JA. 1952. The Excretion of Sodium and Potassium by the Malpighian Tubules of Rhodnius. Journal of Experimental Biology 29(1):110-126.

Ramsay JA. 1954. Active Transport of Water by the Malpighian Tubules of the Stick Insect, Dixippus-Morosus (Orthoptera, Phasmidae). Journal of Experimental Biology 31(1):104-113.

Reagan JD. 1994. Expression Cloning of an Insect Diuretic Hormone-Receptor - a Member of the Calcitonin Secretin Receptor Family. Journal of Biological Chemistry 269(1):9-12.

Reagan JD, Patel BC, Li JP, Miller WH. 1994. Characterization of a Solubilized Diuretic Hormone-Receptor from the Tobacco Hornworm, Manduca-Sexta. Insect Biochemistry and Molecular Biology 24(6):569-572.

Swofford DL. 1993. Paup - a Computer-Program for Phylogenetic Inference Using Maximum Parsimony. Journal of General Physiology 102(6):A9-A9.

Te Brugge VA, Lombardi VC, Schooley DA, Orchard I. 2005. Presence and activity of a Dippu-DH31-like peptide in the blood-feeding bug, Rhodnius prolixus. Peptides 26(1):29-42.

Tobe SS, Zhang JR, Schooley DA, Coast GM. 2005. A study of signal transduction for the two diuretic peptides of Diploptera punctata. Peptides 26(1):89-98.

Veenstra JA. 1989. Isolation and Structure of Corazonin, a Cardioactive Peptide from the American Cockroach. Febs Letters 250(2):231-234.

Veenstra JA, Hagedorn HH. 1991. Identification of Neuroendocrine Cells Producing a Diuretic Hormone in the Tobacco Hornworm Moth, Manduca-Sexta. Cell and Tissue Research 266(2):359-364.

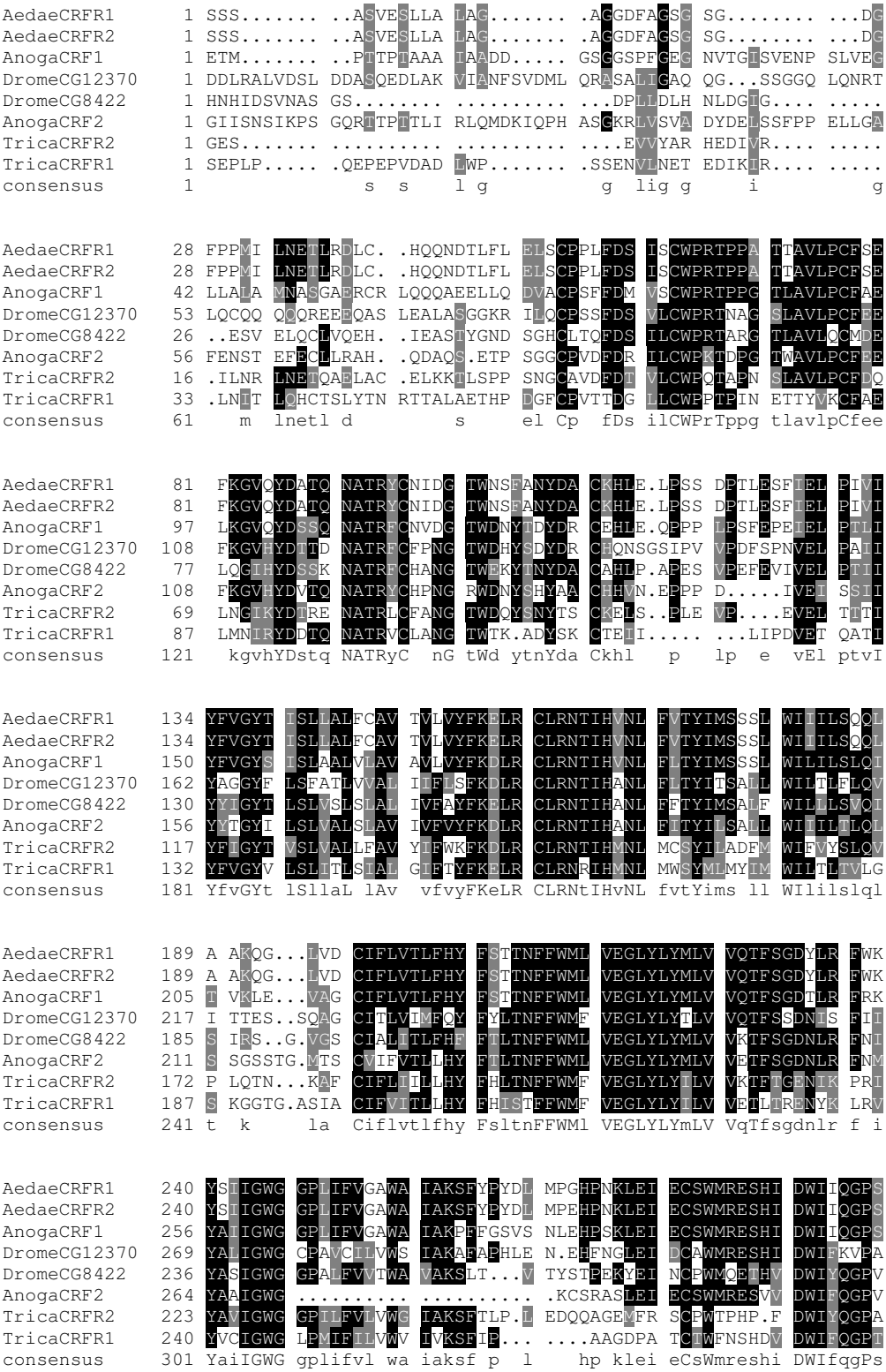
Wiehart UIM, Nicolson SW, Eigenheer RA, Schooley DA. 2002a. Antagonistic control of fluid secretion by the Malpighian tubules of Tenebrio molitor: effects of diuretic and antidiuretic peptides and their second messengers. Journal of Experimental Biology 205(4):493-501.

Wiehart UIM, Torfs P, Van Lommel A, Nicolson SW, Schoofs L. 2002b. Immunocytochemical localization of a diuretic hormone of the beetle

Tenebrio molitor, Tenmo-DH37, in nervous system and midgut. Cell and Tissue Research 308(3):421-429.

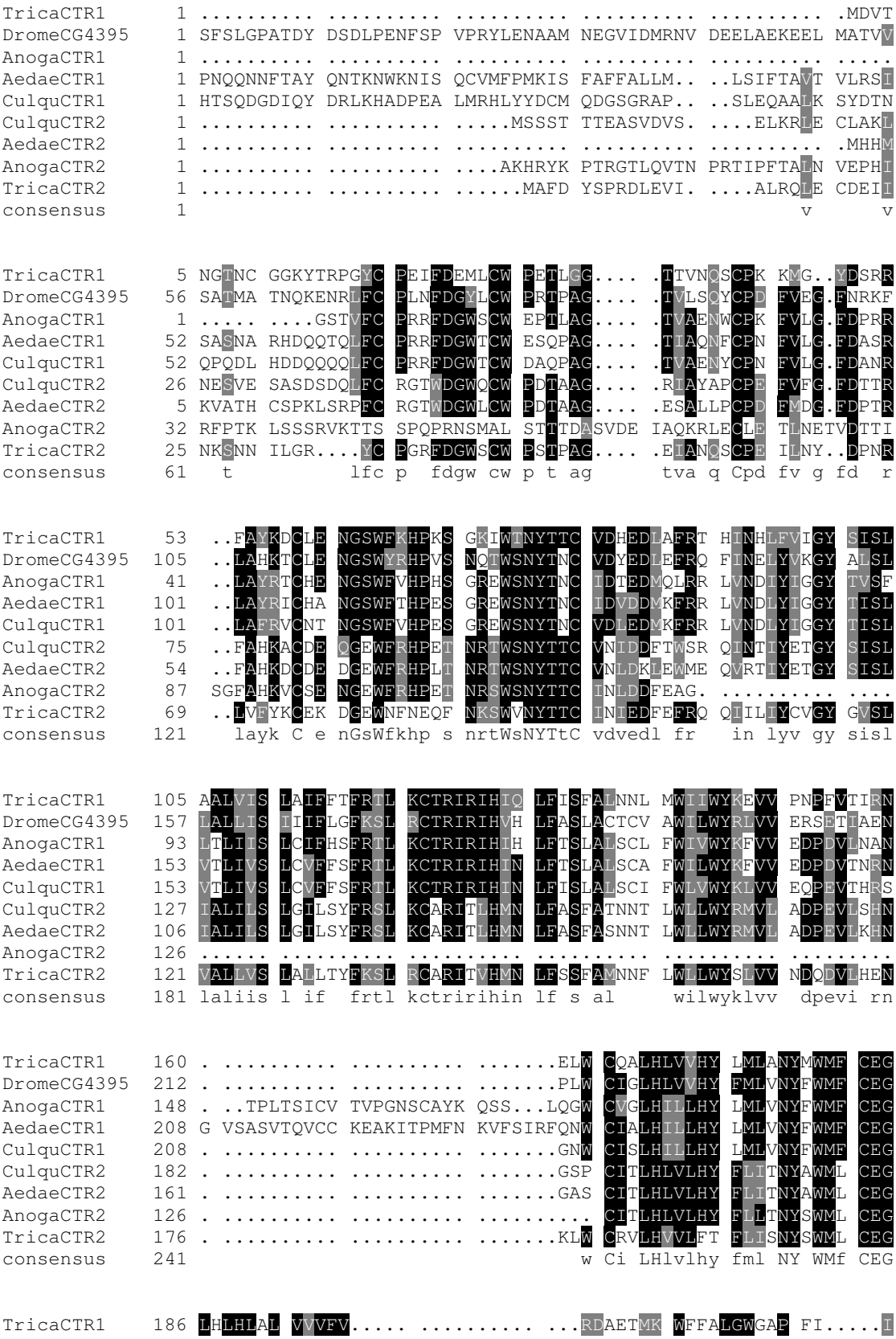
Appendix

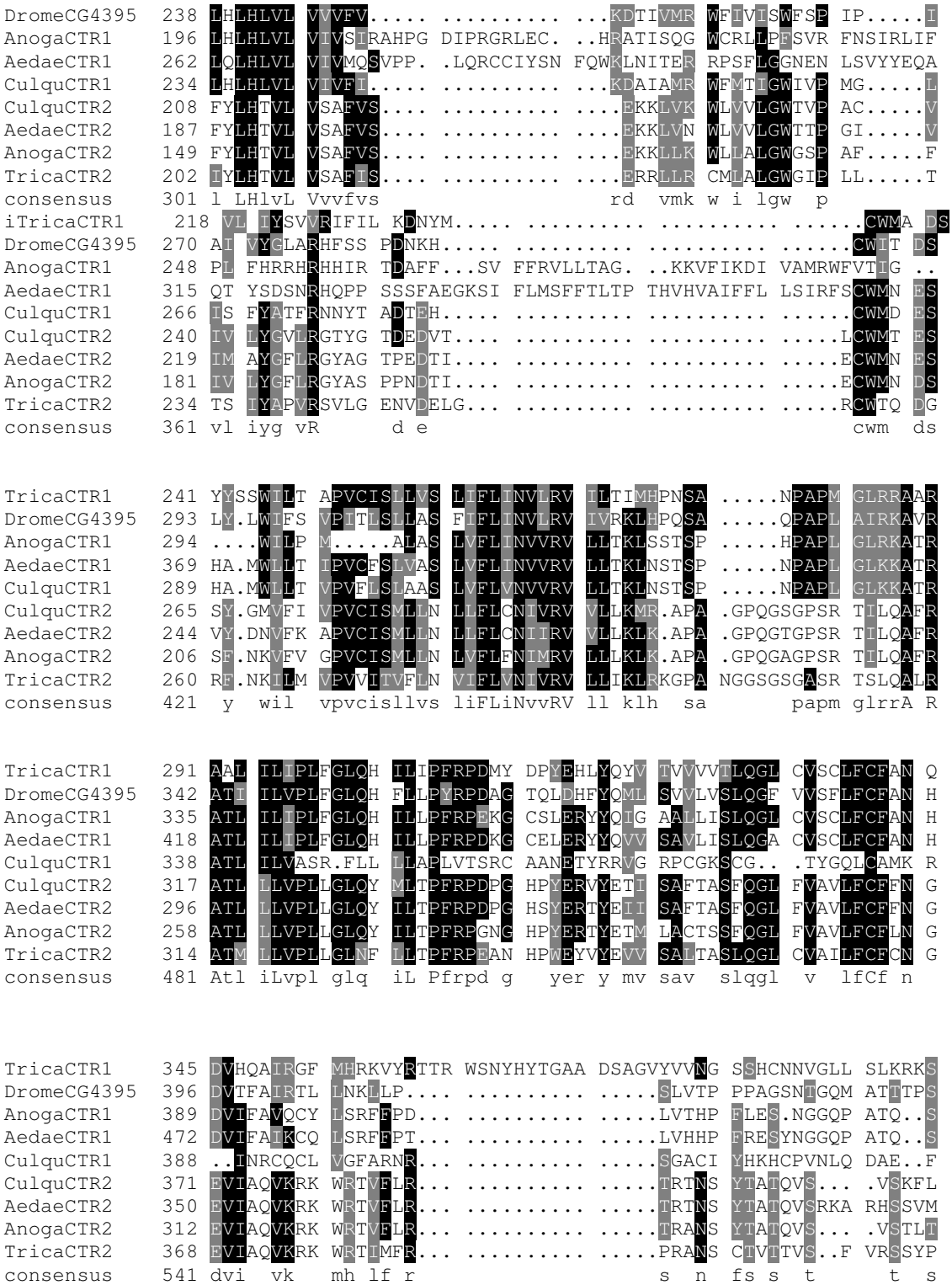
Appendix 1



Alignment of CRF-like receptors from Aedae (*Aedis aegypti*), Anoga (*Anopheles gambiae*), Culqu (*Culex quinquefacius*) and Drome (*Drosophila melanogaster*). Accession numbers: AedaeCRFR1, EU273351.1; AedaeCRFR2, XM_001659059.1; DromeCG12370, NM_165907.3; DromeCG8422, NM_137116.2; AnogaCRF1, XM_315466.4; AnogaCRF2, XM_315468.4; TricaCRFR1, XM_001808544.1; TricaCRFR2, XM_970323.2; CulquCRF1, BK006347.1; CulquCRFR2, EU273352.1.

Appendix 2





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CulquCTR2    404 IKDA RRQ
AedaeCTR2    387 TVTS TYC
AnogaCTR2    345 VLRV ...
TricaCTR2     403 ANGE EKV
consensus     601    v
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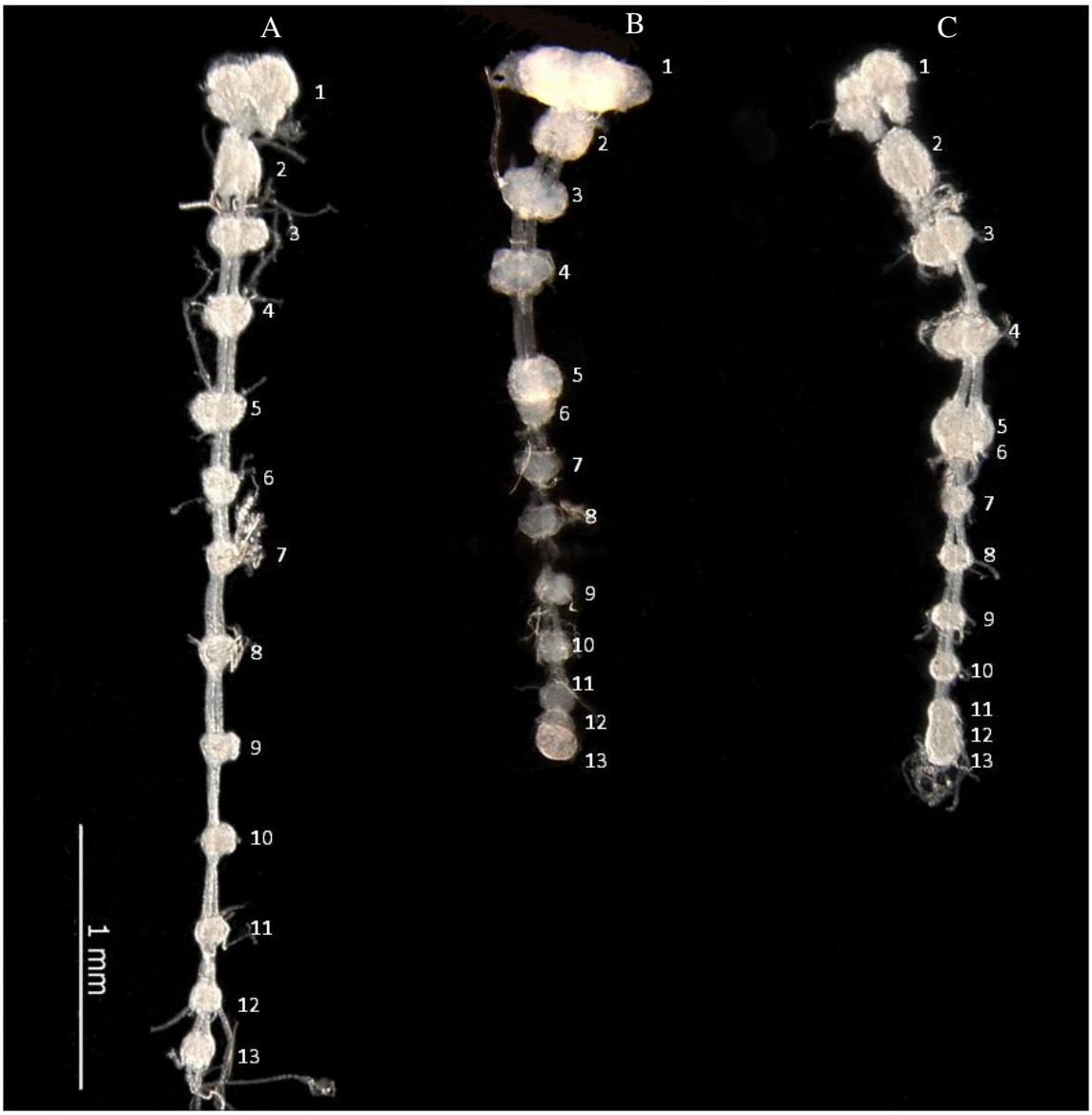
Alignment of CT-like receptors from Aedae (*Aedis aegypti*), Anoga (*Anopheles gambiae*), Culqu (*Culex quinquefacius*) and Drome (*Drosophila melanogaster*). Accession numbers: AedaeCTR1, XM_001651938.1; AedaeCTR2, XM_001660544.1; CulquCTR1, XM_001862151.1; CulquCTR2, XM_001864861.1; DromeCG4395, NM_132615.2; AnogaCTR1, XM_321982.3; AnogaCTR2, XM_318856.3; TricaCTR1, XM_001808544.1; TricaCTR2, XM_963937.1.

Appendix 3



T. castaneum CNS, A, B, C – Larva, Pupa and Adult, respectively: 1. Brain; 2. Subesophageal glanglion; 3, 4, 5. Thoracic ganglion 1, 2 and 3, respectively; 6 to 12. Abdominal ganglia 1 to 7; 13. Terminal abdominal ganglion.