

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 diuresis. 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-crfr2* 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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Table of Abbreviations

<i>Tricadh31</i>	<i>Tribolium castaneum</i> diuretic hormone 31 gene
TricaDH31	<i>T. castaneum</i> diuretic hormone 31 peptide
<i>Tricadh37</i>	<i>T. castaneum</i> diuretic hormone 37 gene
TricaDH37	<i>T. castaneum</i> diuretic hormone 37 peptide
<i>Tricadh47</i>	<i>T. castaneum</i> diuretic hormone 47 gene
TricaDH47	<i>T. castaneum</i> diuretic hormone 47 peptide
<i>Trica-ctr1</i>	<i>T. castaneum</i> calcitonin-like receptor 1 gene
TricaCTR1	<i>T. castaneum</i> calcitonin-like receptor 1 protein
<i>Trica-ctr2</i>	<i>T. castaneum</i> calcitonin-like receptor 2 gene
TricaCTR2	<i>T. castaneum</i> calcitonin-like receptor 2 protein
<i>Trica-crfr1</i>	<i>T. castaneum</i> corticotropin-like receptor 1 gene
TricaCRFR1	<i>T. castaneum</i> corticotropin-like receptor 1 protein
<i>Trica-crfr2</i>	<i>T. castaneum</i> corticotropin-like receptor 2 gene
TricaCRFR2	<i>T. castaneum</i> corticotropin-like receptor 2 protein
LocmiDH	<i>Locusta migratoria</i> diuretic hormone
DippuDH31	<i>Diploptera punctata</i> diuretic hormone 31
TenmoDH37	<i>Tenebrio molitor</i> diuretic hormone 37
TenmoDH47	<i>Tenebrio molitor</i> diuretic hormone 47
RhoprDH31	<i>Rhodnius prolixus</i> diuretic hormone 31
ManseDH41	<i>Manduca sexta</i> 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 Malpighian 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 secrete 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 through 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 sequestration 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 osmoregulation in insects. The first identified peptide of this family was isolated 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 hormone, 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 diuresis 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 5th instar 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 diuresis or which secondary messenger 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 messenger 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 orphanized 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 shown by a large group of peptides, the functional characterization of the receptors is difficult. However, this work became easier due to 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 castaneum

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.

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.

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°C for 3min followed by 30 to 35 cycles of: 94°C, 30s; 55°C, 1min; 72°C, 1min. A final hold of 72°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°C for 1min followed by 35 cycles of: 98°C, 5s; 55 to 60°C depending on the melting temperature of the primers, 3min; 72°C, 1min. A final hold of 72°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°C for 3min followed by 30 cycles of: 94°C, 30s; 55°C, 1min; 72°C, 1min. A final hold of 72°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 Grove, Pennsylvania) in PBST at 4°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 °C 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 coelenterazine 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 Luminometer. 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 cm³ 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 MgCl₂, 2 CaCl₂, 6 NaHCO₃, 4 NaH₂PO₄, 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°C

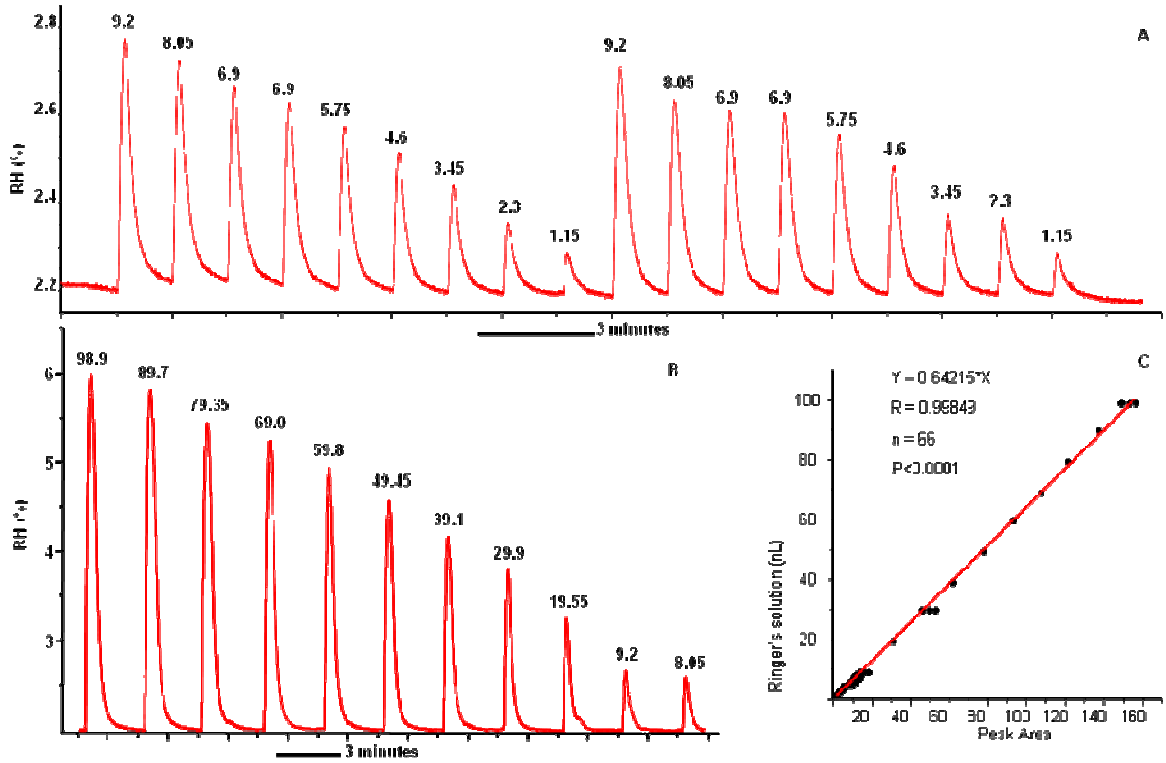


Figure 1. Humidity chamber assay measuring vaporized solution in the airflow. A and B. Different amounts of saline solution were injected in the chamber (1 to 100 nl) and the change in relative humidity (RH) were observed. C. Regression between amount of saline injected in the chamber and the area of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

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

Gene	Forward (5'-3')	Reverse (5'-3')	Use	Amplicon Size (bp)
DH31	915 cctggatactattcgccgat	920 ggaactgtttggaattgaag	RT-PCR	300
DH37 (Exon 1-4)	1001 gacaacatcaccatcggtttctgc	999 tccactctctacatacactgactag	RT-PCR	822
DH37 (Exon 2-4)	1019 gtggcttgttgttcttggtgcg	999 tccactctctacatacactgactag	RT-PCR	683
DH47 (Exon 1-5)	1001 gacaacatcaccatcggtttctgc	913 gtgattggagaaatgccctt	RT-PCR	621
DH47 (Exon 2-5)	1019 gtggcttgttgttcttggtgcg	913 gtgattggagaaatgccctt	RT-PCR	400
DH37	985 ccttcgagcgaagtatcca	999 tccactctctacatacactgactag	<i>In situ</i>	177
DH47	987 aatcccgaagcaaaagagc	914 ttgaagatttcaccgacact	<i>In situ</i>	247
DH31	915 cctggatactattcgccgat	920 ggaactgtttggaattgaag	<i>In situ</i>	300
<i>Trica-crfr1</i>	942 ttaccgtattcctcaacgtg	945 ccgtcaacgctgaaacaact	qRT-PCR	240
<i>Trica-ctr1</i>	967 gagatgcggaacgatgaag	969 gagatgcggaacgatgaag	qRT-PCR	140
<i>Trica-crfr1</i>	935 cgtttacagtttcaggttc	938 ggagtccaagcagctccg	qRT-PCR	297
<i>Trica-crfr2</i>	928 aacgagacgacctatgcaa	931 tccagtccccctttggagc	qRT-PCR	351
<i>Trica-ctr2</i>	940 tgaatggaactttaatgaac	945 ccgtcaacgctgaaacaact	<i>In situ</i>	838
<i>Trica-ctr1</i>	966 ccacatccagctgtttatc	968 ctccggtgtaatggtagtgtg	<i>In situ</i>	612
<i>Trica-crfr1</i>	935 cgtttacagtttcaggttc	939 acgaatatctggttctcgc	<i>In situ</i>	986
<i>Trica-crfr2</i>	930 gaccagccacatgcacttg	933 tccccgaacgtagtctggat	<i>In situ</i>	680
DH37	985 ccttcgagcgaagtatcca	986 taaatTTTTgagatattctcga	dsRNA	137
DH47	987 aatcccgaagcaaaagagc	988 attgtcgttaagtccggaagc	dsRNA	207
DH31	1297 ttctcaagccgccaacatc	1298 cagagagggtgggaaaaggg	dsRNA	448
DH37/47	1479 gttgttggtgaagaagtga	1480 gttgttggtgaagaagtga	dsRNA	484
<i>Trica-ctr1</i>	1377 gagcggaaatgagaataagt	1378 catcatcgtcctcatttacagcg	dsRNA	250
<i>Trica-crfr2</i>	1379 ctctataccaacaggaccac	1380 atgtggaagtaatgtagcag	dsRNA	500
<i>Trica-crfr1</i>	1381 ggcggaattagcttgccaac	1382 tccacataaaatccgcaagg	dsRNA	419
<i>Trica-ctr2</i>	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.

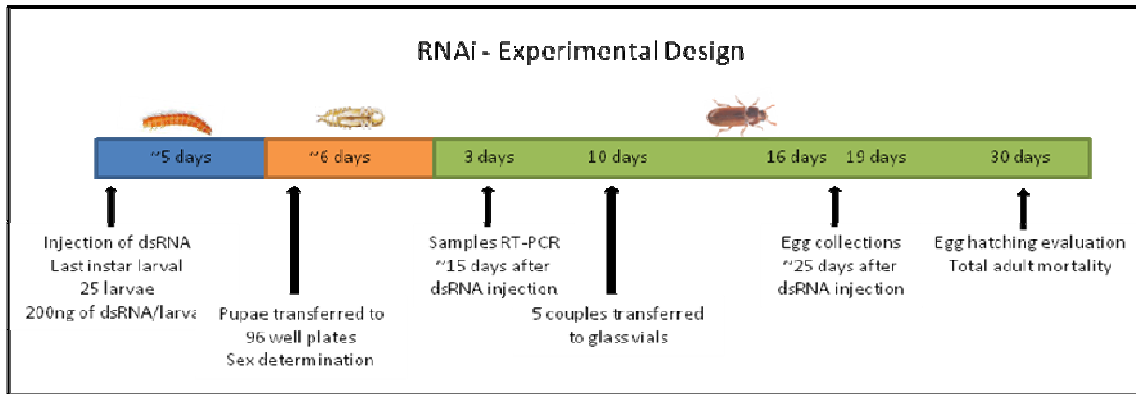


Figure 2. Schematic diagram showing the schedule for examination of RNAi phenotypes. Larvae were injected with 200ng of dsRNA and placed in Petri dishes with food. In the pupal stage, each individual was separated into cells of 96 well plates after the sex was determined. Three days after adult emergence, 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

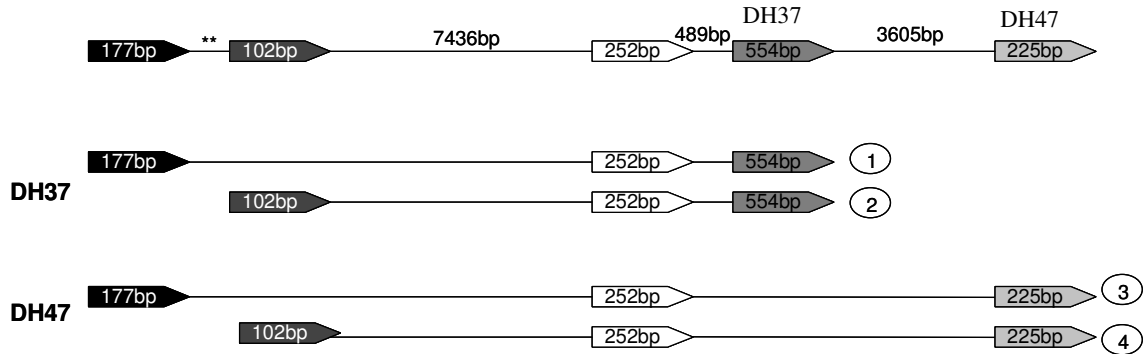
The results section is divided in two parts: in the first section the CRF-like gene/ligands and their receptors are described, and in the second part the calcitonin-like gene/ligand and receptors are described.

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).



CH476295.1 225680

1 ATCAAGTAATGACCGACGCG CATGTCACTGCCTATAAAGA CGCTCTATCACCGTCGCGAC
61 GTCCATAACCTGCGTGATCT TTACCACTAACAGTCGACAA CATCACCATCGGCTTCTGC
121 AACTTTGCGGAGTTTCGTCG CTCGCGACTCGGTGCTCTTT CGATCGAATTGGAGTTGATC
177bp 181 CAAAACCTCCTTCTTCGGCCT TTTTGAAGGGAGATAAGTC CGAAACTTGTTCACCAACCT
241 GTTGAATTTAGCTGCGGCTT TGACGAACAATAAGGTAAGA ACTGGACAAAGTTTATTTT
301 GCTGAGCGATTTTTAATCAT CTTGTTGGCTGGAAAAGTAC ACCTGATTACACGGTTTTCC
**Intron - from CH476295.1 226039 go to CH476317.1 61119
541 TTTTCGTTGTCTAGGTCACA TGACCATTAATCTTCTACAT ATATAATTGTGGAACATTGG
102bp 601 AAACATTTACAAATGTACC ATTTTATCATGGCATTAAACG TCAAAATGTGCCATCGTTTCG
661 CCAAACTGAGTGTAATAATA AAGTGGCTTGTGTTGTTCTTG GTGCGTTTATGAAACATCAA
721 ATGTGAGTAGGACTCGATTT TATATTTTTTAAAAGGGCT TTTTATTGATTCAACCAAA
Intron 7436bp
8101 TGATTAAATTAACCTGCAT ATTTTTGGTAACATTTTTTG AAAATGTGTTTTTCCAGGT
RNAiF
M R V P V Y L V V A A L V V V V R S
8161 CCGTATCATGCGTGTGCCGG TTTACTTGGTATGCGCTGCT TTG**GTTGTTGTTGTAAGAAG**
· S
8221 **TGA**AGAGCGTACGAATTACT ACGGAGGAAGATATTTGGAG CCGTTGATGTAGCTGCTGA
252bp 8281 CCAAGAAACCGTCAGTTATC TCTTACCAAAATTGGCCGCA AAATACAGACCTAACAGTGA
RNAiR
8341 ATGGAGCGGAGTCACTGATC CCAGATTTTACGTTTT**GACT GAAATGGAATCGCAGG**ACAT
8401 CGAAAATCAGGTAAATAACT AAGAAAAATCAAATGACTTA CTCTCAAAATATGAACGAA
Intron 489bp
8821 AAAATATGATTTGTTGCATT CGAGTAGAAAATATTTGTGT TGTTTTTAAATGTCCTGTA
RNAiF
V P S E R S I Q R R S P T I ·
8881 TTAATTTGTATGTTTTAGG **TGCCTCCGAGCGAAGTATC CA**AAGACGGAGCCCTACAAT
· S I T A P I D V L R K T W A K E N M R K ·
8941 TTCCATAACGGCACCAATCG ATGTTTTGCGAAAAACTTGG GCAAAAGAGAACATGCGGAA
· Q M Q
RNAiR
9001 ACAGATGCAGATAAA**TCGAG AATATCTCAAAAATTACAA** TAAATTTGAGACACCGCTGT
9061 GAGAAACAGTGTAGTGTTTA TTGCAAAAGCCCTTCAATAA ATGCTACAGTGTTTAAATCA
9121 ATTTGTGAAAGTATTTTGC CCACTCAAGTACACTAATAA TGCTAGTCACTTTAAATTTA
554bp 9181 AGATAATTGACTTTTTCCA TTTTAAAACAGTATGAAACA GTTCAGTATTTTCGTTACTA
9241 CTATAATGTAAATTAATGC AACTCTAGTCAGTGTATGAG AGAGTGGAAACAAAAATTAG
9301 GATGTGTGTTTGCAGTTTG ATTTAATTTACATTGTACTG TACCAGTTTAGTTTATTGTA
9361 GCCATCGATGATTTTATATA TTATTTATTTTATTATGTTT ATTTGTTACAAAAACAATA
9421 AATGTATAAAAATAAAAAACA CCTCATTTCACTCGTTCTCT TATTTAGTAATAAAAAATTGA
9481 AGCAACTTTATGAGCGATTT TATTTCAATTTTTGTTTTTTT ATCATTATTACCAGAAAATA
Intron 3195bp
12601 AATTAGCCCAGCATTGTGC AAGTGATTTATGTGTTTAAA TTTGCAGAATAAATTGAGC
RNAiF A G A L G E S G A S L S I
225bp 12661 CCC**GAAATCCCGAAGCAAAAG AGC**AGGTGCTTTGGGCGAGT CCGGAGCTTCTCTCTCGATT

V N S L D V L R N R L L L E I A R K K A
 12721 GTGAATTCGTTGGACGTTCT TCGCAACAGACTATTATTGG AAATTGCAAGAAAGAAGGCC
 K E G A
 12781 AAAGAAGGGGCTAACAGAAA CCGACAAATCTTACTCTCAT TAGGAAAAAGGGCATTCTC
RNAiR
 12841 CAATCACGA ***GTTCCGGAAC TTACGACAAT***TAATGTATAAA TCAAGCGAAGAATTCATCGA
 12901 ATTAAATAAGTGTCGGTGAA ATCTTCAATACCTATTGCTT TGTCTTTCTTTAATTTAGT
 Ends at CH476317.1 73538

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.

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Sc_DH44  -----N-KPSLSIVNPLDVLRQRLLEIARROMKENT-RQVELNRAILKNV-
Md_DH44  -----N-KPSLSIVNPLDVLRQRLLEIARROMKENT-RQVELNRAILKNV-
Dm_DH44  -----N-KPSLSIVNPLDVLRQRLLEIARROMKENS-RQVELNRAILKNV-
Zn_DH46  ---TGA-VPSLSIVNPLDVLRQRLLEIARRMRQSQ-DQIQANREMLQTI-
Pa_DH46  ---TGS-GPSLSIVNPLDVLRQRLLEIARRMRQSQ-DQIQANREILQTI-
Dp_DH46  ---TGT-GPSLSIVNPLDVLRQRLLEIARRMRQTO-NMIQANRDFLESI-
Lm_DH46  ---MGM-GPSLSIVNPMDVLRQRLLEIARRRLRDAE-EQIKANKDFLQOI-
Am_DH43  ---IG----SLSIVNSMDVLRQRVLLEIARRKALQDQ-AQIDANRRILETI-
Ad_DH46  ---TG--AQSLSIVAPLDVLRQRLMNEINRRMRRELOGSRIQONRQILLTSI-
Tm_DH47  AGALGESGASSLSIVNSLDVLRNRLLEIARKKAKEGA----NRNRQILLSL-
Tc_DH47 AGALGESGASSLSIVNSLDVLRNRLLEIARKKAKEGA----NRNRQILLSL-

Ms_DH41  -----RMPPSLSIDLPMSVLRQKLSLEKER-KVHALR---AAANRNFLNDI-
Hl_DH41  -----RMPPSLSIDLPMSVLRQKLSLEKER-KVQALR---AAANRNFLNDI-

Tm_DH37  -----SPTISITAPIDVLRKTWEQERARKQMV-----KNREFLNSLN-
Tc_DH37 -----SPTISITAPIDVLRKTWAKENMRKQMQ-----INREYLKNLQ-

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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 phylogenetic 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-crf1* 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/>).

```

1  GTTTTGTTGAAAGAACAGTTTGCACACGTGTGATCATGCATAACCAGCACAAATAATTGT
                                     M D Q G E S E
61  TTCAGGTCATTTTTTCAGTCGGTTTAAATATATGATGGAAAATGGATCAGGGTGAAAGCGAG
   V V Y A R H E D I V R I L N R L N E T Q
121 GTTGTTTATGCCCCCATGAGGACATCGTCCGTATCCTGAATCGTCTCAATGAAACCCAG
      RNAi_F
   A E L A C E L K K T L S P P S N G C A V
181 GCGGAATTAGCTTGCGAACTTAAAAAACACTCTCGCCCCATCGAATGGATGTGCTGTC
   D F D T V L C W P Q T A P N S L A V L P
241 GACTTCGATACGGTTTTGTGTTGGCCGCAAACGGCGCCGAATTCCTTGCTGTTTTACCT
   C F D Q L N G I K Y D T R E N A T R L C
301 TGCTTCGACCAGCTCAATGGCATTAAAGTACGACACGAGAGAAAACGCGACGCGTTTGTGT
   F A N G T W D Q Y S N Y T S C K E L S P
361 TTCGCCAACGGAACCTGGGACCAATATAGCAACTATACCTCGTGCAAAGAATTGTCTCCT
                                     TM1
   L E V P E V E L T T T I Y F I G Y T V S
421 TTGGAGGTGCCCGAAGTGGAAC TAACAACCACAATTACTTCATTGGCTACACCGTCAGC
   L V A L L F A V Y I F W K F K D L R C L
481 CTAGTGGCGCTCCTCTTTGCCGTTTACATCTTCTGGAAGTTCAAAGACTTGGCATGTCTG
                                     TM2
                                     RNAi_R
   R N T I H M N L M C S Y I L A D F M W I
541 CGAAATACCATCCATATGAATCTGATGTGTTCCCTACATCCTTGCGGATTTTATGTGGATT
      ISH_F
   F V Y S L Q V P L Q T N K A F C I F L I
601 TTCGTTTACAGTTTGCAGGTTTCTTTACAAC TAACAAGGCTTTCTGCATATTCCTCATA
                                     TM3
   I L L H Y F H L T N F F W M F V E G L Y
661 ATTCTGCTGCACTACTTCCACCTGACGAACTTTTTCTGGATGTTTGTGGAAGGTTTATAT
   L Y I L V V K T F T G E N I K P R I Y A
721 CTATACATTTTGGTGTGAAAACATTCACCGCGGAGAACATCAAGCCTCGGATCTACGCA
                                     TM4
   V I G W G G P I L F V L V W G I A K S F
781 GTAATAGGTTGGGGGGACCGATTCTGTTTGTACTGGTCTGGGGCATCGCTAAAAGTTTC
   T L P L E D Q Q A G E M F R S C P W T P
841 AACTACCATTAGAGGACCAACAGGCGGGTGAGATGTTCCGGAGCTGCTTGGACTCCG
                                     TM5
   H P F D W I Y Q G P A I A V L I I N V I
901 CATCCTTTGATGGATCTACCAAGGACCCGCAATTGCCGTTCTTATAATTAATGTAATA
   F L C I I M W V L I T K L R S A N N V E
961 TTCCTTGCATAATCATGTGGGTATTAATAACCAAACTCCGGTACGCCAACCAACGTTGAA
                                     TM6
   T Q Q Y R K A A K A L L V L I P L L G V
1021 ACGCAGCAGTACCGGAAAAGCGGCCAAAGCTCTACTAGTCCTTATTCCTCTCCTGGGAGTC
   T Y I L V I V G P T E G I S R R I Y D S
1081 ACTTACATTCTAGTTATAGTGGGGCCACCGAAGGCATCTCCAGACGCATATACGACAGC
                                     TM7
   I R A I L L S T Q G F T V A L F Y C F L
1141 ATTAGAGCCATCCTCTTATCTACACAGGGCTTACAGTGGCGCTTTTTACTGCTTCCTC
   N A E V K N T V R H H Y N S W H T R R T
1201 AACGCTGAGGTGAAGAACACAGTGCGCCACCATTACAACAGTTGGCACACTCGTCGAACT
      ISH_R
   L G S R R T R Y S S S K D W S S Q A R D
1261 TTAGTTCGCGAAGAACCAGATATTCGTCGAGTAAAGATTGGTCGTCGAGGCCAGAGAC
   S M R Y G S K R A K L S T L K Y *
1321 AGCATGCGGTATGGGTCTAAAAGGGCCAAACTTTGACTTTGAAGTACTAACGTTGCGTT

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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 shown in bold font. Sequence shown is from CM000279 10513802 to 10491091. Introns are not shown.

1 CATATATCACCCGTGCAAAC AACTGATAAAGAAAATCTT CGCTCGAATCCACCTCAATT
M S W S E P L
61 GTGTTTCGCAGATTTCGAGTG TCAAAATGCATCATCAACAA TGAGTTGGTCCGAACTCTC
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 AACACTGCACCAGC**CTCTAT**
T N R T T A L A E T H P D G F C P V T T
241 **ACCAACAGGACCAC**AGCCCT CGCAGAAACCCACCCAGATG GGTTCGCCCCGTCACCACC
D G L L C W P P T P I N E T T Y V K C F
301 GATGGCTGCTGTGCTGGCC GCCCAGCCCATCAACGAGA 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 TTATTTGTCGGATACGTTT TAAGTTTAATTACACTGTCG
TM3
I A L G I F T Y F K E L R C L R N R I H
541 ATAGCTTTGGGAATTTTAC ATATTTCAAAGAGCTGCGCT GCTTGAGAAACCGAATTCAC
M N L M W S Y M L M Y I M W I L T L T V
601 ATGAAATCTCATGTGGTCTTA 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 TATTCGTATCAC**CTGCTA**
RNAiR TM4
H Y F H I S T F F W M F V E G L Y L Y I
721 **CATTACTCCACAT**TTCCAC GTTTTTTGGATGTTTGTG AGGGCCTGTATCTTTATATT
L V V E T L T R E N Y K L R V Y V C I G
781 CTGTCGTTGAAACACTGAC CAGGGAGAATTATAAATTGA GGGTTATGTGTGCATCGGG
TM5
W G L P M I F I L V W V I V K S F I P A
841 TGGGGCTTGCCAATGATTTT CATCTCGTTTGGGTGATTG TCAAGAGCTTTATCCCGGCA
ISH_F
A G D P A T C T W F N S H D V D W I F Q
901 GCGGGCG**ACCAGCCACATG** **CACTTGG**TTCAACAGTCATG ACGTGGACTGGATTTTCCAA
TM6
G P T M L V L L L N L A F L L A I M W V
961 GGGCTACAATGCTGTTTCT CCTGCTGAATTTGGCCTTCC TGCTGGCCATAATGTGGGTC
L I T K L R S A N T V E T Q Q Y H K A A
1021 CTCATAACAAA**ACTGCGATC** 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 ACGTTATCACAA**CTACGCC**
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 **TCCAGACTACGTTCCGGGGAG**
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 AATCACGTTTGGCTCGATT 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 shown 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*, *Acyrtosiphon 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

```

25861 CCAAATAAAGTAGTACAAAATAATGTGACATCTCTAACTGATAAAAGCCTTTAGAATAAA
25921 TAAAAATAAATAAATATTGTTTTTTTTTCAATTCAGGAAGATGGTTCCCAACAATGG
25981 AATCTCCCTCTTGATGCTTCTCGTTGCGGGAATGATTTGTTTCAAGCGACTACGACTTA
26041 TGCAGCCCTCATTACCCAGGTAGTAGTGTGTTTCCGAACTTTGCTTGTGCAACTTAT
26101 TATCTAATCTAAATGATTTACCTGCCGAATTGAATTAACCTCGTTAAGTGAATGTATGACA

```

Intron 11517bp

```

37501 ATTTGAGACAGTTTTGTTGCTTTATTTTACAACCTAATGGCGTTATTAATAACATTTTAA
                                     M E G Q .
37561 TAACAAAAATACTTTTTGCAGTTACCTTGATACTATTCCGCCGATATCAATGGAAGGACA
      · N P E Y L L Q T I A R L R Q A L I S D D ·
37621 AAATCCCGAATATCTCTCCAGACAATTGCCAGATTGAGGCAAGCATTGATTTCCGGATGA
      · D L E K ·
37681 TGATTAGAAAAGTAAGTTTGAAGGCACATGTAAAGTAATGTTTACGTTGCATATGACTC

```

Intron 19971bp

```

57601 ATATAATTCAGTAAACGCGTTCATTACGACATAACTGATTAACCTGTAATTGAATAATTC
      · S K R G L D L G L G R G F S G S Q A A ·
57661 CAGTTCCAAACGCGGCTTAGACCTAGGTCTTGGTCTGGCTTCTCTGGTTCTCAAGCCGC
      · K H L M G L A A A N F A G G P G R R R R ·
57721 CAAACATCTGATGGGCTGGCAGCAGCCAACCTTCGCTGGAGGGCCAGGTCGAAGACGTGC
      · S E E E A *
57781 AAGCGAAGAAGAAGCATAAAATGGACACAACAGAATAAATGTAAGTACTTCAATTTCCAAACAG
57841 TTCCTAATGTAAATTTAACCTCTCTCTTTCTCTCTATCATTCTGTGCTTGTGTCAA
57901 GTAGTTAAAACCCCTAAGTAGTTGAATAATACATTTAGAAATAGTAATCGTACACGAAA
57961 AAATGAATAAATTAACCCCTTTCCACCCTCTCTGTATAACAACATTATTTCTGAAAAT
58021 AAAGCACAGGACGCAGTAACAGCACCTTGGTTTTAACTTGTAAATTTATTGTTTGATAGT
58081 TAAGAGATGTATTGTACAGCAATTATATTTCAATTAATAAAGTTTAAAACATCACTTTG
58141 TGCTGCTGTTTTGGAATATTCCTTTCAACATACAGAGTGGTCCAAGCCAGCTCACGTCTT

```

B

Acyrtosiphon_pisum	1	NDGDPEVMLE	LLARIGQNI	RVN	DLNSKRGL	DLGLSRGYS	TOAAK
Nasonia	1	QEDYPEAAQE	LLDRTEENFA	VYA	QEDNAKRGL	DLGLNRGFS	SOAAK
Tribolium	1	EGONPEYLLO	TIARLRQALI	SDD	DLNSKRGL	DLGLGRGFS	SOAAK
Bombyx_mori	1	GOYDPEETID	MLGRIGNLIQ	MER	KMQNYK	NDITSEKRAF	DLGLGRGYS	ALQAK
Drosophila	1	EEVDDILME	LMTRFGRTII	RARN	DLNSKRTV	DFGLARGYS	TCEAK
Anopheles_gambiae	1	KEQPDVLLID	LIARNGHTML	RAKDE	DLNSKRTV	DFGLSRGYS	AQAAK

Acyrtosiphon_pisum	48	HLMGM	AAANFAGGPG	RRRRSE	M L P K	LLTP
Nasonia	47	HLMGL	AAANFAGGPG	RRRRSE	QA..
Tribolium	48	HLMGL	AAANFAGGPG	RRRRSE	EEA..
Bombyx_mori	55	HLMGL	AAANFAGGPG	RRRRSE	Q...
Drosophila	49	HRMGL	AAANFAGGPG	RRRRSE	TDV..
Anopheles_gambiae	49	HRMAM	AAANFAGGPG	RRRRSE	GIML

Figure 7. A. Sequence of *Tricadh31*. The exons are indicated by gray boxes. B.

Multiple sequence alignment of DH31 peptides from different insect

orders: *Acyrtosiphon 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.

```

1  CTCCGAGATTCATGTGGAA  GTTGCCTGAATACGTCTC  CAGGATAATACATCTTGCTC
                               M D V T N G T N C G G
61  ATTGTTGCAGGACTTTTCG  GTCAACAATGGACGTTACAA  ATGGGACTAATGTGGTGGA
   K Y T R P G Y C P E I F D E M L C W P E
121  AAATACACCAGACCTGGTTA  TTGTCCGGAGATTTTCGACG  AGATGCTGTGCTGGCCGGAG
   T L G G T T V N Q S C P K K M G Y D S R
181  ACCCTCGGGGCACCACCGT  GAACCAAGTCCGCGAAAA  AAATGGGATACGACTCTCGC
   R F A Y K D C L E N G S W F K H P K S G
241  CGTTTCGCTACAAAGACTG  CCTCGAAAACGGCTCCTGGT  TCAAGCACCCGAAATCCGGC
   K I W T N Y T T C V D H E D L A F R T H
301  AAAATCTGGACCAACTACAC  CACCTGTGTCGACCACGAGG  ACTTAGCTTTCCGAACTCAC
                               TM1
   I N H L F V I G Y S I S L A A L V I S L
361  ATAAATCACCTGTTTCGTCAT  CGGCTATTTCGATTTTCATGG  CGGCGCTCGTCATCTCGCTC
                               ISH_F
   A I F F T F R T L K C T R I R I H I Q L
421  GCCATTTTCTTCACGTTCCG  GACGCTGAAATGCACCCGCA  TCCGCATCCACATCCAGCTG
                               TM2
   F I S F A L N N L M W I I W Y K E V V P
481  TTTATCTCGTTTCGCCCTCAA  CAACCTCATGTGGATCATCT  GGTACAAAGAAGTCGTCCTC
                               TM3
   N P F V T I R N E L W C Q A L H L V V H
541  AATCCTTTCGTCACCATCCG  CAATGAGCTCTGGTGCAGG  CTCTTCATCTCGTTGTACAT
   Y L M L A N Y M W M F C E G L H L H L A
601  TACTTAATGTTGGCGAATTA  CATGTGGATGTTCTGCGAGG  GCTTGCATCTGCATCTGTG
                               TM4
   L V V V F V R D A E T M K W F F A L G W
661  CTAGTTGTGTCTTTGTGAG  AGATGCGGAAACGATGAAGT  GGTTCGCGCTGGGATGG
                               RNAiF
   G A P F I I V L I Y S V V R I F I L K D
721  GGCGCCCTTTTCATCATCGT CCTCATTACAGCGTTGTGA  GGATATTCATCTTAAAGGAC
                               TM5
   N Y M C W M A D S Y Y S S W I L T A P V
781  AACTACATGTGCTGGATGGC  CGACAGCTACTACAGCTCCT  GGATCCTCACAGCCCCCGTC
   C I S L L V S L I F L I N V L R V I L T
841  TGCATCTCCCTGCTTGTGTC  TCTCATATTTCTGATCAACG  TGCTGCGAGTATTTTGACC
   I M H P N S A N P A P M G L R R A A R A
901  ATTATGCATCCGAATTCGGC  CAATCCGGCTCCCATGGGAC  TGAGACGGGCTGCCAGAGCT
                               RNAiR
                               TM6
   A L I L I P L F G L Q H I L I P F R P D
961  GCACTTATTCTCATTCCGCT CTTCGGGCTCCAGCACATTC  TGATACCGTTTAGACCGGAT
                               TM7
   M Y D P Y E H L Y Q Y V T V V V V T L Q
1021  ATGTACGACCCCTACGAGCA  TCTGTATCAGTACGTCACTG  TCGTCGTTGTACATTGCAA
   G L C V S C L F C F A N Q D V H Q A I R
1081  GGTATGCGTATCGTGCT  GTTCTGCTTCGCCAACCAGG  ACGTCCATCAGGCCATTCCG
                               ISR_R
   G F M H R K V Y R T T R W S N Y H Y T G
1141  GGTTCATGCACCGTAAGGT  TTACCGGACGACTCGCTGGA  GCAACTACCATTACACCGGA
   A A D S A G V Y V V N G S S H C N N V G
1201  GCGCGGACAGCGCCGGGT  TTACGTGGTTAATGGCAGCA  GCCACTGCAACAATGTGGG
   L L S L K R K S T T T V K L *
1261  CTATTACTACTGAAAAGGAA  ATCGACAACCACTGTCAAAC  TGTAATAACGGGATAATTCC

```

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.

```

1   GGAGACCCAAGCTGGCTAGC GTTAAACTTAAGCTTGGTA CCGAGCTCGGATCCACTAGT
61  CCAGTGTGGTGGAAATTCTGC AGATATCCAGCACAGTGGCG GCCGCGGGAATTTCGATTGTG
      M A F D Y   S P R D L E V I A L R Q L
121 AACAAATATGGCATTTCGATTA TTCGCCTAGAGATTTAGAAG TGATAGCACTGCGGCAACTT
      E C D E I I N   K S N N I L G R Y C P G R
181 GAATGCGACGAAATTATAAA TAAATCGAACACATTTTAG GACGTTACTGTCCAGGAAGA
      F D G W S C W   P S T P A G E I A N Q S C
241 TTTGATGGTTGGTCTTGTG GCCTAGCACTCCAGCTGGAG AAATAGCCAATCAATCCTGT
      RNAiF
P E I L N Y D P N R L V F Y K C E K D G
301 CCAGAGATATTGAACTACGA CCCCAACCGTCTCGTCTTCT ACAAATGCGAGAAAGACGGT
      ISH_F
      E W N F N E Q   F N K S W V N Y T T C I N
361 GAATGGAACTTTAATGAACA ATTTAATAAAAGTTGGGTAA ATTACACCACCTGTATTAAC
      TM1
      I E D F E F R   Q Q I I L I Y C V G Y G V
421 ATCGAAGATTTTGAATTTTC CCAACAGATCATATTGATAT ACTGCGTAGGATACGGAGTG
      S L V A L L V   S L A L L T Y F K S L R C
481 TCCTGGTGGCTCTTCTGT GTCACTGCCCCCTCCTCACTT ACTTCAAGTCATTAAGATGT
      TM2
      A R I T V H M   N L F S S F A M N N F L W
541 GCCCGAATTACAGTCCACAT GAATTTATTCTCCTCATTCG CAATGAACAACTTTTTGTGG
      L L W Y S L V   V N D Q D V L H E N K L W
601 CTGTGTGGTACAGTTTGGT GGTTAATGACCAGGACGTCC TCCACGAAAACAAGTTATGG
      RNAiR
      C R V L H V V   L F T F L I S N Y S W M L
661 TGCCGTGTCTCGACGTCGT CCTCTCACCTTCCTCATT CAAATTATTCATGGATGTTG
      C E G I Y L H   T V L V S A F I S E R R L
721 TGCGAAGGCATCTATCTCCA CACCGTGCTGGTATCTGCTT TTATATCAGAAAGGCGTCTC
      TM4
      L R C M L A L   G W G I P L L T T S I Y A
781 CTGCGTTGTATGTTAGCATT AGGTTGGGGCATTCCTCTCC TGACAACTCCATTTACGCT
      P V R S V L G   E N V D E L G R C W T Q D
841 CCTGTGAGGAGCGTCTTGGG AGAGAATGTAGACGAGCTGG GGAGATGTTGGACTCAAGAC
      TM5
      G R F N K I L   M V P V V I T V F L N V I
901 GGCCGCTTTAACAAAATCCT GATGGTGCCAGIGGTCATTA CCGTATTCCTCAACGIGATA
      F L V N I V R   V L L I K L R K G P A N G
961 TTTCTGGTCAACATTGTGAG AGTTCTCCTAATTAATTA GAAAGGGTCCAGCTAATGGC
      TM6
      G S G S G A S   R T S L Q A L R A T M L L
1021 GGATCGGGATCAGGAGCATC CAGAACTTCTCTCCAAGCTC TCAGAGCTACAATGTTGCTG
      V P L L G L N   F L L T P F R P E A N H P
1081 GTGCCACTGTTAGGCTTGAA TTTCCTTTTAACTCCGTTTA GGCCGGAGGCGAACCACCCA
      ISH_R
      W E Y V Y E V   V S A L T A S L Q G L C V
1141 TGGGAGTATGTTTATGAAGT TGTTTCAGCGTTGACGGCTT CTCTAGAGGGTCTTTGTGTG
      A I L F C F C   N G E V I A Q V K R K W R
1201 GCTATTCTGTCTGCTTCTG TAACGGAGAGGTTATAGCCC AAGTTAAAAGGAAGTGGCGG
      T I M F R P R   A N S C T V T T V S F V R
1261 ACTATCATGTTCCGTCCAAG AGCAAATCTTGCACAGTCA CCACCGTATCGTTCGTTCCGC
      S S Y P A N G   E E K V *
1321 TCATCGTATCCTGCAAATGG AGAGGAAAAGGTATGACTGG GGGCAATCGGCCGTCGAGCC

```

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).

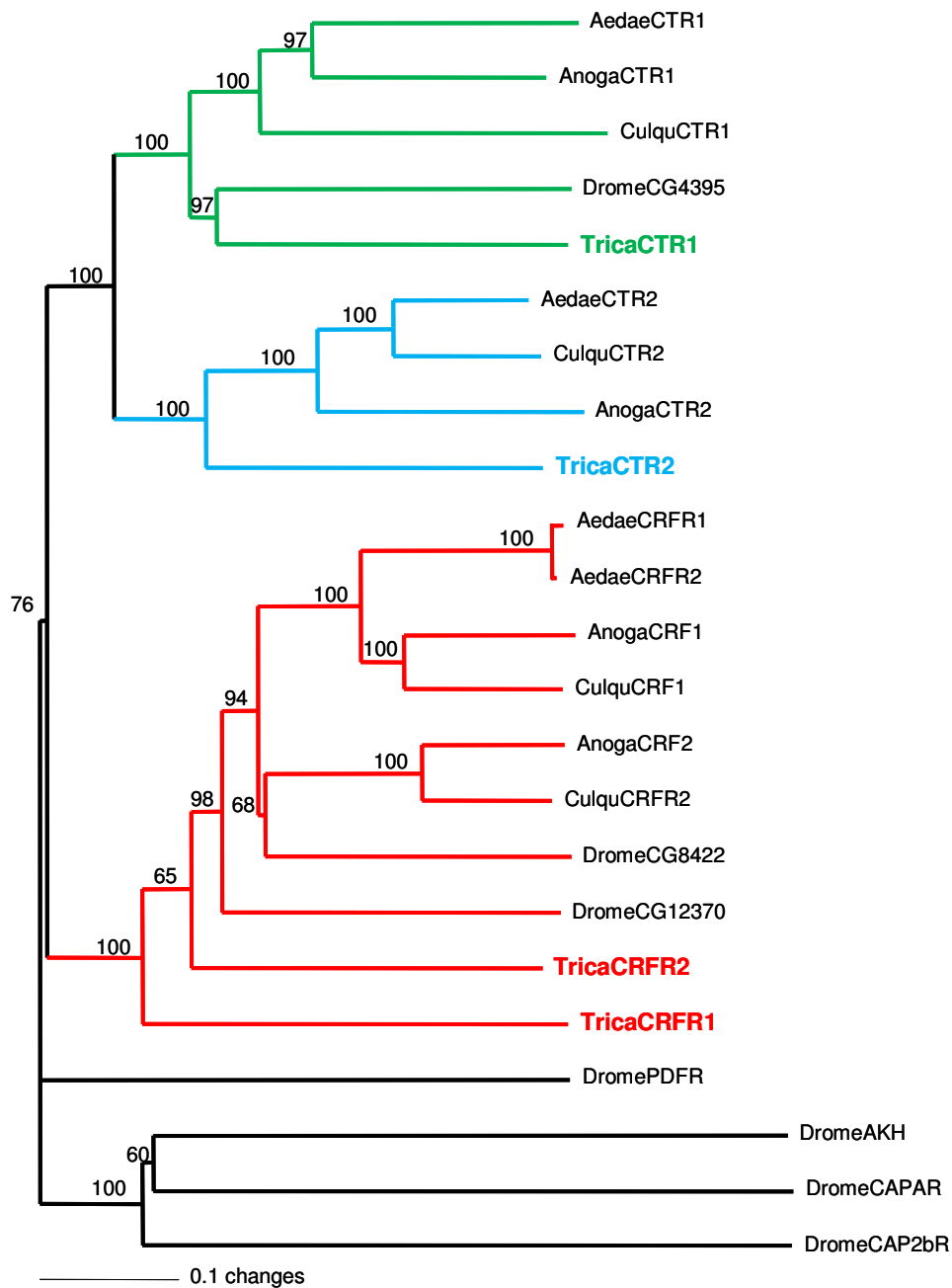


Figure 10. Phylogenetic relationship of *T. castaneum* calcitonin-like receptors and CRF-like receptors with other members of the family B of GPCR (secretin family) from *Aedes aegypti* (Aedae), *Anopheles gambiae* (Anoga), *Culex quinquefasciatus* (Culqu) and *Drosophila melanogaster* (Drome). The outgroups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

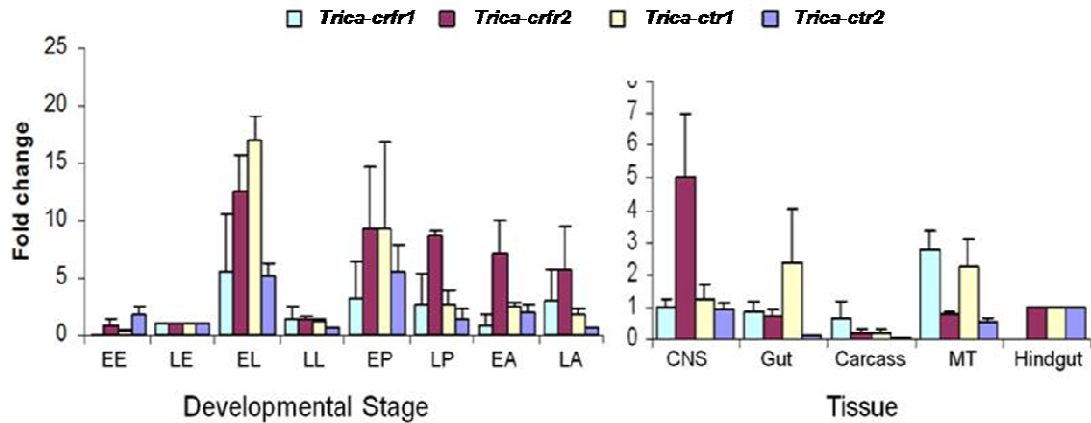


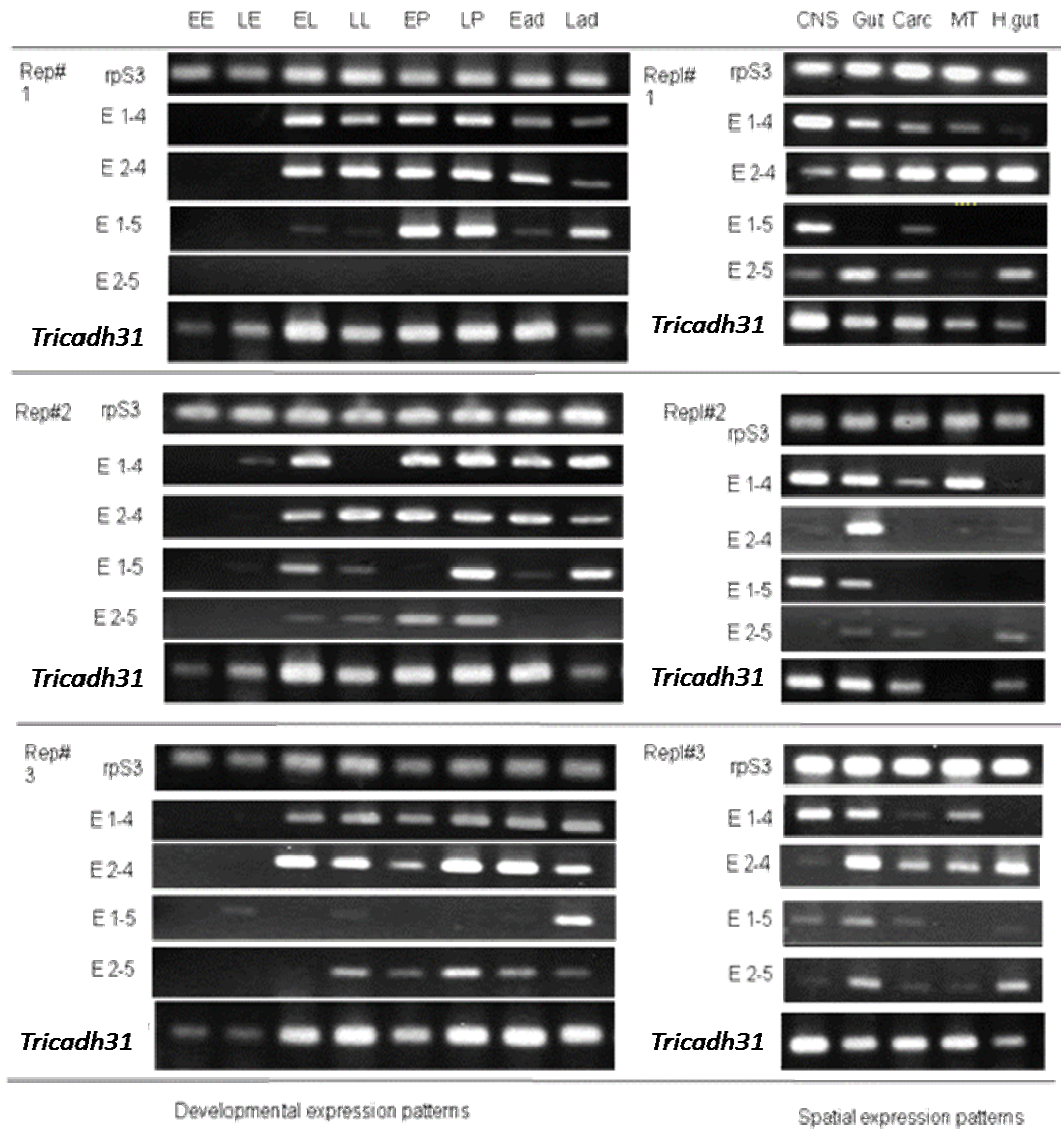
Figure 11. Quantitative RT-PCR in different stages and tissues for diuretic hormone receptors of *T. castaneum*. Pools of tissue dissected from three insects were used for total RNA isolation. The averages and standard deviations of three biological replications are shown. CNS, central nervous system, 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		-	+	++		+		+	++	+++	+++	+	+++
<i>Tricadh31</i>	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++

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 abdominal 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 immunohistochemistry 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 (Figures 16 and 17).

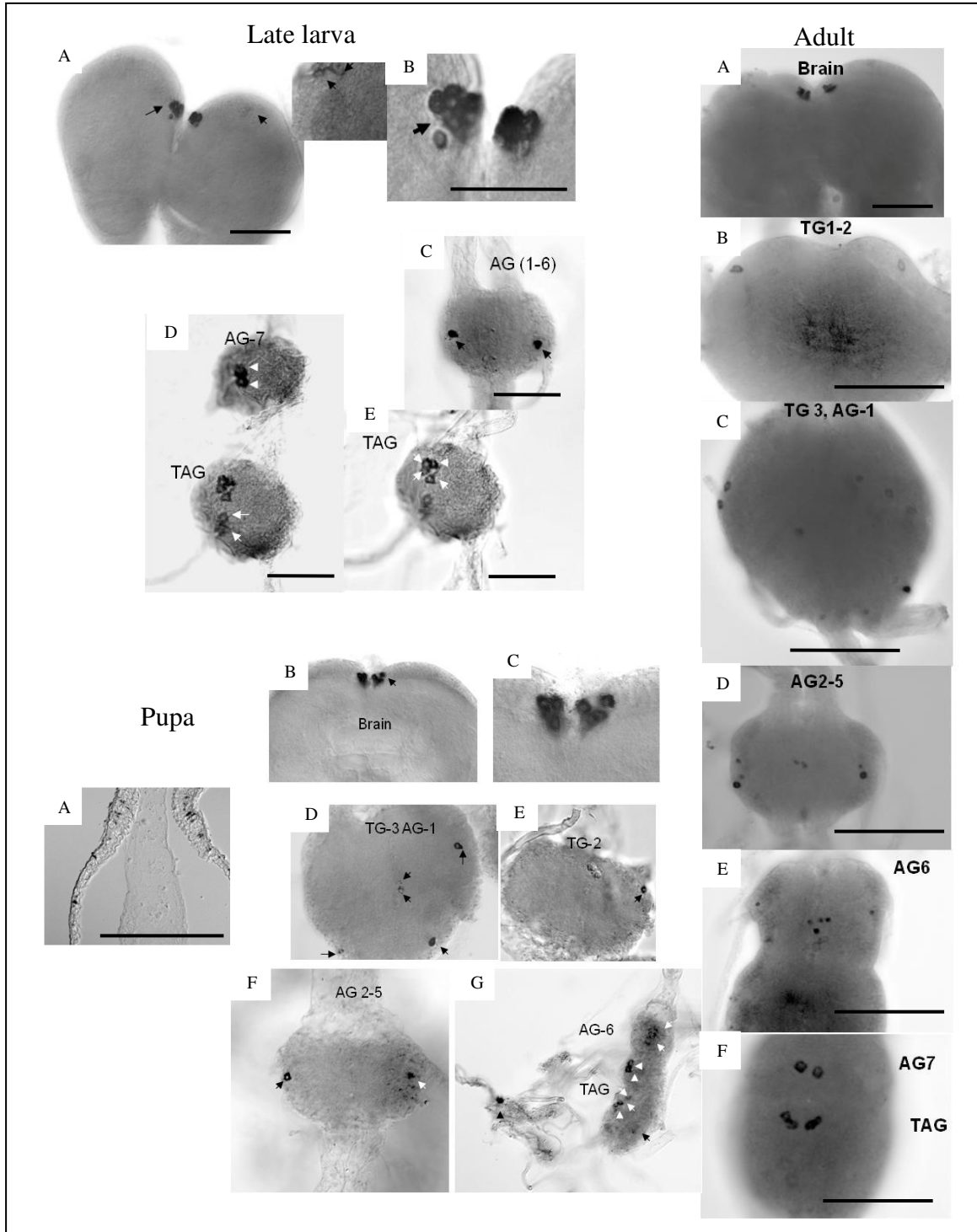


Figure 13. *In situ* hibridazation of *Tricadh37* in larva, pupa and adults of *T. castaneum*. Arrows indicate cells with strong staining. TG, thoracic

glanglion; AG, abdominal ganglion; TAG, terminal abdominal ganglion. **Larva:** A. Brain with 2 anterior centrally 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 strongly stained cells. **Adult:** A. Brain with cluster 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.

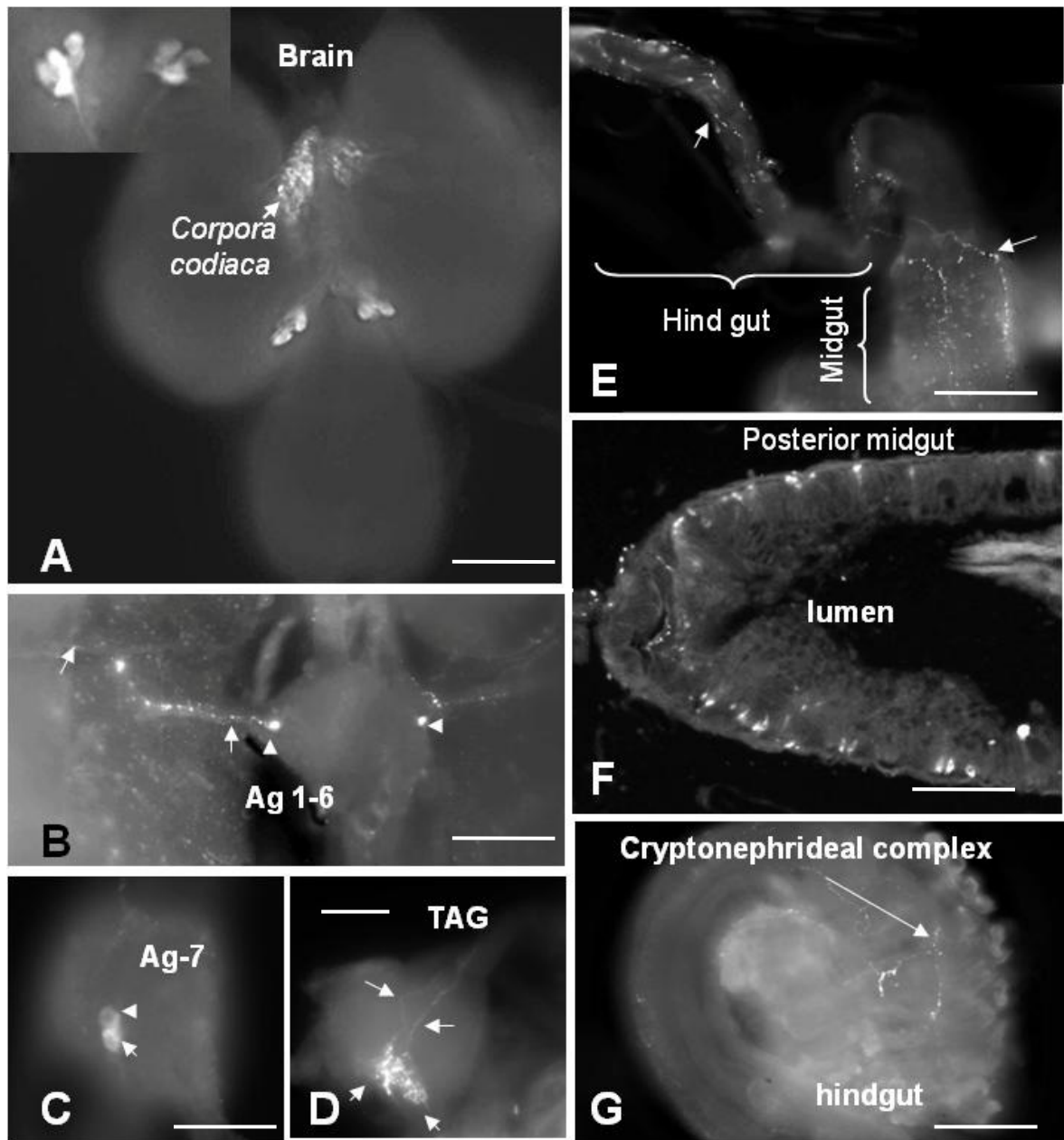


Figure 14. Immunohistochemistry of *TricaDH37* with rabbit anti-TenmoDH37. A.

Immunoreactivity in four pairs of neurons on brain and on Corpora Cardiac cells. B. Abdominal 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 abdominal 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: 100 μ m A, B, C, D and E; 50 μ m F and G.

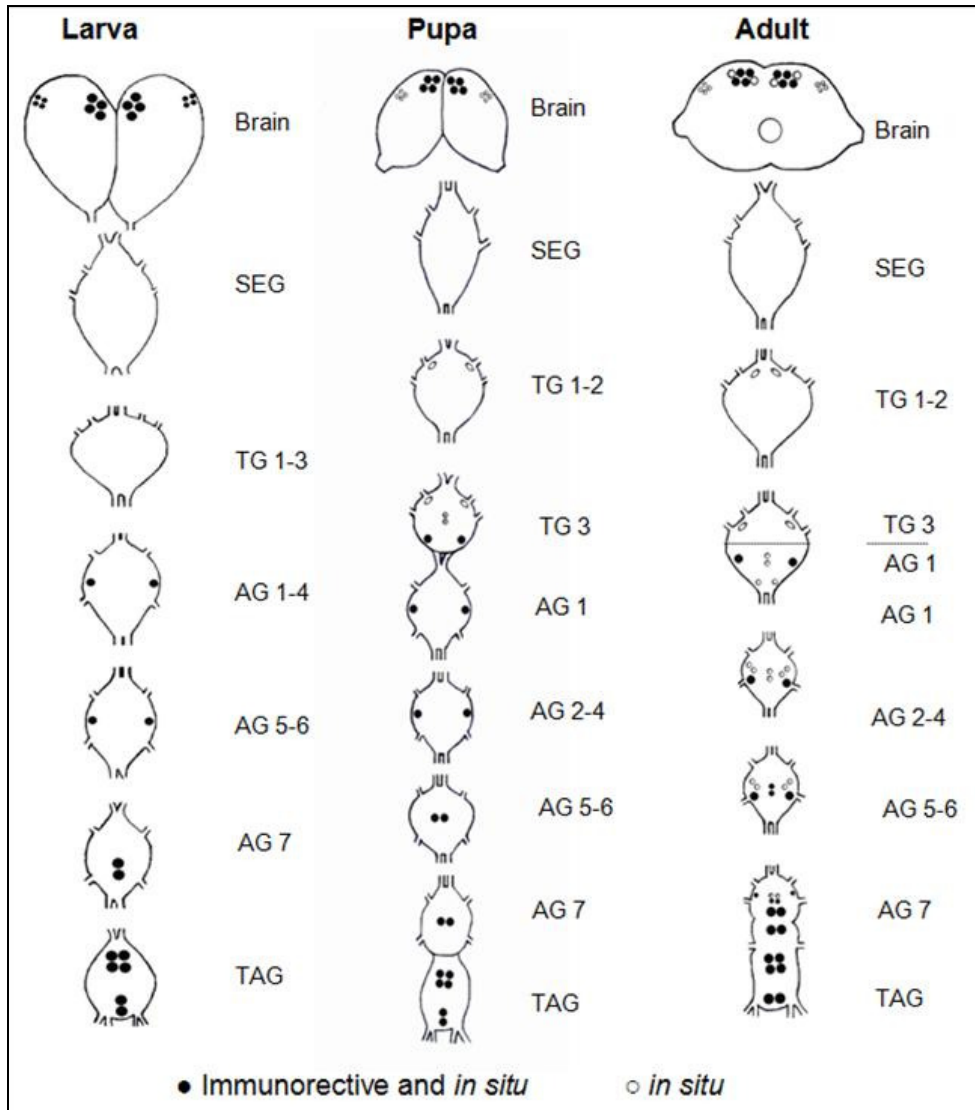


Figure 15. *T. castaneum* immunoreactive cells to rabbit anti-TenmoDH37 and cells stained with specific probes to *Tricadh37* on different stages.

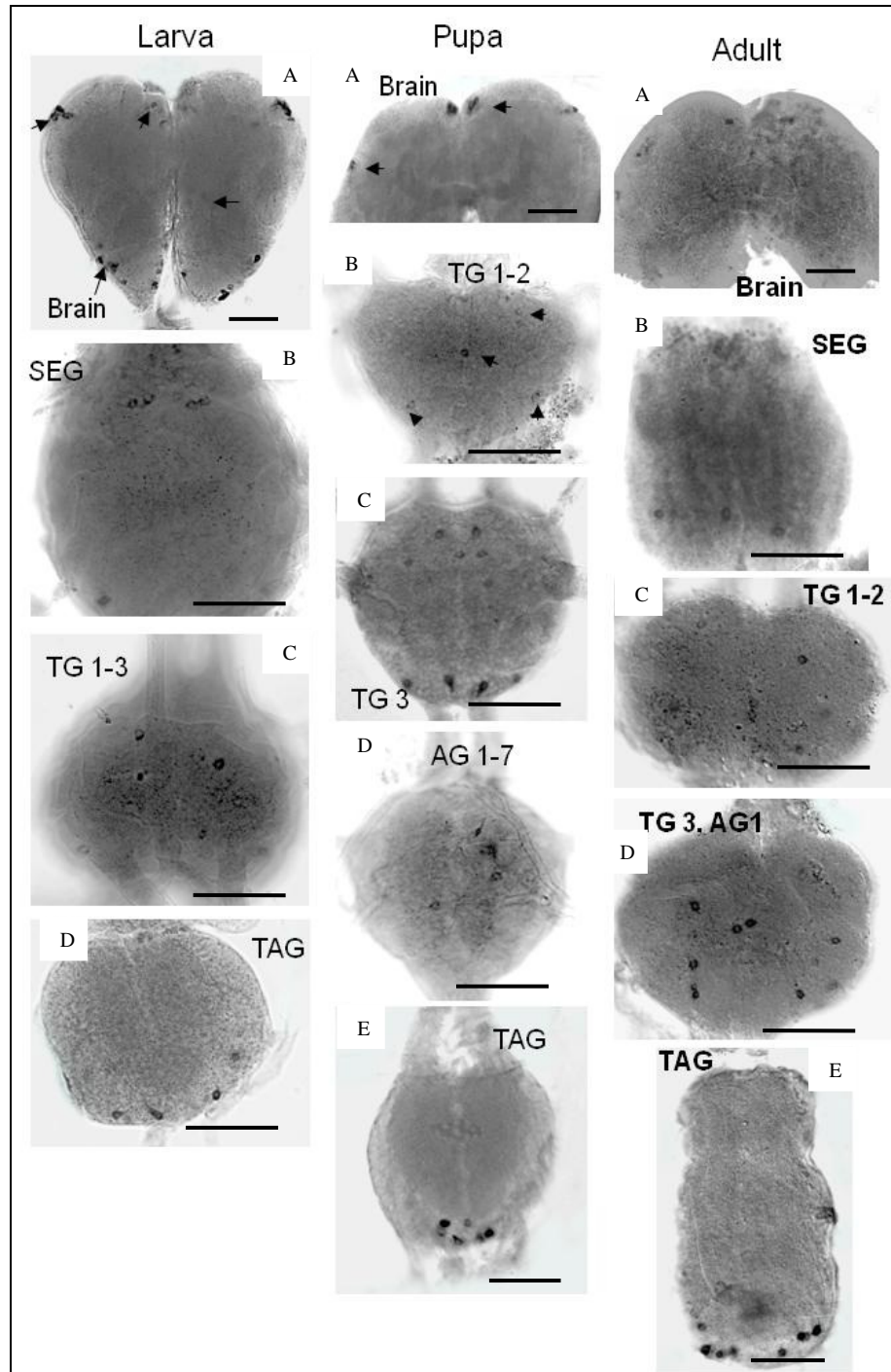


Figure 16. *In situ* hibridazation of *Tricadh47* in larva, pupa and adults of *Tribolium*. Arrows indicate cells with strong staining. SEG, subesophageal ganglion; TG, thoracic ganglion; AG, abdominal ganglion; TAG, terminal abdonominal ganglion. Scale bars 50µm.

Larva: A. Brain with 2 anterior laterally located cluster of cells strongly stained, 2 pairs centrally 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 laterally 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 strongly 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.

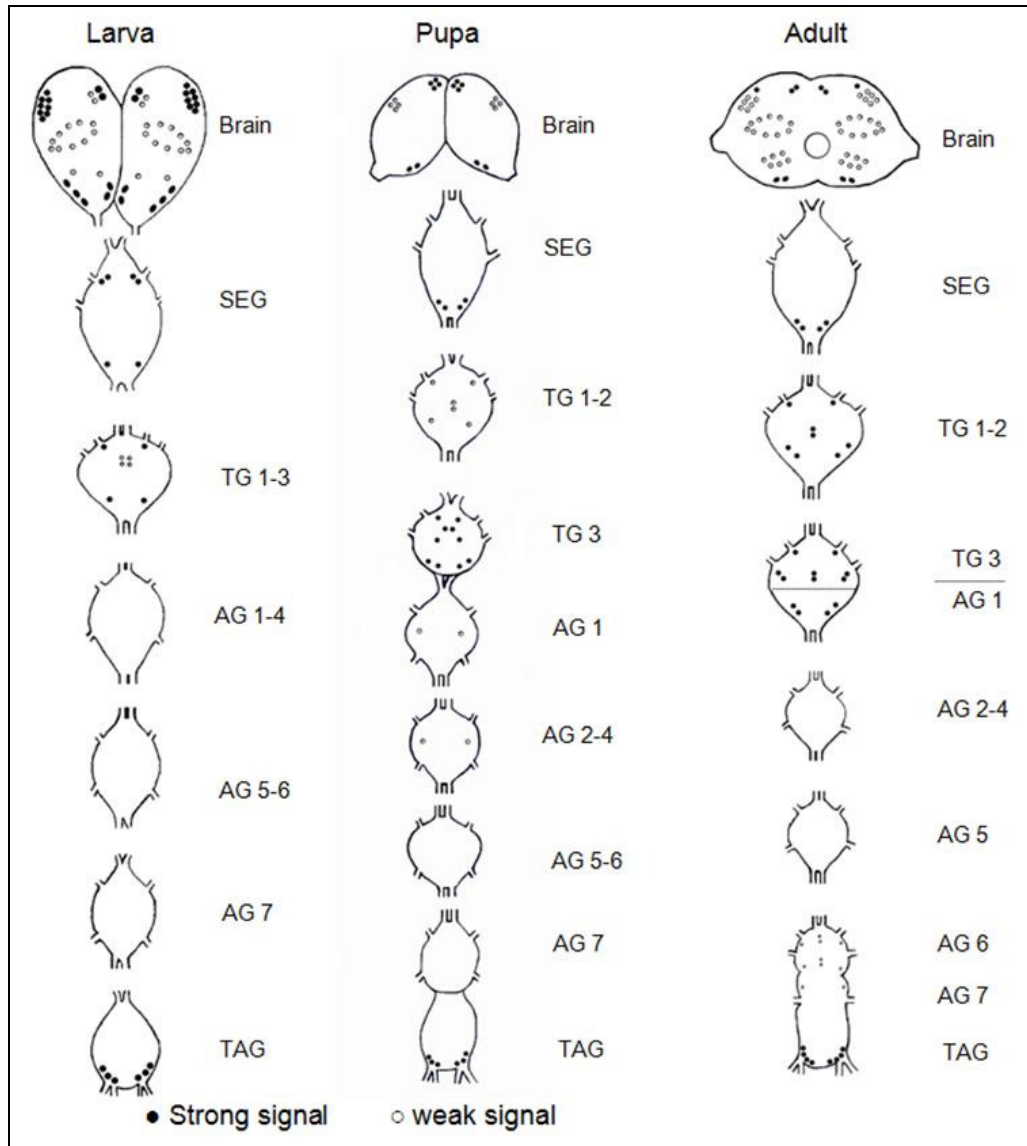


Figure 17. *In situ* hibridazation of *Tricadh47* in larva, pupa and adults of *T. castaneum*.

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.

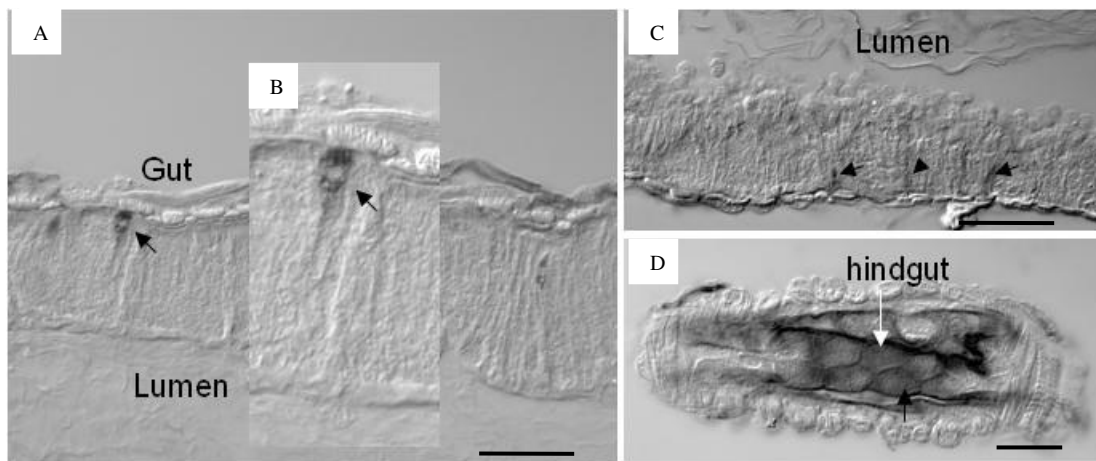


Figure 18. *In situ* hybridization of *Tricadh31* in larval gut. Arrows indicated cells stained. A. Gut; B. Detail showing staining; C. Lumen; D. Hindgut. Scale bars 50 μ m.

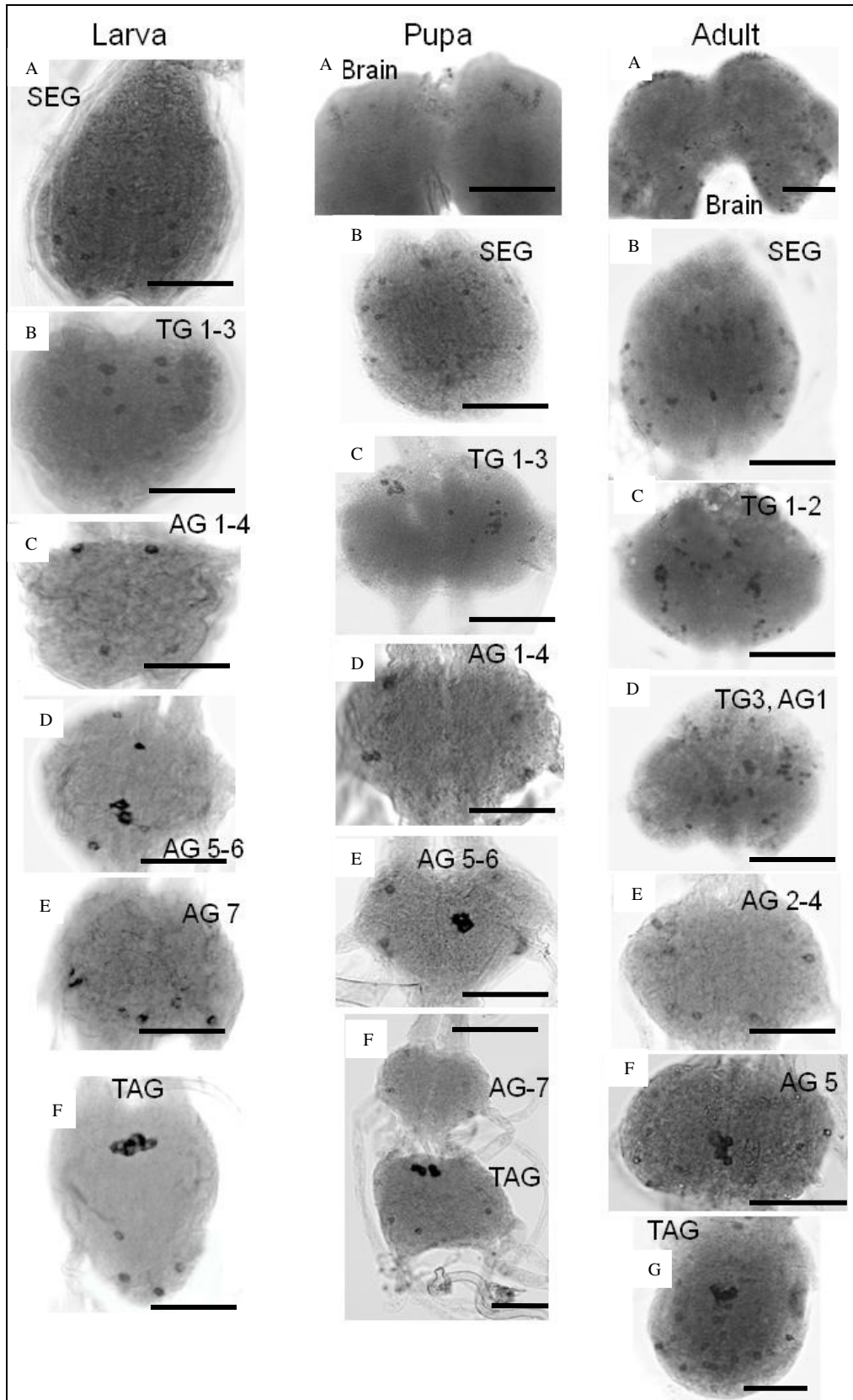


Figure 19. *In situ* hibridazation of *Tricadh31* in larva, pupa and adults of *T. castaneum*. SEG, subesophageal ganglion; TG, thoracic glanglion; AG, abdominal ganglion; TAG, terminal abdonominal ganglion. Scale bars 50µm. **Larva**: A. SEG with 8 pairs of cells stained; B. Represents TG 1-3 showing 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.

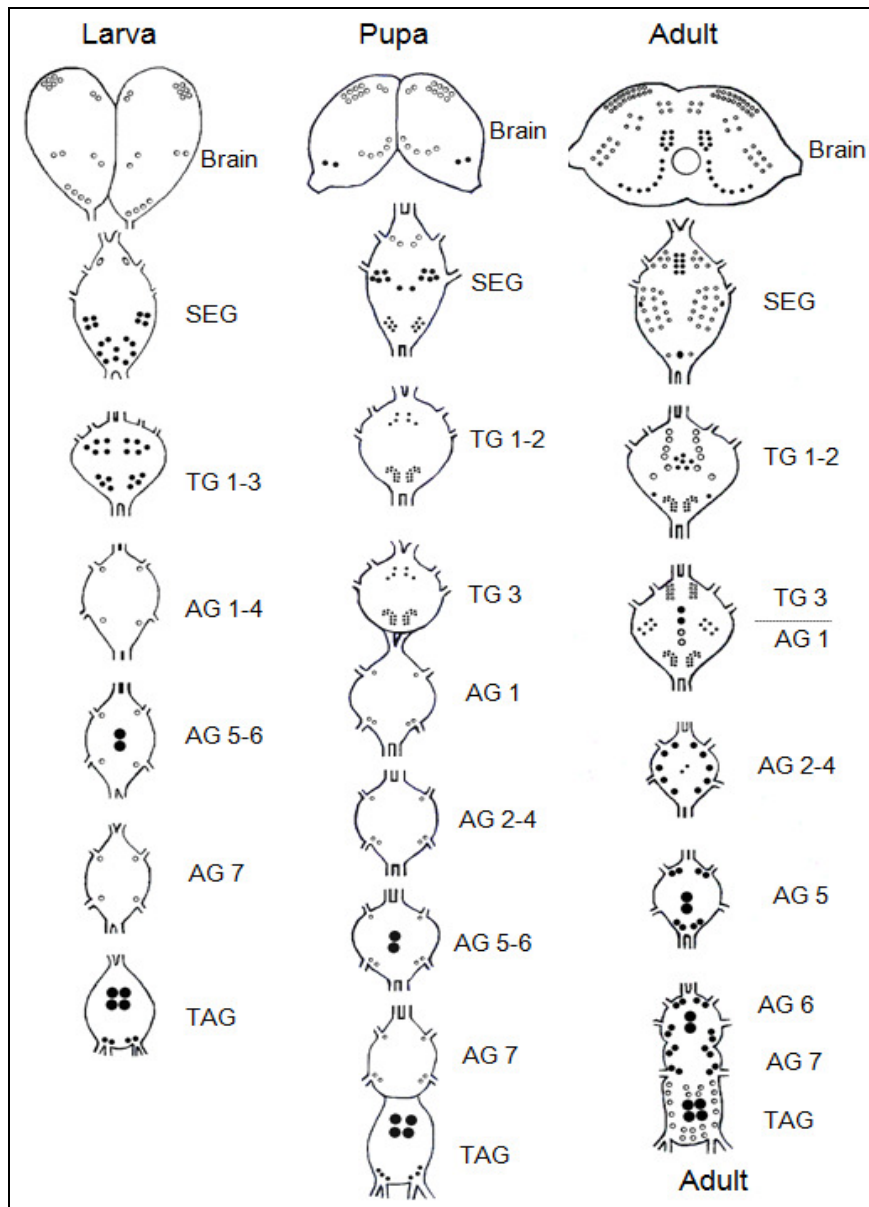


Figure 20. *In situ* hibridazation of *Tricadh31* in larva, pupa and adults of *T. castaneum*.

***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-crfr2* 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).

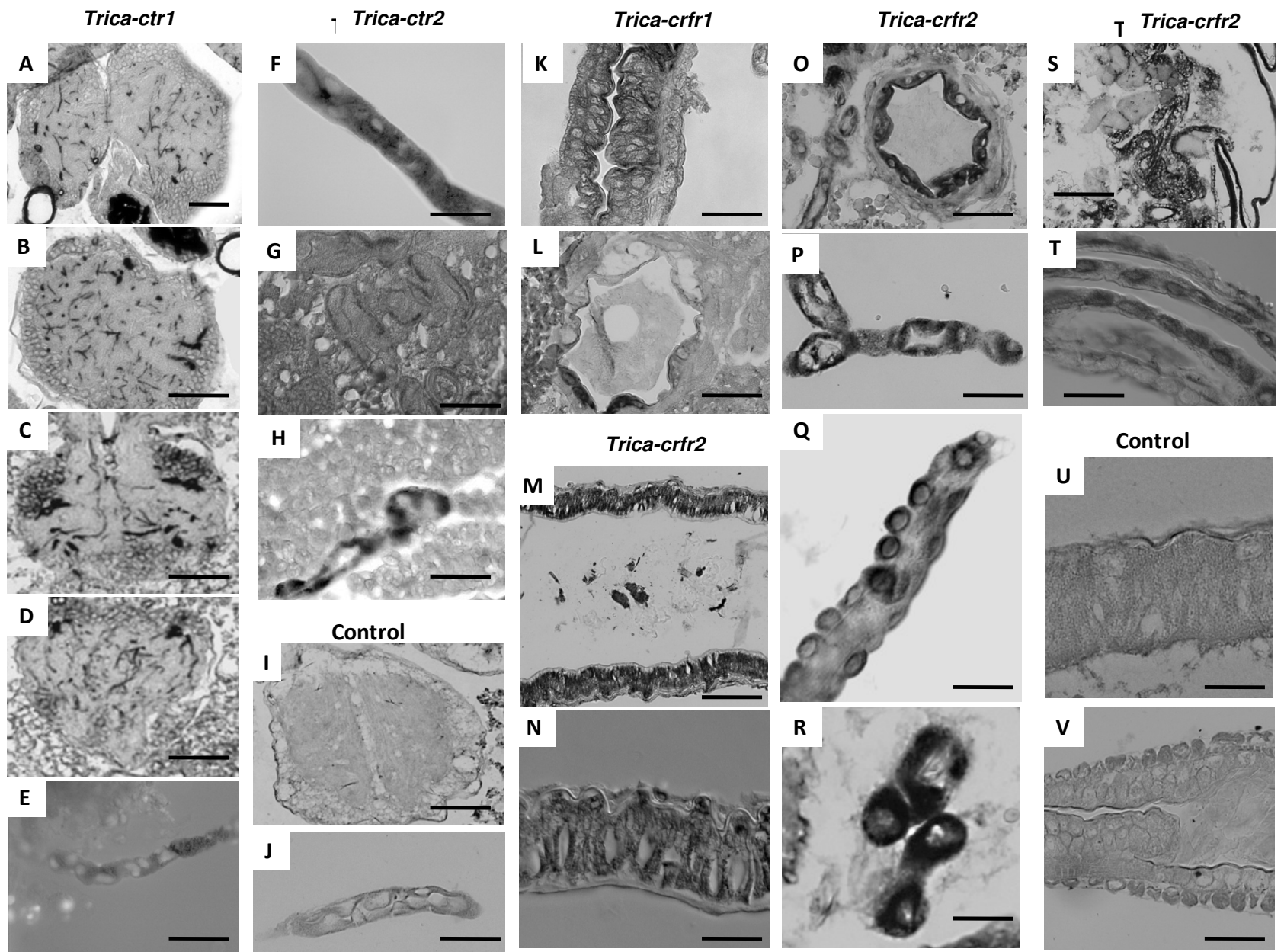


Figure 21. *In situ* hybridization of diuretic hormones receptors of *T. castaneum*.

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. Ileum 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
<i>Trica-ctr1</i>	+	+	
<i>Trica-ctr2</i>		+	
<i>Trica-crf1</i>			+
<i>Trica-crf2</i>			+

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 EC₅₀ was not possible. TricaCRFR1 responded to TenmoDH37 ranging from 2nM to 4000nM (Figure 22). The EC₅₀ 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 EC₅₀ could not be calculated (Figure 23A).

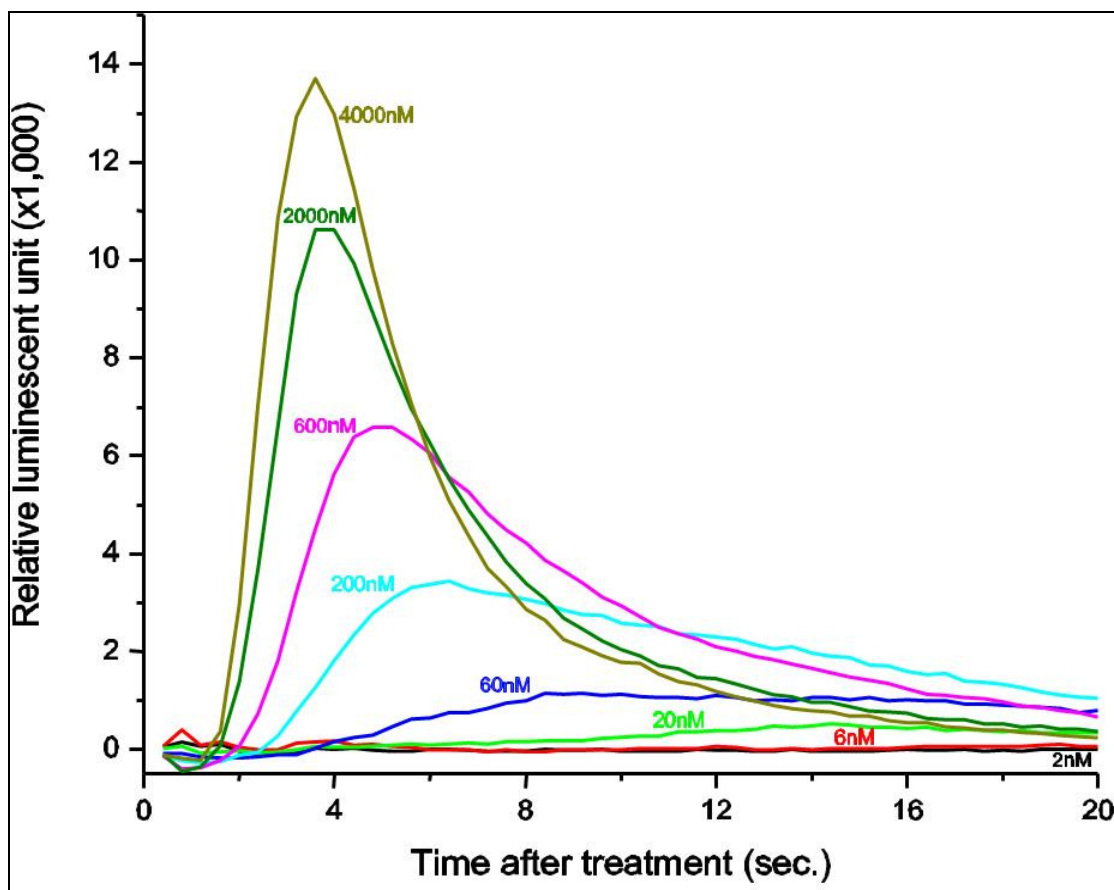
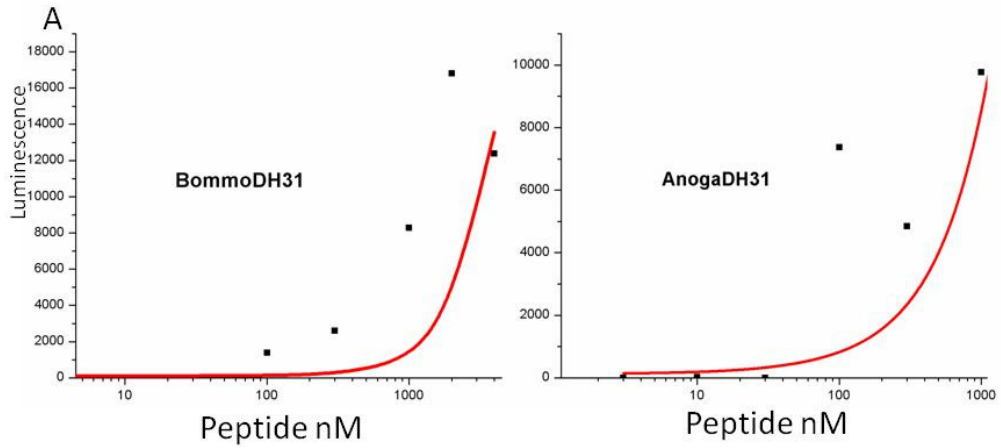


Figure 22. Luminescent reporter assay showing typical responses to varying doses of ligand TenmoDH37.



B

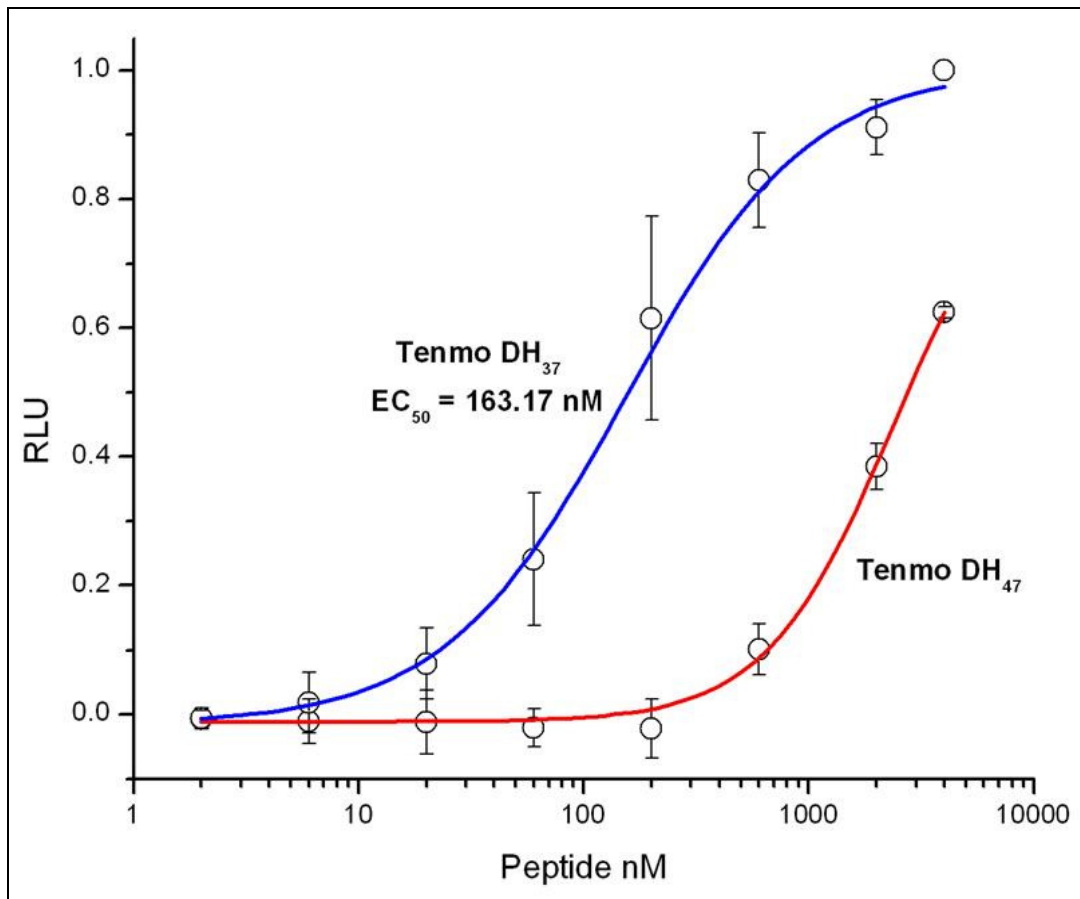


Figure 23. A. *Trica-ctr1* response to BommoDH31 and AnogaDH31, graphs represent average of 3 plates. B. Dose-responses of *Trica-cfr2* to

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 1 μ M (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.

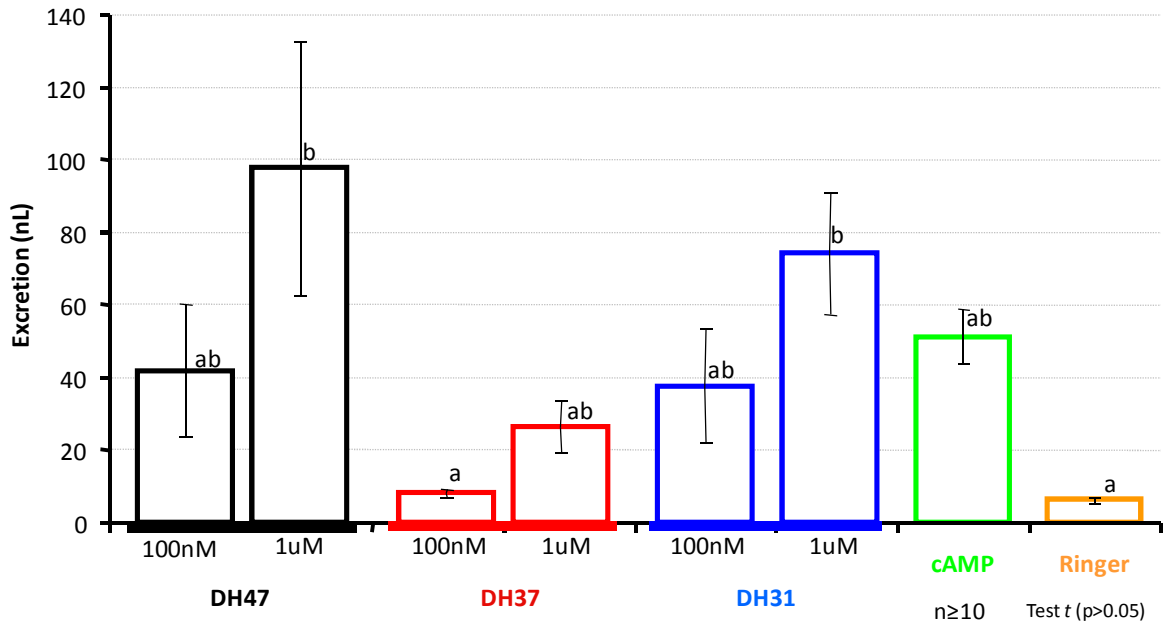


Figure 24. *In vivo* excretions (nL) induced by injections of TenmoDH47, TenmoDH37 and TricaDH31 (50nL) at different concentrations *T. castaneum* adults (10 to 15 days old). The 8-Br cAMP and Ringer's solutions were the positive and negative controls, respectively. The data were obtained for 15 minutes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

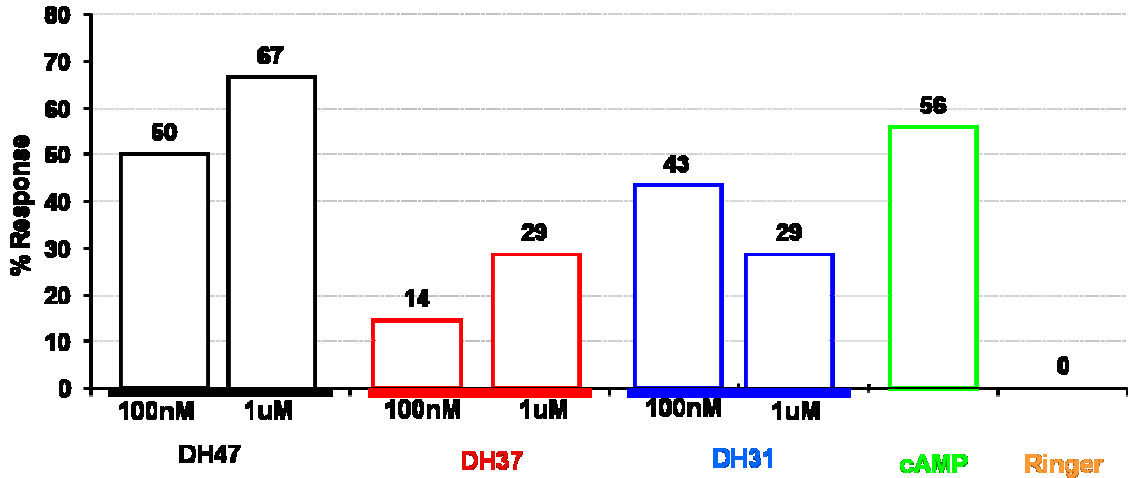


Figure 25. Percentage of response (excretion higher than negative control) of *T. castaneum* (10 to 15 days old) adults to injections of TenmoDH47, TenmoDH37 and TricaDH31 (50nL) at different concentrations. The 8-Br cAMP and Ringer's solutions were the positive and negative controls, respectively. The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

The excretion patterns among insects and according to neuropeptides injected is variable. TenmoDH47 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.

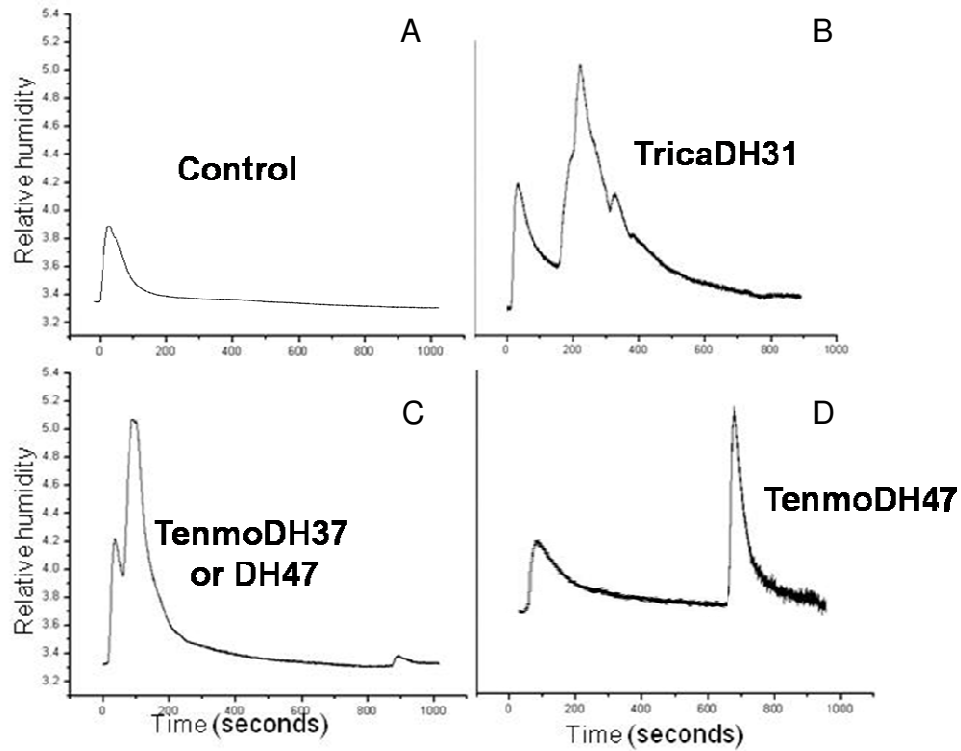


Figure 26. Types of excretions induced by diuretic hormones in *T. castaneum*. A. No response; B. High excretion 5 min after injection of the peptides. C. Two excretions peaks, one before 5 min and one between 10 and 14 min after injection; D. Only one excretion peak between 10 and 14 min after injection.

RNA interference of DH genes and their receptors

Injections of dsRNA of *Trica-crf1* in the last larval instar increased the duration of the pupal stage (Figure 27A), however *Trica-crf2* 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

down almost 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 flattened, 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.

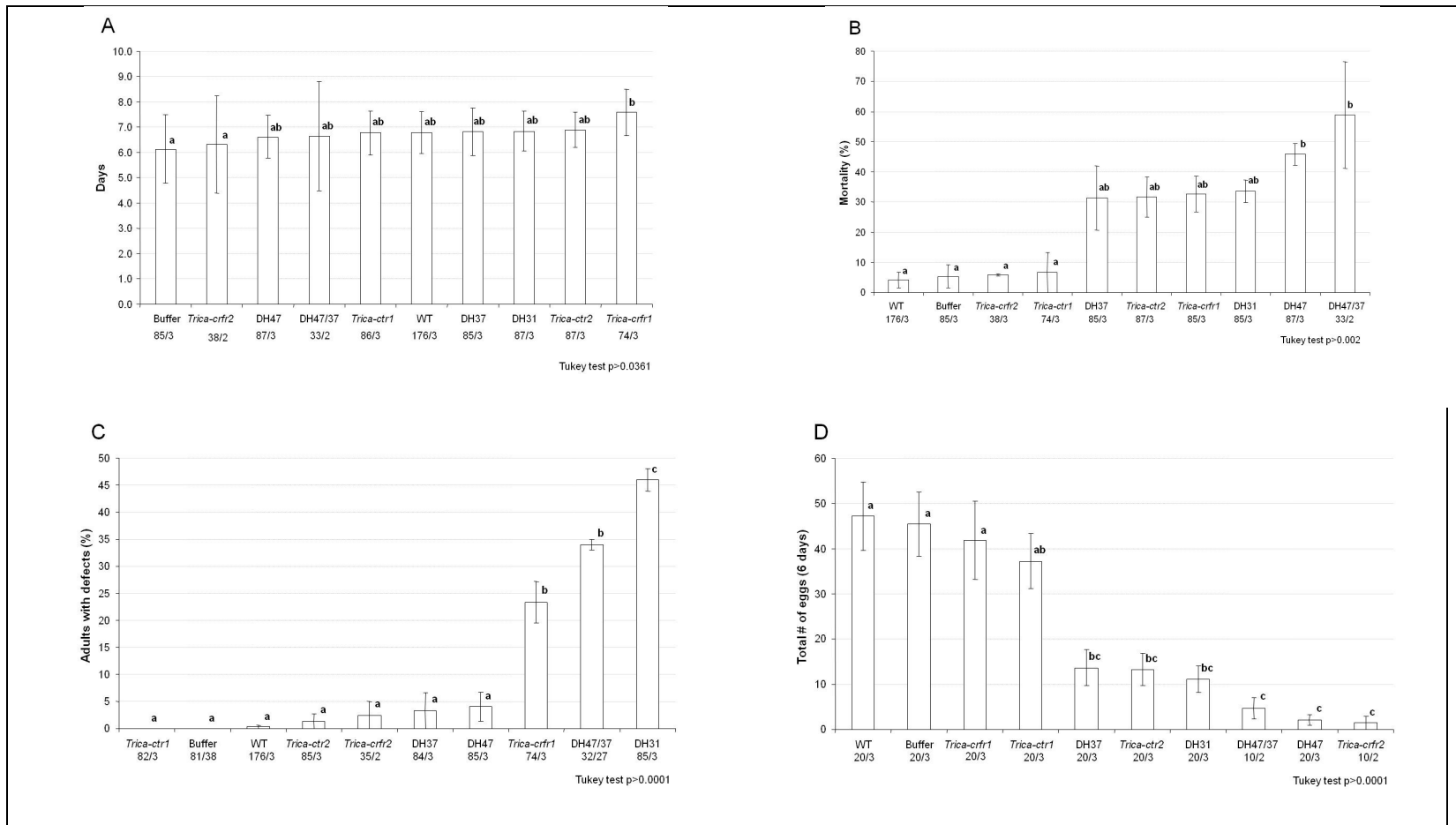


Figure 27. Effects of dsRNA injections of diuretic hormone genes and receptors in *T. castaneum*. A. Duration of pupal stage. B. Total mortality after one month of adult emergence. C. Percentage of adults that emerge with defects. D. Total number of eggs collected 6 days from one couple.

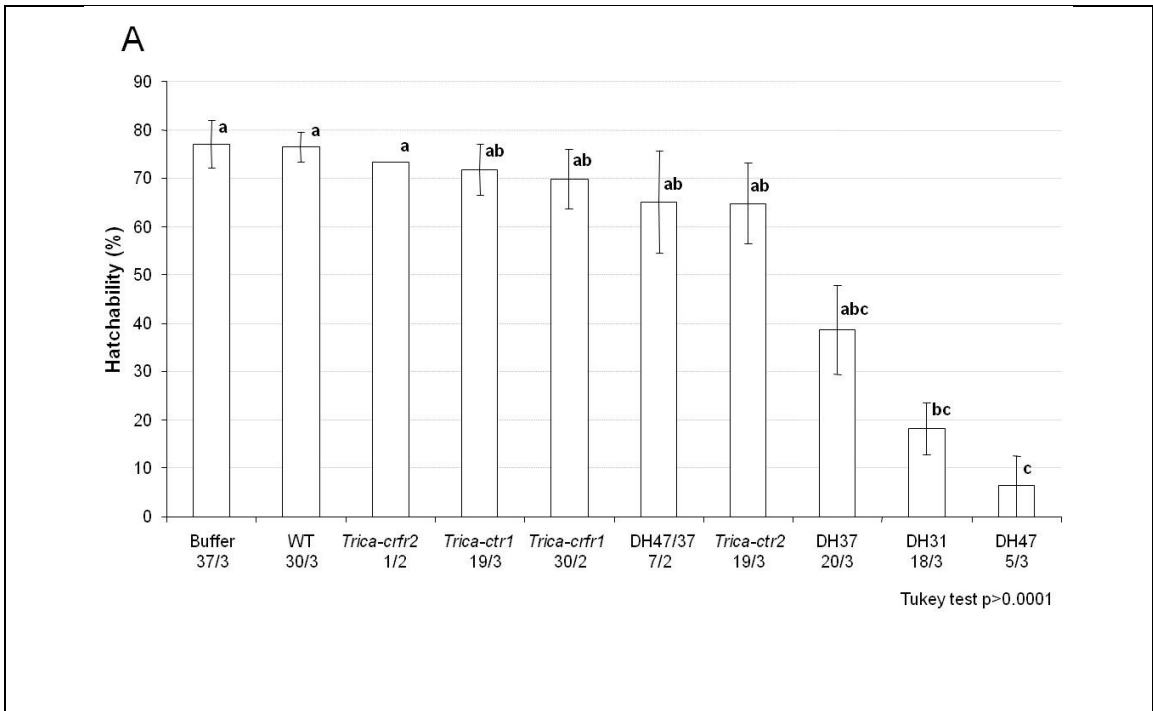


Figure 28. Effects of dsRNA injections of diuretic hormone genes and receptors on the hatchability of eggs of *T. castaneum*.

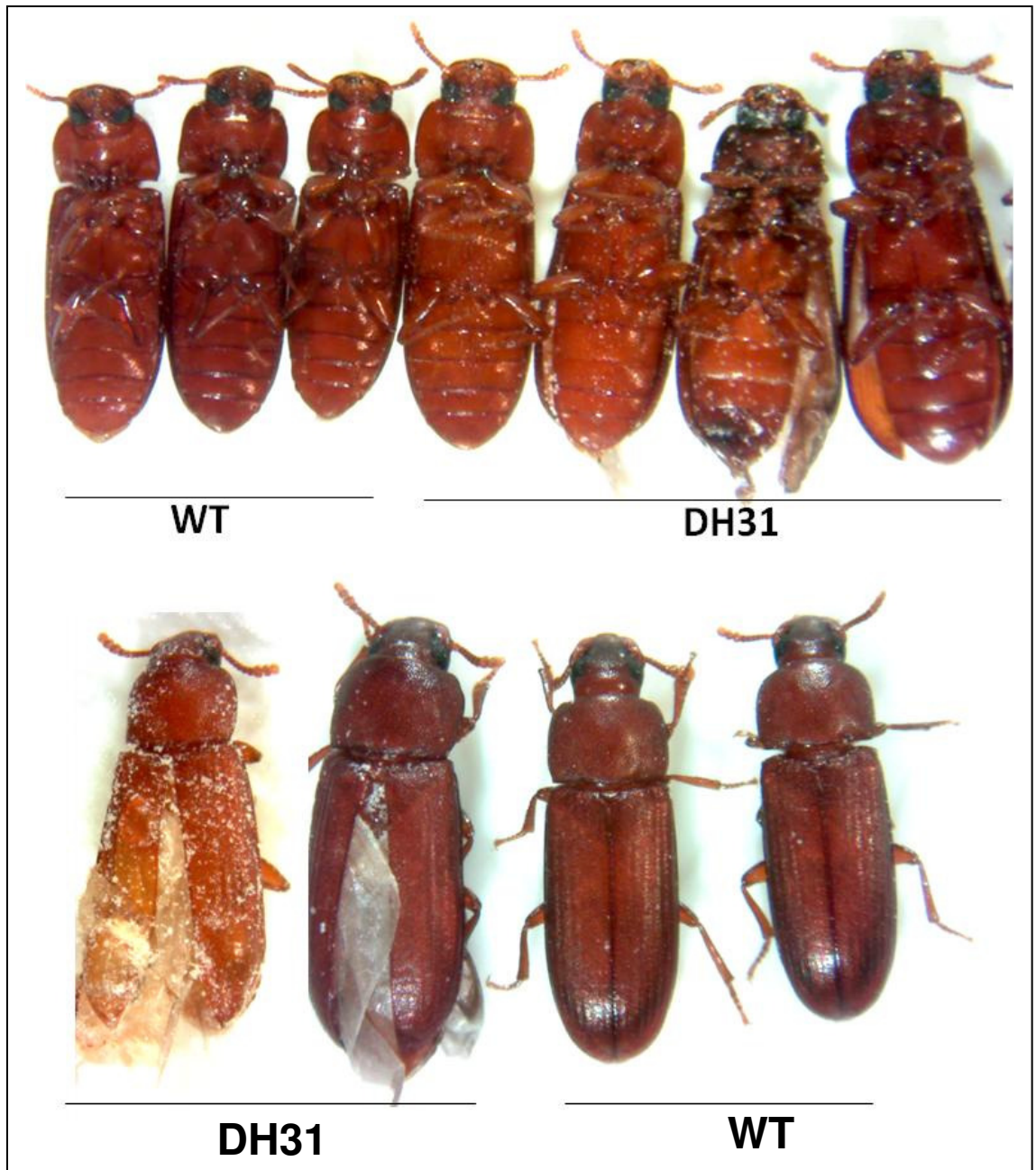


Figure 29. Morphological defects observed in adults when *Tricadh31* dsRNA was injected in the last larval instar of *T. castaneum*.

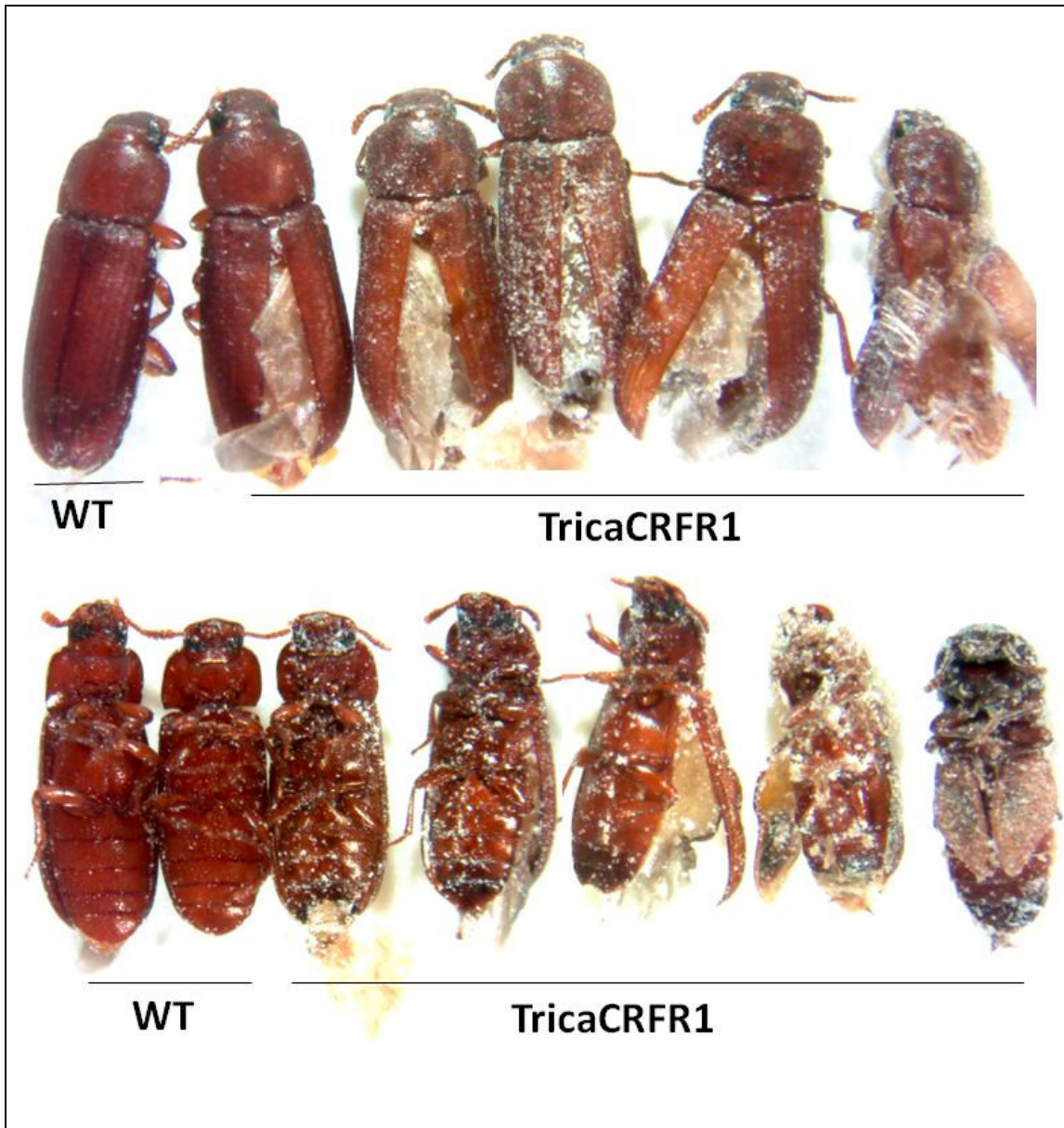


Figure 30. Morphological defects observed in adults when *Trica-crf1* dsRNA was injected in the last larval instar of *T. castaneum*.

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

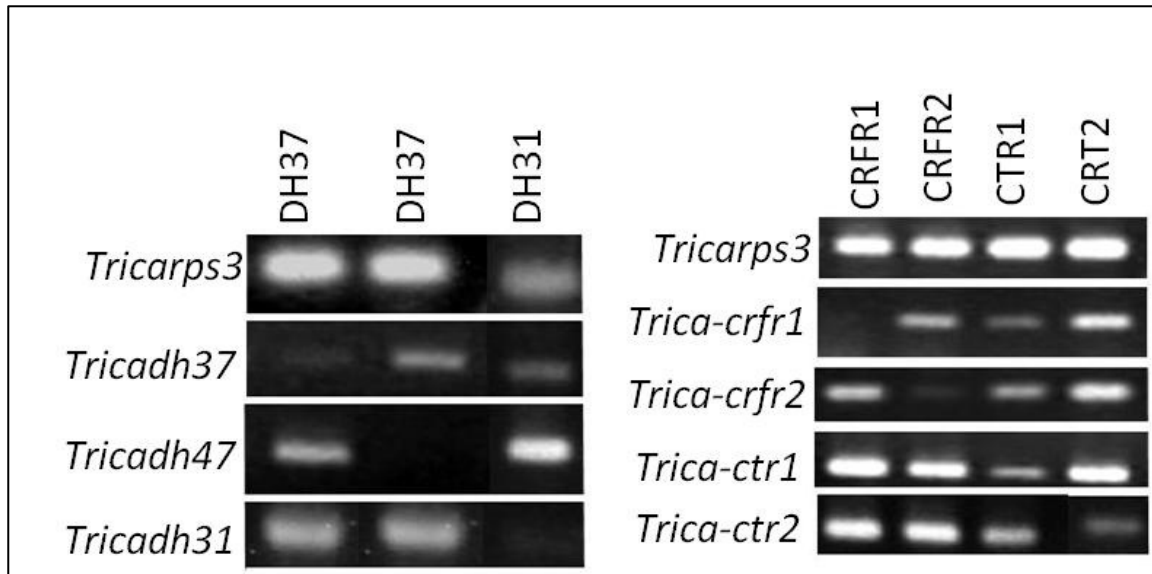


Figure 31. RT-PCR showing the suppression of mRNA levels in dsRNA-injected *T. castaneum*. Each lane contains the RT-PCR product of three individuals treated with dsRNA after 35 PCR cycles. The picture is representative of three biological replications.

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 *Rhodnius prolixus* (Te Brugge et al., 2005). Immunoreaction in *R. prolixus* was found in cell bodies and processes throughout the CNS, hindgut, in the salivary innervation 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 hemolymph 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 diuresis 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 immunohistochemistry. Only antibodies raised against TenmoDH37 were available. Similarly to *D. melanogaster* 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 neurosecretory 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 known where leucokinins are expressed in *T. castaneum*. Similar to *D. melanogaster* (Cabrero et al., 2002) the number of neuroendocrine cells expressing 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 innervating TricaDH37 in the midgut, hindgut and cryptonephridial complex; however we did not find 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 continuously 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 diuresis 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 clustered 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 neuropeptides 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 diuresis. We found staining in the aorta next 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 sensitization were high and we could not calculate the EC₅₀.

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-cfr1* 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-cfr2* 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 addition 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-crf1*. 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.

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Appendix

Appendix 1

AedaeCRFR1	1	SSS.....	..ASVESLLA	LAG.....	.AGGDFAGSG	SG.....	...DG
AedaeCRFR2	1	SSS.....	..ASVESLLA	LAG.....	.AGGDFAGSG	SG.....	...DG
AnogaCRF1	1	ETM.....	..PTPTAAA	IAADD....	GSGGSPFEGG	NVTG	SVENP SLVEG
DromeCG12370	1	DDLRALVDSL	DDASQEDLAK	VIANFVSDML	QRASALIGAQ	QG...	SSGGQ LQNRT
DromeCG8422	1	HNHIDSVNAS	GS.....DPLLDLH	NLDG	G.....
AnogaCRF2	1	GIISNSIKPS	GQRTPITLI	RLQMDKIOPH	ASGKRLVSVV	DYDEL	SSFPF ELLGA
TricaCRFR2	1	GES.....EVVYAR	HEDI	R....
TricaCRFR1	1	SEPLP.....	.QEPEPVDAD	LWP.....	.SSENVINET	EDIK	R....
consensus	1		s s	l g	g lig g	i	g

AedaeCRFR1	28	FPPMI	LNETLRDLC.	.HQQNDTLFL	ELSCPPLFDS	ISCWPRTPPA	TTAVLPCFSE
AedaeCRFR2	28	FPPMI	LNETLRDLC.	.HQQNDTLFL	ELSCPPLFDS	ISCWPRTPPA	TTAVLPCFSE
AnogaCRF1	42	LLALA	MNAGAEERC	LQQAEELLQ	DVACPSFDM	VSCWPRTPPG	TLAVLPCFAE
DromeCG12370	53	LQCQQ	QQQREEQAS	LEALASGGKR	ILQCPSSFDS	VLCWPRTNAG	SLAVLPCFEE
DromeCG8422	26	..ESV	ELQCLVQEH.	.IEASTYND	SGHCLTQFDS	ILCWPRTARG	TLAVLQCMDE
AnogaCRF2	56	FENST	EFCILRAH.	.QDAQS.ETP	SGGCPVDFDR	ILCWPRTPPG	TVAVLPCFEE
TricaCRFR2	16	.ILNR	LNEIQAEFLAC	.ELKKTLSPP	SNGCAVFDI	VLCWPRTPPN	SLAVLPCFDE
TricaCRFR1	33	.LNT	LOHCTSLYTN	RTALAEHP	DGFCPVTTDG	ILCWPRTPIN	ETTYVKCFAE
consensus	61	m	lnetl d	s	el Cp	fdS ilCWPrTppg	tlavlPcfEE

AedaeCRFR1	81	FKGVQYDATQ	NATRYCNIDG	TWNSFANYDA	CKHLE.LPSS	DPTLESFIEL	PIVI
AedaeCRFR2	81	FKGVQYDATQ	NATRYCNIDG	TWNSFANYDA	CKHLE.LPSS	DPTLESFIEL	PIVI
AnogaCRF1	97	LKGVQYDSSQ	NATRFQVNDG	TWDNYTDYDR	CEHLE.QPPP	LPSEFPEIEL	PTII
DromeCG12370	108	FKGVHYDITD	NATRFQVNDG	TWDHYSYDR	CHONGSIPV	VPDFSNVEL	PAII
DromeCG8422	77	LQGLHYDSSK	NATRFCHANG	TWEKYINYDA	CAHLP.APES	VPEFVIVEL	PTII
AnogaCRF2	108	FKGVHYDVTQ	NATRYCHPNG	RWDNYSHYAA	CHHVN.EPPP	D....IVEI	SSII
TricaCRFR2	69	LNGIKYDRE	NATRLCFANG	TWDQYSNYTS	CKELS..PLE	VP....EVEL	TTTT
TricaCRFR1	87	LMNIRYDDTQ	NATRVCLANG	TWTKADYYSK	CTEII.....	...LIPD	VET QATI
consensus	121	kgvhYDstq	NATRYC	nG tWd ytnYda	Ckhl	p lp e vEl	ptvI

AedaeCRFR1	134	YFVGYT	ISLLALFCAV	TVLVYFKELR	CLRNTIHVNL	FVTYIMSSSL	WIIILSQQL
AedaeCRFR2	134	YFVGYT	ISLLALFCAV	TVLVYFKELR	CLRNTIHVNL	FVTYIMSSSL	WIIILSQQL
AnogaCRF1	150	YFVGYT	ISLAALVIAV	AVLVYFKDLR	CLRNTIHVNL	FLTYIMSSSL	WILILSLQI
DromeCG12370	162	YAGGYE	LSFATLVVAL	IIFLSFKDLR	CLRNTIHANL	FLTYITISALL	WIIITFLQV
DromeCG8422	130	YYIGYT	LSLMSLSLAV	IVFAYFKELR	CLRNTIHANL	FFTYIMSALF	WIIILSVQI
AnogaCRF2	156	YYTGYI	LSLVALSLAV	IVFVYFKDLR	CLRNTIHANL	FITYIISALL	WIIILTLQI
TricaCRFR2	117	YFVGYT	ISLVALFCAV	YFVFKFKDLR	CLRNTIHMNL	MCSYILADFM	WIFVYSLOW
TricaCRFR1	132	YFVGYV	LSLITLSLAL	GIFTYFKELR	CLRNTIHMNL	MWSYMLMYIM	WIIITLTVLG
consensus	181	YfvGyt	lslal lAv	vfvyfKeLR	CLRntIHvNL	fvtyims	l1 WIIilslql

AedaeCRFR1	189	A AKQG...LVD	CIFLVTLFHY	FSTTNFFWML	VEGLYLYMLV	VQTFSGDYLR	FWK
AedaeCRFR2	189	A AKQG...LVD	CIFLVTLFHY	FSTTNFFWML	VEGLYLYMLV	VQTFSGDYLR	FWK
AnogaCRF1	205	T VKLE...VAG	CIFLVTLFHY	FSTTNFFWML	VEGLYLYMLV	VQTFSGDILR	FRK
DromeCG12370	217	I TTES..SQAG	CITLVIMFOY	FYLTNFFWME	VEGLYLYTLV	VQTFSSDNLIS	FII
DromeCG8422	185	S IIS..G.VGS	CIALITLFEH	FILTNFFWML	VEGLYLYMLV	VKTFSGDNLR	FNI
AnogaCRF2	211	S SGSSTG.MTS	CVIFVTLFHY	FILTNFFWML	VEGLYLYMLV	VETFSGDNLNR	FNM
TricaCRFR2	172	P LQTN...KAF	CIFLITLHLY	FHLTNFFWME	VEGLYLYTLV	VKTFGENIK	PRI
TricaCRFR1	187	S KGGTG.ASIA	CIFVITLHLY	FHISTFFWME	VEGLYLYTLV	VEITLRENYK	LRV
consensus	241	t k	la Ciflvtlfhy	FsltnFFWml	VEGLYLYmLV	VqTfsgdnlr	f i

AedaeCRFR1	240	YSIIGWG	GPIIFVGAWA	IAKSFVEYDL	MPGHPNKLEI	ECSWMRESHI	DWIIQGPS
AedaeCRFR2	240	YSIIGWG	GPIIFVGAWA	IAKSFVEYDL	MPEHPNKLEI	ECSWMRESHI	DWIIQGPS
AnogaCRF1	256	YAIIGWG	GPIIFVGAWA	IAKPFVFGSVS	NLEHPSKLEI	ECSWMRESHI	DWIIQGPS
DromeCG12370	269	YALIGWG	QPAVCIIVWS	IAKAFVHLE	N.EHFNGLEI	DCAWMRESHI	DWIFKVPA
DromeCG8422	236	YASIGWG	GEALFVITWA	VAKSLT...V	TYSTPEKYEI	NCPWMQETHV	DWIIQGPV
AnogaCRF2	264	YAAIGWGKCSRASLEI	ECSWMRESVY	DWIFQGPV
TricaCRFR2	223	YAVIGWG	GPIIFVIVWG	IAKSFTLP.	EDQQAGEMFR	SCPWTPHP.F	DWIIQGPA
TricaCRFR1	240	YVCIGWG	IPMIFILVWV	IVKSETE...	...AAGDPA	TCIWFNSHDV	DWIFQGPT
consensus	301	YaiIGWG	gplifvl wa	iaksf p l	hp klei	eCsWmreshi	DWIfqgPs

Alignment of CRF-like receptors from Aedae (*Aedis aegypti*), Anoga (*Anopheles gambiae*), Culqu (*Culex quinquefasciatus*) 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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TricaCTR1      1 ..... .MDVT
DromeCG4395   1 SFSLGPATDY DSDLPENFSP VPRYLENAAM NEGVIDMRNV DEELAEKEEL MATVV
AnogaCTR1     1 .....
AedaeCTR1     1 PNQONNFTAY QNTKNWKNIS QCVMFPMKIS FAFFALLM..LSIFTAVT VLRSI
CulquCTR1     1 HTSQDGDIOY DRLKHADPEA LMRHLYDCM QDGSGRAP..SLEQAALK SYDTN
CulquCTR2     1 .....MSSST TTEASVDVS....ELKRLE CLAKL
AedaeCTR2     1 .....MHHM
AnogaCTR2     1 .....AKHRYK PTRGTLQVTN PRITPFTALN VEPHI
TricaCTR2     1 .....MAFD YSPRDLEVI....ALRQLE CDETI
consensus     1 .....v v

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TricaCTR1      5 NGTNC GGKYTRPGYC PEIFDEMLCW PETLGG....TIVNCSCKP KMG..YDSRR
DromeCG4395   56 SATMA TNQKENRLFC PLNFDGYLCW PRTIPAG....TVLSQYCPD FVEG.FNRKF
AnogaCTR1     1 .....GSTVFC PRRFDGWSCW EPTLAG....TVAENWCCK FVLG.FDPRR
AedaeCTR1     52 SASNA RHDQQTQLFC PRRFDGWTCW ESQPAG....TIAQNFQCN FVLG.FDASR
CulquCTR1     52 QPQDL HDDQQQQLFC PRRFDGWTCW DAQPAG....TVAENYCPN FVLG.FDANR
CulquCTR2     26 NESVE SASDSDQLFC RGTWDGWQCW EDTAAG....RIAYAPCPE FVFG.FDTTR
AedaeCTR2     5 KVATH CSPKLSRPF C RGTWDGWLCW EDTAAG....ESALLPCPD FMDG.FDPTR
AnogaCTR2     32 RFPTK LSSSRVKTTS SPQPRNSMAL STTTDASVDE IAQKRLECLE TLNETVDTTI
TricaCTR2     25 NKSNN ILGR...YC PGRFDGWSCW ESTPAG....ETIANQSCPE ILNY..DPNR
consensus     61 t lfc p fdgw cw p t ag tva q Cpd fv g fd r

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TricaCTR1      53 ..FAHKDCLE NGSWFKHKPS GKIWNYTTC VDHEDLAFRT HINHLFVIGY SISL
DromeCG4395   105 ..LAHKTCLE NGSWFRHPVS NQIWSNYTNC VDYEDLEFRQ FINELYVKGY ALSL
AnogaCTR1     41 ..LAYRTCHE NGSWFVHPHS GREWSNYTNC IDTEDMQLRR LVNDIYIGGY TVSF
AedaeCTR1     101 ..LAYRICH A NGSWFHPES GREWSNYTNC IDVDMKFERR LVNDIYIGGY TISL
CulquCTR1     101 ..LAFRVNT NGSWFVHPES GREWSNYTNC VDLEDMKFERR LVNDIYIGGY TISL
CulquCTR2     75 ..FAHKACDE DGEWFRHPT NRTWSNYTTC VNIDDFTWSR QINTIYETGY SISL
AedaeCTR2     54 ..FAHKDCDE DGEWFRHPLT NRTWSNYTTC VNLDKLEWME QVRTIYETGY SISL
AnogaCTR2     87 SGFAHKVCS E NGEWFRHEET NRSWSNYTTC INLDDFEAG.....
TricaCTR2     69 ..LVFYKCEK DGEWNFNEQF NKSWNYTTC INIEDFEFRQ QILIIYCVGY GVSL
consensus     121 layk C e nGswfkhp s nrtWsnYtTc vdvedl fr in lyv gy sisl

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TricaCTR1      105 AALVIS LAIFFTFRIL KCTRIRIHIO LFISFALNNL MWIIWYKEVV PNFVVTIRN
DromeCG4395   157 LALLIS LIIFLGFKSL RCTRIRIHVH LFASLACTCV AWILWYRLVV ERSETIABN
AnogaCTR1     93 LTLIIS LCIFHSFRIL KCTRIRIHVH LFTSLALSCL FWIIVWYKEVV EDPDVNAN
AedaeCTR1     153 VTLIIV LCVFFSFRIL KCTRIRIHIN LFTSLALSCL FWILWYKEVV EDPDVNAN
CulquCTR1     153 VTLIIV LCVFFSFRIL KCTRIRIHIN LFISLALSCL FWLWYKLVV EQPEVTHRS
CulquCTR2     127 IALILS LGILSYFRSL KCARITLHMN LFASFATNNT LWLLWYRMVL ADPEVLSHN
AedaeCTR2     106 IALILS LGILSYFRSL KCARITLHMN LFASFASNNT LWLLWYRMVL ADPEVLSHN
AnogaCTR2     126 .....
TricaCTR2     121 VALLVS LAILTYFKSL RCARITVHMN LFSSFAMNNE LWLLWYSLVV NDQDVIHEN
consensus     181 laliis l if frtl kctririhin lf s al wilwyklvv dpevi rn

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TricaCTR1      160 .....ELW CQALHLVVHY IMLANYMWMF CEG
DromeCG4395   212 .....PLW CIGLHLVVHY FMLVNYFWMF CEG
AnogaCTR1     148 ..TPLTICV TVPGNSCAYK QSS...LQGW CVGLHILLHY IMLVNYFWMF CEG
AedaeCTR1     208 G VSASVTQVCC KEAKITPMFN KVFSIRFQNW CIALHILLHY IMLVNYFWMF CEG
CulquCTR1     208 .....GNW CTSLHILLHY IMLVNYFWMF CEG
CulquCTR2     182 .....GSP CITLHLVLHY FLITNYAWML CEG
AedaeCTR2     161 .....GAS CITLHLVLHY FLITNYAWML CEG
AnogaCTR2     126 ..... CITLHLVLHY FLITNYSWML CEG
TricaCTR2     176 .....KLW CRVLHVVLFT FLISNYSWML CEG
consensus     241 w Ci LHlVlhy fml NY WMf CEG

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TricaCTR1      186 LHLHLAL VVVV.....RDAETMK WFFALGWGAP FI.....I

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DromeCG4395 238 LHLHLVL VVVFV..... ..KDTIVMR WFIIVSWFSP IP.....I
 AnogaCTR1 196 LHLHLVL VIVSIRAHPG DIPRGRLEC. ..HRATISQG WCRLPEFSVR FNSIRLIF
 AedaeCTR1 262 LQLHLVL VIVMQSVPP. .LQRCCIYSN FQWKLNITER RPSFLGGNEN LSVYYEQA
 CulquCTR1 234 LHLHLVL VIVFI..... ..KDAIAMR WFMFIGWIVP MG.....L
 CulquCTR2 208 FYLHTVL VSAFVS..... ..EKKLVK WLVVLGWIVP AC.....V
 AedaeCTR2 187 FYLHTVL VSAFVS..... ..EKKLVN WLVVLGWIVP GI.....V
 AnogaCTR2 149 FYLHTVL VSAFVS..... ..EKKLLK WLLALGWGSP AF.....F
 TricaCTR2 202 IYLHTVL VSAFIS..... ..ERRLLR CMLALGWGIP LL.....T
 consensus 301 l LHLvL Vvfvv rd vmk w i lgw p
 iTricaCTR1 218 VL IYSVVRIFIL KDNM..... ..CWMW DS
 DromeCG4395 270 AI VYGLARHFSS PDNKH..... ..CWIT DS
 AnogaCTR1 248 PL FHRHRHHR TDAFF...SV FFRVLLTAG. ..KKVFIKDI VAMRWFVTIG ..
 AedaeCTR1 315 QT YSDSNRHQPP SSSFAEGKSI FLMSFFTLTP THVHVAIFFL LSIRFSCWMN ES
 CulquCTR1 266 IS FYATFRNNYT ADTEH..... ..CWMD ES
 CulquCTR2 240 IV LYGVLRGTYG TDEVT..... ..LCWMT ES
 AedaeCTR2 219 IM AYGFRLGYAG TPEDTI..... ..ECWMN ES
 AnogaCTR2 181 IV LYGFRLGYAS PPNDTI..... ..ECWMN DS
 TricaCTR2 234 TS IYAPVRSVLG ENVDELG... ..RCWITQ DG
 consensus 361 vl iyg vR d e cwm ds

TricaCTR1 241 YYSSWILT APVCISLLVS LIFLINVLRV ILTIMHPNSANPAPM GLRRAAR
 DromeCG4395 293 LY.LWIFES VPITLSLILAS FIFLINVLRV IVRKLHPQSAQPAPL AIRKAVR
 AnogaCTR1 294WILP M.....ALAS LVFLINVVRV LLTKLSSTSPHPAPL GLRKATR
 AedaeCTR1 369 HA.MWILT IPVCFSLVAS LVFLINVVRV LLTKLNSTSPNPAPL GLKKATR
 CulquCTR1 289 HA.MWILT VPVFLSLAAS LVFLVNVVRV LLTKLNSTSPNPAPL GLKKATR
 CulquCTR2 265 SY.GMVFI VPVCISMLLN LLFLCNIVRV VLLKMR.APA .GPQSGPSR TILOAFR
 AedaeCTR2 244 VY.DNVFK APVCISMLLN LLFLCNIVRV VLLKLR.APA .GPQGTGPSR TILOAFR
 AnogaCTR2 206 SF.NKVFV GPVCISMLLN LVFLFNIVRV LLLKLR.APA .GPQGAGPSR TILOAFR
 TricaCTR2 260 RF.NKILM VPVITVFLN VIFLVNIVRV LLIKLRKGPA NGGSGSGASR TSLOALR
 consensus 421 y wil vpv cisllvs liFl iNvVRV ll klh sa papm glrrA R

TricaCTR1 291 AAL ILIPLFGLQH ILIPIFRPDY DPVEHLYQYV TVVVVTLQGL CVSCLFCFAN Q
 DromeCG4395 342 ATI ILVPLFGLQH FLIPIYRPDAG TQLDHFYQML SVVLVSLOGF VVSFLFCFAN H
 AnogaCTR1 335 ATL ILIPLFGLQH ILIPIFRPEKG CSLERYYQIG AALLISLOGL CVSCLFCFAN H
 AedaeCTR1 418 ATL ILIPLFGLQH ILIPIFRPDKG CELERYYQVV SAVLISLOGA CVSCLFCFAN H
 CulquCTR1 338 ATL ILVASR.FLL ILIPLVTSRC AANETYRRVG RPCGKSCG.. .TYGQLCAMK R
 CulquCTR2 317 ATL LLVPLLGLQY ILIPIFRPDG HPYERVYETI SAFTASFOGL FVAVLFCEFN G
 AedaeCTR2 296 ATL LLVPLLGLQY ILIPIFRPDG HSYERTYEII SAFTASFOGL FVAVLFCEFN G
 AnogaCTR2 258 ATL LLVPLLGLQY ILIPIFRPGNG HPYERTYETM LACTSSFOGL FVAVLFCEFN G
 TricaCTR2 314 ATM LLVPLLGLNF ILIPIFRPEAN HPVEYVYEVV SAITASLOGL CVAILFCFCN G
 consensus 481 Atl iLvpl glq iL Pfrpd g yer y mv sav slqgl v lfcf n

TricaCTR1 345 DVHQAIRGF MHRKVYRTR WSNYHYTGAA DSAGVYVVG SSHCNNVGLL SLKRKS
 DromeCG4395 396 DVTFAIRTL LNKLLP..... ..SLVTP PPAGSNIGQM ATITPS
 AnogaCTR1 389 DVIFAVQCY LSRFFPD... ..LVTHP FLES.NGGQP ATQ..S
 AedaeCTR1 472 DVIFAIKCO LSRFFPT... ..LVHHP FRESYNGGQP ATQ..S
 CulquCTR1 388 ..INRCOCL VGFARNR..... ..SGACI YHKHCPVNLQ DAE..F
 CulquCTR2 371 EVIAQVKKR WRTVFLR... ..TRTNS YTATQVS... .VSKFL
 AedaeCTR2 350 EVIAQVKKR WRTVFLR... ..TRTNS YTATQVSRKA RHSSVM
 AnogaCTR2 312 EVIAQVKKR WRTVFLR... ..TRANS YTATQVS... .VSTLT
 TricaCTR2 368 EVIAQVKKR WRTIMFR... ..PRANS CTVTIVS..F VRSSYP
 consensus 541 dvi vk mh lfr s n fs s t t s

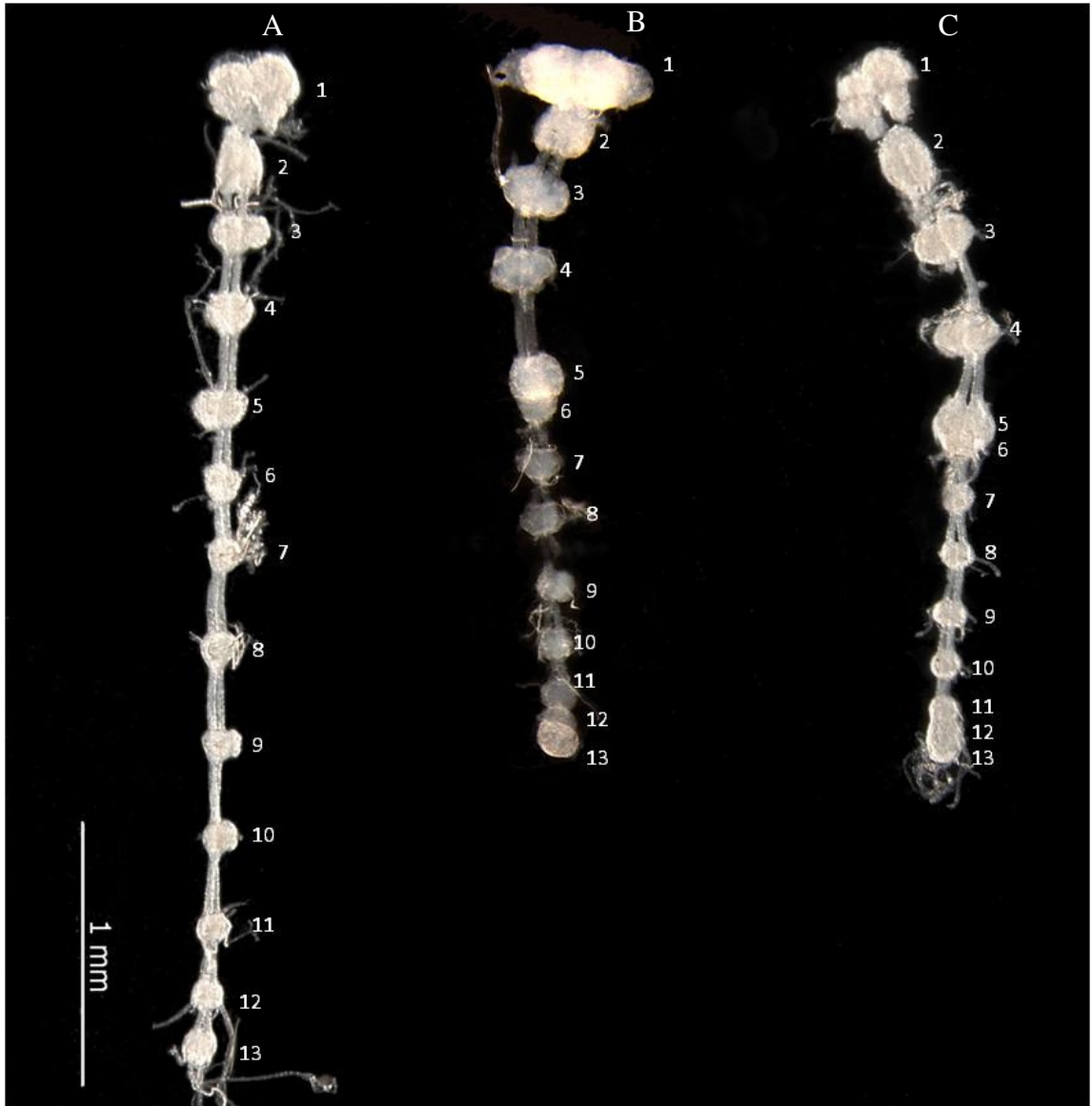
TricaCTR1 400 TTTV KL.
 DromeCG4395 432 RELG V..
 AnogaCTR1 423 RDVV V..
 AedaeCTR1 507 RDMV V..
 CulquCTR1 421 LEFV ...

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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 quinquefasciatus*) 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 ganglion; 3, 4, 5. Thoracic ganglion 1, 2 and 3, respectively; 6 to 12. Abdominal ganglia 1 to 7; 13. Terminal abdominal ganglion.