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Octopamine Receptor Gene Expression in Lepidopteran Insects

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Octopamine Receptor Gene Expression in Lepidopteran Insects

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Abstract

The invertebrate octopaminergic system affects many diverse processes and represents the counterpart to the vertebrate adrenergic/noradrenergic system with the classes of octopamine receptor (OAR) being homologous to those of vertebrate adrenergic receptors. However, there is still little information on the OARs present in different species, and the levels and distribution of these receptors throughout the body. cDNAs sharing high similarity with known insect OARs were cloned in three lepidopteran species: the cabbage looper, *Trichoplusia ni*; the true armyworm, *Pseudaletia unipuncta*; and the cabbage white, *Pieris rapae*. Seven major larval tissues and one adult tissue were examined in *T. ni* using quantitative real-time PCR to determine the relative expression levels of receptor transcripts. A subset of these tissues was also examined in *P. unipuncta* and *P. rapae*. All receptor transcripts were expressed in the nervous system of all three, however, the distribution of the different receptor types varied between species.

Keywords: octopamine, receptors, tyramine, lepidopteran, insect, Malpighian tubule, oviduct

TABLE OF CONTENTS

List of Figures

List of Tables

List of Abbreviations

AC	adenylyl cyclase
ANOVA	analysis of variance
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CRF	corticotrophin-releasing factor
Ct	crossing threshold
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DUM	dorsal unpaired median
E	amplification efficiency
eIF4α	elongation initiation factor 4α
G3PDH	glyceraldehyde 3-phosphate dehydrogenase
GDP	guanosine diphosphate
GOI	gene of interest
GPCR	G protein-coupled receptor
GRK	G protein-receptor kinases
GTP	guanosine triphosphate
H/R	hindgut/rectum
IP_3	inositol 1,4,5-trisphosphate
MB	mushroom body
min	minute
MT	Malpighian tubule
NRQ	normalized relative quantity
NS	nervous system
NTC	no template control
OA	octopamine
OAR	octopamine receptor
Ovi	oviduct
PCR	polymerase chain reaction
PLC	phospholipase C
qPCR	quantitative PCR
RNA	ribonucleic acid
RQ	relative quantity
TA	tyramine
TDC	tyrosine decarboxylase
TM	transmembrane
TβH	tyramine beta-hydroxylase
VUM	ventral unpaired median

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1.1 Introduction

Biogenic amines play a prominent role in invertebrate physiology, and have quite diverse functions in a wide range of processes. For example, the monoamine, octopamine functions as a neurotransmitter in the regulation of endocrine gland secretion, as a neuromodulator in modulating sensory inputs and behaviours such as learning and memory, and as a neurohormone in controlling lipid and carbohydrate metabolism (Blenau and Baumann, 2001).

There are significant similarities between the octopamine signaling system in invertebrates, and the adrenergic system of vertebrates. These include the structure and pharmacology of the respective receptors and transporters with affinity for the active hormones, octopamine and norepinephrine, as well as in the monoamine β-hydroxylases that catalyze formation of these hormones (Caveney et al., 2006; Roeder, 2005). It has, therefore, been suggested that the adrenergic and octopaminergic systems had a common origin prior to the divergence of the protostomes and deuterostomes (Caveney et al., 2006). In addition, the octopaminergic and adrenergic systems also exhibit homology in their function and roles, as the 'fight or flight' response is modulated by the octopamine/tyramine and adrenergic systems in invertebrates and vertebrates, respectively. In this example, octopamine acts upon a wide variety of organs and systems to bring about the fight or flight response and these different effects are accomplished through a number of different octopamine receptor types in each organ. As mentioned above, these receptors show structural and pharmacological similarity to vertebrate adrenergic receptors, and as such have been arranged according to that similarity into

three classes: α adrenergic-like, β adrenergic-like, and octopamine/tyramine receptors (Evans and Maqueira, 2005). Each class of receptor mediates its effects via specific second-messenger systems. Thus, it is necessary to know the distribution of these different receptors throughout the insect in order to understand how they coordinate complex physiological processes.

Plants and their herbivorous predators exhibit complex relationships based on evolutionary adaptations by plants to deter feeding by herbivores (Mello and Silva-Filho, 2002). For example, the octopaminergic system in insects is affected by secondary plant compounds such as cocaine, which is known to affect octopamine transporters and inhibit octopamine re-uptake. Insects feeding on coca plants display hyperactive behaviour leading to leaf walk-off (Nathanson et al., 1993; Roeder, 2005). On the other hand, essential oils such as cinnamic acid, eugenol, trans-anethole and 2-phenylethyl propionate are all thought to target octopamine receptors (Enan, 2005). The exclusivity of the octopaminergic system to invertebrates has made the octopamine receptor a popular target for the development of insecticides with little human health risk (Nathanson, 1985; Enan, 2001). However, in order to develop more effective insecticides it is important to better understand the target of these compounds: the octopamine receptors.

My research seeks to determine the relative distribution of expression of all of the different octopamine receptor types within major tissues of the cabbage looper, *Trichoplusia ni*, as well as further examining octopamine receptor expression in specific tissues of the true armyworm, *Pseudaletia unipuncta* and the cabbage white butterfly, *Pieris rapae,* for comparison to the expression found in *T. ni*.

The three insect species chosen for this study are all common scientific model organisms. They represent two different families within the order Lepidoptera: the first two are moths belonging to the family Noctuidae, and the third is a butterfly in the family Pieridae. *Trichoplusia ni* and *P. rapae* are both specialists feeding primarily on Brassica plants while *P. unipuncta* is a generalist cereal and grass feeder. Specialists are usually better than generalists at dealing with the plant secondary metabolites found within a specific species or genus of host plant utilized (Richards et al., 2010). As all three insect species selected for this study are pests on various crops, their control through the use of insecticides is an important agricultural issue.

1.2 The Octopaminergic System

1.2.1 Synthesis of Octopamine

Octopamine is a biogenic amine primarily synthesized and released from the dorsal and ventral unpaired median neurons (DUM and VUM neurons, respectively) located either in the suboesophageal ganglion or thoracicoabdominal ganglia. These neurons innervate the peripheral muscles, organs and proprioreceptors (Verlinden et al., 2010), and are the main source of octopamine for most parts of the brain.

Both octopamine and tyramine are synthesized from the amino acid tyrosine along the same biochemical pathway. In fact, tyramine was traditionally considered as a precursor to octopamine and only recently has its role as an independent neuroactive molecule been described (Lange, 2009a). The first step in the synthesis of octopamine is the conversion of tyrosine to tyramine by the enzyme tyrosine decarboxylase (TDC).

Tyramine is then converted to octopamine by the enzyme tyramine beta-hydroxylase (TβH). A possible salvage pathway for the synthesis of octopamine exists in which tyrosine is converted by tyrosine hydroxylase to 3,4-dihydroxyphenylalanine, which is then converted to dopamine by the enzyme 3,4-dihydroxyphenylalanine decarboxylase. Dopamine, while a neuroactive molecule in its own right, can be converted to tyramine by the enzyme dopamine dehydroxylase, though the biological relevance of this potential pathway is not well understood (Roeder, 2005).

1.2.2 Roles of Octopamine

Octopamine works in the nervous system to regulate a wide variety of behaviours such as flight, running, swimming, and digging (Roeder, 2005). Octopamine also modulates many sensory systems, both at the site of the sensory organ and in the nervous system. This modulation is achieved through the alteration of sensory receptor sensitivity or density, or by affecting post-synaptic potentials via changes in membrane resistance (Birmingham and Tauck, 2003; Farooqui, 2007). Furthermore, octopamine plays an important role in memory and learning. In *Drosophila*, honeybees and crickets, octopamine can substitute for the sucrose reward during associative (appetitive) learning and this learning is abolished when octopaminergic neurons are blocked (Verlinden et al., 2010).

The regulatory effects of octopamine on behaviour and sensory systems have been studied extensively in honeybees. A wide range of social behaviours exhibited by the honeybee, *Apis mellifera*, appear to be modulated by octopamine, including kin recognition, and foraging and hygienic behaviour (Roeder, 2005). In the visual system,

octopamine enhanced the directional specificity of the antennal reflex of *A. mellifera* to stimuli, while serotonin had the opposite effect (Erber and Kloppenburg, 1995).

Octopamine has also been implicated in fight or flight behaviours in orthopteran insects, and when found at high levels in the haemolymph of crickets, it resulted in aggressive behaviour (Stevenson et al., 2000). The aggregating behaviour of the locust, *Schistocerca gregaria*, during its transition from the solitary to gregarious phase, also seems to be at least partially controlled by octopamine (Verlinden et al., 2010). Sensory systems in these insects are also under octopaminergic control, as injection of octopamine into the prothoracic ganglion increases the sensitivity of the auditory system in the cricket, *Gryllus bimaculatus* (Lühr and Wiese, 1994). And finally, octopamine has been found to modulate tactile sensation, as seen in the *Locusta migratoria* flight behaviour-associated stretch receptor located on the base of each wing (Ramirez and Orchard, 1990).

Octopaminergic modulation has been studied in many other insect species as well, such as in the olfactory system of the moths *Antherea polyphemus* and *Trichoplusia ni*, where octopamine increased the sensitivity to pheromones (Pophof, 2000; Linn and Roelofs, 1986). In the gustatory system of the blowfly *Phormia regina*, octopamine affects the proboscis extension reflex in response to sucrose (Long and Murdock, 1983), and in the cockroach, *Rhyparobia maderae*, octopamine increases feeding behaviour (Cohen et al., 2002). The examples given are representative of the many demonstrated roles of octopamine in insect sensory and behavioural systems and more complete coverage can be found in Farooqui (2007) or Roeder (2005).

Octopamine also modulates most of the peripheral organs in insects. In the fat body, octopamine triggers lipid mobilization to release energy as part of a stress response. This has been studied in *Manduca sexta,* where it was found that octopamine stimulates glycogen phosphorylase (Arrese and Soulages, 2010). Octopamine, along with adipokinetic hormones, also induces the release of diglycerides in the initiation of flight (Arrese and Soulages, 2010). In addition, the stress response initiated by octopamine also occurs during an immune challenge. Octopamine modulates the activity of haemocytes, as well as their locomotory behaviour (Roeder, 2005), suggesting that it plays an integral part in the insect immune response. In the Malpighian tubule, octopamine has been found to increase chloride conductance in *Drosophila*, though it requires high concentrations and is less effective than tyramine, which is probably the *in vivo* effector molecule (Blumenthal, 2003). Thus, it is thought that tyramine is either released as a paracrine signal or obtained from the diet to stimulate post-feeding diuresis. However, in locusts the post-feeding diuresis is stimulated by a corticotropin-releasing factor (CRF)-like diuretic peptide hormone (Blumenthal, 2003), suggesting that the diuretic system is species-specific and related to diet. Octopaminergic regulation of muscle function has been extensively studied using the locust leg muscle. It was found that octopamine increases tension and relaxation rates in the muscle by acting both on the presynaptic and postsynaptic cells (Roeder, 2005). Besides controlling skeletal muscle function, octopamine is also involved in the modulation of visceral muscles, such as in the oviduct and gut muscles.

As mentioned previously, the production of octopamine is controlled by a single enzyme (TβH), and the production of tyramine by, potentially, another single enzyme

(TDC). Therefore, knockout mutants can be studied to ascertain the physiological roles of both amines. Two of these mutants, the *Drosophila* (inactive) *iav* and $TDC2^{RO54}$ mutants, exhibit low levels of octopamine and tyramine due to reduced TDC activity. These mutants display reduced locomotory activity, an inability to become desensitized to cocaine, and impaired egg-laying ability (though not ovulation) (Lange, 2009a). This suggests that normal locomotion is a product of a proper balance of both amines (Lange, 2009a). Furthermore, another *Drosophila* mutant, $T\beta H^{nM18}$, has increased levels of tyramine and low levels of octopamine due to reduced activity of TβH. This mutant is phenotypically normal for the most part, apparently due to compensation by increased tyramine levels for the lack of octopamine. These mutants, however, do display an inability to ovulate and altered flight behaviour (Lange, 2009a). Proper ovulation and egg-laying function, therefore, seem to be controlled by the combined effects of octopamine and tyramine.

Octopaminergic effects on the foregut and hindgut have been shown in *L. migratoria*, where application of octopamine increased foregut contraction frequency while reducing amplitude, and increased hindgut contraction amplitude, but not frequency (Huddart and Oldfield, 1982). In addition, feeding behaviour in *M. sexta* was shown to be impaired due to the elimination of foregut peristaltic activity when octopamine titers in the haemolymph increased (Miles and Booker, 2000). Though the study focussed on a parasite-induced increase in octopamine levels causing an inhibition of feeding and thus of pupation, this reduced gut activity is consistent with a stress-response mediated by octopamine.

1.3 Octopamine Receptors

1.3.1 G Protein-coupled Receptors

Octopamine, like most other biogenic amines, mediates its effects via specific membrane receptors belonging to the rhodopsin-like family of G protein-coupled receptors (GPCR). These receptors, which include the vertebrate adrenergic and olfactory receptor subgroups, share a characteristic 7 transmembrane (TM) domain structure (Fig. 1) and signal by activating intracellular heterotrimeric G proteins. Binding of the agonist causes a conformational change to the receptor, allowing it to interact with and activate different types of G proteins depending on the specific agonist and receptor involved. The G protein, which is made up of α, β and γ subunits, is activated by the agonist-receptor complex, resulting in an exchange of a GDP molecule associated with the α subunit for GTP and separation of the α subunit from the βγ dimer. The activated α subunit then proceeds to interact with various membrane-bound molecules in the propagation of an intracellular signal (Fig. 2) (Pierce et al., 2002; Massotte and Kieffer, 2005).

There are a number of different G proteins with which an activated receptor can interact, and these can, in turn, bind to different effector molecules. Some receptors transmit signals in response to the binding of a ligand by activating a G protein that changes the activity of adenylyl cyclase (AC), an enzyme that catalyzes the conversion of ATP to cAMP. Depending on the receptor, the pathway can act via a stimulatory G protein of the $G_{\alpha s}$ family leading to an increase in AC activity, or an inhibitory G protein ($G_{\alpha i}$) resulting in a reduction of AC activity. The result of increased intracellular cAMP

concentration is the activation of cAMP-dependent protein kinase A, which phosphorylates different target proteins and affects processes such as transcription, metabolism and channel regulation (Blenau and Baumann, 2001; Hwangpo and Iyengar, 2005). Other receptors activate a G protein ($G_{\alpha q}$) that stimulates phospholipase C (PLC) activity, resulting in production of the two second messengers, inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 can cause the release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm, while DAG (in the presence of Ca^{2+}) activates protein kinase C, which has cell-specific functions including roles in contraction and neuronal regulation, as well as transcription and channel regulation (Blenau and Baumann, 2001; Hwangpo and Iyengar, 2005).

There are several different ways that signaling can be terminated following receptor activation and the subsequent activation of both the G protein and downstream effector molecules. Inactivation of signaling can occur at the level of the agonist by dilution and excretion from the body, extracellular enzymatic inactivation, or through uptake by high-affinity transporters (Vauquelin and von Mentzer, 2007). Specific OA and TA transporters responsible for uptake have been well characterized in many insect systems, including *Trichoplusia ni* (Malutan et al., 2002). Other mechanisms for termination of GPCR-mediated signaling target the receptor-agonist complex and result in desensitization of the receptor. $\beta\gamma$ subunits, following dissociation from the α subunit, target G protein-receptor kinases (GRK) and localize them to the membrane. The GRKs cause a conformational change in the receptor-agonist complex and promote the binding of β-arrestin to the receptor, which prevents further interaction between the receptor and additional G proteins (Vauquelin and von Mentzer, 2007)

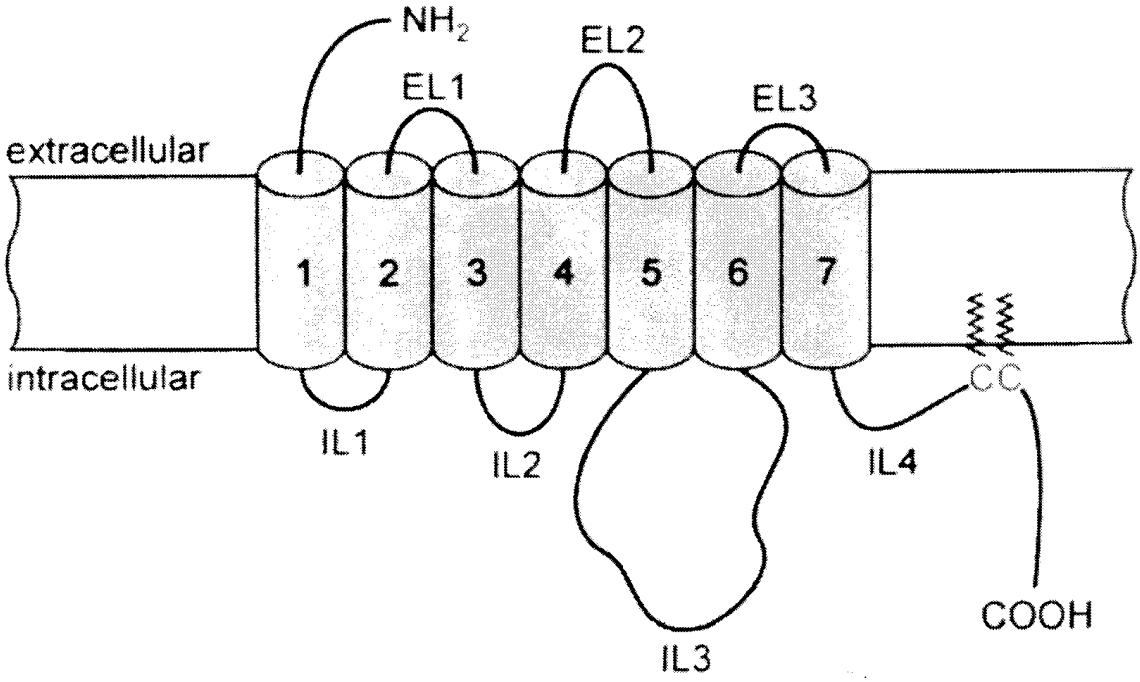

Figure 1. Topography of a biogenic amine receptor showing transmembrane domains (numbered 1-7) as well as alternating intracellular (IL) and extracellular (EL) loops. Postranslational palmitoylation at cysteine residues (C) forms an additional intracellular loop. Modified from Blenau and Baumann (2001).

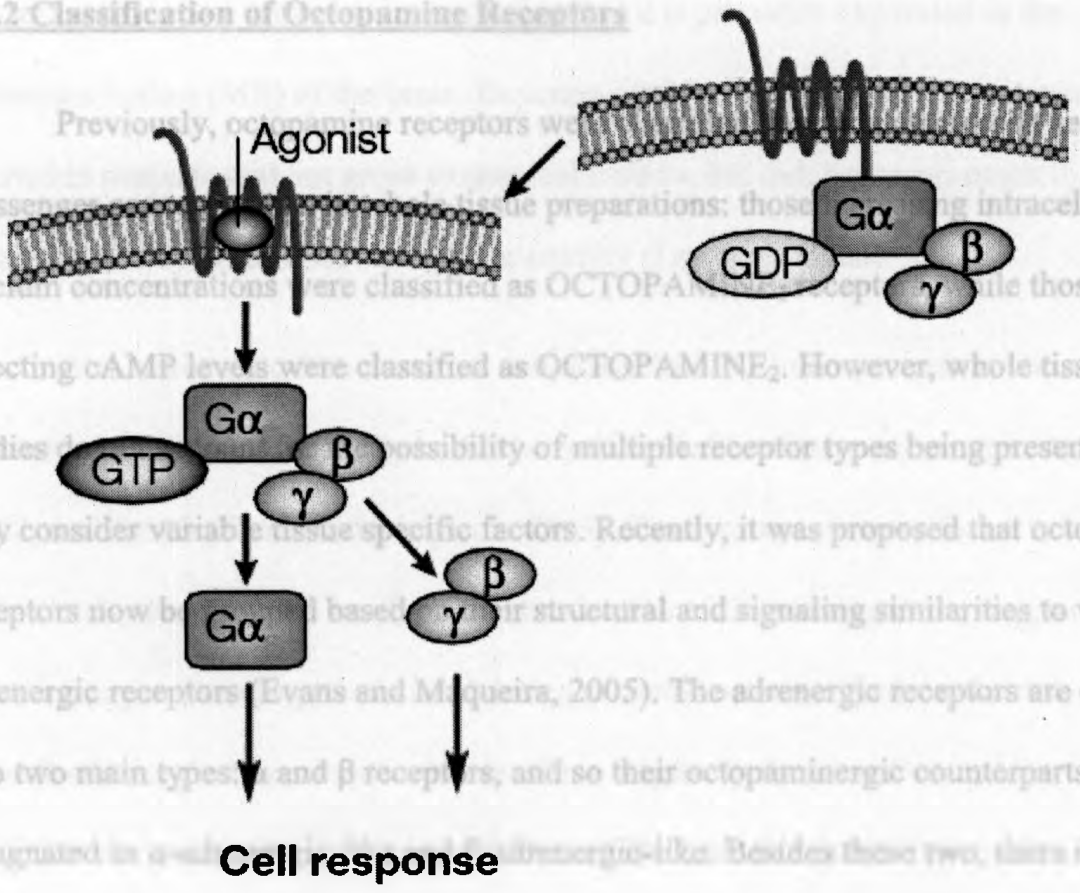

Figure 2. G protein-coupled receptor signalling. Binding of the agonist to the receptor causes an exchange of GTP for GDP and dissociation of the G protein into an α subunit and a βγ dimer, which interact with various molecules in the propagation of an intracellular signal. Modified from Pierce et al., (2002)

1.3.2 Classification of Octopamine Receptors

Previously, octopamine receptors were classified based on changes in second messenger concentrations in whole tissue preparations: those increasing intracellular calcium concentrations were classified as OCTOPAMINE$_1$ receptors, while those affecting cAMP levels were classified as OCTOPAMINE$_2$. However, whole tissue studies do not account for the possibility of multiple receptor types being present, nor do they consider variable tissue specific factors. Recently, it was proposed that octopamine receptors now be grouped based on their structural and signaling similarities to vertebrate adrenergic receptors (Evans and Maqueira, 2005). The adrenergic receptors are divided into two main types: α and β receptors, and so their octopaminergic counterparts are designated as α-adrenergic-like and β-adrenergic-like. Besides these two, there is also a third type of octopamine receptor: the octopamine/tyramine (or tyraminergic) receptor (Evans and Maqueira, 2005). The first two types generally have a higher affinity for octopamine than tyramine, while in the third the inverse is true.

Alpha-adrenergic-like octopamine receptors generally increase calcium concentrations, and although a few increase cAMP concentrations independent of a calcium response, this requires an abnormally high concentration of octopamine and may be a secondary effect of the large levels of calcium released (Grohmann, et al., 2003; Balfanz et al., 2005). To date, receptors in this class have been found in insect brains, thoracicoabdominal ganglia, reproductive organs and mature eggs (Grohmann, et al., 2003; Han et al., 1998; Bischof and Enan, 2004; Ohtani et al., 2006). Of the α-adrenergic-like receptors, perhaps the most extensively studied receptor is the *Drosophila* OAMB, so

named for the initial (mistaken) assumption that it is primarily expressed in the mushroom bodies (MB) of the brain. Experiments in which the OAMB gene was deleted resulted in mutants without gross anatomical defects, but did, however, result in impairment of ovulation and subsequent sterility (Lee et al., 2003).

Beta-adrenergic-like receptors couple exclusively to an increase in the concentration of cAMP and may be further subdivided into three subgroups, Octβ1, Octβ2, and Octβ3 (Evans and Maqueira, 2005). These receptors were found to be preferentially expressed in heads rather than bodies of *D. melanogaster*, suggesting a role of β-adrenergic-like receptors in modulating neuronal activity. To date, the only cloned insect β-adrenergic-like receptors are the three originally found in *Drosophila* (DmOctβ1R, DmOctβ2R and DmOctβ3R), and BmOAR2 in the silkworm, *Bombyx mori*, though many more have been identified using genomic analysis (Verlinden et al., 2010). An examination of the silkworm genome resulted in a DmOctβ3R orthologue that appears to be a pseudogene lacking a start codon, and the absence of a DmOctβ1R orthologue (Chen et al., 2010). Additional β-adrenergic-like receptors have also been found in *Aplysia californica* and *A. kurodial*, as well as *Spisula solidissima* (Maqueira et al., 2005).

The third class of octopamine receptors, the octopamine/tyramine receptors, has sequence similarity with vertebrate α-adrenergic-like receptors and induce a decrease in cAMP concentration through the attenuation of adenylyl cyclase. In most cases, these receptors show a higher affinity for tyramine than octopamine, suggesting that they may couple primarily to tyramine *in vivo*. Their current designation as a dual

octopamine/tyramine receptor is due to findings that in some cases, octopamine is more effective than tyramine in inducing an increase in calcium concentration, pointing to a possible differentiation in receptor downstream effects depending on the specific agonist (Robb et al., 1994). These receptors have been found in insect brain, antenna, nerve cord, oviduct and midgut tissues (Molaei et al., 2005; von Nickisch-Resenegk et al., 1996). This receptor gene is also expressed in the *Drosophila* Malpighian tubule (Blumenthal, 2003), where tyramine and octopamine have been found to regulate fluid secretion (Skaer et al., 2002). Tyramine causes a dose-dependent Cl^- conductance increase, an effect that is not achieved through the application of octopamine. It is thought that tyrosine from the diet is taken up by the principal cells of the Malpighian tubule, converted to tyramine, and secreted as a paracrine signal (Blumenthal, 2003). While this suggests a role for tyramine in stimulating diuresis during food intake through the stimulation of the OA/TA receptor, it may be that octopamine regulates fluid secretion in the Malpighian tubule through a different receptor. A *Drosophila* mutant with reduced levels of OA/TA receptor expression (*honoka*) exhibits a reduced level of avoidance for odor repellents, as well as a reduced capacity for TA-mediated reduction of excitatory junctional potential amplitude in the larval body wall muscle (Kutsukake et al., 2000; Nagaya et al., 2002). This suggests a role for the OA/TA receptor in regulating olfactory sensory input as well as neuromuscular modulation.

A novel tyramine receptor was found in *Drosophila* (Cazzamali et al., 2005), and as it does not interact with octopamine, probably represents a tyramine-specific receptor. This G protein-coupled receptor increases calcium concentrations, presumably through interaction with a $G_{\alpha q}$ protein.

1.4 Insect Tissues

The central nervous system of lepidopteran insects consists of the brain and suboesophageal ganglion in the head, and thoracicoabdominal ganglia along the midline of the body (Nation, 2002). The brain, which is made up of the protocerebrum, deuterocerebrum and tritocerebrum, is the location of the major integrative centres responsible for learning and memory, as well as the processing of sensory information. The suboesophageal ganglion contains sensory and motor connections to the mouthparts and salivary glands, and also influences motor patterns in walking, flying and breathing. The thoracicoabdominal ganglia of the ventral nerve cord consist of sensory and motor neurons innervating the rest of the body. As octopamine plays prominent roles in learning, memory and the modulation of sensory input, as well as in the regulation of locomotory behaviour (Roeder, 2005; Farooqui, 2007), the nervous system is a logical tissue to examine.

In the insect digestive system, the gut is divided into three sections: the foregut, midgut and hindgut (Nation, 2002). The foregut is responsible for breaking up the food, both mechanically and with the aid of secretions from the salivary gland. In lepidopteran larvae the foregut is generally short and almost vestigial. Food is then passed to the midgut where digestive enzymes break the food down further and nutrients are absorbed. From there, undigested food moves into the hindgut where water is reabsorbed and the waste is passed to the rectum before excretion. Closely associated with this process are the Malpighian tubules, blind-ended tubes that open into the hindgut and function as the insect equivalent of the kidney, thus regulating the balance of salts and water by taking

up waste products from the haemolymph and expelling them into the gut (Nation, 2002). The Malpighian tubules are made up of two kinds of cells: the principal cells which transport water, sodium, potassium and hydrogen ions; and secondary cells which transport chloride ions. As mentioned previously, both foregut activity and fluid secretion in the Malpighian tubules are regulated by the octopaminergic system (Huddart and Oldfield, 1982; Blumenthal, 2003).

Another prominent role of octopamine in insect physiology is in the modulation of different muscles where octopamine and tyramine act in conjunction to regulate proper muscle function (Roeder, 2005). The muscle lining the body wall of insects is composed of striated muscle fibers and functions in the same manner as is described by the vertebrate sliding filament model (Nation, 2002). The muscle is attached to the integument, which is composed of chitin, proteins and lipids in two main layers: the procuticle on the inside, and the epicuticle on the outside. The integument provides protection and serves as an exoskeleton for locomotion (Moussian, 2010). Octopamine modulation is also seen in the oviduct. Two lateral oviducts connect the ovaries to the common oviduct, and during ovulation, eggs are moved from the ovaries into the oviduct and fertilized. Muscles in the oviduct are then responsible for egg-laying, a process that appears, along with ovulation, to be under octopaminergic control (Nation, 2002; Lange, 2009b).

The insect fat body is an organ that fulfills many important roles. It functions as a storage organ for nutrients and energy reserves in the form of fat and glycogen. These reserves are then freed up and released into the haemolymph during energy-demanding

processes such as metamorphosis or flight. The fat body also has roles in the insect immune system, endocrine system, and in detoxification of nitrogen metabolism (Arrese and Soulages, 2010). Octopamine has been shown to induce the release of diglyceride during flight initiation in locusts, and is also known to stimulate lipid mobilization in many different insects (Arrese and Soulages, 2010; Roeder, 2005).

1.5 Objectives

Considerable research has been carried out to characterize the classes of octopamine receptors, but very little attention has been given to determining where the different subtypes are expressed within an organism. In my research I examined the relative abundance and pattern of expression of all types of octopamine receptors in major larval and adult tissues of the cabbage looper, *T. ni*. Gaining a better understanding in this area is important as the wide range of processes involving octopamine in different tissues requires that the targeted cells have specific octopamine receptors in order to receive and process this signal. Thus, we would expect that there will be different distributions of receptor types in different tissues, and that this distribution would be related to function. These data will then provide important information regarding the specific physiological roles of each of the various receptor types as well as the basis for eventual analysis of receptor distribution at the cellular level. Following this, I assayed the tissues of interest in the true armyworm, *P. unipuncta*, and the cabbage white, *P. rapae* for the relative expression levels of all octopamine receptor types. I would expect that this information would serve both to support results from the *T. ni* experiments, as

well as to reveal patterns of expression that are potentially related to either the taxonomical differences between moths and butterflies, or the diet-related differences between generalist and specialist herbivores.

To accomplish the above goals, I (i) developed quantitative polymerase chain reaction (qPCR) assays for all *T. ni* octopamine receptor cDNAs identified using the degenerate PCR method, (ii) measured the expression level of each of these receptor genes in a variety of different *T. ni* tissues using qPCR, and (iii) compared the expression levels in tissues of interest with the expression levels of octopamine receptors cloned from *P. unipuncta* and *P. rapae*. I chose to examine the caterpillar as this is the stage responsible for herbivory on crops and thus our findings may have potential impact with respect to subsequent pest management strategies.

2. Methodology

2.1 Insect Rearing

All insects were obtained from lab colonies: *T. ni* from Southern Crop Protection and Food Research Centre in London, Ontario, and *P. unipuncta* from the University of Western Ontario, while *P. rapae* were purchased from Carolina Biological Supply. *Trichoplusia ni* larvae were reared on an artificial wheat germ diet (D'Souza, 2007), while *P. unipuncta* larvae were reared on an artificial pinto bean diet (Shorey and Hale, 1965). The diet for *P. rapae* was a proprietary formulation obtained from Carolina Biological Supply. The insects were raised in growth chambers at 24°C, 16L:8D until they reached the last larval instar, whereupon they were either harvested and dissected or allowed to pupate and emerge as moths. *Trichoplusia ni* and *P. unipuncta* were raised in individual plastic cups, while *P. rapae* were raised in groups of five or six in larger plastic containers.

2.2 Molecular Cloning

2.2.1 RNA Isolation and cDNA Preparation

Total RNA was extracted from the heads of *T. ni* and *P. unipuncta* larvae using a Qiagen RNeasy Kit. Insect heads were removed in 4°C Calpode's insect saline solution (pH 7.2, 10.7 mM NaCl, 25.8 mM KCl, 90 mM glucose, 29 mM $CaCl_2$, 20 mM $MgCl_2$ and 5 mM HEPES) and stored in RNAlater (Ambion) at 4°C prior to homogenization and RNA extraction. cDNA was then synthesized from the RNA using Superscript II reverse

transcriptase with random primers (Invitrogen). In the case of *P. rapae,* total head RNA from adult butterflies was prepared using TRIzol Reagent (Invitrogen). Poly(A^+) RNA was isolated from total head RNA using a Poly(A) Quik oligo(dT) push column (Stratagene), and cDNA synthesized using a cDNA Synthesis Kit (Stratagene), as described in Malutan et al. (2002).

2.2.2 Degenerate PCR

Degenerate primers were designed to amplify a cDNA fragment of each of the receptor types from *T. ni*, *P. unipuncta* and *P. rapae* using highly conserved regions of octopamine receptor sequences known from other insects, and these were used for PCR amplification of cDNA templates. Degenerate primers were also used to clone the three reference genes in this study from *P. unipuncta* and *P. rapae.* The degenerate and unique primers used in this study are listed in Table 1.

Polymerase chain reactions were performed using Platinum Taq DNA polymerase (Invitrogen) in the presence of primers, $MgCl_2$ and dNTPs. The temperature profile consisted of a 2 min denaturation at 95°C, followed by 35 cycles of 30s denaturation at 95°C, 30s annealing at 50-58°C (in order to optimize degenerate primers) and 30s elongation at 72°C, and a final 7 min elongation at 72°C. $MgCl_2$ concentrations were varied from 1.5 to 3mM, and primer concentrations from 100 to 500nM, in order to optimize degenerate primer efficiency.

Table 1. Degenerate primers used to clone OARs and reference genes from *T. ni*, *P. unipuncta*, and *P. rapae*.

Gene	Primer	Sequence
Alpha adrenergic-like receptor	OAR2	GTNGGNTGGAARGAYAA
	OAR4	TTNGCNGCYTTNGTYTCCAT
Octopamine/tyramine receptor	OAR5	GTNCCNGARTGGGARGCNAT
	OAR7	NGGCCARTCRTTCCANCC
Beta adrenergic-like receptor	OCTBETA5	CCNATHTTYATGGGNTGGTA
	OCTBETA7	AARTCNCRRTTRAARTANGC
	OCTBETA7-2	TTGAGAGCGGAGTTGAAG
EIF4A	EIF4a-degen-for1	GAYGARGCNGAYGARATG
	EIF4a-degen-rev1	TCCATNCCRTCRTGCATNGC
G3PDH	G3PDH-3	GGCCAAGGTCATCCATGACAACTT
	G3PDH-6	TAGTGTTTAATTTGTGAGTTTCTGAT
S5	S5-degen-for1	TGYTAYGAYGTNCARGT
	S5-degen-rev1	TTRTTNCKNCCRTGCATCAT

2.2.3 Molecular Cloning

PCR products were separated on 1.2% agarose gels to purify and isolate the desired fragments, gel extracted with the QIAquick Gel Extraction kit (Qiagen) and eluted with 1mM Tris-HCl (pH 8.0). The amplified products were cloned in pGEM-T Easy plasmid vector and transformed into XL1-blue MRF electrocompetent *E. coli* cells that were grown on LB agar plates containing ampicillin, X-Gal and IPTG. Positive clones were cultured in LB broth with ampicillin overnight and plasmid was isolated using a Qiagen Plasmid Mini kit and sequenced at the Southern Crop Protection and Food Research Centre in London, Ontario. In this way, fragments of five unique receptor genes were identified from *T. ni* and *P. unipuncta*, and four from *P. rapae*.

2.3 Quantitative Real-time PCR

2.3.1 qPCR primer design and optimization

For *T. ni,* unique primers and Taqman® (dual-labeled hydrolysis) probes were designed from the sequenced fragments of each receptor subtype as well as for the reference gene, elongation initiation factor 4α (*eIF4α*). The primers and probes for the other reference genes glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) and ribosomal protein *S5* were already available (D'Souza, 2007). In the case of *P. unipuncta* and *P. rapae*, primers and probes were designed for each of the receptor subtypes, as well as for all three reference genes. The probes were labeled with reporter and quencher

fluorophores, in this case 6-FAM™ and BHQ-1, at the 5' and 3' ends, respectively. Primers and probes used for qPCR experiments are listed in Table 2.

Primer melting temperatures, as determined using the Oligo Analysis Tool provided by Operon Biotechnologies (Huntsville, AL), were between 58-60°C, while the probe melting temperature was 10°C higher (68-70°C). Care was taken in designing to minimize the possibility of secondary structures such as primer self-dimerization, formation of "hairpin" loops, or dimerization between primers and probe. The primers were designed such that the resulting amplicon sizes would be between 100-200bp, but in cases where this was not possible due to the lack of optimal primer sites for some sequences, they were kept as small as possible.

The primer and probe combinations for each gene were tested and optimized for quantitative PCR assays. PCR reactions using the primer pairs and linearized plasmid were performed and the resulting PCR reactions separated on a 1.2% agarose gel and the amplicon sizes confirmed visually.

Table 2. Primers and probes used in quantitative real-time PCR experiments

	Gene	Primer	Sequence (5' --> 3')
T. ni	OARalpha	qOAR1-1	TGACGCTCAGAATACATCG
		qOAR1-2	TTAGTGACGTGCTGGTTGA
		PROBE	CCCACGGGTCTCCACTATCAAATGC
	OA/TA	qOAR2-1	TGCGGATCGTACAAAACTTCT
		qOAR2-4	GCATACATATATTCCAAACACCCA
		PROBE	AGTATGCCACATTGAGCGGCAGCACC
	OARbeta1	qOAR3a-FOR	TGGGAATGCGATGCTCA
		qOAR3a-REV	CTCGCTGCTTTGTGTTC
		PROBE	CGACAACGCCCACCAAAGACAGAAAC
	OARbeta2	qOAR3b-FOR2	ATGGAGGCTGATCGACAG
		qOAR3b-REV	ATCTTACTGCTCGCCATC
		PROBE	AGCAGCCTTACTCCTGGACAAACACC
	OARbeta3	qOAR3c-FOR	ATCACAGCTCACATTCACC
		qOAR3c-REV	CTACAGCACGTTTACAGAAG
		PROBE	CCCAATCGACATCACCACTTCTCCAC
	S5	S5-FOR	GTCGACAGCATGCCTTTA
		S5-REV	CACATCCACAGCCTGAC
		PROBE	CATCATCAGAGAGTTTGTCAAGCGCT
	G3PDH	G3PDH-FOR	ATCAAGTACATCCAGACCA
		G3PDH-REV	AGCAATATCCAGAACTGTG
		G3PDH-PROBE	TAACAACATTTACTCTACACTGCTATC
	EIF4a	EIF4a-FOR	CTGGATACGCTGTGTGAC
		EIF4a-REV	ACCTTGCGGCGAGTGTT
		EIF4a-PROBE	TCTCTCCATCGCTCAGGCTGTCATC
P. unipuncta	OARalpha	qOAR1-1	CAACGATGCTGGTTACG
		qOAR1-2	CTCCAGTAGAAGAACAGC
		PROBE	CAGCTCTGGGCTCCTTCTACATCC
	OA/TA	qOAR2-1	AGCACGCGTTCTAAAGTTC
		qOAR2-4	ATGTGCTGCACTTCGTC
		PROBE	CCGTAATCGCCCAATACCTGTCTAATGC
	OARbeta1	qOAR3a-FOR	CTCCATCTATGCTGTATCAG
		qOAR3a-REV	AAACTAGAGCTGGACTGC
		PROBE	CTTGGCATAATGCTGGCTGTTGTCTGG
	OARbeta2	qOAR3b-FOR2	ACTACGCCAACCAAGGAT
		qOAR3b-REV	CCAGCAGAGGATGAAG
		PROBE	AGCTAGAACTCTCGGCATCATCATGGG

	OARbeta3	qOAR3c-FOR	GTGAATGTGAGCTGTGATAC
		qOAR3c-REV	CGCCAGGTCTCTTGTC
		PROBE	CTGGAGACCGAGCTGTTCTTCTGC
	S5	S5-FOR	ATACTTGCCCCATTCTGCT
		S5-REV	TGCATCATAAGGGAGTTGGT
		PROBE	AGTGCCCCATCGTTGAGCGTCTG
	G3PDH	G3PDH-FOR	GTCATCCATGACAACTTCG
		G3PDH-REV	CAGAGCAGGAATGACTTTG
		G3PDH-PROBE	CCTTCTGGAAAGCTGTGGCGTGATG
	EIF4a	EIF4a-FOR	GATTGTGCATGGACTCTG
		EIF4a-REV	AGGAATGGAAGTTAGATACTC
		EIF4a-PROBE	CAATCGACCTTGCGACGAGTGTTACAG
P. rapae	OARalpha	qOAR1-1	CTGTGAGCTGACTAATGAC
		qOAR1-2	GATTAATGGCCTTTGTCGTTC
		PROBE	GTTTGCTCTGCGCTGGGTTCCTTC
	OA/TA	qOAR2-1	CACGCGTTCTAAAGTTCTC
		qOAR2-4	TGCAAGATGTGGCTGAC
		PROBE	TCTGTGATCGCCCAATACCTGTCTAATGC
	OARbeta1	qOAR3a-FOR	GCGTAGAGATAACGAATGG
		qOAR3a-REV	AGCATAGTATCTGTCCACAC
		PROBE	CCTCCGCCTCCATTCTACACTTGTG
	OARbeta2	qOAR3b-FOR2	CTTTCCGCTCTCTCTTC
		qOAR3b-REV	ACAGGAGCAAAGTAGCATC
		PROBE	CTCTGACGTTCTCGCATCACATCACC
	OARbeta3	qOAR3c-FOR	TATGCGATCCACCTGAAC
		qOAR3c-REV	CTGTACATCGACAAAGAGC
		PROBE	GAACAGGACTCGGAGATGGACTGG
	S5	S5-FOR	CCGATATGTCACTTCAGG
		S5-REV	GAATCGTTTGTGGGCATAC
		PROBE	CGCTAAGTACTTGCCCCATTCTGCTG
	G3PDH	G3PDH-FOR	GCACTTAATGGCAAACTCAC
		G3PDH-REV	ATCATAGCTGGCAGGTTTG
		G3PDH-PROBE	CCGTGTTCCAGTTCCTAATGTCTCTGTTG
	EIF4a	EIF4a-FOR	GCATAGATTCAGTCAACCAG
		EIF4a-REV	TTGGAGACCCTATGTGAC
		EIF4a-PROBE	CCTGAGCGATGGAGAGGGTGTC

2.3.2 Experimental design of qPCR

All qPCR experiments were run in triplicate, both with respect to technical and biological replicates. For qPCR experiments, nervous system (brain and nerve cord), midgut, hindgut, muscle, fat body, integument and Malpighian tubule tissues from fifth instar *T. ni* caterpillars, and oviduct tissue from 5-8 day old virgin female moths were dissected in 4°C Calpode's insect saline solution and then stored in RNAlater (Ambion) at 4°C prior to homogenization and RNA extraction as described above. Nervous system, Malpighian tubule and oviduct tissues were similarly dissected from *P. unipuncta* and *P. rapae*. Tissues for each biological replicate were taken from a sample of up to 25 individuals, depending on tissue type. Nervous system, being the smallest of the sampled tissues, required all 25 individuals to ensure an adequate amount of starting tissue for RNA extraction according to the Qiagen RNeasy Mini Kit guideline, which calls for 20-30mg. Larger tissues, such as the midgut, required fewer animals, though no fewer than five individuals were sampled in any instance. RNA quality was confirmed via analysis with an RNA 6000 Nano kit on a 2100 Bioanalyzer (Agilent). cDNA was then synthesized as described above.

In order to determine qPCR amplication efficiency, each experiment included a serial dilution of a linearized plasmid containing the gene fragment from which the primers and probe were designed. These plasmids, obtained during the original molecular cloning of the cDNA, were linearized in overnight enzymatic digestion reactions. The products were then purified and isolated using agarose gel electrophoresis (1.2%) and a QIAquick gel extraction kit (Qiagen). Concentrations were determined using a NanoDrop

1000 spectrophotometer (Thermo Scientific). qPCR optimization experiments were then run using a serial dilution of the linearized plasmid to determine optimal annealing temperature for the experiments. The linearized plasmid was first diluted to 1ng/µl, and subsequently diluted 10-fold using water. The series consisted of 7 dilutions (from 10^{-1} ng/µl - 10^{-7} ng/µl) of which 5 (10^{-3} ng/µl - 10^{-7} ng/µl) were included in qPCR experiments. These dilutions were chosen as they encompassed the expression levels of all three reference genes in their mid to upper range, while allowing for the (assumed) comparatively lower expression levels of the receptor genes.

Template cDNA from each tissue was diluted 1 in 10 with water in order to conserve materials. No template controls (NTC) were also included in each experiment.

2.3.3 qPCR protocol

Experiments were carried out using the Lightcycler Taqman Master kit (Roche) on a Rotor-Gene 3000 thermal cycler (Corbett Life Science). 20µL reactions were run in 0.1mL reaction tubes on a 72-tube rotor and contained 15µL of reaction mix and 5µL of template, each of which were specific for the gene fragment being assayed. The reaction mix contained polymerase, buffer, 0.5µM forward and reverse primers and 0.05µM probe, while the template was either cDNA from tissues diluted 1 in 10, or a linearized plasmid containing the gene fragment diluted as described above. NTCs contained 15µL of reaction mix and 5µL of water. The qPCR run profile consisted of a 10m 95°C activation step, followed by 45 cycles of a three-step process: a 10s 95°C denaturation step, a 30s annealing step at a primer pair-specific temperature, and a 1s 72°C elongation

step. Following the elongation step, a fluorescence reading was taken, measuring the signal generated from the separation of the reporter and quencher fluorophores of the probe by the exonuclease activity of the polymerase.

2.3.4 qPCR Analysis and Statistics

qPCR data were analyzed using the modified ΔΔCt method (Hellemans et al., 2007) to generate a quantity for the expression of the gene of interest (GOI) in each tissue relative to the expression of the GOI in the highest-expressed tissue. Thus, the relative quantity for each gene's expression in a tissue is calculated by the formula:

$$RQ = E_{GOI}^{(\Delta Ct)}$$

where ΔCt is the difference between the crossing threshold (Ct) of a particular tissue and that of the highest-expressed tissue. Reaction efficiency (E) was determined by the Rotor-Gene 6 software (Qiagen) using the slope of the standard curve in each reaction in the equation:

$$E = 10^{(-1/slope)}$$

Thus, an E value of 2 would indicate a 100% efficient PCR reaction, with the amount of PCR product doubling in each cycle. By using an efficiency-corrected model for determining expression, we can account for differences in efficiency between the GOI and the reference genes, which are used to normalize the data.

The relative quantity obtained was then normalized using a normalization factor derived from the expression of the reference genes. This value was calculated for each tissue sample by taking the geometric average of the relative quantities of stably expressed reference genes, in this case *eIF4α* and *S5* (Vandesompele et al., 2002). This process acts as a loading control for the amount of mRNA in each tissue, so proper normalization is essential for obtaining accurate data. Reference gene stability is measured by the M value, which is the average pairwise variation of a particular gene with all other reference genes and was obtained using the geNorm software package (Vandesompele et al., 2002). For this study, three reference genes were tested, the third being G3PDH, and the two most stable genes were chosen for the normalization process (*eIF4α* and *S5*). This yields a normalized relative quantity (NRQ) by the equation:

$$NRQ_{\text{sample (GOI)}} = \frac{RQ_{\text{sample(GOI)}}}{\text{Normalization factor}_{\text{sample(GOI)}}}$$

The data were calculated in two different ways and consequently, so was the statistical analysis. In the *T. ni* experiments, each qPCR run included a linearized plasmid standard curve of the GOI, and one biological replicate of eight tissue samples for the GOI. Further biological replicates were run in separate experiments due to space constraints in the qPCR instrument. Because of this, the NRQs were calculated for each sample in all three biological replicates, and the means were taken to arrive at final NRQ values for each sample. The standard errors for the *T. ni* experiments were calculated from the NRQ values of each biological replicate. For *P. unipuncta* and *P. rapae* experiments, only three tissue samples were included in each run and so it was possible to

fit all three biological replicates into the rotor. For these experiments, the mean Ct value of the biological replicates for each sample was calculated to begin with, and the final NRQ calculated from it. Standard error for each of the samples was therefore calculated according to Hellemans et al., (2007). This method of error propagation makes use of the standard deviation of the mean Ct value for each sample, which makes it unsuitable for the *T. ni* analysis.

A 1-way ANOVA was used to determine if there was a significant difference in expression for each gene across the tissues surveyed, and then a *post hoc* Tukey test was used to determine which expression values were different from the others.

3. Results

3.1 Cloning and Sequence Analysis

Using a degenerative PCR strategy, cDNA fragments of octopamine receptor genes were cloned by first comparing the amino acid sequences of known octopamine receptor proteins to find conserved blocks of sequence and then synthesizing degenerate oligonucleotide primers encoding the deduced sequences. The degenerate primers were then used to amplify the corresponding fragments from cDNA of the desired target species yielding five octopamine receptor gene fragments from *T. ni* and *P. unipuncta*, and four from *P. rapae*. A second fragment of the *P. rapae* beta1 receptor gene was found while searching for the beta2 receptor gene. Phylogenetic analysis of the sequence fragments show that they generally group with known insect octopamine receptors corresponding to the 5 types of octopamine receptor as proposed by Evans and Maqueira (2005) (Fig. 3). The gene fragments cloned from *T. ni*, *P. unipuncta*, and *P. rapae* share generally high protein identity with known and predicted octopamine receptors from other insects, as well as with each other (Table 3).

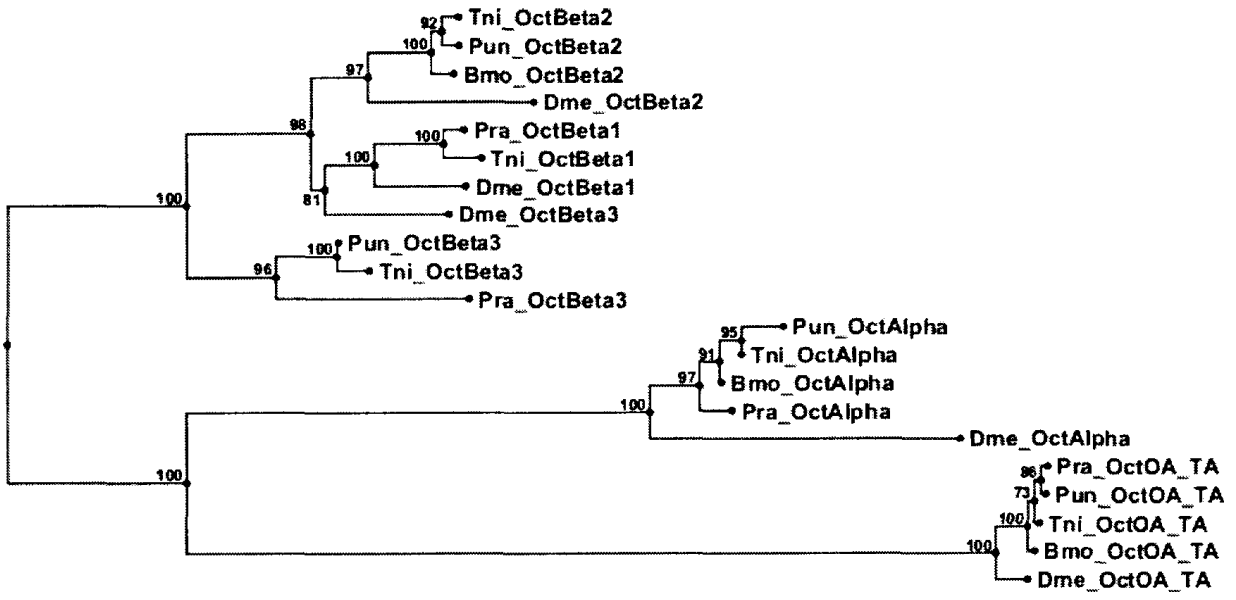

Figure 3. Phylogenetic tree comparison of cloned octopamine receptor gene fragments from *Trichoplusia ni* (Tni), *Pseudaletia unipuncta* (Pun) and *Pieris rapae* (Pra), along with known receptors from *Drosophila* (Dme) and *Bombyx mori* (Bmo). Pun_OctBeta1 is not included as it does not overlap with the Tni and Pra OctBeta1 fragments, preventing proper alignment. The sequences were aligned using CLC Sequence Viewer 6.4 (CLC bio, Aarhus, Denmark) with the software's alignment parameters, and the phylogenetic tree calculated by the Neighbor Joining method (bootstrap=100).

Table 3. Protein identity values between octopamine receptor gene fragments cloned from *T. ni*, *P. unipuncta* and *P. rapae*, and with known and predicted octopamine receptors from other insect species.

Receptor	Accession No.	*T. ni*	*P. unipuncta*	*P. rapae*
OAR alpha				
Bombyx mori BmOAR1	NP_001091748	93%	92%	87%
Manduca sexta MsOAR	ABI33825	92%	90%	88%
T. ni	---	---	95%	87%
P. unipuncta	---	95%	---	85%
P. rapae	---	87%	85%	---
OA/TA				
Mamestra brassicae MbraOAR/TAR	AAK14402	99%	100%	99%
Heliothis virescens K50Hel	Q25188	99%	99%	98%
Spodoptera littoralis TyrRI	ACJ06651	98%	98%	97%
T. ni	---	---	99%	99%
P. unipuncta	---	99%	---	98%
P. rapae	---	99%	98%	---
OAR beta1				
Apis mellifera	XP_397139	72%	75%	79%
Drosophila melanogaster DmOctbeta1R	NP_001163690	64%	74%	69%
Tribolium castaneum	XP_974265	70%	85%	85%
Pediculus humanus corporis GPRoar2	XP_002422997	71%	80%	83%
Harpegnathos saltator OAR beta-1R	EFN87040	47%	79%	78%
T. ni	---	---	87%	91%
P. unipuncta	---	87%	---	95%
P. rapae	---	91%	95%	---
OAR beta2				
Bombyx mori BmOAR2	NP_001171666	89%	90%	
Schistocerca gregaria SgOctbetaR	ADD91575	65%	65%	
T. ni	---	---	92%	
P. unipuncta	---	92%	---	
OAR beta3				
Drosophila melanogaster DmOctbeta3R	NP_001034048	68%	70%	55%
Tribolium castaneum	XP_974238	66%	77%	80%
T. ni	---	---	97%	62%
P. unipuncta	---	97%	---	60%
P. rapae	---	62%	60%	---

Identity values obtained using the NCBI blastx program (http://blast.ncbi.nlm.nih.gov/)

3.2 Quantitative PCR Results

The geNorm software package (Vandesompele et al., 2002) was used to evaluate reference gene stability to ensure accurate normalization of results. The software analyzes the expression of the reference genes across different samples and gives a measure of the average pairwise variation of each gene with all other reference genes (the M value). Reference genes with lower M values have more stable expression across different tissues. In *T. ni*, the M value of the three reference genes in the experiment were: for *G3PDH*, 1.319; for *S5*, 0.989; and for *eIF4a*, 1.113. For *P. unipuncta*, the respective M values were 0.532, 0.720 and 0.526; and for *P. rapae*, they were 0.462, 0.476, and 0.405.

Quanititative Real-time PCR experiments to determine the levels of receptor transcripts in each of the tissues was performed, first on *T. ni* tissues, and then on selected tissues from *P. rapae* and *P. unipuncta*.

3.2.1 Relative Expression Levels of OAR Genes in *T. ni* Tissues

In *T. ni*, the alpha adrenergic-like receptor gene was found to be expressed in all tissues examined, though the expression levels varied, being highest in the muscle and nervous system, and lowest in the midgut (Fig. 4). There was also a moderate level of expression in the oviduct. The expression level of the octopamine/tyramine receptor gene in *T. ni* was significantly higher in the nervous system than in the other tissues examined, between which there was no significant difference (Fig. 5). In contrast, there was a much higher level of expression of the *T. ni* Beta 1 receptor gene in the Malpighian tubule than

in the other tissues (Fig. 6) while in the *T. ni* Beta 2 receptor gene high expression was only observed in the oviduct (Fig. 7). The expression level of the *T. ni* Beta 3 receptor gene was significantly higher in the nervous system than in the other tissues examined (Fig. 8).

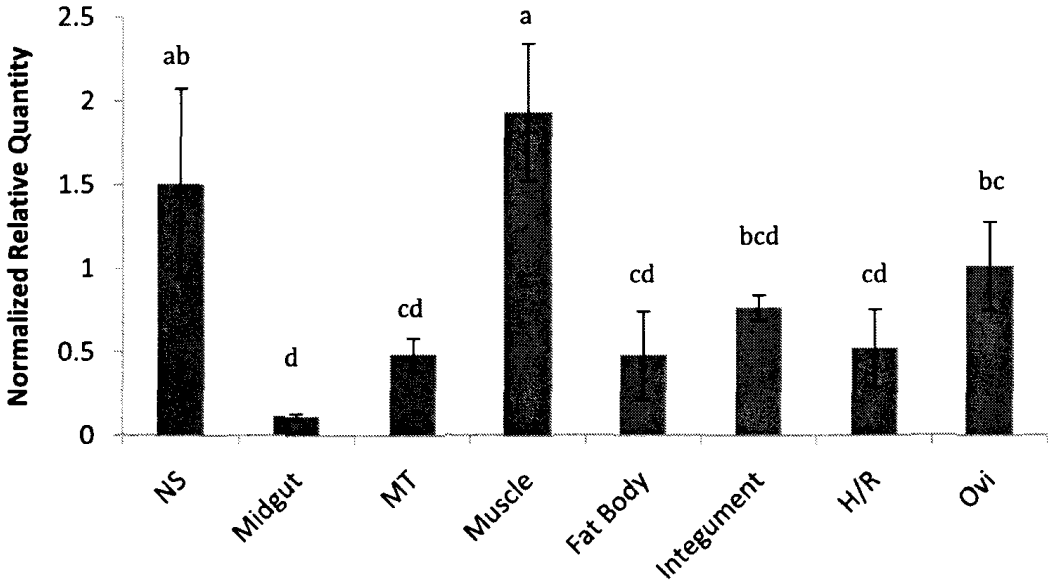

Figure 4. Normalized relative quantity of OctAlpha receptor mRNA in major tissues of *T. ni*. The mRNA quantity is expressed relative to the tissue with the highest level of transcript. The letters denote levels of statistical significance; tissues that share letters with other tissues have no significant difference between them, according to a Tukey test ($\alpha<0.05$). NS = nervous system; MT = Malpighian tubule; H/R = hindgut/rectum; Ovi = oviduct.

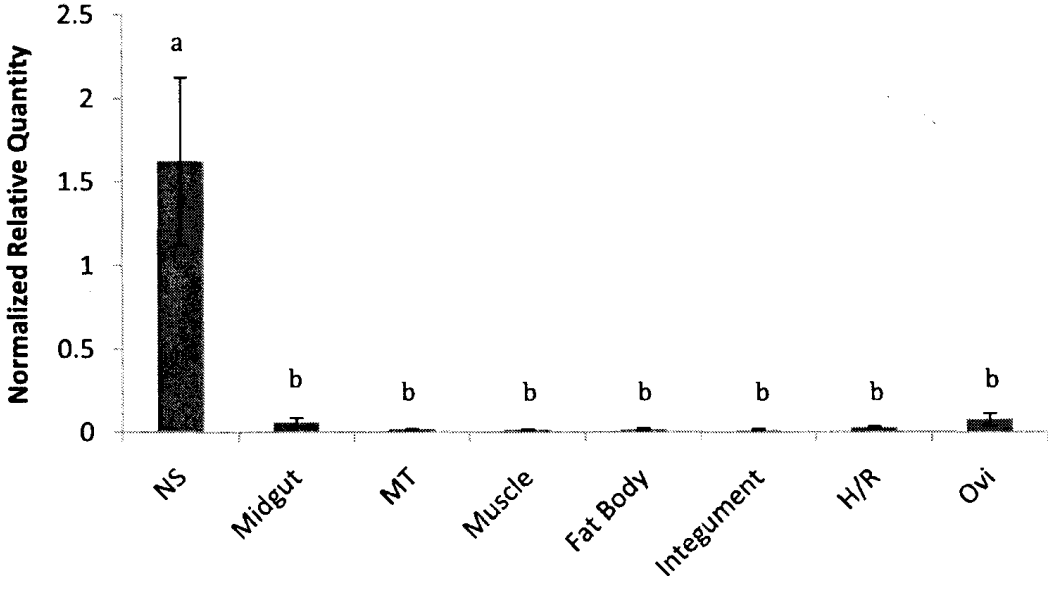

Figure 5. Normalized relative quantity of OctOA/TA receptor mRNA in major tissues of *T. ni*. The mRNA quantity is expressed relative to the tissue with the highest level of transcript. The letters denote levels of statistical significance; tissues that share letters with other tissues have no significant difference between them, according to a Tukey test ($\alpha<0.05$). NS = nervous system; MT = Malpighian tubule; H/R = hindgut/rectum; Ovi = oviduct.

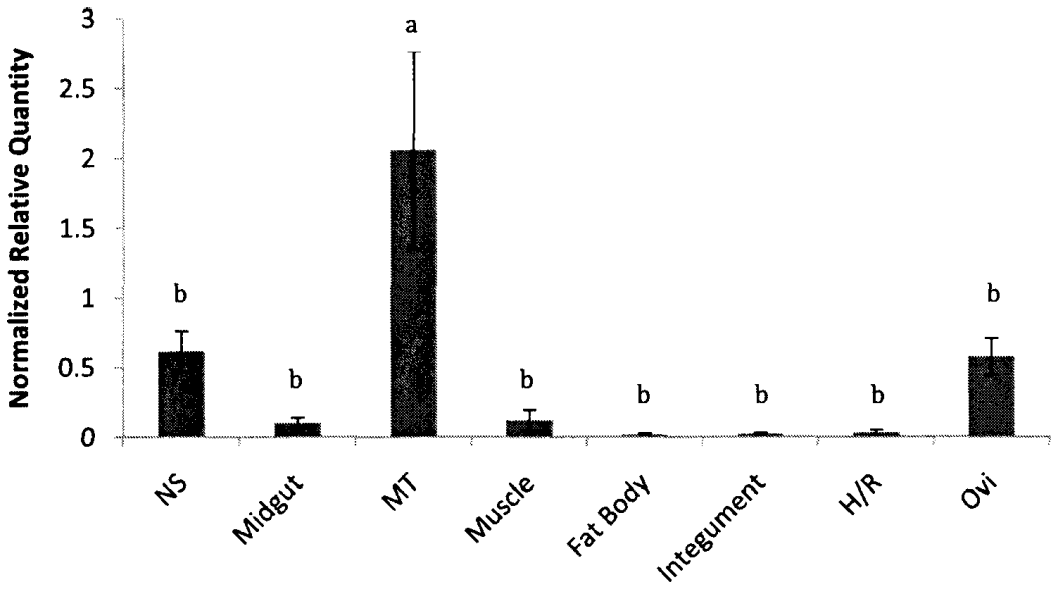

Figure 6. Normalized relative quantity of OctBeta1 receptor mRNA in major tissues of *T. ni*. The mRNA quantity is expressed relative to the tissue with the highest level of transcript. The letters denote levels of statistical significance; tissues that share letters with other tissues have no significant difference between them, according to a Tukey test ($\alpha<0.05$). NS = nervous system; MT = Malpighian tubule; H/R = hindgut/rectum; Ovi = oviduct.

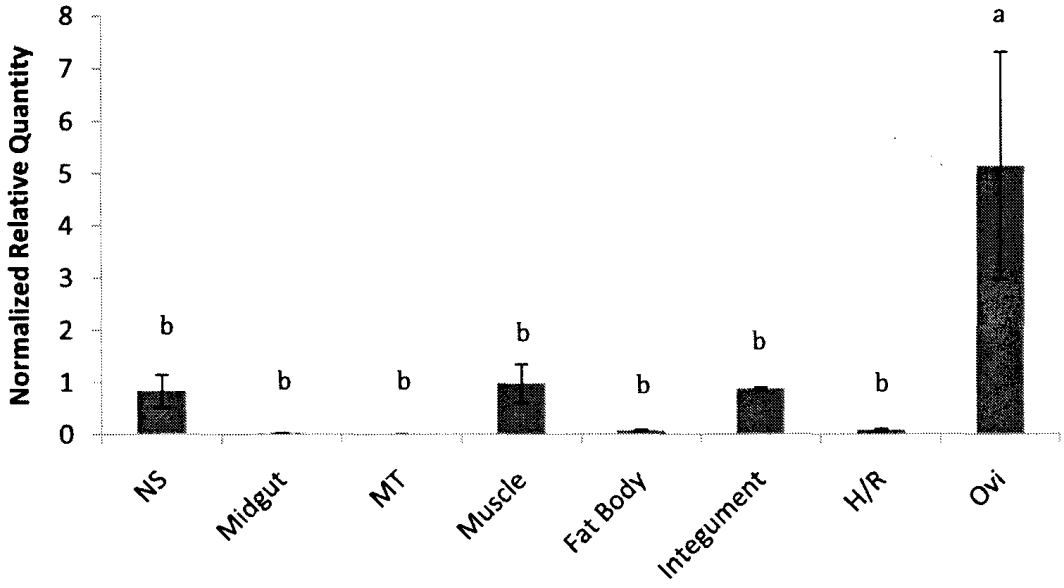

Figure 7. Normalized relative quantity of OctBeta2 receptor mRNA in major tissues of *T. ni*. The mRNA quantity is expressed relative to the tissue with the highest level of transcript. The letters denote levels of statistical significance; tissues that share letters with other tissues have no significant difference between them, according to a Tukey test ($\alpha<0.05$). NS = nervous system; MT = Malpighian tubule; H/R = hindgut/rectum; Ovi = oviduct.

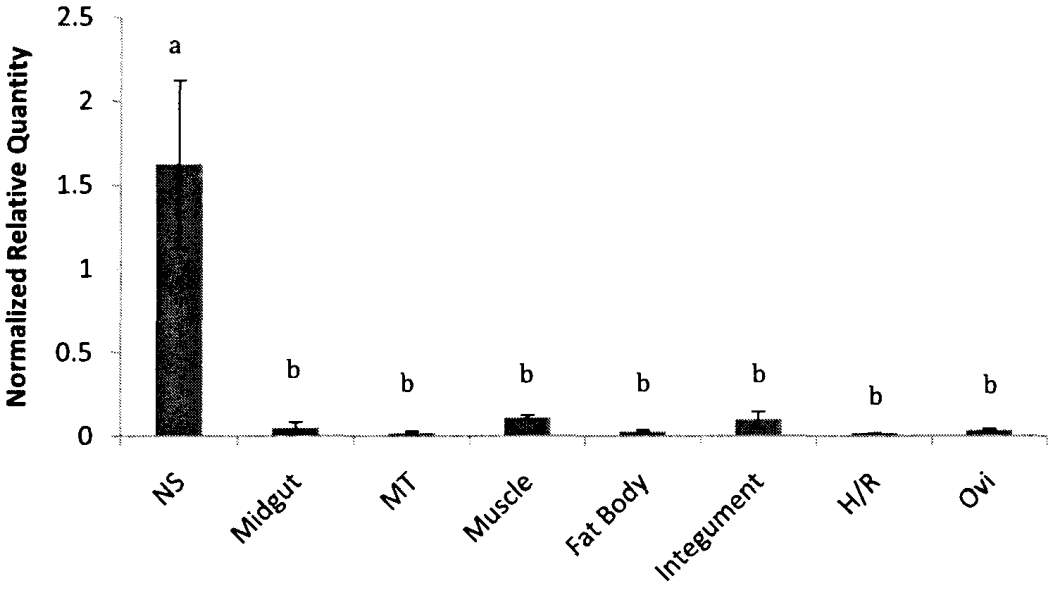

Figure 8. Normalized relative quantity of OctBeta3 receptor mRNA in major tissues of *T. ni*. The mRNA quantity is expressed relative to the tissue with the highest level of transcript. The letters denote levels of statistical significance; tissues that share letters with other tissues have no significant difference between them, according to a Tukey test ($\alpha<0.05$). NS = nervous system; MT = Malpighian tubule; H/R = hindgut/rectum; Ovi = oviduct.

3.2.2 Relative Expression Levels of OAR Genes in *P. rapae* Tissues

In *P. rapae*, the alpha adrenergic-like receptor gene was expressed highest in the nervous system, followed by the oviduct (Fig. 9), with a very low relative level of expression in the Malpighian tubules, while the octopamine/tyramine receptor gene was expressed most highly in the nervous system (Fig. 10). The *P. rapae* Beta 1 receptor gene expression levels were found to be the highest in the nervous system and Malpighian tubules (Fig. 11). The three tissues were also assayed for the expression levels of a second fragment of the *P. rapae* Beta 1 receptor gene, and gave similar results to the first fragment (Fig. 12). In the case of the *P. rapae* Beta 3 receptor gene, no significant difference was found between the expression levels in the tissues examined (Fig. 13).

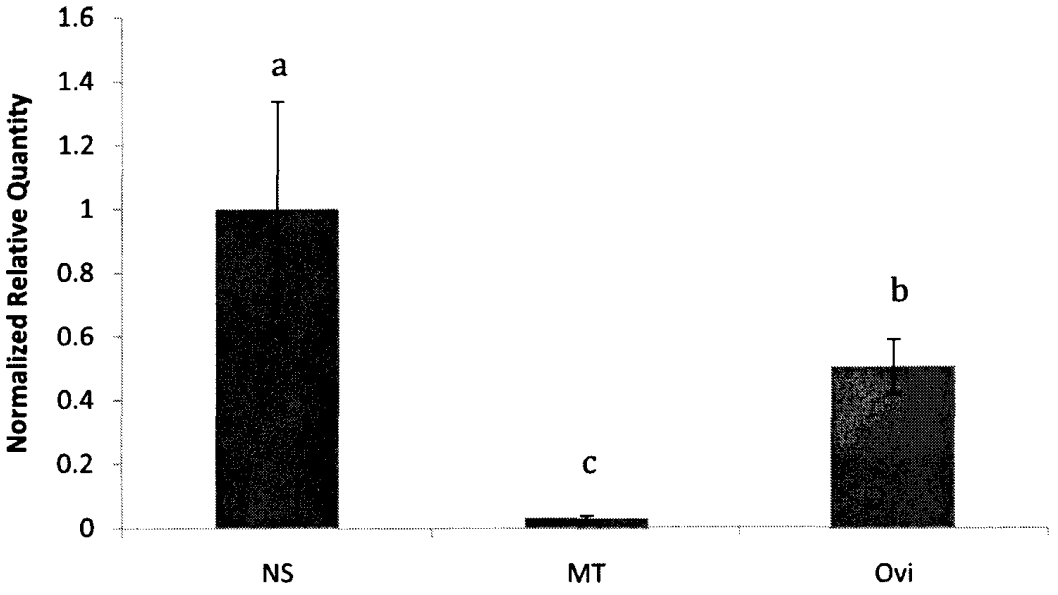

Figure 9. Normalized relative quantity of OctAlpha receptor mRNA in the nervous system, Malpighian tubule, and oviduct of *P. rapae*. The mRNA quantity is expressed relative to the tissue with the highest level of transcript. The letters denote levels of statistical significance; tissues that share letters with other tissues have no significant difference between them, according to a Tukey test ($\alpha<0.05$). NS = nervous system; MT = Malpighian tubule; Ovi = oviduct.

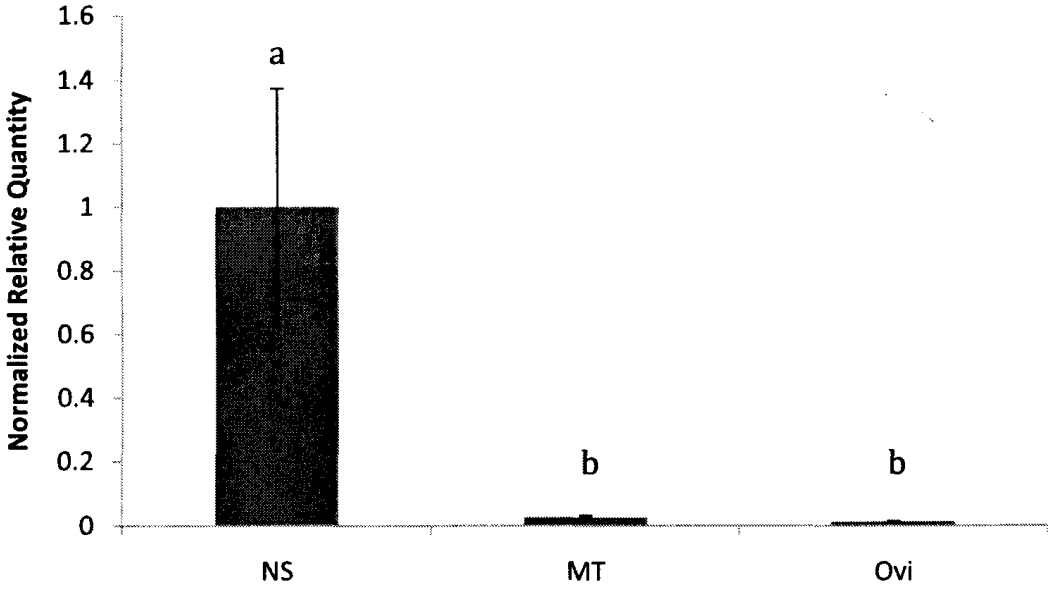

Figure 10. Normalized relative quantity of OctOA/TA receptor mRNA in the nervous system, Malpighian tubule, and oviduct of *P. rapae*. The mRNA quantity is expressed relative to the tissue with the highest level of transcript. The letters denote levels of statistical significance; tissues that share letters with other tissues have no significant difference between them, according to a Tukey test ($\alpha<0.05$). NS = nervous system; MT = Malpighian tubule; Ovi = oviduct.

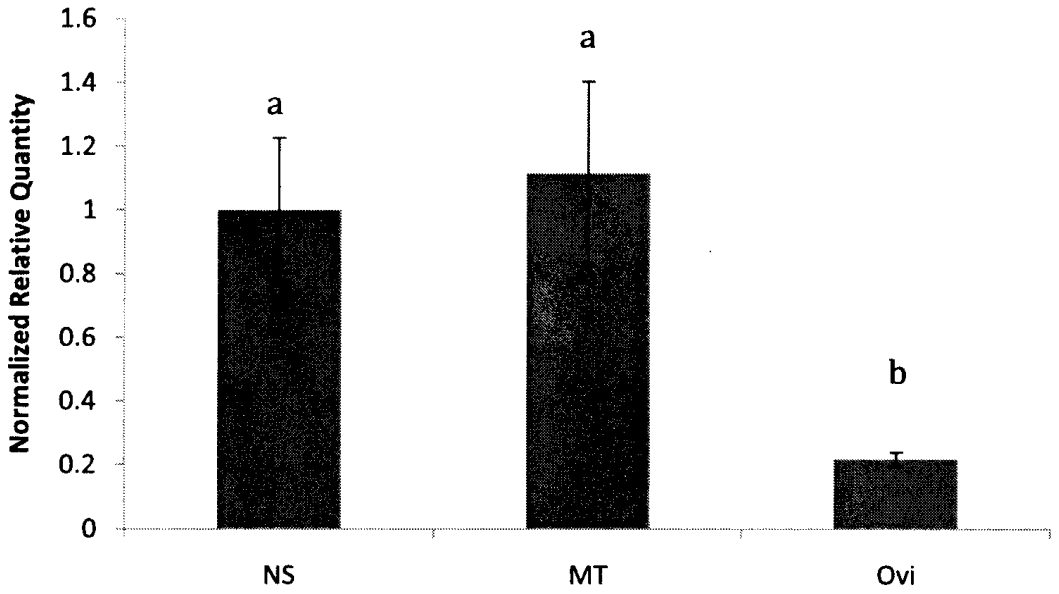

Figure 11. Normalized relative quantity of OctBeta1 receptor mRNA in the nervous system, Malpighian tubule, and oviduct of *P. rapae*. The mRNA quantity is expressed relative to the tissue with the highest level of transcript. The letters denote levels of statistical significance; tissues that share letters with other tissues have no significant difference between them, according to a Tukey test ($\alpha<0.05$). NS = nervous system; MT = Malpighian tubule; Ovi = oviduct.

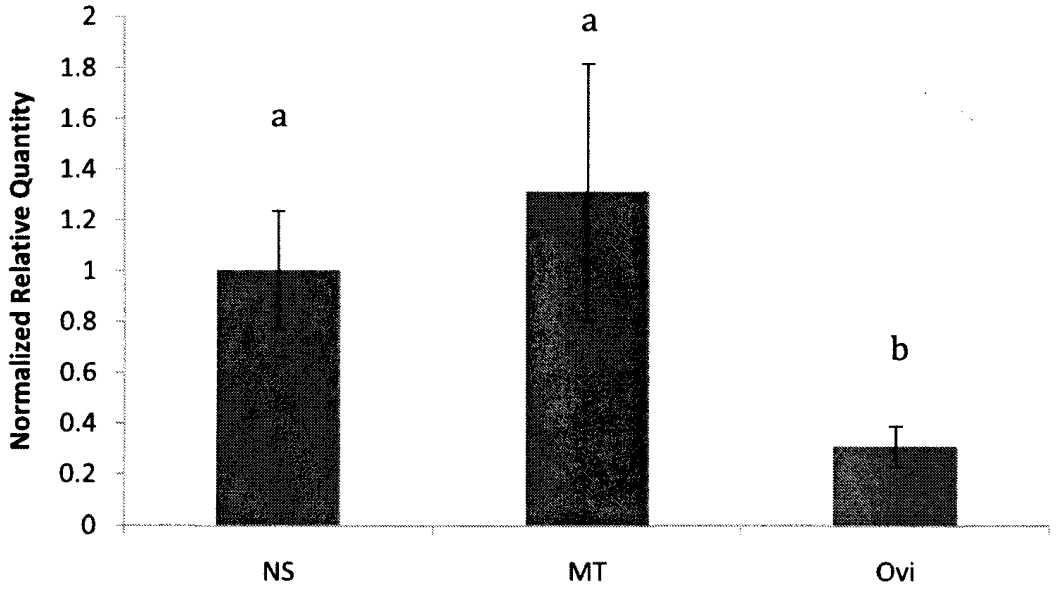

Figure 12. Normalized relative quantity of OctBeta1 receptor mRNA from a second qPCR primer and probe set in the nervous system, Malpighian tubule, and oviduct of *P. rapae*. The mRNA quantity is expressed relative to the tissue with the highest level of transcript. The letters denote levels of statistical significance; tissues that share letters with other tissues have no significant difference between them, according to a Tukey test ($\alpha<0.05$). NS = nervous system; MT = Malpighian tubule; Ovi = oviduct.

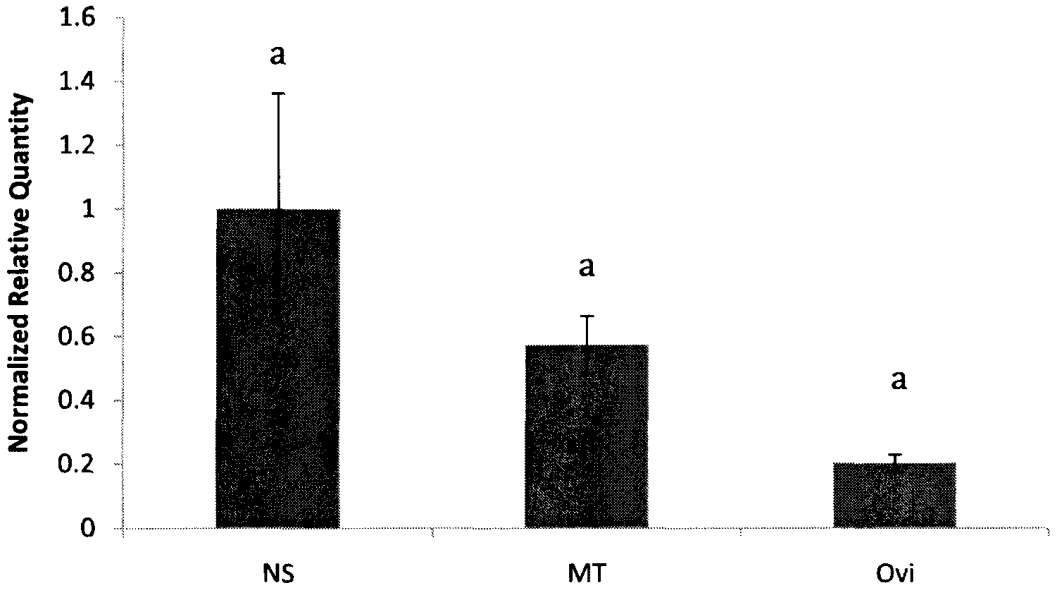

Figure 13. Normalized relative quantity of OctBeta3 receptor mRNA in the nervous system, Malpighian tubule, and oviduct of *P. rapae*. The mRNA quantity is expressed relative to the tissue with the highest level of transcript. The letters denote levels of statistical significance; tissues that share letters with other tissues have no significant difference between them, according to a Tukey test ($\alpha<0.05$). NS = nervous system; MT = Malpighian tubule; Ovi = oviduct.

3.2.3 Relative Expression Levels of OAR Genes in *P. unipuncta* Tissues

In *P. unipuncta*, the alpha adrenergic-like receptor gene was expressed highest in the nervous system, followed by the Malpighian tubule, with little expression in the oviduct (Fig. 14). The expression of the octopamine/tyramine receptor gene was high in the nervous system, but significantly much lower in the Malpighian tubules and oviduct (Fig. 15). The Beta 1 receptor gene expression level was significantly higher in the Malpighian tubule than the nervous system and oviduct (Fig. 16), while the Beta 2 and Beta 3 receptor genes were significantly higher in the nervous system than in the Malpighian tubules and oviduct (Figs. 17 and 18, respectively).

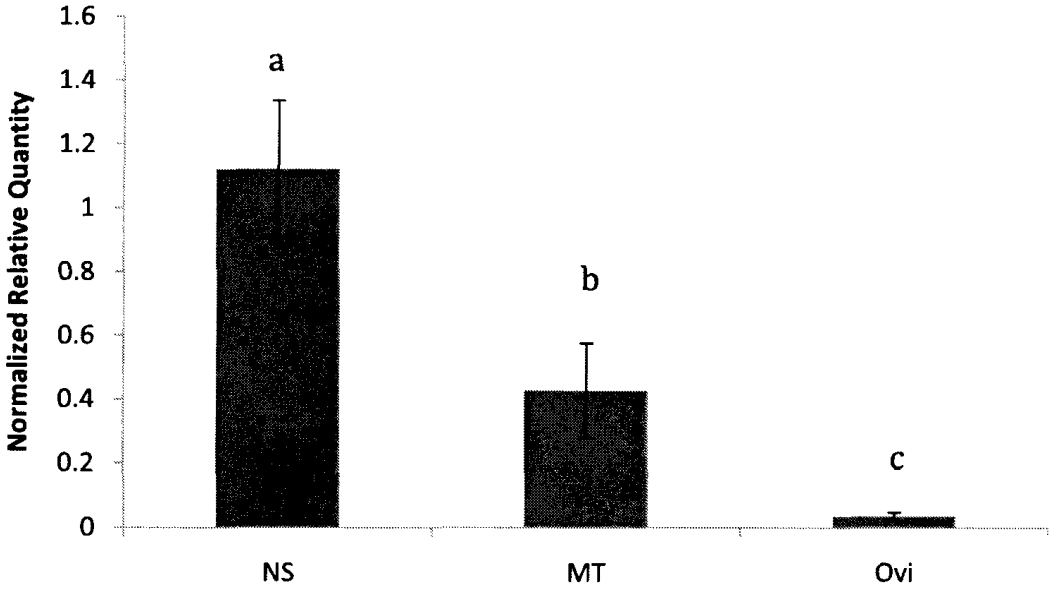

Figure 14. Normalized relative quantity of OctAlpha receptor mRNA in the nervous system, Malpighian tubule, and oviduct of *P. unipuncta*. The mRNA quantity is expressed relative to the tissue with the highest level of transcript. The letters denote levels of statistical significance; tissues that share letters with other tissues have no significant difference between them, according to a Tukey test ($\alpha<0.05$). NS = nervous system; MT = Malpighian tubule; Ovi = oviduct.

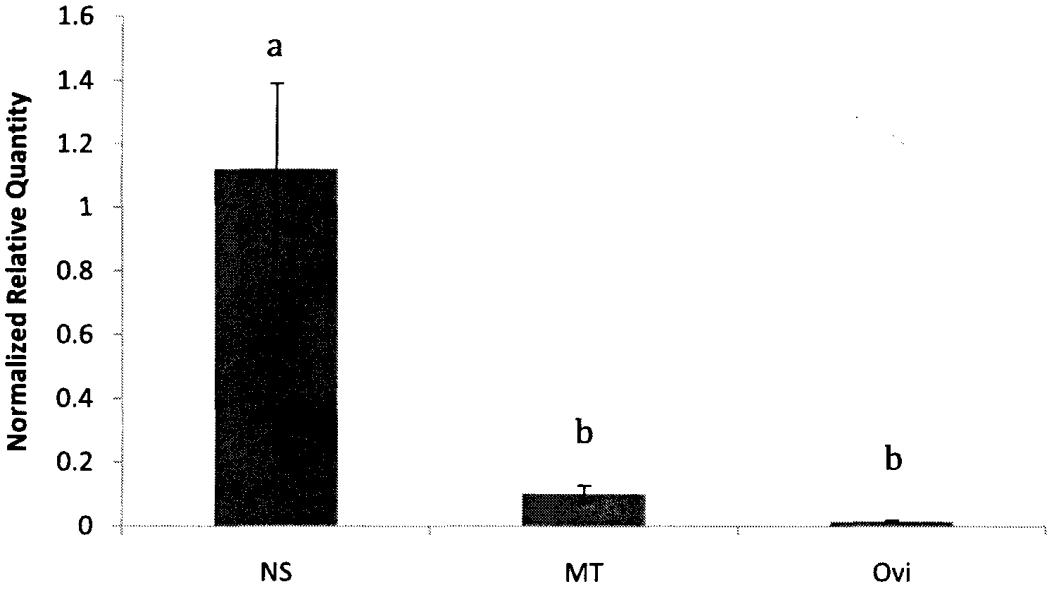

Figure 15. Normalized relative quantity of OctOA/TA receptor mRNA in the nervous system, Malpighian tubule, and oviduct of *P. unipuncta*. The mRNA quantity is expressed relative to the tissue with the highest level of transcript. The letters denote levels of statistical significance; tissues that share letters with other tissues have no significant difference between them, according to a Tukey test ($\alpha<0.05$). NS = nervous system; MT = Malpighian tubule; Ovi = oviduct.

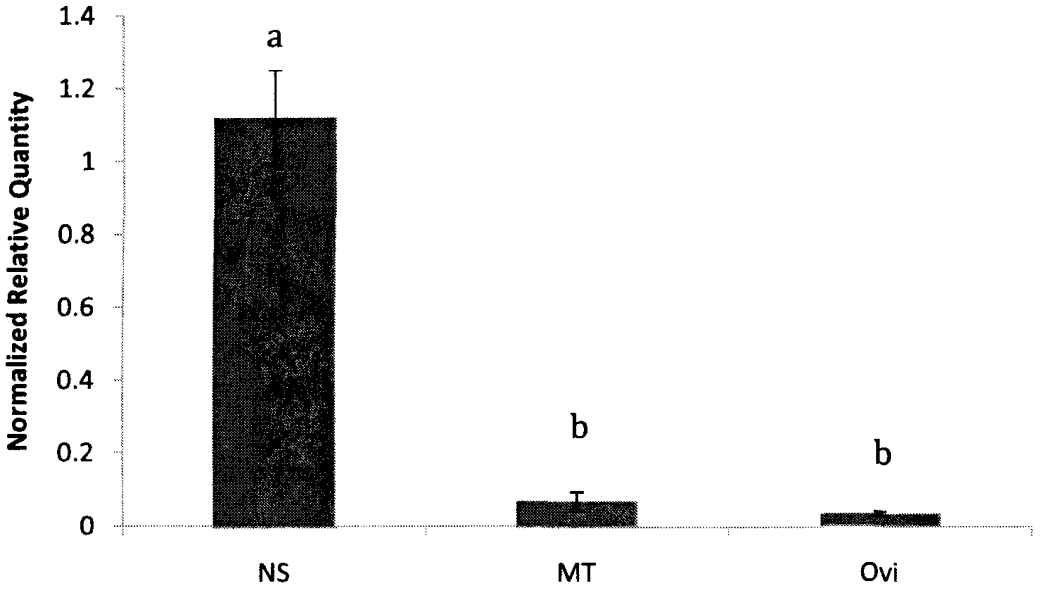

Figure 18. Normalized relative quantity of OctBeta3 receptor mRNA in the nervous system, Malpighian tubule, and oviduct of *P. unipuncta*. The mRNA quantity is expressed relative to the tissue with the highest level of transcript. The letters denote levels of statistical significance; tissues that share letters with other tissues have no significant difference between them, according to a Tukey test ($\alpha<0.05$). NS = nervous system; MT = Malpighian tubule; Ovi = oviduct.

4. Discussion

Molecular Cloning and Sequence Analysis

My first goal was to clone fragments of octopamine receptor (OAR) genes from *T. ni*, *P. unipuncta* and *P. rapae* corresponding to the five octopamine receptor types outlined by Evans and Maqueira (2005): alpha adrenergic-like receptor, octopamine/tyramine receptor, and three beta adrenergic-like receptors. Using degenerative PCR, I was able to isolate fragments corresponding to each receptor type from *T. ni*, *P. unipuncta* and *P. rapae* larvae, except for one OAR gene from *P. rapae* (OARbeta2).

The three lepidopteran OARalpha gene fragments cloned shared a very high identity with known alpha adrenergic-like receptors from other lepidopteran species, although for *P. rapae* it was slightly lower than the other two species. This result is to be expected as the butterfly is part of the Pieridae family in the Papilionoidea superfamily, while both moth species are part of the Noctuoidea superfamily and are more closely related to *B. mori* and *M. sexta*, which are part of the Bombycoidea superfamily (Fig. 19).

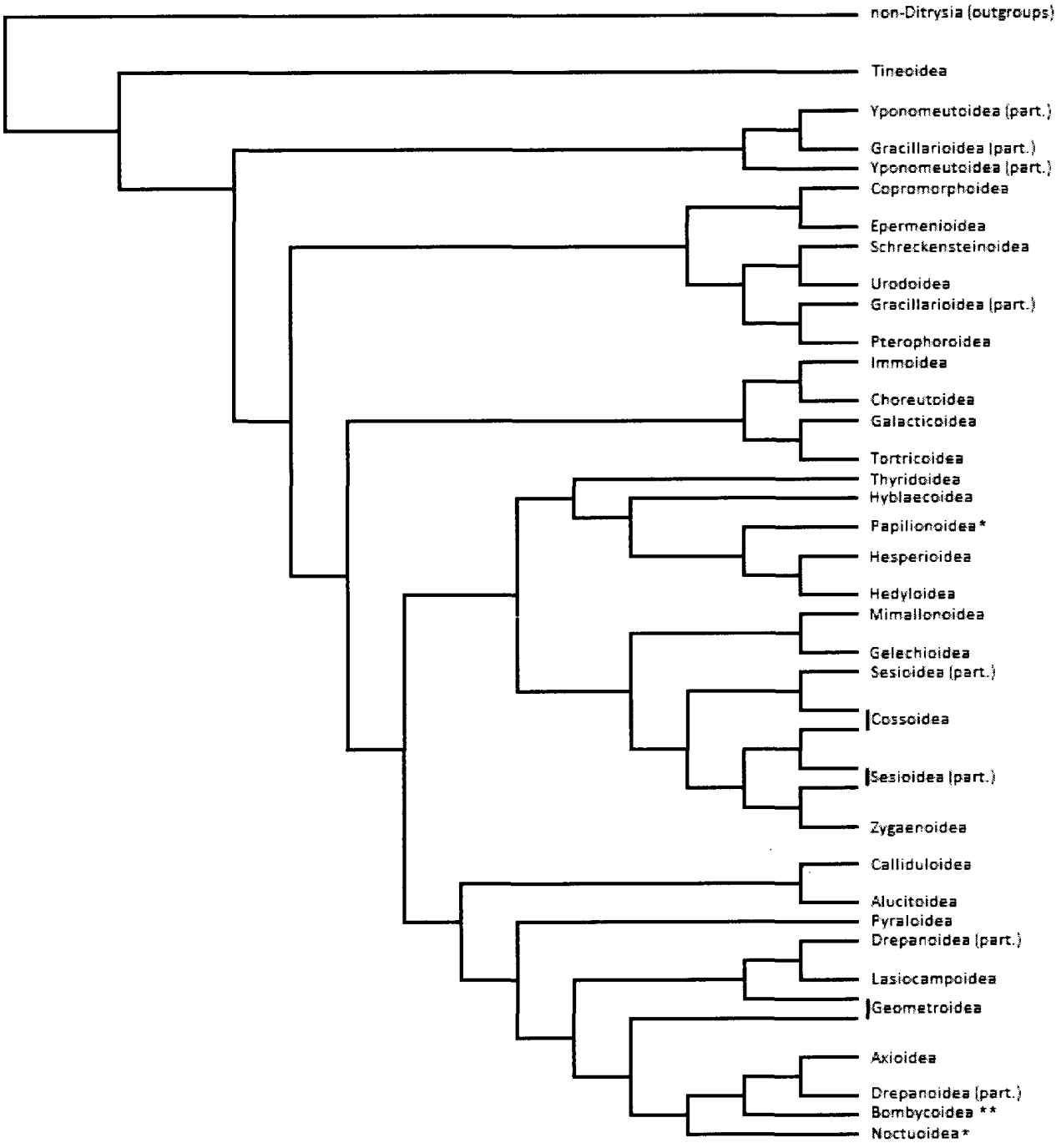

Figure 19. Cladogram of ditrysian superfamiles. Modified from Mutanen et al. (2010).
* indicates superfamilies which are included in this study: Papilionoidea for *P. rapae*, and Noctuoidea for *T. ni* and *P. unipuncta*.
** indicates the superfamily (Bombycoidea) containing *Bombyx mori* and *Manduca sexta*

I found a very high similarity (98-100%) between my OA/TA receptor sequences and those of previously cloned lepidopteran receptors in the moths, *M. brassicae*, *H. virescens* and *S. littoralis*. This indicates that the OA/TA receptor gene is extremely well conserved in the Lepidoptera, more so than any of the other octopamine receptor genes examined.

The three beta adrenergic-like receptor types appeared generally less well conserved, but this may be due to the scarcity of material for comparison as the only other cloned lepidopteran beta adrenergic-like receptor gene is the *Bombyx mori* BmOAR2 receptor, which was identified as an OARbeta2 orthologue by Chen *et al.* (2010). The same study also concluded, following a search of the silkworm genomic database, that *B. mori* lacked complete versions of both the OARbeta1 and OARbeta3 orthologues, the former being completely absent, and the latter being a pseudogene lacking a start codon. In my study, I was able to clone fragments of all three beta adrenergic-like receptor subtypes in both *T. ni* and *P. unipuncta*, and found fragments from two genes (Beta1R and Beta3R) in *P. rapae*. The *P. rapae* Beta2R was not found and a more extensive search is required to confirm if it is really absent. Only four cloned insect beta adrenergic-like receptors are present in the literature, the aforementioned BmOAR2 and the three original *Drosophila* receptors: DmOctBeta1, DmOctBeta2 and DmOctBeta3. However, the cloned fragments do share a generally high identity with a number of predicted receptor genes from other insects. Interestingly, the cloned OARbeta1 and OARbeta3 gene fragments generally share a higher identity with predicted genes from *T. castaneum* than with *A. mellifera* or *D. melanogaster* (Table 3). *Apis mellifera* is in the more distant Hymenoptera order and the lower identity value is not

surprising, but *D. melanogaster* (Diptera) is more closely related to Lepidoptera than *T. castaneum* (Coleoptera) (Simon et al., 2009; Wiegmann et al., 2009). Further work, including complete sequencing of the gene, is needed in order to construct a more accurate phylogeny. Phylogenetic analysis of the cloned OAR sequence fragments along with known receptors from *D. melanogaster* and *B. mori* did not include the *P. unipuncta* OARbeta1 sequence fragment. This is because proper alignment is not possible due to non-overlapping coverage of the OARbeta1 gene between the *P. unipuncta*, and *T. ni* and *P. rapae* fragments and would probably be corrected with complete sequencing of the OARbeta1 genes.

I also found that the sequence identities for the OARbeta3 fragments are lower than for the other beta adrenergic-like receptors. These fragments also group apart from the *Drosophila* orthologue in phylogenetic analysis (Fig. 3), due to the presence of a large insertion of approximately 360 nucleotides that is absent in the *Drosophila* DmOctBeta3 and predicted *T. castaneum* OARbeta3 genes. As mentioned previously, the OARbeta3 orthologue in the closely-related *B. mori* was designated a pseudogene (Chen *et al.*, 2010), and this suggests it may be a more divergent orthologue that is dispensable in some species and less likely to play a highly conserved role in others. The three beta adrenergic-like receptors share similar signaling properties and it is possible that the role of OARbeta3 in *B. mori* is substituted for by OARbeta2.

The cloning of putative OARbeta1 and OARbeta3 orthologues in all three species examined in this study illustrates the potential disadvantages of relying on *B. mori*, a species which has been thoroughly domesticated and lab-reared for decades, as a model

lepidopteran species. Although the finding of differential expression of the transcript in different tissues through the use of qPCR is a promising suggestion of biological relevance, it should be noted that a fifth of pseudogenes surveyed in a recent study were transcribed in a tissue-specific manner (Zheng *et al.*, 2006). It is difficult to draw conclusions using data gathered from cloning only fragments of each of these genes and further cloning will be necessary to determine definitively whether or not these genes encode full-length, functional receptors.

qPCR Experiments to Determine Relative Gene Expression Levels

In selecting the most stable reference genes for this study, three genes were tested, leading to the elimination of the gene with the highest average pairwise variation with respect to the other genes (M value). In this case the G3PDH gene was found to have the highest variation in *T. ni*. Given that G3PDH is a very widely used reference gene in qPCR experiments, this illustrates the importance of proper reference gene validation in these experiments. Ideally, my study would have included a survey of many reference genes, which would then be evaluated and pared down to a 'basket' of the three most stable genes, but this approach is prohibitively costly and time-consuming when using hydrolysis probes as were employed in this study.

As mentioned previously, a second fragment of *P. rapae* OARbeta1 was cloned while searching for OARbeta2, and qPCR experiments run to determine its relative expression levels in the nervous system, oviduct and Malpighian tubule. The relative

expression levels of this second OARbeta1 were nearly identical to that of the first, giving me a strong confirmation of the precision of the qPCR method.

Alpha Adrenergic-like Receptor Gene Expression

An examination of the OARalpha gene fragments cloned from the three lepidopteran species revealed that they were expressed in different patterns. Relative to the nervous system, Malpighian tubule expression was more similar between the two moths, *T. ni* and *P. unipuncta*, while the oviduct expression was more similar between *T. ni* and *P. rapae*.

It is not surprising that the two more closely related moths would have similar expression patterns in the Malpighian tubules. A difference might have been expected due to diet-related differences between generalists and specialists, as a recent study showed that plant secondary compounds can target insect Malpighian tubules, causing toxicity via an impairment of water balance (Stanisçuaski et al., 2009). Studies with the woodrats *Neotoma stephensi* and *N. albigula*, while not necessarily applicable to insects, have found that generalists were more impacted by plant secondary compounds with diuretic effects than specialists (Dearing et al., 2001). Furthermore, both *T. ni* and *P. rapae* are specialists feeding on plants in the Brassicacae family with characteristic defensive compounds, including the isothiocyanates, which can reduce survival and growth (Agrawal and Kurashige, 2003). However from the expression data it appears that if there is an octopaminergic component to the differences in Malpighian tubule adaptation to host plants defenses in these insects, it does not involve OARalpha.

The notion that OARalpha modulates the oviduct agrees with studies done on *Drosophila*, where the alpha adrenergic-like receptor (OAMB) is required for ovulation and female fecundity (Lee *et al.*, 2003). A possible explanation for the difference in oviduct relative expression in *P. unipuncta* compared with the other two species may lie in differences in egg laying. Both *T. ni* and *P. rapae* lay eggs singly while *P. unipuncta* lays clusters of eggs. While the moderate expression level in *T. ni* and *P. rapae* oviduct supports the role of OARalpha as being involved in ovulation, it is possible that ovulation, if mediated by octopamine receptors in *P. unipuncta*, involves different receptor types.

OARalpha gene expression was widely distributed in the remaining tissues of *T. ni*. This contrasts with the other OARs, which were each typically expressed in specific tissues. The relative expression levels were quite varied, however, I can still make some conclusions from the data.

The highest expression of the OARalpha gene appears in the muscle, and with the possible exception of OARbeta2, is the only OAR expressed in this tissue. This result suggests that the OARalpha receptor plays an important role in octopaminergic modulation of muscle, possibly in muscle tension and relaxation as is seen in other insects (Roeder, 2005). Besides the direct modulation of skeletal and visceral (e.g. oviduct) muscle, octopamine also acts on the central nervous system to alter locomotory patterns (Fox et al., 2006), and though the *T. ni* OARalpha receptor is also expressed highly in the nervous system, it is difficult to say which processes it is involved in without further research. Experiments involving *in situ* hybridization would be useful in

determining the expression pattern of the receptor within the nervous system, as this would shed some light into the systems in which they function.

The expression of *T. ni* OARalpha was lowest in the midgut, and this relative lack of expression is unexpected given that there is evidence for octopaminergic control of both long-lasting contractions and spontaneous rhythmic contractions of the midgut (Luffy and Dorn, 1992). However, the latter effect was only seen in midgut preparations that included the ingluvial (ventricular) ganglion, indicating that the octopaminergic control is achieved neuronally rather than through midgut receptors. The former effect, consisting of a single long-lasting contraction, might suggest the presence of octopamine receptors in the midgut, but it was only seen at high doses. Furthermore, the effect was also elicited by serotonin at much lower concentrations than octopamine. In addition, dopamine, noradrenaline and acetylcholine also caused a contraction at high concentrations, leading the authors to suggest that the effect might be a non-specific reaction to high, non-physiological doses. Expression of an octopamine receptor gene has also been found in midgut tissue of the locust (Molaei et al., 2005), though the receptor studied in that case was the OA/TA receptor and will be discussed later.

Moderate levels of expression of the OARalpha gene were found in the fat body, integument and hindgut/rectum samples. Expression in the integument is surprising, given that I was unable to find any published demonstration of octopaminergic control of insect integument tissue. There is documented monoaminergic control in the cuticle of the blood-feeding insect *Rhodnius prolixus* (Barrett et al., 1992; Orchard et al., 1988) and the tick, *Amblyomma hebraeum* (Kaufman et al., 2010). In these cases, it may be involved

in plasticization, a process which allows the cuticle to expand to accommodate a large blood meal, something that would not be required for the insects examined in this study. It is possible that octopamine could play a role in the plasticization of lepidopteran cuticle during ecdysis, and further study is required to verify this proposition.

OARalpha was the only octopamine receptor that showed expression in the hindgut/rectum and fat body tissues of *T. ni*. Octopamine plays a modulatory role on hindgut contractions, though the role may vary with species: increasing amplitude, but not frequency in *L. migratoria* (Huddart and Oldfield, 1982), and increasing frequency, but not amplitude in *Aedes aegypti* (Messer and Brown, 1985). Also, as mentioned previously, octopamine plays a role in lipid mobilization in fat body (Arrese and Soulages, 2010) and OARalpha expression in this tissue would likely be related to lipid mobilization and energy release. Though more work is needed to clarify the significance of the relative expression levels, given the large amount of biological variation seen, OARalpha appears to be a good candidate for the facilitator of octopaminergic control in the hindgut and fat body.

Octopamine/Tyramine Receptor Gene Expression

The OA/TA receptor transcript level was similar in the three insect species, with significant expression only in the nervous system. Again, at this time it is difficult to determine which neurological processes are involved, although studies using the *Drosophila honoka* mutant (reduced OA/TA receptor expression) found that the receptor played a role in olfactory sensory input and neuromuscular modulation (Kutsukake et

al.,2000; Nagaya et al., 2002). As mentioned previously, *in situ* hybridization experiments would be helpful in determining the physiological roles of this receptor type.

I did not find the OA/TA receptor expressed in the Malpighian tubules, which was somewhat unexpected as it had been found in this tissue in *Drosophila* (Blumenthal, 2003). This difference is probably due to the specific diet-related role of the receptor; in the case of *Drosophila* tyramine from the bacteria in the rotting-fruit diet of the fruitfly is presumed to stimulate diuresis through the OA/TA receptor. Furthermore, fermentation by yeast produces ethanol in the fruitfly diet, potentially causing an osmotic imbalance that necessitates specific actions of the Malpighian tubules that would not be required in the lepidopterans under consideration in this study. Octopaminergic modulation of lepidopteran Malpighian tubules is discussed later.

The lack of OA/TA receptor gene expression in the muscle was also unexpected given the evidence for its tyraminergic control. Recall that the *Drosophila* mutant, *honoka* (with reduced OA/TA receptor expression), showed a reduced effect of tyramine on the excitatory junctional potential amplitude in the larval body wall muscle (Kutsukake et al., 2000; Nagaya et al., 2002). It appears from my results that if this effect occurs in the lepidopteran species examined, it would be achieved via the central nervous system and not through the direct modulation of the muscle. Any effects of direct tyramine application on muscle are perhaps mediated by the tyramine-specific (Cazzamali et al., 2005), instead of OA/TA receptors. Differences in larval locomotion between lepidopterans and dipterans may also be important in the differences observed in OA/TA muscle modulation indicated by these expression data. The caterpillars have three

sets of legs as well as several prolegs, while *Drosophila* larvae are legless and move by peristaltic movements followed by turning and head swinging (exploratory) behaviour (Rodriguez Moncalvo and Campos, 2009).

The OA/TA receptor is found in a wide range of tissues in other organisms (Blumenthal, 2003; Molaei et al., 2005; von Nickisch-Resenegk et al., 1996). For example, in *L. migratoria*, RT-PCR and Northern blotting reveals that it is in the midgut, oviduct and nervous system (Molaei et al., 2005). It does appear that the expression is much higher in the nervous system than in the other tissues, which is consistent with my findings. The biological relevance of this low level of expression in non-nervous tissues is uncertain, however, given the similarity of the relative expression levels of the midgut and oviduct in *T. ni* to other tissues (such as the integument) for which no documented octopaminergic or tyraminergic control was found, it appears that the OA/TA receptor in lepidopteran larvae is likely primarily located in the central nervous system. There remain many adult tissues to be assayed for the presence of the OA/TA receptor, and further study is needed in order to determine the extent of its role.

Beta Adrenergic-like Receptor Gene Expression

Little is known regarding the expression of beta adrenergic-like receptors in insects, except that they are expressed at much higher levels in the head than the body of *Drosophila* adults (Evans and Maqueira, 2005). In my study I observed that the expression of *T. ni* OARbeta1 receptor transcript was more than three-fold higher in the

Malpighian tubules than in the nervous system and oviduct, a pattern that is also seen in *P. unipuncta* and *P. rapae.*

Control of fluid secretion in Malpighian tubules by octopamine, and in some cases other biogenic amines, has been well documented in many insect species, increasing fluid secretion rates in the worker ant, *Formica polyctena* (De Decker et al., 1994); the house cricket, *Acheta domesticus* (Coast, 1989); and the tobacco budworm, *Heliothis virescens* (Chung and Keeley, 1989). In these experiments, the diuretic effects of octopamine were compared to that of whole head extracts and the resulting fluid secretion rates differed in degree, as octopamine generated a smaller response. Furthermore, the apparent mechanism of the effect also appeared to be different, as experiments by Chung and Keeley (1989) with starved and feeding larvae showed that octopamine stimulated secretion under both conditions, while head extracts did not affect the feeding larvae which were presumably receiving a high degree of diuretic stimulation due to the high water content of the diet. The authors further found that the effect of head extracts were not caused by biogenic amines in the nervous system as the action was protease sensitive, and concluded that biogenic amines instead mediated a stress-related diuretic effect.

This stress-related effect, however, is not seen in the citrus swallowtail butterfly, *Papilio demodocus* (Nicolson and Millar, 1983), revealing a contradictory picture of lepidopteran Malpighian tubule octopaminergic control. This difference is probably due to the fact that larval tubules were tested in *H. virescens*, while in *P. demodocus* they examined adult tubules. Malpighian tubules are known to be quite different between adult

and larval lepidopterans; unlike adult tubules, the tubules of larvae exhibit high endogenous secretory activity and have no need for diuretic stimulation (Chung and Keeley, 1989). Still another study revealed the lack of octopaminergic action on the Malpighian tubules of the mosquito, *A. aegypti* (Veenstra, 1987), further highlighting the fact that octopaminergic control of Malpighian tubules is not uniform among insects.

The high relative expression of the OARbeta1 receptor gene in the Malpighian tubules of all three of my test species indicates that this receptor is a good candidate for octopaminergic control in this tissue of lepidopteran larvae, rather than the OA/TA receptor as in other insects (Blumenthal, 2003).

The *T. ni* OARbeta2 transcript was expressed highest in a peripheral tissue, especially oviduct, rather than in the head as reported for *Drosophila*. This suggests that the *T. ni* oviduct is potentially under the modulation of at least two different octopamine receptors: OARalpha and OARbeta2. However, an examination of *P. unipuncta* failed to provide any clarity as OARbeta2 expression appeared to be largely confined to the nervous system. As mentioned previously, any difference in oviduct OAR expression may be due to differences in egg-laying between the two species. Furthermore, although locust oviducts treated with octopamine show increased cAMP levels (Lange and Orchard, 1986), an effect which can be achieved by beta adrenergic-like receptors, this can be blocked with phentolamine, which is not an antagonist for beta adrenergic-like receptors and is in fact an agonist (Maqueira et al., 2005; Chen et al., 2010). Further study is needed to determine if the high relative expression of OARbeta2 in *T. ni* oviduct is an artefact, or if oviduct control in *T. ni* is different than in other insects.

Finally, the relative expression levels for *T. ni* and *P. unipuncta* OARbeta3 were low in all tissues except the nervous system. Of the three beta adrenergic-like receptors this is the only one whose expression pattern agrees with that seen in *Drosophila*.

In *P. rapae* OARbeta3 there was considerable variation in the data that was too great to draw any meaningful conclusions. However, it does appear that the nervous system has the highest expression, and additional experiments are needed to clarify this. Additional aspects of the data also need to be clarified, such as the expression of OARbeta1 in *P. unipuncta*, which appears higher in the nervous system than the oviduct, and the expression of OARbeta1 in *T. ni*, where there appears to be a second tier of expression that includes the nervous system and oviduct. These differences in expression were deemed to be statistically insignificant, but nevertheless appear different qualitatively and could turn out to be bona fide if the biological variation can be reduced. As with *T. ni* OARbeta1, there appears to be a second tier of expression in *T. ni* OARbeta2, in this case including the nervous system, muscle and integument that is determined to be not significantly different from the lower expression levels seen in other tissues. Additional experiments would help to determine if an actual difference exists.

Surprisingly, no significant octopamine receptor gene expression was seen in *P. unipuncta* oviduct. It seems improbable that this is due to problems with the tissue itself as all three reference genes were expressed highly. The reproductive physiology of *P. unipuncta* is known to be highly dependent on environmental conditions such as temperature and photoperiodic conditions (McNeil and Tobe, 2001), so perhaps the low

relative levels of octopamine receptor expression in the oviduct is related to any differences in rearing conditions following the procurement of larval individuals. It would be interesting to do further experiments to determine whether or not ovulation in *P. unipuncta* is modulated by octopamine as in other insects, or if the low relative level of expression is in fact biologically relevant. The control of the locust oviduct by biogenic amines has been studied extensively with the conclusion that octopamine plays a very prominent role in the control of oviduct muscle contractions (for a review see: Lange, 2009b). The studies also found that tyramine mimics many of the effects of octopamine and may be an important agent of oviduct control on its own. This mimicry suggests that both tyramine and octopamine act upon the same receptor: the octopamine/tyramine receptor. The absence of a high relative expression level of the octopamine/tyramine receptor in any of the species examined might indicate that oviduct control is achieved in a different manner in the Lepidoptera. OARalpha appears to be a good candidate for oviduct modulation in *T. ni* and *P. rapae* as it appears to be expressed in both species, though OARbeta2 is very highly expressed in *T. ni* and would appear to be involved as well.

I believe that the information gained in this study reveals important insights into the specific physiological roles of different octopamine receptors. However, there remain many tissues to be examined for the expression of octopamine receptors. Adult female tissues like the spermatheca are known to be under octopaminergic control (Clark and Lange, 2003). As mentioned previously, there is also octopaminergic modulation of the foregut contraction (Huddart and Oldfield, 1982). Further studies examining OAR expression in these, and other, tissues would help to determine the receptor(s) responsible

for modulating these organs. Particularly, experiments involving *in situ* hybridization would help to determine the cellular locations of these receptors and reveal specific cell types and regions within tissues in which the receptors play a role. Additionally, immunohistochemical experiments to determine the cellular location of receptors would be useful in further understanding octopamine signaling. A comprehensive picture of the various octopamine receptors present in different tissues will help to determine their specific roles, alone or in combination, in the control of insect physiological processes.

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