รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาคุณลักษณะของฟีโรโมนที่กระตุ้นเพศสัมพันธุ์ในกุ้งก้ามกราม

(Characterization of Reproduction-Related Pheromones in the Fresh Water Prawn, *Macrobrachium rosenbergii*, and Their Possible Applications in Aquaculture)

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สัญญาเลขที่ MRG 5280112

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Abstract (บทคัดย่อ)

In this study, we have identified the structure of olfactory nerve system of giant freshwater prawn, *M. rosenbergii*, and its role in perceiving mating pheromones. The antennules and antennae were dissected and found to be supplied with separately nerve fibers originated from different sources of brain suggesting different functions of these organs. Four types of sensilla setae including long simple, short simple, tuft medium simple and aesthetasc have been identified on the antennules and antenna using scanning electron microscope imaging. The aesthetasc sensilla setae which were previously suspected to be odor receptor was found on the short lateral antennules implicating that this organ may be involved in olfactory perception system. The short lateral antenular nerve from aesthetasc was connected to olfactory receptor neuron conjugated with nerve bundle directly projected to olfactory neurophil of the brain. Immunofluorescense staining with anti-GABA antibody indicated that the olfactory neurons were GABAnergic types and ensured that short lateral antennular neurons connected to the olfactory neuropil in the brain. The functional studies of antennular and antenna neuronal pathway on mating response after female reproductive pheromone attraction was performed by ablation test. Significantly lost of mating behavior response and to female prawn attraction was observed after lateral antennules ablated implicating that this organ was critical for determining reproductive pheromone. Decrease in relative sexual scores was observed in antenna ablated prawn indicated that this organ might be cooperated with antennules in determining female reproductive pheromone.

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Introduction

Giant feshwater prawn (*Macrobrachium rosenbergii*) is one of the most cultivated crustaceans that still command high value for domestic as well as export markets. In the cultivation of this prawn, apart from the problem of diseases, the reproductive behaviors is unpredictable and beyond our control. The ovarian development in captive females is decreased and copulation does not take place properly, even though they are kept together with sexually mature male. Moreover, spawning success and the number of nauplii produced per spawn are also decreased after prolonged captivity. Reproductive pheromones have been shown to be stimulators or modulators in sexual communication and copulation in various crustacean species (Gleeson et al., 1987; Skog et al., 2009; Atema & Cowan, 1986; Corkum & Belanger, 2007). However, detailed knowledge on the reproductive pheromones and how they function in this prawn are still unclear.

Reproductive pheromones are chemical cues released from fully mature female for mating attraction to a conspecific male. Despite to the lack of identified chemical attractants from female crustacean, male receive distal and contact pheromone from molted female and stimulate mating behaviors (Herborg et al., 2006). The courtship behavior and mating behaviors will occur in short period post molting and sperm from male will be deposit onto female before spawning and fertilization occur (Atema & Cowan, 1986). In the evolution of the animals from single cell prokaryote to higher vertebrates, pheromone is important for existing of their generation (Lledo et al, 2005; Hoover, 2010). Sex pheromones have been particular studied in mammal and insects (Matsunami & Amrein, 2003; Wyatt, 2010; Lledo et al., 2005). Previous studies have shown that olfactory system is responsible for sex pheromone perception. The pheromone receptor cells are reported and

localized on vomero-nasal organ (VNO) in mammal and reptiles and it involves in pheromone perception (Touhara, 2008; Kaupp, 2010; DØving & Trotier, 1998; Lledo et al., 2005; Mustaparta, 1996). In insect, this cue is perceived by sensillar-olfactory cell units and its sensillar setae that present on antennae (Matsunami & Amrein, 2003; Mustaparta, 1996). Both of chemoreceptor organs of these are described to be a primary olfactory receptor unit before it sends the axon to synapse with the higher order neurons in the brain. The olfactory system has been reported to be important for reproductive pheromone perception in crustacean (Wyatt, 2003). Odor receptor organs and setae have been reported to be presented on the antennules (Schmidt & Derby, 2005) whereas the antennae are not described. There are two classical chemosensory pathways in crustacean, i.e., aesthetasc-olfactory neuropil (ON) pathway and non-aesthetasc- lateral antenna I neuropil (LAN) pathway. Aesthetasc- ON pathway is involved in chemosensory and pheromone perception whereas non-aesthetasc- LAN pathway is involved in mechanosesory pathway (Horner et al., 2008; Derby & Sorensen, 2008; Schmidt et al., 1992). The specific aesthetasc sensilla were shown to be the pheromone receptor organ which was present on the antennules of spiny lobster (Steullet et al., 2000). However, detailed knowledge on the pheromone perception and pheromone modulation in *M. rosenbergii* is still unknown.

This study aimed at identifying the olfactory organ of male prawn including olfactory receptor, neuronal connection to the brain and the neurotransmitter system. The functional role of olfactory system in pheromone perception was investigated by mating attraction response after ablation of antennules and antennae of male prawn.

Materials and methods

1. Animals

Healthy and sexually mature giant freshwater prawns, *M. rosenbergii* (60- 90g body weight) were collected from commercially available sources in Ayutthaya Province central market, Thailand and reared in laboratory aquarium at the Department of Anatomy, Faculty of Science, Mahidol University Thailand. The animals were maintained in 500 L tanks with aeration at ambient temperature and fed with either life feed or commercial food pellets alternately. The water was changed every day to keep good salinity. Each tank was contained 5 plastic roll cages for avoidances of cannibalism during molt period. Prawns were separated with sexuality and stages in each tank for prevent inter-chemical perceiving between sexes that may effect on experiment interfering. Post-molted female ovarian maturation stage 4, reference (Ra'anan & Sagi, 1985) were used in this behavioral assay. Tissues collection was manipulated after acclimatization for at least 2 days. These tissues were processed in hematoxylin-eosin staining, immunohistochemistry and whole-mount immunofluorescence. The prawns were maintained at a light-dark cycle from natural light for behavioral assay.

2. Tissue processing for histology study

Brains and four types of anterior appendicular filaments (lateral antennules, medial antennules, short lateral antennules and antennae) from mature male prawns were collected by anesthetized on ice and cut the anterior head appendage. The head part was fixed in 4% paraformaldehyde in 0.1M phosphate buffered saline pH7.4 (PBS) at 4°C overnight. Brains and nerves were dissected from hard carapace and cleaned in PBS. The tissues were dehydrated through a series of increasing

concentrations of ethanol, infiltrated with liquid paraffin (Paraplast), and finally embedded in paraffin blocks. The brain blocks were sectioned at 7µm thick, the antennule and antenna filament blocks were section at 7-10 µm depend on hardness of tissues.

The tissue slides were stained with Hematoxylin and Eosin (H&E) before subsequently observed and photographed by a Nikon digital DXM 1200 camera. The anatomical nomenclature of various parts of the brain was based on that described previously by Sanderman et al. (1992).

3. Immunohistochemistry with anti-GABA specific antibody

The tissue sections were run in xylene for deparaffinization and rehydrated in graded series of ethyl alcohol (100%, 95%, 90%, 80%, 70%) respectively for 5 min per each. Sections were then incubated with 1% Lithium carbonate for 15 min followed by 3% H_2O_2 in methanol for 30-45 min at room temperature. All sections were washed two times with PBS 5 min before glycine blocking was done for decrease effect of aldehyde group. Then they were washed twice with TPBS (0.4% triton X-100 in PBS) 5 min and PBS 5 min, respectively. Non-specific binding was reduced by incubated sections in blocking solution (10% normal goat serum TPBS) for 2 h. The sections were incubated with rabbit anti-GABA antibody at dilution 1:500 in blocking solution in moisture chambers for overnight. After primary antibody incubating, the sections were washed twice with TPBS and PBS for 10 min, respectively. Tissue sections were further incubated in secondary antibody goat anti-rabbit HRP conjugated IgG (Sigma Co., St. Louis, MO, USA) in moisture chamber for 2 h at room temperature. After three times of washing, the sections with TPBS were developed signal staining with Nova Red (Vector, Burlingame, CA, USA) for 5-7 min and stop reaction by rinsing through tap water. Counter staining with Mayer's

hematoxylin, washing and closed with permount and coverlid. The sections were subsequently observed and photographed by a Nikon digital DXM 1200 camera. For whole mount immunofluorescence, Brain and antennular nerves were dissected apart from head part of male prawns after fixed in 4% paraformaldehyde in PBS at 4°C for overnight. The tissues were rinsed with PBS for washing and de-sheathed on stereomicroscope. Therefore male prawn's CNS was covered with thick muscle sheath and fat droplets, de-sheathed process was intensively performed to get isolated CNS specimen to minimize low background staining. The brains and each nerve were pre-incubated in 0.1% Sodium azide (NaN_3) in 0.5% triton X-100 in PBST(0.25% tween-20 in PBS) at 4°C for 2 days. Tissues were washed twice with PBST for 15 min and permeabilized with Dent's solution (80% methanol with 20% DMSO) at 20 °C for 4 h. After permeabilization, the tissues whole-mount were washed two times with 0.1M PBS and incubated in primary antibody (rabbit anti- GABA in 10% normal goat serum in PBST) at 4°C for 6 days. The secondary antibody mixture (goat anti-rabbit IgG –Alexa 488 conjugated at dilution at 1: 500, TOPRO-3 for nuclear staining at varies dilution 1:2000-1:4000 and 5% normal goat serum in blocking solution) were added to the tissues after three time washing with 0.1M PBST and PBS, respectively and incubated at 4°C for 4 days in dark moisture chamber prevent from light illumination. Whole mounts were then washed for 1h with PBST, for two times and with addition washing 15 min with PBS two times. Dehydration with serial increasing concentration of ethyl alcohol (from 50%, 70%, 80%, 90%, 95%, 100% two times) and finally, incubated in methyl salicylate for overnight. The tissue were kept them at 4°C before observed and photographed under an Olympus FV 1000 laser – scanning confocal microscope (LSCM).

4. Mating Behaviors of prawn

Sixty male prawn representing mating response to molted female were divided into six groups for ablation experiments. Post-molted stage female prawns (within 12 h after molt) with 3rd-4th ovarian maturation stage which previously reported to secreted contact pheromone (Zhang & Lin, 2006) were applied for mating behaviors. The ablation test were included antennules ablation, short antennules ablation, antennae ablation, sham control with petroleum wax (Vasaline), the first pair swimming legs ablation and control normal prawn. All criteria were ablated in both sites and removed by shape-tipped forceps that not show the effect on shrimp behavior (Zhang & Lin, 2006). The mating behavioral study of *M. rosenbergii*, were collected by video capture recording and analysis the recorded behavioral pattern in each actions (detected by responding) and sum into the group of behavior follow with Seinen Chow report (Chow et al., 1982). The mating process of *M. rosenbergii* was divided in to 4 continuous phases; contract, seizure and mounting, turning and mating (Chow et al, 1982). The ablation tests were studied at 1 h, 20h after ablation. Only approaching and copulation of mating behaviors were characterized as responding to mating and recorded time of response for comparison. All time data were adjusted in time score as shown in table 1. All score in all groups were averaged and statistical analysis was analyzed by SPSS program version 17 with one way ANOVA, Duncan post hoc.

Table1: The time scoring of copulation behavior when changed from raw data

Copulation time (min)	Scores
<1.00	10
1.01-3.00	9
3.01-5.00	8
5.01-7.00	7
7.01-9.00	6
9.01-11.00	5
11.01-13.00	4
13.01-15.00	3
15.01-17.00	2
17.01-19.00	1
>19.01 or non-response	0

Results

Identification of antennular and antenna nerves

There were 3 pairs of antennular filaments and a pair of antennae projecting from the anterior part of the head. The base of antennular filaments was a cone-shaped segment, namely antennular base segment (ABS) (figure 1-A). There were flat cuticle plates bordering the ABS which showed ciliary hair appearance along the lateral sides. The three pairs of antennular filaments were identified as medial antennules (MAnu), lateral antennules (LAnu), and short lateral antennules (short LAnu) (figure 1-A). The short LAnu shared the proximal part with the LAnu. While the 3 pairs of antennules had a common support which was the ABS, the antennae (Ana) were projecting from a separated cone-shape segment located ventro-lateral to the ABS. The nerve bundles projecting to the antennules and the antennae were revealed under a stereomicroscope. There were 4 nerve bundles projecting from the ABS to the antennular filaments, i.e., lateral antennular nerve (LAnNv), medial antennular nerve (MAnNv), outer lateral antennular nerve (OLAnNv), and inner lateral antennular nerve (ILAnNv) (figure 1-A, B). The LAnNv and MAnNv were large nerve bundles projecting toward the LAnu and MAnu, respectively. These two nerves were joined together at the proximal part of the ABS to be a big antennular nerve (AnNv) before connecting to the brain (figure 1-A, B). The OLAnNv ran toward the lumen of short LANu (figure 1-C). It penetrated muscle and ran closely with the ILAnNv that supplied the dorsal surface of the distal ABS. Both OLAnNv and ILAnNv were also joined with the large AnNv. There were another group of nerve fibers projecting to the lateral aspect of ABS and supplying the proximal part of ABS, namely lateral tegumentary nerve (LTNv). The LTNv also jointed the AnNv (figure 1-A).

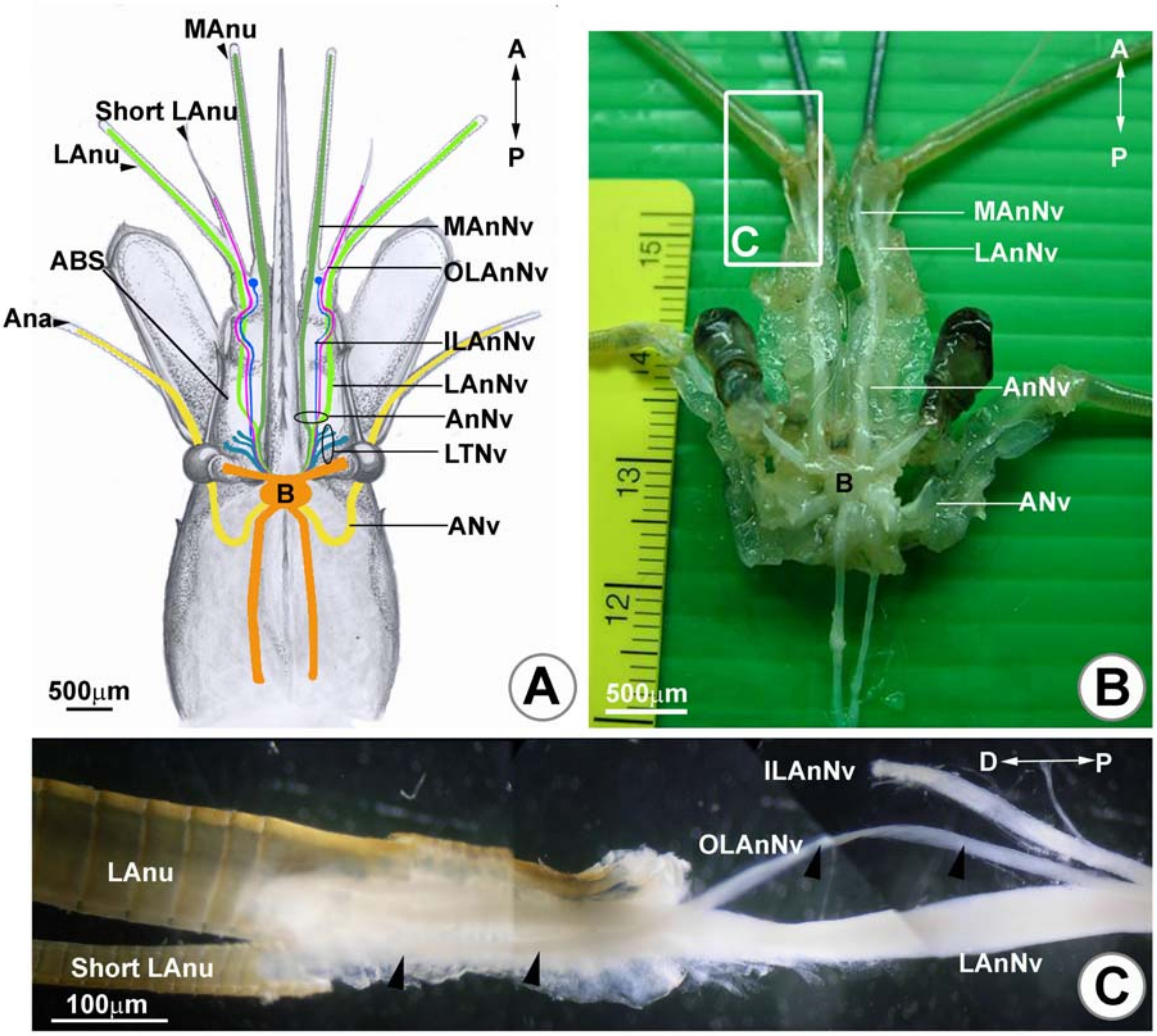

Figure 1. Schematic diagram (A) and photographs (B-C) showing nerve supplies to the antennules and antennae of *M. rosenbergii*. There are 3 pairs of antennular filaments including lateral antennules (LAnu), medial antennules (MAnu) and short lateral antennule (short LAnu). The antennular nerve (AnNv) contains 4 braches, i.e., medial antennular nerve (MAnNv), lateral antennular nerve (LAnNv), outer lateral antennular nerve (OLAnNv) and inner lateral antennular nerve (ILAnNv). The OLAnNv and ILAnNv run parallel to each other. While the OLAnNv runs toward the short LAnu, the ILAnNv finishes at anterior end of the dorsal surface of the antennular base segment (ABS). There are also another group of nerve fibers

projecting to lateral aspect of the proximal ABS and jointing the AnNv, namely lateral tegumentary nerve (LTNv). The antennae have separated nerve supplies, called antenna nerve (ANv). The AnNv runs and connects to the antero-ventral site of the brain (B), whereas the ANv connect to the postero-ventral site of the brain. Stereomicroscopic image of OLAnNv and ILAnNv in panel C showed that the OLAnNv runs toward the short LAnu, while the ILAnNv supplies the dorsal surface of the distal ABS.

Structure and distribution of four types of sensilla setae were characterized on antennules and antennae by SEM

Under SEM, sensilla setae that were distributed on the cuticle surface of the antennules and the antennae were revealed. They could be identified into 4 types including long simple setae, short simple setae, tuft medium setae, and aesthetasc (figure 2). The single long simple setae were present at the center of shallow depression of internode cuticle and they were distributed throughout of the antennules and the antennae (figure 2-A, B). The short simple setae were found only at the lateral part of the short LAnu (figure 2-C, D). The tuft medium setae which were characterized by triple long cuticle hairs that projected from a single hole were distributed on the middle and distal parts of the MAnu and the short LANu (figure 2-E, F). The aesthetasc were present uniquely on the ventral surface of the short LAnu (figure 2-G, H). They were arranged in rows; each row contained 4-5 aesthetascs. Each aesthetasc was appeared as a hallow cylinder with a flattened pad on a tapered end. The lumen of aesthetasc opened at the base of the flatten pad (figure 2-G, H).

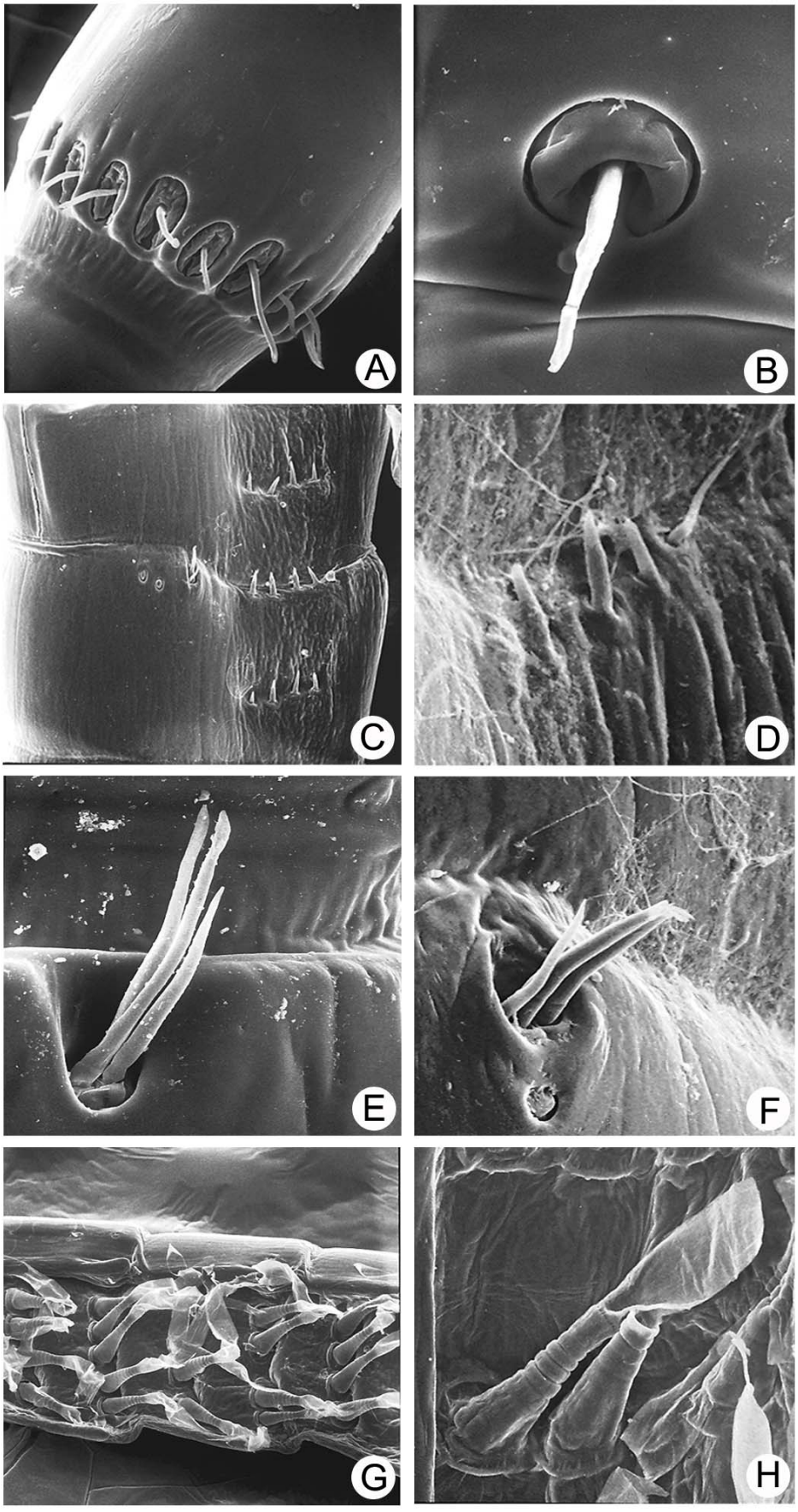
A
B
C
D
E
F
G
H

Figure 2. Scanning electron micrographs of sensilla setae on the antennular and antenna surface. Four setae types are found including simple long setae (A-B), short simple setae (C-D), tuft medium setae (E-F), and aesthetasc (G-H). The simple long setae (A-B) are found throughout the antennae and the antennules including MAnu, LAnu, and short LAnu. The short simple setae (C-D) are found only at the proximal part of short LAnu, specifically at the lateral border. The tuft medium setae (E-F) are distributed on the MAnu and the dorsal surface of the short LAnu surface. The aesthetasc (G-H) are found only at the ventral surface of the short LANu. There are many aesthetascs arranged in rows; each aesthetasc has a central lumen and a flatten pad end.

Aesthetasc on the short lateral antennules were supported with olfactory receptor neurons (ORNs)

Histological study of the antenules showed tuft of epithelial cells lining beneath the cuticle (figure 3-A-D). In MAnu and LAnu, there were particular regions that the cells were packed together and possessed some finely fibers projecting interiorly (figure-3B, asterisk). This structure was found underneath the long simple setae locating on the surface of MAnu and LAnu and could be mechanoreceptive cells or non-olfactory chemoreceptive cells (Schmidt et al., 1992). For the short LAnu that contained the aesthetasc on the ventral side, olfactory receptor neurons (ORNs) were revealed (figure 3-E). The ORNs formed a pack of cells in a globular structure (figure 3-E, inset); it was 8-10 µm in diameter. The length of this structure from the distal pole to the proximal pole was 90-100 µm; the maximum diameter was approximately 50 µm. The proximal end of the ORNs showed the projecting axon extending toward the proximal part of the short LAnu forming the OLAnuNv. Inner dentritic fibers (ID) of the ORNs were found in the area connecting to the lumen of

the aesthetasc with surrounding associated auxiliary cells (Aux) (figure 3-F, G). Outer dendritic fibers (OD) were found in lumen of the aesthetasc (figure 3-H). A diagram of olfactory unit was shown in figure 3-I. The axons of each ORNs were packed together to form OLAnNv.

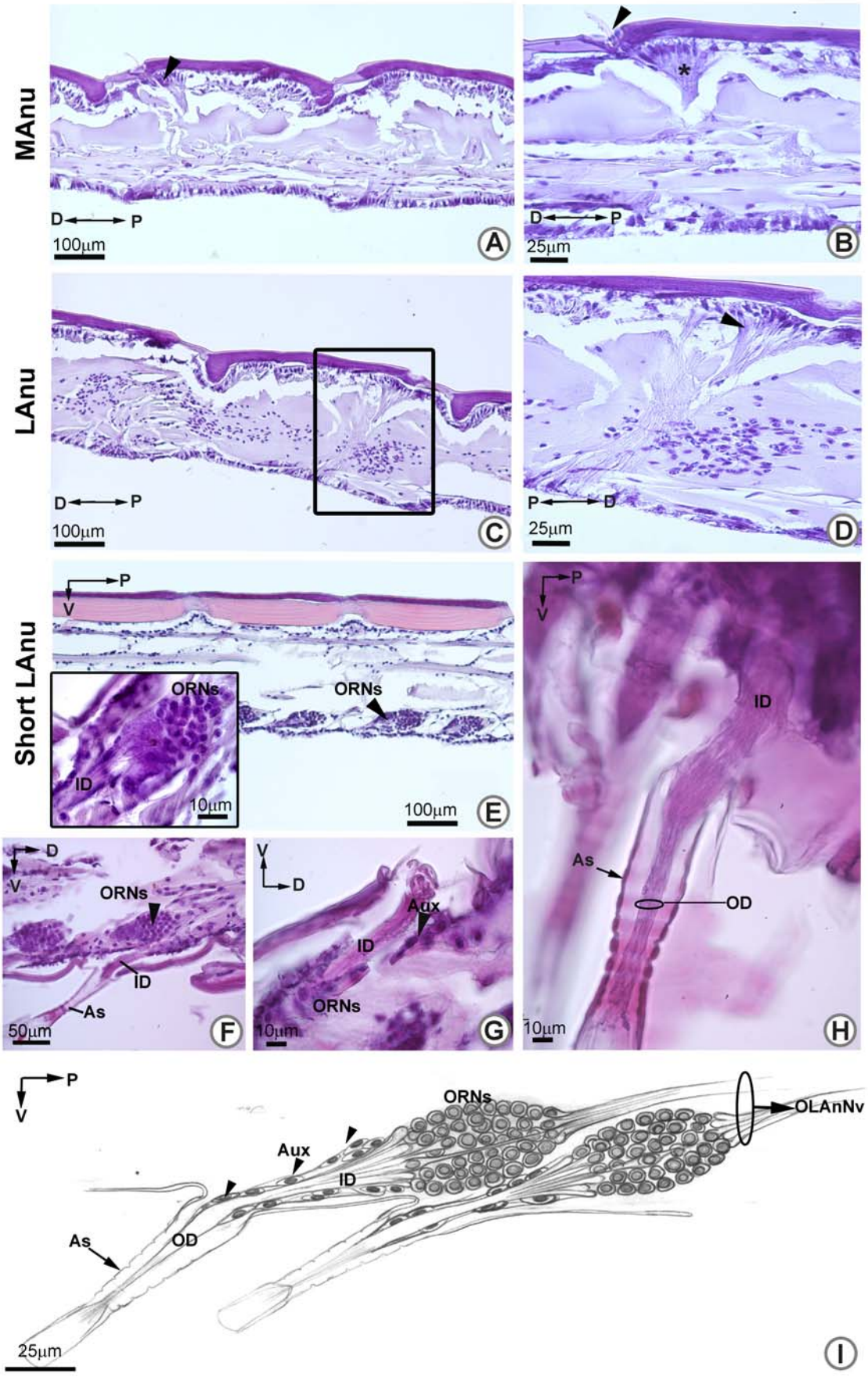
MAnu
LAnu
Short LAnu
D
P
V
100μm
25μm
10μm
50μm
ORNs
ID
As
OD
Aux
OLAnNv
A
B
C
D
E
F
G
H
I

Figure 3. Microscopic images of the antennules including the medial antennule (MAnu) (A-B), the lateral antennule (LAnu) (C-D), and the short lateral antennule (short LAnu) (E-H) showing possible mechanoreceptive cells and olfactory-chemoreceptive cells. Longitudinal sections of the MAnu showed epithelial cell liked receptors cells, located under the cuticle (A, arrow head). This cluster of cells possesses bunch of finely fibers projecting interiorly (B, asterisk). This structure was found underneath the long simple setae locating on the surface of MAnu and LAnu and could be mechanoreceptive cells or non-olfactory chemoreceptive cells (B, arrow head). Longitudinal sections of the LAnu show similar structures as shown in LAnu (C-D). The box in C is the area shown in D. There are also groups of cells underneath the cuticle and their bunch of fibers projecting inside (D, arrow head). Longitudinal sections of the short LAnu show the olfactory receptor neurons (ORNs) which are uniquely found at the ventral site of the short LAnu where the aesthetasc (AS) are located (E, F). The ORNs formed a pack of cells in a globular structure (E, inset). Inner dentritic fibers (ID) of the ORNs are found in the area connecting to the lumen of the AS with the associated auxiliary cells (Aux) surrounding the ID (F, G). Outer dendritic fibers (OD) are found in the lumen of the AS (H). A diagram of olfactory unit is shown in panel I. The axons of each ORNs are packed together to form the outer lateral antennular nerve (OLAnNv).

Nerve fibers from the aesthetasc were directly connected to olfactory neuropil region of the brain

Histology of the AnNv and the brain were investigated. All four branches of the AnNv, including MAnNv, LAnNv, ILAnNv, and OLAnNv, run directly to different areas of the brain. Both MAnNv and LAnNv were packed to be the big bundle which was a part of the AnNv. This big nerve bundles consisted of blue stained small cells embedded in the bundle which was surrounded by pale stained fibers (figure 4-A, B). They projected to the brain by locating medially to OLAnNv, ILAnNv and LTNv and terminated at the lateral antenna I neuropil (LAN), a part of antenna II neuropil (AnN) and the lower margin of the posterior medial protocerebral neuropil (PMPN) (figure 4-A, B). The ILAnNv, which came from the dorsal surface of the distal ABS, and the LTNv showed loosely packed fibers with a few small cells present inside (figure 4-B). Both ILAnNv and LTNv could come from mechanoreceptive organs underneath the ABS. These thin nerve bundles joined together before projecting to medial antenna I nuropil (MAN) and a part of LAN (figure 4-B). Moreover, there were some fibers projecting to the medial side of the olfactory neuropil (ON) where the olfactory globular tract (OGT) projected (figure 4-B). The OLAnNv, which connected the aesthetasc on the ventral side of short LAnNv, run laterally to the large bundle of the MAnNv-LAnNv and medially to the ILAnNv and LTNv (figure 4-C-D). This fiber projected into the ON and could have synapse on the surrounding cap regions of the ON (figure 4-E-F).

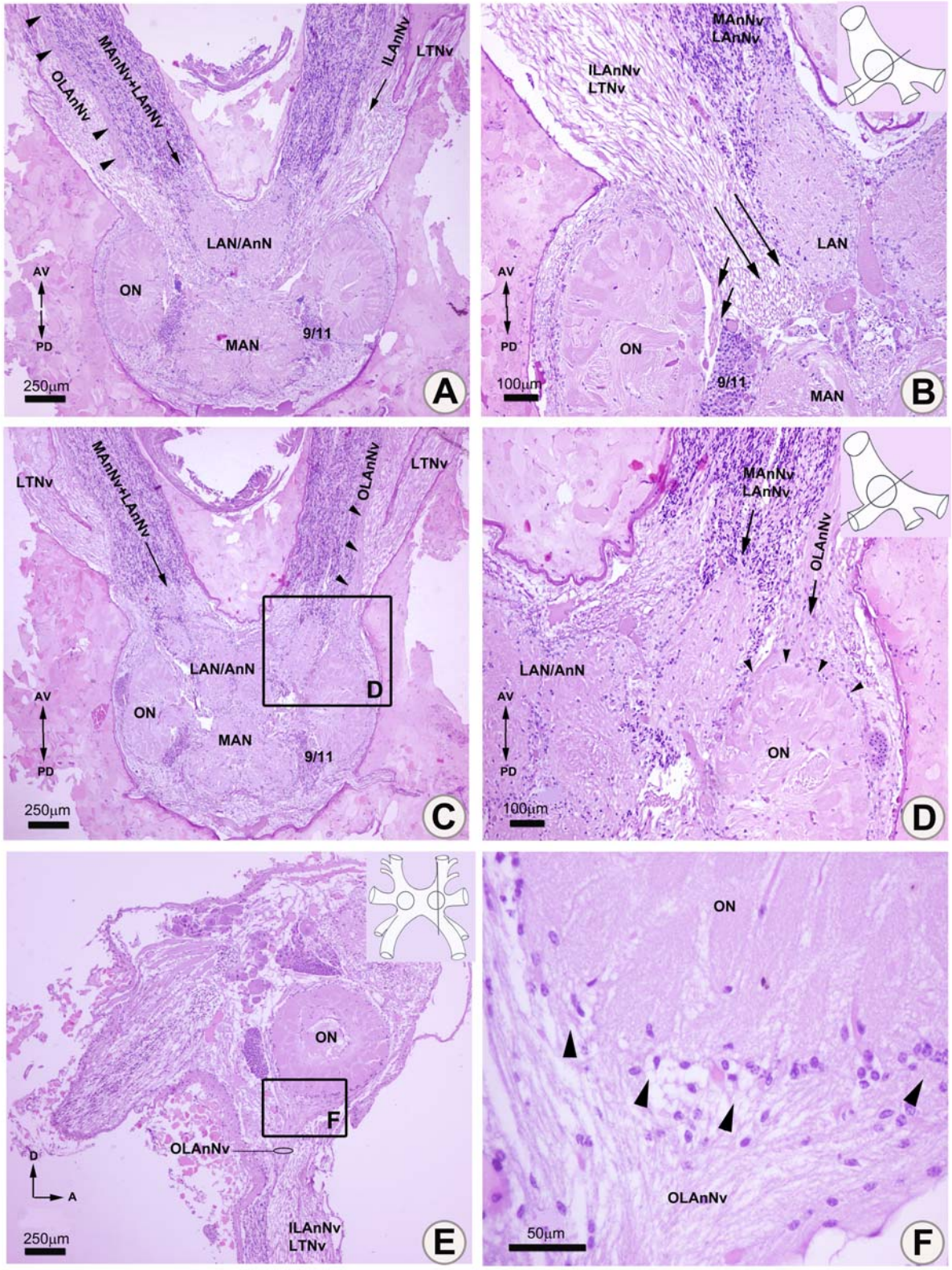
OLAnNv
MAnNv+LAnNv
ILAnNv
LTNv
LAN/AnN
AV
PD
ON
MAN
9/11
250µm
A
MAnNv
LAnNv
ILAnNv
LTNv
LAN
AV
PD
ON
9/11
MAN
100µm
B
LTNv
MAnNv+LAnNv
OLAnNv
LTNv
LAN/AnN
D
AV
PD
ON
MAN
9/11
250µm
C
MAnNv
LAnNv
OLAnNv
LAN/AnN
AV
PD
ON
100µm
D
ON
F
OLAnNv
D
A
250µm
ILAnNv
LTNv
E
ON
OLAnNv
50µm
F

Figure 4. Microscopic images showing the antennular nerve (AnNv) and their connection to the brain (B). The medial and lateral antennular nerves (MAnNv and LAnNv) form a big nerve bundle, which is a part of AnNV, and terminate into the lateral antennular neuropil (LAN), a part of antenna II neuropil (AnN) (A, B) and the lower margin of the posterior medial protocerebral neuropil (PMPN) (image not shown). The inner lateral antennular nerve (ILAnNv) and the lateral tegumentary nerve (LTNv) join together before projecting to the medial antenna I nuropil (MAN) (B, long arrows) and a part of LAN. Moreover, there are some fibers projecting to the medial side of the olfactory neuropil (ON) (B, short arrows). The outer lateral antennular nerve (OLAnNv) (arrow heads in A and C) runs medially to the ILAnNv (A, C, D) and terminates on a cap region of the ON (arrow heads in D). 9/11 = neuronal cluster 9/11.

Olfactory cells and nerve fibers supporting the asthetasc as well as the olfactory neuropils showed GABA immunoreactivity

GABA immunostaining was performed in the olfactory unit locating in the short LAnu, the AnNv, and the brain of male *M. rosenbergii* by immunoperoxidase and immunofluorescence method including the whole mount staining (figure 5, 6). Interestingly, it was demonstrated that the ID fibers of ORNs at the cone of aesthetasc and the Aux showed intense GABA immunoreactivity (GABA-ir) (figure 5-A-F). Moderate intensity of GABA-ir was found in the ORNs globular structure (figure 5-B, G, asterisks). The whole mount immuno-staining showed highly intense GABA-ir in fibers of the ILAnNv and LTNv while the OLAnNv showed moderate intensity of GABA-ir (figure 6-A). Moreover, possible synaptic fibers of the LTNv also showed strong GABA staining (figure 6-B, arrow heads). In contrast, GABA-ir was not present in the MAnNv and the LAnNv (figure 6-A). This result was confirmed with immunoperoxidase staining that fibers in the ILAnNv and LTNv showed intense GABA-ir (figure 6-C, arrow heads). The GABA-ir was also present in the OLAnNv that projected to the ON (figure 6-D). Moreover the staining was found intensely in the whole glomerular punctate of the ON including interior fibers of the ON (figure 6-E, F), OGT, and the olfactory globular tract neuropil (OGTN) that was a part of olfactory deutocerebrum (figure 6-E-G). The control sections where the anti-GABA antibody was omitted showed only background staining (data not shown).

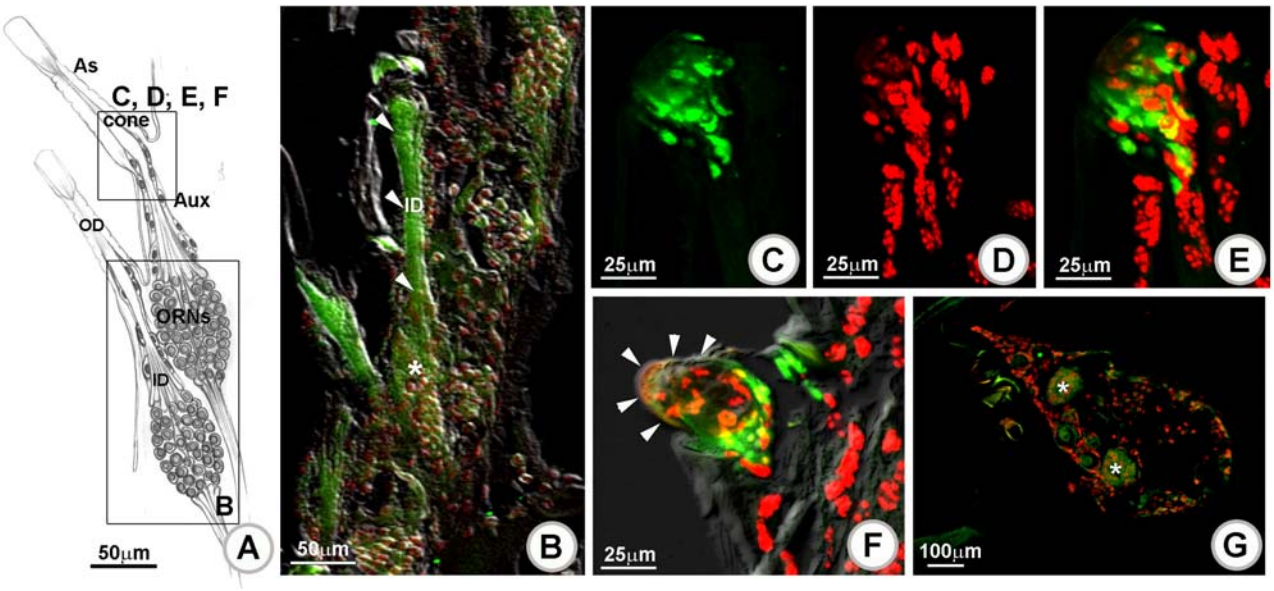

Figure 5. A schematic drawing of olfactory units (A) and fluorescence micrographs showing GABA immunoreactivity in the olfactory units (B-G). The olfactory units consist of olfactory receptor neurons (ORNs) and their projecting fibers toward the aesthetasc (As) lumen, namely the inner dendritic fibers (ID) that are surrounded proximally with auxiliary cells (Aux) (A). The outer dendritic fibers (OD) penetrate into the lumen of As (A). GABA-ir is strongly detected in the ID fibers (arrow heads in B) and moderately detected in the ORNs (asterisk in B). Moreover, the GABA-ir is also present intensely in the aesthetasc cone where the auxiliary cells (Aux) are located (C-F). Panel C shows the GABA-ir (green) in a group of Aux, whereas panel D shows the corresponding image with Tropo-3 staining (red). Panel E is the merged image of C and D. Another image showing GABA-ir (green) in Aux is shown in panel F. Panel G shows GABA-ir in the ORNs (asterisks) in a cross section of short lateral antennules (short LAnu).

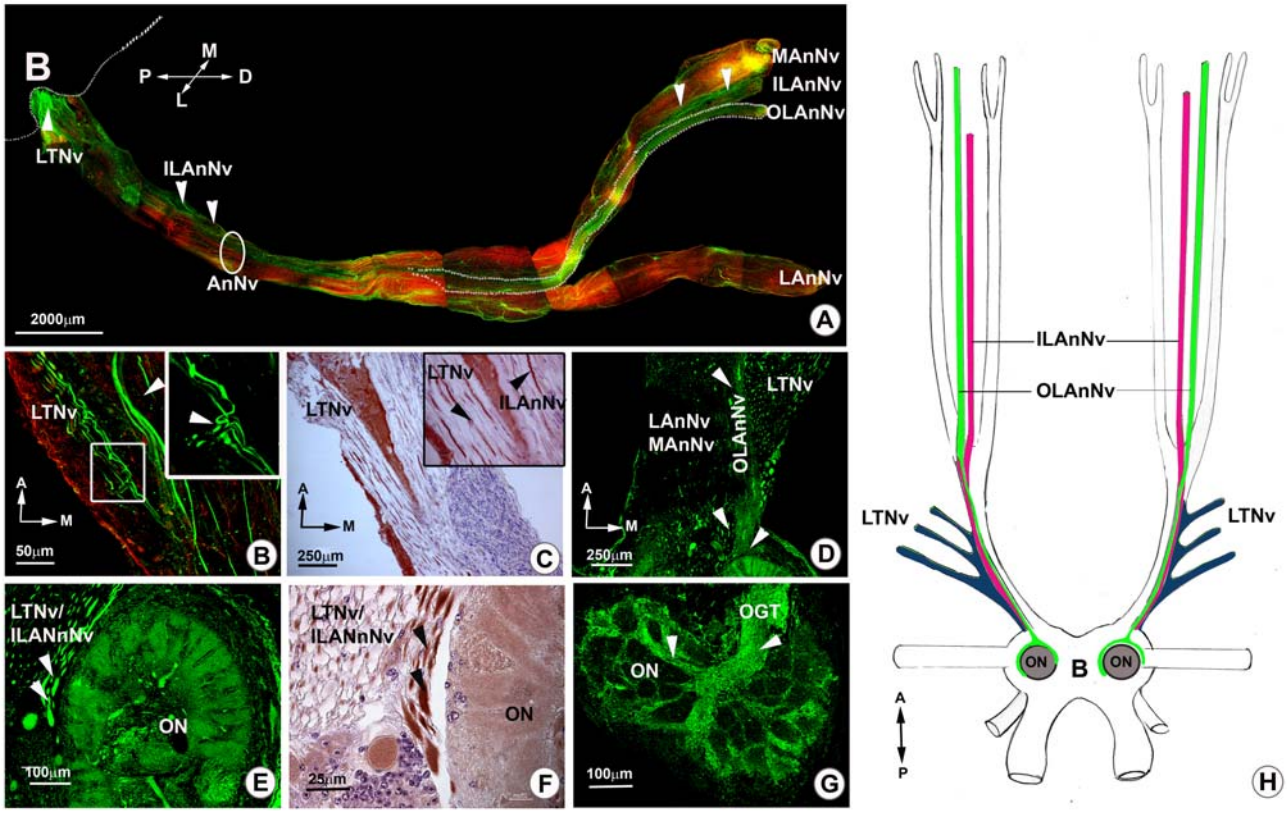

Figure 6. Immunofluorescence images (A-G) and a schematic drawing (H) showing GABA immunoreactivity in the antennular nerves pathway. Whole mount immunofluorescence staining with GABA shows that GABA-ir is strongly found in the inner lateral antennular nerve (ILAnNv) and the lateral tegumentary nerve (LTNv), while it is found moderately in the outer lateral antennular nerve (OLAnNv) (A). At higher magnification, it is shown clearly that GABA-ir is present in fibers of the LTNv and ILAnNv (arrow head in B), and in a possible nerve punctate of LTNv (inset in B). The immunoperoxidase staining of GABA also confirms that fibers of LTNv and ILAnNv are positively stained with GABA (C). The OLAnNv also shows GABA-ir in the synapse at the cap-region of the olfactory neuropil (ON) (D, arrow head). Immunofluorescence and immunoperoxidase staining of GABA in the ON region (E and F, respectively) show intense GABA-ir in the projection of LTNv and ILAnNv fibers to the ON (arrow heads in E and F), and the ON glomeruli punctate.

Olfactory globular tract (OGT), output olfactory ascending pathway, also shows strong GABA-ir (G). The GABA immunolocalization in the AnuNv and their connecting pathway is summarized in the schematic diagram H showing that GABA is present in the OLAnNv, ILAnNv, LTNv, and ON.

Olfactory tract was critical for reproductive pheromone perception and mating response

Functions of the male antennules and antennae on perceiving reproductive pheromone were studied by the ablation test and mating behaviors response. Since contact pheromones have been reported to be secreted from post-molted female to activate male prawn in mating (Zhang & Lin, 2006), the newly molted females were used in this experiment. Newly molted female were kept in separated tanks and immediately introduced into male chambers before mating behaviors of male and the copulation time were recorded. The copulation time of normal males having intact antennules and antennae was 10.29 ± 6.23 min (fastest response time = 1.10 min; slowest response time = 21.30 min), therefore, according to this standard copulation time we set the time scores used in this experiment as shown in table 1. In order to determine the involvement of antennules and antennae in mating response of male, the tested antennules or antennae of male prawns were ablated bilaterally and their mating behaviors, especially the copulation, were determined. All the male prawns used in the experiment were tested for their ability to mate with female prior to cut their antennules or antennae and the copulation time of individual male were recorded as a control in each experimental group. The experimental groups were divided into 5 groups including 1) the male with Anu ablation, 2) the male with short LAnu ablation, 3) the male with Ana ablation, 4) the male with the first swimming legs ablation (Swl ablation; negative control), and 5) the male having petroleum wax covered on the

testing Anu (sham control). For the males that were cut their antennules or antennae, they were rest for 1 h before testing their mating response. In order to ensure the result, each tested male was tested its ability to mate with a newly molted female 2 times by which the second time was performed at 20-h after first mating. The time scores of copulation were compared and the results were shown in Figure 7. The results showed that the copulation time of both first and second tests were significantly decreased ($p<0.05$) in the groups of Anu ablation (copulation time scores of the control, the first test, and the second test were 5.88 ± 2.295 min, 1.13 ± 2.232 min and 1.25 ± 2.053 min, respectively), short Anu ablation (copulation time scores of the control, the first test, and the second test were 4.00 ± 1.00 min, 0.33 ± 0.577 min and 0.33 ± 0.577 min, respectively), and sham control (copulation time scores of the control, the first test, and the second test were 5.25 ± 2.062, 0 and 0, respectively). In contrast, in the Ana and Swl ablation group, the copulation time tested at the second time was not significantly different, even though the copulation time was significantly decreased at first time testing (Ana ablation group: copulation time scores of the control, the first test, and the second test test were 4.86 ± 2.268 min, 1.00 ±1.915 min and 2.67 ± 3.077 min respectively; Swl ablation group: copulation time scores of the control, the first test, and the second test were 5.00 ± 0.816 min, 0.50 ± 1.00 min and 3.75 ± 2.986 min, respectively). The results suggested that the antennules played an important role in mating response of the males possibly by receiving attracting substances released from post-molted females. The significantly decrease in copulation time of the sham control, which the tested antennules were covered with petroleum wax, confirmed that the antennules, without injury, were critical for detecting female attracting substances. The control group that the first swimming legs of males were ablated showed that injury had an effect on the mating response of the

male only in the first period (1 h), however, it did not affect the mating response after 20 h.

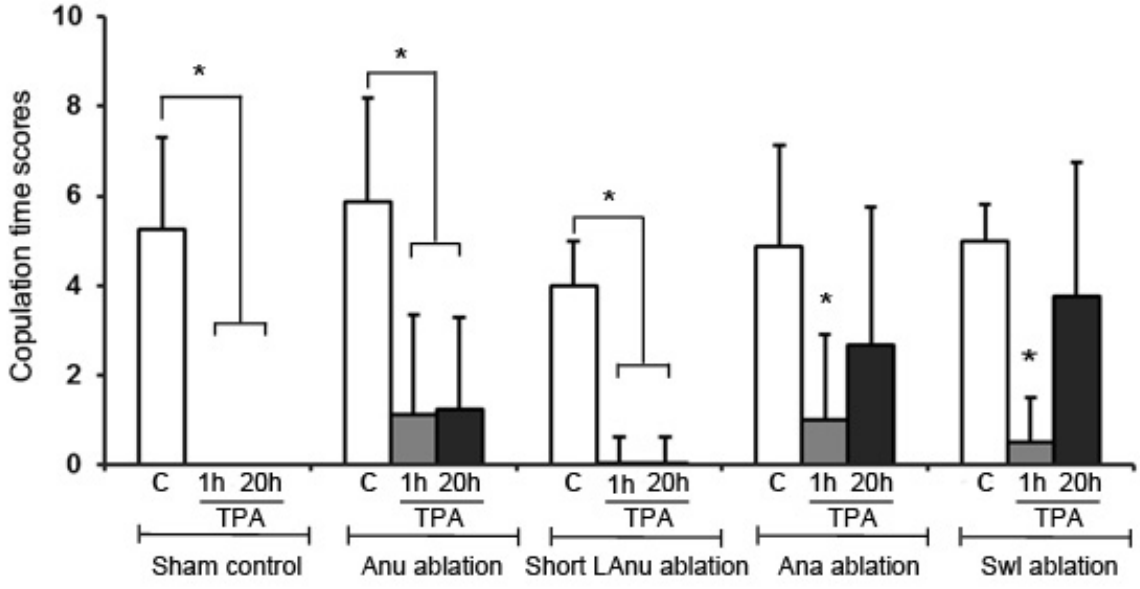

Figure 7. Mating behavioral assay of antennule and antenna ablated male prawn. Reduction of copulation in male is found significantly ($p < 0.05$) in the group of sham control which the tested antennules were covered with petroleum wax, the whole antennules ablation (Anu ablation), and the short lateral antennule ablation (short LAnu ablation) at 1-h and 20-h post ablation. In the group of antennae ablation (Ana ablation) and the first swimming leg ablation (Swl ablation), the copulation time is decreased significantly at 1-h post ablation, however, the copulation time is not decreased at 20-h post ablation.

Discussion

This study we investigated the four antennules nerves tract connection to identify olfactory organ and found that the lateral and medial antenular nerves were connected to lateral antenna I neuropil (LAN) and some part of antenna II neuropil (AnN) whereas the inner lateral antennular nerve was connected median antenna I neuropil (MAN) and some part of LAN. The outer lateral antenulular nerve, from short lateral antennule was connected to olfactory neuropil (ON) in the brain. Four types of sensilla setae on antennules and antennae were characterized by SEM. The aesthetasc setae on short lateral antennule were supported with olfactory receptor units and its nerve bundle directly projected to olfactory neuropils of brain indicated the function of short lateral antennules in olfactory receptor system. We also found that aesthetasc auxillary cells and the olfactory nerves to olfactory neuropil of brain are GABAnergic cells and tracts. The impact of olfactory system on pheromone perception and mating behavior stimulation were performed with ablation and mating tests found that the antennules and antennae are important for female pheromone perception and stimulated mating behavior response.

Therefore, the antennular and antenna nerves in *M. rosenbergii* have never been characterized so the function of these filaments still unclear. The diversity in antennules and antenna structure together with their sensilla setae variation among arthropods showed problem in function prediction of these organs. These filaments were particular study with backfilling method to reveal the terminal sites in the brain together with study of their sensilla setae types prior to postulate their function. The study of antennular nerves termini from sensilla setae to brain was performed in spiny lobster with backfilling method showed that the lateral division of antennular nerve is the nerve bundle diverges to the olfactory lobe. This study indicated that the lateral

antennules contain the afferent nerves that involved olfaction in ON innervations and also receive mechanosensory input with multi-glomeruli innervation of ON (Schmidt & Ache, 2004). The study of nerve connection from antennules of calanoid copepods with backfill method showed different pattern innervations of various setae types in the antennules, indicating that their nerve connection should be had dissimilarities and difference functions. It is suggested that the antennules contain many sensory receptor unit and the nerve supplying also contain many of nerve fibers and neuronal conduction (Bundy & Paffenhofer, 1993). The DiI fills evaluatiton of the Caribbean stomatopod crustacean antennular nerve found that at the junction of the filament also present the two ipsilateral terminal areas in the brain: olfactory lobe (ON) and lateral antenna I neuropil (LAN) (Derby, et al., 2003). Despite of the difficulty in identifying the function of these organs from their structure variations as previously described lead us to study the nerve connection from its filament to brain in *M. rosenbergii*. First of all, we clarify how many nerves innervated and their pattern throughout these filaments. Our results showed that the nerve bundles from three different antennular sources represented their individual disintegrated filaments indicated that they were originated from several nerve bundle fused together to form antennular nerve (AnNv) before terminated into brain. Nevertheless, the short lateral antennules, the unique tiny interested filament also have their individual nerve supplied to brain. From these results suggested that the nerves from antennules and antennae are separately and terminal sites in brain are different hence the function of antennules and antenna should be diverse. To postulate the function of these organs, we analyzed the sensilla setae types, supporting cell and the terminal site of these nerves in the brain.

The function of antennules and antennae were postulated by identification of sensilla setae types on them. The diverse structure and distribution of setae types among decapods had been studied and function of sensilla setae were mentioned.

The sensilla setae can be classified into three types according to their structure and function; mechanosensory receptor, chemosensory receptor and bimodal (mechano- and chemo-receptor function) (Derby & Blaustein, 1988; Schmidt & Gnatz, 1984). The mechanosensilla setae has been shown to be involved in tactile stimuli detection. The electrophysiological study in spiny lobster, *Panulirus argus* on the mechanosensory function of simple setae and cuspidate setae show responding to mechanical stimuli in these setae, and these structures contain displacement-sensitive neurons and bend sensitive neuron, respectively. Moreover, these neurons also response to change of direction and other mechanical stimuli or bending regions (Garm et al., 2004). The other sensory setae types are chemosensory setae included non-olfactory sensilla setae and a special type aesthetasc setae. The chemosensory setae has been shown to be important for odor detection which have impact on many biological behaviors e.g., finding food, determining of enemy and also perception of reproductive pheromones from conspecific specie. The external morphology of sensilla setae types were explored with scanning electron microscopy (SEM) to clarify the exact number of setae types that present on antennules and antennae of *M. rosenbergii*. There were four sensilla setae types found on antennules and antennae. In comparison with spiny lobster, the simple setae which mainly distributed on short LAnu of *M. rosenbergii* were also classified into two type; long simple setae and short simple setae. By comparison of external morphology, the long simple setae which generally found in all type of filaments was postulated to represent mechanoreceptive function or may have bimodal function (chemo-mechanoreceptive) (Cate & Derby, 2001) whereas a short simple and tuft setae which did not show similar structure comparing with other crustacean so, their function still unknown. Aesthetasc which is a special chemosensory perceptive organ that has been reported to be function in pheromone perception (Gleeson, 1982). The olfactory structure of decapods

crustacean had been reported the distribution of asethetasc on classical outer ramus of antennule or lateral antennule filament (Schachtner et al., 2005; Steullet et al., 2000). Therefore, the *M. rosenbergii* aesthetasc structure was firstly described by SEM but the function of aesthetasc of *M. rosenbergii* have never been tested (Hallberg et al., 1992). The aesthetasc sensilla structure quite diverse among species, so it is difficult to identify function from structure (Hallberg et al., 1992, Cate & Derby, 2001). To characterize aesthetasc setae, histology of antennules was analyzed to reveal the cell and neurons types that supported these receptors prior to characterize which setae is actually for olfactory structure.

Previous studies indicated that aesthetasc normally supported with the olfactory receptor units including olfactory receptor neurons (ORNs), their associated cells that innervated by individual ORNs unit. The ORNs are cluster of cells supported underneath aesthetasc structure in the lateral antennules (Schmidt et al., 2006). The aesthetasc morphology is reported in the blue swimming crab, *Callinectes sapidus*, and its internal contents contain outer dendrite connect with ORNs mediate with inner dendrite. Around the dendritic fibers are unsheathed by processes of auxiliary cells (Gleeson, 1996), so the associated structure with each ORNs units were proposed to be olfactory signaling conduction. The role of ORNs were also reported to be pheromone detection units via its free dendrite at the opening end of aesthetasc. The ORNs which showed identical to cell types of spiny lobster's ORNs were found to support with aesthetasc of *M. rosenbergii* (Schmidt et al., 2006). Our histological study also indicated that ORNs sent the dendritic fiber into the lumen of aesthetasc strongly suggested that these setae probably have function in primary olfactory receptive in short lateral antennules.

To ensure that short lateral antennules and aesthetasc have function in olfactory pathway, we showed that histology of nerves from this organ were

terminated in ON of brain. The histology of ON of crustacean brain has been described (Sandeman et al., 1992). The antennular nerves termini in crustacean brain were reported to have different nerve bundles and all bundles are terminated in difference brain regions. The olfactory brain organization of decapod crustacean was divided into two major pathway; lateral antenna I neuropil-medial antenna I neuropil (LAN-MAN) pathway and aesthetasc (chemosensory) pathway (Schachtner et al., 2005). The mechanosensory and non-aesthetasc sensory nerves that run from mechanoreceptive and non-olfactory receptive cell on the antennule are sent to LAN (Schmidt et al., 1992) and may be projected into MAN region of the brain. Both neuropils of the brain, which are described to be involved in mechano- and non-olfactory perception, contain the fiber aborization antennular motor neurons and antennular sensory-motor center (Schmidt & Ache, 1993; Maynard, 1965; Schachtner et al., 2005; Schmidt, 2007). These evidences supposing that the non-aesthetasc and mechanosensory information from antennular nerve into these regions may receive and integrate signal information in several brain regions (Schmidt & Ache, 1996) before ascends the integrating output to the eyestalk ganglion in decapod crustacean (Schachtner et al., 2005). The MAnNv and LAnNv of *M. rosenbergii* were projected from MAnu and LAnu also present the major targeting regions in bilobe structure of LAN and some part of AnN and PMPN. This result suggested that MAnNv and LAnNv may play the functional role in non-aesthetasc chemosensory and/or mechanosensory transduction pathway. The chemo-olfactory brain pathway of many decapods crustacean indicate that the projected axon nerve fibers from olfactory receptor neuron (ORNs) though antennular nerve, synapse and form dense plexus on cap region of olfactory neuropils (ON) of olfactory deutocerebrum and innervate multi-glomeruli occurring (Schachtner et al., 2005; Sendeman et al., 1992), postulate that this tract should be related with chemosensory transduction. The result present the

evident projection pattern of axonal nerve bundle from the ORNs to ON of deutocerebrum suggested that OLAnNv bundle, may be the neuronal tract involving in main olfactory function and possible pheromone perception in male *M. rosenbergii*.

This study indicated that GABA immunoreactivity was found in many region of peripheral and central olfactory system in male *M. rosenbergii*. The peripheral regions of GABA immunoreactivity were localized in dendritic fiber cone of ORNs, the region of auxiliary cell that sheath surround the ORNs dendrite and its neuronal projection to olfactory lobe of brain. Previous studies reported that the olfactory neuromodulation is controlled with many types of neurotransmitters. The several studies reported that γ- aminobutaric acid (GABA) and acetylcholine (ACh) are the two main neurotransmitters important in olfactory regulation in insect antennal lobe. Moreover, this pathway is regulated by several subtypes of biogenic amine; serotonin, dopamine, octopamine and histamine (Python & Stocker, 2002; Schachtner et al., 2005). Recently, γ- aminobutaric acid or GABA is identified to be the inhibitory neurotransmitter in peripheral and central nervous system including play important role in pre-synaptic inhibition of olfactory receptor neurons (Wachowiak et al., 2002). GABA and glutamate are the molecules that mostly reported on olfactory system function in mammal and arthropod groups suggesting that these molecules may be involved in olfaction modulation (Mobley et al., 2008; Schachtner et al., 2005). Glutamate which represents the same function as GABA, are localized in ORNs and auxiliary cell in Caribbean spiny lobsters, *Panulirus argus* and its involved in metabolite function on chemosensory system (Mobley et al., 2008). The GABA immunoreactivity was present in glomerular structure of ON, showed likely result in the glomeruli in the antennal lobe of Cricket *Acheta domesticus* (Strambi et al., 1998). Some evidences in the transcript mRNA by Northern blot analyses and enzymatic activity assays in spiny lobster, *P. argus* show the strong in immunoreactivity to

glutamate synthase (GS) in olfactory lobe and sensory sensilla. It confirm in GS synthesis in olfactory pathway (Linser et al., 1997). GABA may be the chemical signal that modulated the pheromone information from periphery olfactory part to central deutocerebral brain region and this cue may involve in behavioral presentation in courtship and mating in male prawn.

The sex-pheromones are the mediated cues to stimulate courtship and mating behaviors in the animals. The contract pheromone is the strong chemical cues, coated on the female body surface that more efficiency than distance pheromone and lead to mating behavior appear (Zhang & Lin, 2006). Moreover, the Chinese mitten crab (*Eriocheir sinensis*) was investigated on pheromone assay showing that distant pheromones were received by the antennules but the major mating recognition involved in contract pheromone function via physical contact (Herborg, 2006). The impact of antennules on pheromone perception was performed by ablation test and mating assay. Our result has been shown loss of copulation response in short lateral antennules ablation test, which are housed of aesthetasc-ORNs units. It indicated that aesthetascs in short lateral antennules may function in pheromone perception and co-operated with other sensory system in whole antennule. The aesthetasc have been reported to be involved in sex pheromones perception (Greeson, 1982; Horner et al., 2008). The bilateral ablation of aesthetasc tuft of Blue crab, *Callinectes sapidus* showed the exhibiting courtship responses to pheromone presentations are decreased in a highly significant (Greeson, 1982). Both antennae and antennules ablation of a simultaneous hermaphroditic shrimp, *Lysmata wurdemanni* are lose both precopulation behavior and copulation behavior but if inner flagellum and outer flagellum of antennules remain, the copulation behavior will be present (Zhang & Lin, 2006). Moreover, the aesthetasc ablation in spiny robster showed lost of shelter selection behavior to conspecific urine when compare with non-aesthetasc ablation

(Horner et al., 2008). The antennae ablation showed decreasing of mating response to molted female suggesting that they may also be involved in pheromone recognition. The lacking of both aesthetasc and non-aesthetasc antennular setae promote decreasing of mated searching in lobster (Steullet et al, 2002). So it indicated that the olfactory nerve systems rely not only on olfactory tract, but they also integrated with afferent fibers of mechanoreceptive organs and non-aesthetasc fiber in decapod crustacean species (Schatner et al., 2005). This study suggested that the role of olfactory pathway mediated mating behavior may be initiated from complementation and co-operation with non-aesthetasc sensory pathway.

In conclusion, we have characterized the antennules brain nerve tract and shown that short lateral antennule was the house of aesthetasc, special type chemosensillar receptor was projected to olfactory neuropil into the brain of *M. rosenbergii*. The antennules ablation are critical for mating response to female during molt suggested that they function in pheromone perception and the mating behavior was postulated to be conducted from chemical and mechanical stimuli from mature molted female from ORNs to olfactory brain center. When stimulating occurred, GABA was suggested to be modulator of olfactory pathway and may co-functionalized with other neurotransmitters for modulation higher order neurons in olfactory brain region. Lastly, although we have identify the organ and neuronal pathway involving reproductive pheromone perception in male prawn but the active pheromone substances released from female during molt still unknown. The identification of reproductive pheromone and the possible function of antenna in mating behavior will be investigated in next study.

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ข้อเสนอแนะโครงการวิจัยในอนาคต

จากงานวิจัยนี้ได้ค้นพบถึงกลไกการรับรู้ฟีโรโมนของกุ้งก้ามกรามเพศผู้ และระบบประสาทเกี่ยวข้องกับการกระตุ้นการตอบสนองของฟีโรโมน ทำให้ทราบถึงพฤติกรรมการตอบสนองของกุ้งเพศผู้ต่อการกระตุ้นของฟีโรโมนได้ ซึ่งเป็นประโยชน์ต่อแนวทางในการศึกษาในอนาคต เพื่อที่จะสามารถควบคุมพฤติกรรมการผสมพันธุ์ของกุ้งได้ ในการศึกษาต่อไปจะได้ทำการศึกษาถึงการบ่งชี้จำเพาะชนิดของสารออกฤทธิ์ฟีโรโมนที่หลั่งออกมาจากกุ้งเพศเมียระหว่างการลอกคราบ ว่าเป็นสารชีวโมเลกุลประเภทใด อีกทั้งจะทำการทดสอบสารออกฤทธิ์ที่วิเคราะห์ได้ต่อการกระตุ้นการผสมพันธุ์ของกุ้งเพศผู้ และจะได้ทำการศึกษาถึงกลไกการสังเคราะห์สารออกฤทธิ์ฟีโรโมนชนิดนี้ในกุ้งเพศเมียด้วย โดยน่าจะสามารถนำความรู้ที่ได้การศึกษาในเรื่องนี้ไปประยุกต์ใช้ในการกระตุ้นการสืบพันธุ์ของกุ้งได้ ซึ่งจะเป็นประโยชน์ต่ออุตสาหกรรมการเพาะเลี้ยงกุ้งก้ามกรามทั้งต่อการคัดเลือกสายพันธุ์ การเพิ่มผลผลิตลูกพันธุ์ที่มีคุณภาพต่อไปในอนาคต

เอกสารแนบหมายเลข 3

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

   ผลงานวิจัยกำลังอยู่ในระหว่างการตรวจแก้ไขความถูกต้องของ manuscript เพื่อตีพิมพ์ในวารสาร General and comparative neurology

2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
     ยังไม่มี
   - เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)
     ยังไม่มี
   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)
     ยังไม่มี
   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)
     ผลงานวิจัยเรื่องนี้บางส่วนใช้สำหรับวิทยานิพนธ์ของนักศึกษาปริญญาเอก หลักสูตร กายวิภาคศาสตร์และชีววิทยาโครงสร้างจำนวน 1 คน ซึ่งจะเป็นการสร้างนักวิจัยใหม่

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)
   ผลงานวิจัยเรื่องนี้ถูกนำไปเสนอในงานประชุมวิชาการดังนี้
   - **Chotwiwatthanakun, C.,** Vanichviriyakit, R., Kornthong, N., Kruagkum, T., Tinikul, Y., Anuracpreeda, P., Sobhon, P. Structure of the Olfactory Receptor and Its Function in Perception of the Reproductive Pheromones in the Fresh Water Prawn**. งานประชุมนักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส ครั้งที่ 10 14-16 ตุลาคม 2553 ที่ อ.ชะอำ จ.เพชรบุรี (poster presentation)**

- Thanapong Kruangkum, **Charoonroj Chotwiwatthanakun**, Yotsawan Tinikul, Chaitip Wanichanon, Peter J. Hanna, Prasert Sobhon. Olfactory receptor in the antennule and its neural connections with the brain of the giant freshwater prawn, *Macrobrachium rosenbergii*: a possible pathway for pheromone perception. 34th AAT Annual Conference Proceeding of the Anatomy Association of Thailand, 27-29 April 2011, Krabi, Thailand (poster presentation)