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RESEARCH ARTICLE

Indoleamines regulate vitellogenesis via cross-talks with allatotrophe in the American cockroach, *Periplaneta americana*

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Abstract

Vitellogenesis in many insect species is triggered by juvenile hormone (JH) where other factors such as neural or endocrinological ones also play a important roles. Newly emerged adult females of the American cockroach, *Periplaneta americana* were injected with indoleamines such as tryptamine (Tn), *N*-acetyltryptamine (NATn), serotonin (5-HT), *N*-acetylserotonin (NAS) and melatonin (ME) at 20, 30 and 40 µg in the abdomen. Tn and NATn stimulated Vg synthesis, while 5-HT, NAS and ME inhibited it. These amines may exert the effect on either directly on Vg synthesis in the fat body, Vg receptor, follicle cells, or JH synthesis. We first cloned the genes encoding juvenile hormone acid *O*-methyltransferase (JHAMT) and farnesoic acid *O*-methyl transferase (FAMeT) which are involved in JH biosynthesis. qRT-PCR analysis revealed that indoleamines affected the expression of JHAMT and FAMeT in the Br-CA complex. 5-HT, NAS and ME inhibited JHAMT and FAMeT transcription, while Tn and NATn stimulated their transcription. The oocyte length and yolk accumulation showed that, Tn, more likely NATn induced oocyte maturation. Here, the result suggests that intricate mutual interactions induced Vg synthesis in the fat body during oocyte maturation between neurotransmitters/modulator pathway and JH synthesis pathways.

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Introduction:-

Vitellogenesis occurs as an essential step for egg-laying animals both vertebrates and invertebrates. In insects, vitellogenin (Vg) is synthesized in the female fat body, secreted into the hemolymph and then taken up by endocytotic machinery via Vg-receptors (VgRs) complex (Engelmann, 1969; Sappington and Raikhel, 1998; Tufail and Takeda, 2005). The recent evidence has shows that two distinct female-specific proteins Vgs (Vg_1 and Vg_2) and receptor mediate ovarian development in the American cockroach, *Periplaneta americana* (Bell, 1970; Engelmann, 1979; Tufail et al., 2001; Tufail and Takeda, 2005).

In these decades, it was shown that other than JH and 20E, biogenic amines are also involved in insect reproductive regulation (De Loof, 2008; Gruntenko and Rauschenbach, 2008). JH plays a major role in the induction of Vg synthesis in hemimetabolous insects including cockroaches. The action of JHIII or JH analogs upon Vg synthesis has been extensively studied, especially by using cockroaches (Engelmann, 1979; Wyatt and Davey, 1996). *P. americana*, is a model species for examining hormonal regulation of Vg synthesis (Wyatt and Davey, 1996). Moreover ecdysteroids such as 20-hydroxyecdysone (20E), play a gonadotropic role in some insect species (Truman and Riddiford, 2007). Ecdysteroids, synthesized by the ovarian follicular cells, stimulate Yp (an equivalnt to Vg) synthesis in the fat body of *Drosophila melanogaster* and Vg synthesis in *Ades agypti*; Vg and Yp are subsequently taken up by ovaries (Bownes, 1989; Raikhel et al., 2005).

Arylalkylamine *N*-acetyltransferase (aaNAT) is the penultimate enzyme in indoleamine metabolic pathway. Activities of aaNAT have been observed in various organs not only the CNS but also the midgut, and reproductive glands in *P. americana* (Asano et al., 2003; Asano and Takeda, 1998; Hiragaki et al., 2015; Ichihara et al., 1997). The activity changes along oocyte maturation (Asano et al., 2003). We now investigate the causal relationship of aaNAT and its products with Vg synthesis via JH synthesis.

Biogenic monoamines mediate various physiological functions for homeostasis as putative neurotransmitters, neuromodulators, and neurohormones in insects (Evans, 1980). Octopamine (OA), serotonin (5-HT) and dopamine (DA) have been detected in the nervous system of P. americana and their involvement in vital physiological functions in insects (Shafi et al., 1989).

The indoleamine tryptamine (Tn) has received increasing attention as a possible neuroregulatory substance in insects. In *P. americana*, Tn has been detected in the collateral glands by HPLC -fluorometric measurement (Asano et al., 2003) but its functional role has not been investigated.

It has been reported that exogenous indoleamines stimulate the vitellogenesis during the absence of gonadotopic hormones in *Blaberus craniifer* (Goudey-Perrière et al., 1990). Tn has been recognized as the precursor of indole-3-acetic acid (IAA), the principal auxin of higher plants (Wareing and Phillips, 1981) that is also now located in all major brain regions in mammalian species (Philips et al., 1974). It has been reported that it functions as a neoromodulator or neurotransmitter in the mammalian CNS (Frankhuijzen and Bonta, 1974), and the distributions in the CNS have been already elucidated in rat (Philips et al., 1974) and mouse (Juorio and Durden, 1984).

We focus on the effect of indolamines acting as neurohormones on the onset of vitellogenesis to identify the possible functions of Tn in relation to JH synthesis during oocyte maturation.

Material and methods:-

Insects:-

P. americana colony was established by a founder colony of Earth Chemical Company (Ako, Japan) and reared more than 30 years at 25°C under LD 12:12. Water and food were provided *ad libitum*. To trace the age of experimental cockroaches, newly emerged female adults were collected within 24 h of emergence (white roaches) and kept in a transparent plastic jar (14 × 14 × 20 cm).

Chemicals:-

The pharmaceutical grade of indoleamines were purchased from Wako Pure Chemical Industries (Osaka, Japan): Tn, NATn, 5-HT, NAS and ME. After dissolved in ethanol and diluted, the final concentration of ethanol was less than 0.01% and 0, 20, 30 and 40 µg of each amine was injected in the normal and neck ligatured roaches through abdominal tegument and puncture was sealed with instant adhesive, Aron-alpha (TOAGOSEI CO. LTD., Tokyo, Japan). Females used as controls received either distilled water under identical conditions.

Cloning and phylogenetic analysis:-

Total RNA was extracted from the CC-CA complex from fifty females. These CC-CA complexes were extracted by RNAiso Plus (Takara, Japan) according to the supplier's instructions. Poly $(A)^+$RNA was purified using a Gene Elut mRNA miniprep Kit (Sigma, St. Louis, MO, USA). A total of 500 ng of Poly $(A)^+$RNA was used to construct single-strand (ss) cDNA using ReverTra Ace kit (Toyobo Co. Ltd., Osaka, Japan). Degenerate PCR primers were designed based on conserved regions within the JHAMT and FAMeT sequences (published online).

The touchdown PCR conditions were: 10 sec for denaturation at 98°C, 30 sec for annealing at 55°C to 45°C, and 30 sec for extension at 68°C for 35 cycles with KOD DNA polymerase (Toyobo, Japan). The purified fragment was cloned into pBluescript SK^+ vector and sequenced with Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing of the cloned insert showed it to resemble the existing JHAMT and FAMeT sequences in databases.

Sequencing and multiple alignments were conducted by using the GENETYX® Version 5.1.1 (Software Development Co. Ltd., Tokyo, Japan) and BioEdit Sequence Alignment Editor Version 7.0.7 (Hall, 1999). A molecular phylogenetic tree employing the Neighbor-Joining method was constructed by using the Molecular Evolutionary Genetics Analysis (MEGA) Version 6.0 (http://evolgen.biol.metro-u.ac.jp/MEGA/).

RNA extraction and cDNA synthesis for qRT-PCR:-

Four days after injection, the brain-corpus cardiacum-corpus alatum (Br-CC-CA) complex and fat body were dissected in phosphate-buffered saline (PBS; pH 7.4, 2 mM KH_2PO_4, 137 mM NaCl in distilled water), immediately transferred to liquid nitrogen and then kept at -80^oC until use. Total RNA was isolated by using RNAiso Plus reagent (Takara, Japan). For qRT-PCR, the RNA samples was treated with 2 units of DNase I to remove trace amount of genomic DNA. Two hundred fifty nanograms of total RNA with primers using Rever Tra Ace kit (Toyobo Co. Ltd., Osaka, Japan) was used for synthesizing the cDNA.

Measurement of mRNA levels:-

qRT-PCR was performed with the SYBR® Green and THUNDERBIRD^{TM} qPCR Mix (Toyobo Co. Ltd., Osaka, Japan), with the forward and reverse primers designed (Table 1). Cycling parameters were 95°C for 1 min to activate DNA polymerase, and then 40 cycles of the following PCR amplification with primers were performed using the following temperature program; 95°C for 15 sec and 60°C for 2 min. To confirm the specificity of the PCR products, melting curves were determined using the software ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Amounts of amplified products were calculated from cDNA standard curves generated for each PCR run. For expression levels of each transcript, actin (Gene Bank: AY116670.1) mRNA was used as the internal standard.

Table 1:- A list of primers used in the experiments.

Gene	Primer sequence
Vg_1 Forward	CCAGACATTATCAGACCTCCAGTAG
Vg_1 Reverse	TGTAGGTTTGAAGGCCACAATAGTA
Vg_2 Forward	CTTACACGAGGTCGCAAATCAG
Vg_2 Reverse	CTGTCATGTGATACGTGTCTTTGAG
VgR Forward	TGTCTTGTGAAGATGGATTTGTGTG
VgR Reverse	CACTGTTGTCTCCACAATCATCAAA
JHAMT Forward	GAAGCTCTCATAGTATTCGTGGC
JHAMT Reverse	AGGATCTTCTGACTGATGGTAGG
FAMeT Forward	ACTGTATGTAGGACGGGCAAAG
FAMeT Reverse	CCAGTCAGCACCTCATATTCAG
Actin Forward	TGAATCCTAAGGCCAACAGG
Actin Reverse	ACCGGAATCCAGCACAATAC

SDS-PAGE and Western blotting analysis for measurement of oocyte maturation:-

To investigate the effect of various indoleamines in the oocyte maturation, different indoleamine agonists and antagonists of 5-HT receptor were injected. Cockroaches were injected with each reagent at the abdomen on Day 0 by microsyringe at the effective dose of 20 µg. The following indoleamines, Tn, NATn, 5-HT, NAS, and ME, MS (Mianserin) a selective antagonist of 5-HT_2 and 5-HT_{1c} receptor and Li_2CO_3, a depressor of 5-HT pharmacological effect were injected. Control insects were injected with an equivalent volume of distilled water.

After rearing for 4 days, the ovaries of injected animals were dissected out and the pattern of protein bands were analyzed by SDS-PAGE with 7% gel and western-blotting was subjected to a rabbit polyclonal antibody against the cockroach major yolk protein content (97 KDa). After cellulose nitrate membranes were subjected to the standard 3,3`-diaminobenzidine tetra-hydrochloride (DAB) reaction, following the incubation with horseradish peroxidase-conjugated goat IgG fraction against rabbit IgG, those membranes were analyzed by the double-wavelength flying spot scanner (Shimuzu model CS-9000) in the condition that wavelength was 570 nm in linar scanning, beam size 0.4 mm × 2.0 mm, drift line 0, minimum width 2.0 mm and minimum area 2. The experiments (injections) were repeated 10 times for each reagent.

Statistical analysis:-

Results were analyzed by one-way ANOVA followed by post-hoc Turkeys' honestly significant difference (HSD) test using SPSS ver. 15 (IBM, NY, US). The data are shown as mean ± S.E.M. and differences between the groups were considered as significant if $p< 0.05$ or $p< 0.01$.

Results:-

Injections of indoleamines impair or stimulate terminal oocyte growth:-

The injections of indoleamines at 0, 20, 30, and 40 µg affected terminal oocyte length during ovarian development (Fig. 1). 5-HT, NAS and ME at doses higher than 20 µg reduced the oocyte length. However, Tn and NATn significantly increased oocyte length at 20 µg compared to the control, whereas it had no effect as the dose further increased (Fig. 1).

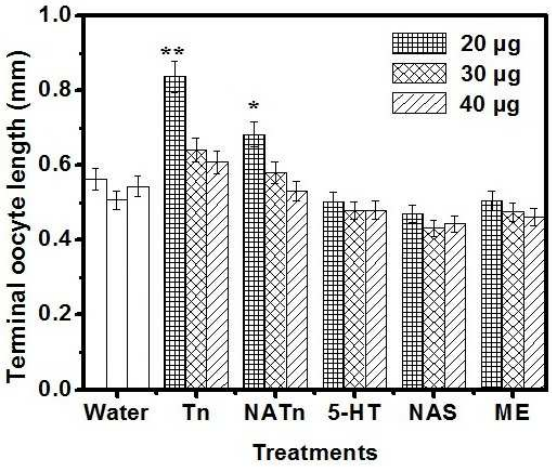

Figure 1:- The length of the terminal oocyte during ovarian development of *P. americana* 4 days after injection of indoleamines (Tn=tryptamine, NATn= *N*-acetyltryptamine, 5-HT= serotonin, NAS= *N*-acetylserotonin and ME= melatonin). Results are expressed as the mean ± S.E and significant differences (Student's *t*-test, **p*<0.05; ***p* < 0.01). Each treatment volume is 5, 7.5, 15 µl, respectively.

Fig. 2 shows the effects of various indoleamines on yolk accumulation in the terminal oocytes. Indoleamines were injected to *P. americana* and their effects on the oocyte maturation were investigated. The oocyte maturation was evaluated as the degrees of the major yolk protein uptake in the terminal oocyte. The degree of yolk accumulation was expressed in mean±SE. The injections of Tn and NATn accelerated the oocyte maturation whereas the the injections of Nac-5-HT and ME inhibited the maturation. The injections of Li_2CO_3 and MS stimulated the yolk accumulation. When MS and Tn were injected simultaneously, yolk accumulation was further stimulated than when MS and Tn were injected singly; whereas the simultaneous injection of MS and 5-HT was not at all effective. The result shows that the pathway leading to ME inhibited the oocyte maturation but the pathway leading to NATn promoted the maturation. The two groups of indoleamines probably have different binding sites/receptors.

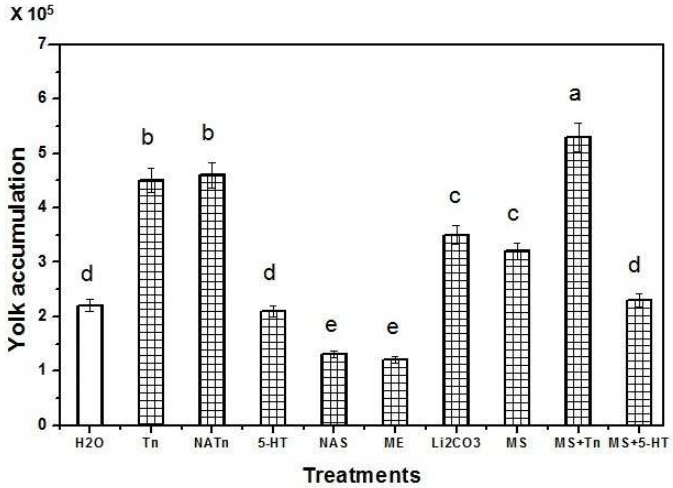

Figure 2:- Effects of various reagents (20 µg) that affect yolk accumulation. The same alphabets represent the same significance level by ANOVA (*p*<0.01). Each column represents the mean of 10 determinations. Vertical bars represent the SEM.

Indoleamines regulate onset of vitellogenesis in adult females:-

The action of indoleamines and their effects on oocyte maturation were investigated in the virgin female of *P. americana* (Fig. 3). White females (within 12 h) were reared on artificial diets and immediately injected with different doses of indoleamines. qRT-PCR was employed to determine Vgs and VgR mRNA levels in the fat body in response to various indoleamine treatments. As shown in Fig. 3, females 4 days after treated with indoleamines demonstrated the effect on Vgs and VgR mRNA level. The injection of Tn and NATn of 20 µg induced significantly increase in Vg_1 and Vg_2 mRNA level whereas the injection of 5-HT, NAS and ME caused significant reduction of Vgs expression in the fat body compared to control (Fig. 3 A, B). The least activity was observed if 30 and 40 µg were injected, which was a significant reduction compared to the control.

VgR mRNA level in the fat body was evaluated after injection of indoleamines. qRT-PCR was also used to determine VgR mRNA levels. The results showed vitellogenin receptor (VgR) mediates the uptaking of vitellogenin by oocytes and plays a significant role in oocyte maturation after injection of Tn and NATn with conrol (Fig. 3C). The result shows that the pathway leading to ME inhibited Vg synthesis but the pathway leading to Tn stimulated Vg synthesis during oocyte maturation.

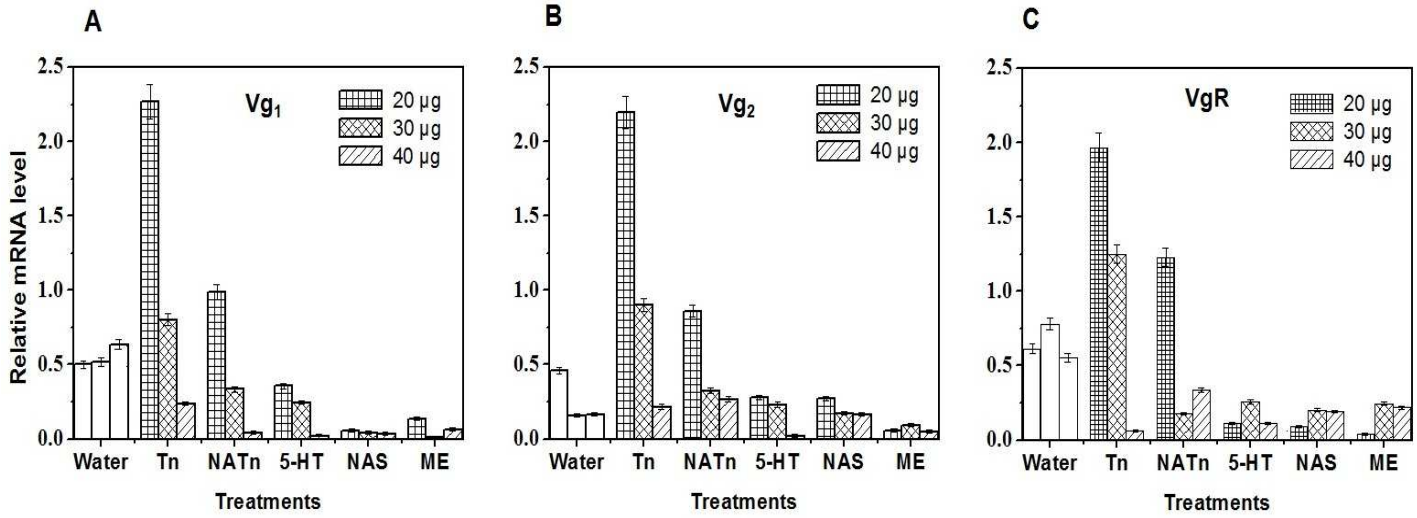

Figure 3:- Transcriptional expression of Vg_1(A), Vg_2(B) and VgR(C) on 4 days after injection of indoleamines in the fat body of *P. americana*.

Phylogenetic analysis in JH synthetic pathway:-

To monitor the effect of indoleamines on JH synthesis, the cDNA sequences encoding JHAMT was first PCR amplified with degenerate primers and the first-strand cDNA was made from CA total RNA. The amplicon was about 648-bp fragment, which encodes a protein of 216 amino acids. The phylogenetic analysis indicated that the deduced amino acid sequence of *Pa*JHAMT (LC164750) had a high identity (60%) to that of *Diploptera punctata* (*Dp*) JHAMT. The *Pa*JHAMT also showed a relatively high amino acid identity (48% and 40%) to those JHAMTs predicted from genomes of *D. melanogaster* and *S. gregaria* (Fig. 4A). It is known that all SAM-dependent methyl transferases have a well conserved motif I, involved in SAM binding, hh(D/E)hGXGXG, where h represents a hydrophobic residue. The motif I of *Pa*JHAMT (VLDLGCGPG) was nearly identical to that of *Dp*JHAMT (VLDVGCGPG) [Data not shown].

Sequencing and BLAST searching revealed that the cDNA sequence shows an 725-bp ORF, encoding a protein of 241 amino acids, i.e, FAMeT (LC164751). Phylogenic tree was constructed based on 14 FAMeT protein sequences, which revealed that the deduced protein sequence of *Pa*FAMeT is an orthoptera homolog, showing 63% identities to FAMeTs from *S. gregaria* and 51% identities to *D. melanogaster*. *Pa*FAMeT also showed 56% identity at the amino acid level to *Nilaparvata lugens* (*Nl*) FAMeT that is the first studied FAMeT from the American cockroach (Figure 4B).

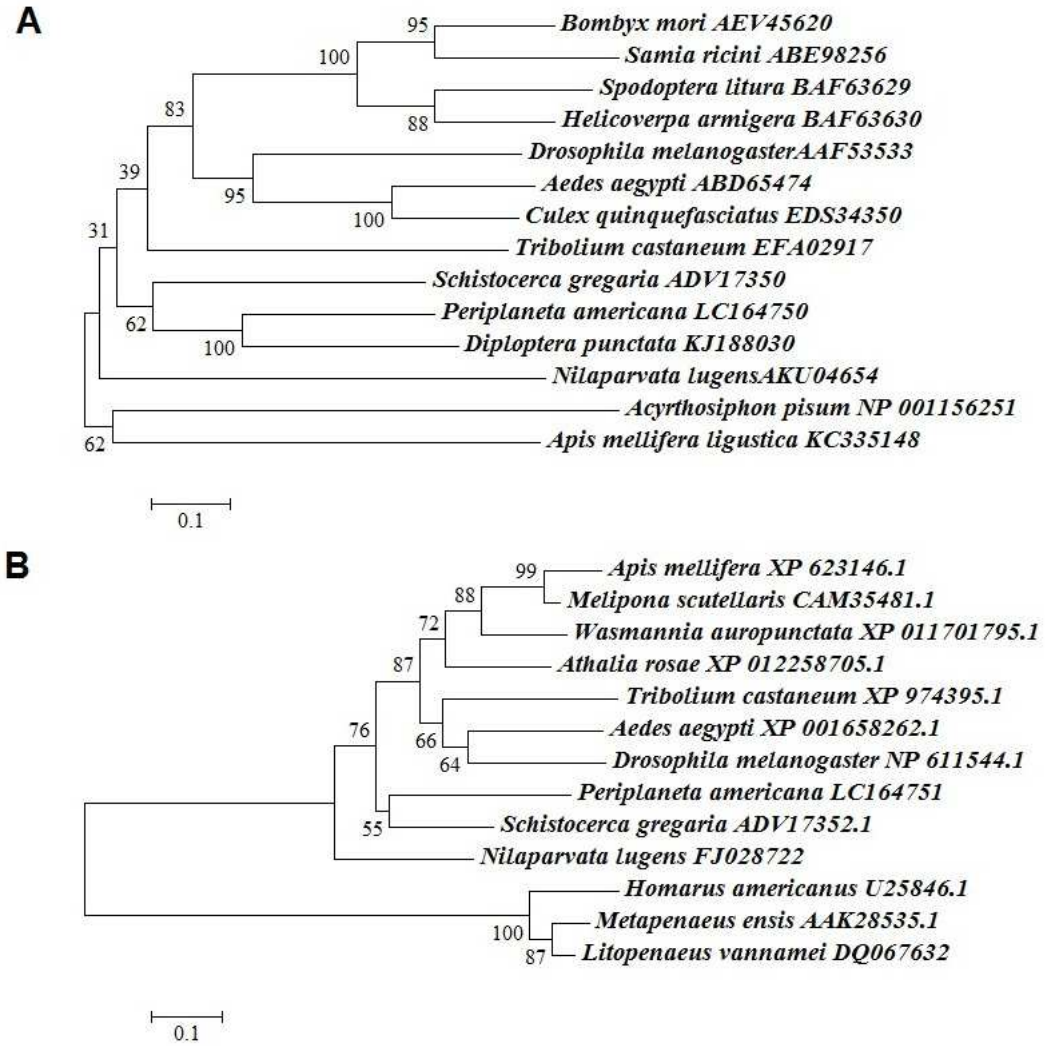

Figure 4:- Phylogenetic analysis was performed by MEGA (version 6.0) program based on the JHAMT (A) and FAMeT (B) amino acid sequences from various species. The phylogenetic tree was constructed using the neighbor-joining algorithm method and bootstrap values (1000 repetitions) of the branches are indicated.

JHAMT and FAMeT expression is regulated by indoleamine signaling:-

To investigate the effect of indoleamines at transcription level of the genes coding for key proteins involved in JH biosynthesis (JHAMT/FAMeT) during oocyte maturation. Gene Specific primers were designed to measure the mRNA expression levels of *Pa*JHAMT and *Pa*FAMeT transcripts in the brain-CC-CA complex of *P. ameicana* adult females, that was analyzed by qRT-PCR. Results showed that indoleamine induced JHAMT/FAMeT production by isolated BR-CA complex in a dose-dependent manner and JHAMT seemed to be slightly more sensitive than FAMeT (Fig. 5). Moreover, the injection of Tn and NATn of 20 µg induced significantly increase in JHAMT/FAMeT expression compared to the control in the BR-CA complex, whereas it had no effect if dose was increased (Fig. 5). Tn was the most effective in inducing the onset of vitellogenesis followed by NATn (Fig. 5 A, B).

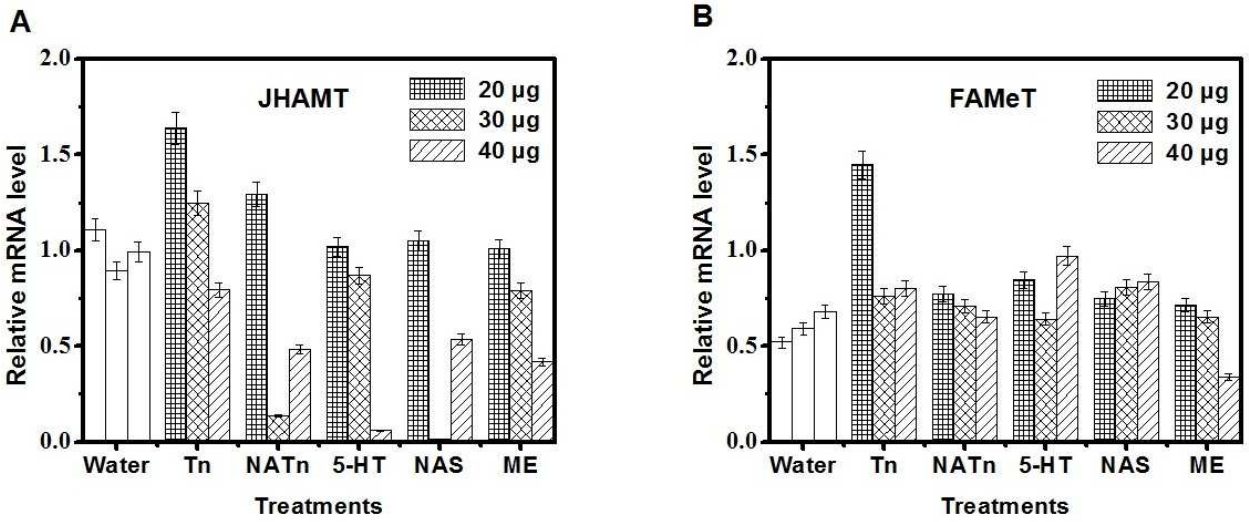

Figure 5:- Relative mRNA expression of JHAMT (A) and FAMeT (B) in the BR-CA complex 4 days after injection of indoleamines in the *P. americana*.

Indoleamine signaling in the neck ligatured anima on vitellogenasis:-

Above result indicates that Tn has significant role during vitellogenesis. To investigate whether the vitellogenesis is trigerred by only JH or mutual interactions with Tn during oocyte maturation. mRNA level detected by q-RTPCR of Tn (20 µg) injected female showed that expression of Vg1, Vg2 and VgR significantly increased in the fat body (Fig. 6). Terminal oocyte length significantly increased compared with the control group after injection of Tn in the neck ligatured female roaches (Fig. 7). The effect of Tn was direct instead of via JH upregulation.

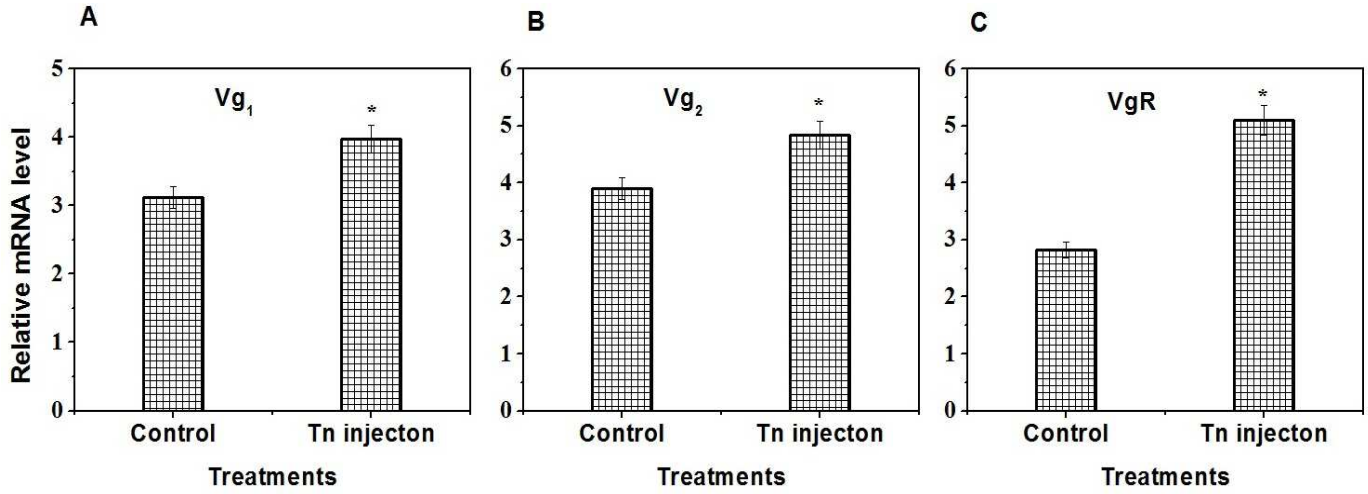

Figure 6:- Transcriptional expression of Vg_1 (A), Vg_2 (B) and VgR (C) 4 days after injection of Tn (20µg) of neck ligatured *P. americana*. Results are expressed as the mean ± S.E and significant differences (Student's *t*-test, *p<0.05;).

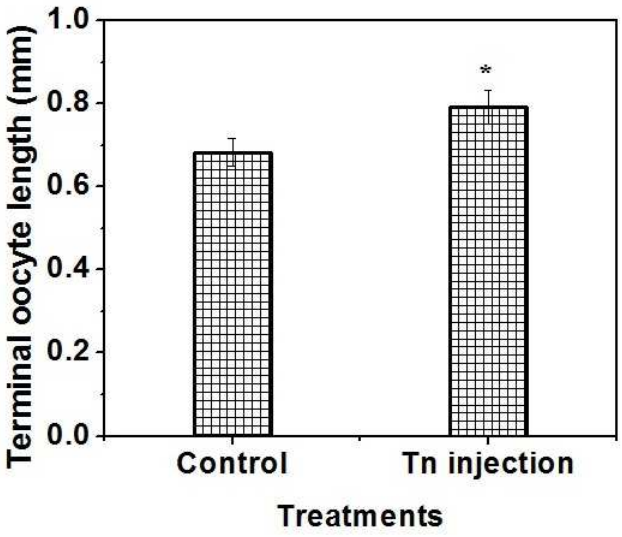

Figure 7:- The length of the terminal oocyte during ovarian development of neck ligatured *P. americana* 4 days after injection of Tn (20µg). Results are expressed as the mean ± S.E and significant differences (Student's *t*-test, *p<0.05;).

Discussion:-

Tn and NATn increased terminal oocyte length (Fig. 1). Although 5-HT had no effect on Vg uptake but NAS and ME inhibited Vg accumulation. Both MS and Li_2CO_3 accelerated the yolk accumulation (Fig. 2). Li_2CO_3 has been used to improve the mental illness especially the manic-depressive psychosis (Schou et al., 1954) by removing the pharmacological effects induced by 5-HT, acetylcholine and noradrenalin in the CNS (Chouinard, 1980). Co-injection of Li_2CO_3 and Tn upregulated Vg accumulation more than Tn or NATn alone, which suggests for a unique Tn receptor. Alternatively, Li_2CO_3 may suppress the effect of 5-HT by deleting the synthesis of 5-HT in the CNS. Although the present data cannot determine whether the effect is caused by blocking 5-HT receptor binding or 5-HT depletion, Li_2CO_3 suppressed the effect of 5-HT and its metabolites. The fact that no 5-HT was detected in the female reproductive system during the oocyte maturation (Asano, 1995) may mean that 5-HT is derived exogenously, probably via nervous system. The fact that NAS and ME injection, but not 5-HT injection, inhibited the oocyte maturation may mean that the action of NAS, if it acts as a ligand and ME are MT (ME receptor) mediated.

Our recent data show that 5-HT promotes the epithelium proliferation of the midgut in *P. americana* and that NAT activity increases when cockroach are starved (Tanigawa unpublished data). The present data suggests that ME inhibits oocyte maturation. These data maybe closely related. If there is a shortage in diet, reproductive maturation is of great risk. Only when they have sufficient quality and amount of diet, oocyte can mature. When the cockroach are starved, NAT converts 5-HT to NAS, and then to ME, respectively. Sequestered NAS and ME may inhibit the ovarian maturation.

In some insects the reproductive state, or the age of the insect, can actually reverse the effect of a biogenic amines on the JH synthesis. Several neurotransmitters are involved in the regulation of JH biosynthesis in cockroaches. OA inhibits JH biosynthesis in the CA in vitro (Thompson et al., 1990). These data suggest that binary regulatory mechanisms exist in the oocyte maturation of *P. americana*; the stimulatory mechanism via the effect of Tn and NATn, and the inhibitory mechanism via the effect of NAS and ME. Although it has not been determined, NAT can regulate both of the systems. Recently it has been suggested that the Tn binding sites have close structural relationship with the 5-HT receptor in vertebrates (Cohen and Wittenauer, 1985). Nevertheless, the existence of the Tn binding sites both in the CNS, fat body and the ovary of *P. americana* was not yet examined.

JH has a central role in the growth, development, and reproduction of insects, especially vitellogenesis and oocyte development, which has also been demonstrated in many insects (Cruz et al., 2003; Guo et al., 2014; Hansen et al., 2015). JH is also involved in the biogenic amine dependent regulation of insect reproduction, but the mechanism of

action remains obscure. The present study addressed to establish whether JH synthesizing enzyme JHAMT and FAMeT in this cockroach are influenced by indoleamines. Any interference in JH biosynthesis, using JH agonists or antagonists, produces anomalous development or disorders on reproduction of the target species (Tunaz and Uygun, 2004).

JH III is the major hormone regulating Vg synthesis in many insect species (Huang et al., 2015; Tufail et al., 2014). In *N. lugens*, JH III not only stimulates Vg expression in the fat body (Tufail et al., 2010), but also VgR is critical for regulating its uptake by the developing oocytes in *N. lugens* (Lu et al., 2015). In *B. germanica*, JH induces the expression of *Bg*Vg both in vivo (Comas et al., 1999) and in vitro (Comas et al., 2001).

For the complete biosynthetic pathway of JH III, we identified two genes encoding for key proteins JHAMT and FAMeT (Figure 4). JHAMT, a key enzyme that is involved in the JH biosynthesis, has been reported for the expression of the Vg gene (Huang et al., 2015; Parthasarathy et al., 2010). In *Ceratitis capitata*, FAMeT also is involved in biosynthetic pathways (Vannini et al., 2010). Indoleamines also affect JH biosynthesis (Figure 3). Biogenic amines are derived from the central nervous system by various distinct routes, e.g. as neurotransmitters via peripheral nervous system, as neurohormones or neuromodulators via neurohemal organs (Davis, 1985; Flanagan and Berlind, 1984; Orchard et al., 1986; Scott et al., 1985) and by enzymatic regulation. Precise network should be disentangled in the future study.

In cockroaches, JH regulates, either directly or indirectly, both the synthesis of Vg, and its incorporation into the oocyte development (Engelmann, 1969; Koeppe et al., 1988). In *Bluberus craniifer*, cockroach, the juvenile hormone, and indolamines may play a combine role in the uptake of haemolymphatic proteins by oocytes (Goudey-Perrière et al., 1991). Our data indicate that indolamines can stimulate JH producing glands (corpora allata), but at the same time Tn injected neck-ligated cockroach showed increased Vgs/VgR expression, indicating that Tn action was direct. In conclusion, (1) Vitellogenesis is upregulated by the injection of Tn and NATn but downregulated by NAS and ME. (2) Action of Tn was both direct and indirect via JH regulation, evidence of cross-talk between monoamines and sesquiterpenoid routes.

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