Final Report

Introduction

Traditionally employed pesticides are poisons that make no difference between damaging and beneficial insects or spiders. Moreover, despite the use of these chemicals individual specimens of the insects generally survive and grow resistant to the pesticide, and their natural predators are often affected much more severely because they exist in significantly fewer numbers.

Insects use a plethora of neuropeptides to regulate physiological events, which may be applicable for selective targeting in pest control. To develop artificial neuropeptides, the researchers have to identify the natural prototypes. Neuropeptides and their receptors are thought to be very well suited for this strategy, as species often have unique neuropeptide sequences. Once these sequences are identified, similar artificial messenger substances can be developed and stabilise them in such a way that the insects cannot break them down quickly.

The periodic release of Pheromone Biosynthesis Activating Neuropeptide (PBAN) is responsible for the synthesis of sex pheromone blend in the pheromone glands (PGs) of female moths to lure conspecific males (Karlson and Butenandt, 1959). These compounds are usually *de novo* synthesized via a modified fatty acid biosynthetic pathways through 16 (or 18):acyl, and undergo chain shortening and/or desaturation, followed by a final reduction to alcohol or acetylation to produce acetate esters or oxidation to produce aldehydes (Rafaeli, 2002; Tillman et al., 1999). Biosynthesis and release typically takes place within the specialized epidermal tissue, the PG, which is located intersegmentally between the 8-9th abdominal segment (Blomquist and Vogt, 2003; Percy and Weatherston, 1974).

These semiochemicals are intensively researched from both basic and applied aspects. The project primarily aimed at focusing on the physiology of blend production in the cabbage moth *Mamestra brassicae* (Lepidoptera; Noctuidae) a pest in Hungary and Eurasia. Behaviour, morphology, biochemistry, chemical analysis studies and molecular biology methods were combined to unravel the process. To expand our knowledge, comparative aspects were introduced namely by working with an other serious pest species, the European Corn Borer (ECB) *Ostrinia nubilalis* (Lepidoptera; Crambidae) also in relation to pheromonogenesis.

Recent investigations have provided a better understanding of the biosynthetic pathways underlying lepidopteran pheromone production and its regulation (Blomquist et al., 2011; Jurenka, 2004; Jurenka and Rafaeli, 2011; Matsumoto et al., 2007; Rafaeli, 2011). Both pheromone production and release are synchronized by the circadian-regulated action of PBAN, which originates from the suboesophageal ganglion (SOG) in mature virgin females (Kitamura et al., 1989; Raina et al., 1989). PBAN is a member of an ancient conserved signalling peptide family characterized by a C-terminal amidated pentapeptide FXPRL-amide (X = S, T, G or V) that is essential for the pheromonotropic activity (i.e. stimulation of pheromone synthesis) of PBAN (Kuniyoshi et al., 1991; Raina and Kempe, 1990). The post-translational processing of the PBAN pre-propeptide yields PBAN as well as four additional FXPRL-amide peptides i.e. diapause hormone (DH) and α-, β- and γ-SOG neuropeptides (SGNPs) that exhibit varying degrees of pheromonotropic activity (Ma et al., 1996, 1994; Sato et al., 1993).

M. brassicae has also been in focus of some pheromonotropic-based investigations. Immunocytochemical studies revealed the presence of PBAN-like peptides in the hemolymph at 100–500 pM during the scotophase (Iglesias et al., 1999). Furthermore, a cDNA encoding *M. brassicae* PBAN with significant homology to *Heliothis zea* PBAN (Helze-PBAN) has been reported (Jacquin-Joly et al., 1998). Earlier a pheromonotropic 18-amino acid (aa) SGNP, referred to as *M. brassicae* (Mambr-PT) (Fónagy et al., 2008, 1998), with close sequence similarity to β-SGNPs identified in *Bombyx mori, Helicoverpa. assulta, H. zea,* and *Pseudaletia separata* (Altstein et al. 2013; Jurenka, 2015; Rafaeli, 2009) has been identified. In *O. nubilalis* moths, previous research has detected PBAN-like biological activity and immunoreactivity in three sets of neurosecretory cells in the SOG and *corpora cardiaca* (Ma and Roelofs, 1995a, 1995b). These observations, along with *in vitro* experiments using isolated PGs, have indicated that PBAN-related neuropeptides in *O. nubilalis* act directly on the PG cells to stimulate sex-pheromone production (Ma and Roelofs, 1995c). Surprisingly, so far the DH-PBAN gene structure has not been described which prompted us to fill in this important knowledge gap.

Pheromone production is initiated by specific binding of PBAN to its cognate receptor (PBANR) expressed at the surface of the PG cells. Although it was demonstrated histochemically in *H. peltigera* PGs (Altstein et al., 2003), the molecular identity of the PBANR proved elusive until sequence similarities between predicted Drosophila G protein-coupled receptors (GPCRs) and mammalian receptors suggested that FXPRL-amide receptors, such as PBANR, belong to the mammalian neuromedin U receptor family (Hewes and Taghert, 2001). Subsequent confirmation of this hypothesis (Park et al., 2002) facilitated homology-based identification of the PBANR in *H. zea* (Choi et al., 2003) and *B. mori* (Hull et al., 2004). Since then PBANRs have been identified in diverse moth species (Cheng et al., 2010; Ding and Löfstedt, 2015; Kim et al., 2008; Lee et al., 2012; Rafaeli et al., 2007; Zheng et al., 2007) including *O. nubilalis* (Nusawardani et al., 2013) with multiple PBANR isoforms (PBANR-As, -A, -B, and -C) shown to be concomitantly expressed in PGs (Kim et al., 2008; Lee et al., 2012). The isoforms are differentiated by the length and composition of their C-termini, which is necessary for the ligand-induced internalization (Hull et al., 2005) . The "short" PBANRs (PBANR-As and -A,) lack a C-terminal extension and exhibit different internalization kinetics as compared to the "long" (PBANR-B and -C) isoforms (Lee et al., 2012). The differing responses of the isoforms, which arise from alternative splicing of the C-terminus (Kim et al., 2008; Lee et al., 2012), have been suggested to reflect the pleiotropic nature of the PK/PBAN signalling system in insects (Nusawardani et al., 2013). Despite these structural differences and variation in the downstream intracellular signal transduction cascade (Hull et al., 2007a; Matsumoto et al., 1995; Rafaeli et al., 1990, 1989), PBANRs are characterized by PBAN-mediated mobilization of extracellular Ca^{2+}, which is a prerequisite for turning the PBAN signal into the biological response of sex pheromone production (see reviews: Matsumoto et al., 2010; Rafaeli, 2009). One of our main objectives was to clone and characterize the PBANR(s) in *M. brassicae*.

Not only the use of synthetic species specific blends may be effective in agriculture, but the employment of results regarding the mechanisms controlling/disrupting pheromone production has similarly great potential (Nachman et al. 2009). An emerging field opens to use synthetic stable neuropeptide analogues or double strand RNA (dsRNA) interfering with the fine mechanism of pheromonogenesis. For exploiting these possibilities a much better understanding

of pheromone production, PBAN binding to specific PBANR and signal transduction cascade is indispensable.

Results and Discussion (according to planned Work packages)

WP I: Different behavioral studies and quantification of pheromone blend on time course basis:

Calling behavior (i.e. protrusion of PG while pheromone is emitted) studies of virgin females (3x20 individuals) was performed where the monitoring period was set at hourly intervals (D0-D8 females, where D0 refers to day of emergence) from the 1st h of scotophase (i.e. Dark) (Sp: 8h) till the 6th h of photophase (i.e. Light) (Pp:16h). D0 females did not show any activity and only 10% of females began calling on the 1st day (last 2 h in the Sp) which continued till the 5th h of Pp. Calling increased daily with roughly10%. Most intensive calling activity was recorded in the older females (D6-7) around 70-80% which behavior remained in the Pp with an almost similar intensity or occasionally higher. D8 females demonstrated less activity. These studies were essential to adjust sampling times of corresponding pheromone blend measurements for further diverse experiments. (Published in Köblös et al. 2015)

Blend (i.e. species specific composition of volatile compounds) content was measured 7x daily (with 2 h intervals from the 1st h of Sp) in 3 replicates. The sampling times were set according to results of preliminary pilot experiments. No pheromone production can be detected on D0 which fully correlated with behavioral findings (no calling and/or coupling). Pheromone production begins with a limited amount on the 2nd full Sp reaching the maximum (above 200 ng *Z*11-16Ac (major component) and almost 20 ng of 16Ac) (minor component) at the end of the 3rd Sp and even after the onset of light for 4 hours. The maximum production with some fluctuation is detectable during D2-D5, while it begins to decline from D6 up till D8 (when samplings were terminated). This is not consistent with the calling behavior, but underlines the hypothesis that pheromone production regulation and calling behavior are under different regulatory mechanisms (Vafopoulou, X. and Steel, 2005). In nature the relatively short, but effective calling (emission of large amounts of lure) increases the fitness of the females.

A new important conclusion is (based on several hundreds of measurements) that the ratio in our colony is different for the extracted components: 93:7 (*Z*11-16Ac: 16Ac) compared the conventionally accepted one (90:10:1) (Attyagale et al, 1987) while *Z*9-16Ac (:1) could not be detected (in our colony and/or under our conditions). This was the first study when both components were monitored by using Gas Chromatography coupled Mass Spectrometry (GC-MS) on a time course basis in *M. brassicae*. (Published in Köblös et al., 2015)

WP II. Morphological studies:

Fluorescence microscopy was performed as described previously (Fónagy et al., 2011). Samples were prepared at emergence, D1-4 at the 5-6th h of Sp, (daily pheromone production is the highest) and one hour prior to onset of Sp (no pheromone production is expected). PGs of D2 decapitated females were studied under similar conditions, as well as *in vivo* samples following incubation with synthetic Mambr-PT as described below (WPIII).

Results indicate that there are no lipid reservoirs at emergence. Moreover, before and during onset of pheromonogenesis there are no lipid droplets formed either under natural (time course basis) or artificial conditions (i.e: PT injection after decapitation). This coincides with the expectations from heliothine moths, where pheromone biosynthesis is most likely under endocrine control via directly triggering the Acyl-CoA-Carboxylase. (Published in Köblös et al., 2015)

WP III: *In vivo* and *in vitro* studies:

1) Two sets of experiments (a, b) were designed to study the effect of Mambr-PT *in vivo*:

a) Females were decapitated on the second day (3rd h of Sp). They were injected on the third day (6th h of Sp) with 5.6 pmol/2µl synthetic 18 aa pheromonotropin (Mambr-PT) (Dep. Medical Chem., Univ. Szeged, Hungary) or only distilled water.

b) Females were decapitated within 4 h after emergence and were maintained until they were injected with 5.6 pmol Mambr-PT on D0, 1 and 2 at the 6th h of Sp, respectively to investigate their pheromone production capacity. PGs were collected 90 min later and blend content was analyzed. It is known from the results of WPI that at the selected sampling time the maximum pheromone production is expected. Results indicate that near normal pheromone production can be restored (including blend ratio).

2) Mating experiments: newly emerged females (5–6) and males (7–8) were placed together into glass jars (see above). PGs were collected and analyzed from D2-5 females during the last hour of Sp as described above. Non-mated D5 old virgin females, sampled at the last hour of Sp served as control, while groups of D5 mated females were injected with Mambr-PT. The pheromone titre drastically decreased after mating compared to that of virgin females, which could be reversed with Mambr-PT injection, indicating that the pheromone producing machinery is readily available, and that mating and egg production most likely suppresses PBAN/PT availability. (Published in Köblös et al., 2015)

The potential effect of multiple mating on fecundity and fertility were checked with hourly intervals. Three types of experiments were set up where males were individually marked. Group A: 1 D1 female was placed into glass jars with 3 D2 males during the monitoring period; Group B: 1 D1 female was grouped with 3 D2 males and after the first copulation the rest was removed; Group C: 1 D1 female was 3 D2 males were offered every second day. Multiple mating was recorded only in Groups B and C. The most active egg laying period is between day 2-4 with an average of 670/female. There is no significant positive effect of multiple mating (in a linear model) on the fecundity, nor there is between egg number and length of copulation. (Unpublished)

3) *In vitro* pharmacological studies related to hypothesized signal transduction cascade:

I must put forward that despite all efforts the *in vitro* studies could not be completed as planned. In consecutive years various and unexpected problems with implementation turned up. Initially we assumed that deteoriation in reproducibility is due to inbreeding of our colony. After refreshing the colony with some natural specimens in 2016, *in vitro* studies could be restored, however during the spring of 2017 results consequently turned out to be totally unreliable again.

Various attempts were performed like preparing fresh stock solutions, Lepidoptera ringer etc. I realized that it's unworthy pursuing a challenge just for the sake of completing a very likely unpublishable series of experiments. Moreover the methodology used gradually became outdated compared to when the project proposal was written in 2012. Last but not least it came to my notion that a Chinese research group M. Du, X. Liu, N. Ma, X. Liu, J. Wei, X. Yin, S. Zhou, R. Ada, Q. Song and S. An will publish their results with the title: Calcineurin-mediated dephosphorylation of acetyl-CoA carboxylase is required for PBAN-induced sex pheromone biosynthesis in *Helicoverpa armigera* in Molecular and Cellular Proteomics (doi: 10.1074/mcp.RA117.000065). This article basically addresses question using State-of-Art methods to unravel PBAN induced signal transduction cascade in heliothine moths (where *M. brassicae* also belongs regarding pheromonogenesis).

As described earlier the aim was to test the pheromone production regulation mechanisms of signal transduction cascade, *in vitro* with various pharmacological agents of individual isolated PGs originating from D2 24h decapitated females. Earlier we used D3 females which also may have added to the viability of the test. The trimmed PGs were incubated in a PCR tube in Lepidoptera ringer (Matsumoto et al. 1995) and placed in an incubator (25°C) with gentle shaking. The glands were individually extracted in *n*-hexane and their blend composition was analyzed with a standardized method (developed at end of the second year of the project): A more suitable internal standard *Z*13-18Ald was chosen instead of 15OH. Since all our components are thermostable -in the required range- we quit from the N gas stream concentration to a thermo-block concentration (at 65°C), thus our flow through has been multiplied. The preparation of samples became faster, reproducible and exact. Individual samples instead of pooled samples (of 3 PGs) could be analyzed. We focused on the exploitation of the resolution capacity of the MS detector, since we were not interested in the monitoring of the full *m/z* spectrum, but only in the respective components, therefore we switched from SCAN mode to SIM measuring (thus significantly improving the sensitivity). The operational running program of the GC was not changed. (This quantification method way was described in our publication Moustafa et al., 2016)

Several experiments were repeated and compared to previous data. The results were more consistent and reproducible (using 5-9 replicates and individual PG pheromone content was measured with the internal standard method). Dose dependence studies revealed that 2.8 pmol Mambr-PT/vial containing 100µl Ringer instead of earlier used 5.6 is more suitable. Moreover the 90 min incubation is the optimal. Beside repeating incubations with earlier used chemicals (and doses), like NaF salt, Tralkoxydim, $LaCl_3$, Ionomycine and Ca^{2+} Ionophore, both stimulants of indispensable Ca^{2+} channels (for Ca^{2+} influx) Trifluoroperazine (Calmodulin inhibitor) (2, 1, 0.5, 0.1 mM concentration) was also tested resulting in significant inhibition (2 and 1 mM) in PGs originating from PBAN deprived individuals incubated in 2.8 pmol Mambr-PT/vial containing 100 µl Ringer.

Earlier conclusions were strengthened that adenylate cyclase is indispensable for the activation (as shown by NaF), tralkoxydim effectively inhibits Acyl-CoA-carboxylase (a key enzyme of *de novo* pheromone synthesis) while inhibition by $LaCl_3$ in presence of Mambr-PT

demonstrates that the influx of Ca^{2+} ions after binding of the PBAN to its receptor is essential. This was reassured by Ca^{2+} ionophore (300 μM) and Ionomycine (50μM) incubations. (Unpublished)

WP IV The cloning of Mambr-PBANR variants and gene expression studies along with functional characterization:

Using degenerate primers designed from known lepidopteran PBANR sequences, a cDNA fragment was amplified by PCR and extended by 3′ and 5′ RACE to obtain the full-length coding sequences. Three PBANR transcript variants (A, B and C) were identified. As for the cloned variants a phylogenic analysis was performed and the resulting maximum-likelhood tree for all known lepidopteran PBANRs strengthens the earlier assumption that *Mambr*-PBANRs show high homology to those from heliothine species.

Specific primers were designed for gene expression studies by quantitative real-time reverse transcription (RT)-PCR. We have found that the PBANR transcript variant C is the most abundant form in *M. brassicae* PG. To evaluate the tissue specificity of expression of genes encoding PBAN receptors, relative transcription levels in PGs of adult virgin females were analyzed along with those in larvae, brain, leg muscles, and fat body of adult females, as well as brain tissues and hairpencil-aedeagus complexes of males. Relatively high receptor gene expression was observed in PGs of adult females, while no mRNA of PBANR was observed in leg muscles and fat bodies of adult females and in larvae. However, genes encoding PBANR variants (A, B and C) were expressed at a relatively high level (only 10 times lower than in PGs of adult females) in male genitalia and brains of male and female moths.

The full-length coding sequences were transiently expressed in cultured *Trichoplusia ni* cells and Sf9 cells for functional characterization. All three isoforms dose-dependently mobilized extracellular Ca^{2+} in response to PBAN analogs with Mambr-PBANR-C exhibiting the greatest sensitivity. Fluorescent confocal microscopy imaging studies demonstrated binding of a rhodamine red-labelled ligand (RR10CPBAN) (Lee et al., 2012) to all three Mambr-PBANR isoforms. RR10CPBAN binding did not trigger ligand-induced internalization in cells expressing PBANR-A, but did in cells expressing the PBANR-B and -C isoforms. Furthermore, activation of the PBANR-B and -C isoforms with the 18 aa Mambr-PT resulted in co-localization with a *Drosophila melanogaster* arrestin homolog (Kurtz), whereas stimulation with an unrelated peptide had no effect. PCR-based profiling of the three transcripts revealed a basal level of expression throughout development with a dramatic increase in PG transcripts from the day of adult emergence with PBANR-C the most abundant.

The biological significance underlying expression of multiple PBANRs in moth PGs remains to be determined. One possibility is that the isoforms provide a mechanism for fine-tuning cellular responsiveness to the pheromonotropic signal. Truncated isoforms of some mammalian receptors have been shown to exert dominant negative effects on signalling when co-expressed with more highly expressed isoforms (Chow et al., 2012; Seck et al., 2005; Zmijewski and Slominski, 2009). Alternatively, the multiple PBANR transcripts may reflect a spatio-temporal dependence of functionality. This hypothesis is especially attractive given the

pleiotropic complexity of PBAN, the multiplicity of reports detailing PBANR activation by multiple FXPRL-amide peptides (Choi et al., 2003; Hariton-Shalev et al., 2013; Kim et al., 2008; Shalev and Altstein, 2015; Watanabe et al., 2007), and the varied tissue/developmental expression profile of PBANR transcripts. The varied spatial expression of PBANR transcripts suggests a pleiotropic role for the receptor in mediating diverse biological functions in Lepidoptera. Last but not least, our identification and demonstration of receptor activation via fluorescent Ca^{2+} responses expand not only our understanding of sex pheromone biosynthesis in *M. brassicae* but also our model of PBANR function. (Published in Fodor et al., 2017)

WP V. Cloning of M. brassicae Δ11-Desat and its expression:

The sequence of a PG-specific *delta11-desaturase* (Δ11Desat) cDNA was first isolated from *Trichoplusia ni* (Knipple et al., 1998). Since that time, a number of Δ11Desats (in addition to other novel desaturases) have been identified from multiple species and shown to function in pheromone biosynthesis (Dallerac et al., 2000; Hao et al., 2002; Liu et al., 2004; Rodríguez et al., 2004; Rosenfield et al., 2001; Yoshiga et al., 2000). Early studies on the stereochemistry of Δ11Desats indicated that most of these enzymes are extremely selective (Boland et al., 1993), while some of the characterized Δ11Desats have been shown to be bi- or multifunctional (Matousková et al., 2007; Moto et al., 2004; Serra et al., 2007, 2006). It was also reported that some of the *desaturase* genes have become functionless (Roelofs and Rooney, 2003). In *M. brassicae,* four *acyl-CoA desaturases* were cloned from PGs (Park et al., 2008). The two most abundant, differentially expressed transcripts encoding acyl-CoA desaturases were studied in more detail. Functional expression experiments demonstrated that the most abundant transcript encodes a Δ11Desat. The other desaturase was a non-functional Δ9-desaturase that had presumably lost functionality because of two mutations (Park et al., 2008).

Since contradictory results have been published in the literature (Bestmann et al., 1989; Iglesias et al., 1999, 1998; Jacquin et al., 1994) regarding the role of PBAN in desaturase activation, we thus aimed to clarify this issue by measuring *Δ11Desat* transcript levels.

Based on the above information first, primers were designed to clone Δ11Desat. The total coding sequence of *M. brassicae* Δ11Desat was ligated into pJET 1.2 blunt cloning vector and transformed in *E. coli* Top 10 cells and sequenced (Gene Bank No:KM283200). Specific primers for real time RT-PCR experiments were designed for Δ11Desat and reference gene beta-actin as well.

In our study, we determined that *Mambr-delta11-desaturase* is expressed in large quantities exclusively in female PGs. The tissue with the next highest expression level, albeit significantly lower than that found in the PG, was the male hairpencil–aedeagus. This structure is involved in male pheromone production (Jacquin et al., 1991), however, it is very unlikely that the Δ11Desat plays role in biosynthesis of the male pheromones, since they are different in chemical structure from the female-produced pheromones. The *Δ11Desat* ORF sequence identified in our study differed from that published by Park et al. (2008) in only 1 out of 1014 nucleotides. This difference does not alter the amino acid sequence.

For gene expression studies by quantitative real-time PCR, total RNA was extracted from 5 PGs in three replicates. *Δ11-Desat* gene expression was calculated with the help of calibration curves and normalized to *beta-actin* gene expression of the same sample.

Time course monitoring (see WPI): 5 PGs from females were collected (pupae, D0-D8 at 1 hour prior to onset of Sp, at 3rd and 7th h in Sp and 3 hours after light on) under liquid N and stored under -80 ^{o}C. *Δ11-Desat* expression in pupae is ~0.1% and in emerging individuals ~10% compared to that in calling females. The gene expression increased up to the end of D1 reaching a plateau without significant fluctuation up to D8.

Expression level was monitored in D2 and in newly emerged decapitated females after 1h or 1.5 h of Mambr-PT treatment. PT treatment induced pheromone production while gene expression was not affected. Studies in relation to mating (see experiment description in WPIII) despite the significant decrease of pheromone production, *Δ11-Desat* gene expression did not show remarkable change, even when injected with Mambr-PT.

In conclusion, the daily fluctuation of the pheromone titre does not correlate with the level of studied gene expression. Results of PT treatments suggest that *Δ11-Desat* gene expression in not under direct PBAN/PT control. (Published in Köblös et al., 2015)

Unplanned projects in relation to the current one:

To perform comparative studies of pheromonogenesis between *M. brassicae* (our target species) and the ECB, *Ostrinia nubilalis* Hbn. (Lepidoptera; Crambidae, Pyraustinae) we have cooperated with another team at Plant Protection Institute, working on project OTKA 100421.

In the early 1970s two pheromonally distinct but otherwise indistinguishable races of *O. nubilalis* were identified both in its native Palearctic range and its introduced range in North America (Klun & Cooperators, 1975). The females produce mixtures of (*Z*)-11- and (*E*)-11-tetradecenyl acetate (*Z*11-14:Ac and *E*11-14:Ac) as their sex pheromone (Klun et al., 1973). The so-called *Z*-strain is characterized by the production and perception of a 97:3 molar ratio of *Z*11-14:Ac and *E*11-14:Ac, whereas the *E*-strain ECB utilizes a 1:99 blend of *Z*11-/*E*11-14:Ac (Kochansky et al., 1975). Substantial research has been conducted to define the genetic basis of pheromone production in *O. nubilalis* from several aspects.

The results showed that Ostnu-PBAN genomic sequence is > 5 kb in length and consists of six exons. The deduced amino acid sequence revealed a 200-residue precursor protein including a signal peptide, a 24-aa diapause hormone, a 37-aa PBAN and three other FXPRLamide neuropeptides. Our *in vivo* assays suggest that the 37-aa synthetic Ostnu-PBAN (CASLO ApS; Technical University of Denmark, Lyngby, Denmark) is hormonally active in the PG in a dose dependent manner when D2 decapitated females are injected. It restores sex pheromone production to normal levels in mated females and decapitated virgins of both E and Z cultures. The results of a real-time PCR analysis indicated that Ostnu-PBAN mRNA levels reached a plateau in the brain-SOG complexes 1 day after eclosion, and mating did not affect the mRNA expression.

In accordance with their close taxonomic relationship, the Ostnu-PBAN precursor showed high sequence conservation among the crambid species. Excluding the signal peptide, it is 87 and 76% identical to DH-PBAN precursors of the bamboo borer (*Omphisia. fuscidentalis*) (GenBank AFP87384), and the legume pod borer (*Maruca vitrata*) (AFX71575), respectively. Lower homology (66–68%) was observed with the DH-PBAN

precursors of *B. mori* and *H. zea*. Last but not least, Ostnu-PBAN had 98.4% nucleotide identity and complete amino acid sequence identity to a DH-PBAN gene fragment (GenBank accession number LC002981) of the Asian corn borer (*Ostrinia furnacalis*).

The benefits of identifying the Ostnu-PBAN precursor are manifold. In physiological studies, synthetic Ostnu-PBAN can be used to trigger *in vivo* and also *in vitro* pheromone production, which may contribute to a better understanding of the endogenous regulation of pheromone biosynthesis. It facilitates deciphering the functional role of the other PBAN family neuropeptides (DH and SGNPs) in the ECB, and can provide new insights into practical means of manipulating insect homeostasis and development. Furthermore, the *O. nubilalis* is an established model organism in evolutionary biology and the Ostnu-PBAN sequence information may shed more light on the divergence and evolutionary origin of the genus *Ostrinia*.

Three international co-operations were launched supported by this project:

1) Bilateral agreement between Academies (Czechia-Hungary): 2 weeks (November 2013), with the participation of the PI and Dr. Kitti Sipos: Measurement of cAMP in the pheromone gland of cabbage moth (*M. brassicae*); Elucidation of signal transduction cascade mechanism of Pheromone Biosynthesis Activating Neuropeptide (PBAN); (Results are not included).

2) Mobility exchange between countries of agreement (France-Hungary; K-TÉT_12_FR-2-2014-0128511): 5-5 participants from each country (2nd July 2014 31st December 2015): Pheromone communication in the cabbage armyworm: a study of membrane receptors as potential new targets in crop protection.

Short background and rational: Thousands of volatile compounds hover in our environment. For a nocturnal moth, some of them carry crucial information about the host plants or conspecific mates. These signals are detected by membrane olfactory receptors (ORs) expressed in sensory neurons housed in dedicated sensilla located on the antennae (Montagne et al., 2015). In moths, mate recognition usually relies on sex pheromones emission and reception by the partners. These molecules are present only in picomolar concentration in the atmospheric mixture, necessitating a highly sensitive and specific detection system (Kaissling 2004; Vogt 2005). One type of ORs, the so-called pheromone receptors (PRs), are delicately tuned to detect the components of the pheromone blend emitted by the conspecific female (Sakurai et al., 2004; Zhang et. al., 2015). To encroach into this process is an enticing strategy for the environmental-friendly pest control management. Our aim was to identify the PRs of the cabbage armyworm (*M. brassicae*) our main working model. In this species these receptors are unknown, although the sophisticated olfactory system is well studied in some other moths (ex: *B. mori, S. littoralis*).

We cloned the mRNA of MbraOR16, the first candidate pheromone receptor of *M. brassicae*. The encoded protein possessed the hallmarks of insect ORs, such as seven transmembrane domains and an expression in the antennae restricted to olfactory sensilla. In addition, the transcript is male enriched and clustered in a phylogenetic analysis with the characterized moth PRs, suggesting that this receptor is a sex pheromone receptor. The female produced sex pheromone has already been identified in *M. brassicae* and currently used to trap

males. To better understand the molecular mechanism of sex pheromone reception in *M. brassicae* male antennae, the assignment of a pheromone ligand to this receptor is the next step. (Manuscript is ready for submission to Journal of Insect Science; G. Köblös, M-C. François, C. Monsempes, N. Montagne, A. Fónagy, and E. Jacquin-Joly: Identification and molecular characterization of a pheromone receptor candidate in the cabbage armyworm *Mamestra brassicae* (Lepidoptera; Noctuidae)

3) Moataz A. M. Moustafa visiting professor from Cairo University (Egypt) Hungarian Scholarship Board, (2015. 02.02–06.30): The study of sublethal effects of bioinsecticides in Lepidopteran pests

Short background and rational: Lepidopteran insect pest management has relied heavily on synthetic chemical pesticides, but their efficiency is declining as a result of emerging insecticide resistance. Recently biopesticides have become very promising products employed in pest management strategies (Chandler et al., 2011). We investigated the sublethal effects of two bioinsecticides, spinosad (Bret et al., 1997) and emamectin benzoate (Jansson et al., 1997), on larval and pupal development, and reproductive activity including calling behaviour, pheromone production, fecundity and fertility of the cabbage moth, *Mamestra brassicae*. To assess sublethal effects, second instar larvae were fed with 0.005, 0.05, or 0.5 μg active ingredient (a.i.) spinosad/g diet or 0.00005, 0.0005, or 0.005 μg a.i. emamectin benzoate/g diet. Both bioinsecticides significantly affected all developmental stages and adult reproductive activity of *M. brassicae*. Percentage of calling behavior was estimated in females that survived after treatment of 2nd instar larvae. Sublethal dose treatments by spinosad affected less the calling behavior showing a remarkable decreased activity only at the highest concentration. The sublethal dose treatments by emamectin benzoate resulted in a very significant to drastic decrease of calling activity. Both groups of females treated by bioinsecticides have relatively high amounts of *Z*11–16Ac (210.3-106.2ng) compared to control females (218.7 ng), while for the 16Ac the results are statistically not significantly different (control: 15.8 ng). The mean blend ratio of *Z*11– 16Ac and 16Ac in both bioinsecticides treated females were basically the same as in control females. These findings suggest that both spinosad and emamectin benzoate are potentially effective compounds for *M. brassicae* control and provide a promising alternative for Integrated Pest Management programs. (Published in Moustafa et al., 2016)

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