

Friedrich-Schiller-Universität Jena

Biologisch-Pharmazeutische Fakultät



seit 1558

Searching for the Right One: Investigating *Manduca sexta* Pheromone
Receptors.

Diplomarbeit zur Erlangung des Grades eines Diplom Biochemikers

Vorgelegt von

Christopher König, geboren am 21.10.1986 in Sonneberg

Angefertigt am

Max-Planck-Institut für chemische Ökologie in der Abteilung evolutionäre
Neuroethologie in Jena

Jena, März 2011

Gutachter:

Prof. Dr. Bill S. Hansson

PD Dr. Dieter Wicher

Content

1.	Introduction	5
2.	Material and Methods	9
2.1.	General Chemicals	9
2.2.	Special Chemicals for Molecular Biology.....	9
2.3.	Animal Rearing	10
2.4.	Devices.....	11
2.5.	Total RNA Extraction	12
2.6.	Gel Electrophoresis	12
2.7.	cDNA Synthesis.....	12
2.8.	Rapid Amplifying of cDNA Ends Polymerase Chain Reaction.....	13
2.9.	Gel Extraction.....	14
2.10.	Cloning	15
2.11.	Plasmid Mini Preparation and Sequencing.....	15
2.12.	Sequence Analysis	16
2.13.	Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)	16
2.14.	Real Time PCR (qPCR).....	17
2.15.	Synthesis of Probes for <i>in situ</i> Hybridization	17
2.16.	Cryosections.....	18
2.17.	<i>In situ</i> Hybridization	18

3. Results	20
3.1. Cloning of Putative <i>Manduca sexta</i> Odorant Receptors	20
3.2. Msex/Orco	22
3.2.1. Transmembrane structure of Msex/Orco	22
3.2.2. Topographic Expression of Msex/Orco in Male Antennae	23
3.2.3. Quantitative Real Time PCR (qPCR) of Msex/Orco	25
3.3. <i>Manduca sexta</i> Putative Pheromone Receptors	26
3.3.1. Sequence Analysis of MsexOR-1 and MsexOR-4	26
3.3.2. Expression Analysis	28
3.3.3. qPCR	29
3.3.4. Topographic Expression of MsexOR-1 and MsexOR-4	29
3.3.5. Topographic Expression of MsexOR-1 and MsexOR-4 with Msex/Orco	33
3.4. MsexOR-31	35
3.4.1. Homology	35
3.4.2. Topographic Expression	36
3.4.3. Expression over the Antennae	38
4. Discussion	39
5. References	42
6. Appendix	45

1. Introduction

The olfactory sense is crucial for the survival of insects, as well as their reproductive success. It enables them to find food, conspecifics, and predators over long distances by identifying emitted volatile compounds. Especially compounds employed for intraspecific communication, so called pheromones, are of great interest. Sex pheromones belong to this group: they are emitted by one individual to attract potential mating partners over long distances. Since the isolation and identification of the first pheromone succeeded in the silkworm *Bombyx mori* (Butenandt et al. 1959) Lepidoptera have been a focus of research on olfaction. The sphingid *Manduca sexta*, which in comparison to the domesticated *Bombyx mori* demonstrates complex behavior as part of natural ecosystems, has become one of the best described model organism in the field of olfaction, with extensive data available on fields like morphology, biochemistry, electrophysiology, ecology and ethology (reviewed in Hildebrand 1995).

Today the morphology of the primary olfactory organ, the antenna, has been described in great detail (Sanes and Hildebrand 1976, Keil 1989, Lee and Strausfeld 1990, Shields and Hildebrand 1999a, b). The antenna can be used to distinguish males and females due to the sexual dimorphism present in *M. sexta*. While male antennae are characterized by long hair-like structures (long sensilla trichodea) which are arranged in a u-shape and are visible to the naked eye, female trichoid sensilla are considerably shorter (Sanes and Hildebrand 1976). Additionally, the cross sections of the antennae differ between males and female. While the female antenna is circular in cross sections, the male antennae are keyhole-like shaped with an extension on which the long trichoid sensilla are located (Sanes and Hildebrand 1976; Fig. 1). Antennae of both sexes offer several types of olfactory sensilla: Two trichoid, two basiconic and one coeloconic (Lee and Strausfeld 1990, Shields and Hildebrand 1999a, b). Of primary interest are the type A trichoid sensilla, because of the obvious difference in appearance in males and females, indicating a function in pheromone detection. Physiologically, this function was demonstrated by Kaissling et al. 1989. The cuticle of trichoid sensilla is perforated. It houses the dendrites of two odorant receptor neurons (ORN) embedded in sensillum lymph (Keil 1989). The neuronal cell bodies are located beneath the sensillum under the cuticle of the antenna (Keil 1989). The axons of the ORN project into the antennal lobe, where information is processed (Keil 1989; Fig. 1).

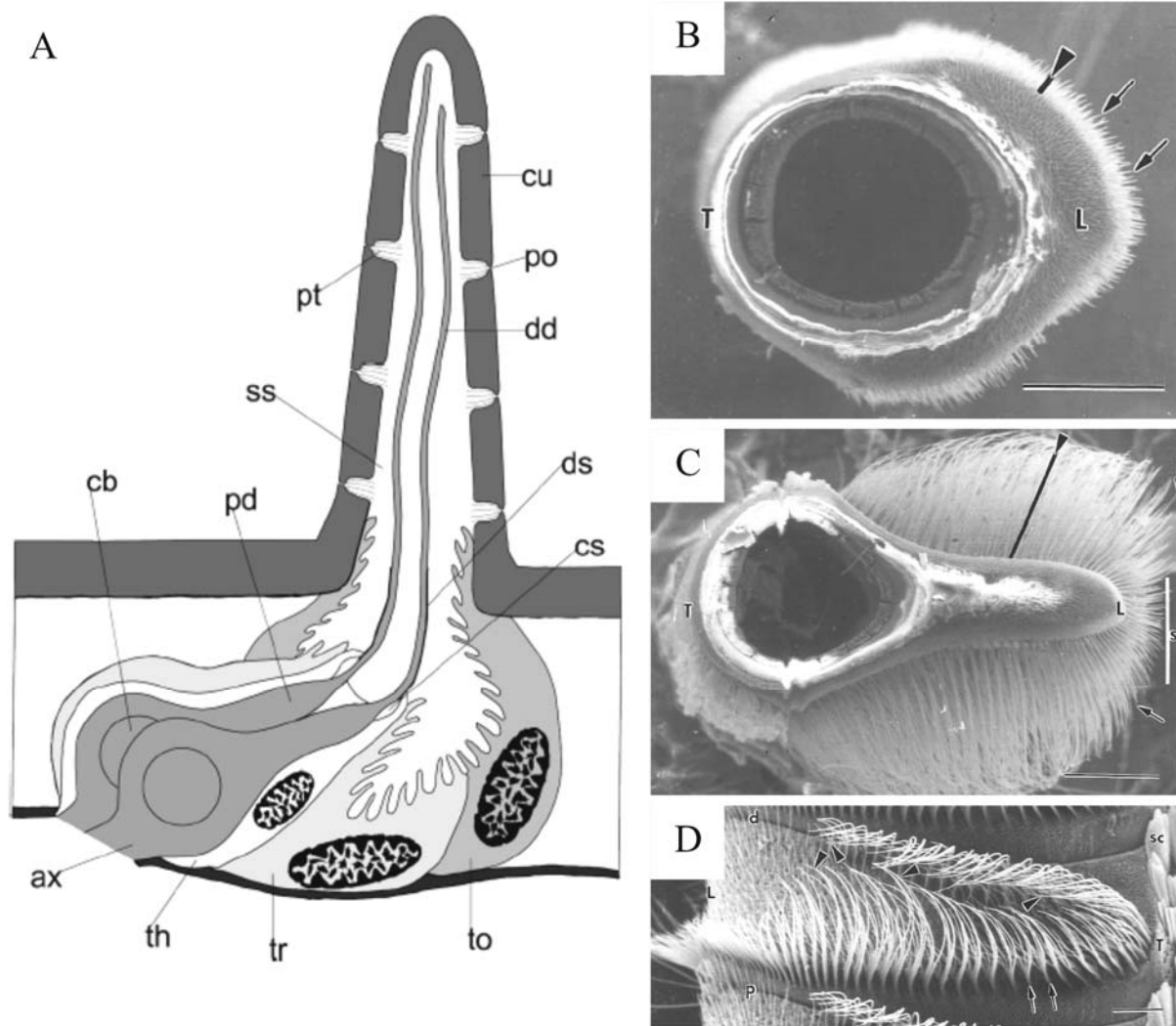


Fig. 1: Morphology of *Manduca sexta* antennae. (A) Schematic drawing of a type A trichoid sensillum. ax, axon; cb, cell body; cs, ciliary sinus; cu, cuticle; ds, dendritic sheath; dd, distal dendrite; ddb, distal dendritic branches; po, pore; pt, pore tubule; pd, proximal dendrite; ss, sensillar sinus; th, thecogen cell; to, tormogen cell; tr, trichogen cell. (B), (C), (D) Scanning electron microscopy pictures of female and male antennae. (B) Cross section of a female antenna demonstrates their oval profile. Scale bar represents 250 μm . (C) The keyhole shaped cross section of male antennae is demonstrated. Notable are the very long trichoid sensilla type A (arrow). Scale bar represents 250 μm . (D) The typical u-shaped order of trichoid type A sensilla of males. Scale bar represents 100 μm . A-D modified from Shields and Hildebrand 1999a.

Based on the results of Tumlinson et al. 1989 and Kaissling et al. 1989 there are supposed to be three pheromone receptors. Female individuals emit a pheromone blend consisting of several components: E10,Z12-16:Al (bombykal), E10,E12-16:Al (EE), E10,E12,Z14-16:Al (EEZ), E10,E12,E14-16:Al (EEE) (Tumlinson et al. 1989). Bombykal and EEZ are necessary to stimulate mating behavior (anemotaxis, approaching and touching the source) in males, which was demonstrated in wind tunnel experiments by Tumlinson et al. 1989. Bombykal is the component with the highest representation in the mix and supposedly the main component (Tumlinson et al. 1989). Tip recordings demonstrate that in each pheromone responding sensillum one odorant receptor neuron is tuned to bombykal (Kaissling et al. 1989). The most frequent pheromone component is detected by the most frequent receptor

cells. EEZ is detected by the most of the second cells in the concerning sensilla (Kaissling et al. 1989). The cells not responding to EEZ showed response to EEE (Kaissling et al. 1989). Concentration of EEZ and EEE in the pheromone blend is not determined exactly (EEZ about 11.3 ng and EEE about 1.2 ng in 1 female gland equivalent) but lower than that of bombykal (23.8 ng in 1 female gland equivalent) (Tumlinson et al. 1989). The incidental components are detected by less frequent cells. However, Kalinova et al. 2001 report on a fourth type of pheromone sensitive sensillum, in which one ORN is tuned to bombykal and the other to EE.

In contrast to our detailed knowledge on morphology there is only sparse information available on the molecular basis of olfaction in *Manduca sexta*. Most studies focused on other insect species like *Drosophila melanogaster* and *Bombyx mori*, because of their role as model organisms of molecular biology, or mosquito species as vectors of human diseases. From these studies we know that odor recognition is mediated by odorant receptors (OR) that are expressed by the ORN (Hallem et al. 2004). The first insect ORs were described by Clyne et al. 1999, Gao and Chess 1999 and Vosshall et al. 1999 in *Drosophila*. All three groups scanned the available genomic sequence data for putative seven transmembrane domain receptors and verified that they are expressed mainly in the ORN on the olfactory organs. Interestingly ORs are highly variant in their sequences, indicating that ORs evolve very quickly (reviewed in Sanchez-Gracia et al. 2009). Due to the low sequence conservation of ORs in insects, it was not possible to identify putative moth OR using sequence similarity comparisons (Krieger et al. 2002). The first ORs of moths were described by Krieger et al. 2002 in *Heliothis virescens* using non-public genomic data to screen an antennal cDNA library. Today several putative ORs of several moth species like *Bombyx mori* (Sakurai et al. 2004, Krieger et al. 2005, Tanaka et al. 2009), *Ostrinia* (Miura et al. 2010) and *Helicoverpa* (Zhang et al. 2010) are identified. Some ORs share more sequence similarity and form subgroups, e.g. the pheromone receptors of Lepidoptera (Krieger et al. 2005). Sequence similarity of these pheromone receptors helped to identify further ones in other Lepidoptera species (Miura et al. 2010, Zhang et al. 2010).

The common OR organization in insects is heteromultimers with a coreceptor called Orco (Larsson et al. 2004, Neuhaus et al. 2005, Vosshall and Hansson 2011). The first Orco was reported as putative OR 83b in *Drosophila* (Vosshall et al. 1999, Vosshall et al. 2000). Krieger et al. 2003 observed the high conservation of 83b homologues in insect species indicating a crucial role in olfaction. It has been demonstrated that a *Drosophila* mutant lacking the Orco gene does not respond to stimulation with odors anymore (Larsson et al. 2004). *In situ* hybridization and histochemistry indicate that Orcos are present in all odorant receptor expressing cells (Larsson et al. 2004). Experiments in *Bombyx mori* demonstrate that coexpressed Orcos improve the pheromone detection of pheromone receptors in

heterologues expression system (Nakagawa et al. 2005). From other experiments it was concluded that the *Drosophila* Orco is necessary for correct membrane integration of odorant receptors (Larsson et al. 2004).

In this study we identify the putative *Manduca sexta* Orco, as well as two putative pheromone receptors.

2. Material and Methods

2.1. General Chemicals

Wheat germ, cholesterol, ascorbic acid, sorbic acid, methyl paraben, nicotinic acid, riboflavin, thiamine, pyridoxine, folic acid, biotin, Tri Reagent and 1-bromo-3-chloropropan were procured from Sigma Aldrich (St. Lois, MO). The manufacturer of RNA storage solution is Ambion (Austin, TX). Biozym (Hessisch Oldendorf, Germany) delivered LE Agarose. Pink Coprecipitant produced by Bioline (London, Great Britain) was used. The manufacturer of Tissue-Tek O.C.T. compound is Sakura Finetek (Tokyo, Japan). Vectabond is produced by Vector Laboratories (Burlingame, CA). Corn meal and soy flour were delivered by Rapunzel (Legau, Germany). Casein was purchased from Thermo Fisher Scientific (Waltham, MA). The Salt Mix was produced by MP Biomedicals (Costa Mesa, CA). Sugar and linseed oil were purchased via Rewe (Köln, Germany). All other general chemicals were delivered by Roth (Karlsruhe, Germany). All experiments were performed with double distilled water (ddH₂O). Diethylpyrocarbonate (DEPC) treated water was gained by incubating a solution of 0.1% DEPC in ddH₂O for 1 h at 37°C followed by autoclaving the solution for 20 min at 121°C.

2.2. Special Chemicals for Molecular Biology

The producer of 2log DNA ladder and all necessary restriction enzymes was New England Biolabs (Ipswich, MA). Invitrogen (Carlsbad, CA) delivered SuperScript III First Strand System for RT-PCR containing DEPC treated water, oligo dt, dNTPs, 10X Reaction buffer, 25 mM MgCl₂, 0.1 M dithiothreitol (DTT), RNase out, SuperScript RT III and RNase H. Additionally, Invitrogen delivered the Topo TA Dual Promotor Cloning Kit (containing pCRII-Topo Vector, Salt Solution) and One shot Top10 chemical competent *E. coli* cells (containing SOC Medium, *E. coli* cells).

For PCR the TAQ DNA Polymerase Kit from Qiagen (Hilden, Germany) containing colored reaction buffer and taq polymerase was used. Also produced by Qiagen is the Rotor Gene SYBR Green PCR Kit containing PCR Master Mix and nuclease free water for real time PCR. Clontech (Mountain View, CA) produced the used SMART RACE cDNA Amplification Kit containing SMART A Oligonucleotide, 3'-RACE CDS Primer A, 5'-RACE CDS Primer A, 5X First Strand Buffer, DTT, Deionized Water, Tricine-EDTA Buffer, 10X Universal Primer A Mix, Nested Universal Primer A and dNTP Mix. Also produced by Clontech was the Advantage 2

PCR Enzyme System containing 50X Advantage 2 Polymerase Mix, 10X Advantage 2 PCR Buffer and 50X dNTPs Mix.

EZNA (Norcross, GA) delivered both the Gel Extraction Kit (containing Binding Buffer, Elution Buffer, Wash Buffer with ethanol added as written in the manual) and the Plasmid Mini Prep Kit (containing Solution I (with RNase A as written in the manual), II and III as well as Equilibration Buffer, Buffer HB, Elution Buffer and DNA Wash Buffer with ethanol added as written in the manual).

Roche Diagnostics (Risch, Switzerland) is the manufacturer of Biotin RNA labeling kit (containing transcription buffer, labeling mix, SP6 Polymerase, T7 Polymerase), Digoxigenin labeling Kit (containing transcription buffer, labeling mix, SP6 Polymerase, T7 Polymerase) and HNPP/Fast Red Detection Set (containing Fast Red, HNPP) as well as enzyme linked anti-Digoxigenin antibodies.

Perkin Elmer (Waltham, MA) delivered the TSA Fluorescence kit containing blocking reagent, enzyme linked Streptavidin, Amplification Diluent and TSA.

Roth supplied dNTPs for RT PCR.

All primers were synthesized by MWG (Ebersberg, Germany).

2.3. Animal Rearing

Manduca sexta adults were allowed to mate in a flight cage containing a *Nicotiana attenuata* plant for oviposition. Eggs were collected from the plant three times per week and transferred into plastic boxes in a climate chamber (day: 26°C, 80% humidity, 16 h light, 100% light intensity; night: 22°C, 80% humidity, 8 h dark). Hatched larvae were placed in new plastic boxes in the same climate chamber with artificial diet (46 g agar, 144 g wheat germ, 140 g corn meal, 76 g soy flour, 75 g casein, 24 g salt, 36 g sugar, 5 g cholesterol, 12 g ascorbic acid, 6 g sorbic acid, 3g methyl paraben, 9 ml linseed oil, 60 ml 37% formalin, 30 mg nicotinic acid, 15 mg riboflavin, 7 mg thiamin, 7mg pyridoxine, 7 mg folic acid and 0.6 mg biotin per 1.8 ml water) on a metal grid and transferred regularly into new boxes with new feed in the same climate chamber. After six weeks the so called wandering larvae were selected and placed into new plastic boxes in another climate chamber (same conditions) to allow pupating. Pupae for rearing were transferred into the flight cage. Remaining animals were allowed to eclose in paper bags and used for experiments.

2.4. Devices

Device	Name/Type	Manufacturer
Pipettes	Pipetman	Gilson (Middleton, WI)
Climate chamber	MC 1000	Snijders Scientific (Tilburg, Netherlands)
Steel beads	4 mm steel bead	Qiagen
Tissuelyzer	Tissuelyzer LT	Qiagen
Vortex shaker	Vortex Genie 2	Scientific Industries (Bohemia, NY)
Centrifuge	Centrifuge 5415 R	Eppendorf (Hamburg, Germany)
Distiller	Mono Dest 3000E	Lenz Laborglas (Wertheim, Germany)
Autoclave	MLS 3781 L	Sanyo (Moriguchi City, Japan)
Photometer	BioPhotometer	Eppendorf
Electrophoresis chamber	Mupid One	Advance (Tokyo, Japan)
Thermocycler	PCR System 9700	Applied Biosystems (Carlsbad, CA)
Heater	Thermomixer comfort	Eppendorf
Incubator	B12	Kendro (Hanau, Germany)
Shaker	AI82K	Infors (Bottmingen, Switzerland)
Real time cyclor	Rotor Gene Q	Qiagen
Cryostat	Microm HM 560	Thermo Fisher Scientific
Hybridization Oven	Hybaid	Thermo Fisher Scientific
Water bath	SW22	Julabo (Seelbach, Germany)
Confocal Laser Scanning Microscope	LSM 501 Meta	Zeiss (Oberkochen, Germany)

2.5. Total RNA Extraction

Antennae of adult *Manduca sexta* were dissected close to the scapulum and transferred into 1 ml of Tri Reagent in a 2.0 ml tube. One 4 mm steel bead was added to the tube and the material was homogenized at 50 Hz in a TissueLyzer for 15 min. 120 µl 1-bromo-3-chloropropan was added followed by incubation at room temperature for 10 min. During incubation the samples were vortexed twice to prevent phase separation and allow RNA extraction. Samples were centrifuged for 20 min at 8°C and 16.1 rcf to induce phase separation. The upper phase was transferred to a fresh 1.5 ml tube containing 0.5 ml of isopropanol and vortexed.

After incubation at -20°C over night the tube was centrifuged for 30 min at 8°C and 16.1 rcf, the supernatant was discarded and the pellet was washed with 0.5 ml of 80% ethanol in DEPC treated water, followed by centrifugation for 15 min at 8 °C and 16.1 rcf. The supernatant was removed and the pellet was dried for 5 min at room temperature. Finally, the pellet was dissolved in 25 µl RNA Storage Solution.

Quality was estimated by gel electrophoresis and photometric determination of absorption to calculate concentration.

2.6. Gel Electrophoresis

Gels were prepared with 1% agarose and 0.005% ethidium bromide in TAE buffer (0.04 M tris, 0.04 M vinegar acid, 1 mM EDTA). If required 6X loading dye (30% glycerol, 70% dH₂O, 2.5 mg per 10 ml ddH₂O bromphenol blue) was added to samples and 2log Ladder was used as marker. Nucleotide separation was performed in an electrophoresis chamber at 135 V for 15 min in TAE buffer.

2.7. cDNA Synthesis

cDNA synthesis was performed using the components of SuperScript III First Strand System for RT-PCR. 1 µg RNA from RNA extraction was filled up to 8 µl with DEPC treated H₂O. 1 µl dNTPs and 1µl oligo dT were added and the tube was incubated at 65°C for 5 min and then cooled on ice. 2 µl Reaction buffer, 4µl MgCl₂ 25 mM, 2 µl DTT 0.1 M, 1µl RNase out and

1µl SuperScript III RT were added. The reaction was performed by incubation for 50 min at 50°C followed by incubation for 5 min at 85°C for termination.

RNA was degraded using 1µl RNase H and incubation for 20 min at 37°C.

2.8. Rapid Amplifying of cDNA Ends Polymerase Chain Reaction

For rapid amplifying of cDNA ends polymerase chain reaction (RACE-PCR) components of the SMART RACE cDNA Amplification Kit and the Advantage 2 PCR Enzyme System were used.

For preparation of 3'-Race ready cDNA 3 µl total RNA, 1 µl 3'-RACE CDS Primer A and 1 µl deionized water were combined followed by incubation at 70°C for 2 min and cooling on ice for 2 min. 2 µl 5X First-Strand Buffer, 1 µl DTT, 1 µl dNTP Mix and 1µl Super Script III RT were added and then incubated at 42°C for 90 min. The solution was diluted with 100 µl Tricine-EDTA Buffer followed by incubation at 72°C for 7 min. The finished cDNA was stored at -20°C.

For preparation of 5'-Race ready cDNA 3 µl total RNA, 1 µl 5'-RACE CDS Primer A and 1 µl SMART II A oligonucleotide were combined followed by incubation at 70°C for 2 min and cooling on ice for 2 min. 2 µl 5X First-Strand Buffer, 1 µl DTT, 1 µl dNTP Mix and 1µl Super Script III RT were added and then incubated at 42°C for 90 min. The solution was diluted with 100 µl Tricine-EDTA Buffer followed by incubation at 72°C for 7 min. The finished cDNA was stored at -20°C.

The 3'-RACE PCR reaction (5'-RACE PCR respectively) consisted of 17.5 µl deionized water, 2.5 µl Advantage 2 PCR Buffer, 2.5 µl UPM A, 0.5 µl Advantage 2 Polymerase Mix, 0.5 forward primer listed in the table (for 5'-RACE PCR reverse primer were used respectively) and 1 µl 3'-RACE ready cDNA (5'-RACE ready DNA respectively). The used PCR program started with 5 cycles 94°C for 30 s, 72°C for 3 min followed by 5 cycles 94°C for 30 s, 70°C for 30 s, 72°C for 3 min and finally 25 cycles 94°C 30 s, 68°C 30 s, 72°C 3 min.

Gene	Primer Name	Primer sequence (5'-3')	T _M [°C]
MsexOR-1	MSRace9F	CAA CAT CAA GGT TTG GCC AAT GGT G	65,8
	MSRace9R	TTT CGT ATT TTG CCG CCA TGC ATT T	62,5
MsexOR-2	MSRace2F	CTT CGG AAA CCG GCT GAT TGA AGA G	67,4
	MSRace2R	AAG TAG GTC ACT ACA GCG CCG AGC A	69,1
MsexOR-4	MSRace3F	TGC AGA AAC GGC TTG GTT GTC ATT G	65,8
	MSRace3R	TCG GCA GTC GTA TCG TCG TAG CAT T	67,4
MsexOR-31	MS29Race1F	TGC GTC GCA ATC TAA AGA CCA TGA G	65,8
	MS29RaceFLR	TTA TGT GTT CGC CCT GTT GAG GAC TGC	61,3

Results of the PCR were visualized with gel electrophoresis and bands were sectioned from the gel with a scalpel to extract DNA fragments.

2.9. Gel Extraction

Gel Extraction was performed with the content of the Gel Extraction Kit. 300 µl Binding Buffer was added to the excised gel slice in a 1.5 ml tube. Gel was melt by incubation at 60°C for 7 min at which the tube was vortexed every 2 min. The DNA Mini Column was placed in the 2 ml collection tube followed by transferring the solved DNA fragment on the column. After centrifuging at 10, 000 x g for 1 min the liquid from the collection tube was discarded and 300 µl Binding Buffer were added to the column followed by another centrifugation step at 10, 000 x g for 1 min. The flow-through was discarded and the column was washed two times with 700 µl Washing Buffer followed each time by centrifugation at 10, 000 x g for 1 min.

The column was dried by centrifugation at 16, 100 x g for 2 min. DNA was eluted with 30 µl Elution buffer through incubation for 1 min followed by centrifugation at 16, 100 x g for 1 min.

2.10. Cloning

For cloning the Topo TA Dual Promotor Cloning Kit with One Shot Top10 chemical competent cells was used. 4 µl of PCR product extracted from agarose gel was used and 1 µl salt solution as well as 1 µl pCRII-TOPO Vector were added. After 5 min of incubation at room temperature the mixture was stored on ice while 25 µl chemical competent cells were thaw for 5 min on ice also.

2 µl of the ligation mix were transferred into the melted cells followed by incubation for 20 min on ice. The cells were exposed to heat shock at 42°C for 1 min and immediately cooled on ice. 250 µl SOC Medium was added and the cells were incubated on a shaker at 37°C for 1 h. Afterwards the cells were plated on LB agarose plates (10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar per 1 l dH₂O) with ampicillin (100 mg per 1 l LB medium) and X-gal (16 mg in 400 µl dimethylformamide per plate) and were allowed to grow over night in the incubator at 37°C.

2.11. Plasmid Mini Preparation and Sequencing

Plasmid Mini Preparation was performed with the corresponding kit. A bacterial culture was grown in 5 ml LB medium with ampicillin (10 g tryptone, 5 g yeast extract, 10 g NaCl, and 100 mg ampicillin per 1 l dH₂O) over night and then centrifuged at 10,000 x g for 1 min. The supernatant was discarded whereas the pellet was resuspended in 250 µl Solution I and then transferred into a 1.5 ml tube.

250µl Solution II was added followed by intensive inverting and incubation for 2 min. The solution was neutralized with 350 µl Solution III and was centrifuged at 16,100 x g for 10 min. Miniprep columns were prepared with 100 µl Equilibration Buffer and centrifuged at 16,100 x g for 1 min. The supernatant of the cell lysate was transferred on the column followed by centrifugation at 16,100 x g for 1 min.

The column was washed once with 500 µl HB Buffer and twice 700 µl DNA Wash Buffer each time followed by centrifugation at 16,100 x g for 1 min. With an additional centrifugation step at 16,100 x g for 2 min the column was dried.

DNA was eluted with 30 µl Elution Buffer and centrifugation at 16,100 x g for 1 min. Concentration was estimated by photometric measurement and the sample was sent to MWG for sequencing.

2.12. Sequence Analysis

Alignment of (putative) odorant receptors of *Manduca sexta*, *Bombyx mori* and *Heliothes virescens* were performed using MAFFT (by Kazutaka Katoh) with the E-INS-i option. Maximum likelihood phylogenetic tree was calculated with FastTree (by Morgan N. Price) based on the alignment. The graphical appearance was developed with MEGA4 as linearized tree (by Sudhir Kumar, Koichiro Tamura, Masatoshi Nei).

For transmembrane domain prediction TMHMM program (version 2.0, by CBS) was used.

2.13. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT PCR reaction consists of 17.5 µl water, 2.5 µl colored reaction buffer, 1 µl dNTPs, 1 µl Primer A, 1 µl Primer B, 1 µl taq DNA Polymerase und 1 µl cDNA with a final volume of 25 µl. Reaction was done in a thermocycler with 94°C for 2 min at the beginning followed by 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 45 s. To check the results samples were loaded on an agarose gel.

The sequences of used primers are supplied in the table.

Gene	Primer name	Primer sequence (5'-3')	Length [bp]	T _M [°C]
RL31	RL31for	GGA GAG AGG AAA GGC AAA TC	224	58,4
	RL31rev	CGG AAG GGG ACA TTT CTG AC		60,5
MsexOR-1	MSPrimer3b	AGT GCA TCG ATT ATC ATC GG	280	56,4
	MS Primer3a	CCG AGG TAC TCC CAT GGA AC		62,5
MsexOR-2	MS02realfor	CTC AAG GCC TGG TAT CCA TT	279	58,4
	MS02realrev	TGG ATC TGG CAC TTT CTC TG		58,4
MsexOR-4	MSPrimer1b	AAT CCT TAC AAA GAC CCT CG	397	56,4
	M2_A08_for	CGG CAA ACA ATA CAC GGA GTC CGT AAG GCC C		76,2
MsexOR-31	OR31realfor	CAT CAG CGC ATT CCT CTT CA	190	58,4
	OR31realrev	GTG GCG AAC ACA TTC GGT AA		58,4

2.14. Real Time PCR (qPCR)

For q PCR the Rotor Gene SYBR Green PCR Kit was used. The Reaction consists of 12.5 µl PCR Master Mix (Rotor Gene SYBR Green PCR Kit), 9.5 µl dH₂O, 1 µl cDNA, 1 µl Primer A, 1 µl Primer B with a final volume of 25 µl. Primers are identical to the primers of normal RT-PCR as written above. Samples run through 94°C for 5 min, 30 cycles of 94°C for 10 s, 58°C for 10 s, 72°C for 10s with the measuring point after elongation phase (72°C) and following melt curve from 50°C to 99°C with 5 s per 1°C. The same primers as for RT-PCR were used. Data is analyzed in the 'Comparative Quantitation' mode of the cycler software which calculates expression levels relative to RL31.

Products of RT-PCR were gel extracted and cloned. Plasmid concentration after mini preparation was measured. An array of five dilutions of these plasmids (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}) were used as standards in the same qPCR.

All samples run as duplicates. To prove that only one PCR product is amplified samples for each primer pair were loaded to an agarose gel. No templates controls were run with every real time PCR.

2.15. Synthesis of Probes for *in situ* Hybridization

To perform double staining it is necessary to produce probes with at least two different marked NTPs. To show the expression of a gene probes have to anneal with the RNA so they have to be antisense RNA probes. The sequences of the putative receptors revealed through sequencing (done by MWG) of cDNA cloned in pCRII vector which has two promoter regions (T7, SP6) in different orientation.

The vector is cut once in a way that the gene cloned in the vector is divided from the second unused promoter region. Enzymatic digestion followed standard protocols with restriction enzymes. After this step the sample was purified through agarose gel electrophoresis and gel extraction with Gel Extraction Kit.

150 ng of purified DNA was filled up to 14 µl with water. To write the probe 2 µl transcription buffer, 2 µl labeling mix and 2 µl Polymerase Sp6 or T7 was added. The sample was incubated for 3 h at 37°C and precipitated with 2.5 µl Pink Coprecipitant and 75 µl 96% ethanol over night at -20°C.

After centrifugation for 30 min at 4°C at 16.1 rcf and discarding of supernatant the pellet was washed with 50µl 70% ethanol and centrifuged again for 30 min at 4°C at 16.1 rcf. The supernatant was removed through pipetting and the dry pellet was solved in 50 µl water.

Fragmentation of the probe was performed with 25 µl probe in 25 µl carbonate buffer (80 mM NaHCO₃, 120 mM Na₂CO₃, pH 10.2) at 60°C for a defined time calculated by a formula from Angerer and Angerer 1992. Reaction was disrupted with 5 µl vinegar acid and 250 µl hybridization buffer (50% formamid, 2X SSC, 10% dextran sulphate, 20 mg/ml yeast t-RNA, 0.2 mg/ml herring sperm DNA).

2.16. Cryosections

Antennae used for *in situ* hybridization were embedded in Tissue-Tek O.C.T. compound diluted with 40 % glucose solution and frozen at -60°C in a cryostat. Sagittal sections were received by cutting at -25°C with a thickness of 14 µm. Cryosections were transferred onto Vectabond treated slides and air dried for 30 min.

2.17. *In situ* Hybridization

After fixation with 4% paraformaldehyde in 0.1 M NaCO₃ (pH 9.5) for 30 min at 4°C the slides were washed in PBS (0.85% NaCl, 1.4 mM KH₂PO₄, pH 7.1) for 1 min. For the denaturation of proteins slides were immersed for 10 min in 0.2 M HCl followed by a washing step (2 min) in PBS with 1% Triton X-100 and two 30 s washes in PBS. Finally, the slides were incubated for 10 min at 4°C in a solution of 50% formamid with 5X SSC (0.15 M NaCl, 0.015 M Na-citrate, pH 7.0). Probes were diluted in hybridization buffer (50% formamid, 2X SSC, 10% dextran sulphate, 20 mg/ml yeast t-RNA, 0.2 mg/ml herring sperm DNA). This mixture was pipetted onto the slides and was incubated at 55°C in a humid box (50% formamid) over night.

After hybridization the slides were washed twice with 0.1X SSC at 60°C for 30 min and then 1 min with TBS (100 mM Tris, pH 7.5, 150 mM NaCl). Unspecific binding sites were saturated with blocking solution (TBS with 0.03 % Triton X-100 and 1% blocking reagent) during incubation for 30 min in a humid box (water) at room temperature. The antibody anti-Dig (linked to alkaline phosphatase) and the streptavidin (linked to horseradish peroxidase) were diluted in blocking solution and after pouring the blocking solution from the slides the antibody solution was added. After a 60 min incubation step at 37°C in a humid box the slides were washed three times for 5 min in TBS with 0.05% Tween 20. The slides were rinsed with DAP and then the HNPP/FastRedTR detection set was used for development of the dye of anti-Dig conjugates for 30 min. After 3 further wash steps with TBS with 0.05% Tween 20 for 5 min the slides were treated with the TSA Fluorescence kit

for 10 min. Afterwards the slides were washed three times with TBS with 0.05% Tween 20 for 5 min and rinsed with aqua dest. Slides were sealed with PBS/glycerol 3:1 and signals visualized with a confocal microscope.

3. Results

3.1. Cloning of Putative *Manduca sexta* Odorant Receptors

The basis of our analysis of *Manduca sexta* odorant receptors was a sequence database of antennal transcripts, a so called EST (expressed sequence tag) database, representing partial and full length sequences of the coding region of expressed genes. To identify putative odorant receptors (ORs) all sequences in the database were compared with sequences of the non-redundant NCBI nucleotide database (nr) using tblastx algorithm. Some sequences exhibited similarity to known ORs of insects and were identified as putative ORs. Additionally, a protein profile analysis using PANTHER using the HMM (Hidden Markov model)-based DecypherHMM was performed. This allowed comparison of newly predicted protein sequences with known OR profiles to identify further candidates.

Identified transcripts were virtually translated into amino acid sequences which were in turn aligned with other amino acid sequences of known putative ORs of *Bombyx mori* and *Heliothis virescens*. To visualize the data a maximum-likelihood dendrogram of sequence similarity was created as shown in Fig. 2.

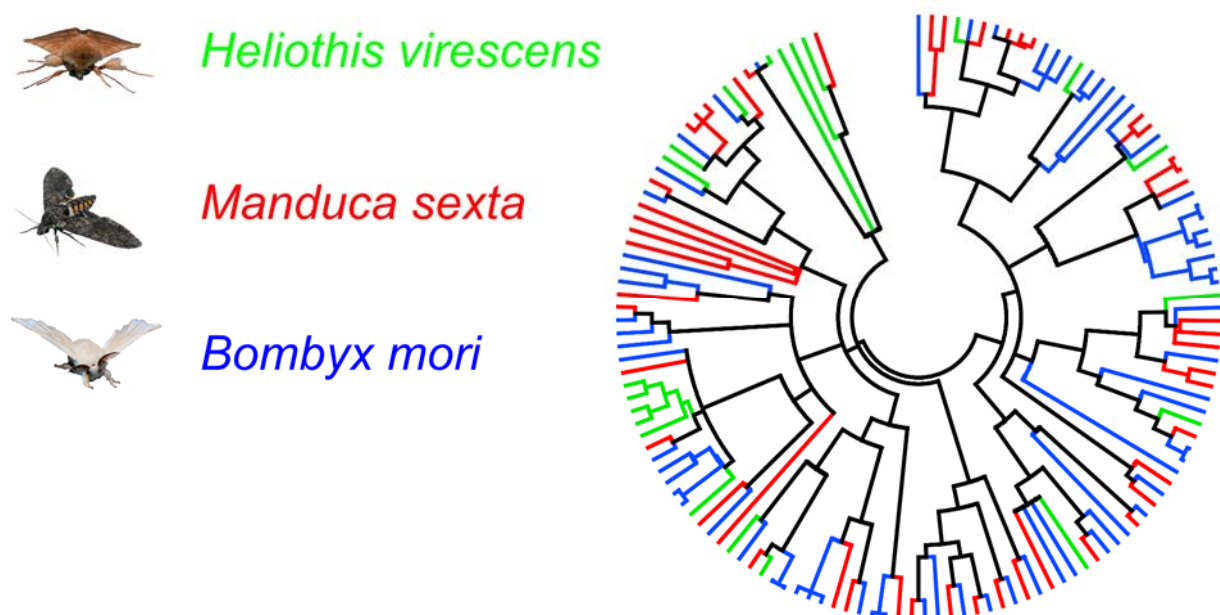


Fig. 2: Sequence similarity dendrogram of predicted amino acid sequences of all known putative ORs of *Manduca sexta*, *Bombyx mori* and *Heliothis virescens*. Most receptor sequences differ strongly not only between the species but also within one species.

The ORs of insects are characterized by low conservation within one species and also between the species (reviewed in Rützler and Zwiebel 2005). However, some putative receptor types exhibit a higher degree of conservation (Fig. 2), indicating a special function of these receptor types.

To facilitate further analysis full length sequences of the more conserved receptor genes were obtained by rapid amplification of cDNA ends PCR (RACE-PCR). For RACE-PCR antennal mRNA of both sexes of *Manduca sexta* was combined with an oligo-dT primer containing an adaptor sequence in a reverse transcription reaction to obtain 3'-RACE-cDNA. This cDNA was used as a template in a PCR with a primer directed against the target transcript and a primer matching the adaptor to amplify the 3' end of mRNA.

For 5'-amplification the antennal mRNA was used together with an oligo-dt primer (without adaptor sequence) and an oligo-dG primer with the adaptor sequence in a reverse transcription reaction. The employed reverse transcriptase characteristically adds oligo-dC to the 3' end of cDNA. The added primer matches this region and contains an overhang (same sequence like in 3' RACE) which is added to the cDNA by the used enzyme performing template switching. In the process the enzyme reaches the end of the template but instead of terminating the synthesis the transcriptase uses the primer matching the new strand as new template generating a 5'-RACE-cDNA. This cDNA was used for PCR with one gene specific primer and a primer matching the overhang sequence to amplify 5'end of mRNA in a PCR.

The PCR products were extracted from the gel, cloned and sequenced. Analyzed sequences revealed the complete open reading frame of Msex/Orco (MsexOR-2), MsexOR-1, MsexOR-4 and MsexOR-31 (Appendix).

3.2. Msex/Orco

Orcos are necessary for olfaction and are highly conserved between insect species (Krieger et al. 2003, Larsson et al. 2004). The *Manduca sexta* Orco has been described previously, but only as partial sequence (Patch et al. 2009; called MsexOR-2). We managed to clone the full length coding region (Fig. 3).

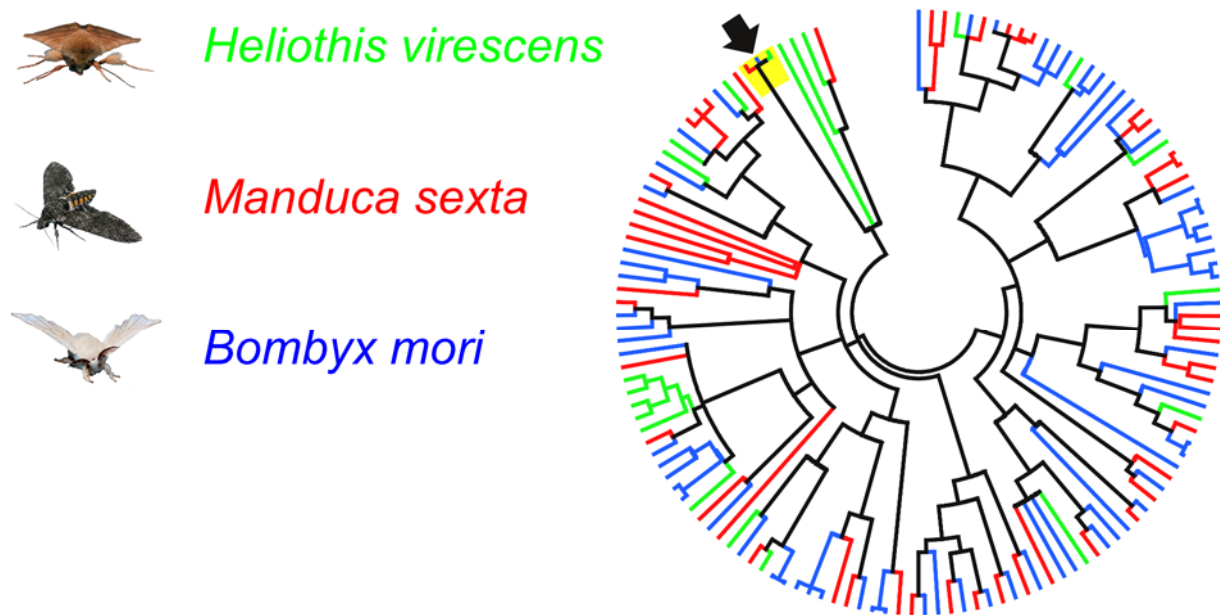


Fig. 3: Sequence similarity dendrogram of amino acids of all known putative ORs of *Manduca sexta*, *Bombyx mori* and *Heliothis virescens*. The arrow marks a group of high conserved proteins in all three species, namely the Orcos of the three species.

3.2.1. Transmembrane structure of Msex/Orco

ORs of insects as well as the Orco are typically predicted to have five to nine transmembrane domains as reported by Vosshall et al. 1999. Hence, the predicted amino acid sequence of Msex/Orco was also analyzed for transmembrane domains. The result is shown in Fig. 4.

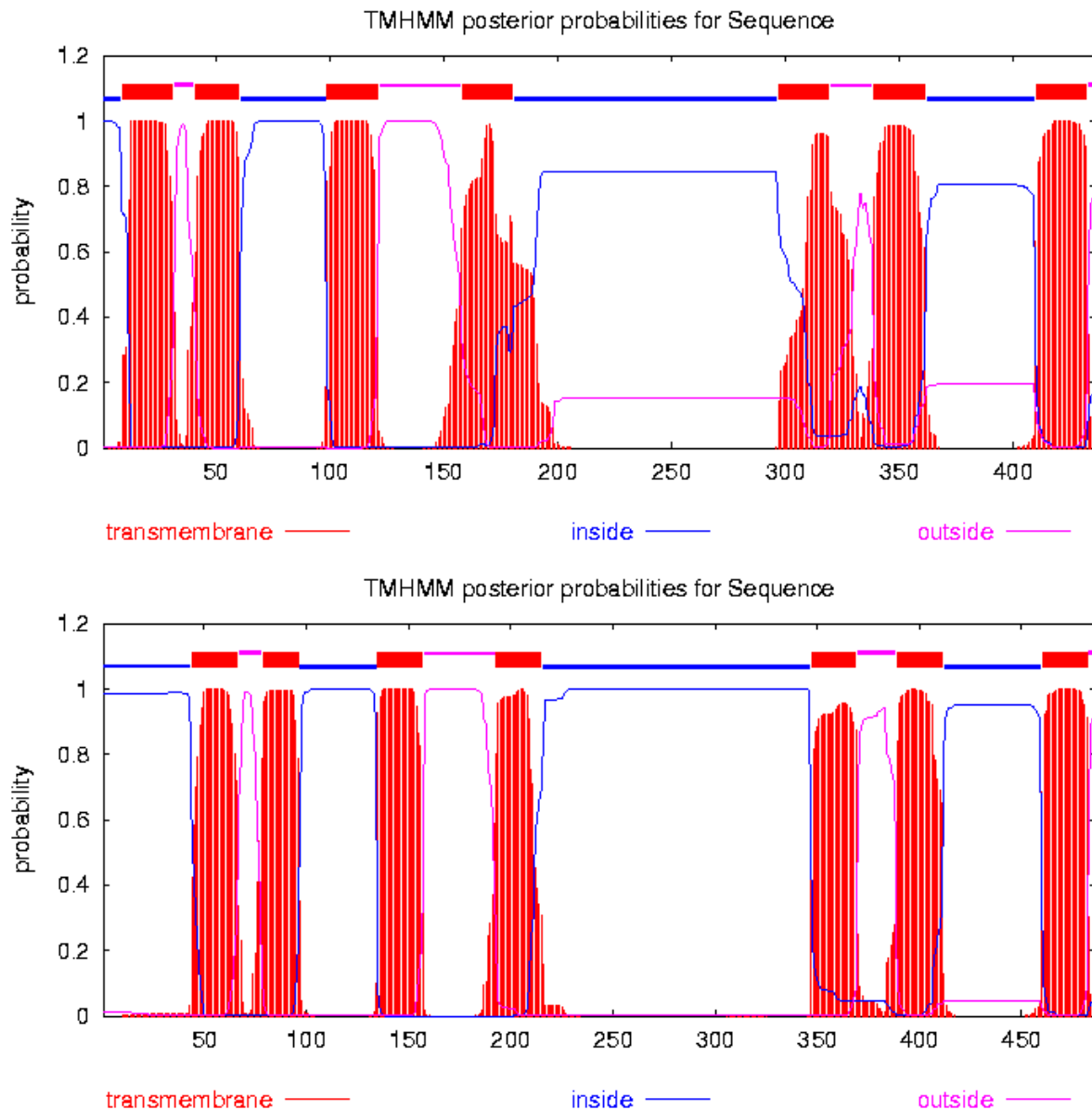


Fig. 4: Predicted transmembrane structure of Msex/ORCO (upper diagram) and Dmel/ORCO (lower picture) amino acid sequence. As is typical for ORCOs MsexOR-2 is predicted to have seven transmembrane domains.

TMHMM predicts seven transmembrane domains in the protein sequence of Msex/Orco with a length of 436 amino acid residues. The same structure was reported for Orcos of other insect species. The cloned Msex/Orco seems to contain the full length open reading frame.

3.2.2. Topographic Expression of Msex/Orco in Male Antennae

Orco expressing cells are typically located beneath the olfactory sensilla. To verify this for Msex/Orco fluorescence *in situ* hybridization (FISH) was performed. A RNA probe directed against the putative Msex/Orco was obtained by RNA polymerization reaction and the usage of labeled ribonucleotides. The Msex/Orco mRNA was hybridized with the RNA probe. The

RNA probe was linked with an antibody which in turn was linked with an enzyme. The enzyme catalyzed a reaction which led to the production of a fluorescent precipitate *in situ*. Using a laser scanning microscope it was possible to visualize labeled cells in sagittal sections of male antennae as shown in Fig. 5.

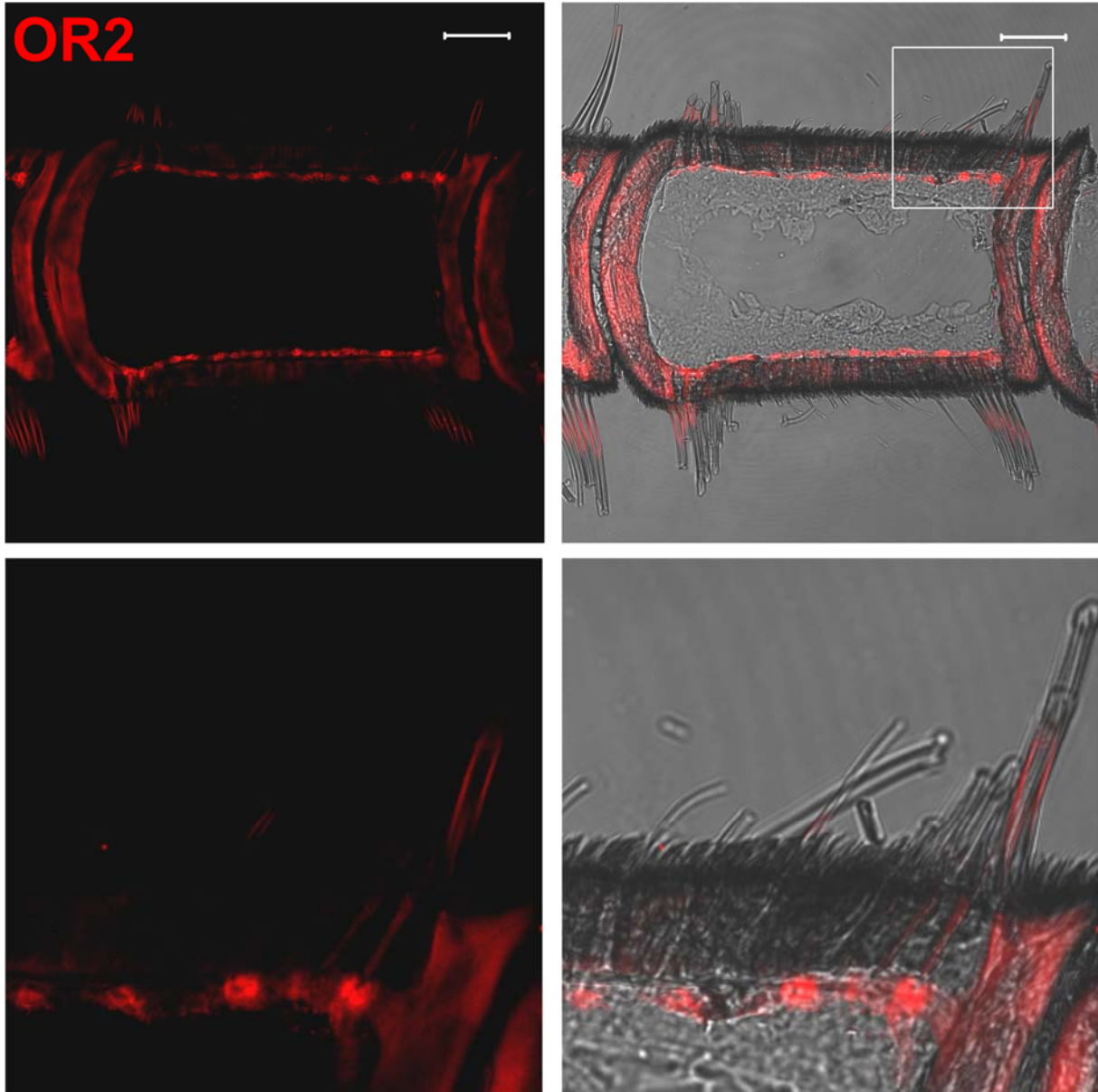


Fig. 5: Fluorescence *in situ* hybridization of Msex/Orco (OR-2) in male *Manduca sexta* antennae. Upper pictures show signals from a Digoxigenin-labeled Msex/Orco probe without (left) and with (right) refracting light in one complete antennal segment. Below is a blow up of the area marked in the upper right picture. The *in situ* hybridization indicates that Msex/Orco expressing cells are located beneath trichoid sensilla. The scale bar represents 50 μ m.

As expected from a Orco-homologue expressing cells are distributed over the whole length of the antenna beneath the trichoid sensilla. No signals were detected in the center of the antenna or in the intersegmental borders.

3.2.3. Quantitative Real Time PCR (qPCR) of Msex/Orco

Using RT-PCR in *Manduca sexta* as well as expression profiling using other methods in *Bombyx mori* as well as *Heliothis virescens* it was demonstrated that Msex/Orco and its orthologues are expressed equally in male and female individuals (Krieger et al. 2002, Krieger et al. 2005, Nakagawa et al. 2005). There is only limited information about the quantities of the expressed mRNAs.

To address this qPCR was performed for Msex/ORCO using Cybergreen. This fluorescent dye intercalates into double stranded DNA resulting in a shift in its emitting profile. By measurement of the fluorescent intensity after each cycle of PCR it is possible to detect the increase of products. Using this method the concentration in the sample can be concluded. Quantification requires PCR runs for the gene of interest (Msex/ORCO) and for a house keeping gene to relate the data. In this study RL31 is used, a gene encoding a ribosomal protein.

Two different methods were chosen for analysis. One directly relates the concentration of Msex/Orco and RL31 eliminating the need for a standard curve or measurement of absolute concentrations (Relative Quantitation). The other one uses standard curves to calculate exact concentrations which in turn are divided by the concentration of RL31 so that a normalized value is gained. Results are shown in Fig. 6.

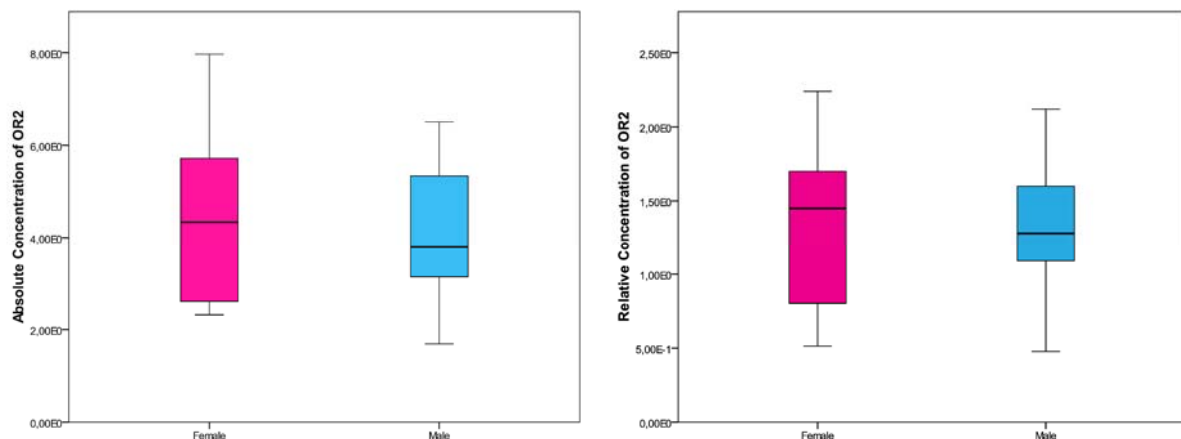


Fig. 6: Boxplot of Msex/Orco (OR-2) mRNA amounts in female and male antennae gained by absolute quantitation (left graph) and relative quantitation (right graph). Results of both graphs show similar amount of expression of Msex/Orco in female and male antennae. There is no significant difference between males and females as a p value of 0.655 resulting from unpaired t-test demonstrates. Values on the y axis mean the ratio of Msex/Orco and RL31.

Both methods indicate that there is no significant difference in expression of Msex/Orco in males and females. Unpaired t-test resulted in a p value of 0.655.

3.3. *Manduca sexta* Putative Pheromone Receptors

3.3.1. Sequence Analysis of MsexOR-1 and MsexOR-4

Based on sequence similarity analysis another group of conserved putative ORs in Lepidoptera can be identified, to which putative as well as functional characterized pheromone receptors belong. MsexOR-1 (Patch et al. 2009) and MsexOR-4 (Große-Wilde et al. 2010) have sequence similarity to the pheromone receptor group (Fig. 7).

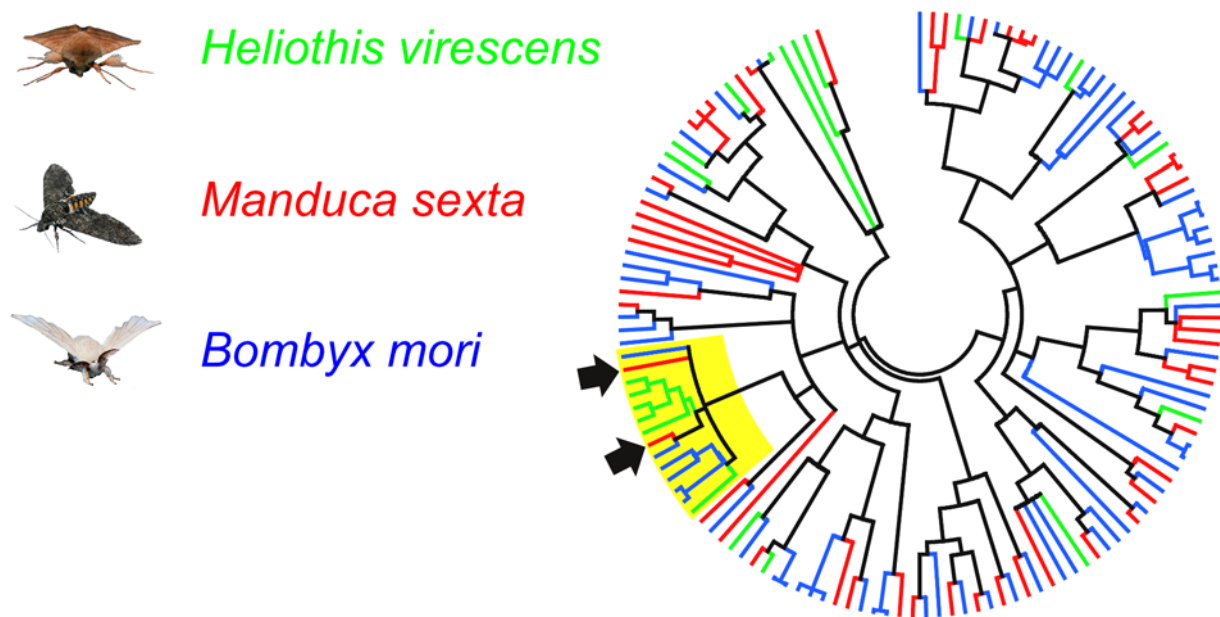


Fig. 7: Sequence similarity dendrogram of amino acids of all known putative ORs of *Manduca sexta*, *Bombyx mori* and *Heliothis virescens*. The upper arrow marks the position of MsexOR-4 next to BmorOR-1. The lower arrow emphasizes the position of MsexOR-1 which belongs in one group with BmorOR-3 and HR-6, HR-16, HR-15, HR-14, HR-11.

MsexOR-4 is an orthologue of BmorOR-1, the bombykol receptor, and MsexOR-1 is similar to BmorOR-3, the bombykal receptor.

We performed prediction for transmembrane domains for MsexOR-1 and MsexOR-4 (Fig. 8, p. 27).

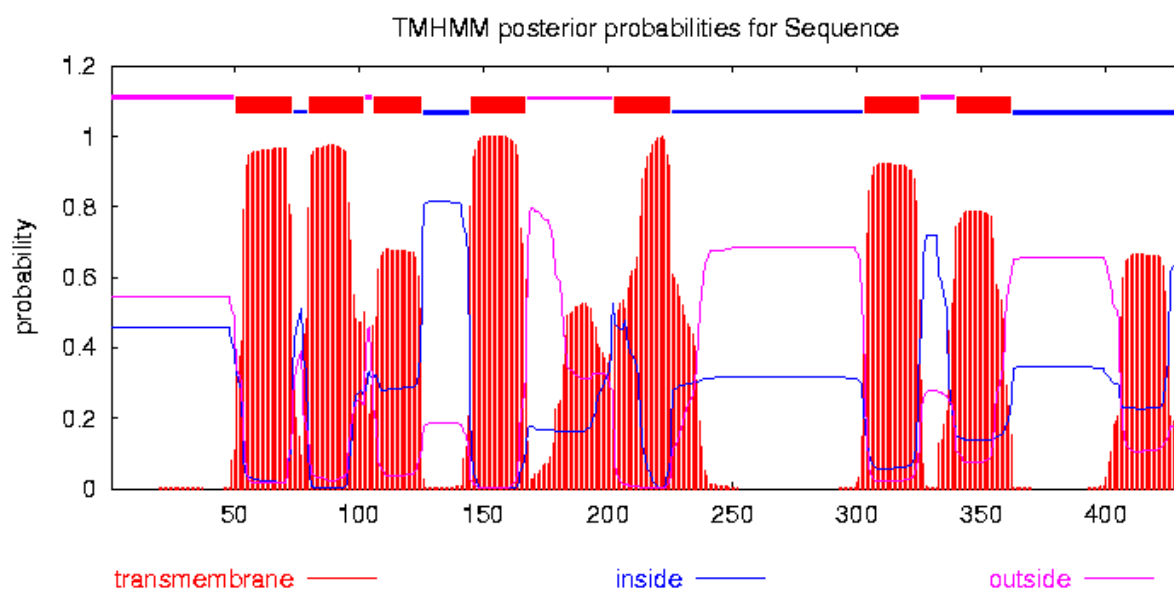
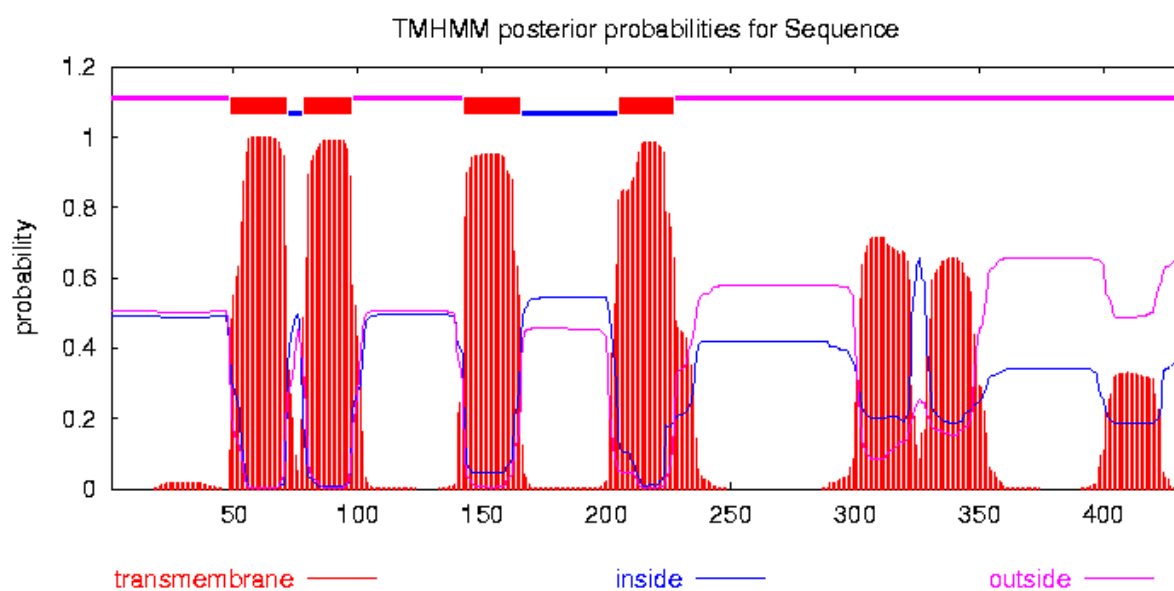
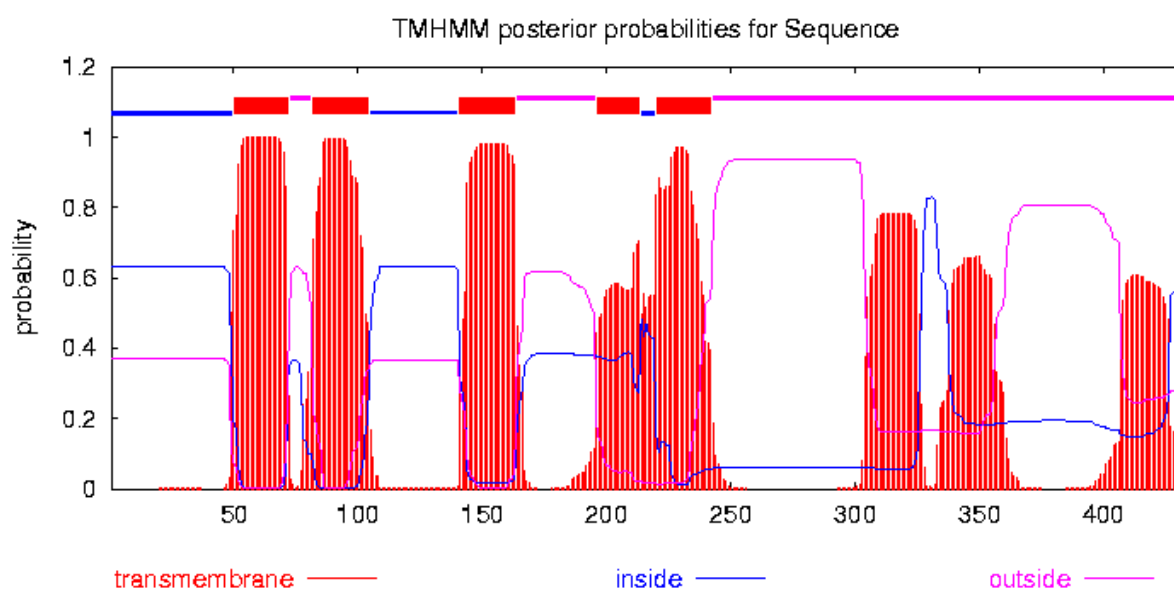


Fig. 8 (p. 27): Predicted transmembrane domains of MsexOR-1 (above) and MsexOR-4 (middle) and BmorOR-1 (lower picture). Profiles of all three receptors seem to be very similar due to conservation of putative receptors in the pheromone receptor group. Four well supported transmembrane domains were found but three other regions have less highly supported further membrane spanning domains.

The predicted transmembrane profile fits to the expected five to nine membrane spanning regions. Thus, the coding sequences seem to be cloned in full length (personal communication E. Große-Wilde).

3.3.2. Expression Analysis

Although the conservation of the pheromone subgroup is an indication for their function there are also some non-pheromone odorant receptors within the subgroup. However, non pheromone receptors differ from pheromone receptors in their expression profile. Pheromone receptors should only be expressed in male individuals not in female ones. For expression analysis it is necessary to detect plenty of molecules. RT-PCR allows detection of very small amounts of cDNA molecules. Due to gene specific primers and an adequate annealing temperature the method is also highly specific.

Primers were designed for RL31, MsexOR-1, and MsexOR-4 to show that the expression is limited to males. PCR products were visualized in agarose gel electrophoresis and ethidium bromide staining as seen in Fig. 9.

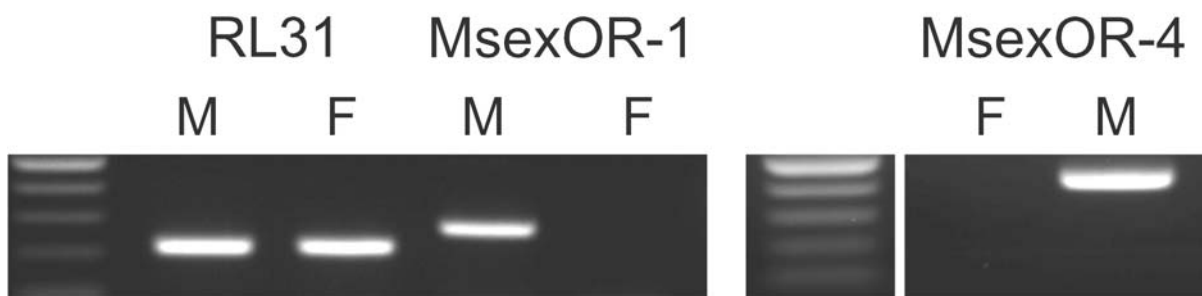


Fig. 9: Agarose gel foto of RT-PCR of female (F) and male (M) cDNA with RL31 (first two bands), MsexOR-1 (next two bands) and MsexOR-4 (the right gel) primers. RL31 cDNA is found in male and female *Manduca* whereas MsexOR-1 and MsexOR-4 cDNA is found only in male individuals. This strengthens the suggestions that MsexOR-1 and MsexOR-4 are pheromone receptors. Marker from the bottom to the top: 100bp, 200 bp, 300 bp, 400 bp, 500bp.

The primers for RL31 led to bands of the expected size in males and females. This is a positive control for successful cDNA synthesis and PCR setup. The primers of MsexOR-1 and MsexOR-4 led to the band of expected size only when using male cDNA not female cDNA. We can assume sex specific expression of both receptors which indeed indicates a function as male pheromone receptors.

3.3.3. qPCR

For detailed expression analysis of MsexOR-1 and MsexOR-4 qPCR was performed. The results of relative and absolute quantization are presented in Fig. 10.

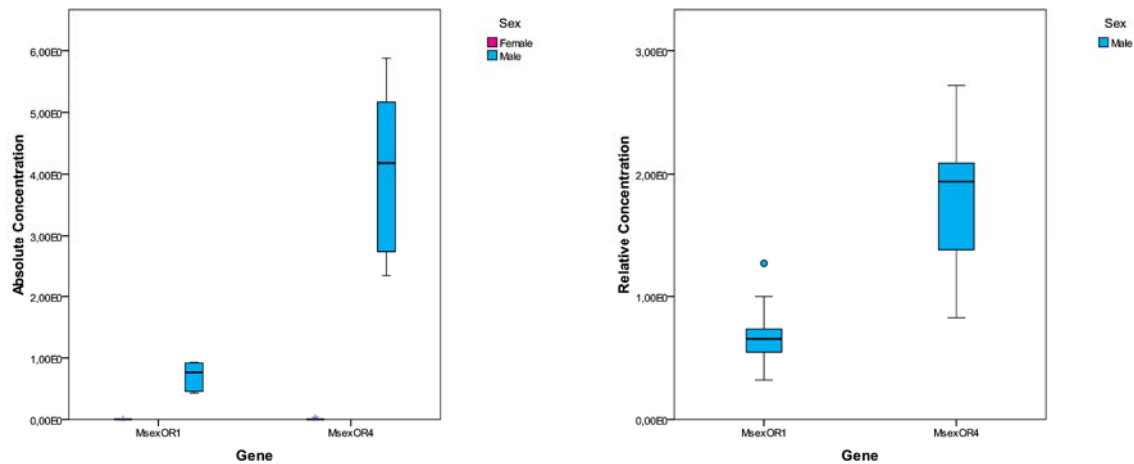


Fig. 10: Quantitation of MsexOR-1 and MsexOR-4 mRNA in female and male antennae through absolute quantitation (left graph) and relative quantitation (right graph, only males). Values on the y axis mean the ratio of MsexOR-1/RL31, MsexOR-4/RL31 respectively. Results of absolute quantitation clearly shows that MsexOR-1 and MsexOR-4 exclusively expressed in male moths and that the expression level of MsexOR-4 is about four times higher than that one of MsexOR-1. The different quantities of male expression can also be seen in relative quantitation. For females this method can not be used due to the problem of division by zero.

Absolute quantitation of MsexOR-1 and MsexOR-4 demonstrates that both putative receptors are expressed in male moths only. Furthermore the expression differs significantly between the both genes. MsexOR-4 is expressed four times higher than MsexOR-1. This fact is also supported in relative quantitation in male moths.

3.3.4. Topographic Expression of MsexOR-1 and MsexOR-4

FISH was performed using probes against MsexOR-1 and MsexOR-4. This allows visualization of cells in the antennae expressing the both receptors as presented in Fig. 11 and Fig. 12.

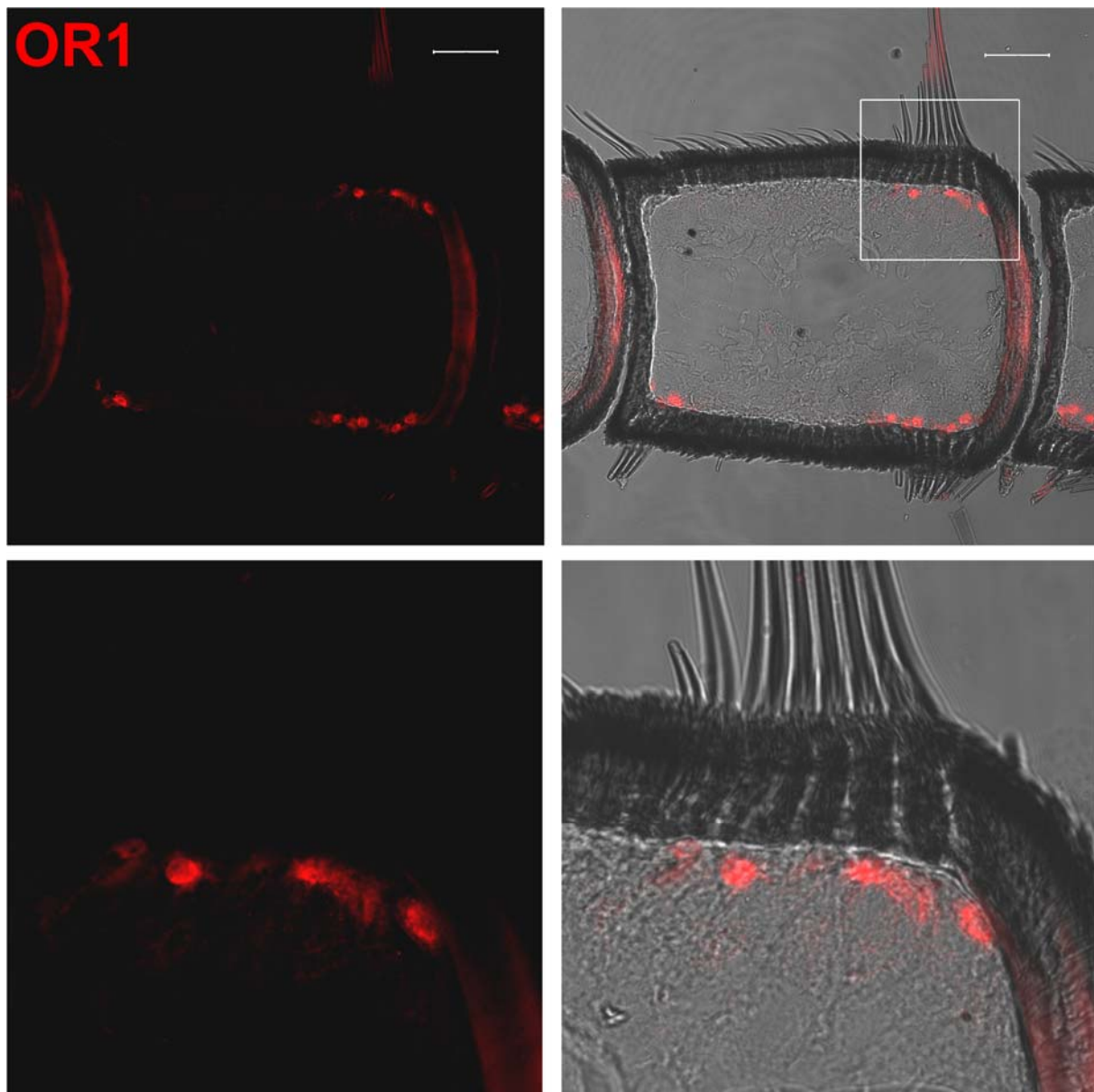


Fig. 11: Fluorescence *in situ* hybridization of MsexOR-1 in male *Manduca sexta* Antennae. Upper pictures show signals from digoxigenin labeled MsexOR-1 probe without refracted light (left) and with refracted light (right) in one complete antenna segment. The box in the upper right picture shows the area blown up in the lower pictures. The *in situ* hybridization indicates the position of MsexOR-1 expressing cells direct under the cuticle only under long trichoid sensilla which are known as pheromone detecting sensilla. The scale bar represents 50 μ m.

MsexOR-1 expressing cells are located directly under long trichoid sensilla in the male antenna. No labels were apparent under the short trichoid sensilla in contrast to MsexOR-2. A very similar pattern is exhibited by MsexOR-4 probe (Fig. 12).

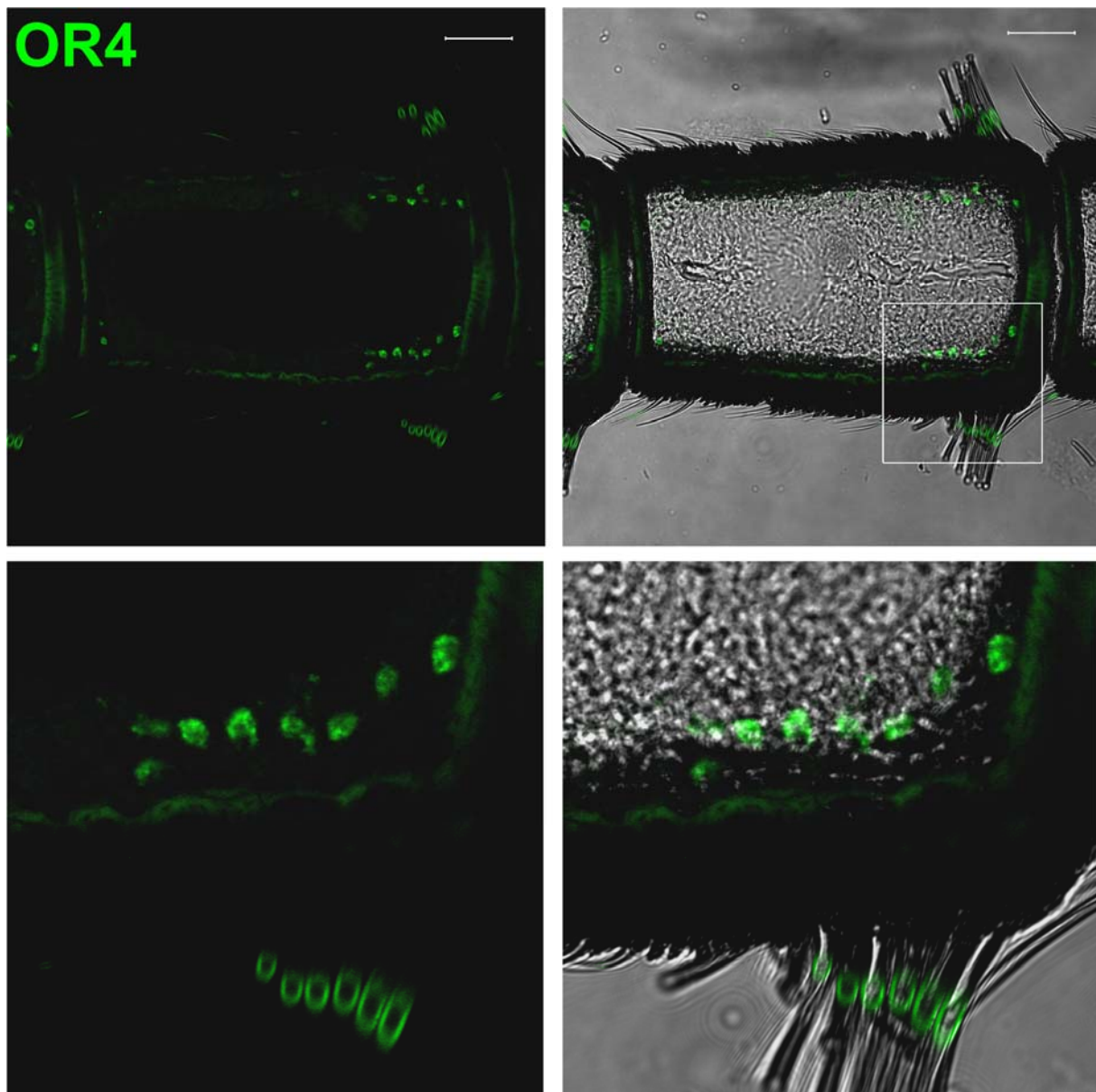


Fig. 12: Fluorescence *in situ* hybridization of MsexOR-4 in male *Manduca sexta* Antennae. Upper pictures show signals from Biotin-labeled MsexOR-4 probe without refracted light (left) and with refracted light (right) in one complete antenna segment. The box in the upper right picture is blown up in the lower pictures. The *in situ* hybridization indicates the position of MsexOR-4 expressing cells underneath long trichoid sensilla which are known as pheromone detecting sensilla. The scale bar represents 50 μ m.

MsexOR-1 and MsexOR-4 seems to be only expressed in cells located underneath long trichoid sensilla in male moths. No signals were detected under short trichoid sensilla. Using this method it was not possible to measure the exact amount of MsexOR-1 and MsexOR-4 expressing cells.

If MsexOR-1 and MsexOR-4 are indeed pheromone receptors they should be expressed in cells, localized closely to each other. To address this hypothesis double staining in-situ hybridizations was necessary to show the location of receptors relative to another. First the putative pheromone receptors MsexOR-1 and MsexOR-4 were investigated (Fig. 13).

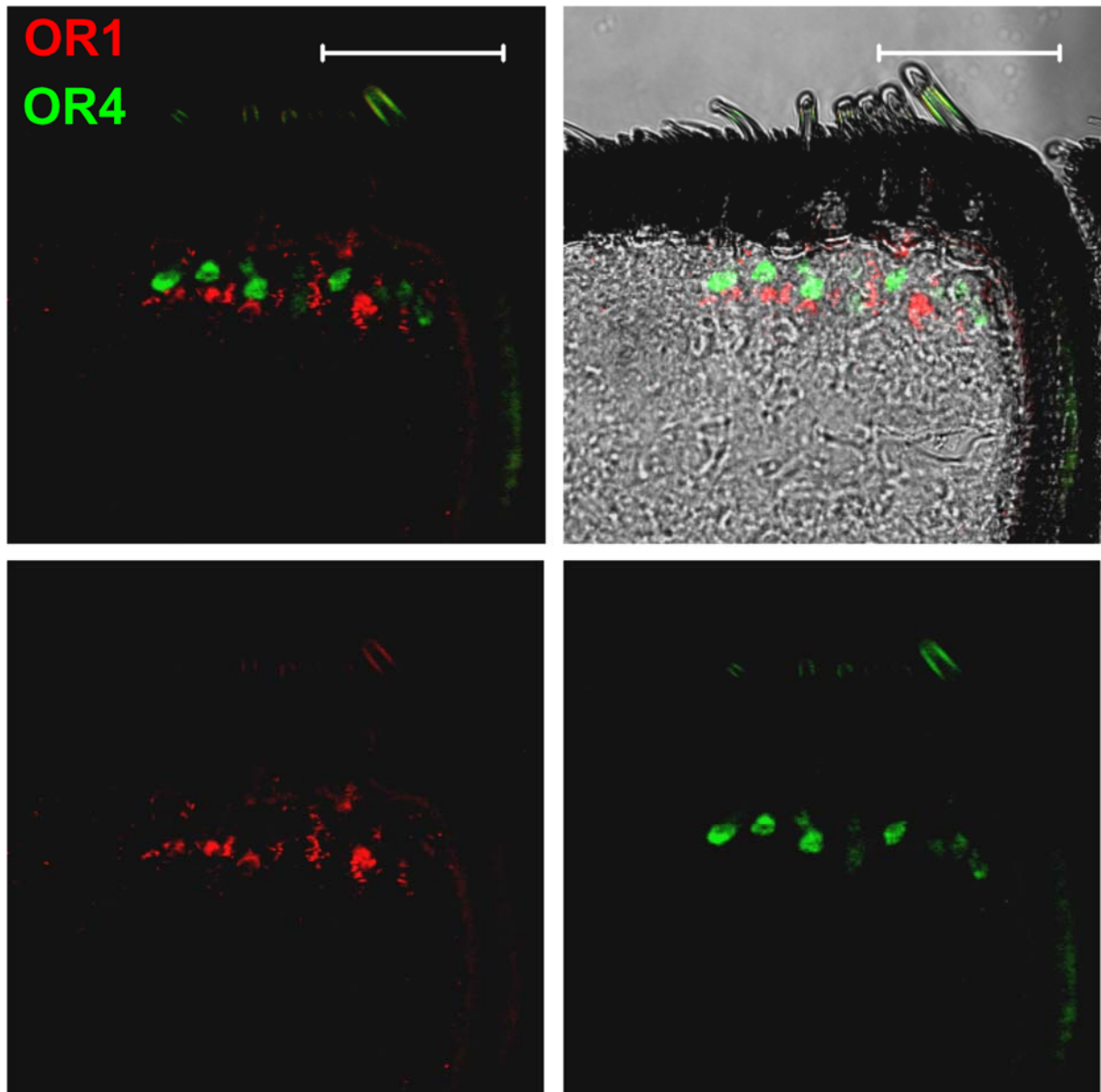


Fig. 13: Fluorescence *in situ* hybridization with probes against MsexOR-1 mRNA (digoxigenin labeled; red) and MsexOR-4 (biotin labeled; green) on sections of *Manduca sexta* male antenna. The upper left picture shows only the both fluorescence signals. Both lower pictures show each signal itself. The upper right picture additionally shows transmitted light, allowing seeing that cells are only located under long trichoid sensilla. Scale represents 50 μ m.

It is expected that MsexOR-1 and MsexOR-4 are not expressed in one cell. The *in situ* hybridization clearly shows that cells expressing MsexOR-1 and cells expressing MsexOR-4 are housed close to each other and are always and only located under long trichoid sensilla which are only present on male antennae.

3.3.5. Topographic Expression of MsexOR-1 and MsexOR-4 with Msex/Orco

Two more double *in situ* hybridizations were performed to show the distribution pattern of MsexOR-2 together with MsexOR-1 and MsexOR-4 respectively. It is not entirely clear if Msex/Orco is a coreceptor for putative pheromone receptors in *Manduca sexta*.

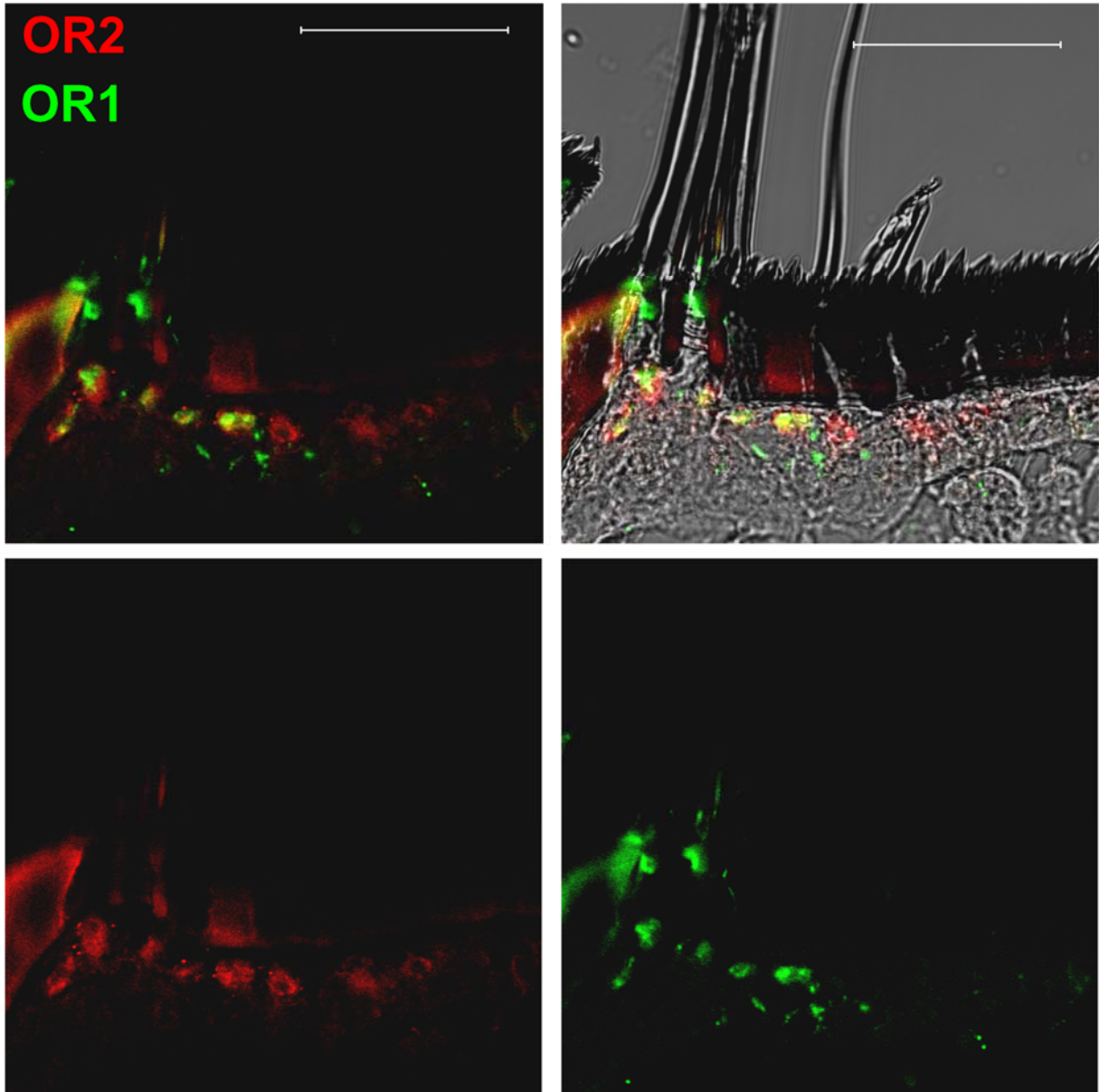


Fig. 14: Fluorescence *in situ* hybridization in sagittal sections of male *Manduca sexta* antenna. Msex/Orco expressing cells are digoxigenin labeled (red) and cells expressing the putative pheromone receptor MsexOR-1 are biotin labeled (green). In the upper right pictures only the fluorescence signals are visible whereas in the upper right additionally the refracted light channel is visible demonstrating that they are located beneath the long trichoid sensilla. The lower pictures are single channel scans. Cells expressing Msex/ORCO and MsexOR-1 seem to be the same. Scale bar represents 50 μ m.

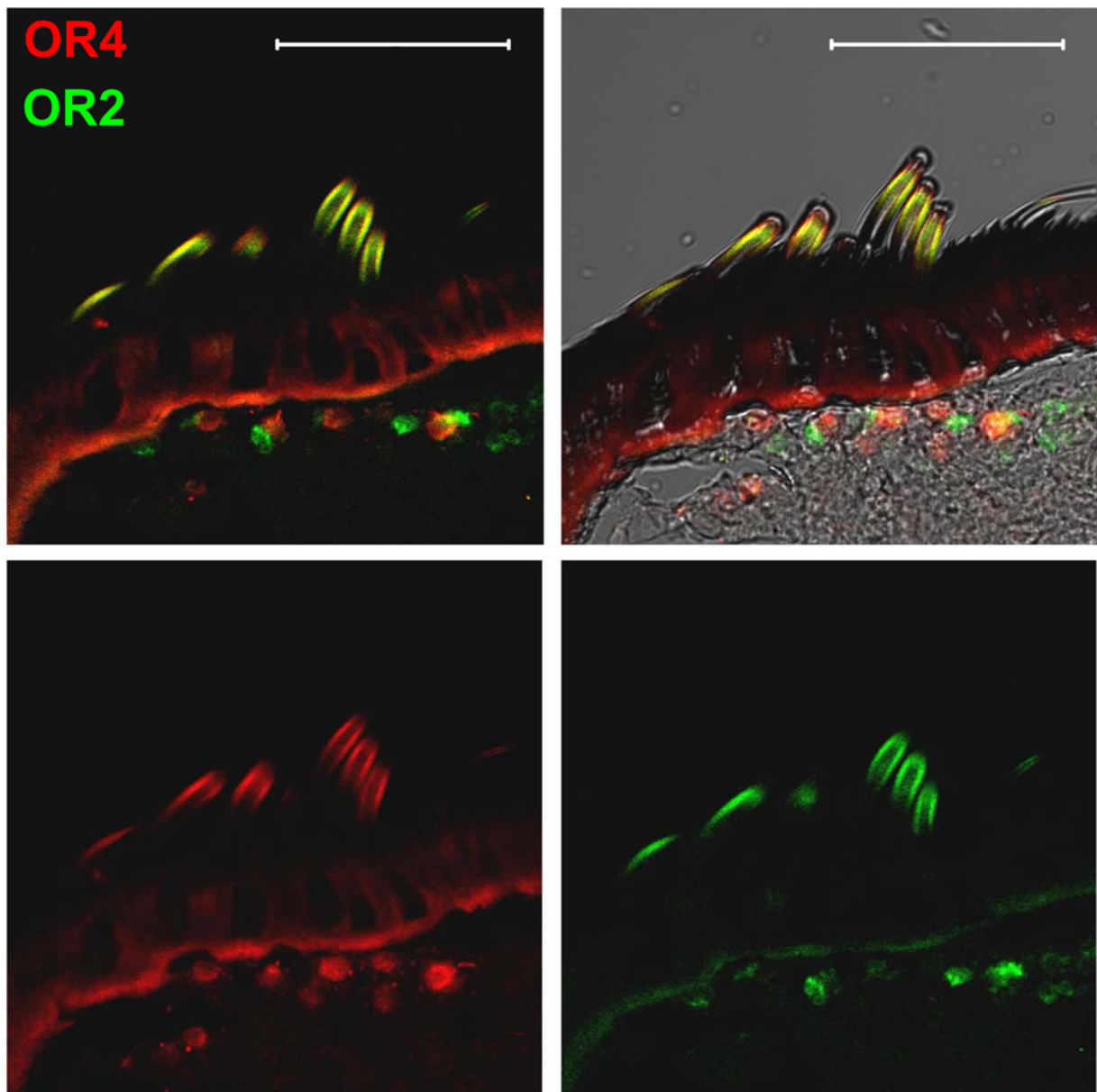


Fig. 15: Fluorescence *in situ* hybridization performed on sagittal sections of male *Manduca sexta* antenna. Upper pictures demonstrate double staining of putative pheromone receptor MsexOR-4 expressing cells (digoxigenin labeled, red) and Msex/ORCO expressing cells (biotin labeled, green) in which the right one is with refracted light and the left one without. The lower pictures present each channel for itself. Cells expressing the common coreceptor ORCO seem not to be identical with cells expressing MsexOR-4. Scale bar represents 50 μ m.

Double FISH analysis (Fig. 14, Fig. 15) indicates that MsexOR-1 and Msex/Orco (MsexOR-2) are coexpressed. MsexOR-4 however seems not to be coexpressed with Msex/Orco.

3.4. MsexOR-31

3.4.1. Homology

Finally we were interested in a highly conserved receptor subtype apparent in all three considered moth species. A complete open reading frame was gained by RACE-PCR.

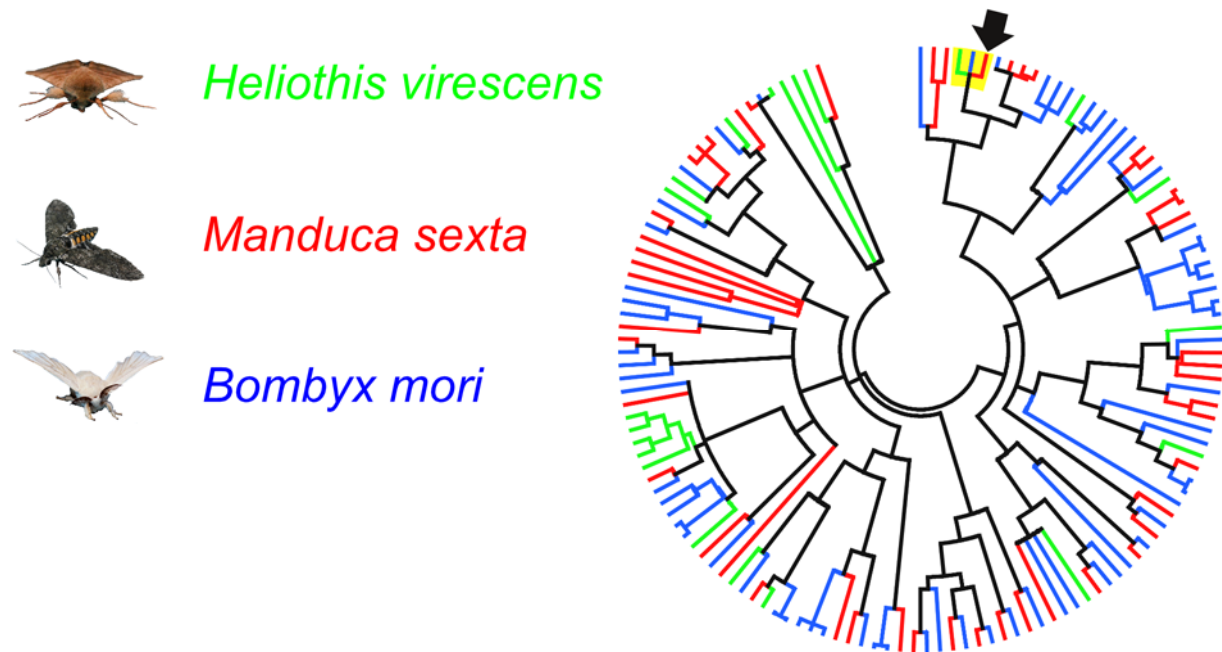


Fig. 16: Sequence similarity dendrogram of amino acids of all known putative ORs of *Manduca sexta*, *Bombyx mori* and *Heliothis virescens*. The arrow highlights another group of conserved putative odorant receptors, containing MsexOR-31, BmorOR-41 and HR10.

Interestingly MsexOR-31 is closely related to BmorOR-41 from *Bombyx mori* and HR10 from *Heliothis virescens*. Another indication whether MsexOR-31 is an OR is the prediction of the transmembrane structure of this gene shown in Fig. 17.

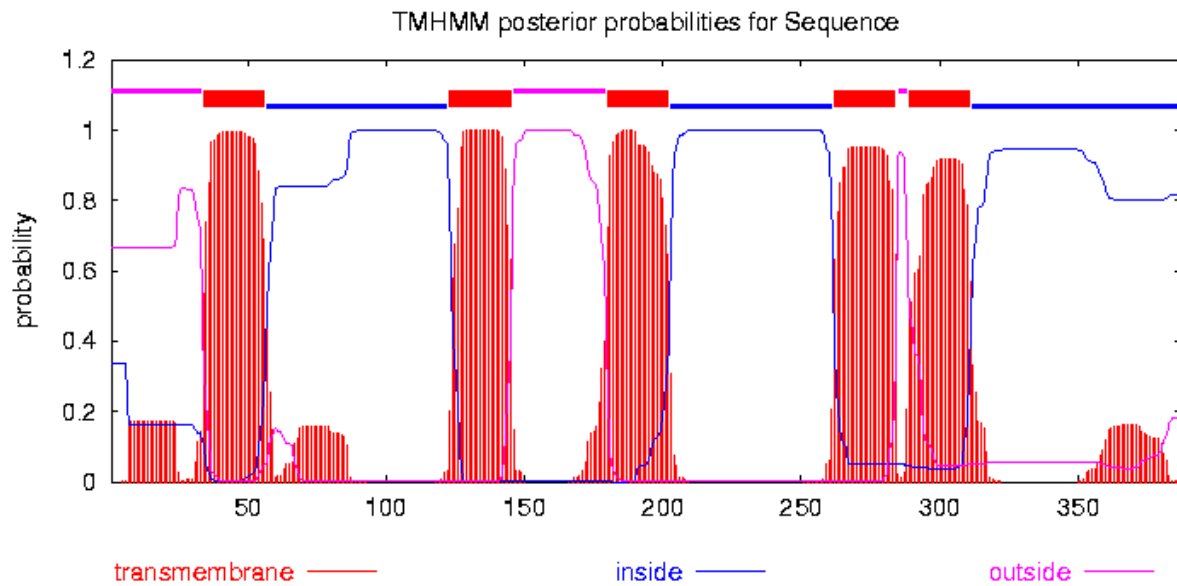


Fig. 17: Predicted transmembrane domains in MsexOR-31. Five domains have high probability for being membrane spanning helices. A usual profile of odorant receptors has seven transmembrane domains.

3.4.2. Topographic Expression

Again RNA *in situ* hybridization was performed as done for MsexOR-1, MsexOR-2 and MsexOR-4 to see where the cells are located that express MsexOR-31. The result is shown in Fig. 18.

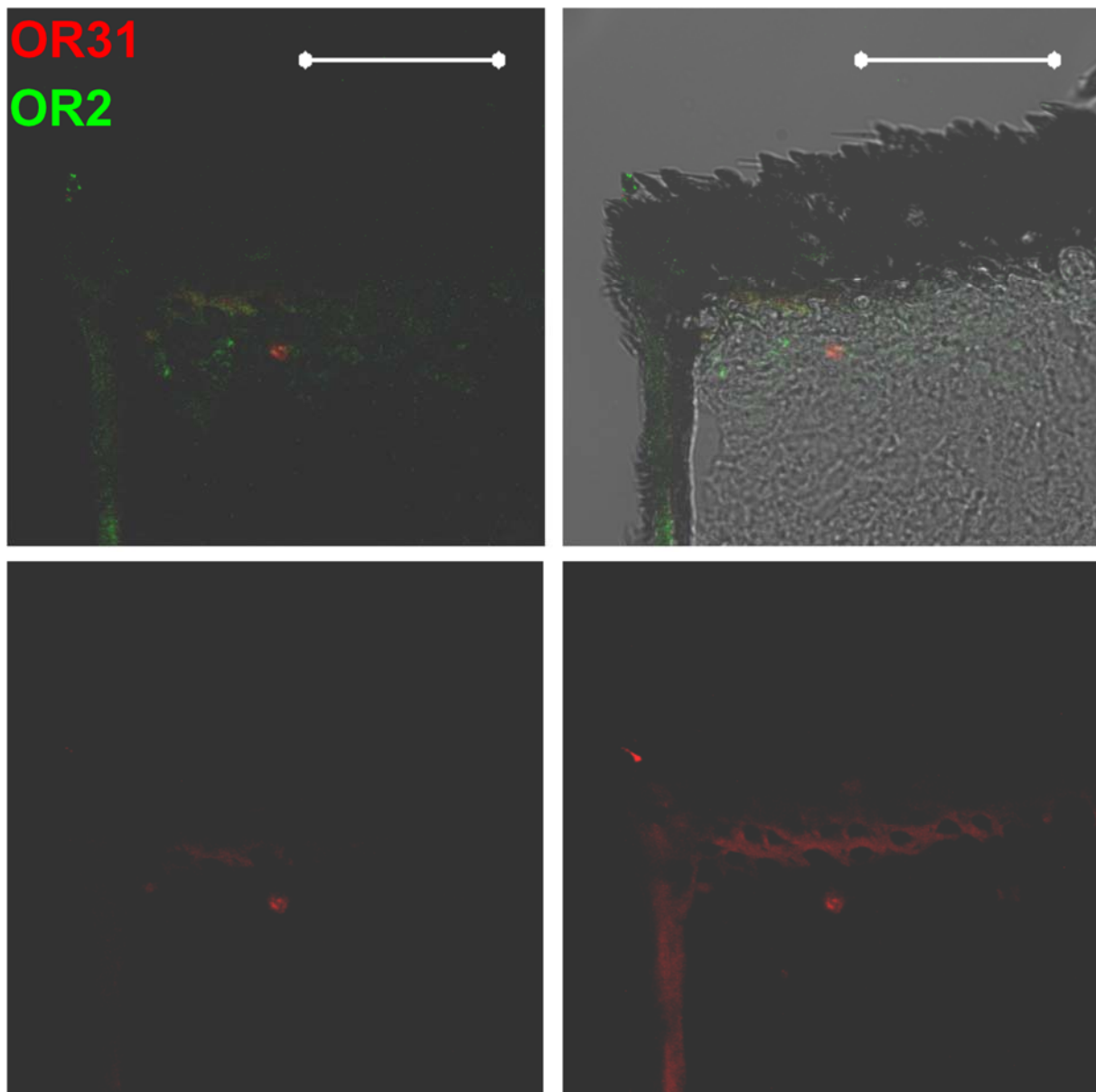


Fig. 18: *In situ* hybridization of MsexOR-31 (digoxigenin labeled; red) with Msex/Orco (biotin labeled; green) in male moth antennae near the tip. In this section is only one red signal visible. The upper left picture shows red and green signals without refracted light, the upper right is the same with refracted light. The lower left picture is the same section with only red signals of MsexOR-31 probe whereas in the right picture is a stack of some layers of the same section. Not many cells express this receptor and they are located primarily in the tip of the antennae. MsexOR-31 expressing cells are located like other odorant receptor cells. Scale bar represents 50µm.

Identifying cells expressing MsexOR-31 is very problematic due to a very low expression level. Only in the segments close to the tip of the antenna was it possible to detect labeling of such cells.

3.4.3. Expression over the Antennae

The result that MsexOR-31 expressing cells are rare and they seemly located only in the tip necessitated to cut the antennae in four pieces (approximately 20 segments) and quantify gene expression in every part using qPCR (Fig. 19).

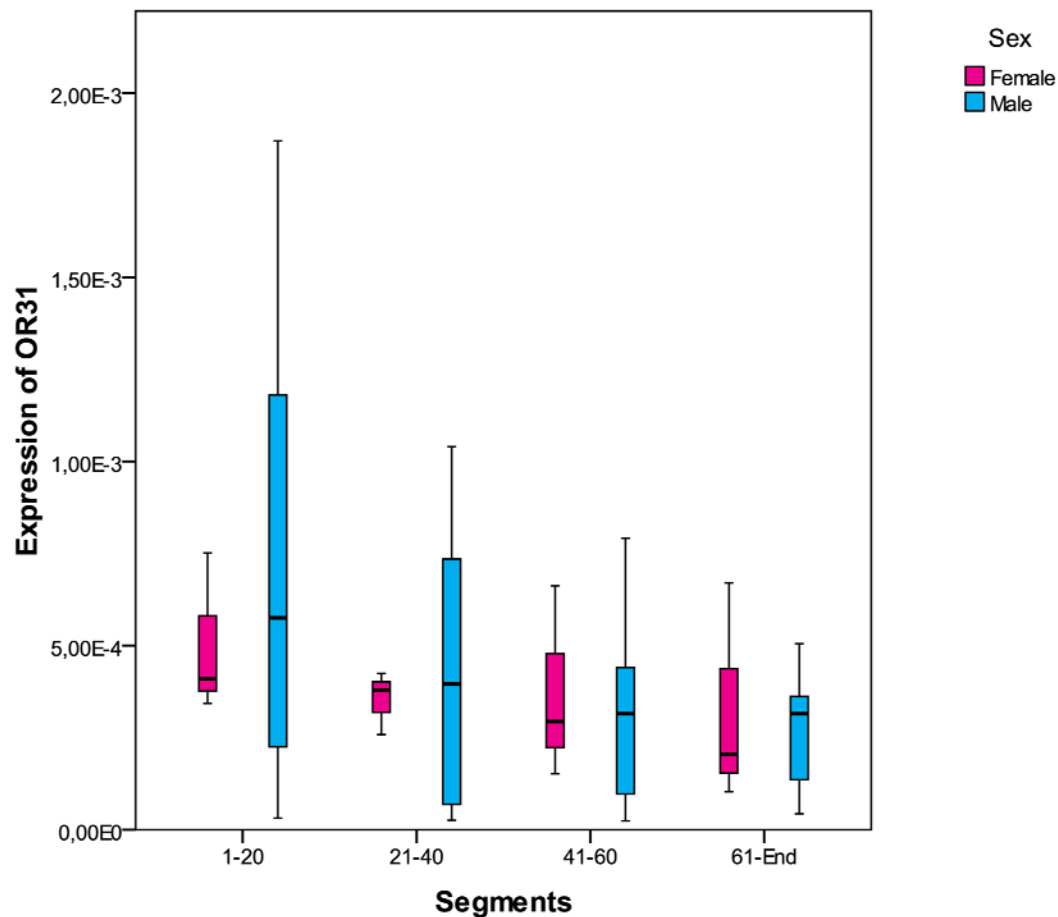


Fig. 19: Results of absolute quantitation of MsexOR-31 normalized to RL31 depending on antennal segments. Expression of MsexOR-31 is nearly 1000 fold lower than expression of MsexOR-2 and MsexOR-4. Also the real time PCR shows that the tip contains higher amount of MsexOR-31 than the rest of the antenna.

Indeed the qPCR indicates a gradient in expression of MsexOR-31 over the length of antenna. The distal segments exhibit a higher expression. Expression of MsexOR-31 decreases near to the head. Male and females seem to have different expression profiles. While females also show highest expression in the tip and lowest near the head the total amount of MsexOR-31 is minor compared with males. Compared with Msex/Orco or MsexOR-4 the expression of MsexOR-31 is 1000 fold lower.

4. Discussion

We were able to identify the odorant coreceptor (Orco), two putative pheromone receptors as well as one conserved Lepidopteran receptor in the Sphingid *Manduca sexta*, which is a model organism for insect olfaction. We extracted full length clones of the four genes and investigated their properties.

Orcos are characterised by high conservation among insect species (Krieger et al. 2003, Vosshall and Hansson et al. 2011) and form a distinct subgroup in sequence similarity comparisons of OR coding genes. Msex/Orco shares high sequence similarity with both BmorOR-2 and HR-2 which belong to this subgroup. Typically Orcos are broadly expressed beneath olfactory sensilla (Krieger et al. 2003, Vosshall & Hansson et al. 2011). Indeed Msex/Orco is expressed over the whole length of the antenna beneath all olfactory sensilla. Additionally Orcos are expressed in male and female individuals of insect species and we were able to demonstrate this also for Msex/Orco.

Our qPCR results indicate a similar expression profile in males and females. In contrast to Patch et al. 2009 we were not able to detect significant differences in expression. This is most likely due to the fact that Patch et al. 2009 used a different approach to deduce the quantity called $2^{-\Delta\Delta C_T}$ -method (described by Livak et al 2001). This method uses the amplification efficiency of the target gene normalized to an endogenous reference gene and calibrated to an internal control gene (house keeping gene). Amplification efficiency of reference and target gene has to be approximately equal. Patch et al. choose MsexOR-2 expression in the foreleg as reference although it was demonstrated that MsexOR-2 is not expressed in the foreleg in the same study. Thus, the assumptions of this method were not fulfilled. To avoid this pitfall we used a method called absolute quantification. Therefore standard curves of the target gene and a calibrator gene (house keeping gene) were established using solutions with known copy number of the PCR products in the same qPCR like the unknown samples. Concentration of samples is deduced from the standard curves. We used the ribosomal subunit RL31 as calibrator. Our result indicates a similar expression of Msex/Orco in males and females. This correlates with our own *in situ* hybridization as well as similar experiments in other moths like *Heliothis virescens* (Krieger et al. 2002) and *Bombyx mori* (Krieger et al. 2005).

Additionally we identified two putative pheromone receptors, namely MsexOR-1 and MsexOR-4, and created full length clones facilitating investigation of their properties. They fulfill common characteristics of odorant receptors: sequence similarity to known insect odorant receptors, seven transmembran domains, antennal expression, and expression in cells located beneath olfactory sensilla. MsexOR-1 and MsexOR-4 belong to the pheromone

receptor group which contains conserved genes encoding subtypes of moth pheromone receptors. MsexOR-1 and MsexOR-4 are exclusively expressed in male individuals. Expressing cells are located beneath long trichoid sensilla which are responsible for pheromone detection in male *Manduca sexta* (Kaissling et al. 1989). Both results indicate a possible role of MsexOR-1 and MsexOR-4 in pheromone detection, prompting additional analysis.

The pheromone blend of *Manduca sexta* consists of several components in which E10,Z12-16:Al (bombykal) is the main component. Both bombykal and E10,E12,Z14-16:AL (EEZ) are sufficient to elicit typical mating behaviour (taxis, approach, hover and touching of paper containing the substances) although higher concentration of the synthetic blend than of gland extract is needed (Tumlinson et al. 1989). Kaissling et al. 1989 report three types of pheromone receptor cells: the abundant type A, type B, and the rare type C detecting bombykal, EEZ, and E10,E12,E14-16:AL (EEE) respectively. There are two receptor cells in each sensillum. In all tested pheromone responding sensilla was one type A receptor cell. Thus, bombykal, the major component is detected by the most pheromone receptor cells. The second receptor type in each sensillum was either type B cell (17 of 20 responded to EEZ) or type C cell (3 of 29 responded to EEE) and detected one of the minor components. qPCR results demonstrate that MsexOR-4 mRNA is about four times more frequent than MsexOR-1 mRNA. This hypothesis is supported by our *in situ* hybridization results. In most cases there is a MsexOR-1 expressing cell (type B) localized beneath the MsexOR-4 expressing cell. This correlates with Kaissling et al. 1989 that most sensilla have a type A and a type B receptor cell. Assuming that mRNA abundance correlates with protein abundance we hypothesize that MsexOR-4 is responsible for detection of bombykal. MsexOR-1 could be necessary for detection of the minor pheromone component EEZ. MsexOR-4 expressing cells are the most abundant pheromone receptor cells (type A). The receptor responsible for EEE detection remains unidentified due to the low number of transcripts in the EST database.

However, we see a very interesting fact that has to be considered. The bombykal receptor BmorOR-3 (Nakagawa et al. 2005, Große-Wilde et al. 2006) shares high sequence similarity with MsexOR-1, the putative EEZ receptor, whereas the putative bombykal receptor MsexOR-4 is a homologue of the *Bombyx mori* bombykol receptor (Nakagawa et al. 2005, Große-Wilde et al. 2006) BmorOR-1. If our hypothesis regarding the function is true, the conservation of MsexOR-4 and BmorOR-1 is not due to their ligand but due to their function as receptor of the main pheromone component. This is an example for evolution of odorant receptors based on their function, not on the molecule they detect. Maybe *Manduca sexta* and *Bombyx mori* have different pheromone blends but the same signal cascade.

Additionally the location of cells expressing Msex/Orco and the pheromone receptors was investigated, motivated by the question if Msex/Orco plays a roll in pheromone detection. Results of Krieger et al. 2005 indicate that both BmorOR-1 and BmorOr-3 are not colocalized with Orco. In contrast, Nakagawa et al. 2005 demonstrate that both BmorOR-1 and BmorOR-3 are colocalized with Orco. Furthermore, they report a stronger response of *Bombyx mori* pheromone receptors expressed in *Xenopus* oocytes with Bmor/Orco than without the coreceptor. It seems that Orcos enforce pheromone response although they are perhaps not essential. Our results indicate that MsexOR-1 and Msex/Orco are coexpressed. This supports the results of Nakagawa et al. for *Bombyx mori*. We did not note a coexpression of MsexOR-4 and MsexOR-2 in contrast to report of Große-Wilde et al. 2010. We have to be aware that *in situ* hybridization is a limited method and does not enable us to definitively clarify the role of Orcos in pheromone detection. Heterologue expression is also limited, if we cannot be sure, that the genes are also expressed in one cell *in vivo*. Demonstrating that Orcos enhance pheromone response is not sufficient if we cannot be sure of that they are coexpressed with pheromone receptors. Additionally the role of pheromone binding proteins (PBP) should not be ignored. Recent studies demonstrate that they are more than simply carrier of pheromones, they are important for tuning of the pheromone receptors to their ligands (Große-Wilde et al. 2006). One approach to conclude the function of Msex/Orco in pheromone detection is to knock down the expression of Msex/Orco using RNAi. The experiments are under way and if we would succeed we could show whether *Manduca* detects pheromones without Msex/Orco.

The putative OR we identified, MsexOR-31, seems to be specific for Lepidoptera. Its notable conservation in the three moth species indicates a very special function in olfaction. We found no homologue in non Lepidoptera species. Even more cryptic is the expression profile of MsexOR-31. qPCR results indicate heterogeneous expression in the antenna. Till now there are no reports about an expression gradient of odorant receptors in any species. Furthermore MsexOR-31 expression level seems to be 1000 fold lower than the level of Msex/Orco. A function as pheromone receptor is unlikely because of the expression in both sexes. Other crucial functions of odor reception are the detection of food and predators as well as the recognition of other dangers.

Our work contributes to the understanding of olfaction in insects and opened new directions in investigation of odorant and pheromone receptors.

5. References

- Angerer LM, Angerer RC. 1992. *In situ* hybridization to cellular RNA with radiolabeled RNA probes. In: DG W, editor. *In situ* hybridization. Oxford: IRL Press. p 17.
- Butenandt A, Beckmann R, Stamm D, Hecker E. 1959. Über den Sexuallockstoff des Seidenspinners *Bombyx mori* - Reindarstellung und Konstitution. Zeitschrift für Naturforschung B 14(4):283-284.
- Clyne PJ, Warr CG, Freeman MR, Lessing D, Kim JH, Carlson JR. 1999. A novel family of divergent seven-transmembrane proteins: Candidate odorant receptors in *Drosophila*. Neuron 22(2):327-338.
- Gao Q, Chess A. 1999. Identification of candidate *Drosophila* olfactory receptors from genomic DNA sequence. Genomics 60(1):31-39.
- Große-Wilde E, Hansson BS. 2011. in press.
- Große-Wilde E, Stieber R, Forstner M, Krieger J, Wicher D, Hansson BS. 2010. Sex-specific odorant receptors of the tobacco hornworm *Manduca sexta*. Front Cell Neurosci 4.
- Große-Wilde E, Svatos A, Krieger J. 2006. A pheromone-binding protein mediates the bombykol-induced activation of a pheromone receptor in vitro. Chemical Senses 31:547-555.
- Hallem EA, Ho MG, Carlson JR. 2004. The molecular basis of odor coding in the *Drosophila* antenna. Cell 117(7):965-979.
- Hildebrand JG, Eisner T, Meinwald J. 1995. Analysis of chemical signals by nervous systems. Chemical ecology: the chemistry of biotic interaction:161-181.
- Kaissling KE, Hildebrand JG, Tumlinson JH. 1989. Pheromone Receptor Cells in the Male Moth *Manduca sexta*. Archives of Insect Biochemistry and Physiology 10(4):273-279.
- Kalinova B, Hoskovec M, Liblikas I, Unelius CR, Hansson BS. 2001. Detection of sex pheromone components in *Manduca sexta* (L.). Chemical Senses 26(9):1175-1186.
- Keil TA. 1989. Fine structure of the pheromone-sensitive sensilla on the antenna of the hawkmoth, *Manduca sexta*. Tissue & Cell 21(1):139-151.
- Krieger J, Große-Wilde E, Gohl T, Breer H. 2005. Candidate pheromone receptors of the silkworm *Bombyx mori*. European Journal of Neuroscience 21(8):2167-2176.
- Krieger J, Klink O, Mohl C, Raming K, Breer H. 2003. A candidate olfactory receptor subtype highly conserved across different insect orders. Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology 189(7):519-526.
- Krieger J, Raming K, Dewar YME, Bette S, Conzelmann S, Breer H. 2002. A divergent gene family encoding candidate olfactory receptors of the moth *Heliothis virescens*. European Journal of Neuroscience 16(4):619-628.
- Larsson MC, Domingos AI, Jones WD, Chiappe ME, Amrein H, Vosshall LB. 2004. Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. Neuron 43(5):703-714.

- Lee JK, Strausfeld NJ. 1990. Structure, distribution and number of surface sensilla and their receptor cells on the olfactory appendage of the male moth *Manduca sexta*. *Journal of Neurocytology* 19(4):519-538.
- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* 25(4):402-408.
- Miura N, Nakagawa T, Touhara K, Ishikawa Y. 2010. Broadly and narrowly tuned odorant receptors are involved in female sex pheromone reception in *Ostrinia* moths. *Insect Biochemistry and Molecular Biology* 40(1):64-73.
- Nakagawa T, Sakurai T, Nishioka T, Touhara K. 2005. Insect sex-pheromone signals mediated by specific combinations of olfactory receptors. *Science* 307(5715):1638-1642.
- Neuhaus EM, Gisselmann G, Zhang WY, Dooley R, Stortkuhl K, Hatt H. 2005. Odorant receptor heterodimerization in the olfactory system of *Drosophila melanogaster*. *Nature Neuroscience* 8(1):15-17.
- Patch HM, Velarde RA, Walden KKO, Robertson HM. 2009. A Candidate Pheromone Receptor and Two Odorant Receptors of the Hawkmoth *Manduca sexta*. *Chemical Senses* 34(4):305-316.
- Rutzler M, Zwiebel LJ. 2005. Molecular biology of insect olfaction: recent progress and conceptual models. *Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology* 191(9):777-790.
- Sakurai T, Nakagawa T, Mitsuno H, Mori H, Endo Y, Tanoue S, Yasukochi Y, Touhara K, Nishioka T. 2004. Identification and functional characterization of a sex pheromone receptor in the silkworm *Bombyx mori*. *Proceedings of the National Academy of Sciences of the United States of America* 101(47):16653-16658.
- Sanchez-Gracia A, Vieira FG, Rozas J. 2009. Molecular evolution of the major chemosensory gene families in insects. *Heredity* 103(3):208-216.
- Sanes JR, Hildebrand JG. 1976. Structure and Development of antennae in a moth, *Manduca sexta*. *Developmental Biology* 51(2):282-299.
- Shields VDC, Hildebrand JG. 1999a. Fine structure of antennal sensilla of the female sphinx moth, *Manduca sexta* (Lepidoptera : Sphingidae). I. Trichoid and basiconic sensilla. *Canadian Journal of Zoology* 77(2):290-301.
- Shields VDC, Hildebrand JG. 1999b. Fine structure of antennal sensilla of the female sphinx moth, *Manduca sexta* (Lepidoptera : Sphingidae). II. Auriculate, coeloconic, and styliform complex sensilla. *Canadian Journal of Zoology-Revue Canadienne De Zoologie* 77(2):302-313.
- Tanaka K, Uda Y, Ono Y, Nakagawa T, Suwa M, Yamaoka R, Touhara K. 2009. Highly Selective Tuning of a Silkworm Olfactory Receptor to a Key Mulberry Leaf Volatile. *Current Biology* 19(11):881-890.
- Tumlinson JH, Brennan MM, Doolittle RE, Mitchell ER, Brabham A, Mazomenos BE, Baumhover AH, Jackson DM. 1989. Identification of a Pheromone Blend Attractive to

Manduca sexta (L.) Males in a Wind Tunnel. Archives of Insect Biochemistry and Physiology 10(4):255-271.

Vosshall LB, Amrein H, Morozov PS, Rzhetsky A, Axel R. 1999. A spatial map of olfactory receptor expression in the *Drosophila* antenna. Cell 96(5):725-736.

Vosshall LB, Hansson BS. 2011. A Unified Nomenclature System for the Insect Olfactory Coreceptor. Chem. Senses in press.

Vosshall LB, Wong AM, Axel R. 2000. An olfactory sensory map in the fly brain. Cell 102(2):147-159.

Zhang DD, Zhu KY, Wang CZ. 2010. Sequencing and characterization of six cDNAs putatively encoding three pairs of pheromone receptors in two sibling species, *Helicoverpa armigera* and *Helicoverpa assulta*. Journal of Insect Physiology 56(6):586-593.

6. Appendix

DNA Sequence of Msex/Orco

ATGACCATGCTTCTGCGGAAGATGTATTCCACGGTGCACGCTATTCTGATCTTTGTTTCAG
TTTGTATGCATGGGCGTCAACATGGCGATGTACGCTGATGAAGTAAACGAGCTGACTGC
AAATACAATCACAGTCCTATTCTTCGCCCACAGTATCATTAAATTGGGATTCTTGGCCTTT
ACCTCCAAAAGTTTTTACAGGACTATGGCTGTGTGGAATCAATCTAACAGCCACCCATTG
TTCACGGAGTCTGACGCTCGCTACCACCAAATTGCTCTTACCAAATGAGAAGGCTTAC
TTACTTCATCTGCTTCATGACTGTTATGTCTGTTGTCAGTTGGGTCACGATTACCTTCTTC
GGCGAGTCAGTGCATGATCGCTAACAAGGAGACGAACGAGACCCTGACAGAACCCG
CGCCACGGCTGCCACTCAAGGCCTGGTATCCATTTGATACCATGAGTGGTTCTATGTAT
GTTTTCGTCTTCGTTTTTCAGATCTACTGGCTCCTCTTCTCCATGTCAATGGCTAATCTAC
TTGATGTTCTATTCTGCGCGTGGTTGATCTTCGCATGTGAGCAACTGCAGCATTGGAAG
GCGATCATGAAACCGCTGATGGAGCTTAGTGCTTCACTAGACACGTACAGACCGAATAC
TGCAGAGCTGTTTCAGAGTTTCTTCGACTGATAAGTCAGAGAAAGTGCCAGATCCAGTAG
ACATGGATATTCGTGGCATATACTCCACGCAGCAAGACTTCGGCATGACGCTGCGAGG
AACTGGTGGAAAACCTGCAGAAATTCGTCCAAAATACGGTCAATCCTAACGGATTGACTC
AGAAGCAAGAGATGCTGGCCAGATCTGCTATCAAGTACTGGGTGAAACGCCATAAGCAC
GTGGTCAGATTGGTGGCGTCCATTGGAGATACGTATGGTACGGCCCTACTGTTCCACAT
GTTAGTTTCTACCATTACTCTGACGCTCCTGGCTTACCAGGCTACCAAGATCAATAGCAT
CAATGTGTACGCGTTCAGCACAATCGGATACTTATGCTACACCCTCGGACAAGTGTTCC
ATTTCTGCATCTTCGGAAACCGGCTGATTGAAGAGAGTTTCTCCGTGATGGAAGCGGCT
TACTCCTGCCAGTGGTACGATGGTTCTGAGGAAGCAAAGACCTTCGTCCAGATCGTTTG
CCAGCAGTGCCAGAAGGCATTGACTATTTCTGGTGCAAAGTTCTTCACGGTGTCCCTGG
ATTTGTTGCTCCTCGGTGCTCGGCGCTGTAGTGACCTACTTCATGGTGTGGTGCAGCTG
AAATAG

DNA Sequence of MsexOR-1

ATGATATTTATGGACGATCCTCTATCAAAGTCTATAAAAGATCCCAGGGACTACCGATAC
ATGAAGTTATTTAGATCTACTTTGAGACTTATTGGATCGTGGCCTGGAAGAGATCTTAAG
GAGGAGGGTGCAACGAAATATGAAATCGCGCCTTTATACTGGGTGCTGGTTATTAAT
CACATGTTTCCTTCTCACAATAATCTATCTCATAGAAAATACAAATAAGCTGGGTTTCTTT
GAAATTGGTCATGTCTACATTACTGTATTTATGACTATGATAACATTGAGCCGTAGCATC
ACTTTGTCCTTAAACCCGAAGTACCGCCGAGTAATGACGAAATATATTACTAAAATGCAT

CTCTTTTACTACAAGGATATGTCTGATATTGCTTTGAAGACTCACATAAGAGTGCATAAA
CTCTCCCATTTTTTTCACAATGTATTTGTCAACTCAAGTAGTATTGGGCACAGTTACGTTCA
ACATTGTCCCAATGTACAACAACACTACAAGGTAGGAAGATTGAAAATAACATATTAGTTA
ACGACTCTTACGAGTTGTCTATATATTTCAAACCCCAACAAAATTCTTATCTACCTTGAA
TGGATACATCGCAATCACCACATTTAATTGGTACTCAAGCTATATTTGCTCTAACTTTTTTC
TGCATGTTTGATCTCGCTCTGTCCCTGTTAATATTTACTGTGTCCGGACATTTCAAATAT
TGATTCATAATTTGAACAACCTTCCCCTGCCTGCTGTGGTGTCCGATTCAAGCAAAGTTT
TAAAAACAGACGAGATACAAGCTCCGTTATACAATAAACTGAAAAGAAAGATATAACAT
TAAGATTGAAACAGTGCATCGATTATCATCGGGAAGTTCTCGAATTCACCCAAGATATTT
CAGAAGCTTTCGGTCCGATGTTATTTGTATACTACTTGTTCATCAGGTCAGCGGCTGC
CTGTTGTTACTGGAATGTTCAAAATGGACGCAGCGGCTTTGATGCGTTACGGCCTTCT
AACAGCTGTTTTGTTCCAGCAACTGATTCAGTTATCTGTTGTCGTTGAGTCTGTTGGAAC
AGTGACCGGCTATCTGAAGGATGCTGTTTATAACGTCCCATGGGAGTACATGGACACAC
AGGACAGGAAGACAGTGTGCATATTTTTGATGAATGTTGAGGAACCAGTGCATATTAATG
CATTAGGACTTGCTAAAGTAGGCGTACAGGCAATGGCTGGAATTCTTAAGACATCGTTTT
CATACTTCGCATTTCTTCGCACCGTTTCTAACTAA

DNA Sequence of MsexOR-4

ATGAAGTTTTTTGTAGACGGCAGCGAAATAGCGCACATAACAAAACCTCAAGATATACAA
TATATGCAAATGTTAAAGTTTTTTACGAATTCGCTAGCAGGTTGGCCTATCGAGGCTGTG
GAAGGGATAGACGGCAAGAAAAATTTTTACTGGAGAAACGGCTTGTTGTATTGCATA
TGCGTATTTTTTCGGACAAGTGTTTTATTTACAGGTATATTAACGATTACACATTTTTA
GTCATGGGTCATTCGTACATCACTGTACTCATGACTATTGTAACAATAGCTCGACACACA
TTACCATACTTCAAATGCTACGACGATACGACTGCCGACTTCGTGCATAATATCCACCTT
TTCAATTATCGAAACAAACCTGGCTACTACAAGGAGTTTCACTTAAAGATACACAAAATAT
CCCACGCTTCTCGGTATACCTGTGCACACTATTGGTGACGGGTCCTAGTATGTTCAAT
GGTATACCACTTTACAACAACACTATGCGTCGGGAGCTTTTAGCTTTAACAGATCGCCTAAC
GTGACCTACGAACAGGCCGTGAGCTTGTTGCTACCTTTCGATGATACAAATAATTTCAA
GGTACTTCGTAGTTTTTCTTGCTAATTGCTGTGTGAGTTATATTTCTCGTGTTGTCTTT
GTATCTATGACCTCCTCCTTTCTCTCATGGTGTTCCATCTTTGGGGACATCTAAAAATCC
TTACAAAGACCCTCGATAACTTCCCGAAGCCAGGATTTCTAAATCCACAAGCTATTGAAG
CGGATCCGAACAAAAGTTTAAAATTTTCTGATGAAGAACTGAAAGTTATTCACAAGAAAC
TTGGTGAATGTGTGGCTCATCATCAGTTGATTTCCAACCTTCTCAACGCGTATGTGAACA
CTTTTGGATTGTCACTGTTTCATCTATTACGGTTTCCATCAGCTGAGCGGCTGCTTGCTGT
TGCTGGAATGCGCACAACTGGAAGCTGCAGCCATTATATGCTATGGGCCGCTTACGTTG
GTCGTGTTTCAACAATTAATACAATTGTCATTTATATTTGAGCTCATAGGGACTGTGAAC

GAGGGCCTTACGGACTCCGTGTATTGTTTGCCGTGGGAGGCTATGGACCAAGGAAACA
AGAAAATAGTATTCACTTTTCTACGTCAATCACAGAAGTCGATGAATTTAAAAGCTTTGAA
CATGCTTAGCATTGGAGTACAGACTATGGCAAAGATTTTGAAGACAACCATGACCTACTT
TTTGATGCTTCAAACAATCGCCAAAGATGAGAGCTAA

DNA sequence of MsexOR-31

ATGGCTCAAAACACAGAATTATTTTTGGGTCGTCCCAAAAAAATATTAACATTTTTTCGGCA
TTTGGCTACCATCGAAACAATATCAAGATTTGGTAAAAATCTACATGATTTTGGTTATGAT
TACTCAATATTCCTTTGTGTTATTCGAAATTGTTTATATAATCAATGTTTGGGGAGATATT
GAGGTAGTTTCTGAGGCGTCTTATTTGCTTTTCACGCAAGCTTCTGTATGTTACAAGACA
ACTGCTTTTCTGATTAAAAACGTAAACTAGTCTTGCTGCTGGAACATATGGAACAGGAG
ATTTTTGCGTCGCAATCTAAAGACCATGAGAAAAATATTACTGGAGCTGTCATTAAGAATT
AAGCGTCTCTGTACGTTTTTCTTGACAAGTGCTATTACGACTTGCACTTTGTGGGCTATG
ATTCCTCTCTTCGATGATGCCGGTCATAAGACTTTTCCGTTTAGGATTTGGATGCCTGTA
ACGGCGGACGAGTCACCAGGCTATGAGTTGGGTACTTGTACCAAATGGTTAGCATATA
CATCAGCGCATTCTCTTCATAGCGGTGGACAGTGTGGCTCTTTCCATGATCATGTTGCG
GATGTGCACAGCTGGACATCATCAAGGACAAAATGCAGAAGGTGCGTGAAGTGCCCAT
ATGTTTAAACGTGGATGAGAAGAAAGAAAAGTTGAAAAGCAATAACGACCTTTTTACCGA
ATGTGTTGCGCACTATCAAATGTAATTAGTTTCGTCGAGCTGGTAGAAGAACTTACCA
CGCAAATATATTTTTCCAGTTGAGTGGCACAGTTGCTATCATATGCAACATTGGACTTCG
TATTTCTATAGTGGACCACCACAGTGTGCAGTTCTTCTCGATGTTAACTACATGGTGAC
CATGTTGTCTCAACTGTTCTGTATTGCTGGTGCGGCAACGAGCTCACTATACGGAGCG
AGGTCTTACGTGATTGGATGTACCAGTGTCCATGGTACGACCAAGATACCAAGTTCAAG
AGAACTCTATGGATCACCATGGAGCGTATGAAGAAACCCATTATATTTAAAGCCGGCCA
CTATATCTCGCTCTCTAGGCCTACATTTGTTGCTATTCTGCGCTGTTTCGTA CTACTT
CGCAGTCCTCAACAGGGGCGAACACATAA

Acknowledgment

I owe thanks to Bill Hansson as leader of the department and Dieter Wicher for the opportunity to investigate the *Manduca sexta* pheromone receptors in this department with its employees and its equipment.

I do not know how to thank Ewald Große-Wilde as mentor of this work. He introduced me to the group, the lab, and the topic. I learned much from him and he encouraged me in being scientist. I am deeply grateful for his support.

Regina and Sascha, I thank both of you for your help in the lab and the conversations. I owe thanks to Sylke for *Manduca* rearing. I am grateful for the animals, which were donated by Anna. I would like to thank Robin, Katja, Sandra, and Sarah, the four students from Kassel, which helped to establish real time PCR and Martin Kaltenpoth for his advice to improve real time PCR. Katrin, I thank you for your help in the lab, the discussions, and for finding a good title for the diploma thesis. I thank Linda for the pictures of the moth species.

Special thanks to all co workers. I really enjoyed the coffee breaks, the conversations with all of you and it was a pleasure to work in this group. I will prepare a Frankfurter Kranz or two for you all.

Eidesstattliche Erklärung

Hiermit erkläre ich, Christopher König, dass ich die vorliegende Arbeit selbständig und ausschließlich unter Verwendung der angegebenen Quellen und Hilfsmittel verfasst und noch keiner anderen Prüfungsbehörde vorgelegt habe.

Jena, 27. März 2011