



Identification of pheromone-like compounds in male reproductive organs of the oriental locust *Locusta migratoria*



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ABSTRACT

Despite the great economical interest of locusts in agriculture, knowledge on their chemoreception systems is still poor. Phenylacetone nitrile is recognised as a pheromone of the desert locust *Schistocerca gregaria*, triggering gregarization, promoting aggregation and inhibiting courtship. However, in the other major locust species, *Locusta migratoria*, pheromones have not been reported. We have identified the two isomers of naphthylpropionitrile from the male reproductive organs of *L. migratoria*. Chemical synthesis has confirmed the identity of the two compounds. Both isomers show significant affinity to CSP91, a protein reported in the testis, but not to three other proteins of the same family (CSP180, CSP540 and CSP884) expressed in female accessory glands. The striking similarity of these compounds with phenylacetone nitrile and the unusual nature of such chemicals strongly suggest that naphthylpropionitrile could be pheromones for *L. migratoria*, while their site of expression and binding activity indicate a role in communication between sexes.

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1. Introduction

Locusts are responsible for enormous losses in agriculture all over the world. The desert locust *Schistocerca gregaria* is mainly active in Africa, the oriental locust, *Locusta migratoria*, in Asia. Despite their exceptional economical interest, very little is known on their chemoreception system, both regarding semiochemicals and the proteins involved in their recognition. However, in other insect species pheromones and other semiochemicals are efficiently used to control their populations. Locusts are particularly interesting in this respect as they use pheromones to undergo a physiological shift from the “solitary” to the “gregarious” phase, accompanied by some major morphological modification changes, including colour. It is only in the gregarious phase that locust develop the capacity of aggregating into swarms of millions of individuals that cause enormous damage to crops.

Most of the information on locust pheromones is available for the desert locust *S. gregaria* [1–3], where phenylacetone nitrile has been identified as the chemical involved in the transition to the gregarious phase and aggregation of “gregarious” locusts [4]. The same compound can also acts as a courtship-inhibiting pheromone and as a self-marking volatile in order to avoid homosexual attacks [5–7]. Cuticular hydrocarbons have been also identified and suggested to play some role in phase transition [8].

In the oriental locust no clear information on pheromones or semiochemicals has been reported so far, apart from some electrophysiologically active phenols and derivatives, identified in the faeces [9]. Although their ligands are still unknown, two binding proteins seem to be involved in the phase transition mechanism [10]: a chemosensory protein, previously reported as CSP-I [11] and another binding protein called “take-out”. At the central level, serotonin and dopamine have been reported as the neurotransmitters mediating the phase transition in *S. gregaria* and *L. migratoria*, respectively [12,13].

In a previous paper, we reported on the expression of 17 chemosensory proteins (CSPs) in female reproductive organs of *L. migratoria*, only one of which was also present in male organs, specifically in testis [14]. Such large number of these carrier proteins [15] suggest the presence of semiochemicals in the reproductive organs of locusts. In fact, often pheromones are bound at their production sites to soluble proteins, such as OBPs (odorant-binding

Abbreviations: OBP, odorant-binding protein; CSP, chemosensory protein; 1-NPN, N-phenyl-1-naphthylamine; GC/MS, gas-chromatography/mass spectrometry.

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protein) and CSPs, that help solubilise these hydrophobic compounds and assist their release in the environment. In vertebrates, the best known examples of carrier proteins with such functions are the urinary proteins of mice [16,17] and the salivary proteins of pigs [18,19], proteins that also occur in the olfactory area, where they are involved in detecting the same chemicals. In insects, the pheromone glands of *Lepidoptera* and the mandibular glands of the honeybee contain proteins of the OBP [20] and CSP families [21,22], that are also found in chemosensory organs [11,15,23,24].

Reproductive structures also express proteins of the above families, whose presence in some cases has been put in relationship with semiochemicals [25–27]. The male seminal fluid of some insects, including *Orthoptera*, contains chemicals able to produce physiological or behavioural effects on conspecifics [28,29]. Sex pheromones and other semiochemicals are also produced by males in their seminal fluid and could be transferred to females during copulation [27,30].

Here we report on the identification and synthesis of two pheromone-like compounds in the reproductive organs of *L. migratoria*, 3-(1-naphthyl)propionitrile and 3-(2-naphthyl)propionitrile, and on their affinity to chemosensory proteins expressed in the same organs [14].

2. Materials and methods

2.1. Materials

Locusts (*L. migratoria*) were raised in the Department of Grassland, (China Agricultural University, Beijing, China) at 28–30 °C, relative humidity 60%, and photoperiod of 18 h:6 h (light:dark). Chemicals, unless otherwise stated, were from Sigma–Aldrich, reagent grade.

2.2. Synthesis of naphthylpropionitriles (1) and (2)

¹H NMR (250 MHz) and ¹³C NMR (62 MHz) were performed on a Bruker Avance-II in deuteriochloroform solutions.

1-Bromomethylnaphthalene (4). A mixture of 1-methylnaphthalene (3) (Aldrich, 1.4 g, 10 mmol), N-bromosuccinimide (NBS, 1.8 g, 11 mmol) in carbon tetrachloride (20 ml) was heated under reflux in a Pyrex flask while exposed to a sun lamp (Osram UV 300 W) for 30 min. The resulting mixture was partitioned between hexane and diluted thiosulphate; the organic phase was dried over magnesium sulphate, filtered through a short column of silica gel, and evaporated to afford crude (4) (1.8 g, 81% yield) as an oil, which was used in the next step without further purification. ¹H NMR: δ 4.98 (s, 2H), 7.52 (m, 1H), 7.64 (m, 1H), 7.87 (m, 1H), 8.17 (m, 1H). ¹³C NMR: 31.99, 123.9, 125.6, 126.4, 127.8, 127.9, 129.0, 130.0, 131.3, 133.4, 134.2.

2-Bromomethylnaphthalene (6) was obtained in 85% from 2-methylnaphthalene (5) following the same procedure adopted for compound 3. ¹H NMR: 4.68 (s, 2H), 7.45–7.55 (m, 3H), 7.75–7.90 (m, 4H). ¹³C NMR: 34.2, 126.7, 126.8, 126.9, 127.1, 128.1, 129.4, 135.2, 139.1.

3-(1-Naphthyl)propionitrile (1). To a solution of butyllithium (10 mL of a 1.6 M solution in hexane, 16 mmol) in anhydrous tetrahydrofuran (25 mL) stirred under argon in a dry ice/acetone bath acetonitrile (3.2 mL, 60 mmol) was added dropwise. After 20 min, bromide (4) (1 g, 5 mmol) dissolved in hexane (5 mL) was added; after 30 min stirring and –78 °C, the reaction was allowed to equilibrate with the room temperature, quenched with saturated ammonium chloride solution and partitioned between water and ethyl acetate. The organic phase was washed with brine, dried over magnesium sulphate, and evaporated to leave a residue from which (1) (0.38 g, 42% yield) was obtained by flash-chromatogra-

phy with hexane–ethyl acetate 4:1. ¹H NMR: 2.71 (t, J = 7.5 Hz, 2H), 3.38 (t, J = 7.5 Hz, 2H), 7.40–7.60 (m, 4H), 7.80 (d, J = 7 Hz, 1H), 7.90 (m, 2H). ¹³C NMR: 18.5, 28.8, 119.5, 122.8, 125.7, 126.1, 128.1, 129.3, 131.2, 134.1.

3-(2-Naphthyl)propionitrile (2) was prepared as described above using bromide (6) (60% yield). ¹H NMR: 2.71 (t, J = 7.5 Hz, 2H), 3.12 (t, J = 7.5 Hz, 2H), 7.34 (d, J = 7 Hz), 7.47 (m, 2H), 7.51 (s, 1H), 7.75 (m, 3H). ¹³C NMR: 19.5, 31.9, 119.4, 126.1, 126.5, 126.6, 127.1, 127.8, 127.9, 128.8, 132.7, 133.6, 135.6.

2.3. Analysis of volatiles

Samples of reproductive organs and other parts of the body were dissected and stored at –20 °C until use. Extraction was performed by homogenisation in dichloromethane, followed by centrifugation. The organic phase was concentrated to small volume and 1 μ L was used for analysis. The GC–MS system was constituted by a Focus Thermo-Finnigan gas-chromatograph equipped with a Varian CP-WAX-52 capillary column (60 m \times 0.32 mm; coating thickness 0.5 μ m) and a DSQ Thermo-Finnigan quadrupole mass detector. Analytical conditions were: injector and transfer line at 250 °C; oven temperature was programmed from 50 °C (10 min) to 240 °C at 10 °C/min; carrier gas helium at 1 mL/min; splitless injection. Identification of the constituents was based on comparison of the retention times and mass spectra with those of authentic samples, analysed in the same conditions.

2.4. Protein expression and purification

For the bacterial expression of CSP180, CSP540 and CSP884, constructs were prepared in pET5b containing the genes encoding mature proteins with the addition of an initial methionine. The following oligonucleotides were used to amplify the relative genes:

CSP180-Nde: AACATATGTACACCACCAAGTACGA;
CSP180-Eco: GTGAATTCTTACGCGGAGACCCTCTTGAG;
CSP884-Nde: AACATATGTACACCACCAAGTACGA;
CSP884-Eco: GTGAATTCTTACTCGAAGGGTGATCCAGA;
CSP540-Nde: AACATATGCAGGACAAGTACACCAC;
CSP540-Eco: GTGAATTCTTACGACTTGAGCTTCTTGAT.

Expression and purification of recombinant proteins was accomplished along with standard protocols, previously described for similar proteins [14].

2.5. Fluorescence measurements

Emission fluorescence spectra were recorded on a Jasco FP-750 instrument at 25 °C in a right angle configuration, with a 1 cm light path quartz cuvette and 5 nm slits for both excitation and emission. The protein was dissolved in 50 mM Tris–HCl buffer, pH 7.4, while ligands were added as 1 mM methanol solutions.

2.6. Fluorescence binding assays

The affinity of fluorescent ligand N-phenyl-1-naphthylamine (1-NPN) to recombinant proteins was measured by titration of a 2 μ M solution of the protein in 50 mM Tris–HCl, pH 7.4, with aliquots of 1 mM ligand in methanol to final concentrations of 2–16 μ M. The probe was excited at 337 nm and emission spectra were recorded between 380 and 450 nm. Binding constant was calculated using Prism software. The affinity of other ligands was measured in competitive binding assays, using 1-NPN as the fluorescent reporter at 2 μ M concentration in a 2 μ M solution of the protein in Tris buffer, and each competitor at 2–16 μ M. Dissociation constants of the competitors were calculated from the corresponding IC₅₀ values (the concentration of each ligand halving the initial value of fluorescence), using the equation: $K_D = [IC_{50}]$

$1 + [1\text{-NPN}]/K_{1\text{-NPN}}$, $[1\text{-NPN}]$ being the free concentration of 1-NPN and $K_{1\text{-NPN}}$ being the dissociation constant of the complex Protein/1-NPN.

3. Results and discussion

Based on the occurrence of semiochemicals and their binding proteins in sex organs of some insects [25–30], we decided to search for pheromones of the oriental locust *L. migratoria* in its reproductive organs.

Fig. 1 reports a section of the gas-chromatographic profile of a dichloromethane extract from male reproductive organs. The major peaks in the whole chromatogram have been identified as fatty acids, their esters and several long-chain hydrocarbons, along with our previous report [14]. Among the less abundant components, our attention was attracted by two compound that showed the same unusually simple mass spectrum (Fig. 1). The base peak ($m/z = 141$) is only accompanied by a much weaker molecular ion ($m/z = 181$) and another at $m/z = 115$, apart from trace peaks. The spectrum was best visualised after subtracting

the strong background and the relative chromatographic peaks are more evident when the abundance of a single ion ($m/z = 141$) is plotted instead of the total ion current (Fig. 1). The simple mass spectra indicate two isomeric aromatic compounds with a very stable core and a chain easy to fragment, giving a highly stabilised ion, similar to the tropylium cation. On the other hand, a loss of 40 mass units from the molecular ion was strongly suggestive of an acetonitrile radical. Only two structures appeared to be reasonably compatible with such data, the two positional isomers of 3-naphthylpropionitrile (1) and (2).

We therefore decided to synthesise both isomers in order to assign the correct identification to the two peaks. The synthesis was easily accomplished through the reactions reported in Fig. 2. The two isomeric 1- and 2-naphthylpropionitrile were synthesized from commercially available 1- and 2-methylnaphthalene by allylic bromination to afford the corresponding brominated compounds, which were finally condensed with lithiated acetonitrile (Fig. 2). Both compounds have been characterised by NMR and their structures confirmed.

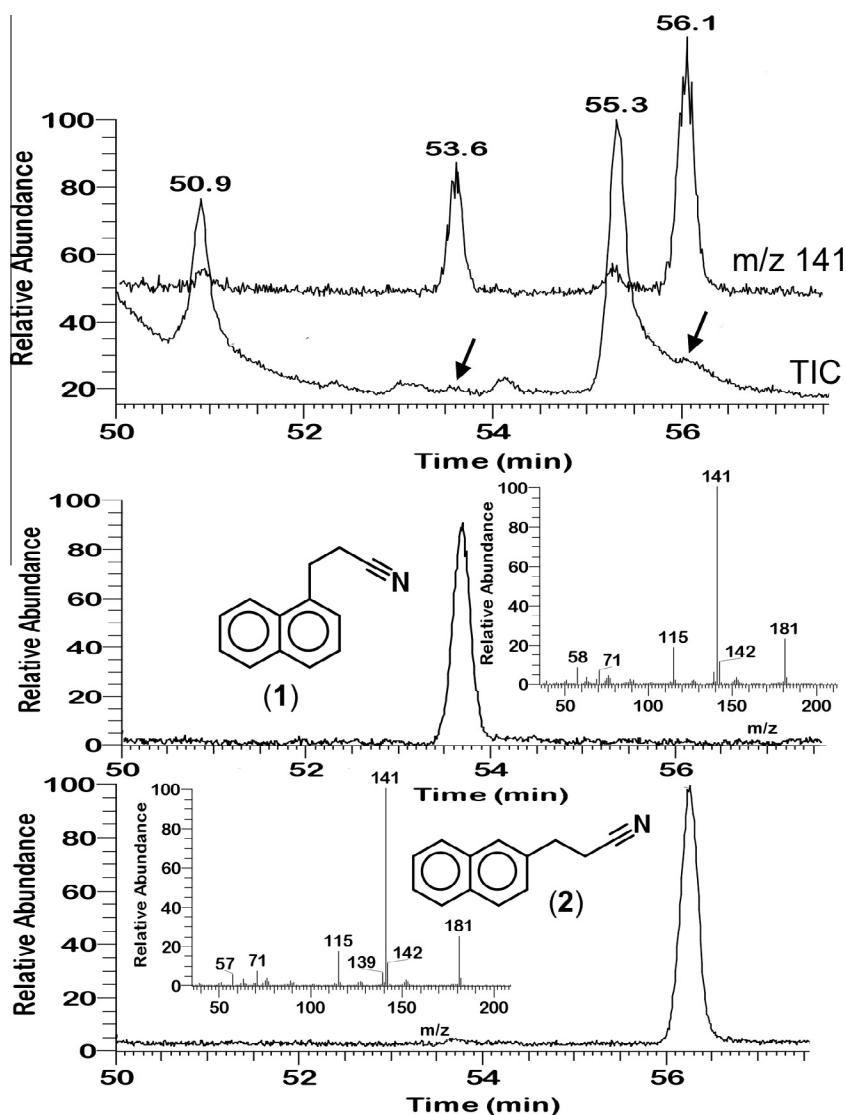


Fig. 1. Upper panel: gas-chromatography/mass spectrometry (GC/MS) analysis of a crude dichloromethane extract from male reproductive organs of *L. migratoria* adults. The lower trace reports the total ion current (TIC) the upper one shows the current relative to a single ion ($m/z = 141$), representing the base peak in the mass spectra of both naphthylpropionitriles. Arrows indicate the peaks in the TIC chromatograms relative to the compounds of interest. Lower panels: GC/MS of authentic samples of the two isomeric naphthylpropionitriles.

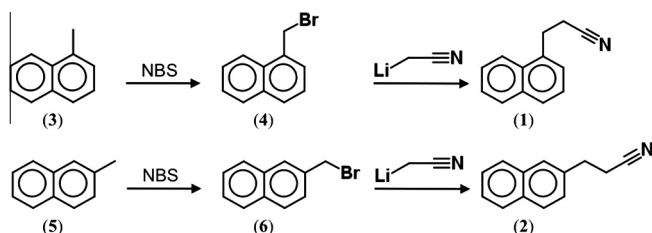


Fig. 2. Synthesis of the two isomeric naphthylpropionitriles. Either methyl-naphthalene was brominated with *N*-bromo-succinimide and the product reacted with lithium acetonitrile. In both cases the reactions proceeded with good yields and the products were characterised by NMR spectroscopy.

Gas-chromatographic analysis of the two purified compounds, performed in the same conditions as the gland extract (Fig. 1) has revealed that the first peak in the gland extract, eluted at 53.6 min in our GC conditions, is 3-(1-naphthyl)propionitrile (**1**), while the second, eluted at 56.1 min, is the other isomer (**2**).

The structures of the two naphthylpropionitriles are strongly reminiscent of phenylacetone, the chemical recognised as the aggregation and gregarization pheromone in the desert locust *S. gregaria* [1] suggesting that the naphthylpropionitriles might act as pheromones for the oriental locust *L. migratoria*. We then performed binding experiments with recombinant CSP91, the only CSP among the 17 found in female sex organs to be also present in males. We had previously characterised the binding properties of this protein [14], that showed good affinity to linear alcohols, acids and esters with a strong dependence on length of the chain, best ligands being molecules of 14 carbon atoms.

This protein also binds well both isomers of naphthylpropionitrile with dissociation constants of 3.3 and 6.0 μM for the **1** and **2** derivatives, respectively (Fig. 3). A model of the protein [14] reveals the presence of five aromatic residues inside the binding pocket (two phenylalanine, two tyrosine and one tryptophan), supporting the good affinity found for our aromatic nitriles.

To investigate the selectivity of these ligands towards the CSPs expressed in the locust, we have selected three additional members of this family, CSP180 (acc. No. CAB65180), CSP540 (acc. No. CO849540) and CSP884 (acc. No. CO835884) among those identified in female reproductive organs [14]. The bacterial expression and purification of these three CSPs was accomplished along standard protocols and is reported in Fig. S1. Their amino acid sequences are aligned in Fig. S2. None of them binds any of the two isomers significantly (Fig. 3).

A structural similarity of naphthylpropionitriles with phenylacetone, a pheromone of the desert locust *S. gregaria* with courtship inhibiting effect [5], and their occurrence in male sex organs suggest similar functions for the new compounds here described in the oriental locust *L. migratoria*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2013.07.015>.

References

- [1] D.J. Nolte, A pheromone for melanization of locusts, *Nature* 200 (1963) 660–661.
- [2] D.J. Nolte, I.R. May, B.M. Thomas, The gregarisation pheromone of locusts, *Chromosoma* 29 (1970) 462–473.
- [3] A. Hassanali, P.G.N. Njagi, M.O. Bashir, Chemical ecology of locusts and related acridids, *Annu. Rev. Entomol.* 50 (2005) 223–245.
- [4] M.P. Pener, Y. Yerushalmi, The physiology of locust phase polymorphism: an update, *J. Insect Physiol.* 44 (1998) 365–377.
- [5] K. Seidelmann, H.J. Frenz, Courtship inhibition pheromone in desert locusts, *Schistocerca gregaria*, *J. Insect Physiol.* 48 (2002) 991–996.
- [6] K. Seidelmann, K. Warnstorff, H.J. Frenz, Phenylacetone is a male specific repellent in gregarious desert locusts, *Schistocerca gregaria*, *Chemoecology* 15 (2005) 37–43.
- [7] E. Rono, P.G.N. Njagi, M.O. Bashir, A. Hassanali, Concentration-dependent parsimonious releaser roles of gregarious male pheromone of the desert locust, *Schistocerca gregaria*, *J. Insect Physiol.* 54 (2008) 162–168.
- [8] Y. Heifetz, I. Boekhoff, H. Breer, S.W. Applebaum, Cuticular hydrocarbons control behavioural phase transition in *Schistocerca gregaria* nymphs and elicit biochemical responses in antennae, *Insect Biochem. Mol. Biol.* 27 (1997) 563–568.
- [9] X. Cui, C. Wu, L. Zhang, Electrophysiological response patterns of 16 olfactory neurons from the trichoid sensilla to odorant from fecal volatiles in the locust, *Locusta migratoria* manilensis, *Arch. Insect Biochem. Physiol.* 77 (2011) 45–57.
- [10] W. Guo, X. Wang, Z. Ma, L. Xue, J. Han, D. Yu, L. Kang, CSP and takeout genes modulate the switch between attraction and repulsion during behavioral phase change in the migratory locust, *PLoS Genet.* 7 (2011) e1001291.
- [11] L.P. Ban, A. Scalon, A. Brandazza, S. Angeli, L. Zhang, Y.H. Yan, P. Pelosi, Chemosensory proteins of *Locusta migratoria*, *Insect Mol. Biol.* 12 (2003) 125–134.
- [12] M.L. Anstey, S.M. Rogers, S.R. Ott, M. Burrows, S.J. Simpson, Serotonin mediates behavioral gregarization underlying swarm formation in desert locusts, *Science* 323 (2009) 627–630.
- [13] Z. Ma, W. Guo, X. Wang, X. Wang, L. Kang, Modulation of behavioral phase changes of the migratory locust by the catecholamine metabolic pathway, *Proc. Natl. Acad. Sci. USA* 108 (2011) 3882–3887.
- [14] X.H. Zhou, L.P. Ban, I. Iovinella, L.J. Zhao, Q. Gao, A. Felicioli, S. Sagana, G. Pieraccini, P. Pelosi, L. Zhang, F.R. Dani, Diversity, abundance, and sex-specific

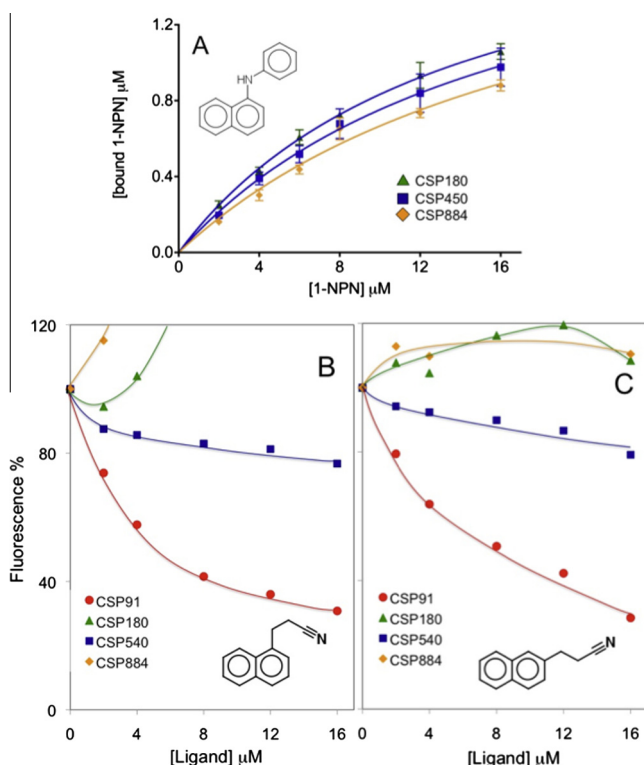


Fig. 3. (A) Binding of 1-NPN to recombinant CSP180, CSP540 and CSP884. Dissociation constants were 14.1, 16.7 and 20.3 μM respectively for the 3 proteins. (B) and (C) Competitive binding experiments of the two isomeric naphthylpropionitriles to the above proteins and to CSP91. Both compounds bind significantly to CSP91 (dissociation constants of 3.3 and 6.0 μM for isomers **1** and **2**, respectively) but exhibit poor or no affinity for the other three proteins.

- expression of chemosensory proteins in the reproductive organs of the locust *Locusta migratoria manilensis*, *Biol. Chem.* 394 (2013) 43–54.
- [15] P. Pelosi, J.-J. Zhou, L.P. Ban, M. Calvello, Soluble proteins in insect chemical communication, *Cell. Mol. Life Sci.* 63 (2006) 1658–1676.
- [16] A. Cavaggioni, C. Mucignat-Caretta, Major urinary proteins, alpha(2U)-globulins and aphrodisin, *Biochim. Biophys. Acta* 1482 (2000) 218–228.
- [17] J.L. Hurst, C.E. Payne, C.M. Nevison, A.D. Marie, R.E. Humphries, D.H. Robertson, A. Cavaggioni, R.J. Beynon, Individual recognition in mice mediated by major urinary proteins, *Nature* 414 (2001) 631–634.
- [18] S. Marchese, D. Pes, A. Scaloni, V. Carbone, P. Pelosi, Lipocalins of boar salivary glands binding odours and pheromones, *Eur. J. Biochem.* 252 (1998) 563–568.
- [19] S. Spinelli, F. Vincent, P. Pelosi, M. Tegoni, C. Cambillau, Boar salivary lipocalin: three-dimensional X-ray structure and androstenol/androstenone docking simulations, *Eur. J. Biochem.* 269 (2002) 2449–2456.
- [20] I. Iovinella, F.R. Dani, A. Niccolini, S. Sagona, E. Michelucci, A. Gazzano, S. Turillazzi, A. Felicioli, P. Pelosi, Differential expression of odorant-binding proteins in the mandibular glands of the honey bee according to caste and age, *J. Proteome Res.* 10 (2011) 3439–3449.
- [21] E. Jacquín-Joly, R.G. Vogt, M.C. François, P. Nagnan-Le Meillour, Functional and expression pattern analysis of chemosensory proteins expressed in antennae and pheromonal gland of *Mamestra brassicae*, *Chem. Senses* 26 (2001) 833–844.
- [22] F.R. Dani, E. Michelucci, S. Francese, G. Mastrobuoni, S. Cappellozza, G. La Marca, A. Niccolini, A. Felicioli, G. Moneti, P. Pelosi, Odorant-binding proteins and chemosensory proteins in pheromone detection and release in the silkworm *Bombyx mori*, *Chem. Senses* 36 (2011) 335–344.
- [23] S. Angeli, F. Ceron, A. Scaloni, M. Monti, G. Monteforti, A. Minnocci, R. Petacchi, P. Pelosi, Purification, structural characterization, cloning and immunocytochemical localization of chemoreception proteins from *Schistocerca gregaria*, *Eur. J. Biochem.* 262 (1999) 745–754.
- [24] M. Calvello, N. Guerra, A. Brandazza, C. D'Ambrosio, A. Scaloni, F.R. Dani, S. Turillazzi, P. Pelosi, Soluble proteins of chemical communication in the social wasp *Polistes dominulus*, *Cell. Mol. Life Sci.* 60 (2003) 1933–1943.
- [25] S. Li, J.F. Picimbon, S.D. Ji, Y.C. Kan, C.L. Qiao, J.J. Zhou, P. Pelosi, Multiple functions of an odorant-binding protein in the mosquito *Aedes aegypti*, *Biochem. Biophys. Res. Commun.* 372 (2008) 464–468.
- [26] L.K. Sirot, R.L. Poulson, M.C. McKenna, H. Girnary, M.F. Wolfner, L.C. Harrington, Identity and transfer of male reproductive gland proteins of the dengue vector mosquito, *Aedes aegypti*: potential tools for control of female feeding and reproduction, *Insect Biochem. Mol. Biol.* 38 (2008) 176–189.
- [27] Y.L. Sun, L.Q. Huang, P. Pelosi, C.Z. Wang, Expression in antennae and reproductive organs suggests a dual role of an odorant-binding protein in two sibling *Helicoverpa* species, *PLoS One* 7 (2012) e30040.
- [28] W.E. Wagner Jr, C.J. Harper, Female life span and fertility are increased by the ejaculates of preferred males, *Evolution* 57 (2003) 2054–2066.
- [29] L.W. Simmons, Allocation of maternal- and ejaculate-derived proteins to reproduction in female crickets, *Teleogryllus oceanicus*, *J. Evol. Biol.* 24 (2011) 132–138.
- [30] R.J. Bartelt, A.M. Schaner, L.L. Jackson, Cis-vaccenyl acetate as an aggregation pheromone in *Drosophila melanogaster*, *J. Chem. Ecol.* 11 (1985) 1747–1756.