Molecular characterization of a new adult male putative calycin specific to tergal aphrodisiac secretion in the cockroach *Leucophaea maderae*

Richard Cornette, Jean-Pierre Farine, Brigitte Quennedey, Rémy Brossut*

Développement-Communication chimique, Unité Mixte de Recherche 5548 du Centre National de la Recherche Scientifique, Université de Bourgogne, 6 Bd Gabriel, 21 000 Dijon, France

Received 25 September 2001; accepted 5 October 2001

First published online 17 October 2001

Edited by Jacques Hanoune

Abstract Lma-p18 is an epicuticular surface protein specific to the tergal gland aphrodisiac secretion of *Leucophaea maderae* adult males. Native Lma-p18 was purified and the complete cDNA sequence was determined by RT-PCR using primers based on Edman degradation fragments. Northern blot and in situ hybridization analyses showed that Lma-p18 is expressed exclusively in the anterior part of male tergal gland, which is exposed only during sexual behavior. Sequence analysis indicated that Lma-p18 belongs to the calycin superfamily and is very similar to Lma-p22, the first known male-specific tergal protein in *L. maderae*. Lma-p18 and Lma-p22 were proposed to bind different sexually attractive compounds as other calycins.

Key words: Cockroach; Secretion; Tergal gland; Calycin; Sex behavior

1. Introduction

Chemical communication is remarkably developed in insects and involves two main types of compounds. Volatile pheromones play a role mainly in sexual attraction and distance communication whereas heavier cuticular hydrocarbons are essential for contact recognition. Until now, the cuticular hydrocarbons were considered as the main fraction of a complex mixture of compounds commonly called cuticular waxes which cover the whole cuticle of insects. The presence of exocrine proteins in this layer was demonstrated in honeybees [1] and cockroaches [2] but little is known about their function. In cockroaches, proteinaceous secretions are found on the abdomen with different levels of specificity: surface proteins are secreted onto the whole cuticular surface in both sexes [2,3], whereas other proteins are specific to glandular regions on the male's tergites, which are involved in sexual behavior [4,5]. In *Leucophaea maderae*, the male volatile sex pheromone produced by the sternal glands attracts the female from a distance [6]. Once both sexes are in contact, the male raises its wings displaying its tergal glands and their proteinaceous secretion, and the female climbs onto its back to feed on the tergal secretion. The female is then in a proper position for mating and the male grasps its genitalia. Proteinaceous secretions are not present onto the abdomen of larvae whereas four major proteins were identified in adult abdominal secretion [2]. Lma-p54, a major protein secreted by both sexes, was characterized as an inactive member of the aspartic proteases family (Cornette, unpublished). Lma-p72 is male-specific and the last two proteins, Lma-p18 and Lma-p22, are exclusively found in the abundant male tergal secretion. Lma-p22 was cloned and characterized as belonging to the lipocalin family [7]. Lipocalins are members of the calycin group of proteins [8]. Those proteins are known to share a structure with a large cup-shaped cavity that is able to bind small hydrophobic molecules such as retinol [9]. Odorant binding proteins (OBPs) of mammals, which also belong to the lipocalin family, are involved in olfactory perception [10] and similarly, Lma-p22 was proposed to bind volatile compounds of the sexual pheromone in *L. maderae* [7]. Here, we report the characterization of Lma-p18, which is the second protein specific to the male tergal secretion. The complete nucleotide sequence encoding the protein was obtained and the localization of its expression is reported in this paper.

2. Materials and methods

2.1. Insects

The conditions for rearing insects and the methods to analyze protein secretion are the same as previously described [2].

2.2. Protein microsequencing

The tergites surface proteins, separated on 10% SDS–PAGE [11], were electroeluted to a PVDF membrane [12]. Lma-p18 protein containing bands were cut out, digested in situ by trypsin endopeptidase and the resulting peptides were separated by reverse phase high-performance liquid chromatography on a C18 column (Lichrospher, 100RP-18 (5 μm), Merck). Fractions containing each peptide were sequenced by an automatic protein sequencer (Applied Biosystems).

2.3. cDNA cloning

Poly(A)$^+$ RNAs (6 μg) from the second tergite were reverse-transcribed using the Superscript preamplification system kit (Life Technologies).

Lma-p18 cDNA was amplified directly by rapid amplification of 3′ cDNA ends (3′ RACE) [13] using a degenerate oligodeoxynucleotide deduced from the peptide sequence YLNENLDDFVK (GARAAYYTNGAYGAYTTYGT, (N = A/T/C/G, Y = C/T and R = A/G)) and an oligo(dT) adaptor primer (GAATTC(T)18(V)2, V = A, C or G). Each cycle comprised 1 min at 94°C, 1 min at 48°C and 2 min at 72°C, the last cycle was a 10 min extension step at 72°C. Reaction was performed in 50 μl containing 50 pmol of each primer, 0.2 mM deoxynucleotides and 1 U of Taq polymerase (Life Technologies). The 350 bp fragment was subcloned in a T/A cloning plasmid (pGEM-T easy, Promega) and was sequenced on both strands by the dideoxyribonucleotide method [14]. Based on the sequence of this fragment, a specific oligonucleotide primer was synthesized and used for 5′ RACE [13].

In order to obtain the 5′ end, the following protocol (5′ RACE system, Life Technologies) was used. The first strand cDNA was poly(C)-tailed using terminal transferase and was used as template

*Corresponding author. Fax: (33)-3-80 39 62 89.
E-mail address: remy.brossut@u-bourgogne.fr (R. Brossut).

PII: S0014-5793(01)02997-0

for PCR using the oligo(dG/dI) adaptor primer (GGCCACGCGTC-GACTAGTACGGGIIGGGIIGGGIIG) and an internal 5′ specific primer (CTGGGACTGATTGGAAGTTC). This product was then used as template for a second nested PCR using an Abridged Universal Amplification Primer (GGCCACGCGTCGACTAGTAC) and a second 5′ specific primer (CCATTTTAAGGACAATTCAGTTT).

The 5′ fragment was subcloned in a T/A cloning plasmid and sequenced on both strands.

2.4. Northern blotting

Total RNA was extracted from male tergal and sternal glands, whole tergal glands from females and last stage male larvae. The obtained total RNA (15 μg) was run on a denaturing formaldehyde agarose gel, transferred to a nylon membrane (Hybond N+, Amersham), hybridized with a random primed α-^{32}P-labeled cDNA probe in hybridization buffer (SCC 6×, formamide 50%, SDS 0.5% and salmon sperm DNA 100 μg/ml) at 42°C overnight, washed in 0.2×SSC, 0.1% SDS at 42°C and then autoradiographed.

2.5. In situ hybridization

The 5′-CTGGGACTGATTGGAAGTTC-3′ oligonucleotide was end-labeled by T4 polynucleotide kinase and used as a probe for in situ hybridization. Cryosections were deposited on poly(L-lysine)-coated slides. After three washes in phosphate-buffered saline (130 mM NaCl, 7 mM $NaPO_4$, 3 mM NaH_2PO_4, pH 7), sections were incubated in 0.2 M HCl for 10 min and acetylated using acetic anhydride, and then dehydrated in increasing concentrations of ethanol (50, 75, 95 and 100%). Desiccated sections were incubated for 1 h at 25°C in prehybridization buffer (4×SSC; 1×Denhardt). The probe was dissolved in hybridization buffer (40% (v/v) formamide; 10% (w/v) dextran sulfate; 1×Denhardt; 4×SSC; 0.5 mg/ml salmon sperm DNA) and deposited on each section for 16 h at 25°C (15°C under the calculated melting temperature). After six washes (20 min each) in 4×SSC, and one wash (45 min) in 2×SSC, slides were desiccated, coated with Ilford K5 emulsion, and exposed for about 1 week before revelation.

3. Results

3.1. Isolation and characterization of Lma-p18 cDNA

A 3′ and 5′ RACE-PCR approach was developed to isolate the full length cDNA encoding the Lma-p18 surface protein specific to the male tergites. Electrophoretic analysis of the selected amplified cDNA on 1% agarose gel showed a 3′ RACE-PCR single band of 333 bp. Specific primers deduced from the corresponding sequence were then used for the amplification of the 5′ end of the corresponding cDNA. The complete cDNA sequence (GenBank™ accession number AF411081) presents an open reading frame of 534 nucleotides, comprised between 5′ and 3′ untranslated sequences of, respectively, 78 and 288 nucleotides in length (Fig. 1). The deduced protein is composed of 178 amino acids with a calculated molecular weight of 19.8 kDa and an isoelectric point (p*I*) of 7.18. The sequence obtained after Edman degradation of the protein extracted from the male tergal secretion was found from positions 161 to 171. The hydrophobicity profile [15] revealed that the first 20 amino acids are hydrophobic and they probably correspond to a signal peptide with a putative cleavage site [16] between residues 20 (glycine) and 21 (aspartic acid). Protein sequence alignment showed that Lma-p18 was very close (75% of similarity) to Lma-p22, the lipocalin previously characterized from *L. maderae* [7]. Further alignment was performed, using the Clustal program, with different lipocalins. The percentage of identity falls to 19% with gallerin (EMBL Q24996, unpublished), 18% with insecticyanin B from *Manduca sexta* [17] and 11% with Bla g 4 [18], a cockroach allergen from *Blattella germanica* (Fig. 2). The

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  1                                                                  AGA  3
  4 ACTCACGATATAATAACTGTATTACTATTCTATATTTTACGGCGGCAGTGTGATAAGTTTCAACGCCAGAAAAAA  78

 79 ATG AAT TCA GTG GCT GTC TTA CTG ACA TGT TTT TCT GTT TCC ATT TTA AAT GTC AAT  135
  1 Met Asn Ser Val Ala Val Leu Leu Thr Cys Phe Ser Val Ser Ile Leu Asn Val Asn  19

136 GGG GAC TGC AAT CTT GGA CCT ATA TTT CGG TTT CAT CCA AAT GGG ATG GAA AAC ACG  192
 20 Gly Asp Cys Asn Leu Gly Pro Ile Phe Arg Phe His Pro Asn Gly Met Glu Asn Thr  38

193 TGG TAT TTC ATC TAC TCA TCC CCT TCT ATG TTC GAT GAG GCT AAC AAT ATA TTT GCT  249
 39 Trp Tyr Phe Ile Tyr Ser Ser Pro Ser Met Phe Asp Glu Ala Asn Asn Ile Phe Ala  57

250 AAA TAT AAG CTT GAA GGA CAC TCA CAC TAT GAG GCA CAT GCT GGA GCT ACT TTT GTA  306
 58 Lys Tyr Lys Leu Glu Gly His Ser His Tyr Glu Ala His Ala Gly Ala Thr Phe Val  76

307 AAC ACA GGC TCA CCT AAA GAA GTT AAT GCT ACA TTG ACA GCG CTA GAC TAT GGC ACC  363
 77 Asn Thr Gly Ser Pro Lys Glu Val Asn Ala Thr Leu Thr Ala Leu Asp Tyr Gly Thr  95
                                    *
364 AGG TTC AGT GTA CAG ATT CCT CAA TGG AGT AAA TAC AAT GGA ATG TAC CGA GTG ACA  420
 96 Arg Phe Ser Val Gln Ile Pro Gln Trp Ser Lys Tyr Asn Gly Met Tyr Arg Val Thr  114

421 GCA TTA GAG TAT GGG AGT TAT TTA ATT CTA AAA GGT TGT CGT GGA GAC TCT ACA ATT  477
115 Ala Leu Glu Tyr Gly Ser Tyr Leu Ile Leu Lys Gly Cys Arg Gly Asp Ser Thr Ile  133

478 GAA TCC CTT ACG ATC GTC ATG TTC AGT AAG AAA TGT CCC GAT GAA GCA TCA GTG GGA  534
134 Glu Ser Leu Thr Ile Val Met Phe Ser Lys Lys Cys Pro Asp Glu Ala Ser Val Gly  152

535 GCA GCA AGA GCA GCT CTT AAG AAA TAC CTG AAT GAG AAC CTA GAT GAC TTT GTT AAG  591
153 Ala Ala Arg Ala Ala Leu Lys Lys Tyr Leu Asn Glu Asn Leu Asp Asp Phe Val Lys  171

592 GAT ACA AAA CTG AAT TGT CCT TAAAATGGAGTGAAATATGAGTATGACAGATATTATAACAATTATTA  659
172 Asp Thr Lys Leu Asn Cys Pro                                              178

660 ATTGTTTTTTTTTAGATATTATAACAATGTTTATTGCTTTGATTTGAACTTCCAATCAGTCCCAGATCATAATTTG  734
735 CTGTATGTACACTTTGCTTGAACCACGTATTATGTGCTCCCATGGAATGTAAATCTGGCATTATTTTATTGATTT  809
810 CTTATCAGTGCCAGAGTACTGAAGCTTATTGTATGTAGATAATAATGAATTTAATAAATTGACAAGCATACACAA  884
885 AAAAAAAAAAAAAAAA                                                          900
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Fig. 1. Nucleic acid and deduced amino acid sequences of cDNA encoding Lma-p18. The arrow corresponds to the putative cleavage site of the predicted signal peptide. The putative polyadenylation site is in bold letters. Amino acid sequence obtained after Edman degradation is underlined (*, putative *N*-glycosylation site).

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                                  SCR1
Lma-P18      -------DC--NLGPIFRFHPNGMENTWYFIYSSPSMFDEAN-NIFAKYKLEGHSHYEAH  70
Lma-P22      -------DC--DFGPIFRFHPNWMENTWYVVYSSPSAFDEAN-NVSFSYELQGHSHYVAH  70
GMGall 1     ASAVHEGKCP-DFKPVDNFNLTAYQGVWYEISKTPNDAEKNGKCGQAEYKLEGE---VVK  70
Icy B        GDIFYPGYCP-DVKPVDDFDLSAFAGAWHEIAKLPLENENQGKCTIAEYKYDGK---KAS  73
Bla g 4      -----NEDCFRHESLVPNLDYERFRGSWIIAAGT-SEALTQYKCWIDRFSYDDA----L-  61

                                                          SCR2
Lma-P18      AGATFVNTGSPK--EVNATLTALDYGT---RFSVQIPQWSK-YNGMYRVTALEYGSYLIL  124
Lma-P22      VGITFADIGTAK--EINGTVTALDFGT---KFNVQLPEWSK-YSGTYRVTALEYGNYLIA  124
GMGall 1     VKNSHVVDGVQKYVEGTAKFAEDANKS—AKLLVTLTYGAVNRESPLNVIATDYQNYAIA  128
Icy B        VYNSFVVNGVKEYMEGDLEIAPDAKYTKQGKYVMTFKFGQRVVNLVPWVLATDYKNYAIN  133
Bla g 4      VSKYTDSQGKNR--TTIRGRTKFEGNK----FTIDYNDKGKAFSAPYSVLATDYENYAIV  115

                  SCR3
Lma-P18      KGCR--GDSTIESLTIVMFSKKCPDEASVGAARAALKKYLNENLDD--FVKDTK—LNCP  178
Lma-P22      KGCP--EKSTVKSFTVVMFSKKCPDEASVGAARAALKKYLNENLEH--YAKDTF—LNCP  178
GMGall 1     YTCKYDEKSKSHNDSIWILSRAKKLEGDAKTAVDNYLKEHAKEIDASKLVQTDFSEEACK  188
Icy B        YNCNYHPDKKAHSIHAWILSKSKVLEGNTKEVVDNVLKTFSHLIDASKFISNDFSEAACQ  193
Bla g 4      EGCPAAANGHVIYVQIRFSVRRFHPKLGDKEMIQHYTLDQVNQH—KKAIEEDLKHFNLK  173
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Fig. 2. Amino acid sequence alignment of Lma-p18 with lipocalins. Sequence alignment was performed between Lma-p18, Lma-p22, gallerin (GMGall 1), *Manduca* insecticyanin (Icy B) and *Blattella* human allergen (Bla g 4). Amino acids identical to Lma-p18 are shown by shaded boxes and the SCRs defining the lipocalin family are indicated (SCR1, -2 and -3). Gaps (–) were introduced to improve the alignment.

three structurally conserved regions (SCRs) defining the lipocalin family are shown with the alignment. Secondary structure prediction shows that Lma-p18 would be mainly made of β-strands (9) and would contain only one α-helix [19]. Tertiary structure prediction with the program 3D-PSSM [20] concludes that Lma-p18 presents a lipocalin-type folding.

3.2. Expression of Lma-p18 transcript

The first obtained 333 bp cDNA was used as a probe to check the male specificity of Lma-p18 by Northern blot analysis. As shown in Fig. 3A, a single mRNA of approximately 900 nucleotides was detected in adult male tergite extracts, while no signal was observed in control tergites from females and larvae, neither in sternites from males. Lma-p18 transcript was mainly expressed from the second to sixth male tergite (Fig. 3B). The highest amount of transcript was found in the second tergite and decreased progressively to the sixth tergite.

3.3. Histological localization of Lma-p18 transcript

In situ hybridization was realized on sections of abdominal tergites and sternites. As expected from Northern blot analysis, no labeling of male sternites was observed (Fig. 4A) whereas the male tergites were labeled. The expression of Lma-p18 was mainly observed in the glandular region located in the anterior part of the tergite (Fig. 4B). This glandular region, anterior to the median ridge shaped in cuticle, is usually covered by the anterior tergite. In contrast with this strongly labeled region (Fig. 4D), the glandular area, under the depression situated before the knob-like structure, where secretion accumulates, was not or faintly labeled (Fig. 4C). In more sagittal sections of the second tergite, the knob-like structure is well developed and Lma-p18 expression is still limited to the anterior part of the gland (Fig. 4E). Consequently, Lma-p18 seems to be expressed uniformly in all the width of the anterior part of the tergal gland.

4. Discussion

Among the four main proteins present in the abdominal surface secretion of *L. maderae*, two are specific to male's tergites [2]. Those two low molecular weight proteins were named Lma-p22 and Lma-p18. The first one was characterized and its complete cDNA was cloned and sequenced [7]. Our results show that the Lma-p18 sequence is very close to Lma-p22 and both proteins have the same size in amino acids and the same deduced molecular weight. The difference of apparent molecular weight observed in SDS–PAGE [2] could be explained by an additional putative glycosylation site in Lma-p22. Both proteins belong to the calycin superfamily which gathers binding proteins sharing a similar cup-shaped folding pattern such as lipocalins and fatty acid binding proteins [8,21]. Lma-p22 was proposed to belong to the lipocalin family [7]. Lipocalins constitute a large and diverse group of proteins (sequence identity often falls under 20%) sharing common tertiary structure. Eight β-strands are fold so as to

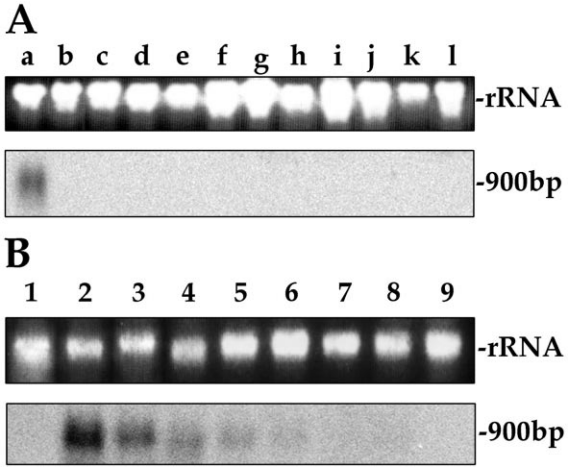

Fig. 3. Northern hybridization analysis of *L. maderae* RNA with Lma-p18 cDNA. Northern blot (A) was performed with total RNA (15 μg) from male tergites (a), male sternites (b), male midguts (c), male larval tergites (d), male larval sternites (e), male larval midguts (f), female tergites (g), female sternites (h), female midguts (i), female larval tergites (j), female larval sternites (k) and female larval midguts (l). Northern blot (B) was performed with total RNA (10 μg) from male first to ninth tergite (1–9). Ethidium bromide-stained ribosomal RNAs indicate that approximately equal amounts of RNA were loaded.

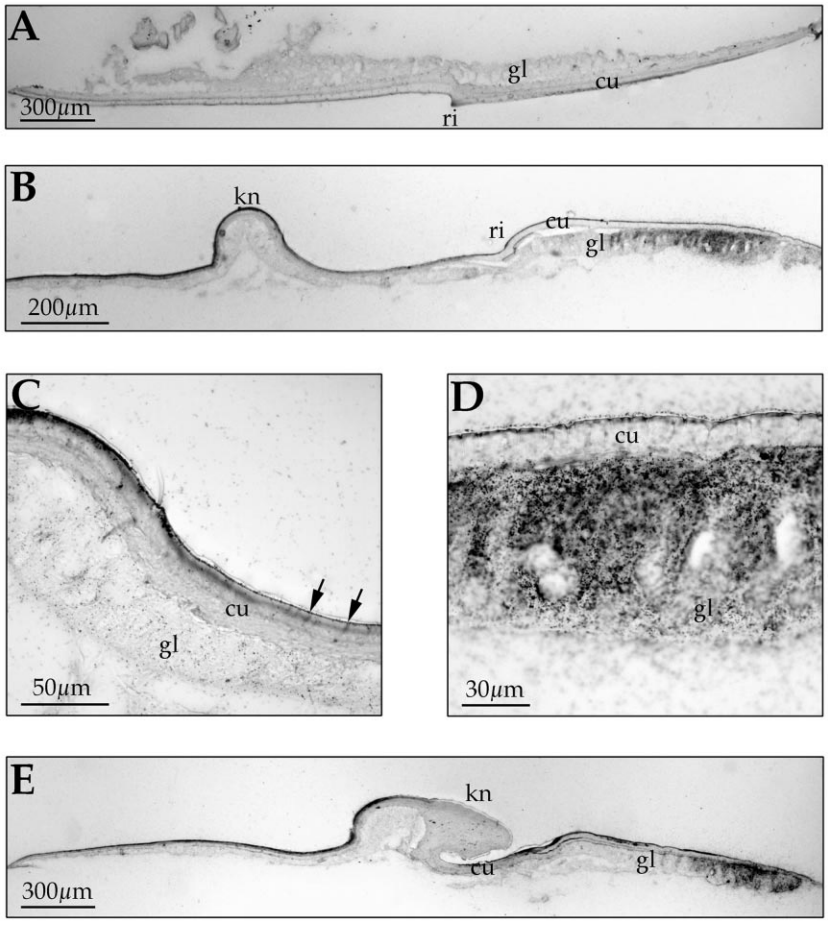

Fig. 4. In situ hybridization analysis with Lma-p18 radioactive probe. Cryosections from sternites and tergites of male *L. maderae* hybridized with the γ-^{32}P-labeled Lma-p18 oligonucleotide probe. A: Parasagittal section of a second sternite as a negative tissue. The glandular area (gl) is not labeled. B: Parasagittal section of a second tergite. The general shape of the cuticle (cu) forms an anterior ridge (ri) and a posterior knob-like structure (kn). Labeling is restricted to the glandular region (gl) anterior to the ridge (ri). C: Enlargement of the knob-like structure of second tergite. Ducts (arrows) from which secretion flows outside are seen in the cuticle (cu). The secretory cells of the tergal gland (gl) are not labeled. D: Enlargement of the anterior region of the second tergite showing strong labeling of the gland (gl). E: Sagittal section of a second tergite with a well developed knob-like structure (kn), showing the anterior expression of Lma-p18 transcript in the tergal gland (gl).

form a non-polar pocket able to bind hydrophobic ligands [10]. Lipocalins can bind a large variety of ligands and most of them are known to be carrier proteins. Some lipocalins can also bind to cell-surface receptors as it is the case for Retinol Binding Protein and α_1-microglobulin [10,22]. Lipocalins are generally considered as extracellular transport proteins, but they also present very different functions [10,23]. According to Flower et al., although Lma-p22 presents similarity to bilin binding protein and apolipoprotein D, they are at great phylogenetic distances from each other, even for lipocalins, and Lma-p22 may not be a lipocalin [21,24]. Nevertheless, as well Lma-p18 as Lma-p22 present predicted secondary and tertiary structures compatible with a lipocalin-type folding and should be binding proteins closely related to the lipocalin family. As a precaution, we will hereafter consider Lma-p18 and Lma-p22 as calycins. An other cockroach protein, the allergen Bla g 4 from *B. germanica*, was qualified as a calycin, although this protein contained each of the three SCRs characterizing the lipocalin family [18]. Along with Bla g 4, many lipocalins are responsible for most of the important respiratory allergies [25]. The function of Lma-p18 and Lma-p22 remains to be determined. The male specificity of both proteins is congruent with a role in sexual behavior. Moreover, the expression of Lma-p18 increases from posterior male tergites up to second tergite, where the females stop and feed on male secretion just before mating. Lma-p18 could create an orientated chemical scent for the female on male's tergites. Surprisingly, Lma-p18 presents an expression pattern similar to the expression of Lma-p22 transcript whereas the corresponding second protein is specific to the first two tergites [2,7]. These observations could be explained by the fact that Lma-p22 probe was obtained by cDNA random priming, and could then contain sequence parts identical to Lma-p18. Antibody raised against Lma-p22, which is also cross-reactive with Lma-p18, showed that proteins are secreted by the tergal gland and are poured out on the cuticle surface [2]. Both proteins are produced by glandular class 3 cells, which were in general described as producing mainly volatile compounds [26]. Immunohistological analysis showed that the calycins were present in the whole glandular surface of the second tergite [2]. Our in situ hybridization studies showed that Lma-p18 expression is restricted to the anterior region of the second tergite; so, immunostaining of the posterior region, including the knob-like structure, should therefore be imputed mainly to Lma-p22. The anterior part of male tergites, where Lma-p18 is expressed, is usually covered with the posterior edge of preceding tergites and is

only discovered during sex behavior, when the male raises its wings and extends its abdomen, inviting the female to feed on its tergal secretion. This enables us to put forward the hypothesis that Lma-p18 would bind and carry some pheromonal compound at the cuticular surface, then releasing the olfactory signal slowly as described for mouse major urinary proteins (MUPs), which increase the longevity of scent marks [27]. MUPs are present in large quantities in mouse or rat male urine and bind various odorant molecules, among which 2-*sec*-butyl-4,5-dihydrothiazole and dehydro-*exo*-brevicomin are the most abundant [27]. Volatile molecules play a role in chemical signaling and, together with MUPs, can act as a primer pheromone, by accelerating female puberty [27]. Mammalian OBPs, which belong to the lipocalin family, can bind a wide range of volatile molecules such as aliphatic, aromatic or heterocyclic odorants and terpenoids [28]. In other respects, bovine OBP was shown to bind an insect attractant, 1-octen-3-ol, as natural ligand [29]. Components of the sexual pheromone of *L. maderae* such as hydroxy-3-butanone-2, 2,3-butanediol or senecioic acid [30] could be good candidates for the putative ligands of Lma-p18 and Lma-p22. Amino acid substitutions between both proteins occur, not in the α-helix, but mainly at the level of the β-barrel constituting the central pocket of calycins, where there are interactions with the ligand. This could be related to differential ligand specificity for Lma-p18 and Lma-p22. Pheromone binding among lipocalins was also recorded for hamster aphrodisin [27]. Aphrodisin is a protein isolated from female hamster vaginal discharge, which binds with high affinity to dimethyl disulfide [31]. This volatile component is known to function as a sex attractant and thus, aphrodisin would act as a pheromone carrier [31]. Nevertheless, after removing all possible ligands, aphrodisin still elicits sexual behavior in males, suggesting that aphrodisin could be a pheromone by itself [27]. In the same way, *L. maderae* calycins could also present an intrinsic pheromonal role when detected or ingested by the female and actually bind to no natural ligand. Lma-p18 and Lma-p22 could constitute a gustative cue by themselves for male recognition when the female licks its tergites. To conclude, the characterization of proteins from *L. maderae* secretion gives new bases for the study of the mechanism of action of male tergal secretion and its involvement in the courtship behavior. Calycins are likely to constitute, if binding a pheromonal compound, an odorant or, if acting by themselves, a gustative scent on the male's back so as to immobilize the female in a proper position for mating. Binding tests using recombinant protein and behavioral tests will enable us to verify all these hypotheses by screening the putative ligands for both proteins.

Acknowledgements: This work was supported by Centre National de la Recherche Scientifique and the Université de Bourgogne. We are grateful to H. Bouhin, J.P. Charles and A. Quennedey for helpful discussions and critical reading of the manuscript.

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