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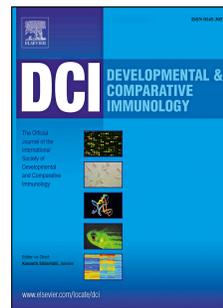


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1 **Molecular characterization of a c-type lysozyme from the desert locust, *Schistocerca gregaria***  
2 **(Orthoptera: Acrididae)**

3 **Amr A. MOHAMED<sup>1,\*</sup>, Long ZHANG<sup>2</sup>, Moataza A. DORRAH<sup>1</sup>, Mohamed ELMOGY<sup>1</sup>, Hesham A.**  
4 **YOUSEF<sup>1</sup>, Taha T.M. BASSAL<sup>1</sup> and Bernard DUVIC<sup>3</sup>**

5  
6 <sup>1</sup> **Department of Entomology, Faculty of Science, Cairo University, P. O. Box 12613, Giza, Egypt**

7 <sup>2</sup> **Key Lab for Biological Control of the Ministry of Agriculture, Department of Entomology, China**  
8 **Agricultural University, Beijing 100193, P. R. China**

9 <sup>3</sup> **Unité DGIMI UMR INRA-UM 1333, Université Montpellier, Place Eugène Bataillon, Montpellier,**  
10 **France**

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23 **\* To whom correspondence should be addressed: [mamr@sci.cu.edu.eg](mailto:mamr@sci.cu.edu.eg)**

24

25 **Abstract**

26 Lysozymes are bacteriolytic peptides that are implicated in the insect nonspecific innate immune  
27 responses. In this study, a full-length cDNA encoding a c-type lysozyme from *Schistocerca gregaria*  
28 (*SgLys*) has been cloned and characterized from the fat body of immune-challenged 5<sup>th</sup> instar. **The**  
29 **deduced mature lysozyme is 119 amino acid residues in length, has a calculated molecular mass of**  
30 **13.4 kDa and an isoelectric point of 9.2.** *SgLys* showed high **identities** with other insect lysozymes,  
31 ranging from **41.5%** to **93.3%** by BLASTp search in NCBI. **Eukaryotic *in vitro* expression of the *SgLys***  
32 **ORF (*rSgLys*) with an apparent molecular mass of ~ 16 kDa under SDS-PAGE is close to the**  
33 **calculated molecular weight of the full-length protein.** *rSgLys* displayed growth inhibitory activity  
34 against Gram-negative and Gram-positive bacteria. 3D structure modeling of *SgLys*, based on  
35 comparison with that of silkworm lysozyme, and sequence comparison with the helix-loop-helix ( $\alpha$ -  
36 hairpin) structure of hen egg white lysozyme (HEWL) were employed to interpret the antibacterial  
37 potencies. Phylogenetic alignments indicate that *SgLys* aligns well with insect c-type lysozymes that  
38 expressed principally in fat body and hemocytes and whose role has been defined as immune-  
39 related. Western blot analysis showed that *SgLys* expression was highest at 6-12 h post-bacterial  
40 challenge and subsequently decreased with time. Transcriptional profiles of *SgLys* were determined  
41 by semi-quantitative RT-PCR analysis. *SgLys* transcript was upregulated at the highest level in fat  
42 body, hemocytes, salivary gland, thoracic muscles, and epidermal tissue. It was expressed in all  
43 developmental stages from egg to adult. These data indicate that *SgLys* is a predominant acute-  
44 phase protein that is expressed and upregulated upon immune challenge.

45

46

47 **Keywords**48 *Schistocerca gregaria* – C-type lysozyme – cDNA – Innate immunity – Antibacterial activity –

49 Recombinant expression – Transcriptional profiles

## 50 Introduction

51 Insects have an innate immune system that allows them to be protected against a wide range  
52 of microbial pathogens. This system is highly developed and comprises cellular and humoral  
53 components that can be activated by invasion of pathogens (Tsakas and Marmaras, 2010). The  
54 humoral defense response of insects takes effect by over-expressing an array of potent antimicrobial  
55 peptides and proteins (AMPs) in order to kill invaders at the time of pathogenic infection. Among the  
56 large number of inducible antimicrobial peptides and proteins, lysozyme, a bacteriolytic enzyme, is  
57 the most ubiquitous antibacterial factor and is widely distributed in vertebrate and invertebrate  
58 animals. In invertebrates, it is well known that the lysozyme expression is regulated and responds to  
59 a bacterial challenge (Jiang et al., 2010; Mohamed et al., 2013). Both hemocytes and fat body,  
60 primarily the latter, of various insects have been reported to synthesize and release lysozyme to  
61 hemolymph (Lemaitre and Hoffmann, 2007; Mohamed et al., 2013; Zachary and Hoffmann, 1984).  
62 However, other tissues, such as epidermal tissues, may also participate in lysozyme production (Lee  
63 and Brey, 1995; Mulnix and Dunn, 1994).

64 Since the first insect lysozyme was reported in honey bees (Mohrig and Messner, 1968), more  
65 than fifty lysozyme genes have been identified from several insects, including the orthopterans  
66 *Locusta migratoria* (Zachary and Hoffmann, 1984), and *Gryllus bimaculatus* (Schneider, 1985), and  
67 the lepidopterans including *Spodoptera littoralis* (Jolles et al., 1979), *Hyalophora cecropia* (Engström  
68 et al., 1985), *Manduca sexta* (Mulnix and Dunn, 1994), *Bombyx mori* (Abraham et al., 1995),  
69 *Trichoplusia ni* (Kang et al., 1996), *Heliiothis virescens* (Lockey and Ourth, 1996), *Hyphantria cunea*  
70 (Park et al., 1997), *Antheraea mylitta* (Jain et al., 2001), *Samia cynthia ricini* (Fujimoto et al., 2001),  
71 *Spodoptera litura* (Kim and Yoe, 2003), *Artogeia rapae* (Bang and Yoe, 2005; Yoe et al., 1996),  
72 *Ostrinia furnacalis* (Wang et al., 2009), and *Spodoptera frugiperda* (Chapelle et al., 2009). It has also  
73 been reported, by sequence mining, from both the coleopterans *Tribolium castaneum* (Altincicek et

74 al., 2008) and *Sitophilus zeamais* (Anselme et al., 2008) **and by immunity-related transcriptome**  
 75 **analysis from *Harmonia axyridis* (Vilcinskas et al., 2013)**, and the hymenopteran, *Camponotus*  
 76 *floridanus* and *Harpegnathos saltator* (Bonasio et al., 2010). In dipterans, the overwhelming majority  
 77 of the studied lysozymes, isolated gene and/or cDNA structure were the digestive or involved in  
 78 blood feeding (Cançado et al., 2008) whereas, a basic lysozyme with muramidase activity, the Lys c-1,  
 79 is expressed in most tissues of adult mosquitoes and upregulated during bacterial infections (Kajla et  
 80 al., 2010). In Diptera, the lysozyme genes cluster is a multi-gene family which resulted in a large  
 81 expansion of the c-type lysozyme gene family in this insect order. For example, in the mosquito *A.*  
 82 *gambiae* there are eight c-type lysozyme genes (*Lys c1-8*) (Li et al., 2005; Waterhouse et al., 2007).  
 83 **Likewise, in Lepidoptera, four c-type lysozyme homologues have been predicted in *Galleria***  
 84 ***mellonella* (Vogel et al., 2011) and two lysozymes have been identified in the *Manduca sexta***  
 85 **genome (He et al., 2015).**

86 The survey on insect lysozymes sequenced to date shows that most of them are belonging to  
 87 holometabolous ones, except for the isopteran *Reticulitermes speratus*, and the hemipterans  
 88 *Rhodnius prolixus* (Ursic-Bedoya et al., 2008), *Triatoma brasiliensis* (Araujo et al., 2006) and *T.*  
 89 *infestans* (Balczun et al., 2008). Interestingly, lysozymes of hemimetabolous insects have digestive  
 90 function and **almost** belong to c-type group. **However, the genome of the pea aphid *Acyrtosiphon***  
 91 ***pisum* contains three i-type (invertebrate) lysozymes encoding genes (Gerardo et al. 2010).** To date,  
 92 the isolation and/or molecular cloning of *Schistocerca gregaria* lysozyme (*SgLys*) has not been  
 93 reported.

94 The desert locust, *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae) remains the most  
 95 feared of all locusts and is one of the most notorious insects in the world (Lecoq, 2004). Desert  
 96 locusts, and possibly other hemimetabolous insects, differ in their biological and developmental  
 97 aspects from holometabolous insects, and therefore, different defense mechanisms against

98 pathogens may be expected. Accordingly, there is a longstanding interest in understanding the  
99 molecular mechanisms of the innate response of the hemimetabolous insects to microorganisms.

100 In order to understand the antimicrobial mechanism of *S. gregaria* lysozyme, we recently  
101 purified and characterized this antimicrobial molecule from the plasma of this insect (Elmogly et al.,  
102 2015; Mohamed et al., 2013). In the present work, we report, for the first time, the isolation and  
103 cloning of a complete cDNA encoding *S. gregaria* lysozyme (*SgLys*) and demonstrate the protein's  
104 primary, secondary and three dimensional structures. The directional cloning, *in vitro* expression and  
105 activity of r*SgLys* were also examined. In addition, *SgLys* was compared with other insect and non  
106 insect lysozymes and a molecular phylogenetic tree was constructed. The protein synthesis of the  
107 mature form by western blot and levels of lysozyme transcript in different tissues, and at different  
108 developmental stages post bacterial challenge were analyzed.

## 109 **Materials and Methods**

### 110 **Insect rearing**

111 The desert locust, *Schistocerca gregaria* (Forskål), was from a well-established laboratory  
112 colony at the Entomology Department, Faculty of Science, Cairo University, Egypt. Detailed  
113 descriptions of *S. gregaria* colony and the rearing techniques are given by Tanaka and Maeno (2008)  
114 and Mohamed et al. (2013).

### 115 **cDNA cloning**

116 Total RNA was extracted from the fat body tissue of *E. coli*-immunized 5<sup>th</sup> instar *S. gregaria*  
117 using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the supplier's instructions. Genomic  
118 DNA contamination was removed by Dnase I (Fermentas Life Sciences, Burlington, ON, Canada)  
119 treatment. Poly(A)<sup>+</sup>RNA was purified from total RNA by oligo-dT cellulose (Amersham-Pharmacia  
120 Biotech, Buckinghamshire, UK) according to the manufacturer's instructions. Extracted mRNA was  
121 used to synthesize cDNA first strand with 200 units of RevertAid™ M-MuLV Reverse Transcriptase

122 from the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas Life Sciences) and an oligo (dT)  
123 primer [a cDNA synthesis primer (10 μM)] with the dNTP mixture (10 mM). The degenerate primers  
124 Lys-F1 and Lys-F2, designed from a highly conserved amino acid sequence (DYGL/IFQI) found in insect  
125 lysozymes (Fujita et al., 2002; Regel et al., 1998), were first used in PCR to get a partial sequence. All  
126 primers used in this study are listed in Supplementary Table S1.

127 The 3'-terminal end of lysozyme cDNA was amplified by 3' RACE (rapid amplification of cDNA  
128 ends). The first PCR reaction was amplified with Lys-F1 (10 μM), 2 μL of poly-T primer (5'-  
129 GCGAATTCGTCGACAAGC(T)<sub>17</sub>-3') (10 μM) (Fermentas Life Sciences). The cDNA amplification was  
130 performed in a Master cycler Thermocycler (Eppendorf, Germany) based on the following program:  
131 Initial denaturation of 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min,  
132 annealing at 55°C for 1 min and extension at 72°C for 1.5 min. The second PCR (nested) was identical  
133 to the first, except that the reaction mixture comprised 1 μL of the first PCR product and 37.0 μL of  
134 nuclease free water; also Lys-F2 and T-amp (5'-GCGAATTCGTCGACAAGC-3') primers were used and a  
135 final extension was carried out at 72°C for 20 min. Then, 5 μL of the final PCR product was analyzed  
136 by 1.5 % agarose gel electrophoresis (Biobasic Inc., Toronto, Canada) and a 450 bp amplified  
137 fragment was detected and purified by the PCR purification kit (Norgen Biotek Corporation, Canada)  
138 as per manufacturer's instructions. The purified products were cloned into pTZ57R/T vector with the  
139 InstAclone™ PCR Cloning Kit (Fermentas Life Sciences) for sequencing.

140 The 5'-terminal end of lysozyme cDNA was amplified by 5' RACE (5' RACE System for Rapid  
141 Amplification of cDNA Ends, Version 2.0, Invitrogen) and the gene-specific primers Lys-R1, Lys-R2,  
142 and Lys-R3, and AAP (5'-GGCCACGCGTCTGACTAGTACGGGATGGGATGGGATG-3'), AUAP (5'-GGCC  
143 ACGCGTCTGACTAGTAC-3'). The gene specific primers were designed based on the sequence obtained  
144 from 3' RACE. 5' RACE-PCR reactions were carried out according to the instructions of the  
145 manufacturer. Amplification conditions employed for dC tailed target cDNA were initial denaturation  
146 of 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min

147 and extension at 72°C for 1.5 min. Nested amplification were carried under the same conditions  
148 except that denaturation comprised 40 cycles and a final extension at 72°C for 20 min. The amplified  
149 5' RACE-PCR products were cloned into pTZ57R/T vector by InsTAclone™ PCR Cloning Kit (Fermentas  
150 Life Sciences. The PCR was carried out same way as the nested amplification with the primers AUAP  
151 and Lys-R3 except that 0.5 µL of plasmid was used as template and the final extension was 5 min  
152 instead of 20 min. We named *SgLys*, the lysozyme encoded by the cDNA.

153 Finally, the complete (end to end) sequence of *SgLys* was amplified by the specific primers Lys-  
154 tF1 and Lys-tR1 designed on the sequences obtained from 3' RACE and 5' RACE. The amplification  
155 was performed with the following program: Initial denaturation of 94°C for 2 min followed by 40  
156 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min.  
157 Final extension was carried out at 72°C for 20 min. The amplified complete coding sequence of *SgLys*  
158 was cloned into pTZ57R/T vector by InsTAclone™ PCR Cloning Kit (Fermentas Life Sciences), and  
159 sequenced.

160 All nucleotide sequences were determined by automated DNA sequencer 3037x/ DNA analyzer  
161 from Applied Biosystems using BigDye® Terminator v3.1 cycle sequencing Kit (Applied Biosystems)  
162 and T7 as forward primer and M13 as a reverse primer.

### 163 **Production of recombinant *S. gregaria* lysozyme**

164 The complete sequence encoding *SgLys* was amplified by PCR using specific primer pairs  
165 designed based on the sequences obtained from 3' RACE and 5' RACE. The restriction sites for EcoRI  
166 and NotI were introduced into the forward (*SgLysEcoRI*) and reverse (*SgLysNotI*) primers,  
167 respectively, as indicated in Supplementary Table S1. The amplification was performed in a Master  
168 cycler® Thermocycler (Eppendorf, Germany) using the following program: initial denaturation of 94°C  
169 for 2 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and  
170 extension at 72°C for 1 min. Final extension was carried out at 72°C for 20 min. The PCR product was

171 digested with EcoRI and NotI and separated by electrophoresis on 1.5 % agarose gel. The ~480 bp  
172 fragment was purified using PCR purification kit (Norgen Biotek Corporation, Canada) according to  
173 the manufacturer's instructions. The purified PCR product was cloned into EcoRI and NotI digested  
174 *Pichia pastori* expression vector pPIC9K (Invitrogen) to create in frame fusion of lysozyme gene with  
175  $\alpha$ -factor signal peptide. **The purpose of cloning full-length lysozyme (native SgLys) in *Pichia***  
176 **with alfa factor signal sequence (MF- $\alpha$  Prepro) is to improve secretion based on human**  
177 **lysozyme expression studies (Oka et al. 1999).** Representative clones containing complete  
178 coding sequence of lysozyme (pPIC9K-Lys) were confirmed by sequencing using  $\alpha$ -factor (5'-  
179 TACTATTGCCAGCATTGCTGC-3') and 3'AOX1 (5'-GCAAATGGCATTCTGACATCC-3') primers  
180 (Supplementary Figure S1). The plasmid pPIC9K-Lys was linearized by digestion with BglII and  
181 transformed into *P. pastoris* strain GS115 by spheroplasting method according to Invitrogen Pichia  
182 expression kit manual.

183 To check that the gene of interest was integrated into the *Pichia* genome, genomic DNA  
184 isolated from the recombinant *Pichia* GS115 was analyzed by PCR using either  $\alpha$ -factor/3'-AOX1  
185 primers or 5'-AOX1 (5'-GACTGGTTCCAATTGACAAGC-3')/3'-AOX1 primers. The expected size of  
186 amplicon for pPIC9K-Lys is ~972bp with  $\alpha$ -factor/3'-AOX1 primers and ~675bp with 5'-AOX1/3'-AOX1  
187 primers.

188 *P. pastoris* GS115 transformed with pPIC9K-SgLys were grown in BMGY medium at 30°C with  
189 shaking at 250 rpm (buffered glycerol complex medium) for 48 h. The cells were harvested by  
190 centrifugation at 10,000 for 5 min. To induce expression, the cells were resuspended in 1/5 the  
191 original volume of culture with BMMY medium (buffered methanol complex medium) and grown for  
192 4 days with shaking at 30°C. At every 24 h, 100% methanol was added to a final concentration of  
193 0.5% to maintain induction. The induced culture was centrifuged at 10,000 rpm for 30 min and the  
194 culture supernatant containing rSgLys was used (Supplementary Figure S2A).

**195 Antibacterial assay**

196 Activity of rSgLys was determined by the zone inhibition test as described in Mohamed et al.  
197 (2013). In this approach, a suspension of 1 mg lyophilized *Micrococcus luteus* (ATCC4698) cells  
198 (Sigma-Aldrich) in 50 mM phosphate buffer pH 8.0 mixed in 1% agarose was poured in Petri dishes. A  
199 well was done by puncture with an inverted Pasteur pipette, and known amounts of expressed  
200 rSgLys or, as control, hen egg white lysozyme (HEWL) were applied. Finally, the plates were  
201 incubated overnight at 37°C and the activity was recorded by measuring the diameter (mm) of the  
202 clear zone. Muramidase activity of rSgLys was detected (Supplementary Figure S2B).

**203 Sequence analysis**

204 Nucleotide sequence analysis and prediction of the open reading frame were performed with  
205 Genetyx software version 7.3.0 (GENETYX, Tokyo, Japan). The nucleotide homology search for the  
206 DNA sequence and protein homology search for the translated amino acids were performed by  
207 BLAST on the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequence alignment analysis was  
208 performed with the ClustalW program (Thompson et al., 1994) and BioEdit v7.1.3 program  
209 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). Phylogenetic analysis was performed with  
210 MEGA6 (<http://www.megasoftware.net>). The molecular weight and isoelectric point of the protein  
211 were estimated with the use of ProtParam tool (<http://www.expasy.org/tools/protparam.html>). The  
212 signal peptide was predicted by SignalP (<http://www.cbs.dtu.dk/services/SignalP/>). The domain  
213 search was performed by the CD-search in NCBI  
214 (<http://www.ncbi.nlm.nih.gov/structure/cdd/cdd.shtml>). For 3D analysis of SgLys, alignment of  
215 mature lysozymes without signal peptides was performed by ClustalW and *Bombyx* lysozyme (*BmLys*,  
216 PDB1G6A) was used as a suitable template in the homology modeling analysis using CPH models 3.0.

**217 SDS-PAGE and Western blotting**

218 Samples from the hemolymph of *E. coli*-immune challenged *S. gregaria* 5<sup>th</sup> instar nymphs  
219 collected at different time intervals, purified lysozyme from *S. gregaria* plasma (Mohamed et al.,  
220 2013) or rSgLys were subjected to 12% SDS-PAGE, according to Laemmli (1970)(Laemmli, 1970) and  
221 stained with Coomassie brilliant blue or transferred from the gel onto polyvinylidene difluoride  
222 membrane (Sigma-Aldrich) by electroblotting using semi-dry transfer cell (Bio-Rad) at 15 V for 1 h at  
223 room temperature in transfer buffer (25 mM Tris pH 8.3, 192 mM glycine, 20% (v/v) methanol). Non-  
224 specific binding sites on the PVDF membrane were blocked in a 5% skim milk in 1x Tris-buffered  
225 saline, 0.1% Tween 20 [TBS-T] solution and then incubated with a 1:2000 dilution of a polyclonal  
226 antiserum to SgLys (Elmogy et al., 2015) as primary antibody, in 1% skim milk–TBS–T for 90 min. Blots  
227 were washed with TBS–T containing 1% skim milk (TBS-T-M) and incubated with a 1:2000 dilution of  
228 a goat anti-rabbit horseradish peroxidase conjugated antibody (Sigma) as secondary antibody for  
229 another 90 min. Blots were washed three times in TBS–T-M. Finally, color development was carried  
230 out by adding a diaminobenzidine (DAB) solution (5 mg of 3,3'-diaminobenzidine (Sigma) in 100 mL  
231 of 50 mM Tris-HCl, pH 7.2) and 2  $\mu$ L of 30% H<sub>2</sub>O<sub>2</sub>. Control experiments were performed to check the  
232 specificity of the antiserum and that no cross reactive bands were found on the membrane  
233 (Supplementary Figure S2A).

#### 234 **Analysis of developmental and tissues expression of SgLys transcripts by RT-PCR**

235 Tissue expression patterns of SgLys was conducted on total RNA from cuticular epidermis  
236 (whole integument), fat body, hemocytes, midgut, salivary glands, and thoracic muscles by semi-  
237 quantitative RT-PCR. On the other hand, SgLys expression analysis during locust development was  
238 performed on total RNA from eggs, fat body tissues of different instars (1<sup>st</sup> to 5<sup>th</sup>), and adults (male  
239 and female) stages.

240 Briefly, total RNA (3  $\mu$ g extracted from each tissue) was used to synthesize cDNA with the  
241 RevertAid™ first Strand cDNA Synthesis Kit and an oligo (dT) primer (Fermentas Life Sciences). **The**

242 total RNA preparations were treated with DNase I (RNase-free, Thermo Fisher Scientific) in order  
243 to digest possible contaminating genomic DNA. The RT-PCR primers used were Lys-tF1 and Lys-tR2  
244 (Supplementary Table S1) which gave a PCR product of 479 bp. The PCR conditions consisted of an  
245 initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min,  
246 annealing at 56°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

247 The expression of the housekeeping gene  $\beta$ -actin (Genbank accession number: HQ851398) was  
248 used as an internal control with the forward primer 5'-GTGGGGCGCCCCAGGCACCA-3' and the  
249 reverse primer 5'-CTCCTTAATGTCACGCACGATTTC-3' and under the same PCR conditions as above.  
250 PCR products from three parallel experiments were separated on a 1.5% agarose gel. The gels were  
251 stained with ethidium bromide and the quantitation of RT-PCR produced band intensity was carried  
252 out in Image J software (<http://rsbweb.nih.gov/ij/download.html>). Finally, the *SgLys*/ $\beta$ -actin ratios  
253 were calculated.

## 254 Results and discussion

### 255 Structure of the *SgLys* gene

256 The full-length cDNA sequence was obtained by combining sequences of the 3'-RACE and 5'-  
257 RACE. The complete (end to end) nucleotides sequence and the deduced amino acids of *S. gregaria*  
258 lysozyme are presented in Figure 1. The results show that the cDNA is consisted of 659 bp. It contains  
259 untranslated sequences (UTR) in the 5' region (26 bp upstream from start codon) and in the 3' region  
260 downstream of stop codon at positions 453-659. The 3'UTR is ended with an 18 poly(A) tail. The  
261 5'UTR has moderate G+C content (65%), while the 3' UTR has low G+C content (41%). The sequence  
262 flanking the putative initiator codon follows the rule of eukaryotic initiation sites (Kozak, 1981).  
263 Hence, the first ATG at positions 27–29 is most probably the initiation codon. The TGA at positions  
264 450–452 was assigned as the termination codon, and thus an open reading frame (ORF) of 423 bp  
265 was predicted (Figure 1). The sequence contained a possible polyadenylation signal (AATAAA) at the

266 3' end (double underlined in Figure 1). The obtained cDNA sequence of *S. gregaria* lysozyme was  
 267 designated as SgLys, submitted to NCBI GenBank and allotted the accession number JQ012999.

### 268 **Characterization of the predicted *S. gregaria* c-type lysozyme**

269 The encoded amino acid sequence of SgLys was also deduced with the Genetyx-Win version  
 270 7.0.3 software. The SgLys gene contains a 423 bp ORF with a possible initiation codon at position 27-  
 271 29 of the nucleotide sequence which confirms our analysis above. The ORF encodes a 141 amino acid  
 272 sequence composed of 22 residues at the N-terminal side predicted as a signal peptide (bold letters  
 273 in Figure 1) and 119 residues as a mature peptide. The predicted 22 residues of the signal peptide are  
 274 mostly hydrophobic amino acids, a feature common to the signal peptides of both eukaryotes and  
 275 prokaryotes (von Heijne, 1990). Based on these predictions, the molecular weight and isoelectric  
 276 point of the full-length SgLys were estimated to be 15,702 Da and 9.09, respectively, **whereas the**  
 277 **molecular mass and Ip of mature lysozyme were 13.4 kDa and 9.2, respectively.** Also, the partial N-  
 278 terminal amino acid sequence of the biochemically purified lysozyme (Elmogly et al., 2015),  
 279 **KLQR\*EIVSALKRHHGITSDLRNWV\*LVESESGGRTDKRGPRNKNGSY** obtained by Edman degradation, is  
 280 closely matches the deduced amino acid sequence of the mature protein, based on the cDNA data  
 281 (underlined in Figure 1). **In Elmogly et al. (2015) some amino acids could not be determined during**  
 282 **the Edman degradation and therefore were supposed to be cysteines (\* in the above sequence).**

283 BLASTp search on NCBI (<http://blast.ncbi.nlm.nih.gov/>) using SgLys deduced amino acids  
 284 sequence as query showed that the SgLys has moderate homology (from 43% to 93% identities) to  
 285 several reported insects and ticks lysozymes. It has 62% amino acid identity with *Papilio xuthus* (Li et  
 286 al., 2015), 60% with *Manduca sexta* (Rosenthal and Dahlman, 1991), 59% with *Simulium nigricornum*,  
 287 57% with *G. mellonella*, *Samia cynthia ricini* (Fujimoto et al., 2001), *Hyalophora cecropia* (Engström et  
 288 al., 1985), *Ostrinia nubilalis* (Khajuria et al., 2011), and 55% with *Bombyx mori* (Matsuura et al., 2002)

289 and *O. furnicalis*. On the other hand, lysozymes from Orthoptera are highly conserved since the  
290 percent identity of *SgLys* with that of *Locusta migratoria* reaches 93%.

291 Alignment of the mature *SgLys* amino acid sequence with nineteen mature lysozymes from  
292 different insects and ticks was done using Clustal W and is shown in Supplementary Figure S3.  
293 According to the alignment, the *SgLys* has the 20 amino acid residues (asterisks in Supplementary  
294 Figure S3) highly conserved in the vast majority of c-type lysozymes (Fujita et al., 2002; Grunclová et  
295 al., 2003; Ursic-Bedoya et al., 2008) including the 8 cysteine residues, Cys<sup>6</sup>, Cys<sup>26</sup>, Cys<sup>61</sup>, Cys<sup>70</sup>, Cys<sup>74</sup>,  
296 Cys<sup>88</sup>, Cys<sup>108</sup> and Cys<sup>119</sup> (full circles in Supplementary Figure S3). The high conservation of the eight  
297 cysteine residues indicates their importance in the formation of disulfide bridges necessary for the  
298 stabilization of the molecule. Moreover, the alignment of the two Orthopteran lysozymes presented  
299 in Figure 2 shows that *SgLys* possesses all of the features of a c-type lysozyme with the two residues,  
300 Glu<sup>31</sup> and Asp<sup>49</sup> of the catalytic site (arrow heads in Figure 2 and Supplementary Figure S3), the 11  
301 residues Ser<sup>30</sup>, Glu<sup>31</sup>, Gly<sup>33</sup>, Gly<sup>40</sup>, Gln<sup>54</sup>, Asn<sup>56</sup>, Tyr<sup>59</sup>, Ile<sup>92</sup>, Arg<sup>95</sup>, Gly<sup>100</sup>, and Trp<sup>101</sup> that compose the  
302 lysozyme catalytic cleft (Figure 2, squares) and the residues Asn<sup>79</sup>, Asp<sup>84</sup> and Asp<sup>85</sup> of the Ca<sup>2+</sup> binding  
303 site (Figure 2, asterisks), a characteristic of most lactalbumin and few lysozymes (McKenzie, 1996).  
304 These three features form the conserved domain called LYZ1. Together with the two catalytic  
305 residues, Glu<sup>31</sup> and Asp<sup>49</sup> of the *SgLys*, the 8 cysteines are fundamental for the three dimensional  
306 structure and the biological activity of the lysozymes (Prager and Jolles, 1996). One major difference  
307 in the active site residues in terms of hydrophobicity between *SgLys* and the other c-type lysozymes  
308 occurs at Gly<sup>100</sup> (hydropathy index: -0.4) of *SgLys*, which is Ala (hydropathy index: 1.3) in all other  
309 lysozymes but that of *Reticulitermes speratus*.

310 The active site of HEWL (Kumagai et al., 1992) consists of 6 subsites from A to F which are able  
311 to bind 6 sugar residues (Maenaka et al., 1994; Maenaka et al., 1995). These subsites are present in  
312 the *SgLys* as Ser<sup>30</sup>, Gly<sup>33</sup>, Tyr<sup>59</sup>, Trp<sup>60</sup>, Arg<sup>95</sup>, and Trp<sup>101</sup> (Figure 2) and are believed to interact with  
313 sugar rings placed in the subsites E, F, B, C, A and D, respectively. However, the *SgLys* shows some

314 amino acids substitutions in these subsites when compare to HEWL. Indeed, the HEWL subsites have  
315 Phe<sup>44</sup>, Asn<sup>37</sup>, Trp<sup>62</sup>, Trp<sup>63</sup>, Asp<sup>101</sup> and Trp<sup>108</sup>, respectively. The substitutions of the conserved residues  
316 with others were supposed to modulate the enzymatic activity (Jolles and Jolles, 1984). For example,  
317 when Asn<sup>37</sup> and Trp<sup>62</sup> of HEWL were replaced by site-directed mutagenesis with Gly and Tyr,  
318 respectively, an enhanced bacteriolytic activity was observed (Kumagai et al., 1987; Kumagai and  
319 Miura, 1989). As a matter of fact, such substitutions occur naturally in *SgLys*, since the sequence of *S.*  
320 *gregaria* contains a Gly in position 33 and a Tyr in position 59, and indeed, *SgLys* exhibits about 2-3  
321 fold higher bacteriolytic activity than HEWL (Mohamed et al., 2013). This observation is in agreement  
322 with those reported in human and rat (Mulvey et al., 1973; Mulvey et al., 1974) showing the  
323 presence of Gly and Tyr of subsites F and B, respectively, and that these lysozymes exhibit higher  
324 bacteriolytic activity than HEWL (4-fold in the case of human lysozyme).

325  $\alpha$ -helical hairpin structures were reported in c-type lysozymes of chicken, equine, and human  
326 by computer-assisted structural analysis (Ibrahim et al., 2001; Nakajima and Kikuchi, 2007), and of *B.*  
327 *mori* (Matsuura et al., 2002), *O. furnicalis* (Wang et al., 2009) as well as in bactericidal and cytolytic  
328 pore-forming peptides (Andreu et al., 1985; Boman et al., 1991; Chen et al., 1988; Engelman and  
329 Steitz, 1981; Song et al., 1994; Song et al., 1991; Srisailam et al., 2000). They vary in length,  
330 conformation, net charge and in its distribution, and hydrophobicity, whose characteristics seem to  
331 be main factors leading to variations in their activities (Ibrahim et al., 2001). The helix-loop-helix  
332 (HLH) of HEWL comprises the amino acid residues 87-114, of which residues 87-100, 101-106 and  
333 107-114 form the N-terminal helix (H1), the loop (L), and the C-terminal helix (H2), respectively  
334 (Figure 3A). When the amino acid sequence of *SgLys* was aligned with the corresponding sequence of  
335 the HEWL HLH, homologies of 78%, 91% and 85% were revealed (Figure 3B). Moreover, comparison  
336 of a generated three dimensional structures of *SgLys* with that of *BmLys* (Matsuura et al., 2002),  
337 which shows 66.6% identity of active site residues with *SgLys* (Figure 3C), reveals the presence of an  
338  $\alpha$ -helical hairpin motif formed of two  $\alpha$ -helices joined by a loop stabilized by glycine (Figure 3D).

339 Pellegrini et al. (1997) reported that a peptide composed of residues 98-112 of HEWL, released from  
340  $\alpha$ -helical hairpin motif by digestion with clostripain (an ArgC-endoproteinase), was shown to have  
341 antimicrobial activity against both Gram-positive and Gram-negative bacteria and that the  
342 replacement of Asn<sup>106</sup> with a positively charged Arg generated an increased antimicrobial activity of  
343 this peptide. As a matter of fact, an arginine residue is naturally present in *SgLys* (as Arg<sup>99</sup>)(Figure 3B)  
344 and may also contribute in the observed elevation of *SgLys* antimicrobial activity over that of HEWL  
345 (Mohamed et al., 2013).

346  $\alpha$ -Helical hairpin of HEWL (Ibrahim et al., 2001) and of antimicrobial peptides (Hung et al.,  
347 1999; Srisailam et al., 2000; Wang et al., 1999) are active against both Gram-negative and Gram-  
348 positive bacteria by channel formation in the bacterial membrane. *SgLys* has two tryptophan  
349 residues Trp<sup>101</sup> and Trp<sup>104</sup> homologous to Trp<sup>108</sup> and Trp<sup>111</sup> of HEWL which are located in the C-  
350 terminal helix. When either Trp<sup>108</sup> or Trp<sup>111</sup> of HEWL was replaced with the less hydrophobic tyrosine,  
351 the antimicrobial activity was reduced (Pellegrini et al., 1997). In this respect, Ibrahim et al. (2001)  
352 suggested that the amphipathic N-terminal  $\alpha$ -helix could account for membrane insertion that places  
353 the C-terminal  $\alpha$ -helix (containing the tryptophan residues) close to the polar membrane interface.  
354 This could be supported by the higher hydrophobicity of the N-terminal  $\alpha$ -helix over that of the C-  
355 terminal one. The Trp residues are responsible for inducing functionally active conformation of the  
356 channel due to their specific preference for the interface (Cross et al., 1999; Hu et al., 1993; Kelkar  
357 and Chattopadhyay, 2007). The time-dependent rate of passage of the cytoplasmic  $\beta$ -galactosidase  
358 into the incubation medium (Elmogy et al., 2015) indicates a proceeding process of the inner  
359 membrane permeabilization by the *SgLys* which is most probably due to the referred insertion ability  
360 and pore formation.

361 ***SgLys* domains and signatures analysis**

362 The predicted *Sg*Lys mature protein was subjected to analysis using ScanProsite (release 20.79)  
363 to identify protein domains, families, and functional sites as well as associated patterns and profiles.  
364 The scan results indicated the existence of  $\alpha$ -lactalbumin/lysozyme C family profile (residues 1–119)  
365 and Alpha-lactalbumin/lysozyme C signature with the following consensus pattern C-x(3)-C-x(2)-  
366 [LMF]-x(3)-[DEN]-[LI]-x(5)-C (70-88). The scan also determined the positions of the four disulfide  
367 bridges to exist between the cysteine residues 6-119, 26-108, 61-74 and 70-88.

### 368 **Phylogenetic analysis**

369 The phylogenetic relationship of the *Sg*Lys with c-type lysozymes from different insect orders  
370 and ticks was inferred using the Neighbor-joining method (Saitou and Nei, 1987) to determine the  
371 closest associations among these lysozymes. The analysis conducted with MEGA6 was performed on  
372 amino acid sequences of mature lysozymes and HEWL was used as out-group. The data presented in  
373 Figure 4 show a general separation of lysozymes into two main groups likely based on their function.  
374 Lysozymes from *Lepidoptera* and *Nematocera* (including mosquitoes and black flies) of immune-  
375 related function are grouped together in a large clade while those from bugs and cyclorrhaphan flies,  
376 which are of digestive function and/or midgut origin, group together under two branches in another  
377 clade. In many nematocerans, lysozymes are found in the hemolymph but not in the gut (Lemos and  
378 Terra, 1991), and thus the recruitment of lysozymes as digestive enzymes, and their adaptation to an  
379 acidic midgut, may have occurred after the divergence of *Cyclorrhapha* from *Nematocera*. The  
380 mosquito lysozymes are expressed in the salivary gland (Kang et al., 1996; Moreira-Ferro et al., 1998)  
381 and it has been suggested that they might serve to control the microbial population ingested with a  
382 sugar meal (Rosignol and Lueders, 1986). This apparent dichotomy between recruitment of  
383 lysozyme for a digestive function (mainly in *Cyclorrhapha* flies) and as an immune peptide  
384 (*Lepidoptera*), was previously reported (Ursic-Bedoya et al., 2005; Ursic-Bedoya et al., 2008).

385 Tick lysozymes of immune-responsive function like those of the hard ticks *Amblyomma*  
386 *maculatum*, *Dermacentor andersoni*, and *Haemaphysalis longicornis* (Simser et al., 2004; Tanaka et  
387 al., 2010) are grouped together in a distinct clade whereas the lysozyme of the soft tick *Ornithodoros*  
388 *moubata* whose function has been described as digestive (Grunclová et al., 2003) separates in  
389 another major branch. Lysozymes from the coleopterans *Sitophilus zeamais* (Anselme et al., 2008)  
390 and *Tribolium castaneum* (ACV32411) are grouped together in their own clade. **In these latter, the**  
391 **multi-lysozyme domains, with the so-called H-branch of lysozymes share a histidine residue (H)**  
392 **which replaces the conserved tyrosine residue (Y) adjacent to the catalytic aspartic acid residue (D)**  
393 **in the majority of known c-type lysozymes (Beckert et al., 2015).**

394 The SgLys, whose activity is greatest in the fat body (Mohamed et al., 2013), is found clustered  
395 with the lysozyme of the other orthopteran, *Locusta migratoria*, in a clade of molecules whose  
396 function has been described as immune-related, including from distantly related *Lepidoptera* and  
397 *Diptera*. It is grouped with the subtree of the hymenopteran lysozymes, but in a separate branch, and  
398 both are diverging from the lepidopteran clade.

#### 399 **Expression of SgLys protein**

400 We have previously reported that a bacterial challenge with *E. coli* induced an increased  
401 lysozyme activity in the hemolymph of 5<sup>th</sup> instars larvae of *S. gregaria* and a maximum of activity was  
402 found 6 h after the injection of the bacteria (Mohamed et al., 2013). In the present study, by western  
403 blot analysis and the use of a polyclonal antiserum directed towards SgLys (Elmogly et al., 2015 and  
404 Supplementary Figure S2), a single protein band with a molecular weight of about 15.7 kDa was  
405 stained in the hemolymph of control larvae (not injected with *E. coli*)(Figure 5) which size  
406 corresponds to the lysozyme with its signal peptide still attached. **We suggest that this apparent**  
407 **molecular mass of around 16 kDa is likely due to an unusual migration of the protein upon**  
408 **reduction of disulphide bridges rather than of the non-cleavage of the signal peptide. A**

409 **phenomenon that was reported before by Koganesawa et al. (2001) for the recombinant *B. mori***  
410 **lysozyme.** As a matter of fact, Liu et al. (2004) reported the presence of two forms of lysozymes, one  
411 of 14.4 kDa and the other of 16.4 kDa, in salivary gland homogenates of *Helicoverpa zea*. These  
412 authors showed that the lower molecular weight form was the processed mature lysozyme while the  
413 higher molecular weight peptide was considered as the predicted unprocessed, unsecreted form of  
414 lysozyme which might be the case in our analysis for *SgLys*. Figure 5 also shows a remarkable change  
415 in the levels of *SgLys* protein in the hemolymph of 5<sup>th</sup> instars *S. gregaria* following injection with *E.*  
416 *coli*. The maximal protein level was detected 6 h post injection which is in agreement with our  
417 previous observation (Mohamed et al., 2013).

#### 418 **Developmental and tissue-specific transcriptional expression of *SgLys***

419 Transcriptional expression of *SgLys* at different developmental stages of *S. gregaria* was  
420 examined by semi-quantitative RT-PCR (semiQ RT-PCR) on total RNA from eggs or RNA from fat body  
421 for the different instars and the adults. The results are presented in Figure 6. They show a  
422 progressive and strong *SgLys* expression throughout the insect development with a gradual increase,  
423 starting from the 2<sup>nd</sup> instar up to the adult. Therefore, *SgLys* appears to be developmentally  
424 regulated, as this is the case for *M. sexta* (Russell and Dunn, 1991) and *H. armigera* (Zhang et al.,  
425 2009). In *Aedes aegypti*, the expression of LysE in the larval and pupal stages, as well as defensin  
426 expression in pupae, was supposed to prevent bacteria-induced septicaemia within these stages  
427 (Ursic-Bedoya et al., 2005). In *D. melanogaster*, LysX is only expressed in early pupae and was  
428 proposed to play a similar protective role (Daffre et al., 1994).

429 Transcriptional expression of *SgLys* was analyzed on different tissues by semiQ RT-PCR (Figure  
430 7). A constitutive expression of *SgLys* was found in epidermis, fat body and salivary glands but could  
431 not be detected in hemocytes and thoracic muscles. After injection of *E. coli*, *SgLys* transcripts were  
432 detected in all tissues but midgut with higher levels in epidermis, fat body and salivary glands.

433 Therefore, it appears that a bacterial challenge triggers an up-regulation of *SgLys* in a tissue-specific  
434 pattern. In the fat body, *E. coli* elicited a 2-fold increase in *SgLys* transcription 6 h post-injection.  
435 Consequently, the fat body, salivary gland, epidermis and hemocytes seem to be capable of  
436 discriminating the microbial stress factor. Substantial expression of *SgLys* in thoracic muscles was  
437 observed which may be due to a contamination of this sample with infiltrated hemocytes in this  
438 tissue at the site of injection. This phenomenon was reported during the course of lysozyme gene  
439 expression in *M. sexta*, following treatment with peptidoglycans (Mulnix and Dunn, 1994).

440 The expression profile of *SgLys* in the different immune-related tissues reported here is similar  
441 to that of other recognized immune lysozymes. Generally lysozymes are rapidly expressed in  
442 response to microbial infection in specific tissues, mainly fat body and hemocytes (Bulet and Stocklin,  
443 2005; Callewaert and Michiels, 2010; Fujita, 2004; Hultmark, 1996). In *M. sexta*, low levels of  
444 transcripts in fat body of non-infected larvae increased rapidly after treatment with peptidoglycans  
445 and remained elevated over several days (Mulnix and Dunn, 1994). These authors also reported a  
446 very low lysozyme transcript level in salivary glands of this insect. In contrast, *H. zea* salivary gland  
447 lysozyme is not only present at high transcription levels but is also one of the most abundant  
448 proteins in saliva (Liu et al., 2004).

449 **Whereas typical c-type immune-related lysozymes are usually basic enzymes with Ip around 8**  
450 **or higher, the recruitment of lysozymes for digestive purposes like the *Drosophila* midgut**  
451 **lysozymes is much related to their acidic Ip and/or pH optima of gut lumen (Hultmark, 1996).** In  
452 insects, lysozymes have a major role in immune responses to pathogens whereas other reports have  
453 identified them from larval and adult digestive tracts and from salivary glands, suggesting their role  
454 in digestion. **Taking together, c-type lysozymes have been postulated to display immunity- or**  
455 **digestion-related functions. For instance, bacterial-challenge of *H. axyridis* boosted the basic c-lys4**  
456 **(Ip= 8.18) gene expression 8-fold in the gut, whereas the acidic c-lys3 (Ip= 5.46) gene was**  
457 **expressed at comparable levels in both naïve and challenged beetles (Beckert et al. 2015). These**

458 **two *H. axyridis* c-type lysozymes possessing muramidase activity, may help to control the gut flora,**  
459 **and thus are likely to play an important role in the innate immune system.**

460 The data presented here suggest a defensive role of the lysozyme produced by fat body,  
461 salivary glands, thoracic muscles, hemocytes and epidermis in *S. gregaria*. *SgLys* is constitutively  
462 expressed and is up-regulated after bacterial challenge. It is expressed throughout all developmental  
463 stages, suggesting a key role for this gene in *S. gregaria* innate immunity. This is especially true in  
464 orthopterans when taking into account that no antimicrobial peptides, such as those inducible after  
465 the activation of the two signaling pathways Toll and Imd, have been reported yet in these insects  
466 with the exception of locustin (Accession number [P83428](#), Bulet et al., 2002, unpublished).

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## Figures legends

**Figure 1.** cDNA sequence (upper) and predicted amino acid sequence (one letter code, below) of *SgLys*. Numerical designation of the nucleotide and deduced amino acid sequences are shown on the left and right, respectively. Assigned initial and terminal codons are in bold-italic. The termination codon is marked with an asterisk. The bold face amino acid sequence indicates the predicted signal peptide whereas the N-terminal amino acid sequence confirmed by the Edman degradation method is single underlined. The putative polyadenylation signal (AATAAA) is bold-double underlined. The cDNA sequence has been deposited in GenBank with the accession number JQ012999.

**Figure 2.** Alignment of sequences from two orthopteran lysozymes, *Schistocerca gregaria* and *Locusta migratoria*. Amino acids that are different between the two sequences are boxed. The 11 residues that compose the lysozyme catalytic cleft on the conserved LYZ1 domain are indicated with black squares. The two residues, Glu<sup>31</sup> and Asp<sup>49</sup>, of the putative lysozyme catalytic site on conserved LYZ1 domain are marked with arrow heads. Asterisks indicate Ca<sup>2+</sup> binding site on conserved domain LYZ1.

**Figure 3.** (A) Ribbon representation of the fold of  $\alpha$ -helical hairpin, i.e. helix-loop-helix (HLH), motif of hen egg white lysozyme (HEWL). The secondary structural elements are as follows:  $\alpha$ -helix 1 (H1) Asp<sup>87</sup> to Ser<sup>100</sup> - loop (L) Asp<sup>101</sup> to Asn<sup>106</sup> -  $\alpha$ -helix 2 (H2) Ala<sup>107</sup> to Arg<sup>114</sup> (from Ibrahim et al., 2001). The tightness of the loop between the two helices is constrained by Gly<sup>102</sup> and Gly<sup>104</sup>. (B) Pairwise alignment of amino acid sequences of the  $\alpha$ -helical hairpin motifs of *SgLys*, *BmLys* with HEWL. Large bold letters represent the identical residues while the partially homologous ones are represented by large regular letters. To the right, percent identity of both *SgLys* and *BmLys* with HEWL. (C) Pairwise alignment of mature lysozymes of *SgLys* and *BmLys* without signal peptide using clustalW showing *BmLys* as a suitable template in CPH models. Black boxes are identical amino acids while grey boxes indicate conserved amino acids. (D) Three dimensional structures of *SgLys* and *BmLys* (PDB1GD6A) using CPH models-3.0.

**Figure 4.** Multiple phylogenetic analysis. 36 amino acid sequences of mature c-type lysozymes from different insect orders (Coleoptera, Diptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera and Orthoptera) and ticks (Ixodida) were aligned by ClustalW and a phylogenetic tree was constructed by Neighbor-Joining criteria using MEGA6. Only bootstrap values higher than 50% are indicated for each root. *Gallus gallus* lysozyme was used as the out-group. *Schistocerca gregaria* lysozyme is written bold face letters. The sequences used in this analysis are listed in Supplementary Table S2. Lysozymes with digestive function are underlined while full circles indicate acidic lysozymes.

**Figure 5.** Kinetics of expression of *SgLys* in 5<sup>th</sup> instar of *Schistocerca gregaria* after bacterial challenge with *Escherichia coli*. The lysozyme (15.7 kDa) was detected by western blot analysis using a polyclonal antiserum directed against *SgLys* (Elmogy et al., 2015). Hemolymph proteins were withdrawn at different time intervals post-injection with *E. coli* as indicated. Each lane contains 30  $\mu$ L of the hemolymph. C: non-immunized hemolymph (control).

**Figure 6.** Developmental expression of *SgLys*. RNA was extracted from fat body tissues of 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup>, 5<sup>th</sup> instars, male and female insects 6 h post-injection with *E. coli* beside egg homogenate. Statistical analysis of 3 parallel RT-PCR results of *SgLys* expression was performed. Error bars represent the SD of 3 independent PCR amplifications and quantifications (n= 3). Columns, with the same letter(s) are homogenous (not significantly different at  $p>0.05$ ), whereas those with different letters are significantly different at  $p<0.001$ . The relative expression level (was expressed as the ratio of *SgLys* RT-PCR product band intensity to that of the house keeping  $\beta$ -actin) calculated from the gel picture using Image J software.

**Figure 7.** Tissue expression of *SgLys*. Total RNA was extracted from the different tissues 6 h post-injection of bacteria and from native insects. (u) unchallenged larvae and (c) larvae injected with *E. coli*. He, hemocytes; Ep, epidermis; MG, midgut; FB, fat body; SG, salivary glands; TM, thoracic

muscles. To the left, DNA molecular size markers. The results shown are from 3 independent PCR amplifications.

**Supplementary Figure S1.** Sequence analysis of *SgLys* cloned in *Pichia pastoris* expression vector pPIC9K. **The primers used for the cloning of *SgLys* ORF are double-underlined. Bold/Italic:**  $\alpha$ -factor primer, **Italic/single-underlined:** 3'AOX1 primer.

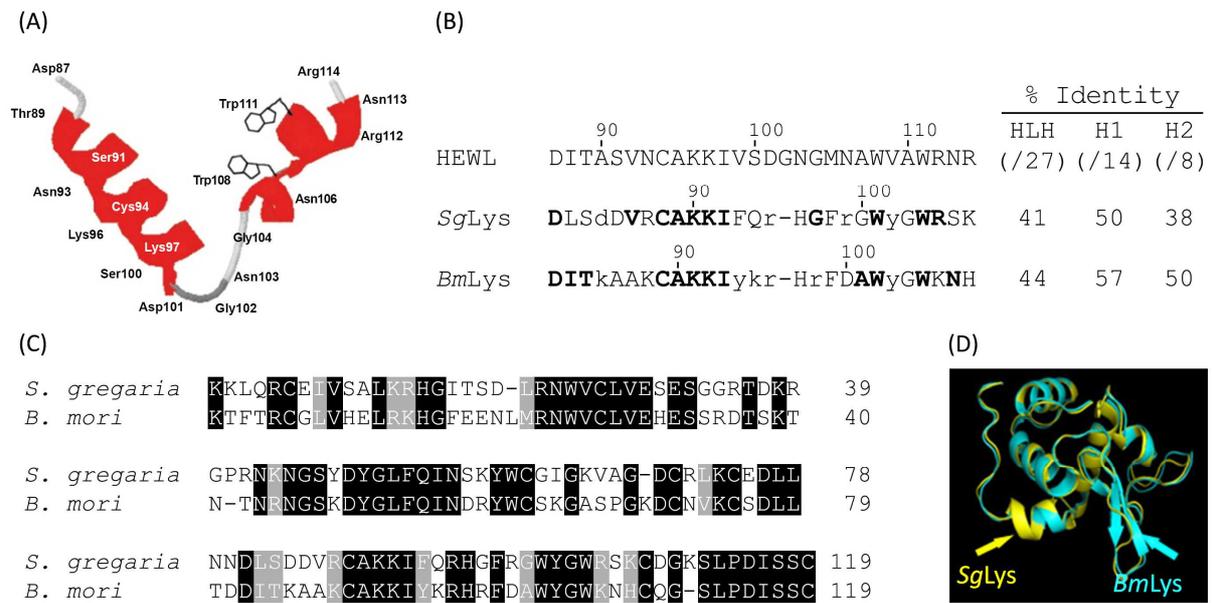
**Supplementary Figure S2.** Production of recombinant *SgLys*. (A) SDS-PAGE analysis of culture of *Pichia pastoris* GS115 transformed with pPIC9K-*SgLys* after 0 h, 24 h, 48 h, 72 h and 96 h of incubation (lanes 1 to 5) and Western blot of the purified r*SgLys* immune-stained with anti-*SgLys* polyclonal antiserum (lane 6). **Prior to SDS-PAGE, fractions (1-2 ml) were boiled for 5 min in denaturation buffer containing 65 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, and 5% (v/v) 2-mercaptoethanol.** (B) Zone inhibition assay showing lysozyme muramidase activity of r*SgLys* compared to that of hen egg white lysozyme (HEWL). (C) Cross reactivity of anti-*SgLys* polyclonal antiserum against the r*SgLys* as revealed by Ouchterlony double immune-diffusion assay. Center well (anti-*SgLys*), anti-*SgLys* polyclonal antiserum. Upper peripheral wells (r*SgLys*), purified r*SgLys*. Agarose plate was stained with Coomassie brilliant blue.

**Supplementary Figure S3.** Alignment of the deduced amino acid sequence of *S. gregaria* lysozyme with those of other insects and ticks (Supplementary Table S2). Sequences of mature proteins were aligned using ClustalW. Dashes indicate gaps inserted in the alignment. Numbers of amino acids are indicated to the right of the sequences. Asterisks indicate 20 conserved amino acid residues. The 8 conserved cysteine residues are marked by arrow heads and the two catalytic residues E and D with black squares. Signal peptides were predicted using SignalP 4.0 server and removed. The multiple sequence alignment was edited to define the sets of residues of high homology (shadowed).





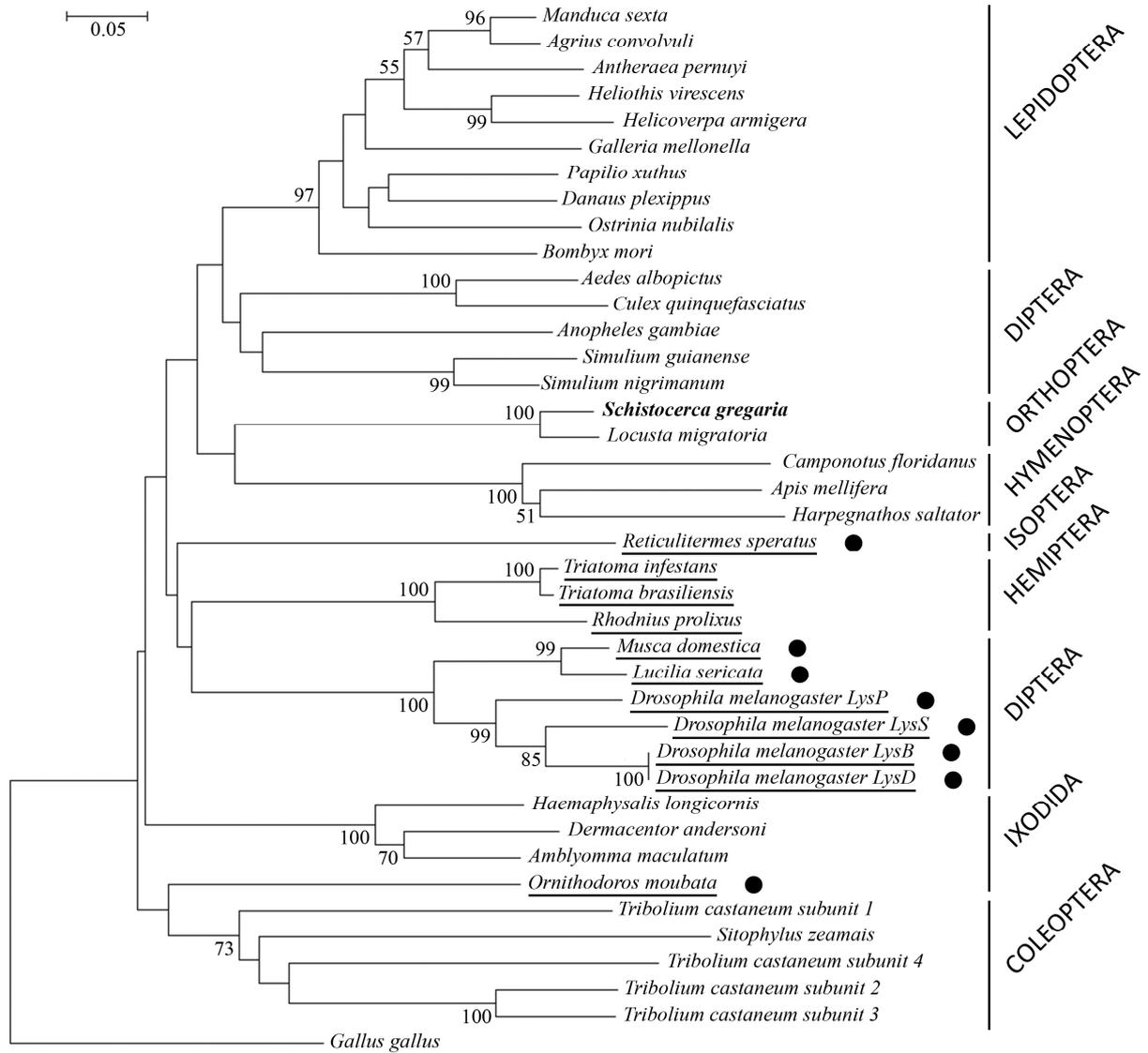
Figure 3



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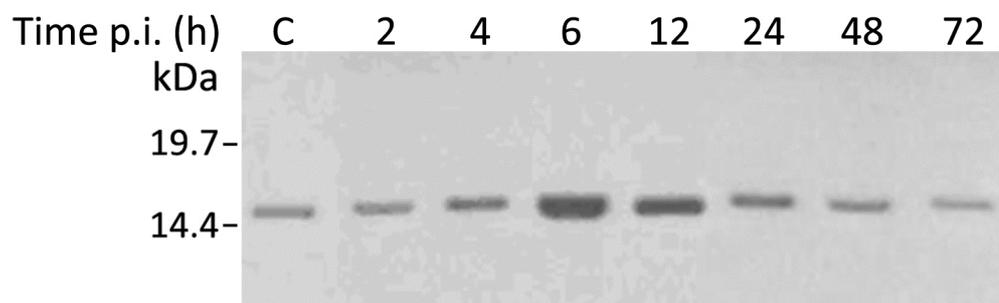
Figure 4



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Figure 5

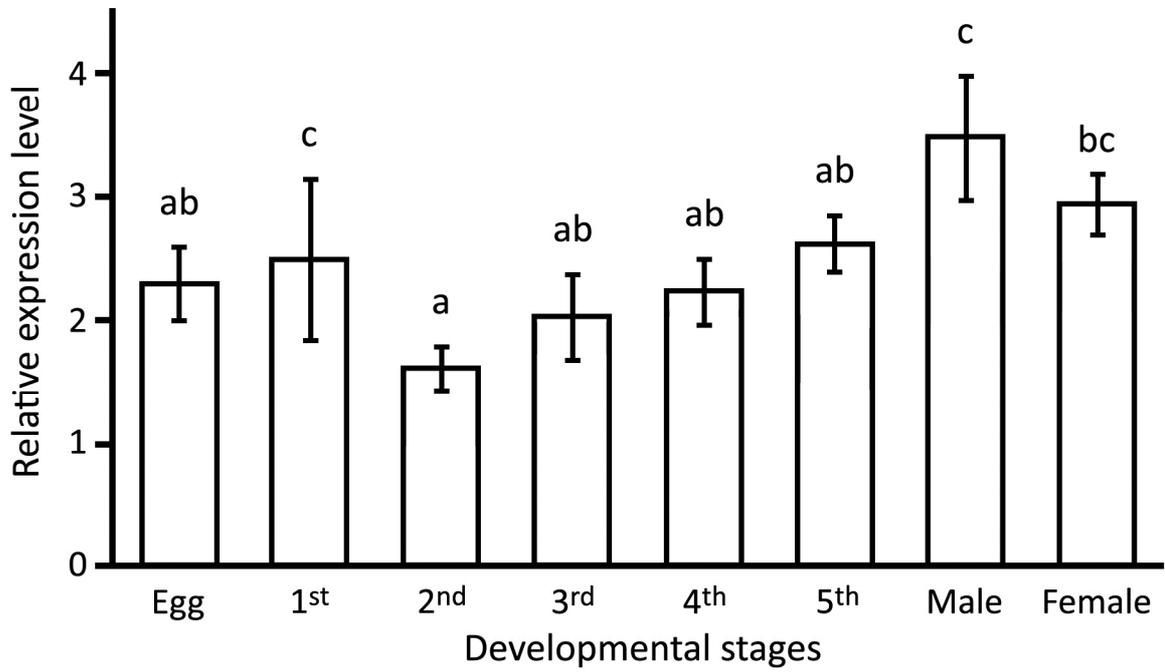


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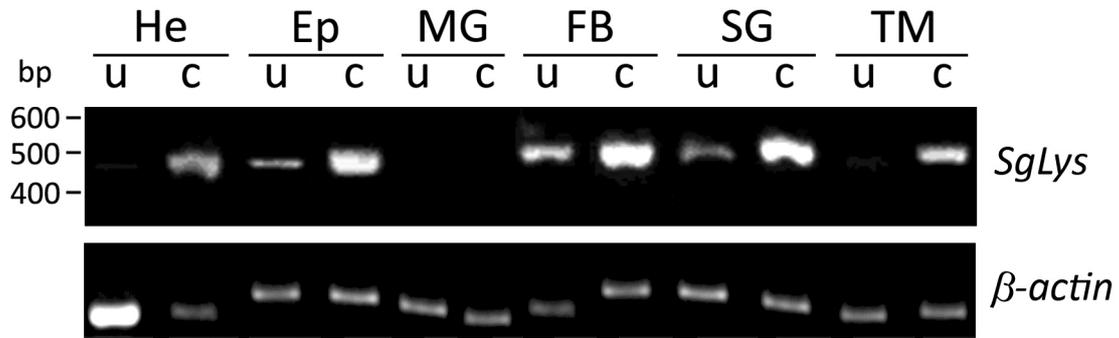
Figure 6



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Figure 7



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**Highlights**

- A lysozyme from fat body of the orthopteran *Schistocerca gregaria* was cloned.
- It contains all the features of a c-type lysozyme.
- *SgLys* is expressed at all developmental stages from egg to adults.
- *SgLys* is strongly upregulated in several tissues but midgut after *E. coli* injection.
- *SgLys* is likely a predominant acute-phase protein.

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