

The salivary gland salivation stimulating peptide from *Locusta migratoria* (Lom-SG-SASP) is not a typical neuropeptide

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The salivary gland salivation stimulating peptide was identified from the salivary glands of the migratory locust by its ability to stimulate cAMP production in the same tissue. The gene coding for this peptide has recently been identified and been shown to code for a precursor consisting of a signal peptide, several copies of the peptide separated by Lys-Arg doublets and a few other peptides. These data are consistent with it being a neuropeptide. However, antiserum raised to this peptide labels the acini of the salivary glands while RT-PCR only gives positive results in the salivary gland, but not in any ganglion of the central nervous system. Thus, this peptide is not a typical neuropeptide as previously assumed.

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20 Abstract

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22 The salivary gland salivation stimulating peptide was identified from the salivary glands
23 of the migratory locust by its ability to stimulate cAMP production in the same tissue. The gene
24 coding for this peptide has recently been identified and been shown to code for a precursor
25 consisting of a signal peptide, several copies of the peptide separated by Lys-Arg doublets and a
26 few other peptides. These data are consistent with it being a neuropeptide. However, antiserum
27 raised to this peptide labels the acini of the salivary glands while RT-PCR only gives positive
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36 Key words : SG-SASP ; PC1 ; PC2 ; RT-PCR ; antiserum ; immunohistology

37 1. Introduction

38

39 Insect neuropeptides are interesting for two different reasons. On the one hand, insects
40 are protostomes, while vertebrates are deuterostomes. Thus, comparing insect with vertebrate
41 neuropeptide regulatory systems provides insight as to how structures and functions may have
42 changed during evolution. On the other hand, many insect species are pests and vectors of
43 disease and thus responsible for serious agricultural damage as well as the transmission of human
44 disease. As insects are becoming increasingly resistant to classical pesticides novel insecticides
45 are constantly needed and it has repeatedly been suggested that agonists or antagonists of
46 insect neuropeptide receptors might offer a solution (*e.g.* Audsley et al., 2015 ; van Hiel et al.,
47 2010). Inhibition or at least disruption of feeding by insects would be very attractive as it would
48 presumably avoid or diminish economic damage, or in the case of disease vectors, might reduce
49 transmission of infectious agents. As production of saliva is usually a first and necessary step in
50 feeding the regulation of salivation by neuropeptides is particularly interesting.

51 A pentadecapeptide was isolated from the salivary glands of the migratory locust by its
52 ability to stimulate the production of cAMP in the same glands at concentration of 10^{-6} M
53 (Veelaert et al., 1995). As it also stimulates salivation (Veelaert et al., 1995), its characteristics
54 suggests it to be neuropeptide that likely acts as a neurotransmitter rather than a hormone. If it
55 were a hormone, one would expect it to stimulate the production of cAMP and salivation in the
56 nanomolar , rather than in the micromolar range and it would be expected in a neurohemal organ,
57 rather than in the salivary gland itself (Veelaert et al., 1995). Unlike most insect neuropeptides
58 orthologs of this peptide have not been identified from any other arthropod, suggesting that it
59 may not be universally present in insects. This might be advantageous as any pesticide based on
60 it could be relatively selective. The genome sequence combined with the RNAseq data of the
61 migratory locust (Wang et al., 2014) made it possible to predict a likely precursor for this peptide
62 (Veenstra, 2014). There is single amino acid mismatch between the predicted precursor and the
63 identified peptide, but the peptide sequence is bounded by putative convertase cleavage sites
64 (Fig. 1) and it is thus highly likely that this is indeed the precursor of this peptide. The precursor
65 has all the hallmarks of a typical neuropeptide precursor: a signal peptide and a propeptide
66 encoding multiple copies of the peptide separated by Lys-Arg convertase cleavage sites. It thus
67 appeared of interest to study this putative neuropeptide in more detail, but as reported here the

68 peptide turned out not to be a typical neuropeptide, in spite of the data suggesting otherwise.

69

70 **2. Materials and methods**

71

72 2.1 Locusts.

73 Adult and fifth instar *Locusta migratoria* were purchased at a local pet store. They were
74 kept for five to seven days at 25 °C and fed fresh grass once a day before being used. Tissues
75 were dissected under saline and either frozen immediately at -80 °C for subsequent RNA
76 extraction, or used for immunohistology.

77

78 2.2 Immunohistology

79 I choose the SVTVREVGDLFQEWLQGNVN sequence for making antisera, as this
80 sequence is present in multiple copies on the precursor (Fig. 1). Two mg (purity 84 %,
81 Proteogenix, Schiltigheim, France) were conjugated to 5 mg of bovine serum albumin using
82 difluorodinitrobenzene as the conjugation reagent as documented by Tager (Tager, 1976).
83 Polyclonal mouse antisera were raised in three six week-old NMRI female mice as described
84 previously (Veenstra and Ida, 2014). Tissues were fixed for 1 to 2 hrs at room temperature. All
85 other immunohistological procedures are the same as described (Veenstra and Ida, 2014).
86 Primary antiserum was diluted 1: 2,000, the secondary antiserum, DyLight-488-conjugated goat
87 anti-mouse IgG that was from Jackson ImmunoResearch Europe (Newmarket, Suffolk, UK),
88 1:1,000.

89

90 2.3 Bioinformatics.

91 cDNA sequences coding for the *L. migratoria* orthologs of vertebrate PC1 and PC2 are
92 not present in the databases and were therefore obtained by using a combination of the published
93 genome sequence and Trinity on sequences extracted from the various short read archives
94 (SRAs) for this species available at NCBI (SRR014351, SRR014352, SRR058432, SRR058446,
95 SRR058447, SRR058448, SRR058449, SRR058450, SRR058451, SRR058452, SRR058453,
96 SRR058454, SRR058455, SRR058456, SRR058457, SRR058458, SRR058488, SRR058489,
97 SRR058490, SRR058491, SRR058492, SRR058493, SRR058494, SRR058495, SRR058496,
98 SRR058497, SRR058498, SRR058499, SRR058500, SRR058501, SRR058502, SRR058503,

99 SRR1032161, SRR1032192, SRR167712, SRR513208, SRR513209, SRR513210 and
100 SRR513211) using methodology described in detail elsewhere (Veenstra, 2016). Protein and
101 cDNA sequences for *L. migratoria* PC1 and PC2 are provided in the supplementary data.

102

103 2.4 RT-PCR

104 The following tissues were dissected : brain, suboesophageal ganglion, pro- and meso-
105 thoracic ganglia combined, the meta-thoracic ganglia combined with all abdominal ganglia,
106 salivary gland, fat body and midgut. For the analysis of the expression of PC1 and PC2
107 Malpighian tubules and flight muscle were also analyzed. At least two samples were processed
108 completely independently for each of these tissues. Each sample containing tissues from at least
109 four different animals. From salivary glands, fatbody, Malpighian tubules and flight muscle,
110 small parts were taken from four different animals. For the midgut four longitudinal half midguts
111 were processed individually. RNA extraction was performed using mini spin columns from
112 Macherey-Nagel. Next RNA (800-1000 ng) was reverse transcribed in a 20 µl reaction using
113 Moloney Murine Leukemia Virus Reverse Transcriptase (New England Biolabs, Evry, France)
114 and random primers. One µl of the resulting cDNA was next amplified by PCR using OneTaq
115 Quick-Load DNA Polymerase (New England Biolabs) with specific primers for each mRNA.
116 Primers used are : for the salivary gland salivation stimulating peptide: 5'-
117 GCCTTCCTGCTAGTCGTCTG-3 and 5'-TACCTTTTGCCCACTCTTGG-3'; for actin: 5'-
118 AGGGCTGTTTTCCCTCAAT-3' and 5'-GAAGGTGTGGTGCCAGATTT -3'; for PC1: 5'-
119 ACAACCACGTGCACAAGAAG-3' and 5'-TGAATGCGACTAAGCCACAG -3' ; and for PC2:
120 5'-GGTGGACTACCTGGAACACG-3' and 5'-TGTGGATATTCTCCCCAGGT -3' . PCR
121 profiles consisted of 90 second denaturing at 94°C followed by 32 cycles of 30 seconds at 94°C,
122 15 seconds at the annealing temperature and 15 seconds at 68°C the amplification was followed
123 by 5 min extension at 68°C. Annealing temperatures were 55°C, 64°C, 64°C and 60°C for the
124 salivary gland salivation stimulating peptide, actin, PC1 and PC2 respectively. Controls in which
125 water replaced the cDNA showed no PCR amplification.

126

127 3. Results and Discussion

128

129 I anticipated that an antiserum would allow the identification of neurons expressing this

130 peptide. However all three mice produced antisera that labeled the acinar cells of the salivary
131 glands (Fig. 2), while immunoreactive material was completely absent from the central nervous
132 system. Preimmune antisera from the same mice or diluted antisera that were absorbed with
133 SVTVREVGDLFQEWLQGNVN (over night, 10 μ g/ml) were not immunoreactive (Fig. 3), thus
134 showing that the observed immunoreactivity is specific. This suggested that the peptide was
135 made by the salivary gland itself, rather than by the nervous system. Intron spanning primers
136 were designed to look for expression of the gene coding the peptide and results revealed
137 amplification only in the salivary glands (Fig. 4). The PCR-amplified band was sequenced using
138 the primers for amplification and the sequencing results confirmed the expected sequence (Fig.
139 S1). Thus there is independent confirmation that this gene is expressed in the salivary gland, but
140 neither in the central nervous system, the fatbody nor the midgut. RT-PCR confirmed strong
141 expression of this gene as (1) amplicons become visible after 20 cycles (Fig. 4) and (2) the
142 slightest contamination of thoracic ganglia with a small piece of salivary gland (these tissues are
143 closely associated with one another) leads to false positives. The integrity of the cDNA samples
144 were checked by looking for expression of actin as a control.

145 The Lys-Arg convertase cleavage sites in the precursor are identical to those commonly
146 found in neuropeptide precursors and thus suggested the presence of a neuropeptide specific
147 convertase in the salivary gland. The cDNA sequences of the two locust orthologs of vertebrate
148 PC1 and PC2 were obtained using Trinity on the various short read archives (SRAs) for this
149 species available at NCBI (Fig. S2) and intron spanning primers were designed to look for their
150 expression in the salivary gland. PC1 was found not only in the salivary gland, but also in other
151 peripheral tissues (Fig. 5), but PC2 was absent from the salivary glands. It thus appears that the
152 tissue distribution of PC1 in migratory locusts and perhaps other arthropods is much broader than
153 in vertebrates (Seidah et al, 2013).

154 A question that remains to be answered is what the function of this peptide might be.
155 Salivary glands typically secrete digestive enzymes in an exocrine fashion, but in principle, it
156 could also be secreted on the basolateral membrane into the hemolymph. Only the latter
157 possibility could explain how it could stimulate the salivary glands to produce cyclic AMP. In
158 spite of this and the precedent for autocrine stimulation by dopamine in the tick salivary gland
159 (Kočič, Šimo & Park, 2014), there are several arguments to suggest that the peptide is secreted as
160 an exocrine rather than an autocrine or paracrine. First, the situation in ticks remains highly

161 unusual. Secondly, in the tick dopamine is made only in a minor cell type, while SASP is made
162 in the major cell type. Thirdly, SASP is made as a protein precursor and as such should be
163 expected to be co-secreted with the digestive enzymes in the same granules (this is not the case
164 for dopamine, which is present in different granules). It would be very surprising if such
165 digestive enzyme containing granules would be secreted into the hemolymph. Fourth, although
166 dopamine in the tick salivary gland is present in relatively high concentrations, it remains a
167 minor product, while SASP as suggested by the immunohistological data as well as the RT-
168 PCR seems to be a major product of the salivary gland. Fifth, it is to be expected that the Lys-
169 Arg cleavage sites in the SASP precursor are cleaved efficiently, however for the peptide
170 identified by Veelaert et al. (1995) to be produced, this site needs to be still there, for a single
171 Arg residue is not easily cleaved by convertase. Hence, relatively small amounts of the active
172 peptide are expected to be made, while the likely much larger amounts of the peptide used here
173 as antigen, were not identified as a stimulator of cyclic AMP production. This seems to suggest
174 that the stimulation of cyclic AMP production is an accidental artifact, as perhaps also suggested
175 by the high concentrations needed of this peptide to do so.

176 Even if this may suggest an exocrine function for this peptide, it does not answer the
177 question what this function entails. As saliva is generally reabsorbed with the food, its tempting
178 to speculate that it somehow facilitates digestion or perhaps functions

179 In the last decade a large number of putative neuropeptide genes have been identified in
180 genome sequences of a wide variety of invertebrate species, sometimes based exclusively on the
181 presence of signal peptide and Lys-Arg cleavage sites that separate the presumptive
182 neuropeptides in the precursor. In many cases the predicted peptides show clear homology to
183 known neuropeptides, while in other cases they have subsequently been shown to activate G-
184 protein coupled receptors (*e.g.* Bauknecht et al., 2015). However, as illustrated here the presence
185 of a signal peptide, reputable convertase cleavages sites and multiple copies of the same or a
186 very similar peptide do not make a neuropeptide precursor.

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188 4. References

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Figure 1

Conceptual translation of the of the predicted open reading frame of SG-SASP from *Locusta migratoria*

Conceptual translation of the SASP mRNA predicted from transcriptome and genome sequences (Veenstra, 2016). Amino acid sequences highlighted in yellow indicate the signal peptide, Lys-Arg doublets highlighted in red are predicted convertase cleavage sites. In blue are the multiple copies of SVTVREVGDLFQEWLQGNVN, the major peptide encoded by the SASP gene. The amino acid sequence obtained by Veelaert et al. (1995) is not present in this sequence, but there is a single copy of EVGDLFEEWLQGNMN that is highlighted in black. The molecular mass of the EVGDLFEEWLQGNMN differs by less than 1 dalton from that of EVGDLFKEWLQGNMN. It seems plausible that an incorrect interpretation of the Edman degradation is responsible for the difference between the peptide sequence as experimentally determined and that deduced from the various DNA sequences. Similar minor differences were found for other *Locusta* neuropeptides (Veenstra, 2014).

EVGDLFEEWLQGNMN is predicted to be cleaved from its precursor by typical neuropeptide convertases. The Lys-Arg doublet at its C-terminal in the precursor is the canonical neuropeptide precursor cleavage site, while cleavage at the single Arg residue at its N-terminal is supported by the presence of another Arg residue at the -6 position (Veenstra, 2000). The latter Arg residue is part of another Lys-Arg doublet, that is the preferred convertase substrate and should thus be expected to be cleaved rapidly on exposure of the precursor to convertase. Once this site has been cleaved, the single Arg residue can no longer be cleaved. Consequently, it must be expected that little of EVGDLFEEWLQGNMN will be produced as most of the time the Lys-Arg site will be processed preferentially and once this has occurred there is no longer an Arg residue in position -6 to support cleavage at the single Arg residue. Based on the precursor sequence and known convertase cleavage preferences in insects (Veenstra, 2000) one would expect it be processed mostly into

SVTVREVGDLFQEWLQGNVN, the peptide used for raising an antiserum.

MALKTLAAFLLVVCLAQLTCASPAPAPNRRSTDESTADSSSATVTTSANNATS
DADTISSNDTSSDDIFGEIGEKVEGFFGHLFGKRDIIEIPSDVFTKLYQEWAKG
RPSRSVTVRDTGKNFTVGDLFQEWLQGNVNRKRSVTVREVGDLFQEWLQGNVNRK
RSVTVREVGDLFQEWLQGNVNRKRSVTVREVGDLFQEWLQGNVNRKRSVTVREVG
DLFQEWLQGNVNRKRSVTVREVGDLFQEWLQGNVNRKRSVTVREVGDLFQEWLQ
GNVNRKRSVTVREVGDLFQEWLQGNVNRKRSVTVREVGDLFQEWLQGNVNRKRSVTV
REVGDLFQEWLQGNVNRKRSVTVREVGDLFQEWLQGNVNRKRSVTVRDTDNSSTM
GDLIKEWLKGNLNERSVTVRDTGKNFTVGELFQEWLQGNVNRKRSVTVRDTDNS
STMNERSVTV

Figure 2

Immunohistological localization of SG-SASP.

Immunoreactive salivary gland peptide in the adult salivary gland of *L. migratoria*. Note the strong labeling in the acinar cells. Scale bar 250 μm .

*Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.

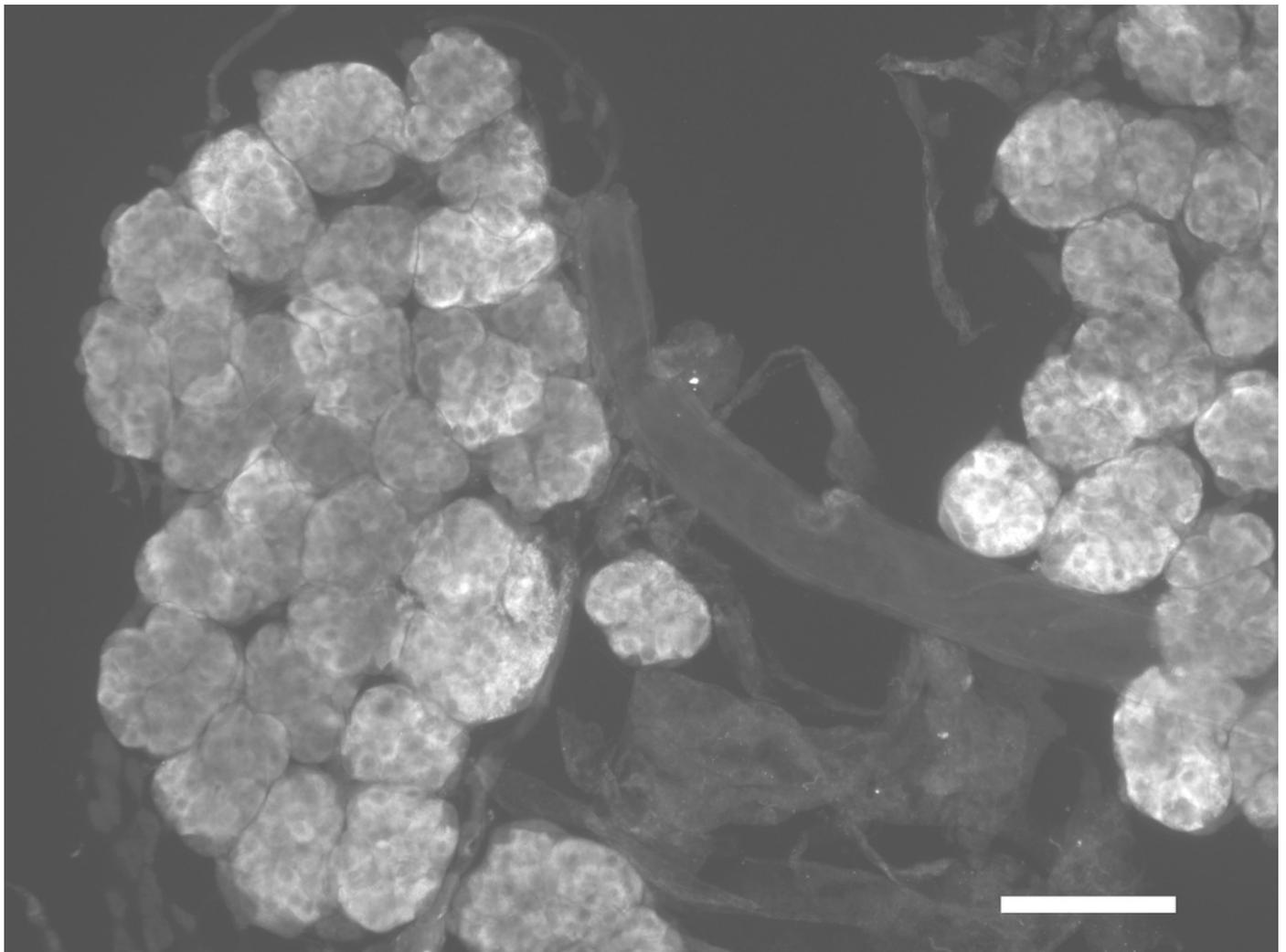


Figure 3

Antiserum specificity

Different pieces of the same salivary gland were exposed to pre-immune serum (a), immune serum (b), or immune serum preabsorbed with SVTVREVGDLFQEWLQGNVN. Note that no immunoreactivity is found in the preimmune serum and the immunoreactivity is abolished after preabsorption to SG-SASP.

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

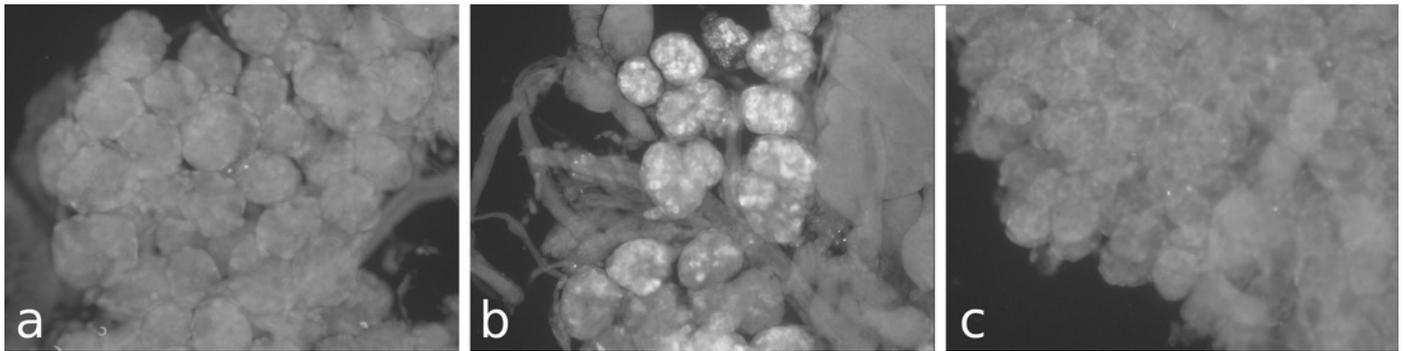


Figure 4

RT-PCR of the salivary gland peptide.

RT-PCR on different tissues from *L. migratoria* for the expression of SG-SASP and actin.

Numbers indicate the number of PCR cycles employed. Note that 20 cycles is sufficient to show expression in the salivary gland, while 35 cycles do not show any expression in the nervous system. TG 1 & 2, the pro- and meso-thoracic ganglion combined, TG3 & AGs the methathoracic ganglion together with all the abdominal ganglia. Complete gels with markers are shown in the supplementary data.

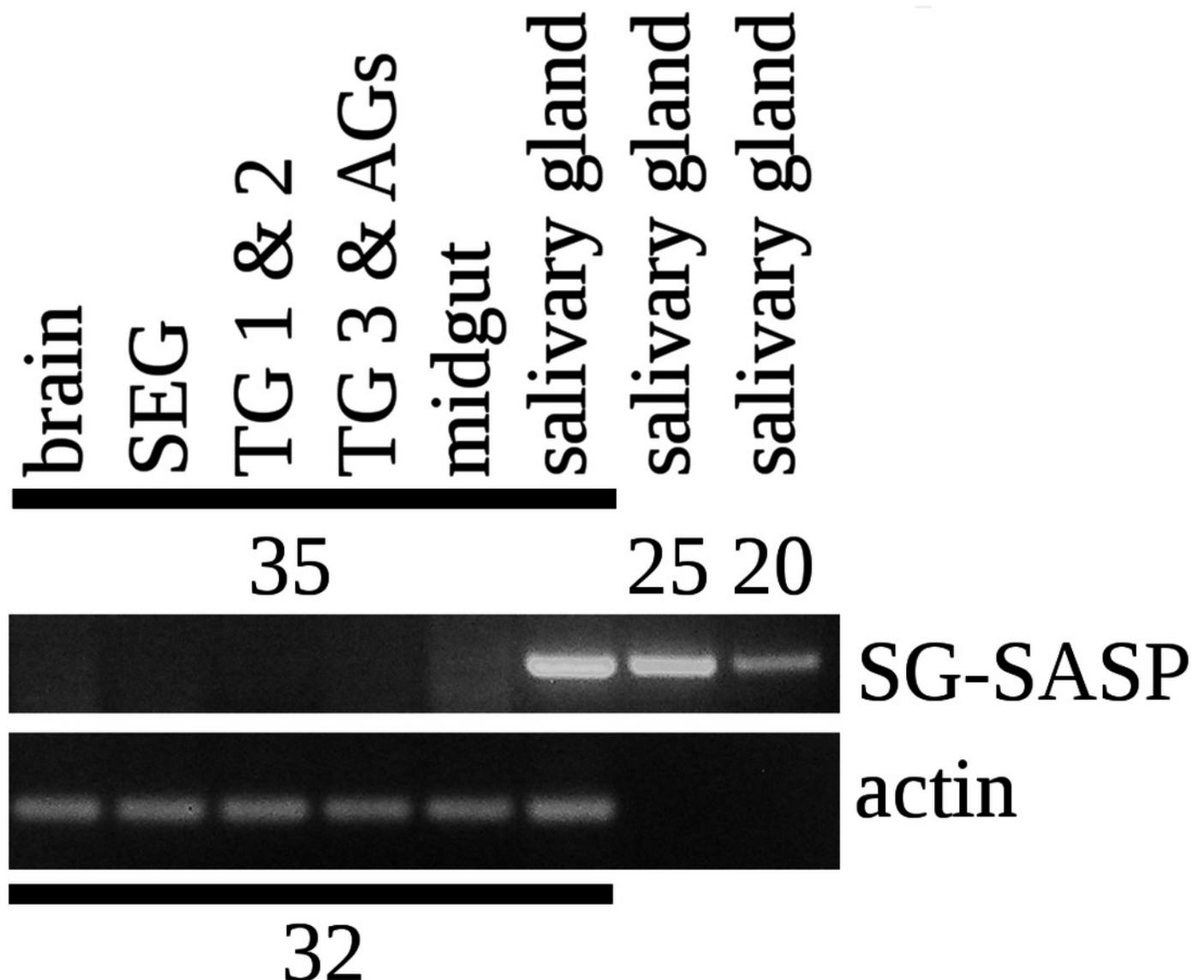
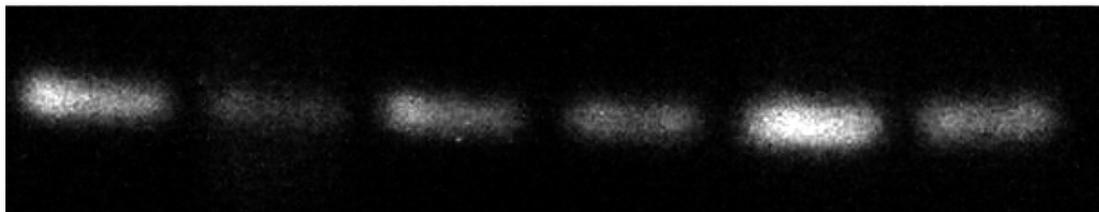


Figure 5

RT-PCR of PC1 and PC2.

RT-PCR on different tissues from *L. migratoria* for the expression of PC1 and PC2 convertaes with actin as a control. Note that PC1 is widely expressed, including in the salivary gland, while the expression of PC2 is much more limited and not expressed in the salivary gland. Thirty two cycles of PCR in each sample. Complete gels with markers are shown in the supplementary data.

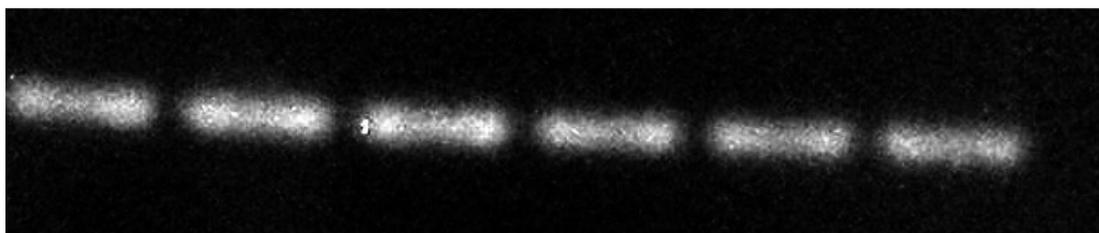
brain
midgut
Malpighian tubules
fat body
salivary gland
flight muscle



PC1



PC2



actin