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

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INTRODUCTION

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

A pentadecapeptide was isolated from the salivary glands of the migratory locust by its ability to stimulate the production of cAMP in the same glands at concentration of 10^{-6} M (Veelaert et al., 1995). As it also stimulates salivation (Veelaert et al., 1995), its characteristics suggests it to be neuropeptide that likely acts as a neurotransmitter rather than a hormone. If it were a hormone, one would expect it to stimulate the production of cAMP and salivation in the nanomolar , rather than in the micromolar range and it would be expected in a neurhemal organ, rather than in the salivary gland itself (Veelaert et al., 1995). Unlike most insect neuropeptides orthologs of this peptide have not been identified from any other arthropod, suggesting that it may not be universally present in insects. This might be advantageous as any pesticide based on it could be relatively selective. The genome sequence of the migratory locust (Wang et al., 2014) showed that the predicted precursor encoding this peptide has all the hallmarks of a classical neuropeptide precursor: a signal peptide and a propeptide encoding multiple copies of the peptide separated by Lys-Arg convertase cleavage sites (Veenstra, 2014). It thus appeared of interest to study this putative neuropeptide in more detail, but as reported here the peptide turned out not to be a neuropeptide after all, in spite of the data suggesting otherwise.

MATERIALS & METHODS

2.1 Locusts.

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

2.2 Immunohistology

Two mg of SVTVREVGDLFQEWLQQNVN (purity 84 %, Proteogenix, Schiltigheim, France) were conjugated to 5 mg of bovine serum albumin using difluorodinitrobenzeze as the conjugation reagent as documented by Tager (Tager, 1976). Polyclonal mouse antisera were raised in three six week-old NMRI female mice as described previously (Veenstra and Ida, 2014). Tissues were fixed for 1 to 2 hrs at room temperature. All other immunohistological procedures are the same as described (Veenstra and Ida, 2014). Primary antiserum was diluted 1: 500, the secondary antiserum, DyLight-488-conjugated goat anti-mouse IgG that was from Jackson ImmunoResearch Europe (Newmarket, Suffolk, UK), 1:1,000.

2.3 Bioinformatics.

cDNA equences coding for the *L. migratoria* orthologs of vertebrate PC1 and PC2 are not present in the databases and were therefore obtained by using a combination of the published genome sequence and Trinity on sequences extracted from the various short read archives (SRAs) for this species available at NCBI (SRR014351, SRR014352, SRR058432, SRR058446, SRR058447, SRR058448, SRR058449, SRR058450, SRR058451, SRR058452, SRR058453, SRR058454, SRR058455, SRR058456, SRR058457, SRR058458, SRR058488, SRR058489, SRR058490, SRR058491, SRR058492, SRR058493, SRR058494, SRR058495, SRR058496, SRR058497, SRR058498, SRR058499, SRR058500, SRR058501, SRR058502, SRR058503, SRR1032161, SRR1032192, SRR167712, SRR513208, SRR513209, SRR513210 and SRR513211) using methodology described in detail elsewhere (Veenstra, 2016). Protein and cDNA sequences for *L. migratoria* PC1 and PC2 are provided in the supplementary data.

2.4 RT-PCR

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

RESULTS & DISCUSSION

I anticipated that an antiserum would allow the identification of neurons expressing this peptide. However all three mice produced antisera that labeled the acinar cells of the salivary glands (Fig. 1), while immunoreactive material was completely absent from the central nervous system. This suggested that the peptide was made by the salivary gland itself, rather than by the nervous system. Intron spanning primers were designed to look for expression of the gene coding the peptide and results revealed amplification only in the salivary glands (Fig. 2). The PCR-

amplified band was sequenced using the primers for amplification and the sequencing results confirmed the expected sequence (Suppl. Data). Thus there is independent confirmation that this gene is expressed in the salivary gland, but neither in the central nervous system, the fatbody nor the midgut. RT-PCR confirmed strong expression of this gene as (1) amplicons become visible after 20 cycles (Fig. 2) and (2) the slightest contamination of throcacic ganglia with a small piece of salivarygland (these tissues are closely associated with one another) leads to false positives. The integrity of the cDNA samples were checked by looking for expression of actin as a control.

The Lys-Arg convertase cleavage sites in the precursor are identical to those commonly found in neuropeptide precursors and thus suggested the presence of a neuropeptide specific convertase in the salivary gland. The cDNA sequences of the two locust orthologs of vertebrate PC1 and PC2 were obtained using Trinity on the various short read archives (SRAs) for this species available at NCBI (Suppl. Data) and intron spanning primers were designed to look for their expression in the salivary gland. PC1 was found not only in the salivary gland, but also in other peripheral tissues (Fig. 3), but PC2 was absent from the salivary glands. It thus appears that the tissue distribution of PC1 in migratory locusts and perhaps other arthropods is much broader than in vertebrates (Seidah et al, 2013).

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

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Figure 1

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.*

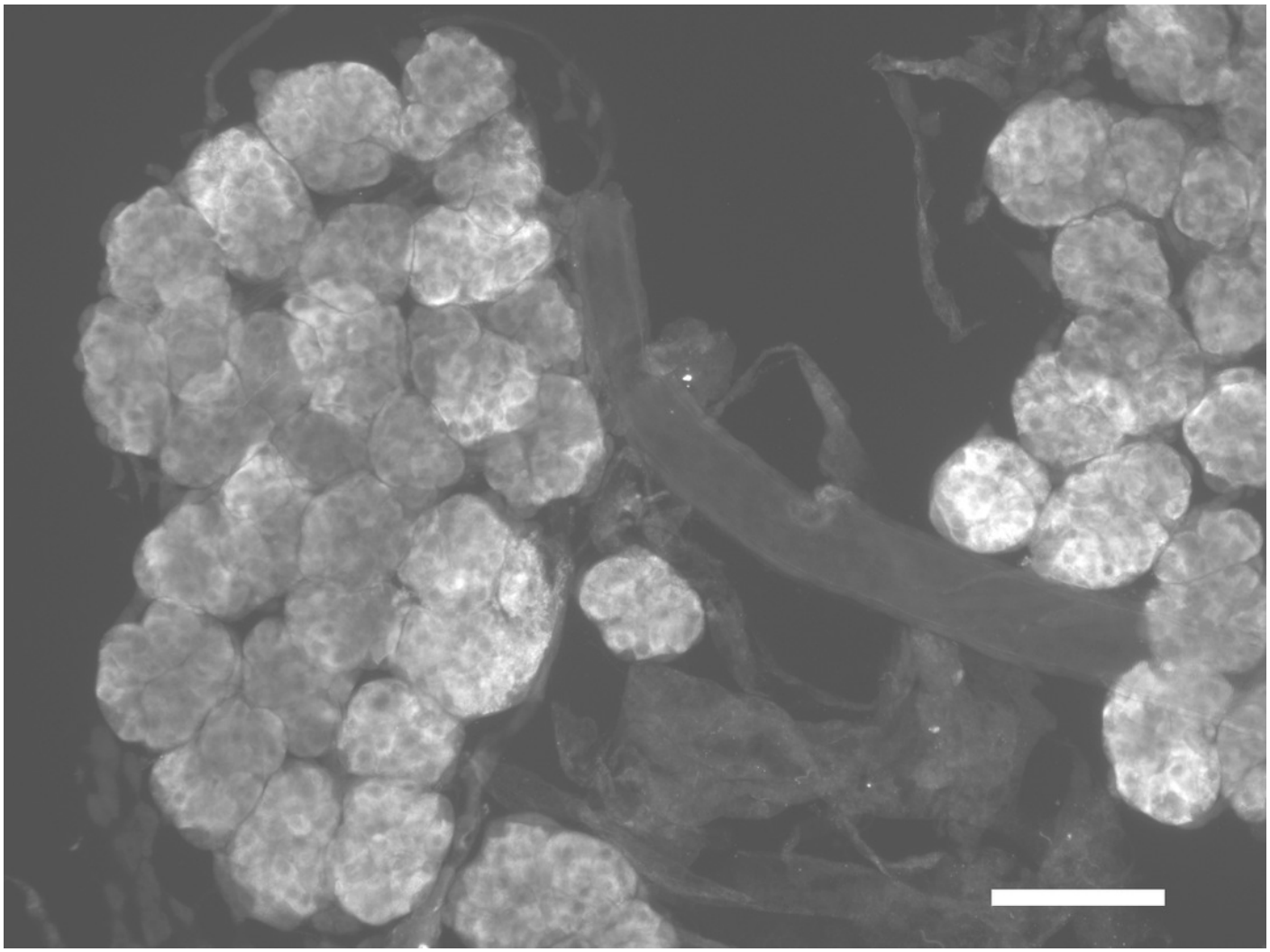


Figure 2

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.

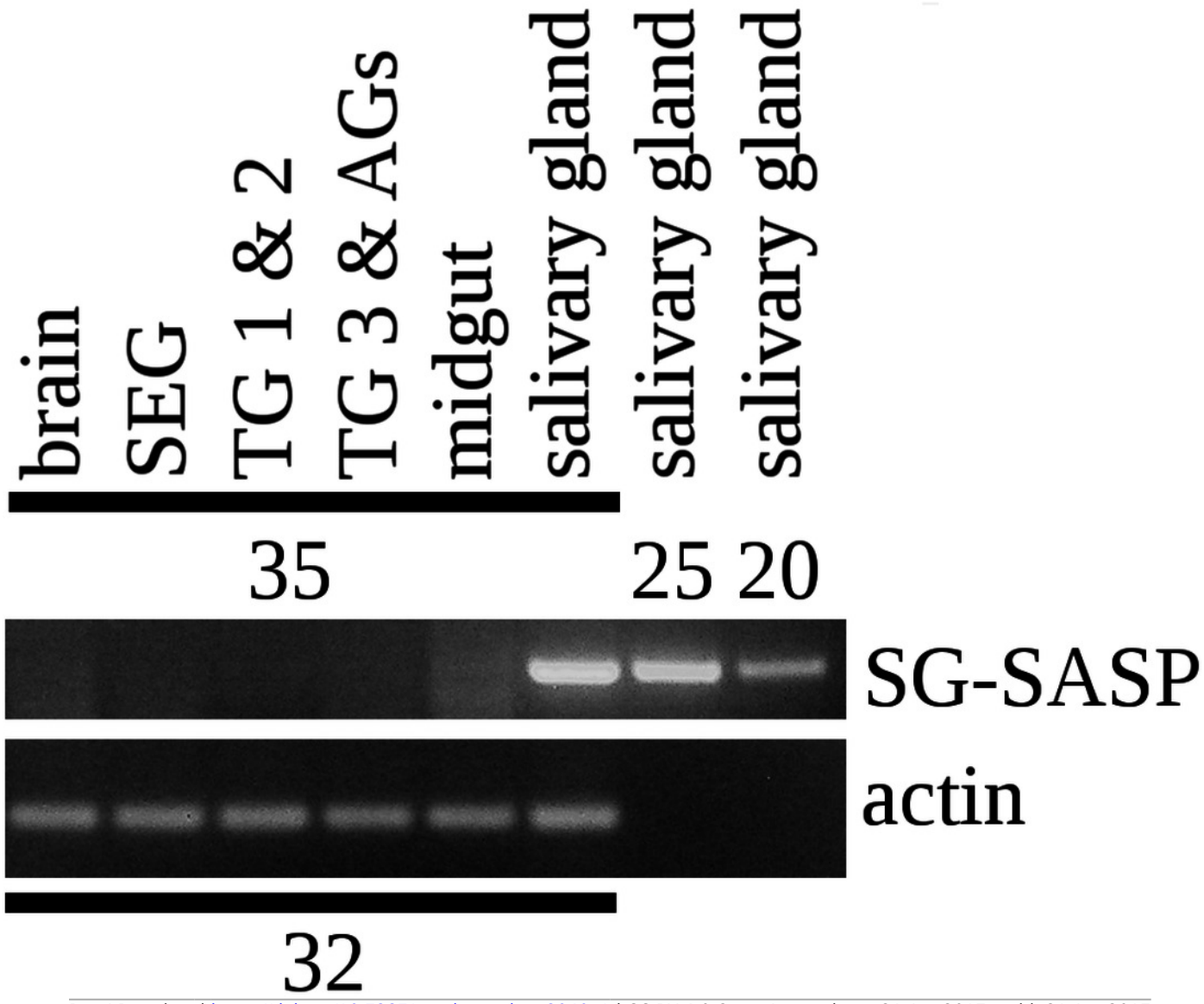
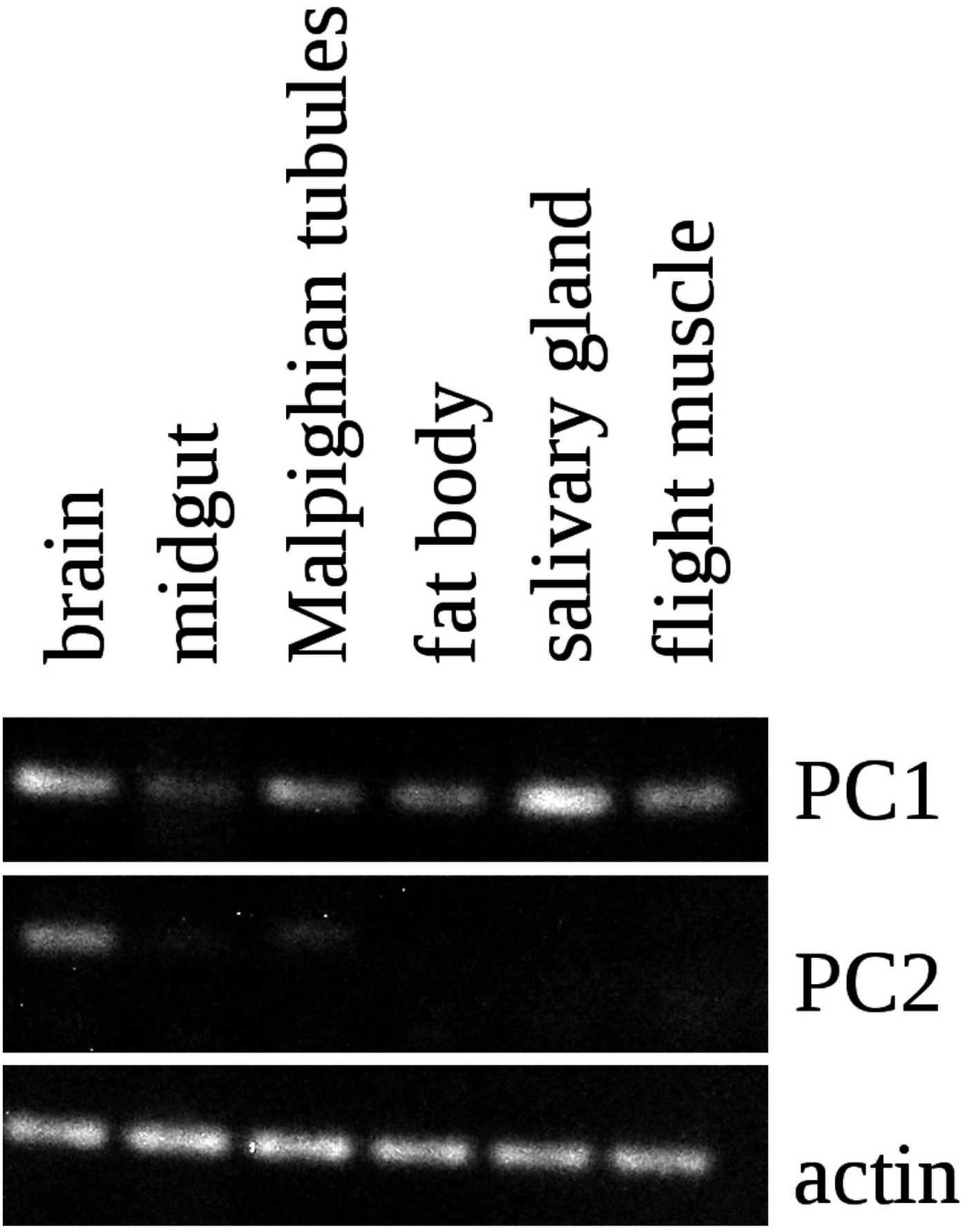


Figure 3

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.



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