

Short communication

# Characterization of a serine carboxypeptidase in the salivary glands and fat body of the orange wheat blossom midge, *Sitodiplosis mosellana* (Diptera: Cecidomyiidae)

Omprakash Mittapalli<sup>a</sup>, Ian L. Wise<sup>b</sup>, Richard H. Shukle<sup>a,c,\*</sup>

<sup>a</sup>Department of Entomology, Purdue University, West Lafayette, IN, USA

<sup>b</sup>Cereal Research Centre, Agriculture and Agri-Food Canada, 195 Dafoe Road, Winnipeg, Man., Canada R3T 2M9

<sup>c</sup>USDA-ARS, Department of Entomology, Purdue University, West Lafayette, IN, USA

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## Abstract

A full-length cDNA encoding a serine carboxypeptidase (designated *SmSCP-1*) was recovered from an ongoing salivary gland EST project of the wheat midge. The deduced 461-amino acid sequence had a putative signal sequence at the amino terminus, indicating it was a secreted protein. The protein shared homology with serine carboxypeptidases from other insects, mammals, plants, and yeasts. *SmSCP-1* mRNA was expressed in all stages of development and detected in salivary gland and fat body tissues but not in midgut tissue. Expression analysis and quantitative real-time PCR assays in male and female wheat midges and the fat body tissue of adult midges revealed that *SmSCP-1* was up-regulated nearly four-fold in the female midges compared to males and nearly two-fold in female fat body compared to male fat body. The wheat midge serine carboxypeptidase (*SmSCP-1*) most likely has a dual function. As a secreted digestive enzyme, it could play a role in mobilizing host-plant seed reserves for feeding larvae and as expressed in fat body could function as an exopeptidase in degradation of vitellogenin and/or in post-translational processing of other enzymes.

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**Keywords:** Wheat midge; *Sitodiplosis mosellana*; Salivary glands; Fat body; Serine carboxypeptidase; Wheat

## 1. Introduction

The orange wheat blossom midge, *Sitodiplosis mosellana* (Géhin), is the most important insect pest of spring wheat, *Triticum aestivum* L., in western Canada (Lamb et al., 2000) and is also a pest of wheat in the United States, Europe, Russia, and China (Berzonsky et al., 2003). Alternate hosts of the wheat midge in the family Gramineae include rye, *Secale cereale* L., and barley, *Hordeum vulgare* L. Adult female flies oviposit on the surfaces of wheat heads ('spikes' or 'ears') from head emergence until anthesis. The hatched larvae feed on developing seeds for 10–12 days, resulting in significant

yield and crop grade losses annually (Lamb et al., 2000). Damage to the developing wheat seed is due entirely to the first two feeding larval instars, while the third instar is a non-feeding stage. Adults live for only a few days, do not feed (Pivnik and Labbe, 1993) and are thought to not have functional salivary glands (I.L. Wise, unpublished data). The short lifespan of adults is directed towards reproduction.

Wheat midge is controlled in western Canada (Saskatchewan and Manitoba) and the United States (North Dakota) mainly by insecticide applications. Ding and Ni (1994) in China and Barker and McKenzie (1996) in Canada initially identified genetic resistance to wheat midge in winter wheat. Resistant wheats have few damaged seeds and their infested seeds often contain dead larvae (antibiotic resistance) (Barker and McKenzie, 1996; Lamb et al., 2000). The host plant resistance is associated with elevated levels of phenolic acids (p-coumaric and ferulic),

\*Corresponding author. Department of Entomology, Purdue University, West Lafayette, IN, USA. Tel.: +1 765 494 6351; fax: +1 765 496 1219.

E-mail address: [shukle@purdue.edu](mailto:shukle@purdue.edu) (R.H. Shukle).

which are rapidly induced by feeding larvae, on infested seed surfaces (Ding et al., 2000). The phenolic acid levels soon return to normal, low levels after the chemical defense has been effective and the seeds mature (Lamb et al., 2000). To date, only one wheat midge resistance gene *Sm1* has been identified and characterized (McKenzie et al., 2002). Efforts to incorporate *Sm1* into spring wheat cultivars in Canada and to safeguard its breakdown by virulent wheat midge populations are being undertaken (Smith et al., 2004).

Identifying and characterizing genes that are important in the biology of the wheat midge will provide a more comprehensive understanding of this pest at the molecular level. Serine carboxypeptidases participate in several physiological and cellular processes, including protein digestion in the guts of animals (Bown et al., 1998), mobilization of seed storage proteins in wheat plants (Mikola, 1986), degradation of yolk proteins in mosquito oocytes (Cho et al., 1991), and post-translational processing of other enzymes (Galjart et al., 1990). These enzymes are exopeptidases and function by cleaving a single amino acid residue from the C-terminus of a protein or peptide substrate. All known serine carboxypeptidases are characterized by the presence of a conserved catalytic triad of Ser-Asp-His similar to serine proteases (Lehfeldt et al., 2000).

Here, we provide the first report of a wheat midge gene encoding a secreted serine carboxypeptidase. The gene, designated *SmSCP-1*, was identified from an on-going EST project for the salivary glands of the wheat midge. The deduced amino acid sequence of *SmSCP-1* contained a conserved catalytic triad and shared homology with other Dipteran, mammalian and plant serine carboxypeptidases. The potential relevance of the serine carboxypeptidase in the biology of the wheat midge is discussed.

## 2. Materials and methods

### 2.1. Insect material

Adult wheat midges were obtained from cultures that had been established by collecting mature larvae from the field or laboratory (Lamb et al., 2000). The mature larvae were maintained in moist soil at 2.5 °C for at least 180 days to allow them to complete their diapause. Emerged adult midges were added to wheat, cv. 'Roblin', with newly emerged spikes, inside meshed cages (Wise et al., 2001). After 2–3 days, the plants, grown hydroponically in tubular plastic pots, were transferred to a greenhouse at 20 °C with an 18-h photoperiod. Larvae were extracted from the spikes at the desired larval instar.

### 2.2. Larva and adult midge dissections and RNA isolation

Two hundred salivary gland pairs were dissected from early and mid-2nd-instar larvae (5–8 days old) immersed in ice-cold 1 × phosphate buffered saline (PBS). Salivary

glands were removed by first pinching off the anterior tip of a larva and then gently compressing the body at the posterior end to expel the entire alimentary tract from the body. Each salivary gland was gently removed from the anterior end of the foregut with a pair of fine-tipped forceps. Midguts were dissected from the remaining part of the alimentary tract by carefully separating the midgut with its contents intact from the foregut and hindgut. All dissected tissues, including salivary glands, midguts and larval carcasses (mainly fat body), were immediately transferred to separate 1.5-ml Eppendorf microcentrifuge tubes containing 200 µl of ice-cold 1 × PBS, flash frozen in liquid nitrogen and stored at –80 °C until RNA was isolated. Fat body from adult midges was isolated by first separating the abdomens from the remaining body and then the fat body tissue was collected by dissecting the abdomens. Total RNA was extracted from the isolated tissues and from all development stages with the RNeasy<sup>®</sup>-4PCR kit from Ambion following the manufacturer's protocol.

### 2.3. Comparison of *SmSCP-1* with other serine carboxypeptidases

Results for each genomic clone were aligned, and a consensus was formed with the SeqWeb sequence analysis program (<http://silverjack.genomics.purdue.edu>) to correct sequencing errors or errors introduced by the *Taq* polymerase. Sequence similarity and annotations were determined with available BLAST programs (Altschul et al., 1990) on the National Center for Biotechnology Information (Bethesda, MD) website (<http://www.ncbi.nlm.nih.gov/>).

### 2.4. Salivary gland cDNA library construction

A cDNA library was constructed from the total RNA isolated from all 200 pairs of salivary glands with a 'SMART<sup>™</sup>' cDNA library construction kit from BD Biosciences Clontech (Palo Alto, CA, USA) following the manufacturer's protocol with one modification. Instead of cloning the PCR fragments into the original phage vector provided with the kit, the fragments were cloned directly into the pCR<sup>®</sup>4-TOPO<sup>®</sup> vector included in a TOPO TA cloning<sup>®</sup> for sequencing kit from Invitrogen. Plasmid DNA was isolated with a Qiagen BioRobot 3000 and sequenced from both ends with T7 forward, T3 reverse and sequence-specific primers in an ABI 3700 DNA analyzer.

### 2.5. Fluorescence in situ hybridization (FISH)

Salivary glands of late 2nd-instar wheat midge larvae were dissected in ice-cold Schneider's insect medium (Sigma-Aldrich) and fixed in 40% acetic acid. Polytene chromosome spreads were prepared as described by Pardue (1986) for the salivary polytene chromosomes of

*Drosophila melanogaster*. FISH for *SmSCP-1* was done following the protocol described by Chen et al. (2004) with two modifications. Probe for FISH was prepared by labeling a genomic clone of *SmSCP-1* with digoxigenin-conjugate. For detection of the signal, a layered technique was used wherein two secondary antibodies were used instead of a single antibody. The first secondary antibody used was anti-digoxigenin-sheep and the second one was anti-sheep-mouse antibody. This technique would enhance the intensity of a true hybridization signal.

## 2.6. Expression analysis of *SmSCP-1* mRNA

Reverse Transcription—PCR (RT-PCR) was performed to assess expression of *SmSCP-1* mRNA in different larval tissues of late 1st-instars and early 2nd-instars and during development with the SuperScript™ One Step RT-PCR System with Platinum® *Taq* DNA polymerase from Invitrogen. RNA extracted from salivary glands, midgut tissue, larval carcasses and from different stages of development (1st-, 2nd-, 3rd-instars, pupae and adults) was used as the template in the RT-PCR reactions. Each reaction was performed three times with 0.5 µg of RNA. The RNA was initially reverse transcribed at 50 °C for 30 min. The PCR amplification included 35 cycles of 30 s denaturing at 94 °C, 30 s annealing at 47 °C, and 1 min extension at 72 °C in a PTC-Dyad Disciple™ thermocycler (MJ Research, Reno, NV). The primer sequences used for the expression analysis were: *SmSCP-1F*: 5'-ATGCGTTT GATTTTGTTATTGTTCAA-3'; *SmSCP-1R*: 5'-TCAT GTCAGCGTTTTCAAGTAGTGA-3'. Five microliters of each PCR reaction was assessed on a 1% agarose gel and stained with ethidium bromide (0.5 µg/ml). DNA bands were photographed and band intensity was determined with a UVP BioDOc-It™ system (Bioimaging Systems, Upland, CA). RT-PCR with primers designed to amplify a 105-bp fragment of a wheat midge ribosomal protein gene (*SmRPL-1*), similar to *D. melanogaster RPL11* gene, was used as the positive control. The primer sequences used were: *SmRPL-1F*, 5'-CGTTGCCCA CAAACGCAAGAAGA-3'; *SmRPL-1R*, 5'-TGCTTTGC TGTTCAAGATGATAC-3'. Also, RT-PCR without reverse transcriptase to generate ssDNA template was used as the negative control to test for possible contaminating DNA.

To study the differential gene expression profile of *SmSCP-1* in male and vitellogenic female flies and in the fat body tissue of both adult midges, RT-PCR with different number of amplification cycles (20, 25, 30, 35 cycles) was done to assess whether PCR products had been taken to saturation. Each RT-PCR reaction was performed three times with equal amounts of RNA (0.5 µg) and also with equal volumes (2 µl) of single stranded DNA (ssDNA) samples that were normalized with a radio-labeled tracer technique (described below). PCR reactions were performed and the products analyzed as mentioned above.

## 2.7. Quantitative real-time RT-PCR

RNA was quantified by the radio-labeled tracer technique according to Puthoff et al. (2003) with modifications. Total RNA (4.35 µg in 10 µl of water) was treated with DNase with the DNase free kit from Ambion (Austin, TX) following the manufacturer's protocol. The reverse transcription reaction was carried out with Superscript First Strand Synthesis kit for RT-PCR from Invitrogen (Carlsbad, CA). One microliter of oligo d(T) primer and 1 µl of dNTPs were added to the 10 µl sample of total RNA that had been treated with DNase. The mixture was heated at 65 °C for 5 min and then put on ice. RT reactions were performed by adding the following on ice: 2 µl 10 × first strand buffer, 2 µl 50 mM MgCl<sub>2</sub>, 2 µl 0.1 M DTT, 1 µl RNase Out, and 1 µl SuperScript II. From each reaction, an aliquot of 5 µl was taken and mixed with 1 µl of <sup>32</sup>P-dCTP (Amersham, Piscataway, NJ) diluted 1:5 (<sup>32</sup>P: water). This step assumed that each reaction had the same amount of time to incorporate <sup>32</sup>P. The remaining 15 µl of the RT reaction and the 5 µl samples containing the isotope were incubated at 42 °C for at least an hour. Reactions were stopped by heating samples to 70 °C for 15 min. After cDNA synthesis was complete, the 15-µl reactions were placed on ice, while the <sup>32</sup>P reactions were used to measure the amount of cDNA synthesized in each sample. From the latter reactions an aliquot of 5 µl was spotted on a filter (DE-81 from Fisher, Fairlawn, NJ). The filters were washed 4 times (1 min/wash) with 0.5 M sodium phosphate buffer after allowing them to dry for 10 min at room temperature. These filters were used to assess the scintillation counts. Depending on the measured counts, the samples were normalized to yield a final concentration of 10 ng/µl. For each PCR reaction, 2 µl of the normalized cDNA, 0.25 µM of each primer, and 10 µl of 2 × SYBR Green Mix ABI (Foster city, CA) were used. PCR reactions were done in an ABI 7000 machine. Cycling parameters included 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, and 60 °C for 1 min.

In the analysis of the relative fold change, the male sample, which is one of the experimental samples, was taken as the calibrator or 1x sample (Pfaffl, 2001). Thus, the relative fold change was assessed by comparing the expression level of *SmSCP-1* in female flies to that in males. The male sample was set at '1' for ease of presentation and female samples were expressed relative to the male.

## 3. Results and discussion

In this study, we report the first characterized gene in the wheat midge, *S. mosellana*: a full-length gene *SmSCP-1* that encodes a serine carboxypeptidase. The lengths of nucleotide and deduced amino acid sequences of the recovered serine carboxypeptidase gene (GenBank accession no. AY962406) from the wheat midge were similar to other serine carboxypeptidases. The similarity of gene

sequences for *SmSCP-1* with lower and higher eukaryotes indicated the presence of a catalytic triad (Ser-201, Asp-339, and His-392) and an active pocket, the PROSITE motif (Appel et al., 1994) of serine carboxypeptidases, (LIVM)-X-(GT)-E-S-Y-(AG)-(GS). The PROSITE motif harbors the active site serine residue (Mahoney et al., 2001) and the sequence for this conserved motif in *SmSCP-1* is VTGESYGG.

Results from the BLAST program indicated that SmSCP-1 protein had several domains conserved in serine carboxypeptidases. SmSCP-1 showed greatest similarity with a serine carboxypeptidase from *D. melanogaster* (AAF55705) with a high amino acid identity (65%) followed by an *Anopheles gambiae* carboxypeptidase (XP\_308370, 58% identity). SmSCP-1 also showed similarity to a vitellogenic carboxypeptidase from *Aedes aegypti* (A41612, 53% identical), a serine carboxypeptidase from *Arabidopsis thaliana* (AAG51389, 41% identical) and to a vitellogenic-like carboxypeptidase from *Mus musculus* (XP\_132566, 40% identical). Determining the biochemical activity of recombinant SmSCP-1 protein to confirm it has carboxypeptidase activity was outside the scope of the present study; however, the high level of identity shared with serine carboxypeptidases from other Dipterans is in agreement with the hypothesis that *SmSCP-1* encodes a serine carboxypeptidase.

A cytological analysis by FISH revealed a single hybridization signal on only one salivary gland polytene chromosome (Fig. 1). These results suggest that *SmSCP-1* is encoded in the wheat midge genome and is most likely present at a single copy. The results from multi-layered FISH also indicate its value in this species to physically anchor a genetic map and to compare synteny with other Dipterans.

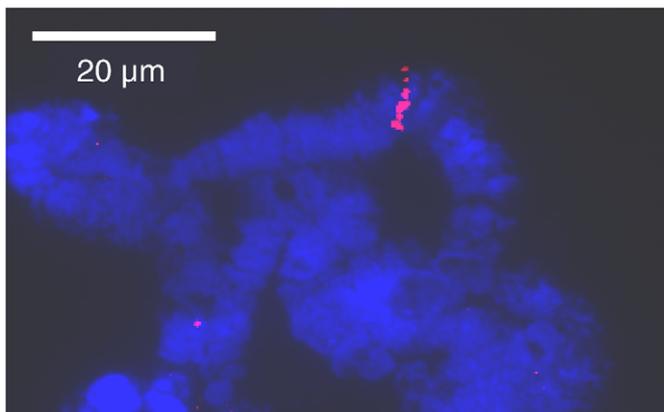


Fig. 1. Fluorescent in situ hybridization (FISH) of *SmSCP-1* to wheat midge polytene chromosomes. A genomic clone of *SmSCP-1* labeled with digoxigenin was used as the probe. Two secondary antibodies, anti-digoxigenin-sheep and anti-sheep-mouse were used to amplify the signal (multi-layering). A strong linear hybridization signal (red) on only one of the polytene chromosome was observed suggesting that *SmSCP-1* is present at a single copy.

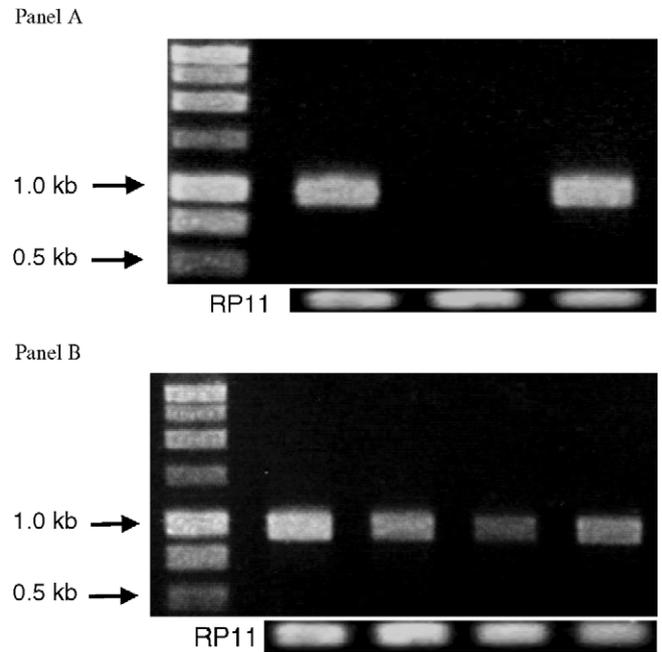


Fig. 2. Reverse transcription polymerase chain reaction (RT-PCR) analysis of *SmSCP-1* with RNA from different tissues of wheat midge larvae and stages of development. Panel A: RT-PCR analysis of *SmSCP-1* in different larval tissues. A PCR product was detected for *SmSCP-1* with RNA from salivary gland tissue (Lane 2) and fat body tissue (Lane 4). No PCR product for *SmSCP-1* was detected with RNA from midgut tissue (Lane 3). Panel B: RT-PCR analysis of *SmSCP-1* with RNA from different life stages of the wheat midge. A PCR product was detected for *SmSCP-1* with RNA from 1st-instars (Lane 2), 2nd-instars (Lane 3), 3rd-instars (Lane 4), and pupae (Lane 5). In both panels, 5- $\mu$ l of PCR reaction (50- $\mu$ l reaction volume) was loaded per lane and a Promega 1 kb DNA ladder was run in Lane 1. RT-PCR product obtained with primers designed to a wheat midge ribosomal protein (SmRPL-1) was used as an internal control for each RT-PCR reaction and is shown as an insert.

We found *SmSCP-1* to be expressed both in salivary gland and fat body tissues (Fig. 2, Panel A) and in all stages of development (Fig. 2, Panel B). Its expression in the salivary glands of larvae and the presence of a secretory signal peptide at the amino terminus in the deduced amino acid sequence indicates the encoded serine carboxypeptidase (SmSCP-1) is secreted. The length of the putative signal peptide associated with SmSCP-1 agreed with those for serine carboxypeptidases from other organisms including insects (Cho et al., 1991; Mahoney et al., 2001).

While expression of the *SmSCP-1* transcript in salivary glands and fat bodies of *S. mosellana* has been documented, caution must be exercised in interpretation of our results since protein blot analyses to reveal SmSCP-1 protein is expressed in these tissues has not been undertaken in the present study. Additionally, we have not expressed recombinant SmSCP-1 protein and revealed in biochemical analysis it indeed has carboxypeptidase activity. These proteomics are outside the scope of the present short communication, but are part of our developing program directed toward elucidating genes important in the biology of *S. mosellana* and its adaptation to

wheat as a host plant. However, we feel our results are not in disagreement with the hypothesis the putative wheat midge serine carboxypeptidase is secreted by salivary glands and involved in extra-cellular digestion of storage proteins within the developing wheat kernels by feeding larvae. While a digestive role for carboxypeptidases in the midgut of other insects has been demonstrated (Bown et al., 1998), our results revealed that *SmSCP-1* was not expressed in the midgut of feeding larvae (Fig. 2, Panel A).

Carboxypeptidases can be grouped into two types depending on their functions. The first types are involved in the complete or partial degradation of a wide array of proteins, whereas the second types are very selective and involved in hydrolysis of a particular protein or peptide (Mahoney et al., 2001). The expression we observed with *SmSCP-1* in fat body, a tissue equivalent to liver in higher animals, suggests that it is involved in roles apart from digestion such as processing and or degradation of other proteins or peptides.

The novel role of a serine carboxypeptidase synthesized in the fat body of female *Aedes aegypti*, accumulated by developing oocytes, and activated to mobilize vitellogenin for the developing embryo has been well documented (Cho et al., 1991). A differential expression profile of *SmSCP-1* in adult male and female wheat midges was observed in the current study (Fig. 3). The *SmSCP-1* transcript was not detected in both male and female flies following 20 cycles of PCR amplification (Fig. 3, Panel A). However, after 25 cycles of amplification, *SmSCP-1* amplicon was detected only in females. Differential expression of *SmSCP-1* was most prominent with RNA from both genders following 30 cycles of PCR amplification (Fig. 3, Panel A). The expression level of *SmSCP-1* was much higher in female flies when compared to its expression in male flies after 30 cycles of PCR amplification. Saturation of the transcripts was observed in both male and female flies following 35 cycles of PCR amplification. With consideration of the cautions alluded to earlier (*vide supra*) concerning the expression and biological activity of *SmSCP-1* protein taken into account, the up-regulation of *SmSCP-1* in the order of a four-fold increase observed in female wheat midge flies compared to male flies (Fig. 3, Panel B).

A differential expression profile for *SmSCP-1* was also observed in the adult female fat bodies relative to adult male fat bodies (Fig. 4). Expression analysis revealed that *SmSCP-1* was expressed nearly two-fold greater in female fat bodies than in the male fat bodies. These observations suggest that *SmSCP-1* in female *S. mosellana* could function in a similar phenomenon to that described in *A. aegypti* for the breakdown of vitellogenin in developing embryos. The expression of *SmSCP-1* observed in adult male *S. mosellana* and in their fat bodies suggests that *SmSCP-1* has a different function, such as a possible role in immune responses (Galjart et al., 1990), from that described in *A. aegypti* for mobilization of vitellogenin reserves during embryogenesis (Cho et al., 1991). The expression of *SmSCP-1* in the salivary glands of adults was

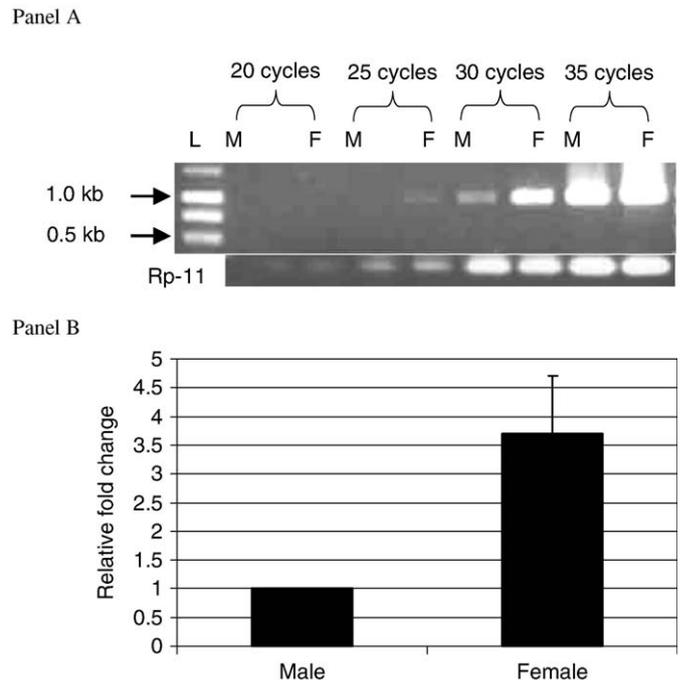


Fig. 3. Differential expression of *SmSCP-1* in male and vitellogenic female flies. Panel A: Reverse transcription results with different number of amplification cycles. A PCR product was detected in female flies after 25 cycles of amplification, while there was no PCR product detected in male flies after 25 cycles of PCR amplification. A PCR product in females was observed that was much greater in amplitude than in males after 30 cycles of PCR amplification. The *SmSCP-1* transcript approaching saturation was detected with 35 cycles of amplification in both male and female flies. Five microliters of each PCR (50- $\mu$ l reaction volume) was loaded per lane. A Promega 1 kb DNA ladder was run in Lane L. RT-PCR product obtained with primers designed to a wheat midge ribosomal protein gene (*SmRPL-1*) was used as an internal control for each RT-PCR reaction and is shown as an inset. Panel B: Quantitative real-time RT-PCR (qPCR) assays in male and female flies. A 3.7-fold increase of the *SmSCP-1* transcript in females was observed as compared to the males. Total RNA was isolated from pools of both sexed flies. First strand cDNA synthesis was performed and then subjected to qPCR in triplicate. The relative fold change was assessed by comparing the expression level of *SmSCP-1* in females to that in males. The male sample was set at '1' (Pfaffl, 2001) for ease of the presentation and female samples were expressed relative to the male. The standard error of the mean for three technical replicates is represented by the error bar.

not undertaken because the adult flies do not feed (Pivnik and Labbe, 1993) and are thought to have non-functional salivary glands (I. L. Wise, unpublished data).

In conclusion, we report here the characterization and expression of a wheat midge gene encoding a secreted serine carboxypeptidase. We postulate the serine carboxypeptidase has a dual function in the wheat midge. Expression in the salivary glands of feeding larvae could indicate involvement in the extra-cellular digestion of storage proteins in the developing wheat kernels by larvae. Expression in fat body taken together with greater expression in females relative to males could suggest a function similar to the vitellogenic serine carboxypeptidase in *Aedes aegypti*.

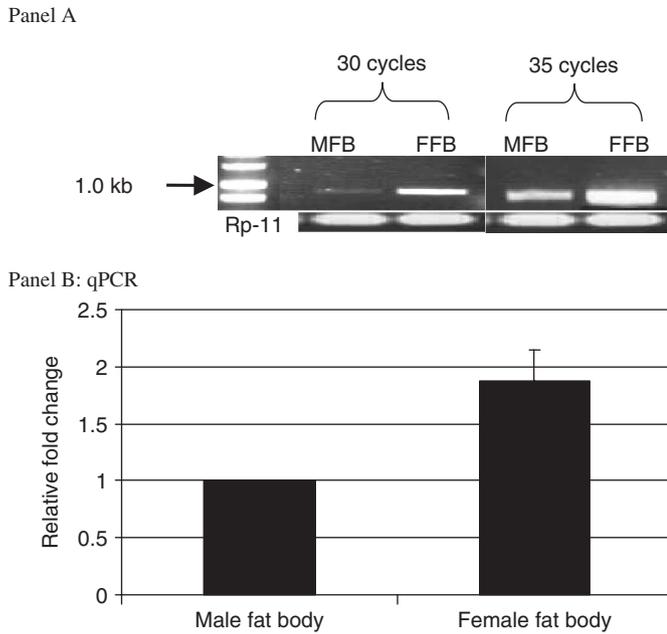


Fig. 4. Differential expression of *SmSCP-1* in the fat body tissue of male and vitellogenic female flies. Panel A: Reverse transcription results with different number of amplification cycles. A PCR product in female fat body (FFB) was observed that was much greater in amplitude than in the male fat body (MFB) after 30 cycles of PCR amplification. The *SmSCP-1* transcript approaching saturation was detected with 35 cycles of amplification in both male and female fat body tissues. Five microliters of each PCR (50- $\mu$ l reaction volume) was loaded per lane. A Promega 1 kb DNA ladder was run in Lane L. RT-PCR product obtained with primers designed to a wheat midge ribosomal protein gene (SmRPL-1) was used as an internal control for each RT-PCR reaction and is shown as an insert. Panel B: Quantitative real-time RT-PCR (qPCR) assays in the fat body tissue of male and female flies. A 1.9-fold increase of the *SmSCP-1* transcript in the female fat body tissue was observed as compared to the male fat body tissue. First strand cDNA synthesis was performed and then subjected to qPCR in triplicate. The relative fold change was assessed by comparing the expression level of *SmSCP-1* in female fat body to that in male fat body. The male fat body sample was set at '1' (Pfaffl, 2001) for ease of presentation and female fat body samples were expressed relative to the male fat body. The standard error of the mean for three technical replicates is represented by the error bar.

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