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# Diet-specific salivary gene expression and glucose oxidase activity in *Spodoptera exigua* (Lepidoptera: Noctuidae) larvae

Khashayar Afshar, Philippe J. Dufresne, Li Pan, Magali Merkx-Jacques, Jacqueline C. Bede *

*Department of Plant Science, McGill University, 21,111 Lakeshore, Ste-Anne-de-Bellevue, Québec, Canada H9X 3V9*

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ABSTRACT

Saliva secreted during caterpillar feeding contains enzymes to initiate digestion or detoxify noxious plant compounds. Activity of some salivary enzymes is diet-dependent and may be transcriptionally regulated. In this study, cDNA-amplified fragment length polymorphism was used to identify beet armyworm, *Spodoptera exigua* Hübner, labial salivary genes that are differentially expressed in response to diet. In addition, *SeGOX* was sequenced based on homology and characterized to confirm that the transcript encodes a functional enzyme. Three labial salivary transcripts, encoding glucose oxidase (GOX) and two proteins of unknown function (Se1H and Se2J), were expressed in a diet-specific manner. Since diet, particularly the protein to digestible carbohydrate levels and ratio, may affect labial salivary enzyme activity, the influence of nutritional quality on gene expression was determined. Transcript levels of the labial salivary genes *Se1H*, *Se2J* and *SeGOX* increased with dietary carbohydrate levels, regardless of protein concentrations. In contrast GOX enzymatic activity increased with increasing dietary carbohydrates when caterpillars were fed protein-rich diets, but not when caterpillars were fed protein-poor diets. Our results suggest that dietary carbohydrates affect *SeGOX*, *Se1H* and *Se2J* transcription, but dietary protein or amino acid levels affect translational and/or post-translational regulation of the enzyme GOX.



## 1. Introduction

Generalist caterpillars that feed on numerous host plant species potentially face high variation in dietary nutritional quality as well as exposure to a diverse range of defensive phytochemicals (Behmer et al., 2002; Hoy et al., 1998). Therefore, caterpillars have mechanisms, such as salivary enzymes, to deal with the noxious chemicals and nutritional imbalances they encounter. During feeding, plant tissues are mixed with saliva secreted from the labial and mandibular salivary glands which, in addition to initiating digestive processes, may be first-line defenses against plant defensive compounds (Eichenseer et al., 1999; Musser et al., 2002, 2005a,b; Zong and Wang, 2004). Lepidopteran salivary proteins are not as well characterized as other arthropods, such as Hemiptera, Diptera, or Ixodida (Alarcon-Chaidez et al., 2007; Calvo et al., 2004; Francischetti et al., 2002, 2005; Li et al., 2001; Miles, 1999; Ribeiro et al., 2004; Valenzuela, 2004; Valenzuela et al., 2003). Even so, glucose oxidase (GOX), lysozyme and ascorbate peroxidase have been identified in caterpillar labial saliva and the first two enzymes are diet-dependent (Babic et al., 2008; Hu et al., 2008; Liu et al., 2004; Mathews et al., 1997; Merkx-Jacques and Bede, 2005; Peiffer and Felton, 2005).

Insect digestive gene expression and enzyme activity are often tightly regulated in response to feeding and dietary nutritional quality. In *Anopheles gambiae*, temporal- and diet-specific transcription is associated with intake of a blood or sugar meal (Dana et al., 2006). Predatory two-spotted stink bug, *Perillus bioculatus*, nymphs have lower transcript expression of genes encoding tyrosine-3-monooxygenase and a chitin binding protein when they fed on *Trichoplusia ni* caterpillars compared to artificial diet (Coudron et al., 2006). *Helicoverpa armigara* midgut amylase isoforms and activity reflect the host plant or artificial diet that the caterpillars are feeding on (Kotkar et al., 2009). In a closely related species, labial salivary lysozyme transcript levels also reflect diet; expression is absent when *Helicoverpa zea* caterpillars are restricted to artificial diet, weak on tobacco and strong on cotton and tomato plants (Liu et al., 2004).

In *Spodoptera exigua* caterpillars, labial salivary GOX (EC 1.1.3.4.) activity increases temporally as caterpillars are transferred from plant to a meridic artificial diet and may be related to the composition of the diet (Babic et al., 2008; Merkx-Jacques and Bede, 2005). For many insect, dietary nutritional quality, particularly the protein to digestible carbohydrate ratio (p:c) are tightly

* Corresponding author. Tel.: +1 514 398 7860; fax: +1 514 398 7897.
*E-mail addresses:* Khashayar.afshar@mail.mcgill.ca (K. Afshar), Philippe.dufresne@mail.mcgill.ca (P.J. Dufresne), Panli920@yahoo.com (L. Pan), Magali.merkx-jacques@mail.mcgill.ca (M. Merkx-Jacques), jacqueline.bede@mcgill.ca (J.C. Bede).

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linked to caterpillar performance. As a consequence, many Lepidopteran species self-regulate their intake of nutrients, a behaviour known as "dietary self-selection" (Waldbauer and Friedman, 1991). When given dietary choices, *S. exigua* caterpillars select a dietary P:C ratio of 22p:20c (Merkx-Jacques et al., 2008); restriction on this diet leads to favourable performance when criteria such as survival, developmental time and pupal size and lipids are evaluated. Nutritional quality also affects *S. exigua* labial salivary GOX activity (Babic et al., 2008).

Understanding the basis of this dietary regulation is critical since labial salivary enzymes may perform roles beyond "simple" digestion. For example, numerous functions have been proposed for labial salivary GOX in Noctuid caterpillars (Eichenseer et al., 1999). Musser et al. (2002, 2005a) proposed that $H_2O_2$ produced by GOX is an important strategy used by caterpillars to circumvent the induction of plant defenses since $H_2O_2$ may activate pathways that interfere with octadecanoid-dependent plant responses (Diezel et al., 2009; Weech et al., 2008). In addition, since this enzyme converts glucose to gluconate, a carbohydrate that cannot be utilized by the insect, GOX may allow caterpillars to cope with detrimental dietary carbohydrate excesses that they encounter when feeding on herbaceous plants (Bede et al., 2007; Matteson, 1980; Scriber and Slansky, 1981; Warbrick-Smith et al., 2006). Since GOX is proposed to be a caterpillar-mediated strategy to undermine the plants' ability to mount anti-herbivore defenses, understanding the regulation of GOX and other labial salivary proteins is of key importance.

The overall objective of our study was to investigate diet-dependent gene expression in the labial salivary glands of *S. exigua* Hübner (Lepidoptera: Noctuidae) caterpillars. These genes were identified in caterpillars fed on different diets by the robust differential display technique cDNA-amplified fragment length polymorphism (cDNA-AFLP). In addition, *SeGOX* was sequenced based on homology to the honeybee gene and expressed to confirm functionality. Caterpillars were then restricted from 3rd to 4th instars on defined diets varying in protein to digestible carbohydrate (p:c) to determine how nutritional quality affects expression of these salivary genes. These artificial diets reflected the crude protein to non-structural carbohydrate ratios that may be encountered by caterpillars feeding on protein-rich crops, such as alfalfa, but do not have the confounding effects of the plant secondary compounds (Chatterton et al., 1972; Michaud et al., 2001). In addition to transcriptional profiling, GOX activity was measured to further understand the relationship between transcript expression and activity of this salivary enzyme.

## 2. Materials and methods

### 2.1. Caterpillar colony and Medicago truncatula maintenance

*S. exigua* Hübner (Lepidoptera: Noctuidae), obtained originally from the R.T. Gast Insect Rearing Facility, were reared for multiple generations on a meridic wheat germ-based artificial diet (Bio-Serv) in a growth chamber (16:8 light:dark hours; 28.5 °C). *Medicago truncatula* (Gaertn.) cv. Jemalong, was grown in pasteurized soil (16:8 light:dark hours, 22 °C, 260 $mEm^{-2}s^{-1}$) and watered with dilute fertilizer (N20:P20:K20) three times a week.

### 2.2. Identification of diet-regulated salivary genes

#### 2.2.1. cDNA-amplified fragment length polymorphism

Labial salivary glands (100 pairs) were dissected from 4th instar caterpillars, reared on Bio-Serv artificial diet or plants or a subset of caterpillars reared on plants and transferred to artificial diet for 4 h, placed in RNAlater (Ambion) and stored at 4 °C until use. Two independent biological replications were performed for each diet.

mRNA was extracted from the labial salivary glands using the mTRAP mRNA purification kit (Active Motif). After the removal of RNAlater and washes in sterile DEPC-treated PBS, pH 7.0, salivary glands were homogenized in lysis buffer. After incubation at 45 °C, the lysate was passed 4–5 times through a sterile 21-gauge needle. The mTRAP mRNA purification kit uses a peptide nucleic acid oligo-dT probe to recognize the polyA-mRNA; this allows binding at higher temperatures and lower salt conditions than conventional oligo-dT probes. Streptavidin beads were used to bind the biotin molecule on the peptide nucleic acid probe. After wash steps and DNAse treatment, mRNA was eluted and precipitated using Touchdown reagent. mRNA concentration was determined using RiboGreen (Invitrogen) and quality was assessed by electrophoresis on a 0.8% agarose gel.

First strand cDNA synthesis was performed using the Active Motif Reflection kit. One microgram polyA-mRNA was added to 50 pmol of oligo-dT primer followed by heating to 65 °C for 10 min. To this, 5 mM DTT, a mixture of 1 mM dNTPs, ribonuclease inhibitor and 20 U reverse transcriptase was added and incubated for 1.5 h at 42 °C. This was followed by second strand cDNA synthesis. On ice, 2 mM dNTPs, DNA ligase (10 U, Invitrogen), T4 DNA polymerase (50 U, Invitrogen) and Ribonuclease H (1.6 U, Invitrogen) were added and incubated for 2 h at 16 °C until the reaction was stopped by the addition of 0.03 M EDTA. cDNA was extracted in phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated from the aqueous phase using 1/10th volume of 3 M sodium acetate and three volumes cold ethanol (100%, −20°C) after incubation at −20 °C for 2 h.

cDNA-AFLP was conducted following Bachem et al. (1998) with minor modifications. Fifty nanogram cDNA was digested for 2 h at 65 °C with 5 U *Taq*I (NEB), which generates 3′ overhangs. This was followed by digestion by 10 U of *Mse*I (NEB) at 37 °C for 2 h. Enzymes were then denatured by incubation at 80 °C for 20 min. A negative control which did not contain sample was included. Adapters designed to the overhang sequences generated by *Mse*I and *Taq*I were ligated onto the digested cDNA strands. *Mse*I adapters (5′-GACGATGAGTCCTGAG-3′ and 5′-TACTCAGGACTCAT-3′) and *Taq*I adapters (5′-GACGATGAGTCCTGAC-3′ and 5′-CGGTCAGGACTCAT-3′) mixed in equimolar amounts (2 pmol), denatured by heating to 90 °C and cooled to room temperature were added to digested cDNA and incubated with 1 U T4 DNA ligase at 20 °C for 2 h.

The primary template was amplified using 100 ng of each primer (5′-GATGAGTCCTGAGTAA-3′, 5-GACGATGAGTCCTGACCGA-3′) directed against the ligated adapters in the presence of 0.2 mM dNTPs, 1 U Taq DNA polymerase (NEB) using the following program: 25 cycles of 94 °C for 30 s, followed by 55 °C for 30 s and 72 °C for 1 min. The resultant amplicons were separated by electrophoresis on a 1% gel and after ethidium bromide staining, showed a smear between 50 and 500 bp. The remaining sample was diluted 1:10 with DEPC-treated water and stored at −20 °C.

This template was amplified using normal and infrared-labeled (LiCor) primers directed against adapters that had two selective nucleotide base extensions at the 3′-end. Different primer combinations used were as follows (*Taq*I:*Mse*I): AC:AG; AC: GC; GA:AG; GA:CT; TC:TG; TG:CA; TG:GA. Using similar PCR reaction mixture as explained above, selective populations of cDNA were amplified using the following program: 12 cycles of 94 °C for 30 s followed by 65 °C for 30 s (this step was reduced by 0.7 °C for every subsequent cycle) and then 72 °C for 1 min. This was immediately followed by the PCR program: 30 cycles of 94 °C for 30 s, followed by 55 °C for 30 s and 72 °C for 1 min.

After denaturation at 90 °C for 3 min, PCR products were separated on a 10% polyacrylamide (Long Ranger), 7% urea sequencing gel (25 cm long × 0.25 mm thickness) by electrophoresis on a 4300 LI-COR automated DNA sequencer. cDNA fragments ranged from 50 to 350 bp. Two putative differentially expressed bands and one constitutive band were sliced from the polyacrylamide gel and extracted with the QIAquick gel extraction kit (Qiagen). Fragments were reamplified using selective *Taq*I and *Mse*I primers. After confirmation of purity and size on a 2% agarose gel, cDNA fragments were inserted into the TOPO-TA pCR 2.1 vector (Invitrogen) and used to transform DH5$\alpha^{TM}$-T1 cells (Invitrogen). Plasmids were isolated using the FastPlasmid$^{TM}$ Mini kit (Eppendorf) and sent to the Genome Québec Innovation Center for sequencing using M13F and M13R universal primers.

### 2.2.2. 5′- and 3′-Rapid amplification of cDNA ends (RACE)

To identify gene fragments, 5′- and 3′-RACE was performed using the SMART-RACE cDNA amplification kit (Clontech). First strand cDNA, prepared as described above from 1 μg mRNA, was amplified using 20 pmol of a gene-specific forward primer and a universal primer mix under the following PCR conditions: 5 cycles of 94 °C for 30 s followed by 72 °C for 3 min; 5 cycles of 94 °C for 30 s, then 70 °C for 30 s followed by 72 °C for 3 min; 25 cycles of 94 °C for 30 s, then 68 °C for 30 s and 72 °C for 3 min. A second amplification was performed using the same conditions and amplicons subjected to electrophoresis on a 1% agarose gel. A single band was purified by Qiaquick gel extraction kit and sent to the Genome Québec Innovation Center for sequencing. Gene-specific primers designed to *Bombyx mori* β-tubulin (gi:NP001036888) and *Apis mellifera* GOX (gi:ABO22904) were used to identify *S. exigua* homologues.

### 2.2.3. Validation of diet-dependent differential gene expression

Newly moulted 4th instar *S. exigua* caterpillars which had been reared on *M. truncatula* plants were maintained on the plants or transferred to an artificial diet (Bio-Serv) for 4 h. Labial salivary glands were dissected from caterpillars placed into RNAlater and stored at −80 °C and gene expression analyzed by quantitative real time-polymerase chain reaction (qRT-PCR, Section 2.2.3.1). Three independent biological replications were performed.

*2.2.3.1. Quantitative real time-polymerase chain reaction (qRT-PCR).* mRNA was purified from 50 pairs of pooled caterpillar salivary glands homogenates using the mTRAP mRNA isolation kit (Active Motif). After DNase I (Invitrogen) treatment to eliminate genomic DNA contamination, samples were amplified using primers 5′-CTGAAGCCAATAAGCTGTCCA-3′ and 5′-GCAAGCCTG TTTGGTGTAGAG-3′; this amplicon spans an intronic region to confirm the absence of genomic contamination. RNA levels were quantified using a NanoDrop spectrophotometer. A cDNA copy was generated from 1 μg mRNA using the QuantiTech Reverse Transcription kit (Qiagen).

Gene expression was analyzed by qRT-PCR using a Stratagene Mx3000p thermal cycler. Primers were designed against *Se1H*, *Se2J*, *SeUbi*, *SeGox* and *SeTub* (Table 1). A standard curve for targeted and reference genes were generated on each plate. Amplicons used to make the standard curve were sequenced for verification. qRT-PCR was performed in duplicate using the Brilliant One-Step qRT-PCR kit (Strategene). Reactions contained 1 × SYBR green I, 0.375 nM ROX, 200 nM each of gene-specific forward and reverse primers (Table 1), 1.25 ng cDNA and Mastermix which contains dNTPs, $MgSO_4$ and Taq polymerase. Thermocycler conditions were as follows: 95 °C for 10 min; 40 cycles of 56 °C for 1 min, then 72 °C for 30 s, followed by 95 °C for 1 min; 1 cycle of 56 °C for 30 s and 95 °C for 30 s. A dissociation curve is generated from the last cycle to assess amplicon purity. The reference gene, *SeTub*, and non-template controls were included on every plate and two plate replicates were performed.

Gene copy number was estimated from standard curves using the MxPro v3.20 software. Standard curve $r^2$ values were >0.96 and the reaction efficiency was between 90 and 110%. Relative gene expression was normalized by *SeTub* gene expression.

### 2.2.4. Gene prediction

Transcript sequences and their putative protein sequences were compared against non-redundant sequences deposited in NCBI Genbank databases using BLASTx, BLASTn and BLASTp and annotated according to their highest BLAST scores with an *E*-value $<10^{-4}$ (Altschul et al., 1990). As well, sequences were compared to the silkworm (SilkDB) and ButterflyBase databases (Papanicolaou et al., 2008; Wang et al., 2005). Conserved protein domains were identified by BLASTp and ExPASy Prosite (http://www.expasy.org) (Altschul et al., 1997; Marchler-Bauer et al., 2007; Schäffer et al., 2001). Protein sequences were submitted to SignalP (Center for Biological Sequence Analysis) (Emanuelsson et al., 2007) and iPsort and PSORT II (http://ims.u-tokyo.ac.jp) for the bioinformatic analysis of putative secretory signal peptides. Sequences were aligned using CLUSTALW (Thompson et al., 1994).

### 2.2.5. SeGOX functional characterization

*SeGOX* cDNA subcloned into a TOPO-TA vector by PCR was used to transform DH5α cells. After plasmid purification using the Maxi plasmid purification kit (Qiagen), the stop codon was removed by PCR-directed mutagenesis and replaced by a KpnI restriction endonuclease consensus sequence for further cloning. Primers used to remove the stop codon and generate the KpnI restriction site are as follows: 5′-GTAACGGCCGCCAGTGTGCTGGAATT-3′ and 5′-AGCTCGGTACCTTGCCAGGCTTCTTT-3′, respectively. The new insert was cloned into a modified pEGFP-N3 vector using KpnI and EcoRI restriction enzymes 5′-upstream and in-frame to a flag-tag sequence. Following plasmid amplification and purification, mouse fibroblast NIH3T3 cells were transiently transfected with the new construct and an empty vector control using Lipofectamine 2000 (Invitrogen) ($1.2 \times 10^6$ cells, 10 μg plasmid). Expression was driven by a constitutively active CMV promoter. Cells were harvested for Western blot detection or enzyme assays.

Cell-free protein extracts prepared by homogenizing cells in lysis buffer (25 mM Tris–HCl, pH 7.4 containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM dithiothreitol and 1% Triton x-100). After centrifugation (14,000 rpm for 10 min at 4 °C), soluble proteins were separated by SDS-PAGE (4% stacking and 8% resolving gels). After transfer to a nitrocellulose membrane (Hybond-C extra, Amersham Biosciences), blots were blocked for 1 h in PBS containing 3% milk powder and 0.05% tween at room temperature. Membranes were incubated with primary antibodies overnight at 4 °C; an anti-flag M2 monoclonal antibody (mouse; Sigma) was used to detect the flag-tagged GOX (dilution 1:3000 in 3% milk

**Table 1**
Primers used for qRT-PCR expression.

| Gene | Forward primer (5′–3′) | Reverse primer (5′–3′) | Amplicon size (bp) |
|---|---|---|---|
| *Se1H* | TCCAAGGACTAACACCTCGGC | CGATCTGTACTCCAGCAACGT | 161 |
| *Se2J* | ACTGGCTGCGGCATTAGAAGA | AAGATGCGGCGCAAGACGATG | 251 |
| *SeGox* | CCTCTTTACGGGCCTTCACGA | ATAACGGCCAGCGTTACACCA | 180 |
| *SeUbi* | AGTGGTAGCACGGGGGTGA | GAGGGAATCCCACCAGACCA | 229 |
| *SeTub* | GCCGTCGTGTTACCGATAAACG | CCATCTTCCGTGGTCGCATGT | 175 |

powder in PBST) and anti-actin antibody (goat; Santa Cruz) was used to detect actin (dilution 1:5000 in 3% milk powder in PBST). After extensive washing, HrP-linked secondary anti-mouse and HrP-linked anti-goat antibodies diluted 1:5000 in 3% milk powder in PBS were incubated while rocking at room temperature for 1 h. After additional washing with PBST, protein bands were visualized using SuperSignal West Dura (ThermoScientific). Film was exposed to the membrane for ~3 min before developing.

In parallel experiments, three independent NIH3T3 cell lines transfected with either *SeGOX*::pEGFP-N3 vector or the vector-only control were homogenized in lysis buffer and the cell-free extracts were assayed for GOX activity as detailed below (Section 2.3.3). A boiled homogenate was also included to verify that activity was due to a protein.

### 2.3. Effect of dietary nutritional quality on gene expression and enzyme activity

#### 2.3.1. Chemically defined artificial diets

Five diets ranging in the P:C levels were used in these experiments: 22p:20c, 22p:40c, 30p:20c, 33p:30c, 30p:40c. They were prepared according to Simpson and Abisgold (1985), using cellulose as the non-digestible filler. Unless specified, diet ingredients were purchased from Sigma. The carbohydrate source was glucose and the protein/amino acids were prepared from a 3:1:1 ratio of casein (bovine milk, Bio-Serv), peptone and albumen (egg white, Bio-Serv). The amino acid composition of this protein mixture mimics wheat seedlings (Lee et al., 2003). Constituents of all diets were as follows: 0.55% cholesterol (Bio-Serv) solubilized in 0.5% linoleic acid, 2.5% Wesson's salts, 1% ascorbate, 0.5% choline chloride, 0.5% sorbic acid, 0.35% methyl paraben (Bio-Serv), 0.285% thiamine, 0.285% ribovlavin, 1.14% nicotinamide, 0.285% pyridoxine, 0.285% folic acid, 2.85% *myo*-inositol, 0.57% calcium panthothenic acid, 0.285% *p*-aminobenzoic acid and 0.011% biotin (Bio-Serv). The diet was prepared in a 2% agar.

#### 2.3.2. Effect of nutritional quality on salivary gene expression

*S. exigua* caterpillars were reared on Bio-Serv diet until 2nd instar at which time larvae were carefully transferred to the baseline diet 22p:20c. Early 3rd instar caterpillars were then transferred individually to Petri dishes containing a block of the chemically defined artificial diets (22p:20c, 22p:40c, 30p:20c, 33p:30c, 30p:40c). Labial salivary glands (50 pairs) were dissected from mid-4th instar caterpillars and stored in RNAlater at 4 °C until analysis. Gene expression was measured by qRT-PCR as described above (Section 2.2.3.1) and normalized against *SeTub*. For each diet, three independent biological replications were performed.

#### 2.3.3. Glucose oxidase (GOX) activity

Early 2nd instar *S. exigua* caterpillars reared on Bio-Serv diet were transferred to the self-selected diet (22p:20c) which has low carbohydrate and protein levels; this ensured minimal labial salivary GOX background activity. Third instar larvae were transferred individually to Petri dishes containing a block of the chemically defined artificial diets (22p:20c, 22p:40c, 30p:20c, 33p:30c, 30p:40c). Labial salivary glands were dissected from mid-4th instar caterpillars (approx. 48 h) and assayed for GOX activity and soluble protein levels (Merkx-Jacques and Bede, 2005). After cooling on ice, caterpillars were placed ventral-side up and rinsed with cold, sterile Nathanson's saline (150 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$, 10 mM TES, 20 mM $MgCl_2$, pH 6.9 (Christensen et al., 1991). The head was carefully removed to avoid damage to labial salivary glands which were pooled (8 pairs) and homogenized in Nathanson's saline and broad-spectrum proteinase inhibitors (Sigma). After centrifuge-filtration using 0.22 μm low protein binding membrane filters (Millipore), GOX activity was assayed using the *o*-dianisidine-horseradish peroxidase coupled enzymatic assay (Bergmeyer, 1974). A reaction mixture of 10% β-D-glucose, 0.0066% *o*-dianisidine in 0.1 M potassium phosphate buffer, pH 7.0 was brought to 35 °C. Salivary gland homogenate and horseradish peroxidase (final concentration 3 U, Sigma) were added and GOX activity was measured spectrophotometrically at 460 nm. Samples were measured in duplicate. Controls included the reaction mixture except for salivary gland homogenate (blank) and boiled homogenate (negative control). A positive control of fungal GOX (Sigma) was also used.

Soluble protein levels were determined according to the Bradford method (Bradford, 1976) using bovine serum albumin as the protein standard. Soluble protein levels and GOX enzyme activity were measured in six independent biological replicates of pooled labial salivary glands.

### 2.4. Statistics

For gene expression analysis and GOX assays, statistical differences were determined by one-way ANOVA followed by Tukey HSD post hoc test using SPSS version 16 (SPSS Inc.). Greater than a 2-fold difference in gene expression was used as appropriate criteria to identify significant differences in transcript expression (Yang et al., 2002).

## 3. Results and discussion

### 3.1. Identification of diet-regulated salivary genes

Studies have shown that labial salivary enzyme activity or transcript expression, such as GOX activity or lysozyme expression, respectively, reflects the diet that the caterpillars are feeding upon (Babic et al., 2008; Merkx-Jacques and Bede, 2005; Peiffer and Felton, 2005). To identify genes differentially regulated by diet, *S. exigua* caterpillars were reared on either artificial diet (Bio-Serv) or *Medicago truncatula* plants. A group of caterpillars was also transferred from the plant to artificial diet for 4 h. Diet-dependent differentially expressed labial salivary genes were identified by cDNA-AFLP (Fig. 1). For each selective primer set, approximately 40 bands, ranging from 50 to 350 ntd were observed. As expected, salivary transcript expression was generally consistent across diets and conditions tested. Two bands (*Se2J*; *Se1H*) that showed distinct diet-related differences and one constitutive gene (*SeUbi*) were

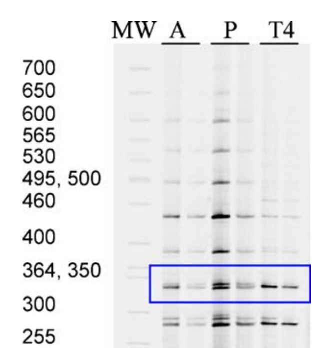


**Fig. 1.** cDNA-AFLP profile of salivary gene expression of *Spodoptera exigua* caterpillars reared on different diets. Duplicate lanes represent gene expression in salivary glands isolated from 4th instar caterpillars reared on Bio-Serv artificial diet (A), plants (P) or transferred from plants to artificial diet for 4 h (T4). Lane 1: molecular weight marker (MW; 100 bp ladder); lanes 2–7: mRNA digested with *Taq*I and *Mse*I and fragments amplified with selective primers with 3′-AT and -TC overhangs, respectively. Boxed area indicates a lower constitutive gene fragment. The higher band indicates diet-specific gene expression; the gene is expressed when insects are on the plant but lower transcript levels are seen when caterpillars are fed artificial diet.

**Table 2**
Caterpillar salivary genes identified by cDNA-AFLP or based on homology with insect orthologues. Gene sequences were compared to NCBI Genbank, butterfly base and silkworm genome database and the best hit represented below (Altschul et al., 1997; Papanicolaou et al., 2008; Wang et al., 2005).

| Gene | Size (bp) | Accession # | Homology | *E*-value |
|---|---|---|---|---|
| Differentially expressed: | | | | |
| *Se1H* | 881 (partial) | GU983916 | Unknown protein (*Helicoverpa armigera*; ABU98617.1) | $8.0 \times 10^{-11}$ |
| *Se2J* | 796 | GU983914 | No significant matches | |
| *SeGox* | 2133 | GU983912 | Glucose oxidase (*H. armigera*; ACC94296) | 0.0 |
| Reference genes: | | | | |
| *SeTub* | 683 (partial) | GU983915 | β-Tubulin (*Bombyx mori*; NP001036888.1) | $8.0 \times 10^{-36}$ |
| *SeUbi* | 544 | GU983913 | Ubiquitin-53 aa extension protein (*S. exigua*; AAN15743.1) | $4.0 \times 10^{-65}$ |

isolated and sequenced by 5′- and 3′-RACE. Full length transcript sequences were obtained for *Se2J* and *SeUbi*, whereas only partial sequences were obtained for *Se1H* and *SeTub*. Two labial salivary genes (*Se2J* and *Se1H*) are novel genes of unknown function (Table 2, Fig. 2a and b); database searches did not generate significant matches for Se2J and Se1H shows homology with an unknown protein identified in the gut proteome of the closely related Noctuid, *Helicoverpa armigera* (gi:ABU98617.1) (Pauchet et al., 2008).

*SeGox* was sequenced based on homology with the honey bee, *Apis mellifera*, enzyme present in the hypopharyngeal gland of adult workers, since this was the only sequence available when this project was initiated (gi:NP001011574) (Iida et al., 2007). At the protein level, SeGOX shares 72% similarity with the honey bee enzyme and greater than 93% similarity with *H. armigera* and *H. zea* GOX (gi:ACC94296 and gi:ACJ71598) (Table 2, Fig. 2c). This protein has two signature domains which are characteristic of glucose–methanol–choline oxidoreductases (Kiess et al., 1998).

*SeUbi* was isolated by cDNA-AFLP to represent a constitutive, reference gene. *SeUbi* encodes a highly conserved ubiquitin polypeptide linked to a 53 amino acid extension protein on the C-terminus (Fig. 2d) (Catic and Ploegh, 2006). In eukaryotic systems, the C-terminal extension polypeptide (CEP) associates with ribosomal RNA sequences in the large ribosomal subunit and is generally believed to be constitutively expressed even if the cell experiences stressful conditions (Muller and Schwartz, 1995; Van Zeveren et al., 2007). *SeTub* was sequenced based on homology with silkworm, *Bombyx mori*, *BmTub* (gi:NP001036888) (Fig. 2e); only a partial sequence for *SeTub* was obtained, representing ~49% of the gene.

Labial salivary genes were analyzed for conventional and non-classical 5′-secretory peptides and cellular localization; none of these proteins are predicted to be secreted by conventional ER/Golgi routes. Se2J is predicted to be targeted either to the mitochondria or a nuclear protein. SeUbi is also predicted to be nuclear. However, proteins may also be secreted by non-conventional routes (Nickel, 2003). For example, endosomal release of secretory granules is associated with some salivary systems (Castle, 1998). SeGOX, which is present in caterpillar LS secretions, is predicted to be secreted through a non-classical route (Bendtsen et al., 2004; Peiffer and Felton, 2005). Further biochemical analysis must be performed to determine this mechanism.

### 3.2. Functional characterization of SeGOX

To date, the functional characterization of the putative lepidopteran *GOX* gene has not been reported. Strong GOX protein levels with a predicted size of ~70 kDa were observed in mouse fibroblast cells lines transfected with the *SeGOX*::pEGFP-N3 vector as compared to the empty vector control (Fig. 3a). The molecular mass difference between this protein and GOX isolated from *H. zea* probably reflects the glycosylation of this enzyme (Eichenseer et al., 1999). In cell-free homogenates, GOX activity in the *SeGOX*-transfected extracts was significantly higher than the boiled or vector-only controls (GOX: $F = 50.1$; df = 2,8; $P < 0.01$) (Fig. 3b). These results indicate that *SeGOX* does indeed encode a functional enzyme.

### 3.3. Effect of nutritional quality on labial salivary gene expression

Differential transcript patterns of labial salivary genes in caterpillars reared on *M. truncatula* plants or transferred to Bio-Serv artificial diet for 4 h were confirmed by qRT-PCR (data not shown). This experiment verified that *SeTub* is an appropriate reference gene since its expression was constant across treatments (diet: $F = 0.6$; df = 4,14; $P = 0.68$) and stable relative to the constitutive gene (*SeUbi*) identified by cDNA-AFLP (Brunner et al., 2004).

Once diet-specific patterns of gene expression were validated, caterpillars were restricted to chemically defined AD that ranged in nutritional quality. Diet nutritional quality, particularly the protein (p) to digestible carbohydrate (c) ratio, affects caterpillar labial salivary enzyme activity (Babic et al., 2008; Merkx-Jacques and Bede, 2005). Third instar caterpillars were transferred for one stadium from a basal diet (22p:20c) to one of five diets varying in p:c levels and ratio: 22p:20c, 22p:40c, 30p:20c, 33p:30c, 30p:40c. The basal diet represents the self-selected diet of *S. exigua* caterpillars (Merkx-Jacques et al., 2008). Two diets, 22p:20c and 33p:30c, have the same nutrient ratio but different P and C levels. Two diets have a lower protein content (22p:20c and 22p:40c) compared with the second set (30p:20c, 33p:30c and 30p:40c). Diet sets also have a constant protein level with a low or high carbohydrate level (22p:20c vs. 22p:40c and 30p:20c vs. 30p:40c).

As expected, constitutive expression of *SeUbi* was not affected by diet (data not shown). In comparison, low gene expression of *Se2J*, *Se1H* and *SeGOX* observed in caterpillars restricted to carbohydrate-poor diets increased as the dietary carbohydrate levels increased, irrespective of protein content (*Se2J*: $F = 4.5$; df = 4,14; $P = 0.01$; *Se1H*: $F = 3.8$; df = 4,14; $P = 0.03$; *SeGox*: $F = 3.2$; df = 4,14; $P = 0.04$) (Fig. 4a–c). For example, at the same protein level, transcript levels increased over 2-fold when caterpillars are fed carbohydrate-rich diets (22p:20c vs. 22p:40c and 30p:20c vs. 30p:40c). This implies that transcription of *Se2J*, *Se1H* and *SeGOX* is regulated in a similar manner.

### 3.4. Effect of nutritional quality on labial salivary glucose oxidase enzyme activity

To understand the relationship between *SeGOX* gene expression and enzyme activity, labial salivary GOX activity was measured in caterpillars restricted to the same diets. Unexpectedly, the enzyme activity pattern did not match that of gene expression (Figs. 4c and 5a). Basal levels of labial salivary GOX activity was observed in caterpillars restricted to protein-poor diets, irrespective of the carbohydrate content (22p:20c and 22p:40c) and only reflected carbohydrate levels when caterpillars were fed protein-rich diets (diet; $F = 1.3$; df = 4,29; $P < 0.01$) (Fig. 5a). Protein is a limiting nutrient for caterpillars and, presumably, only when sufficient protein resources have been obtained can the caterpillar afford to

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(A) S. exigua 2J    MRRKTMLLLIKQKRATLRMPRKTMQPLIQHKQATLRMPRKTMQPLIQHKQAILKMPFKM
                    MLPRKKATLRMPRKTVALLMTQPVLIPSTQLPKLFLKTTDSLLF

(B) S. exigua 1H    MKYGFNLFGMGSKVFIDLPRTNTSAVDKGWQSMNRPRRLSGYCPLQLWCPTDDYTVCIH
                    TDETGYVAGVQIAIPVAKFNSTFDMKEQGXXVWTTIFNSKIVRFWVTSLYFVSPDRQAR
                    DSLDADKVLRDDNVYFNSFNGVKGNVSTSLDKLSSLYTKQACIGWLGRQY

(C) S. exigua       MILAPEDCGCPIVEEGPSLVNSPVCSGNFLFMVLLQSYLWGRCSISTPCKRIESVEETEQ
    H. armig.       MILAQQDCGCATVVEGASILNSTACSGTYLFMVLLQGYLWGRCEIATPCKRIESIDETES

    S. exigua       EYDFIVVGAGSAGSIVAGRLSENTTYKVVLLEAGGPEPLGVRVPSFYRTFWDNPAVDWQI
    H. armig.       EYDFIIVGAGSAGSIVAGRLSENTSYNVLLLEAGGPEPLGARVPSFYKTFWGHDEVDWQG

    S. exigua       RTVP-ADYCLDQEGLGCKWPLGKGLGGTSQLNGMMYHRGHHADYTCDWVEAGAKGWSWDE
    H. armig.       RAVPDPNFCRDQGELGCQWPLGKSLGGSSLLNGMMYHKGHAADYET-WVEEGAEGWSWDE

    S. exigua       IKPFMDLTEGNKQIGSLVDGKYHSDIGPLPIQTFRYQPLALYDLMDAINQTGLPLIKDMN
    H. armig.       VKPFMDLAEGNRQVGSLVDGKYHSETGRMPIQTFNYQPPQLRDLIEAINQTGLPIITDMN

    S. exigua       NPNTPEGFVIAQAFNDNGQRYTTARAYLPPQSERPNLNIKLHAHVTKVLFRRKKAIGVEY
    H. armig.       DPNTPDGFVVAQTFNDNGQRYTTARAYLAPKSERPNLSVKLYAHVTKVLFDGKKAVGVEY

    S. exigua       VDENGNTKVVKARKEVILSAGALTSPKILMHSGVGPKETLEPLGIKVIEDLPVGKNLKNH
    H. armig.       VDKNGNTKTVKTTKEVIVSAGPLTSPKILMHSGVGPKEVLEPLGIPVVADVPVGKRLRNH

    S. exigua       CGATLYFILKKVKNTQVLDWSALTEYLLQNDGPMSSTGLTQLTGLLYSSYAKKELKQPDL
    H. armig.       CGATLNFLLKKSNNTQSLDWSAMTDYLLELDGPMSSTGLTQLTGLLYSSYADKSRKQPDL

    S. exigua       QFFFNGFYAECSKTGAIGEPAIECPNSGYNVSANAVYLLPRSVGYMTINSTDPFEQAIYD
    H. armig.       QFFFNGLYADCSKTGVIGEPAEDCS-DGYKISANAVALLPRSVGHVTINSTDPFKSALFY

    S. exigua       PNFFSDPVDMEVIKEGLEYLRQIFNSELLQEQYEIELDPTYTEKCDKVAPAWSDDWKECM
    H. armig.       PNFFSHPDDMNIVMEGVDYLRQIFESEVLQEKYKVELDPEYTKECDDYE-AWSRDWKECM

    S. exigua       IRVHTDPQNHQLGTCAIGKVVDPELRVYNLKALRVCDAGSMPSQPTGNPQGAIMAVAERC
    H. armig.       IRLHTDPQNHQLATNAIGKVVDPQLRVYDVKNLRVCDAGSMPSPPTGNPQGAIMVVAERC

    S. exigua       AHFIKEAWQ
    H. armig.       AHFIKQTWQ

(D) AAN15743        MQIFVKTLTGKAITLEVEASDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGR
    S. exigua 2M    MQIFVKTLTGKTITLEVEASDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGR

    AAN15743        TLSDYNIQKESTLHLVLRLRGGTIEPSLRILAMKYNCDKMICRKCYARLHPRAT
    S. exigua 2M    TLSDYNIQKESTLHLVLRLRGGTIEPSLRILAMKYNCDKMICRKCYARLHPRAT

    AAN15743        NCRKTKCGHTNNLRPKKKLKD
    S. exigua 2M    NCRKTKCGHTNNLRPKKKLKD

(E) S. exigua       ----------------------------------------------------------
    B. mori         MREIVHLQAGQCGNQIGAKFWEIISEEHGIDPTGVYRGTSDLQLERISVY

    S. exigua       ----------------------------------------------------------
    B.mori          YNEASVATAESGGKYVPRAILLDLEPGTMDAVRSGAYGQLFRPDNFVF

    S. exigua       ----------------------------------------------------------
    B.mori          GQSGAGNNWAKGHYTEGAELVDAVLDVVRKECENCDCLQGFQLTHSL

    S. exigua       ------------------------MNTYSVVPSPK VSDTVVEPYNAVLSI
    B.mori          GGGTGSGMGTLLISKIREEYPDRIMNTYSVVPSPKVSDTVVEPYNAVLSI

    S. exigua       HQLVENTDETYCIDNEALYDICYRTLKVPNPTYGDLNHLVSLTMSGVTT
    B. mori         HQLVENTDETYCIDNEALYDICYRTLKVPNPTYGDLNHLVSLTMSGVTT

    S. exigua       CLRFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLTSRGSQQYRALTVP
    B.mori          CLRFPGQLNADLRNWSVNMVPFPRLHFFMPGFAPLTSRGSQQYRALTVP

    S. exigua       ELTQQMFDAKNMMAACDPRHGRYLTVAAIFRGRMSMKEVDEQMLSIQ
    B.mori          ELTQQMFDAKNMMAACDPRHGRYLTVAAIFRGRMSMKEVDEQMLSIQ

    S. exigua       NKNSSFIVEWIPNNVKTAVCDIPPKGLKMSSTFIGN
    B.mori          NKNSSFFVEWIPNNVKTAVCDIPPKGLKMSSTFIGNTTAIQELFKRISEQFS

    S. exigua           ------------------------------------------------------
    B.mori          AMFRRKAFLHWYTGEGMDEMEFNEAESNVNDLVSEYQQYQEATAEDD

    S. exigua       ---------------------------------
    B.mori          TEFDQEDLEELAQDEHHD
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**Fig. 2.** Predicted protein sequences of diet-specific and constitutive labial salivary genes. (A) Predicted protein sequence of Se2J based on the longest open reading frame. (B) Predicted protein sequence of Se1H based on the longest open reading frame. (C) Protein sequence alignment of *S. exigua* and *Helicoverpa armigera* glucose oxidase (gi:ACC94296). Proteins share 75.6% identity (in blue) and 17.5% similarity (in green) in amino acids. Two conserved glucose–methanol–choline signature domains are underlined. (D) Protein sequence alignment of SeUbi and *S. exigua* ubiquitin-53 amino acid C-terminus extension protein (gi:AAN15743). Proteins share 99% identity; sequence difference is shown in blue. Ubiquitin polypeptide (———) is underlined. (E) Protein sequence alignment of Setub and *Bombyx mori* β-tubulin (gi:NP001036888). Proteins share 98% identity; differences are highlighted in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

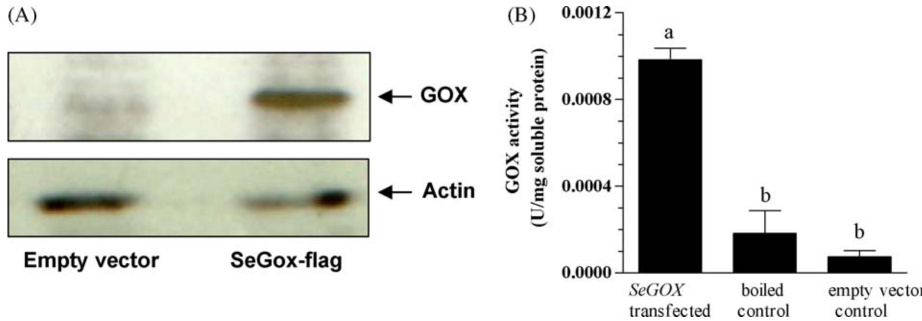


**Fig. 3.** Expression and functional characterization of SeGOX. *SeGox* cDNA cloned in-frame into the flag-tag pEGFP-N3 vector or the empty vector control were transfected into mouse fibroblast lines. *SeGOX* expression was driven by a constitutive CMV promoter. Cell-free homogenates were used for (A) Western blot detection, using a mouse anti-flag M2 or goat anti-actin (loading control) or (B) GOX activity assay was determined by the *o*-dianisidine-horseradish peroxidase coupled reaction. Bars represent the means of three independent transfections ± SD. Significant differences ($P < 0.05$) are indicated by different alphabetical letters.

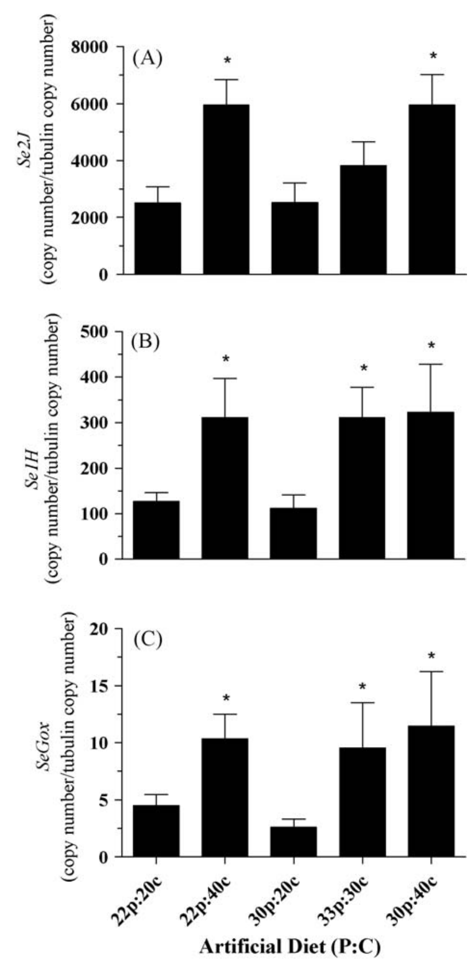


**Fig. 4.** Effect of dietary nutritional quality on caterpillar labial salivary gene expression. From the basal 22p:20c AD, early 3rd instar *S. exigua* caterpillars were transferred to one of the following chemically defined diets: 22p:20c, 22p:40c, 30p:20c, 33p:30c, 30p:40c. Salivary glands were dissected from 4th instar caterpillars and transcript expression of four genes, A. *Se2J*, B. *Se1H*, C. *SeGOX* and *SeTub*, were analyzed by qRT-PCR using gene-specific primers (Table 1). Expression levels were normalized using *SeTub* as the reference gene. For each treatment, three independent biological replications of 50 pooled pairs of labial salivary glands were used. Data represents means ± SE. A >2-fold increase in relative gene expression as compared to insects maintained on 22:20 is denoted by an asterisk (*).

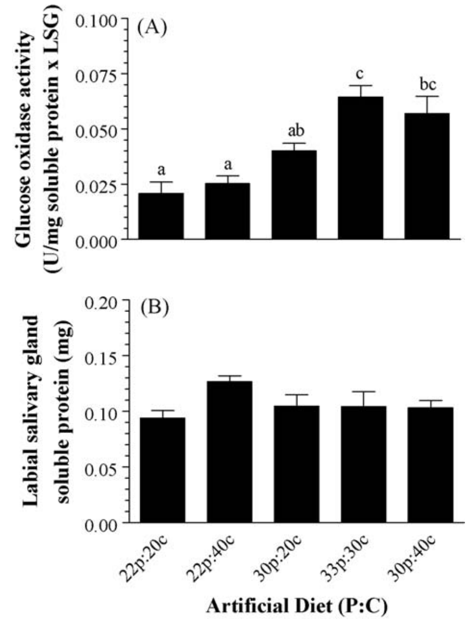


**Fig. 5.** Effect of dietary nutritional quality on caterpillar salivary glucose oxidase activity. From the basal 22p:20c AD, early 3rd instar *S. exigua* caterpillars were transferred to one of the following chemically defined diets: 22p:20c, 22p:40c, 30p:20c, 33p:30c, 30p:40c. Salivary glands were dissected from 4th instar caterpillars. (A) GOX activity was measured using a horseradish peroxidase-*o*-dianisidine coupled assay. (B) Salivary gland soluble protein level. For each diet, six independent biological replications of eight pooled pairs of labial salivary glands were used. Data represents means ± SE. Significant differences ($P < 0.05$) are indicated by different alphabetical letters.

invest in secreted enzymes, such as GOX (Babic et al., 2008; Hu et al., 2008). Therefore, this study suggest that there are at least two mechanisms involved in the regulation of these caterpillar labial salivary proteins; transcriptional regulation mediated by dietary carbohydrate levels and translational or post-translational regulation mediated by amino acid/protein levels. At present, the basis for this diet-dependent post-transcriptional regulation is unknown; possible mechanisms include activation of zymogens through proteolysis or covalent modifications to activate the enzymes (Bayés et al., 2003, 2006; Ramalho-Ortigão et al., 2005; Wang et al., 2004).

## 4. Conclusion

Our study shows that *SeGOX* and two other genes, *Se1H* and *Se2J*, which encode uncharacterized proteins, are transcriptionally regulated in a diet-specific manner. In comparison to these genes where expression increased when caterpillars fed on carbohydrate-rich diets, GOX enzyme activity also reflected the protein content of the diet, indicating this enzyme also undergoes translational or post-translational regulation. This begs the question of how information from the diets translates into changes in insect digestive physiology (Bede et al., 2007; Borovsky, 2003; Terra and Ferreira, 1994). This may be a straightforward reflection of nutritional resources, the cellular amino acid and carbohydrate pools; however, endocrine hormones which act to integrate physiological status and dietary nutritional quality may also be involved (Bede et al., 2007). For some insect species, stretch receptors in the gut may be involved in stimulating digestive processes, such as the transcriptional regulation of digestive enzymes, and/or the cessation of feeding (Borovsky, 2003; Briegel and Lea, 1975; Gelperin, 1971; Klowden and Lea, 1979). Since these receptors are regulated by volumetric feedback, presumably, they do not exert diet-specific physiological effects. Nutrient specificity is more likely regulated by paracrine and/or prandial mechanisms (Bede et al., 2007; Lehane et al., 1995; Rana and Stanley, 1999). Diet compounds may affect the types of digestive enzymes released. Caterpillars alter their gut trypsin proteases in response to ingestion of plant trypsin proteinase inhibitors (Bown et al., 2004; Broadway, 1995; Jongsma and Bolter, 1997; Paulillo et al., 2000). Endocrine-like cells in insect guts may also be responsible for monitoring gut nutrient content and insect physiological status and influencing the secretion of digestive enzymes in neighbouring cells through hormone release (Bede et al., 2007; Fusé et al., 1999).

Yet, in our study, nutritional content of the diet affected gene expression in the labial salivary gland, a discrete organ from the caterpillar gut. Again, one can speculate that hormones released from endocrine-like cells in the gut may be transported in the haemolymph to the salivary glands (Bede et al., 2007). Haemolymph also contains the disaccharide trehalose which may directly mediate information on the insect nutritional status (Thompson, 2003). Neural or neurohormonal signals could arise from innervated salivary glands and affect cellular transcription of digestive enzymes (Gris, 1989; Vegliante, 2005). One or more of these signals conveying information on the insect physiological status and dietary contents must be integrated at the organ level to affect labial salivary gene transcription.

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