



Lectin expression in grasshopper (*Melanoplus differentialis*) fat body, ovary and testis tissue  
by Layla Gedik

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in  
Biochemistry

Montana State University

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Abstract:

Lectins play an important role in the humoral and cellular defense response of invertebrates. The carbohydrate-binding capacity of these proteins mediates recognition of specific carbohydrates on the surface of microorganisms, leading to their opsonization.

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The objective of this study is to determine lectin expression in grasshopper fat body, ovarian and testicular tissue using molecular biology techniques. Results would confirm or contradict previous published data that indicates synthesis of agglutinins in the three respective tissues (Stiles et al. 1988).

Total RNA and polyA<sup>+</sup> RNA were isolated from pre-dissected and frozen fat body, ovary and testis tissue, for use in Northern analysis and reverse transcription-polymerase chain reaction (RT-PCR). Northern analysis using a <sup>32</sup>P-labeled lectin cDNA probe was successful with fat body polyA<sup>+</sup> RNA, indicating lectin transcription in this tissue. RT-PCR with clone 3- and clone 4-specific primers gave expected bands with fat body, ovary and testis polyA<sup>+</sup> RNA. Southern analysis of the RT-PCR products with a radioactively labeled lectin cDNA probe showed that the RT-PCR products were a result of amplification of lectin mRNA. Interpretation of RT-PCR and Southern analysis results indicate that genes encoding clone 3 and clone 4 are expressed in fat body, ovary and testis tissues. Additional lectin-specific bands with the three tissues in Southern analysis data suggest expression of other lectin genes. Overall results confirm that grasshopper fat body, ovarian and testicular tissues are synthesizing lectin molecules.

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FAT BODY, OVARY AND TESTIS TISSUE

By

Layla Gedik

A thesis submitted in partial fulfillment  
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MONTANA STATE UNIVERSITY  
Bozeman, Montana

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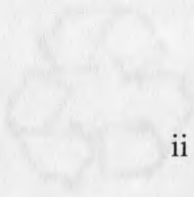
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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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## ABSTRACT

Lectins play an important role in the humoral and cellular defense response of invertebrates. The carbohydrate-binding capacity of these proteins mediates recognition of specific carbohydrates on the surface of microorganisms, leading to their opsonization.

Agglutinin that binds specific carbohydrates has been isolated from hemolymph of grasshopper, *M. differentialis*. This protein is a C-type lectin, requiring calcium for sugar binding activity. Two related lectin cDNA clones have been isolated, namely clone 3 and clone 4. The full length cDNAs of clone 3 and clone 4 are 2.1 Kb and 2.0 Kb, respectively, and they show 80% sequence identity within the open reading frame.

The objective of this study is to determine lectin expression in grasshopper fat body, ovarian and testicular tissue using molecular biology techniques. Results would confirm or contradict previous published data that indicates synthesis of agglutinins in the three respective tissues (Stiles et al. 1988).

Total RNA and polyA<sup>+</sup> RNA were isolated from pre-dissected and frozen fat body, ovary and testis tissue, for use in Northern analysis and reverse transcription-polymerase chain reaction (RT-PCR). Northern analysis using a <sup>32</sup>P-labeled lectin cDNA probe was successful with fat body polyA<sup>+</sup> RNA, indicating lectin transcription in this tissue. RT-PCR with clone 3- and clone 4-specific primers gave expected bands with fat body, ovary and testis polyA<sup>+</sup> RNA. Southern analysis of the RT-PCR products with a radioactively labeled lectin cDNA probe showed that the RT-PCR products were a result of amplification of lectin mRNA. Interpretation of RT-PCR and Southern analysis results indicate that genes encoding clone 3 and clone 4 are expressed in fat body, ovary and testis tissues. Additional lectin-specific bands with the three tissues in Southern analysis data suggest expression of other lectin genes. Overall results confirm that grasshopper fat body, ovarian and testicular tissues are synthesizing lectin molecules.

## INTRODUCTION

Insect (Invertebrate) Immunity

Insects have been very successful in evolution, populating all niches apart from the seas (Hoffman 1995). This achievement is largely due to the insects' ability to defend themselves against many different microorganisms such as bacteria and fungi, as well as viruses and parasites. The defense is both external, that includes avoiding discovery, resisting attack and physical barriers such as the insects' cuticle, or internal, involving the insects' innate immune system.

Insects lack the immunoglobulin-like recognition molecules and immune memory characteristic of the vertebrate immune system (Lowenberger 1996). However, there are similarities between vertebrate and invertebrate immune responses. Insects use cellular and humoral components of their hemolymph to fight harmful microorganisms (Strand and Pech 1995, Glinski and Jarosz 1995). With the cellular facet, it is understood that most insects have several types

of cells in the open circulatory system that remove foreign material by phagocytosis or encapsulation (Lowenberger 1996, Hoffman 1995). However, little is known of the molecular mechanism of the cellular response involved in the recognition of the foreign particles. The humoral response mechanism is better grasped. This involves the induction of two proteolytic cascades - the hemolymph coagulation cascade and prophenoloxidase cascade (Hoffman et al. 1996). The cascades result in clotting and melanization of encapsulated pathogens. Another humoral response to a septic injury is the synthesis, predominately by the insect fat body, of antimicrobial proteins and peptides that are released directly into the circulating hemolymph (Lowenberger 1996, Hoffman et al. 1996). The response has similarities to the vertebrate acute-phase response.

Lectins are recognition molecules that have suspected roles in insect defense against pathogens. They will be described below.

### Lectins

Lectins are ubiquitous and diverse proteins or

glycoproteins that bind to carbohydrates and glycoconjugates with considerable specificity (Rini 1995, Kennedy et al. 1995). Many lectins have been isolated from plants, animals and microorganisms (Sharon 1993). They serve to mediate biological recognition events through the binding of ligands to their one or more carbohydrate recognition domains (Rini 1995, Kennedy et al. 1995). Agglutinins are lectins in which binding of ligands at the carbohydrate binding sites leads to precipitation or agglutination of the cell or glycoconjugate (Lis and Sharon 1986).

There are four classes of animal lectins: C-type, S-type (galactins), I-type and P-type (Rini 1995, Drickamer 1993; Powell and Varki 1995). The C-type lectins require one calcium ion at each carbohydrate-recognition domain (CRD) to directly coordinate with the carbohydrate (Kolatkar and Weis 1996, Weis et al. 1992). Additional domains, if any, of the protein dictate the role and location of the lectin molecules (Drickamer 1993). Most C-type lectins bind to galactose and its derivatives (Gal-type ligands) or to D-mannose, D-glucose and derivatives (Man-type ligands) (Kolatkar and Weis 1996). Selectins, collectins, endocytic glycoprotein receptors and the macrophage mannose receptor

are all members of the C-type family (Rini 1995, Weis et al. 1992).

C-type vertebrate lectins are found in serum, membranes and extracellular matrix (Drickamer 1993). For example, type II endocytic receptors that mediate endocytosis of glycoproteins have been described and include the mammalian hepatocyte asialoglycoprotein receptor, which is the most understood of the Gal-binding C-type lectins (Kolatkar and Weis 1996). The rat serum mannose-binding protein (MBP) is the best described Man-binding C-type lectin and its crystal structure is known (Weis et al. 1991). By recognizing self from nonself; MBP functions independently of immunoglobulins in host defenses against mannosidic-containing pathogens.

Agglutinins have been discovered in many species of invertebrates (Mori et al. 1992). It has been proposed that they play an important role in the humoral and cellular immunity in insects (Ratcliffe et al. 1985; Wheeler et al. 1993). Lectins in the invertebrates *Sarcophaga peregrina* (flesh fly; Komano et al. 1983), *Periplaneta americana* (cockroach; Kawasaki et al. 1996), *Drosophila melanogaster* (fruit fly; Natori et al. 1996), *Bombyx mori* (silkworm; Mori

et al. 1992), *Manduca sexta* (tobacco hornworm; Minnick et al. 1986) and *Spodoptera exigua* (beet armyworm; Boucias and Pendland 1993) have been described and they all possess hemagglutinin activity. The expression of the *Sarcophaga* lectin is induced by injury to the body wall of the larvae (Sugiyama and Natori 1991). Two *Periplaneta* hemolymph lectins show enhanced expression after injection of *E.Coli* into the body cavity of the adult (Kawasaki et al. 1996). These lectins act as opsonins and have been implied to have functions in insect defense systems.

The *Sarcophaga*, *Periplaneta* and *Spodoptera* lectins are synthesized in the fat body of the insect (Mori et al. 1992, Komano et al. 1983). The fat body is an amorphous tissue that lines the hemocoel of the insect (Lowenberger 1996). One of its roles is to synthesize immune compounds that are then released into the hemolymph. The hemocyte is the site of synthesis of the lectins in *B. mori* (Mori et al. 1992).

Although it appears that certain lectins are involved in insect immunity, this is not their only role. Lectins are involved in cell-cell interactions. For instance, the *Sarcophaga* lectin and *Drosophila* lectin are suspected to function in development as well as host defense, since their

expression is induced during embryonic and pupal stages of the fly (Sugiyama and Natori 1991, Natori et al. 1996). In most hemimetabolous invertebrates, agglutinins occur constitutively and are apparently not induced by foreign material. These agglutinins probably have roles in various tissue interactions and developmental processes (Lis and Sharon 1989). A well-studied lectin from sea urchin sperm binds carbohydrates on the egg surface in a species specific manner, prerequisite to fertilization (O'Rand 1988).

#### Previous Work

Previous work has given indication of agglutinin synthesis in grasshopper fat body, ovary and testes (Stiles et al. 1988). Agglutinin release from hemocytes was not detected, although hemagglutinin was located to hemocytes via potential hemocytic membrane receptors (Bradley et al. 1989). The work involved two techniques on primary cultures of the respective tissues, (1) the use of ELISA assays to determine whether primary cultures of the three tissues were releasing agglutinin into the medium, and (2) metabolic labeling of newly synthesized proteins using L-<sup>[35S]</sup>

methionine followed by immunoprecipitation with agglutinin-specific antibody to determine if the cultured cells were synthesizing agglutinin in addition to releasing it from cellular storage.

Agglutinin release from fat body was not stimulated by the addition of microbial cell wall components to fat body cultures or injection of these constituents into the grasshopper hemocoel. It appears that the agglutinin is constitutively present in the hemolymph and is an opsonin toward *Beauveria bassiana* blastospores, since it stimulates adhesion to hemocytes leading to removal of the fungal blastospores from the circulation (Wheeler et al. 1993). The agglutinin is not opsonic toward certain bacteria, protozoa or asialo erythrocytes, indicating strict selectivity (Bradley et al. 1989).

More recently, two positive lectin clones were isolated and sequenced from a *Melanoplus differentialis* grasshopper cDNA  $\lambda$ gt11 library, namely clone 3 and clone 4 (Rognlie, unpublished results). The clones were subsequently inserted into Promega pGem<sup>®</sup>-7zf(+) plasmid vector (see Figure 1). It was found that the two clones were only partial length

representing incomplete copies of the two clones. Clone 4 was missing the extreme 5' end and clone 3 was absent approximately 30 percent of the 3' end. It has been recently shown that both truncated clones were the result of cleavage at internal EcoRI sites during library construction. Currently, using RACE (Rapid Amplification of cDNA Ends) methods, the extreme 5' end of clone 4 and the 3' portion of clone 3 were isolated and sequenced in this laboratory (Radke, unpublished results). This work resulted in full length cDNAs of approximately 2.1 Kb and 2.0 Kb for clone 3 and clone 4, respectively. Clone 3 and clone 4 code a protein of 324 and 326 amino acid residues, respectively. The open reading frames of clone 3 and clone 4 show 80% sequence identity. See Figure 2 for alignment of clone 3 and clone 4 nucleotide sequence, indicating homology between the two clones. Each clone contains two CRDs that are 30% identical to each other as well as other invertebrate C-type lectins, indicating considerable time since divergence of the ancestral form (Doolittle 1995). There are two potential N-glycosylation sites in each clone.

Homology modeling of one clone 3 CRD has been performed using the three-dimensional x-ray crystal structures of rat

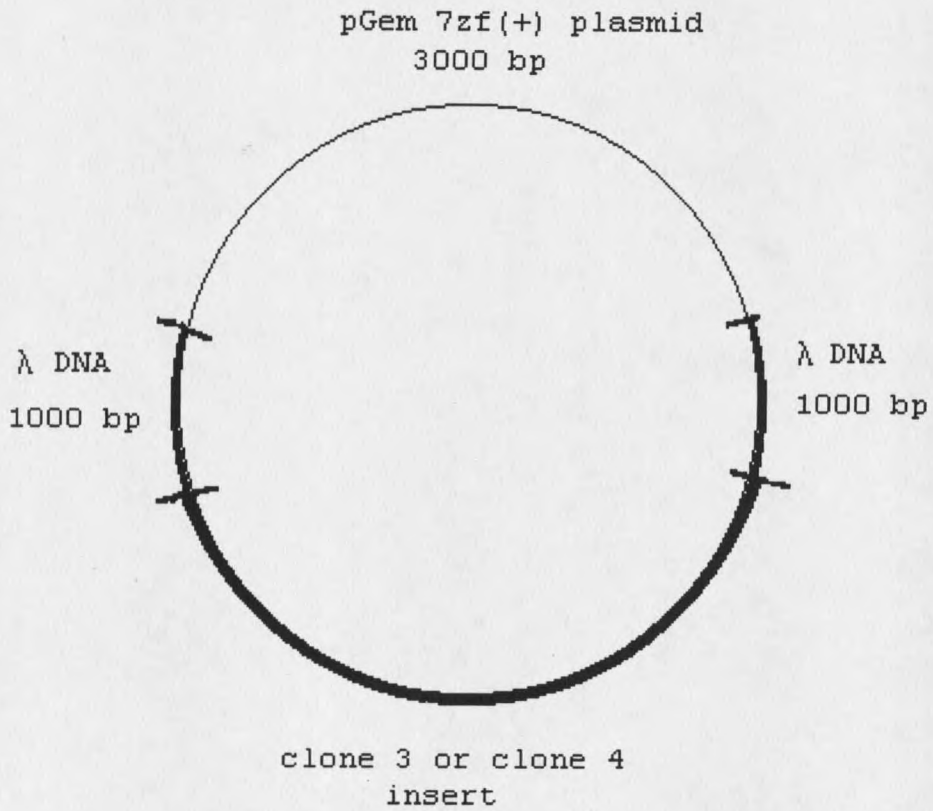


Figure 1: Plasmid vector pGem 7zf(+) with clone 3 or clone 4 inserts (bracketed with approximately 1000 bp of  $\lambda$  DNA from previous vector).



```

TGTGGAAGGTGACTTCGTTACCGAAACAGGAAGACACCTAAAAGGCATGGAATTCAGGT      891
** ***** **
TGCGGAAGGTGACTTCGTTGCCGAAACAGGAAGACACCTAAGAGACATGGAATTCGAAGT      866

GTGGAGCCCTGGTGAGCCAAATAACGACGTCGATGGGAAGCCCGAGAATTGCCTAGCCTT      951
***** ** * ***** ** ** **
GTGGAAACCTGGTGAGCCAAATAACAACCTTTCTTGGGAAGCCCGAGAAGCTTCTAGGCTT      926

TTCGGGCCGTGGCTACTACGGCGACAGGAGCTGCGAAGTGGAGCTGCCCTTCATCTGTGA      1011
* **** ***** ** * ***** **
TGGGGGCAAAGGCTACTACGACGACAAGAGCTGCGATTTGGAGCTGCCCTTCATCTGCGA      986

GATAGCGCCCTGACCGTGGCGCTCTCGGGACACAATTCTGAGGACGCAACAACCTGCGAATG      1071
***** ***** ** * *****
GATAGCGCCCTGACCGTGGCGCTCTCGTGACAG--TCTGAGGACGCAACAACCTGCGAATG      1046

GAAACGACGTACGCGGAGAGCATGGACTCGTGCGAAGCCGAGTGACATTCAGAAGAACTT      1131
***** **** ** ***** ** * ** * **
GAAACGACGTACTCGGAAAGTATGGACTGTTGCGAGGCAGAGTTACGTTCAGAAGACGTT      1106

GTATAATTTATATGTGAATAAATATTTCGTTAGCAACCCCTAAAAAAAAAAAAAAAAAAAAA      1191
***** ***** *****:**** ** * *****
GTATAATTGATATGCGAATAAATATTTCGTTAGTAACC----TAAAAAAAAAAAAAAAAAAAAA      1166

GTACTAGTCGACCATATGGGAGAGCTCCC      clone 3      1220
** * ** * ** **
AAAC--GGAATCC-----GCGGAATTCCG      clone 4      1226

```

Figure 2: Sequence alignment of clone 3 (top) and clone 4 (bottom), using Genepro (Version 5.00, Riverside Scientific, WA). Numbers on the right correspond to nucleotide numbers starting from the 5' end. Asterisks indicate identical nucleotides between the two clones. The potential initiation site is within the triangle. The termination codon is surrounded by a rectangle.

MBP (Weis et al. 1991) and E-selectin (Graves et al. 1994) as reference proteins (Radke, unpublished results).

Mass determination of a purified grasshopper lectin, GHA, has been performed using SDS-PAGE and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS) (Wenzlick, unpublished results). The protein is a disulfide dimer of 72 kDa with identical-sized monomers of 36 kDa. It has been demonstrated through enzymatic removal of carbohydrate that the lectins are glycoproteins (Wenzlick, unpublished results). The two monomers are apparently identical.

Molecular biology work underway in the laboratory, in addition to that presented in this thesis, includes Southern analysis to reveal the number of lectin genes and their intron character (Gedik T, unpublished results).

### Rationale and Approaches for this Research

Grasshoppers (*Melanoplus differentialis*) are a pest in Montana and other western states because of the expensive damage they cause to crops. Control of the grasshopper populations is therefore a practical and economic goal.

Insect parasites, by attacking their host, reduce the population number of the host. Increased knowledge of the natural immunity of the insect could lead to new methods of pest control that rely on manipulations of its defense systems. This research is concerned with lectin proteins that have a putative role in the immune response of the grasshopper. Knowing the site of synthesis of the lectins will give information as to which tissues to target when manipulating the defense system of the grasshopper. Since there is more than one lectin in *M. differentialis*, expression of each lectin may not be simultaneous and could require differential manipulation. In some insect species, genital tract cells synthesize antimicrobial proteins (Hoffman et al. 1996). It would be meaningful to investigate lectin expression in the genital organs of the grasshopper, as well as in fat body.

Examining the expression of lectin genes in fat body, ovary and testis is the major goal of this research. Two techniques are used to accomplish this aim - (RNA hybridization analysis (Northern) and Reverse Transcription - Polymerase Chain Reaction (RT-PCR) of mRNA. These methods are described below.

### Northern Analysis

Northern analysis is the most commonly used method for determination of gene expression. It allows for the analysis of a particular mRNA sequence in an assortment of mRNA sequences (KroczeK 1993). The approach entails the separation of total RNA by gel electrophoresis, transfer of the RNA onto a membrane, and detection of sequences of interest via hybridization to a specific, labeled probe. DNA probes are typically used, labeled with a radioactive ( $^{32}\text{P}$ ) label or a non-radioactive tag (biotin). Through use of a selected probe of lectin cDNA, synthesized by PCR-amplification of clone 3, Northern analysis will be used to determine lectin expression.

The results will confirm or challenge previous immunological work that indicated agglutinin synthesis in grasshopper occurs in fat body, ovary and testis (Stiles et al. 1988).

### Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

RT-PCR is a method that is becoming increasingly

popular for RNA analysis (Mullis and Faloona 1987). PCR is highly sensitive, so extremely low abundance mRNA may be detected (Souaze et al. 1996). Reverse transcriptase is used to convert mRNA into first strand cDNA (McPherson et al. 1993). Target DNA can then be amplified via PCR utilizing two sequence-specific primers that are complementary to the 3' ends of the target DNA duplex. The polymerase enzyme used for DNA synthesis is heat-stable *Taq* DNA polymerase. Amplified cDNA can be detected by gel electrophoresis and Southern hybridization with a labeled DNA probe (Raval 1994). The specific PCR product visualized on the gel is diagnostic for the presence of the corresponding mRNA in the original tissue.

### Goals

The general goal of this project is to investigate lectin expression in grasshopper fat body, ovarian and testicular tissues. Specific goals are:

- 1) to confirm the expression of lectin genes in the respective tissues of the grasshopper

- 2) successfully apply Northern analysis and RT-PCR to the problem
- 3) confirm or contradict previous published work that showed lectins to be synthesized in grasshopper fat body, ovary and testes tissues
- 4) specify if clone 3 and/or clone 4 lectins are being expressed.

## MATERIALS AND METHODS

Grasshopper Dissection and RNA IsolationInsects

Grasshoppers (*Melanoplus differentialis*) were provided by the USDA/ARS Rangeland Insect Laboratory (Montana State University, Bozeman, Montana) and were a gift from Elaine Oma. The grasshoppers were insectory-raised and had a diet of bran and lettuce. Female and male adult insects were used for dissection.

Avoiding Ribonuclease Contamination

Ribonucleases (RNases) break down RNA and can be found in all places around the laboratory. Care must be taken in avoiding contaminating RNA preparations with RNases. Within the laboratory, precautions were undertaken to avoid contamination. Water was obtained from the MILLI-Q® Water Purification System (Millipore, Bedford, Massachusetts) and

treated with diethyl pyrocarbonate (DEPC) and used throughout this work. DEPC reacts with histidine residues of proteins and inactivates RNases. Gloves were worn when handling reagents.

RNase-free microcentrifuge tubes (Ambion, Inc., Austin, TX) were used throughout and dissection instruments were pre-baked for 4 hours at 400°C. Instruments that could not be baked were treated with RNase ZAP™ (Ambion) and rinsed with DEPC water.

### Dissection

Fat body, ovary and testis tissue was dissected from grasshoppers and frozen for future RNA isolation. Fat body was obtained from both sexes of adult grasshopper, while ovary and testis tissues were removed from adult female and male grasshoppers; respectively.

Grasshoppers were chilled, then washed sequentially in H<sub>2</sub>O containing detergent, H<sub>2</sub>O containing 5% bleach and H<sub>2</sub>O only. The insects were pinned and dissected with a ventral cut from anus to the last abdominal segment. The gut was removed by cutting it at the anus and lifting upward toward

the head. When dissecting the fat body, that lines the dorsolateral walls of the exoskeleton, reproductive organs were removed first. The open carcass was bathed in 0.15 M NaCl saline solution containing 16 units placental RNase inhibitor (BRL Life Technologies™, Inc., Gaithersburg, MD) while tissues were removed using a dissecting scope and forceps. Dissected tissue was placed on a pre-weighed container on dry ice to inactivate any RNases. The tissue samples were weighed and stored at -70°C.

#### RNA Isolation

Total RNA and polyA<sup>+</sup> RNA was extracted from dissected tissues using TRI-Reagent™ (Molecular Research Center, Inc., Cincinnati, OH). This reagent contains water-saturated phenol and the protein denaturant guanidinium thiocyanate that solubilizes cells and inactivates ribonucleases. The manufacturer's protocol was followed, which is based on the method of Chomczynski and Sacchi (1987). Tissues (100-300 mg) from the grasshopper were manually homogenized in TRI-Reagent™. Insoluble material that remained was removed by centrifugation at 13000 g for 5

minutes at 4°C. The centrifugation was an additional step, not described in the manufacturer's protocol, and it was performed to remove solid material that could interfere with the remaining procedure. Chloroform was added and the mixture centrifuged again. The mixture separated into a biphasic system, with RNA in the upper, aqueous phase. The upper phase was removed and the RNA precipitated with isopropanol (0.5 ml/100 mg tissue) at room temperature, resolubilized in 20 µl water and stored at -70°C.

PolyA<sup>+</sup> RNA was isolated using the TRI-Reagent™ manufacturer's protocol. Not all of the steps of total RNA isolation were followed. Once RNA was in the aqueous phase it was mixed with 0.1 volume of 1 M Tris (free base) and 0.8 volume of isopropanol and then applied onto a column that was packed with 50 mg of oligo (dT) cellulose (New England Biolabs, Inc., Beverly, MA). An additional step not described in the protocol was performed. This involved reapplication of the flow through solution to the column in an attempt to acquire more polyA<sup>+</sup> RNA. The polyA<sup>+</sup> RNA that eluted from the column was precipitated with isopropanol, in the presence of 15 µl Microcarrier Gel supplied in the kit.

The kit recommended the use of 6  $\mu$ l of Microcarrier Gel, but a greater amount of polyA<sup>+</sup> RNA was collected with 15  $\mu$ l. The polyA<sup>+</sup> RNA was resolubilized in 20  $\mu$ l water and stored at -70°C.

#### Determination of RNA concentration

To determine RNA concentration, an aliquot of the prepared RNA samples was analyzed in a UV-Vis Spectrophotometer (Varian Techtron Ltd, Australia). Absorbance was read at 260 and 280 nm wavelengths - RNA/DNA and proteins absorb at these wavelengths, respectively. The ratio of OD<sub>260</sub>/OD<sub>280</sub> estimates the purity of the RNA preparation. A 'clean' RNA solution with no contaminants should have a ratio of 1.9-2.2 (Berger and Kimmel 1987)..

The amount of RNA in the preparation is calculated using the following equation (Berger and Kimmel 1987):

$$\text{Concentration of RNA in sample} = (\text{OD}_{260}) (40) (\text{reciprocal dilution}) \cdot \mu\text{g/ml of RNA sample}$$

Probes and PrimersNorthern and Southern Analysis

In both Northern and Southern analyses, a specific DNA probe is used that binds to target DNA or RNA sequences. A 580 bp cDNA lectin probe was prepared in the laboratory by Tanya Gedik by PCR amplification of pGem 3.0 using primers 5'B and 3'D, for use in the Northern analysis. The position of the 580 bp probe (and primers) within clone 3 are included in Figure 3, Page 27. After PCR amplification, the 580 bp PCR product was run on an agarose gel and purified using Prep-a-Gene DNA Purification System kit (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's protocol. The final concentration of 580 bp product, after purification, was estimated by gel electrophoresis and comparison to a known amount of  $\lambda$  DNA.

A 413 bp DNA probe for use in Southern analysis was also prepared by Tanya Gedik with PCR amplification, using primers 3152 and 3'NT on pGem 3.1. Figure 3 shows the position of the primers on pGem 3.1. The techniques used

with the 580 bp probe were followed for the preparation and purification of the 413 bp probe.

The probes are considered to be 'lectin-specific' since they should bind to clone 3 and clone 4 cDNA or transcripts, and any additional lectin cDNA or transcripts to which the probe is homologous.

#### Control Beta-actin Probe

A commercial chicken  $\beta$ -actin cDNA probe (Oncor, Inc., Gaithersburg, MD) was used as a control probe for Northern analysis of chicken brain polyA<sup>+</sup> RNA (Clontech Laboratories, Inc., Palo Alto, CA). The 770 bp  $\beta$ -actin probe was radioactively labeled with <sup>32</sup>P (see procedure below), then hybridized against electrophoresed chicken brain polyA<sup>+</sup> RNA.

#### Probe Labeling

The 580 bp cDNA probe, 413 bp cDNA probe and  $\beta$ -actin control probe were radioactively labeled with <sup>32</sup>P by random priming using Ready-To-Go™ DNA labeling beads (Pharmacia

Biotech Inc., New Jersey), according to the manufacturer's protocol.

Before labeling, 25 ng of double stranded probe was denatured by heating 5 minutes at 95°C. The probe and 50  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dCTP were added to the reaction mixture (in the bead) that contained FPLCpure<sup>®</sup> Klenow fragment, buffer, dATP, dGTP, dTTP and random oligodeoxyribonucleotides, primarily 9-mers. The reaction was incubated at 37°C for 30 minutes.

After incubation, the reaction mixture was applied to a P-30 Bio-gel<sup>®</sup> (Bio-Rad Laboratories, Hercules, California) spin column to remove unincorporated nucleotides. It is helpful to remove unincorporated nucleotides, since any [ $\alpha$ -<sup>32</sup>P]dCTP that is not incorporated into the probe may bind to the membrane, causing background fogging on the subsequent autoradiograph.

An instrument detecting Cerenkov radiation (Bioscan, Inc., Washington, DC) was used to ascertain the specific activity (dpm/ $\mu$ g) of the labeled probe. Cerenkov radiation is given off in the wall of the scintillation vial by energetic <sup>32</sup>P particles (Berger and Kimmel 1987).

PCR Primers

The primers used for PCR were 20 nucleotides long and purchased from National Biosciences, Inc. (Plymouth, MN). The primers were used to amplify a specific region of DNA in a PCR reaction, resulting in a specific length of PCR product. The primers are 100% complementary to a region of either clone 3 and/or clone 4. See primer map (Figure 3) for the position of the primers on clone 3 and/or clone 4. Table 1, below, shows the nucleotide sequence of the primers.

Primer	Nucleotide sequence (5'-3')	T <sub>d</sub>
3152	TCTACAAGGTGCACGCCGAG	68.7°C
3'NT	GTCGTTTCCATTTCGCAGTTG	65.6°C
4052	ACAAAACGTGTCAAAAAGCC	62.1°C
3053	CACCACAGGGACTCGACGAC	67.5°C

Table 1: Primer nucleotide sequence and T<sub>d</sub> (dissociation temperature).

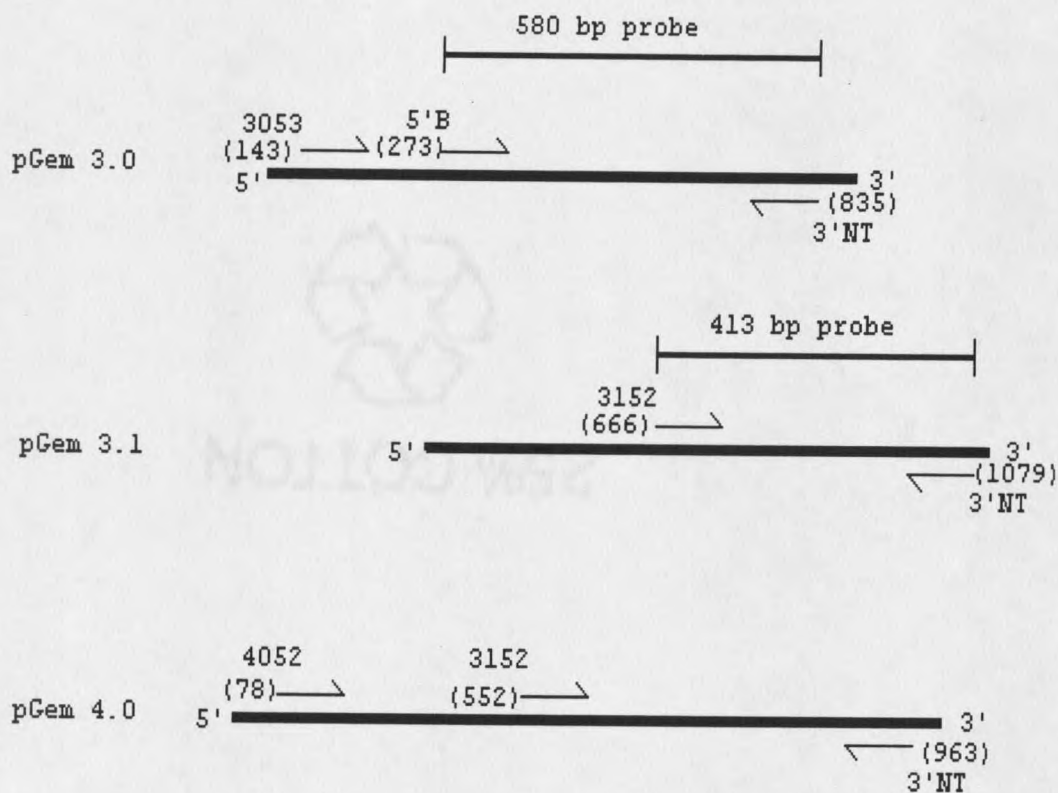


Figure 3: Map showing where primers (3053, 5'D, 3'D, 3152, 3'NT and 4052) and probes (580 bp and 413 bp) anneal to plasmids pGem 3.0 and pGem 3.1, that contain clone 3, and pGem 4.0 that contains clone 4. Only the clone insert within the plasmid is shown. All primers are 20 nucleotides long. Numbers show annealing position of the 5' end of each primer.

$T_d$  was calculated using the nearest neighbor method utilizing the Oligo computer software (National Biosciences). The program also predicted that the primers form insignificant 3'-terminal dimers and/or hairpin loops.

### Northern Analysis

Gel electrophoresis in a 1% agarose-formaldehyde denaturing gel was performed to separate RNA according to its length (Sambrook et al. 1989). Formaldehyde prevents re-formation of RNA secondary structures (Kroczek 1993). The gel was submerged in 1 X MOPS buffer (25 mM MOPS (3-(N-morpholino) propane-sulfonic acid), 8 mM sodium acetate, 1 mM EDTA) containing 0.22 M formaldehyde and electrophoresis was run at 4-5 V/cm of gel for 2 hours.

RNA that was loaded onto the gel included total RNA, polyA<sup>+</sup> RNA and commercial RNA ladder. Preparation of the RNA samples was adapted from Sambrook et al. (1989). MOPS buffer (1 X), 7% formaldehyde, 50% formamide and 1  $\mu$ g ethidium bromide were added to the RNA samples. DNA samples

loaded onto the gel included positive control pGem 3.0 and negative control pGem plasmid. PGem 3.0 refers to pGem 7zf(+) plasmid with clone 3 879 bp insert (see Figure 1). PGem plasmid is pGem 7zf(+) plasmid with no insert. An aliquot of 5  $\mu$ ls of 0.4 M sodium hydroxide/0.6 M sodium chloride solution was added to the pGem DNA samples. Gel loading buffer (2  $\mu$ l; 95% formamide, 0.5 mM EDTA, 0.025% SDS, 0.025% Bromophenol Blue, 0.025% Xylene cyanol) was added to both DNA and RNA samples. DNA samples were denatured by incubating 5 minutes at 100°C and then chilled on ice. RNA samples were denatured by incubating 5 minutes at 70°C and then chilled on ice.

After electrophoresis, a photo of the gel was taken under UV light using a video graphic printer (Ultra Lum, Inc., Carson, CA). The gel was then was soaked in H<sub>2</sub>O to remove the formaldehyde, followed by 15 X SSC (1.5 M NaCl, 0.15 M sodium citrate). Upward transfer (Southern 1975) onto a Hybond<sup>TM</sup>-N<sup>+</sup> positively-charged nylon membrane (Amersham Life Science Inc., Cleveland, OH) was carried out for 18 hours, using 15 X SSC as transfer buffer.

After transfer, dot blots of positive control pGem 3.0

and negative control pGem plasmid were spotted onto the membrane, which was then baked at 80°C for 1 hour. The baking was carried out to fix the RNA onto the membrane. Prehybridization in 50 ml hybridization buffer (7% SDS, 1% casein, 1 mM EDTA, and 0.25 M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) was performed in a rotating hybridization tube (Robbins Scientific Corporation, Sunnyvale, CA) for 24 hours at 65°C.

A 30 ml aliquot of hybridization buffer was removed, since hybridization is more efficient with less buffer (Sambrook et al. 1989). The radioactively-labeled probe that was synthesized (see preparation of probe on Page 23) was added to the hybridization buffer and the reaction incubated 22 hours at 65°C.

After hybridization, three separate washes were carried out, each 20 minutes at 65°C, using 1 X SSC/0.1% SDS solution. The washes eliminate probe that has bound to non-specific targets on the membrane. An X-ray film (Fuji Photo Film Co., Japan) was exposed to the membrane, then developed.

### Southern Analysis

Southern analysis is a very similar procedure to Northern analysis - the differences are due to DNA being analyzed, rather than RNA. Southern analysis using the 413 bp lectin cDNA probe was used to confirm that PCR-amplified products (described below) were due to amplification of lectin cDNA. Differences between Northern and Southern analysis will be described in this section.

Aliquots of 1  $\mu$ l PCR-amplified products and DNA size markers (described below) were electrophoresed in a 1% agarose non-denaturing gel containing 2  $\mu$ g of ethidium bromide (Sambrook et al. 1989). The samples loaded onto the gel also contained 2  $\mu$ l gel loading buffer. Electrophoresis was carried out in 0.5 X TBE buffer (0.045 M Tris-borate, 0.001 M EDTA) at 4-5 V/cm of gel for 2 hours.

After electrophoresis, the gel was put through a series of washes to denature the DNA. These were 0.6 M NaCl/ 0.4 M NaOH solution for 15 and 30 minutes, followed by 10 X SSC for 2 X 15 minutes, all at room temperature.

Upward transfer was carried out using 10 X SSC for 13

hours. Prehybridization in 50 ml hybridization buffer (0.7% SDS, 1% casein, 1 mM EDTA, and 0.25 M  $\text{Na}_2\text{HPO}_4$ , pH 7.4) was carried out at 65°C for 2 hours. Hybridization with 413 bp probe was performed at 65°C for 22 hours. Washing the membrane in 1 X SSC/0.1% SDS after hybridization and subsequent X-ray film exposure was performed as with Northern analysis.

As explained above, DNA size markers were electrophoresed in an agarose gel, along with PCR-amplified products. These markers were DNA fragments of known length, to which a portion of the 413 bp probe could hybridize (see Figure 3 for the overlap of the probe to PCR-amplified products), resulting in the markers being observed on a subsequent autoradiograph. Comparison of the distance traveled during electrophoresis of these markers to the distance traveled of unknown bands allows determination of the size of unknown bands. The DNA fragments were acquired by specific enzyme restrictions to pGem 3.0 plasmid. The Sambrook et al. (1989) restriction protocol was followed, employing Kpn I (Promega), Acc I (Promega), EcoR I (New England Biolabs) and a combination of Kpn I and Sac I (New England Biolabs) restriction enzymes. Plasmid digests with

each restriction enzyme resulted in more than one fragment, with only one of these fragments that had an area of complementarity to the 413 bp probe. To acquire the restricted fragment that was complementary to the probe, the restriction products were electrophoresed on a 1% agarose gel and the desired fragment cut out. The fragment was purified using Prep-a-Gene<sup>®</sup> DNA Purification System kit (Bio-Rad Laboratories), following the manufacturer's protocol. The lengths of DNA size markers obtained were 5825, 2863, 1757, and 879 bp.

### Polymerase Chain Reaction

Reverse transcription PCR was carried out on polyA<sup>+</sup> RNA isolated from grasshopper fat body, ovary and testis.

For the reverse transcription reaction, GIBCO 3' RACE System for Rapid Amplification of cDNA Ends (BRL Life Technologies<sup>™</sup>, Inc., Gaithersburg, MD) was used, according to the manufacturer's protocol. A kit-provided adaptor primer and Superscript II<sup>™</sup> reverse transcriptase were used on polyA<sup>+</sup> RNA. Adaptor primer contains a poly(dT) tail, and

selects for polyadenylated mRNAs while it primes first strand cDNA synthesis. The reaction mixture also included 1 x PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), 2.5 mM MgCl<sub>2</sub>, 500 μM of each deoxyribonucleotide (dNTP) and 10 mM dithiothreitol (DTT) and was incubated 50 minutes at 42°C. A Perkin Elmer DNA Thermal Cycler (Foster City, CA) was used to obtain the temperatures needed during the reactions. To terminate the reaction, the mixture was incubated 15 minutes at 70°C. With this temperature, reverse transcriptase is denatured and primers dissociate from the template.

RNase H was added to the reaction tubes and incubated for 20 minutes at 37°C to degrade the RNA. The tubes were stored at -20°C until PCR amplification was performed.

To amplify a target cDNA fragment, specific primers were used. Figure 3 shows the position of the primers on clone 3 and/or clone 4, while Table 1 gives the nucleotide sequence of the primers. A modified version of the protocol from the GIBCO 3' RACE System for Rapid Amplification of cDNA Ends was followed. The conditions of PCR were optimized, concerning the amount of template cDNA, concentration of MgCl<sub>2</sub> and primer annealing temperature and optimal conditions were utilized in the subsequent

experiments.

The reaction mixture contained AmpliTaq Gold™ DNA polymerase (Roche Molecular Systems Inc., Branchburg, NJ), that is inactive until heated to 94°C, thus providing a 'hot start'. Also in the reaction mixture was 1 X PCR buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 0.001% (w/v) gelatin; 500 mM of each dNTP; 0.2 μM of each primer; variable amounts of template (see 'Results') and 2.5 mM MgCl<sub>2</sub>, unless otherwise stated in the 'Results' section. PGem 3.1 or pGem 4.0 were used as positive controls templates for the PCR reaction.

PCR amplification was performed using the following conditions: 94°C for 10 minutes, then 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 2 minutes. The temperature of the first 10 minutes allows activation of AmpliTaq Gold™ and denaturation of DNA template, followed by annealing of the primers at 55°C and extension of DNA between the primers using the optimal *Taq* temperature of 72°C.

After PCR-amplification, an aliquot of each PCR product was run on a polyacrylamide gel (see 'Polyacrylamide Gel

Electrophoresis, Page 37). Aliquots of PCR products were also used in Southern analysis (see 'Southern Analysis', Page 30), or restricted with restriction endonucleases (see below).

### Restriction Analysis

PCR amplification was performed on grasshopper fat body, ovary and testis first strand cDNA. The PCR primers used were complementary to either clone 3 or clone 4. To confirm that the clone 3 specific primer only amplified clone 3, the PCR products were digested with a restriction endonuclease that restricts clone 3 but not clone 4. Restriction fragments of the expected restricted size indicated that the primers amplified only clone 3. A restriction enzyme that cuts clone 4 but not clone 3 was used to confirm the clone 4 specificity of the clone 4 specific primer.

Firstly, the PCR products were spin-purified using QIAquick PCR Purification Kit (QIAGEN Inc., Chatsworth, CA) to remove PCR buffer that may interfere with the restriction reactions. The manufacturer's protocol was used. Binding

buffer was added to PCR products and then applied to a spin column containing silica-gel. The column was centrifuged and DNA absorbed onto the silica-gel while salts and enzymes flowed through. Washing with ethanol-containing Buffer PE removed more salts. DNA was eluted with 50  $\mu$ l of 10 mM Tris/HCl (pH 8.5), that is, basic conditions and low salt concentration.

For restriction reactions, the manufacturer's protocol was used. The restriction endonucleases used were Aat II and Alw NI (New England Biolabs) that cut clone 3, and Eco0109 I (New England Biolabs) and Sal I (Promega) that cut clone 4. Buffer D (6 mM Tris-HCl, 6 mM MgCl<sub>2</sub>, 150 mM NaCl, 1 mM DTT, pH 7.9) was used with Sal I, while buffer 4 (20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT, pH 7.9) was used with Alw NI, Aat II and Eco0109 I. Bovine serum albumin to a final concentration of 100  $\mu$ g/ml was added to the reaction mixture containing Eco0109 I. The reactions were incubated at 37°C for 0.5-17 hours. An aliquot of 35  $\mu$ l of the restricted product was electrophoresed on a polyacrylamide gel.

### Polyacrylamide Gel Electrophoresis

PCR samples were run on a polyacrylamide gel to detect small quantities of PCR-amplified product and to resolve any short fragments that were a result of restriction reactions.

A 4% polyacrylamide gel, 1.0 mm x 8.2 cm x 10.2 cm containing 1 X TBE (0.09 M Tris-borate, 0.002 M EDTA), 0.07% ammonium persulfate and 0.04% v/v TEMED (N,N,N',N'-tetramethylethylene) was prepared (Sambrook et al. 1989). A 10 µl aliquot of each PCR-amplified product or 35 µl of restriction analysis product was removed and gel loading buffer (Promega) containing orange G, bromophenol blue and xylene cyanol was added to give a final concentration of 1 X gel loading buffer. The samples were loaded onto the gel and run at 65 volts in 1 X TBE buffer utilizing a Bio-Rad mini-PROTEAN® II gel apparatus. After electrophoresis, the gel was soaked in ethidium bromide solution (1 µg/25 ml) so that the PCR bands could be visualized under a UV light source (Spectroline® Longlife Filter (312 nm)). A photo of the gel was made using an Ultra Lum video graphic printer.

## RESULTS

RNA Integrity

Northern analysis and RT-PCR techniques were used to investigate the expression of lectin genes in grasshopper fat body, ovarian and testicular tissues. Total RNA and polyA<sup>+</sup> RNA were isolated from these tissues for use in the two respective techniques. The RNA was extracted from pre-dissected frozen tissues using TRI-Reagent™ according to a protocol based on the method of Chomzynski and Sacchi (1987). High yield, non-degraded pure RNA is desired, giving maximum mRNA targets for Northern analysis and RT-PCR. The RNA isolation protocol was optimized to improve RNA yield. The optimization included removal of insoluble material by centrifugation after homogenization of tissues in TRI-Reagent™. During polyA<sup>+</sup> RNA isolation, oligo(dT) cellulose flow through solution was reapplied to the column in an effort to increase yield of polyA<sup>+</sup> RNA. Additionally, the inclusion of more RNA carrier Microcarrier Gel than the protocol recommended resulted in higher RNA yields. Total

RNA yields increased from 80  $\mu\text{g}/100$  mg tissue to 140  $\mu\text{g}/100$  mg tissue, which is 30% of the expected yield stated by the protocol. PolyA<sup>+</sup> RNA yields increased dramatically from 5  $\mu\text{g}/100$  mg tissue to 40  $\mu\text{g}/100$  mg tissue after performing the optimizations. This increased yield is 200% of the expected yield if pure mRNA is isolated, however, the sample still contains residual rRNA, as seen in Figure 4.

A final 'clean' RNA solution, with no contaminants, is desirable and the RNA purity is measured using absorbance ratio  $\text{OD}_{260}/\text{OD}_{280}$ . An acceptably pure RNA solution has a ratio of 1.9-2.2 (Berger and Kimmel 1987) and typically these ratios were obtained.

In order to analyze the extent of any degradation of prepared RNA, the RNA was electrophoresed on a denaturing agarose gel (Figure 4). Figure 4 is a scanned photo of the agarose gel showing total RNA and polyA<sup>+</sup> RNA. There is a little smearing with the total RNA sample at lower molecular weight, below 0.24 Kb indicating less than 1% degradation of the total RNA. This is an insignificant amount of degradation and the RNA was considered usable for analysis with Northern hybridization. There is no smearing at lower

molecular weight with polyA<sup>+</sup> RNA indicating that the mRNA had not been degraded, at least not seriously. mRNA should be seen as a very faint smear that runs from approximately 5 Kb to 0.4 Kb (Kroczek and Siebert 1990) and this appeared on the original gel with both total RNA and polyA<sup>+</sup> RNA samples.

The use of ethidium bromide to stain RNA in Northern analysis decreases the final hybridization signal by 11-18% compared with unstained RNA (Kroczek 1989). However, the stain was used since critical information is available from stained RNA, such as size separation and RNA integrity.

There are no ethidium bromide bands with pGem 3.0 and pGem 7zf(+) plasmid samples in Figure 4. At least 25 ng of DNA is needed to visualize DNA on an agarose gel with ethidium bromide stain and neither pGem 3.0 nor pGem 7zf(+) plasmid samples contain this much DNA. Samples pGem 3.0 and pGem 7zf(+) were included as positive and negative controls for Northern hybridization which will be discussed later.

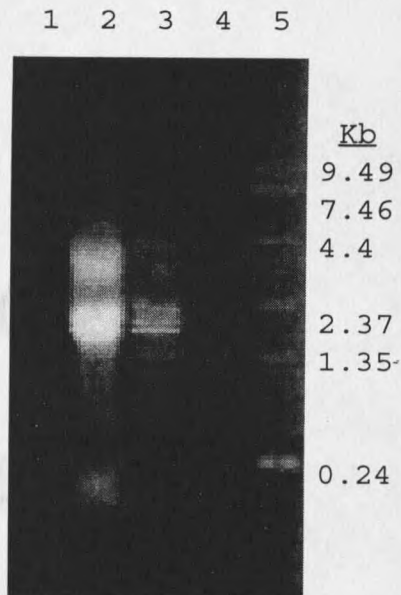


Figure 4: Agarose electrophoresis of fat body RNA. Lane 1, pGem 3.0 (250 pg); Lane 2, fat body total RNA (15  $\mu\text{g}$ ); Lane 3, fat body polyA<sup>+</sup> RNA (5  $\mu\text{g}$ ); Lane 4, pGem 7zf(+) plasmid (500 pg); Lane 5, RNA ladder (3  $\mu\text{g}$ ). Ethidium bromide was added to the samples before electrophoresis, but ethidium bromide bands cannot be seen in lanes 1 and 4 due to inadequate amount of DNA. mRNA was seen as a faint smear on the original gel that runs from approximately 5 Kb to 0.4 Kb with total RNA and polyA<sup>+</sup> RNA samples. Lack of an intense band at low molecular weight, below 0.24 Kb, with total RNA and polyA<sup>+</sup> RNA samples indicates that RNA has not been severely degraded and is useful for Northern analysis.

Optimization of Northern Hybridization

Northern analysis was used to detect lectin encoding mRNA prepared from grasshopper fat body, ovary and testis tissue. The Northern hybridization technique was optimized by performing modifications to the procedure. These changes included upward transfer (Southern 1975) and downward alkaline transfer (Chomczynski 1992) as different methods of transferring RNA from gel to membrane. Also, hybridization temperatures and wash temperatures of 45°C, 50°C, 55°C and 65°C were used (results not shown). Upward transfer (rather than downward) and hybridization/wash temperature of 65°C were found to be optimal conditions, resulting in the most intense signals and least background on the autoradiograph compared to the other conditions mentioned above, and were used in subsequent experiments. Also, the conditions were used in a control Northern analysis experiment to confirm that the Northern analysis procedure was working and giving expected signals on the resultant autoradiograph.

In the control Northern analysis, chicken brain polyA<sup>+</sup> RNA was probed with chicken <sup>32</sup>P-labeled chicken β-actin

probe. Chicken brain polyA<sup>+</sup>RNA should contain mRNA encoding the constitutive housekeeper protein  $\beta$ -actin. The probe is expected to bind to the 2 Kb  $\beta$ -actin mRNA in the chicken brain polyA<sup>+</sup> RNA sample, giving a corresponding signal on the autoradiograph.

Chicken brain polyA<sup>+</sup> RNA, an RNA ladder and a negative control pGem 3.0 which is a recombinant plasmid with no nucleic acid homologous to the  $\beta$ -actin probe, were samples used in the Northern analysis experiment. RNA ladder was included as both a negative control and as a size marker. The probe should not bind to RNA ladder, but the size markers are visible on ethidium bromide-stained agarose gel and also on the membrane after transfer. The position of the size marker bands were penciled on the membrane. The resulting autoradiograph was oriented to the membrane and the position of the pencil marks used to determine the position of all bands on the autoradiograph. Chicken brain polyA<sup>+</sup>RNA, RNA ladder and pGem 3.0 were electrophoresed with identical conditions to samples shown in Figure 4. The electrophoresis results are not shown since there was not enough chicken brain polyA<sup>+</sup>RNA and pGem 3.0 to be seen as

ethidium bromide bands on the agarose gel. After transfer of the samples onto a nylon membrane, the membrane was incubated with radiolabeled  $\beta$ -actin cDNA probe. The probe was labeled by random priming, incorporating [ $\alpha$ - $^{32}$ P]dCTP into the probe during its synthesis. Labeling of the probe usually resulted in a high specific activity of  $1.9 \times 10^9$  dpm or above. Figure 5 shows the resulting autoradiograph of the control Northern analysis. Chicken brain polyA<sup>+</sup> RNA has an expected 2 Kb signal on the autoradiograph, showing that the probe bound to target  $\beta$ -actin mRNA. This result indicates that the Northern hybridization procedure is successful. That is, the preparation of samples, gel electrophoresis, capillary transfer, baking, probe labeling and hybridization were satisfactory, resulting an expected signal on the autoradiograph.

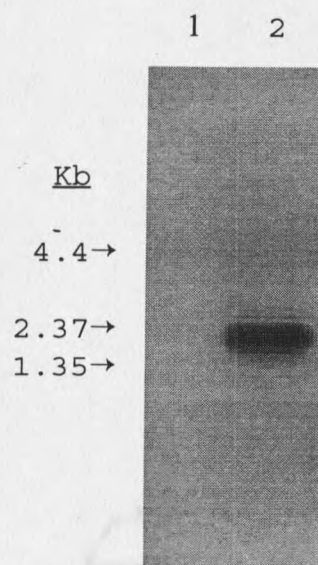


Figure 5: Northern analysis of commercial chicken brain polyA<sup>+</sup> RNA probed with <sup>32</sup>P-labeled chicken β-actin cDNA. Lane 1 is pGem 3.0 negative control (250 pg); Lane 2 contains chicken brain polyA<sup>+</sup> RNA (250 ng). Specific activity of the probe was 4.4 x 10<sup>9</sup> dpm/μg. The intense, signal of 2 Kb with chicken brain polyA<sup>+</sup> RNA is due to hybridization of the probe to 2 Kb β-actin mRNA, as expected, indicating that the Northern hybridization procedure worked.

Northern Analysis of Fat Body RNA

Northern hybridization was used for detection of mRNA coding for lectin in mRNA prepared from grasshopper fat body. Positive results will confirm previous data indicating synthesis of lectin by grasshopper fat body (Stiles et al. 1988).

In the Northern hybridization experiment, samples in Figure 4 were transferred to a nylon membrane and probed with <sup>32</sup>P-labeled 580 bp cDNA probe synthesized from clone 3. The 580 bp probe was prepared by Tanya Gedik. Briefly, the 580 bp probe was made by PCR-amplification of pGem 3.0 using primers 5'B and 3'D (see Figure 3 for position of primers on clone 3), followed by purification of the probe using Prep-a-Gene DNA Purification System kit. The probe was labeled by random labeling, as described in the previous section. The probe is expected to bind to clone 3 mRNA, as well as clone 4 mRNA, since there is 90% homology between clone 3 and clone 4 where the probe binds (Radke, unpublished results).

Figure 6 shows the resulting autoradiograph of the Northern analysis experiment. The positive control pGem 3.0

sample has the expected signal on the autoradiograph of 6 Kb since pGem 3.0 is 6 Kb in length and contains clone 3 insert to which the probe can bind. The negative control pGem plasmid sample does not have a signal on the autoradiograph, which is expected, since the plasmid contains no sequence homologous to the probe. RNA ladder has no signal indicating lack of non-specific binding of the probe.

There is a signal on Figure 6 for grasshopper polyA<sup>+</sup> RNA at approximately 1.2 Kb. This result indicates that the probe hybridized to RNA of 1.2 Kb. The band is likely due to a lectin transcript and implies that fat body is expressing a lectin gene. The two lectin clones available in the laboratory, namely clone 3 and clone 4, have recently been determined to be 2.1 and 2.0 Kb in length, respectively. This suggests that the 1.2 Kb band is due to neither clone 3 nor clone 4 full transcripts. Potential explanations for the 1.2 Kb band will be addressed in 'Discussion'.

Also with polyA<sup>+</sup> RNA, a signal of 3 Kb is seen, though it is much fainter than the 1.2 Kb signal. This signal is probably too large to be target mRNA, and yet its position does not correspond to ribosomal RNA, that is, the signal is

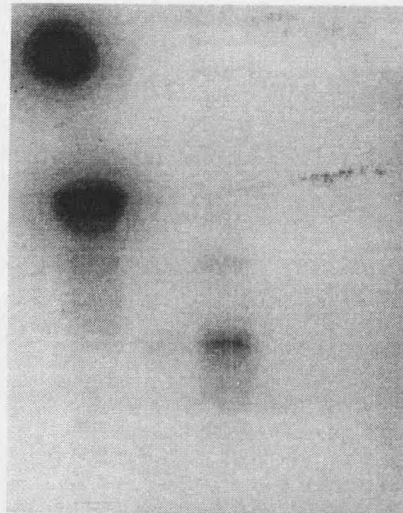
apparently not due to non-specific binding. The signal is too large to be due to clone 3 or clone 4 transcripts, though the signal could be due to a larger lectin. Other possible reasons for the signal will be addressed in 'Discussion'.

There is a very faint 1.2 Kb signal on the original autoradiograph with fat body total RNA. The band is more intense with polyA<sup>+</sup> RNA than total RNA, because of the much higher concentration of mRNA in the polyA<sup>+</sup> RNA sample. PolyA<sup>+</sup> RNA is 1-2% of total RNA, suggesting that the 15 µg total RNA sample may contain approximately 750 ng of mRNA.

Northern analysis with ovary and testis polyA<sup>+</sup> RNA gave no bands on the autoradiograph (results not shown). The RNA had not been degraded severely, as shown by the lack of smearing at low molecular weight when analyzed by gel electrophoresis and optimal Northern hybridization conditions were used, as with fat body. The 1.2 Kb and 3 Kb signals seen with fat body (Figure 6) do not appear on autoradiographs of ovary and testis which seems to indicate that the 1.2 Kb and 3 Kb transcripts were either not present

pGem 3.0

↓ 1 2 3 4 5



← pGem 7zf(+) plasmid

Kb

9.49

7.46

4.4

2.37

1.35

0.24

Figure 6: Northern analysis of fat body RNA. A  $^{32}\text{P}$ -labeled 580 bp cDNA probe was hybridized to samples transferred from the gel in Figure 4. Lane 1, linearized positive control pGem 3.0 (250 pg); Lane 2, fat body total RNA (15  $\mu\text{g}$ ); Lane 3, fat body polyA<sup>+</sup> RNA (5  $\mu\text{g}$ ); Lane 4, negative control pGem 7zf(+) plasmid (500 pg); Lane 5, RNA ladder (3  $\mu\text{g}$ ). After transfer, pGem 7zf(+) plasmid (500 pg) and pGem 3.0 (250 pg) were spotted onto the membrane before baking. The arrows indicate where the spots were applied. The specific activity of the 580 bp probe was  $1.9 \times 10^9$  dpm/ $\mu\text{g}$ . A strong signal is seen for fat body polyA<sup>+</sup> RNA at approximately 1.2 Kb, indicating the presence of lectin mRNA of this size and the expression of this lectin gene in fat body tissue.

or present in non-detectable amounts in ovary and testis tissues. The more sensitive technique of RT-PCR was employed with polyA<sup>+</sup> RNA isolated from grasshopper ovary and testis instead, to determine lectin expression in these tissues.

#### Lectin Expression using RT-PCR with primers 3152 and 3'NT

RT-PCR is a technique whereby mRNA is initially reverse-transcribed to cDNA followed by amplification of cDNA target defined by two sequence-specific primers. Amplified DNA is agarose gel electrophoresed and the PCR products visualized with ethidium bromide. An amplified band of predictable size suggests that the corresponding mRNA is present in the tissue from which the RNA is isolated. The conditions of PCR were optimized, concerning the amount of template cDNA used, concentration of MgCl<sub>2</sub> and primer annealing temperature and these optimal conditions are described in 'Materials and Methods'.

RT-PCR was performed on polyA<sup>+</sup> RNA isolated from grasshopper fat body, ovary and testis tissue to examine the expression of lectin genes in these tissues. Total RNA was

not used for RT-PCR since it contains abundant rRNA which may interfere with the RT-PCR reaction.

Primers 3152 and 3'NT were used during PCR amplification. See Figure 3 for the annealing position of these primers. The primers are 100% complementary to both clone 3 and clone 4. PCR amplification using these primers is expected to result in a 413 bp product for clone 3 and 411 bp product for clone 4. Figure 7 displays the results of the RT-PCR experiment. The negative control that lacks template cDNA shows no bands on the gel, indicating no PCR-amplification and no contamination of target cDNA. PCR-amplification of fat body, ovary, testis as well as positive control pGem 3.1 result in a band at approximately 413/411 bp. A difference in 2 bp cannot be differentiated on the gel, so it cannot be determined whether the band is 413 bp or 411 bp. The result with pGem 3.1 is expected since the plasmid contains a clone 3 insert to which the primers should bind. The expected 413/411 bp band of amplified fat body, ovary and testis cDNA show that clone 3 and/or clone 4 cDNA was amplified during PCR. It cannot be determined whether one or both clones were PCR-amplified since the resolution on the gel is inadequate to give clear separation

of the 413 bp and 411 bp bands. The results suggest that clone 3 and /or clone 4 mRNA is present in fat body, ovary and testis tissues, and indicates that these tissues are expressing the corresponding genes. Southern analysis on these PCR products using a cDNA lectin probe should confirm whether the bands are, in fact, due to amplification of cDNA coding for lectin.

Fat body and positive control pGem 3.1 show a band of approximately 620 bp in Figure 7. Testis has a band of this size and other bands of approximately 680 bp, 750 bp and 880 bp. The bands may be PCR artifacts or amplification of a lectin or non-lectin cDNA. Southern analysis of the PCR samples using a cDNA lectin probe would indicate whether the bands are a result of lectin cDNA amplification (described below). Corresponding signals on the autoradiograph to those on the gel would indicate that the PCR product is due to amplification of lectin cDNA.

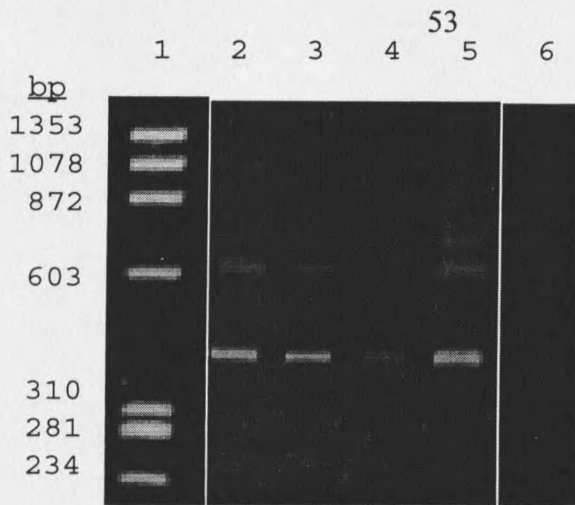


Figure 7: RT-PCR amplification of 1  $\mu$ g mRNA from fat body (lane 3), ovary (lane 4) and testis (lane 5), using primers 3152 and 3'NT (see Figure 3, Page 27 for location of primers). Lane 2 is positive control pGem 3.1; lane 6 is negative control with no template cDNA; lane 1 contains  $\phi$ X174 DNA size standards. Ovary and testis samples contained 1.5 mM  $MgCl_2$  as an optimum concentration for PCR. Samples were electrophoresed in a 4% polyacrylamide gel, and stained with ethidium bromide. The expected 411 bp and/or 413 bp product, based on clone 4 and clone 3 nucleotide sequences, respectively, is seen in all three tissues examined. The results suggest that fat body, ovary and testis tissues are expressing clone 3 and/or clone 4 lectin gene(s).

Southern Hybridization to ConfirmLectin-Identity of PCR Product

The 413/411 bp bands seen on the gel in Figure 7 are of expected size for PCR amplification of clone 3 and/or clone 4 lectin cDNA. However, the bands may have been the result of primer binding to non-lectin template cDNA that coincidentally resulted in a 413/411 bp amplified product. To determine if the ethidium bromide-stained bands were a result of amplification of lectins rather than other cDNAs, Southern hybridization was carried out with <sup>32</sup>P-labeled 413 bp probe from clone 3 (see Figure 3, Page 27 for position of the probe on clone 3). The probe was synthesized by Tanya Gedik using PCR with primers 3152 and 3'NT on pGem 3.1. This 413 bp probe was selected because it should bind to cDNA from PCR-amplified clone 3 and clone 4 cDNA.

Aliquots of the PCR-amplified products were electrophoresed on an agarose gel and Southern analysis performed as described in 'Materials and Methods'. The results of the Southern analysis are shown in Figure 8. Fat body, ovary, testis and positive control pGem 3.1, all give visible signals on the autoradiograph at the expected size

of 411/413 bp. The result with PCR-amplified pGem 3.1 is expected since the primers should anneal to the clone 3 insert of pGem 3.0 plasmid and amplify specific target to which the 413 bp probe can bind. Negative control gives no signal, a result that is expected since there was no template cDNA for PCR amplification and, therefore, no target for the probe to bind. The 411/413 bp signals on the autoradiograph with fat body, ovary and testis confirm that this PCR-amplified fragment, as seen in Figure 7, is due to amplification of lectin cDNA and that the three respective tissues contain mRNA encoding lectin. The results strongly suggest that clone 3 and/or clone 4 lectins are being expressed in fat body, ovary and testis of the grasshopper.

The 620 bp bands seen on the ethidium bromide gel in Figure 7 with fat body, testis and pGem 3.1 samples do not appear on the autoradiograph (Figure 8). The lectin probe did not hybridize to the band. It can be concluded that the 620 bp band was not due to amplified lectin and was probably a PCR artifact. There is a faint band at approximately 470 bp with testis PCR-amplified product on the original autoradiograph, though it cannot be clearly seen in Figure 8. This signal, which is larger than the 411/413 bp signal,

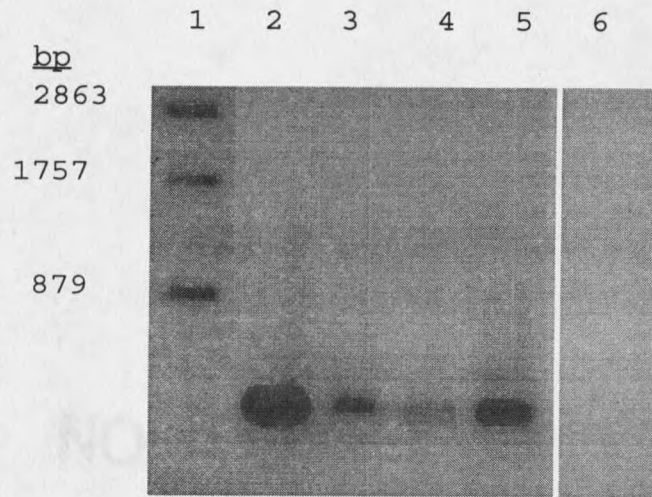


Figure 8: Southern analysis of RT-PCR products using primers 3152 and 3'NT. RT-PCR results are shown in Figure 7. A  $^{32}\text{P}$  labeled 413 bp cDNA probe was hybridized to the transferred RT-PCR products. Lane 1, DNA size standards; lane 2, pGem 3.1 positive control; lane 3, fat body; lane 4, ovary; lane 5, testis; lane 6, negative control. Strong 411/413 bp signals are seen for fat body and testis and a weaker signal is shown by ovary. The results suggest that the 411/413 bp RT-PCR product represents amplification of clone 3 and/or clone 4 lectin mRNA and that one or both of the corresponding genes are expressed in fat body, ovary and testis tissue.

suggests amplification of a larger lectin than clone 3 and /or clone 4. Other possible causes for this signal will be mentioned in 'Discussion'.

#### Clone 3 and/or Clone 4 Expression

It cannot be concluded from RT-PCR results using primers 3152 and 3'NT whether both clone 3 and clone 4 cDNA, or only one, is amplified, since the expected-size 411 bp and 413 bp bands cannot be differentiated or separated to give clear bands on a 4% polyacrylamide gel. To investigate whether clone 3 or clone 4 cDNA, or both cDNAs, were amplified with primers 3152 and 3'NT, PCR-amplified products of fat body, ovary and testis were restricted with 'clone-specific' restriction endonucleases.

Aat II restriction enzyme is considered clone 3-specific, since it cuts clone 3 at nucleotide 917, while it does not cut clone 4. Restriction with this enzyme of PCR products that are due to amplification of clone 3 cDNA would give restriction fragments of 251 bp and 162 bp. Clone 4-specific restriction enzyme Eco0109I does not cut clone 3,

but restricts clone 4 at position 654. Incubation of clone 4 PCR-amplified products with Eco0109I would result in 102 bp and 309 bp fragments. Restriction fragments of 102/309 bp or 162/251 bp would indicate whether clone 4 cDNA template or clone 3 cDNA template was PCR amplified with fat body, ovary and testis samples. This result would determine whether the corresponding mRNA was present in the three tissues.

Restricted PCR products are shown in Figure 9, with (A) representing restriction with Aat II and (B) indicating Eco0109 I restriction. Unrestricted 411/413 bp PCR products are seen with positive control pGem 3.1 in Figure 9(A). Also, a 411/413 bp band is seen with positive control pGem 4.0 indicating unrestricted pGem 4.0 PCR product, in Figure 9(B). If the restriction enzymes had completely restricted the control PCR-amplified products, the unrestricted bands of 411/413 bp would not be present. Since the band does appear with pGem 3.1 and pGem 4.0 samples, this indicates that neither Aat II nor Eco0109I restrictions have gone to completion. The incomplete restriction does not effect restriction analysis of fat body, ovary and testis samples since unrestricted bands can be seen on the gel.

Fat body, ovary and testis samples incubated with Eco0109 I show a restricted band at approximately 309 bp in Figure 9(B). The other expected restricted band of 102 bp is too small and of inadequate quantity to be visualized with ethidium bromide. Since Eco0109 I is clone 4-specific, the restricted bands with these samples signify that clone 4 was amplified during the PCR reaction using 3152 and 3'NT primers with the three respective tissues. It can be concluded from this that clone 4 was originally present as mRNA in these tissues.

Testis and ovary samples restricted with Aat II show ethidium bromide bands at the expected restricted sizes of 251 bp and 162 bp (Figure 9(A), although ovary restricted bands are faint and can only be clearly seen on the original gel. These results demonstrate that clone 3 was amplified during RT-PCR of testis and ovary mRNA and that clone 3 lectin was expressed in these tissues. Fat body sample has no visible restricted bands in Figure 9(A), which could be due to an inadequate restriction reaction. However, in another experiment, restriction was performed for 17 hours with fat body and positive control pGem 3.1 samples. The restriction went to completion with pGem 3.1, yet restricted

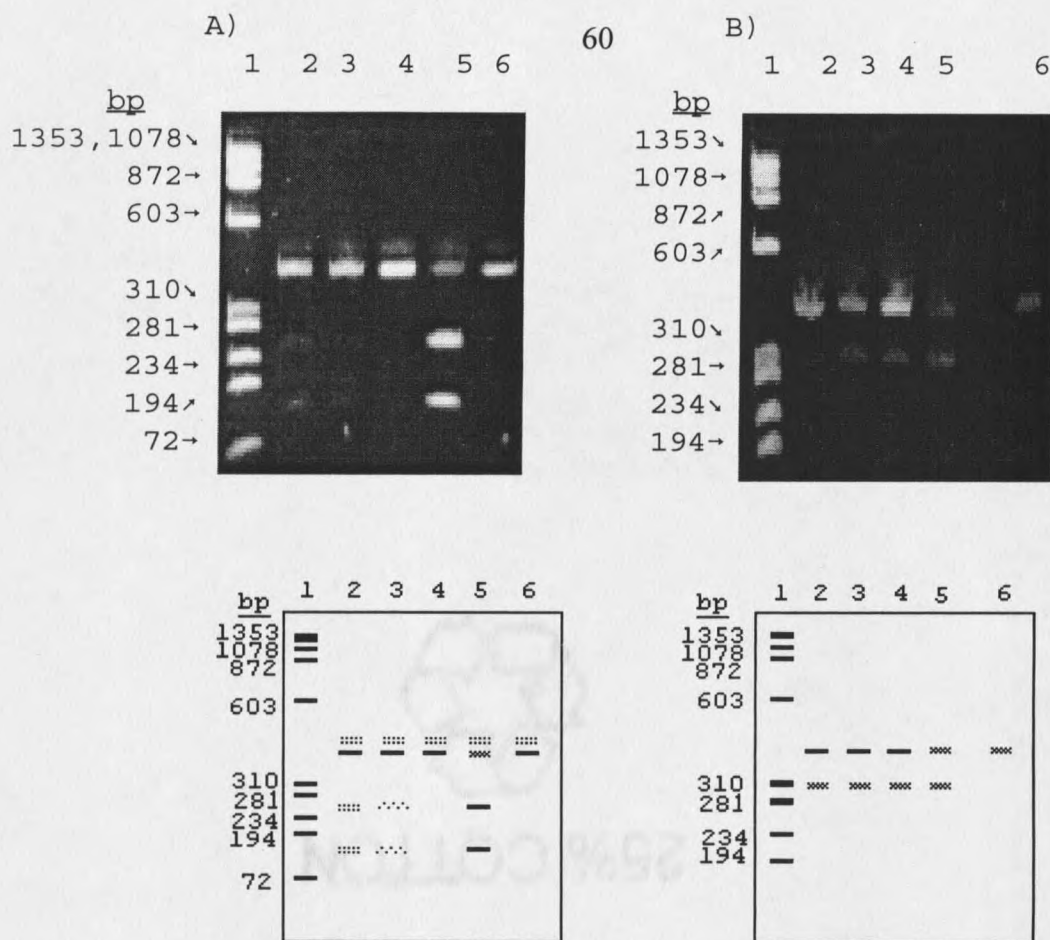


Figure 9: Restriction of RT-PCR products with clone 3-specific Aat II (A) and clone 4 specific Eco0901 I (B). The RT-PCR products are shown in Figure 7. Lane 1 (A,B),  $\phi$ x174 DNA size standards; lane 2 (A,B), testis; lane 3 (A,B), ovary; lane 4 (A,B), fat body; lane 5(A), pGem 3.1; lane 6(A), unrestricted pGem 3.1; lane 5(B), pGem 4.0; lane 6(B), unrestricted pGem 4.0. The samples were electrophoresed on a 4% polyacrylamide gel, and stained with ethidium bromide. A representation of the original gel is shown below each Figure. Faint restricted bands are seen in Figure (A) with testis and ovary, but not with fat body. Weak restricted bands are seen in Figure (B) for all three tissues. The results suggest that ovary and testis, but not fat body, is expressing clone 3 lectin gene, while all three tissues are expressing the clone 4 lectin gene.

bands could not be visualized with fat body (not shown). The results suggest that the fat body PCR-amplified sample does not contain clone 3 amplified product because of no clone 3 mRNA in the original tissue. Another suggestion is that perhaps a small amount of clone 3 mRNA is present in fat body tissue, but not enough to see on a polyacrylamide gel after amplification. It can be inferred that clone 3 lectin gene is either not expressed at all, or only in small quantities in fat body.

Clone 4 Lectin Expression using RT-PCR  
with Primers 4052 and 3'NT

Restriction of RT-PCR products with a clone-specific restriction endonuclease was used as a tool to investigate clone 3 and clone 4 expression in fat body, ovary and testis tissue, and was described in the previous section. As a possibly more sensitive technique to directly analyze clone 4 expression, RT-PCR was performed with a clone 4-specific primer, 4052. Primer 4052 is 100% complementary to clone 4, while only 60% complementary to clone 3. It is called a clone 4 specific primer, although it is noted that it may

anneal to, and perhaps amplify, another lectin cDNA template. Determining the clone 4-specificity of the primer by restriction analysis will be shown in a later section. Primer 4052 in combination with 3'NT were used during PCR-amplification of fat body, ovary and testis cDNA. See Figure 3 for annealing position of the primers to clone 4.

The results of the PCR reaction are displayed in Figure 10. A PCR-amplified product of 885 bp is expected if clone 4 cDNA is amplified and a band of this approximate size is seen in fat body, ovary, testis and positive control pGem 4.0 samples on the gel. The band actually migrates a little further in the gel than expected with fat body, ovary, testis and pGem 4.0 samples, but it is assumed that a difference in buffer composition with these samples and the DNA size standards results in this difference in migration. When the samples are electrophoresed on an agarose gel, the electrophoresis is less sensitive to differences in sample buffer and the RT-PCR product migrates to give a band of 885 bp (results not shown). The results indicate that clone 4 is being amplified during PCR and was originally present as mRNA in the three tissues. The result also confirms data obtained with PCR amplification of fat body, ovary and

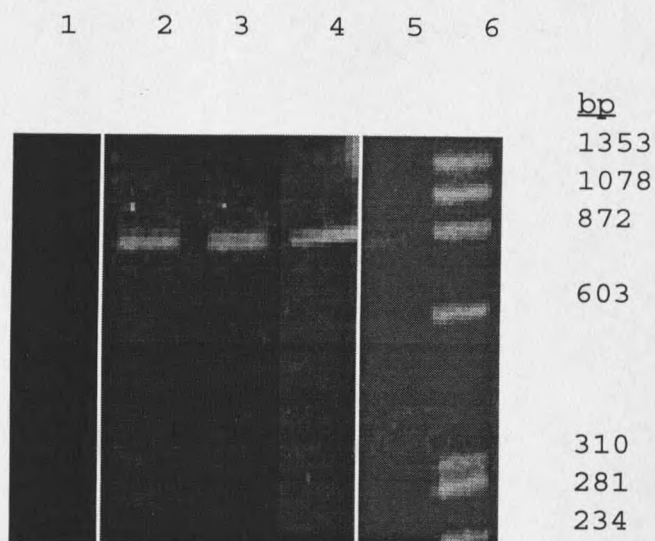


Figure 10: RT-PCR amplification of 1  $\mu$ g of mRNA from fat body (lane 5), ovary (lane 4) and testis (lane 3), using clone 4 specific primer 4052 and 3'NT (see Figure 3, Page 27 for location of primers). The expected 885 bp product, based on clone 4 nucleotide sequence, is seen in all three tissues examined. Lane 1 is negative control with no template cDNA; lane 2 is positive control pGem 4.0; Lane 6 contains  $\phi$ X174 DNA size standards. Samples were electrophoresed in a 4% polyacrylamide gel, then stained with ethidium bromide. The results indicate that fat body, ovary and testis tissues are expressing the clone 4 lectin gene.

testis cDNA using primers 3152 and 3'NT that resulted in a 411/413 bp band (Figure 7) that suggested PCR amplification of clone 4 cDNA.

#### Confirmation of Lectin-Identity of PCR product

Southern analysis was performed on an aliquot of the PCR products, amplified using primer combination 4052 and 3'NT, to confirm that a lectin cDNA template was amplified rather than a non-lectin cDNA. Radioactively labeled 413 bp lectin probe was used. Figure 11 shows the resulting autoradiograph. Fat body, ovary, testis and positive control pGem 4.0 samples give signals of the expected 885 bp size, showing that lectin probe hybridized to this PCR-amplified product. The results confirm that a lectin cDNA was amplified with the three tissues using primers 4052 and 3'NT during PCR, and that the expected 885 bp band on the gel in Figure 10 is probably due to amplification of clone 4 lectin cDNA.

A band of approximately 650 bp appeared with ovary, testis and positive control pGem 4.0 in Figure 11, however, with ovary sample the band is faint on the original

autoradiograph. This band is lectin-specific, since lectin probe hybridized to the product, but is not of expected size for a clone 4-specific product. Plasmid pGem 4.0 is a positive control for the PCR reaction using primers 4052 and 3'NT, since it contains clone 4 insert to which the primers can anneal and amplify. PCR with pGem 4.1 would result in a clone 4-specific product and a corresponding signal on the Southern analysis autoradiograph using lectin probe. The 650 bp signal on Figure 11 with PCR amplified pGem 4.0 is, therefore, clone 4-specific. It is likely that the PCR-amplified fragment is due to another priming site within clone 4 to which either 4052 or 3'NT is homologous, resulting in amplification of the 650 bp. It is assumed that the 650 bp signal with ovary and testis samples is also due to another priming site within clone 4, and not due to primers amplifying a different lectin cDNA.

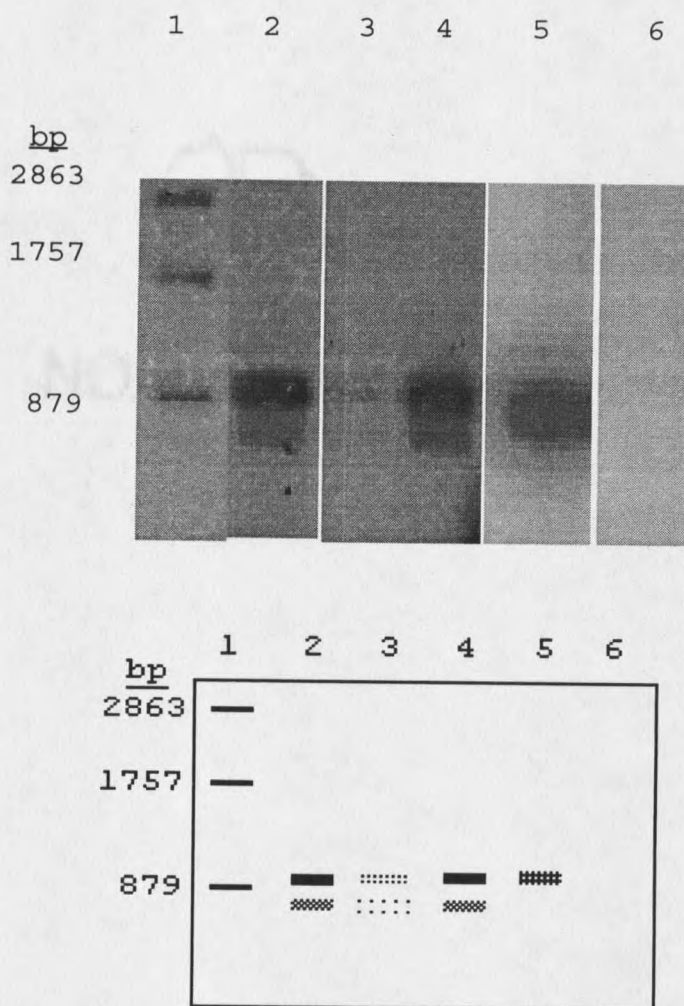


Figure 11: Southern analysis of RT-PCR reactions using clone 4-specific primer, 4052 and 3'NT. RT-PCR results are shown in Figure 10. A  $^{32}\text{P}$ -labeled 413 bp cDNA probe was hybridized to the transferred RT-PCR products. Lane 1, DNA size standards; Lane 2, pGem 4.0 positive control; Lane 3, ovary; Lane 4, testis; Lane 5, fat body; Lane 6, negative control. The drawing is a representation of the original autoradiograph. Strong signals are seen for testis and fat body, and a weak signal is shown for ovary. The results indicate that the 885 bp RT-PCR product represents amplification of clone 4 mRNA expressed from a lectin gene.

Clone 4 Specificity of Primer 4052

Expected PCR results with primer 4052, shown in the two previous sections, suggest that primer 4052 is clone 4-specific, resulting in clone 4 cDNA amplification during PCR. To confirm the clone 4 specificity of primer 4052, fat body cDNA was PCR-amplified with 4052 and 3'NT primers and then restricted with a clone 4-specific enzyme. Sal II restriction endonuclease was used that cuts clone 4 at nucleotide 431, but does not restrict clone 3. The size of restricted bands expected are 353 bp and 532 bp. The result of restriction is shown in Figure 12.

After restriction, fat body PCR-amplified product shows a band of approximately 532 bp, in Figure 12, with no band remaining at the unrestricted size of 885 bp. The other restricted band at 353 bp appeared as very weak signal on the original gel that cannot be seen in Figure 12. The restriction result strongly suggests that 4052 primer is annealing to and amplifying only clone 4 cDNA template during PCR, indicating that it is clone 4 specific. This also implies that RT-PCR products of 885 bp obtained with primer combination 4052 and 3'NT with fat body, ovary and

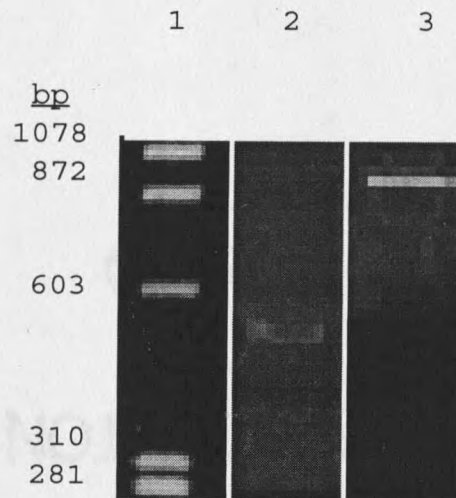


Figure 12: Restriction of fat body RT-PCR products with clone 4-specific Sal I restriction endonuclease. PCR used primers 4052 and 3'NT. Restricted fat body PCR product (lane 2), unrestricted fat body PCR product (lane 3) and  $\phi$ X174 DNA standards (lane 1) were electrophoresed on a 4% polyacrylamide gel, and stained with ethidium bromide. The expected restriction fragment of 532 bp with restricted fat body indicates the clone 4-specificity of the 885 bp PCR product and confirms the clone 4-specificity of the 4052 primer.

testis cDNA, as shown in Figure 10, are due to clone 4 cDNA amplification.

Clone 3 Lectin Expression using RT-PCR  
with Primers 3053 and 3'NT

RT-PCR, which is a very sensitive technique, was performed on fat body, ovary and testis mRNA, using clone 3-specific primer 3053 and 3'NT, to investigate clone 3 lectin expression. The annealing position of primer 3053 to clone 3 is shown in Figure 3. Primer 3053 is 100% complementary to clone 3 and is 85% complementary to clone 4, and is referred to as a clone 3 specific primer, although it may still anneal to clone 4 or other lectin cDNAs. A PCR-amplified fragment of 936 bp is expected if these primers amplify a clone 3 cDNA template.

Figure 13 shows the PCR-amplification results after electrophoresis on a polyacrylamide gel. There is no positive control sample, since a clone to which the primers can both anneal and amplify is not available in the laboratory. Primer 3053 anneals to clone 3 insert in pGem 3.0 plasmid, while primer 3'NT anneals to clone 3 insert in

pGem 3.1 plasmid (see Figure 3). There are products of the expected size of 936 bp in Figure 13 with fat body, ovary and testis samples. This result implies that clone 3 cDNA template was amplified during PCR and clone 3 mRNA was present in the original mRNA isolation, suggesting that the three tissues express clone 3 transcript.

The three tissues also have bands representing a smaller fragment than 936 bp of approximately 650 bp, although the band is very faint with ovary sample. This band is either due to binding of the primer to lectin or binding to another protein cDNA followed by amplification and resulting in a PCR-amplified product of 650 bp. Whether the 650 bp fragment is lectin-specific will be addressed by Southern analysis using a lectin probe, and is described below.



Figure 13: RT-PCR amplification of 1  $\mu$ g mRNA from fat body (lane 3), ovary (lane 4) and testis (lane 5), using clone 3-specific primer 3053 and 3'NT (see Figure 3, Page 27 for location of primers). Samples were electrophoresed on a 4% polyacrylamide gel, and stained with ethidium bromide. The expected 936 bp product, based on clone 3 nucleotide sequence, is seen in all three tissues examined. Lane 1 is  $\phi$ X174 DNA size standards; Lane 2 is negative control with no template cDNA. A band of 936 bp with fat body, ovary and testis tissues indicates that these tissues are expressing clone 3 lectin gene.

Lectin-Identity of PCR Productfrom Primers 3053 and 3'NT

PCR amplified fat body, ovary and testis cDNA using primers 3053 and 3'NT, were analyzed in a Southern hybridization experiment with lectin probe, to confirm that PCR-amplified products were due to amplification of lectin cDNA. A signal on the autoradiograph would indicate that lectin 413 bp cDNA probe had hybridized to lectin cDNA. Figure 14 shows the Southern analysis autoradiograph. The expected 936 bp PCR product appears as a signal on the autoradiograph with fat body, ovary and testis PCR-amplified products. The results show that the lectin probe hybridized with amplified lectin cDNA of 936 bp, the expected size of amplified clone 3 cDNA. It can be indicated from these results that clone mRNA was being transcribed in the original fat body, ovary and testis tissues.

There is a signal at 650 bp on the autoradiograph (Figure 14) with fat body, ovary and testis samples, although with ovary the band is very faint and can only be seen on the original autoradiograph. The band represents the 650 bp PCR product seen with the three respective

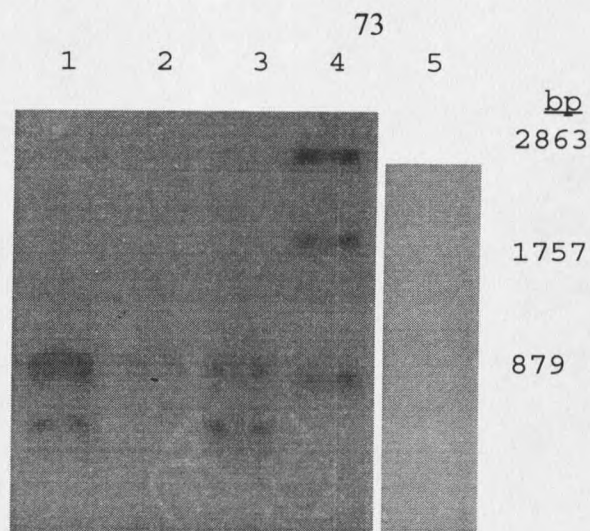


Figure 14: Southern analysis of RT-PCR products, shown in Figure 13, from clone 3-specific primer 3053 and 3'NT. A <sup>32</sup>P-labeled 413 bp cDNA probe was hybridized to the transferred RT-PCR products. Lane 1, fat body; Lane 2, ovary; Lane 3, testis; Lane 4, DNA size standards; Lane 5, negative control. Signals of 936 bp and 650 bp are seen with fat body, ovary and testis, although the 650 bp signal can only be seen in the original autoradiograph with ovary. The results indicate that the 936 bp RT-PCR product represents amplification of clone 3 mRNA expressed from a lectin gene.

tissues in Figure 13. The band could be due to binding of the lectin probe to non-lectin PCR product, which would probably have very little homology with the probe, but since the signal is very intense, this hypothesis is unlikely. The 650 bp signal probably represents an authentic lectin PCR product, signifying either another priming site within clone 3 cDNA or a different lectin cDNA.

#### Clone 3-specificity of Primer 3053

Fat body cDNA that was previously PCR-amplified with primer combination 3053 and 3'NT was incubated with Alw NI, a clone 3-specific restriction endonuclease. This was carried out to confirm that primer 3053 is specific for clone 3 and does not bind clone 4. Alw NI cuts clone 3 at nucleotide 976, giving restricted fragments of 833 bp and 103 bp, but does not cut clone 4. If the expected PCR product of 936 bp obtained with primers 3053 and 3'NT is restricted with clone 3-specific Alw NI, the result will strongly suggest that primer 3053 amplified clone 3 cDNA.

The results of restriction of fat body PCR product are shown in Figure 15. The unrestricted PCR product in lane 2

is expected to be 936 bp, however, comparing its position with the size markers in lane 3, the former does not seem to be 936 bp, since it has migrated too far in the gel. It has been found in previous experiments that a slight difference in buffer composition or volume of the sample can alter the migration rate in the gel of one sample compared to another. A difference in buffer composition may be the reason for the unexpected migration of the 936 bp fragment.

The 833 bp restriction fragment is seen with restricted fat body PCR product in lane 1. The other expected restriction fragment of 103 bp did not appear on the gel since there was inadequate quantity to see with ethidium bromide. The restriction reaction did not go to completion, since a faint 936 bp band representing unrestricted PCR product is seen in lane 1. Although restriction was not completed, the fragment of 833 bp shows that the fat body PCR-amplified product was digested with the clone 3-specific enzyme. This indicates that the PCR product was a result of amplification of clone 3 cDNA and confirms the clone 3 specificity of the 3053 primer. It can be implied that the 936 bp PCR product seen in Figure 13 is specifically due to clone 3 amplification.



Figure 15: Restriction of fat body RT-PCR product using clone 3-specific Alw NI restriction endonuclease. PCR was performed with primers 3053 and 3'NT. Restricted fat body PCR product (lane 1), unrestricted fat body PCR product (lane 2) and  $\phi$ X174 DNA size standards (lane 3) were electrophoresed on a 4% polyacrylamide gel and then stained with ethidium bromide. The drawing on the right is a representation of the original gel. The expected restriction fragment of 833 bp with fat body (lane 1) indicates that 936 bp PCR product is clone 3-specific and confirms the clone 3-specificity of the 3053 primer.

Presence of Non-Clone 3/Clone 4 Lectins

In this report, investigation into lectin expression in grasshopper fat body, ovarian and testicular tissues has concentrated on clone 3 and clone 4 lectin expression, but the tissues may be expressing other lectins and, perhaps more dominantly than clone 3 or clone 4. Stiles et al. (1988) noted lectin synthesis in the three respective tissues, but it was not determined which particular lectins were present - perhaps they were something other than clone 3 or clone 4 lectins.

Northern analysis of grasshopper fat body polyA<sup>+</sup> RNA with lectin probe gave a 1.2 Kb band on the autoradiograph, shown in Figure 6. This band probably corresponds to lectin mRNA that is neither clone 3 nor clone 4 mRNA, which are 2.1 Kb and 2.0 Kb in length, respectively. The result implies that fat body expresses another lectin gene in addition to clone 3 and clone 4. Northern analysis was unsuccessful with ovary and testis polyA<sup>+</sup> RNA, so this technique could not be used to detect non-clone 3/clone 4 lectin mRNAs in these two respective tissues.

As an alternative technique, RT-PCR was performed on

polyA<sup>+</sup> RNA prepared from ovary and testis tissue, as well as fat body. PCR utilized the primer combination 3152 and 3'NT. These primers are complementary to clone 3 and clone 4 cDNA, and it was considered that they may be homologous to other lectin cDNAs, enabling amplification of these putative lectin cDNAs. The result of RT-PCR is shown in Figure 7. Only the clone 3/clone 4 PCR product of 413/411 bp appears on the gel with fat body, ovary and testis samples, so if other lectin cDNAs were amplified, their length would have to be similar to clone 3/clone 4 PCR products, therefore, indistinguishable on the gel.

To determine if other lectin PCR products of similar length to clone 3 and clone 4 products were present, the PCR products of fat body, ovary and testis were incubated with clone 3- and clone 4-specific restriction endonucleases. This double digest included Aat II, that restricts clone 3, and Eco0109 I, which restricts clone 4. Any unrestricted product may be due to PCR-amplification of a different lectin than clone 3 and clone 4, providing that these putative other cDNAs lack identical restriction sites to clone 3 or clone 4.

Restricted PCR products are shown in Figure 16.

Samples pGem 3.1 and pGem 4.0, representing restricted clone 3 and clone 4 PCR products, respectively, show no unrestricted bands of 411/413 bp, indicating that restriction had gone to completion. Any unrestricted bands with fat body, ovary and testis would, therefore, not be due to an incomplete restriction reaction. The original gel, as represented by the drawing in Figure 16, shows a very faint unrestricted band of 411/413 bp with ovary and testis.

These results suggest that ovary and testis template cDNA contain lectin cDNA that is neither clone 3 nor clone 4, with homologous priming sites to clone 3 and clone 4, but not the same restriction sites as the two respective clones. The unrestricted band of 411/413 bp does not appear with fat body, that is, all of fat body PCR products were restricted with clone 3- and clone 4-specific restriction endonucleases. This result suggests that fat body cDNA does not contain lectin cDNA that is neither clone 3 nor clone 4. However, many assumptions are used in this RT-PCR and restriction experiment. Fat body could be synthesizing non-clone 3/clone 4 lectin mRNA that has no homologous priming sites to clone 3 and clone 4, or identical restriction sites so is not differentiated from the other restricted

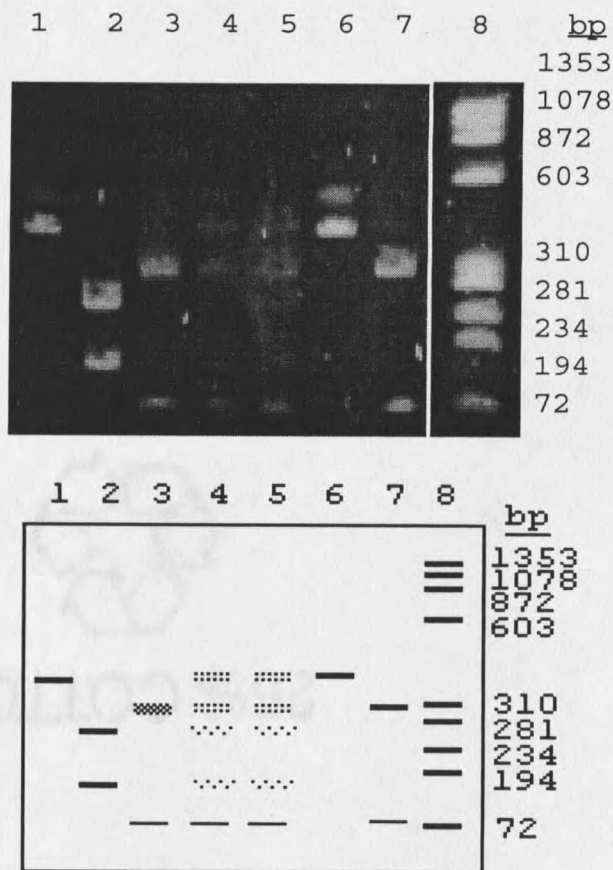


Figure 16: Double digest of RT-PCR products shown in Figure 7, with both clone 3-specific Aat II and clone 4-specific Eco0109 I restriction endonucleases. Lane 1, unrestricted pGem 3.1; lane 2, pGem 3.1; lane 3, fat body; lane 4, ovary; lane 5, testis; lane 6, unrestricted pGem 4.0; lane 7, pGem 4.0; lane 8,  $\phi$ x174 DNA size standards. The samples were electrophoresed on a 4% polyacrylamide gel, then stained with ethidium bromide. The drawing is a representation of the original gel. Faint unrestricted bands are seen on the original gel with testis and ovary, but not with fat body. The results suggest that, with RT-PCR, a lectin mRNA other than clone 3 and clone 4 mRNA is amplified with ovary and testis, but not fat body, and indicates the expression of another lectin gene with the two former tissues.

products. It cannot be concluded from the experiment whether fat body contains other lectin cDNAs that are neither clone 3 nor clone 4. It may be expected however, that fat body would contain mRNA coding for GHA - the major lectin in hemolymph. GHA, however, could have priming and restriction sites homologous to those in clone 3 and/or clone 4.

Overall results suggest that genes for both clone 3 and clone 4 are expressed in *M. Differentialis* fat body, ovarian and testicular tissues. Also, all three tissues seem to be expressing additional lectin genes.

## DISCUSSION

Perspective

This research utilized the molecular biology techniques of Northern analysis and RT-PCR to investigate the expression of two similar lectins termed clone 3 and clone 4, in fat body, ovarian and testicular tissue from *M. Differentialis*. For convenience, the genes representing the two clones will be called gene 3 and gene 4.

In Northern analysis, polyA<sup>+</sup> RNA from fat body was fixed on a membrane and probed with radiolabeled lectin. 580 bp probe from clone 3. RT-PCR used polyA<sup>+</sup> RNA from fat body, ovary or testis tissue to synthesize cDNA and then utilized clone 3- and clone 4- specific primers to amplify target lectin cDNAs. Both techniques were optimized, with modifications made to the protocols described in the 'Materials and Methods' section.

The previous section reported the results obtained with Northern analysis and RT-PCR. The key results, as well as uncertainties in the interpretation of some results will be

discussed in the following sections.

#### Lectin Expression in Fat Body

Lectin expression in *M. differentialis* grasshopper fat body tissue was investigated by means of Northern analysis and RT-PCR techniques. Northern analysis results will be discussed first.

Fat body total RNA and polyA<sup>+</sup> RNA were electrophoresed, fixed on a positive nylon membrane and probed with radiolabeled 580 bp probe (see Figure 3, Page 27 for probe position). The results of the Northern analysis experiment are shown in Figure 6. There is a strong 1.2 Kb and a weaker 3 Kb signal on the autoradiograph for polyA<sup>+</sup> RNA and a weak 1.2 Kb signal for total RNA. This result has been repeated in several other experiments. The two signals seen with polyA<sup>+</sup> RNA indicates that fat body expresses at least two lectin genes, approximately 1.2 Kb and 3 Kb in length, that bind the lectin probe.

It cannot be concluded which particular lectin gene is being expressed in fat body. The 1.2 Kb and 3 Kb bands are presumably not due to 3 or clone 4 mRNA, because the sizes

do not correspond with that expected from clone 3/4.

A 5' RACE product representing the 5' 1300 bp of clone 3 has been synthesized by J. Radke (unpublished). When combined with the known 3' structure of clone 3, the total length of the corresponding mRNA is approximately 2.1 Kb. This size is intermediate to the signals observed on the Northern blot, suggesting the observed bands are due to mRNA other than that coding for clone 3.

The length of clone 4 mRNA is 2 Kb. This length was determined by combining the length of a clone 4 5' RACE product with known structure of clone 4 (Radke, unpublished results). The length of clone 4, similarly, does not correspond to the 1.2 Kb and 3 Kb signals seen on the Northern blot.

It appears that the two bands on the Northern blot are not due to clone 3 or clone 4 mRNA. Although it cannot be concluded which particular lectin mRNAs correspond to the signals, reasons for the signals can be proposed. The 1.2 Kb and 3 Kb signals are not due to non-specific binding of the probe to a large quantity of RNA, such as abundant rRNA, because of the lack of signals corresponding to rRNA on the Northern blot. It can be concluded that the probe bound to

homologous RNA.

The weak 3 Kb signal could be due to a rare lectin transcript with either a large untranslated region or a larger open reading frame coding for a larger protein, or both. *Periplaneta* C-type lectin transcript (PL25) is large - mRNA is over 2.8 Kb with a long 5' non-translated region of more than 663 nucleotides (Kawasaki et al. 1996). The 3 Kb signal is much fainter than the 1.2 Kb signal, suggesting that the former transcript either has lower expression or less homology with the probe than the 1.2 Kb transcript.

Whichever lectin the 1.2 Kb signal is due to, an estimate of its abundance can be made. mRNAs that are 0.1% or more of the mRNA population can usually be seen on a Northern blot using 10-20  $\mu$ g total mRNA (Sambrook et al. 1989). Since the 1.2 Kb signal is seen with total RNA on the Northern blot (Figure 6), it can be approximated that the lectin transcript is 0.1% of the mRNA population in fat body. It is noted, however, that the 1.2 Kb signal may be due to more than one lectin mRNA species of this size. It may also be possible that the 1.2 Kb signal represents a degraded or alternative form of the 3 Kb signal.

A hemagglutinin protein (GHA), has been isolated in the

laboratory and is the major lectin found in the hemolymph of the grasshopper (Stebbins and Hapner 1985). It may be that either the 1.2 Kb or 3 Kb lectin is due to GHA transcript. Either would be large enough to code for the 36 Kda protein chain. However, no GHA-specific probes are available for detection of GHA transcript.

The results from Northern analysis confirm previous work that showed lectins to be synthesized by *M. differentialis* fat body using ELISA assays and immunoprecipitation with agglutinin-specific antibody on primary cultured fat body tissue (Stiles et al. 1988). This tissue is also the site of synthesis of lectins in other invertebrates. *Sarcophaga* and *Periplaneta* lectins were determined by molecular biology techniques of Northern analysis and RT-PCR, respectively (Kawasaki et al. 1996; Takahashi et al. 1985). *Spodoptera* lectins were discovered to be synthesized in fat body using isotope incorporation followed by immunoprecipitation with monoclonal antibodies (Boucias and Pendland 1993).

Gene 3 Expression in Fat Body

Northern analysis results gave indication of lectin gene expression in fat body, but did not show gene 3 or gene 4 expression. Investigation into gene 3 and gene 4 expression in fat body utilized RT-PCR. To individually detect the presence of clone 3 transcript in fat body mRNA, PCR was performed using clone 3-specific primer 3053 and primer 3'NT (Figure 13). The clone 3-specificity of primer 3053 was confirmed by restriction of PCR-amplified product with clone 3-specific restriction endonuclease, yielding expected restriction fragments (Figure 15).

Fat body PCR-amplified product in Figure 13 shows a band of 936 bp, which is the expected size of a clone 3 product. The 936 bp PCR-amplified product probed with lectin cDNA probe during Southern analysis gave a signal on the autoradiograph, indicating the product was due to amplification of lectin cDNA. These results imply that clone 3 cDNA was available for PCR-amplification and was originally present as mRNA in fat body. The results confirm that gene 3 is expressed in fat body.

The results of a different RT-PCR experiment seem to

contradict the conclusions of the above experiment. RT-PCR amplification was performed with primers 3152 and 3'NT followed by incubation with a clone 3-specific restriction endonuclease. Primers 3152 and 3'NT are complementary to both clone 3 and clone 4 and should amplify both clone cDNAs. Incubation with clone 3-specific restriction enzyme should restrict clone 3 PCR products. The results of restriction are shown in Figure 9. PCR-amplified fat body was not restricted, indicating lack of clone 3 PCR product and, therefore, no RT-PCR amplification of clone 3 mRNA. The results infer that gene 3 is not being expressed in fat body. By combining this result and the seemingly contradictory result mentioned above, it can perhaps be concluded that fat body is transcribing a small amount of clone 3 mRNA, that was not preferentially amplified during RT-PCR with primers 3152 and 3'NT, but was amplified with the clone 3-specific primer, 3053. Clone 4 cDNA may have been preferentially amplified using primers 3152 and 3'NT. Clone 4 expression in fat body will be dealt with in a later section.

Clone 3 was obtained from a cDNA library from fat body polyA<sup>+</sup> RNA (Rognlie 1991). The results obtained in RT-PCR

experiments discussed above suggest that the expression of gene 3 in fat body is low. A larger amount of clone 3 transcript may have been available when the library was constructed as compared to recent work, increasing the probability of obtaining a clone 3 cDNA from the library. If this hypothesis is true, then it implies that the expression of gene 3 is not constant, that is, it is not constitutively present in fat body and may be under regulation. *Sarcophaga* lectin and *Drosophila* lectin have enhanced expression due to injury, while two *Periplaneta* hemolymph lectins are induced by injection of *E. Coli* into the adult cockroach (Sugiyama and Natori 1991, Natori et al. 1996, Kawasaki et al. 1996). Lectin release in *M. Differentialis* is not stimulated with the addition of microbial cell wall components to fat body cultures or injection of these constituents into the hemocoel (Stiles et al. 1988). Microbial invasion does not seem to be the reason of increased lectin expression in grasshopper.

Fat body polyA<sup>+</sup> RNA used for both cDNA library construction and RT-PCR was isolated from adult grasshoppers. The grasshoppers were at the same stage in development, so it cannot be stated that changes in gene 3

expression are a result of differential expression during separate ontogenetic stages of the grasshopper. RT-PCR would need to be carried out on earlier stages of the grasshopper to investigate any development-related changes in gene 3 expression.

#### Gene 4 Expression in Fat Body

Gene 4 expression in fat body was examined using RT-PCR with clone 4-specific primer 4052 and 3'NT. The clone 4-specificity of the primer was confirmed by restriction of PCR-amplified fat body cDNA with a clone 4-specific restriction endonuclease, yielding expected restriction fragments (Figure 12).

The primer combination 4052 and 3'NT in the PCR reaction gave expected sized bands of approximately 885 bp with fat body (Figure 7). This experiment was repeated by Jay Radke with identical results (unpublished). The 885 bp band is assumed to be the product of amplification of clone 4 cDNA. A corresponding 885 bp signal was seen on the Southern blot using lectin cDNA probe. This result proves that the 885 bp product is due to amplification of lectin

cDNA and suggests that fat body is synthesizing clone 4 mRNA.

Another experiment was carried out to determine gene 4 expression in fat body. This procedure involved RT-PCR amplification of fat body polyA<sup>+</sup> RNA with primers 3152 and 3'NT, followed by incubation with a clone 4-specific endonuclease (Figure 9). The restriction reaction gave a restriction fragment of 309 bp, the size expected when a clone 4 PCR product is restricted. The result suggests that clone 4 mRNA was present in the isolated polyA<sup>+</sup> RNA from fat body tissue.

Overall results from Northern analysis and RT-PCR with fat body imply that this tissue expresses the genes of two, unknown lectins as well as gene 3 and gene 4. The results confirm previous data that detected, via antibody, lectin synthesis in *M. differentialis* fat body, although exactly which lectin(s) was detected is uncertain (Stiles et al, 1988).

#### Gene 3 Expression in Ovary and Testis Tissue

Northern analysis was performed on polyA<sup>+</sup> RNA isolated

from grasshopper ovarian and testicular tissues to investigate gene 3 expression in ovarian and testicular tissue. A 580 bp lectin cDNA probe was used. No bands were seen on the Northern blots (results not shown), and suggested that lectin expression was low in the two respective tissues.

As an alternative method, RT-PCR was performed. PolyA<sup>+</sup> RNA from ovary and testis was used to obtain cDNA via reverse transcription, followed by RT-PCR using clone 3-specific primer 3053 and 3'NT. The same procedure was used to detect clone 3 lectin expression in fat body, as described earlier. A band of approximately 936 bp was seen with both ovary and testis PCR products after electrophoresis on a polyacrylamide gel (Figure 13). This size of product is expected with amplification of clone 3 cDNA and, therefore suggests that this clone was amplified in the PCR reaction. Southern analysis of the ovary and testis PCR products with a radiolabeled 413 bp lectin cDNA probe (Figure 14) resulted in a signal on the autoradiograph corresponding to the 936 bp PCR product on the polyacrylamide gel. This result proves that the 936 bp band is due to amplification of lectin cDNA. Combined results

imply that mRNA was present in the original ovary and testis tissues and both tissues were expressing clone 3 lectin gene.

#### Gene 4 Expression in Ovary and Testis Tissue

Expression of clone 4 lectin gene in grasshopper ovary and testis was examined with RT-PCR. A clone 4-specific primer, 4052, was used in combination with primer 3'NT (see Figure 3, Page 27 for primer position on clone 4) and the PCR products electrophoresed (Figure 10). Upon staining with ethidium bromide, an expected band of 885 bp appeared on the gel. This 885 bp band also appeared on a Southern blot when probed with 413 bp lectin cDNA probe, proving the lectin specificity of the PCR product. Combined RT-PCR and Southern analysis results imply that mRNA encoding gene 4 was present in the original ovary and testis tissues and that clone 4 lectin was being synthesized in the two tissues.

Gene 3 and gene 4 are shown to be synthesized in ovary and testis of the grasshopper, confirming previous data that indicated lectin synthesis in these two tissues (Stiles et

al. 1988). It is noted that the lectin synthesis suggested by Stiles et al. may not be due to gene 3 or gene 4 expression. Synthesis of other lectins, such as GHA, may have been detected in the previous published experiments. There is no other literature that describes lectin expression in invertebrate ovary or testis tissue, so comparisons of lectin expression in *M. Differentialis* cannot be made with other sources.

Relative Expression of Gene 3 and Gene 4 in  
Fat Body, Ovary and Testis

Overall RT-PCR results show that grasshopper fat body, ovary and testis synthesize lectins, both clone 3 and clone 4. Some information about relative expression of gene 3 and gene 4 with respect to each other as well as between the three tissues can be obtained. In order to reach any conclusions about relative expressions of lectin genes, two assumptions need to be made concerning the techniques used. Firstly, the UV spectrophotometric measurements of isolated polyA<sup>+</sup> RNA yields are assumed to be accurate, to ensure consistent amounts of polyA<sup>+</sup> RNA for RT-PCR. Secondly, it

is assumed that the extent of reverse transcription and PCR is equal for the three tissues, leading to equal amounts of cDNA and PCR product, respectively.

Any variability in results caused by potential inconsistencies mentioned above were examined. Different stocks of polyA<sup>+</sup> RNA and cDNA were used in reverse transcription and PCR experiments, respectively.

Experimental repeats with different stocks resulted in consistent results and implies that the potential inconsistencies in polyA<sup>+</sup> RNA measurements and RT-PCR are only minor.

The amount of testis template cDNA used in all PCR experiments shown in this report was twice that of fat body and ovary template cDNA. When the amount of testis cDNA was not diluted and was the same amount as used with the other two tissues, no PCR products were obtained, suggesting that there was an inadequate amount of target cDNA with testis (results not shown). The results imply that testis tissue produces less clone 3 and clone 4 transcript than ovary and fat body.

Fat body has the most intense ethidium bromide bands of expected size in all RT-PCR experiments, followed by ovary

and, lastly testis. These results indicate fat body has the most target mRNA followed by ovary and then testis. Since the RT-PCR experiments detected clone 3 and clone 4 mRNA, it is implied that these transcripts are most abundant in fat body and least abundant in testis. If the lectins encoded by gene 3 and gene 4 are involved in immunity, it is not surprising that expression of these lectins is greater in fat body than the reproductive organs, with its major role of synthesizing immune compounds (Lowenberger 1996).

The dominant expressed lectin gene in fat body does not seem to be clone 3, as discussed in a previous section. Restriction of PCR products, that include amplified clone 3 and/or clone 4 cDNA, with clone 3- and clone 4-specific restriction enzymes yields clone 4 restriction fragments only (Figure 16). Based on these results, clone 4 lectin gene appears to be predominately expressed in fat body compared to clone 3. It is realized that other lectins, such as GHA, may be more greatly synthesized than clone 4.

The experiment described above was performed with ovary and testis PCR products (Figure 16). Restriction resulted in clone 3 and clone 4 fragments of equal intensities, indicating that target clone 3 and clone 4 cDNAs were

equally abundant. It can be concluded that clone 3 and clone 4 lectin genes are similarly expressed in ovary and testis.

#### Presence of Non-Clone 3/Clone 4 Lectins

Previous published data indicated lectin synthesis in fat body, ovary and testis tissue (Stiles et al. 1988). It is not known which particular lectins were being synthesized. This project has determined the expression of gene 3 and gene 4 in the three tissues, however, these lectins may not correspond to those found by Stiles et al. It appears that at least three C-type lectin genes exist in the grasshopper's genome (Radke, J., Gedik, T., Hapner, K.D. unpublished results) and they may all be expressed in fat body, ovary and testis tissues.

Northern analysis and RT-PCR of fat body gave indication of two unknown lectin transcripts and transcripts of gene 3 and gene 4, respectively. An RT-PCR experiment was performed to detect non-clone 3/clone 4 lectin genes in fat body, ovary and testis and will be discussed below.

RT-PCR was carried out on fat body, ovary and testis

cDNA, using 3152 and 3'NT primers that are complementary to both clone 3 and clone 4. It was considered that perhaps lectin cDNA template that did not correspond to clone 3 or clone 4 was PCR-amplified, resulting in PCR product of similar size to clone 3 and clone 4 PCR product. Clone 3 and clone 4 PCR products were cut with restriction endonucleases (Figure 16). The rationale of the experiment was that lectin PCR product without identical restriction sites to clone 3 or clone 4 would not be restricted and remain as unrestricted 411/413 bp. This unrestricted band remained in ovary and testis samples, but not fat body. This result implies that ovary and testis tissues contain additional mRNA that is neither clone 3 nor clone 4 mRNA. This mRNA may be lectin mRNA because of similar primer annealing sites to clone 3 or clone 4, and suggests that an additional lectin(s) is synthesized in the reproductive organs of the grasshopper. The lack of unrestricted fragment with fat body insinuates that this tissue does not express the gene encoding this putative lectin(s), or at least not in significant amounts. The lectin may have functions in the reproductive system of the grasshopper, that are not required in fat body.

RT-PCR followed by Southern analysis with a 413 bp cDNA lectin probe detected the presence of other lectins in fat body, ovary and testis. Figure 8 is a Southern blot of PCR-amplified products using 3'52 and 3'NT primers. Testis PCR-amplified product has a signal at approximately 470 bp, a band that is larger than expected clone 3 and clone 4 PCR products. This result has been repeated and implies either PCR-amplification of lectin cDNA that is neither clone 3 nor clone 4 cDNA. Results imply expression of a non-gene 3/gene 4 lectin in testis tissue.

An experiment similar to the one above, except clone 3-specific primer 3053 and 3'NT were used during PCR, resulted in the detection of other lectins in fat body, ovary and testis. The Southern blot (Figure 14) shows a band at approximately 650 bp with fat body, ovary and testis PCR-amplified samples and this band is smaller than the PCR-amplified products of clone 3. The band is either due to a lectin PCR-amplified product that is not clone 3, or another priming site within clone 3. The former conclusion suggests the presence of another lectin in fat body, ovary and testis tissue.

It is noted that the 413 bp cDNA probe used in Southern

analysis experiments was PCR-amplified from clone 3 using primers 3152 and 3'NT. These primers were also used to amplify fat body, ovary and testis cDNA. The probe would be better designed if it was amplified using primers that did not correspond to those used in the RT-PCR experiments, otherwise perhaps only primer sites of probe and RT-PCR product anneal to give a false positive result showing lectin cDNA amplification. Primer sites on the probe and PCR product may anneal if there are low stringency conditions during the hybridization and wash steps of Southern analysis, especially since the  $T_d$ 's of the primers and hybridization temperature of 65°C are similar (see Table 1 for  $T_d$ 's of primers). Primers that anneal outside of the PCR-amplified region of 3152 and 3'NT should be used to synthesize a lectin cDNA probe in the future. The hybridization and wash temperatures used during Southern analysis in this research are stringent, so hybridization of probe to PCR products would be due to homology between the probe and PCR product and not a result of primer sites annealing.

The overall results obtained from RT-PCR indicate that

testis is expressing at least two other lectin genes besides gene 3 and gene 4, while ovary is expressing at least one additional lectin gene. An additional lectin could perhaps be GHA. Northern analysis and RT-PCR results suggest that there are at least two non-gene 3/gene 4 lectin genes being expressed in fat body. As mentioned earlier, Southern analysis results on grasshopper genomic DNA indicate the presence of multiple lectin genes, perhaps a lectin family (Gedik, T., unpublished results). Data in this project imply the existence of at least 4 lectin genes.

#### Future Work

This work has been successful in determining gene 3 and gene 4 expression in grasshopper fat body, ovary and testis tissue. Additional lectins also seem to be synthesized. Sequencing unexpected RT-PCR products may lead to discovery of new lectins. Perhaps the sequence of GHA, a major lectin in grasshopper hemolymph would be discovered by this method.

In order to fully determine relative expression of gene 3 and gene 4 in fat body, ovary and testis tissues that has been described in this report, an internal control is

needed, such as  $\beta$ -actin. The protein is useful because it is synthesized in every tissue with similar expression levels. As a control for mRNA precipitation, primers of the *Drosophila*  $\beta$ -actin gene can be used with PCR (Kawasaki et al. 1996). The expression of  $\beta$ -actin would be expected to be the same in each tissue used. It is noted that expression levels can still slightly vary between tissues and during different developmental stages of the organism, however, it is expected that an internal control gives important information and is employed in RT-PCR in published papers dealing with relative expression levels of proteins.

## CONCLUSIONS

The primary objective of this project was to investigate and confirm expression of lectin genes in fat body, ovarian and testicular tissue from the grasshopper. This goal was successfully achieved through Northern analysis and RT-PCR amplification of lectin gene transcripts from the respective tissues. Conclusions that can be reached from this research are:

- 1) RT-PCR is a more sensitive technique than Northern analysis for detection of grasshopper mRNA (in this work)
- 2) Fat body synthesizes at least four lectin transcripts, two of which correspond to clone 3 and clone 4. Ovary and testis are synthesize clone 3 and clone 4 lectin transcripts. Ovary is expressing at least one additional lectin gene, while testis is expressing at least two other lectin genes.
- 3) Gene 3 and gene 4 seem to have similar expression in

testis, and also in ovary. With fat body, relatively more clone 4 lectin is being synthesized than clone 3 lectin.

4) Fat body is expressing more gene 3 and gene 4 than ovary, followed by testis, as indicated by relative intensity of bands on RT-PCR polyacrylamide gels.

5) Overall results, utilizing molecular biology methods, confirm previous published data in this laboratory that showed lectins to be synthesized in *M. differentialis* grasshopper fat body, ovary and testis tissue.

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