2003

INHIBITORY PROPERTIES OF *MICROPLITIS CROCEIPES* TERATOCYTE SECRETORY PRODUCTS AND THE RECOMBINANT PROTEIN TSP14 ON PROTEIN SYNTHESIS

Francis Anthony DiLuna
*University of Kentucky*, tonydiluna@alumni.bates.edu

Click here to let us know how access to this document benefits you.

Recommended Citation
https://uknowledge.uky.edu/gradschool_theses/280

This Thesis is brought to you for free and open access by the Graduate School at UKnowledge. It has been accepted for inclusion in University of Kentucky Master's Theses by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.
ABSTRACT OF THESIS

INHIBITORY PROPERTIES OF *MICROPLITIS CROCEIPES*
TERATOCYTE SECRETORY PRODUCTS AND THE RECOMBINANT PROTEIN TSP14
ON PROTEIN SYNTHESIS

Microplitis croceipes is a solitary endoparasitic wasp that oviposits in the hemocoel of Heliothis virescens larvae. Upon parasitization, the host larvae’s physiology is altered; resulting in a compromised immune system and a decrease in the production of some vital proteins resulting in a terminal post-wandering prepupal state. Teratocytes, cells derived from the extra–embryonic serosa of the parasitic wasp, mimic symptoms of parasitization when injected into host larvae, independent of other factors like polydnavirus and venom. Some of the inhibition of protein synthesis can be attributed to proteins secreted by the teratocytes (teratocyte secretory proteins or TSP). A fraction of TSP between 3–30 kDa inhibits protein synthesis in vivo, in the in vitro fat body and testes assays, and in the rabbit reticulocyte lysate and wheat germ extract assays. This fraction, however, has no effect on nucleic acid synthesis. Its effect on protein synthesis is dose dependent and exposure time sensitive. A 13.9 kDa protein isolated from TSP and expressed in a baculovirus system seems primarily responsible for the inhibition. Although TSP14 production was low, it did bind to the cell surface, enter the cell, and inhibit protein synthesis as the 3–30 kDa factor did.

KEYWORDS: Microplitis croceipes, Heliothis virescens, Teratocyte Secretory Proteins, TSP14, Protein Synthesis Inhibition

Francis Anthony DiLuna

December 2, 2003

Copyright 2003, F. Anthony DiLuna
INHIBATORY PROPERTIES OF *MICROPLITIS CROCEIPES*
TERATOCYTE SECRETORY PRODUCTS AND THE RECOMBINANT PROTEIN TSP14
ON PROTEIN SYNTHESIS

By

Francis Anthony DiLuna

Douglas L. Dahlman
Director of Thesis

Kenneth V. Yeargan
Director of Graduate Studies
RULES FOR THE USE OF THESES

Unpublished theses submitted for the Master’s degree and deposited in the University of Kentucky Library are as a rule open for inspection, but are to be used only with due regard for the rights of the authors. Bibliographical references may be noted, but quotations or summaries of parts may be published only with the permission of the author, and with the usual scholarly acknowledgements.

Extensive copying or publication of the thesis in whole or in part also requires the consent of the Dean of the Graduate School of the University of Kentucky.
THESIS

Francis Anthony DiLuna

The Graduate School
University of Kentucky
2003
INHIBITORY PROPERTIES OF *MICROPLITIS CROCEIPES*
TERATOCYTE SECRETORY PRODUCTS AND THE RECOMBINANT PROTEIN TSP14
ON PROTEIN SYNTHESIS

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Agriculture at the University of Kentucky

By
Francis Anthony DiLuna
Lexington, Kentucky

Director: Dr. Douglas Dahlman, Professor of Entomology
Lexington, Kentucky

2003

Copyright 2003, F. Anthony DiLuna
ACKNOWLEDGEMENTS

The following thesis, while an individual work, benefited from the insights and direction of several people. First, my advisor, Douglas Dahlman, from whom I have learned so much about life and people as well as science. It was Dr. Dahlman’s guidance, encouragement, and above all, his perseverance that enabled this project to be completed. Next, I would like to thank Bruce Webb, not only for his guidance but for opening up his lab and his resources to me, allowing me to finish things up. I would also like to thank Adria Elskus for joining my committee on short notice, and Kenneth Yeargan for all his administrative work to get the degree granted. Though too numerous to name individually, I would like to acknowledge the support and assistance I received from my fellow graduate students, faculty, post-doctoral scholars, visiting scholars, undergraduates, lab technicians and support staff in both the Entomology and Toxicology Departments.

In addition to the technical and scientific assistance above, I also benefited from the love and support of my friends and family. I would like to thank my parents, Frank and Lisa DiLuna, who under difficult circumstances made the completion of this project a priority. They never stopped believing in me, even when I doubted myself. I also benefited from the unwavering support of my mother and stepfather Jeanne and Gene Grindlinger and sister Alysa. I have also been greatly influenced by my grandfathers, Frank DiLuna and William “Bud” Mills who I miss every day, and by my grandmothers Mary DiLuna and Mary Mills, whose strength and courage inspired me to see this project to the end. I must also thank Peggy, Hobbie and Dale Appling, who under some odd circumstances welcomed me into their home…you are family to me. Last, but certainly not least, John “Pick” Pickles, who has always been there for me…I am at a loss for words, thank you!
TABLE OF CONTENTS

Acknowledgments........................................................................................................................................ iii
List of Tables.................................................................................................................................................. vi
List of Figures............................................................................................................................................... viii
List of Files.................................................................................................................................................. xii

LITERATURE REVIEW................................................................................................................................. 1
  Host-Parasite System................................................................................................................................... 1
    *Heliothis virescens*................................................................................................................................. 1
    *Microplitis croceipes*............................................................................................................................ 2
  Teratocytes.................................................................................................................................................. 3
  Teratocyte Secretory Products.................................................................................................................... 5
  Eukaryotic Protein Synthesis....................................................................................................................... 7
    Eukaryotic Initiation Factor Type–1.......................................................................................................... 7
    Eukaryotic Initiation Factor Type–2.......................................................................................................... 7
    Eukaryotic Initiation Factor Type–2B........................................................................................................ 8
    Eukaryotic Initiation Factor Type–3.......................................................................................................... 8
    Eukaryotic Initiation Factor Type–4A........................................................................................................ 8
    Eukaryotic Initiation Factor Type–4B........................................................................................................ 9
    Eukaryotic Initiation Factor Type–4C........................................................................................................ 9
    Eukaryotic Initiation Factor Type–4D........................................................................................................ 9
    Eukaryotic Initiation Factor Type–4E........................................................................................................ 9
    Eukaryotic Initiation Factor Type–4F........................................................................................................ 10
    Eukaryotic Initiation Factor Type–5......................................................................................................... 10
    Eukaryotic Initiation Factor Type–6......................................................................................................... 10
    Elongation Initiation Factor Type–1α...................................................................................................... 10
    Elongation Initiation Factor Type–1 βγ.................................................................................................... 11
    Elongation Initiation Factor Type–2...................................................................................................... 11
    Release Factor......................................................................................................................................... 11
    Initiation................................................................................................................................................ 12
    Elongation............................................................................................................................................... 13
    Termination............................................................................................................................................ 14
    Regulation............................................................................................................................................... 14
  Toxic Inhibition of Protein Synthesis........................................................................................................ 16
    Diphtheria Toxin................................................................................................................................. 17
    Ricin..................................................................................................................................................... 18

HYPOTHESES............................................................................................................................................... 21

MATERIALS AND METHODS...................................................................................................................... 23
  General.................................................................................................................................................... 23
<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maintenance and Care of <em>Heliothis virescens</em></td>
<td>23</td>
</tr>
<tr>
<td>Maintenance and Care of <em>Microplitis croceipes</em></td>
<td>24</td>
</tr>
<tr>
<td>Maintenance and Care of <em>Manduca sexta</em></td>
<td>25</td>
</tr>
<tr>
<td>Production of Teratocytes</td>
<td>25</td>
</tr>
<tr>
<td>Production and Collection of TSP</td>
<td>27</td>
</tr>
<tr>
<td>Effects and Properties of TSP on <em>H. virescens</em> Testes</td>
<td>27</td>
</tr>
<tr>
<td>General Procedures for Protein Inhibition Tissue Assay</td>
<td>27</td>
</tr>
<tr>
<td>Inhibition of Protein Synthesis</td>
<td>28</td>
</tr>
<tr>
<td>Left vs. Right Testes</td>
<td>28</td>
</tr>
<tr>
<td>Incubation Times</td>
<td>28</td>
</tr>
<tr>
<td>Dose Response</td>
<td>28</td>
</tr>
<tr>
<td>Nucleic Acids</td>
<td>29</td>
</tr>
<tr>
<td>Insect Injections</td>
<td>29</td>
</tr>
<tr>
<td>Statistics</td>
<td>29</td>
</tr>
<tr>
<td>Effects of TSP on Translation of mRNA in Cell Free Systems</td>
<td>30</td>
</tr>
<tr>
<td>Dissection to Collect Tissues</td>
<td>30</td>
</tr>
<tr>
<td>Isolation of Total RNA</td>
<td>30</td>
</tr>
<tr>
<td>Isolation of Messenger RNA</td>
<td>31</td>
</tr>
<tr>
<td>Agarose–Formaldehyde Electrophoresis for RNA</td>
<td>32</td>
</tr>
<tr>
<td>Rabbit Reticulocyte Lysate Assay</td>
<td>33</td>
</tr>
<tr>
<td>Wheat Germ Extract Assay</td>
<td>34</td>
</tr>
<tr>
<td>TSP14 Production</td>
<td>35</td>
</tr>
<tr>
<td><em>Sf–9</em> and <em>Tn–5</em> Cell Culture Maintenance</td>
<td>35</td>
</tr>
<tr>
<td><em>In vitro</em> Production of Recombinant TSP14</td>
<td>36</td>
</tr>
<tr>
<td><em>In vivo</em> Production of Recombinant TSP14</td>
<td>37</td>
</tr>
<tr>
<td>Localization of TSP</td>
<td>38</td>
</tr>
<tr>
<td>Synthesis of a Radio–labeled Probe</td>
<td>38</td>
</tr>
<tr>
<td>Northern Blot</td>
<td>39</td>
</tr>
<tr>
<td>SDS–Polyacrylamide Gel Electrophoresis for Proteins</td>
<td>40</td>
</tr>
<tr>
<td>Western Blot</td>
<td>40</td>
</tr>
<tr>
<td>Dehydration and Infiltration of <em>H. virescens</em> for Histology</td>
<td>41</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>42</td>
</tr>
<tr>
<td>RESULTS</td>
<td>44</td>
</tr>
<tr>
<td>Effects of TSP on Protein Synthesis in <em>H. virescens</em> Testes</td>
<td>44</td>
</tr>
<tr>
<td>Effect of TSP on Nucleic Acid Synthesis in <em>H. virescens</em> Testes</td>
<td>55</td>
</tr>
<tr>
<td>Effects of TSP on Translation of mRNA in Cell Free Systems</td>
<td>55</td>
</tr>
<tr>
<td>TSP14 Production</td>
<td>75</td>
</tr>
<tr>
<td>Efficacy and Stability of TSP14</td>
<td>78</td>
</tr>
<tr>
<td>Localization of TSP</td>
<td>85</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>100</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>106</td>
</tr>
<tr>
<td>VITA</td>
<td>122</td>
</tr>
<tr>
<td>Table 1:</td>
<td><em>In vitro</em> incorporation of $[^{35}\text{S}]$–methionine by matched pairs of <em>H. virescens</em> testes (dpm / µg fresh weight tissue ± standard error) (t = 8.79; p = 0.0; n = 50). TSP treatment received 3 LE of crude TSP for 4 hr incubation period.</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Table 2:</td>
<td>A comparison of matched pairs of <em>H. virescens</em> testes <em>in vitro</em> incorporation of $[^{35}\text{S}]$–methionine (mean dpm/µg tissue ± SE) when one of each pair was incubated with 3 larval equivalents of crude TSP for 4 hours (n = 22).</td>
</tr>
<tr>
<td>Table 3:</td>
<td>Effect of time on TSP inhibition and percent reduction of <em>in vitro</em> incorporation of $[^{35}\text{S}]$–methionine by matched pairs of <em>H. virescens</em> testes. (3 LE crude TSP / testis) (dpm / testis ± SE) (n = 20).</td>
</tr>
<tr>
<td>Table 4:</td>
<td>Effect of crude TSP dose on the percent reduction of $[^{35}\text{S}]$–methionine incorporation on <em>H. virescens</em> testes (4 hour incubation) (n = 15).</td>
</tr>
<tr>
<td>Table 5:</td>
<td>Effect of TSP dose on the percent reduction of $[^{35}\text{S}]$–methionine incorporation on <em>H. virescens</em> testes (1 hr incubation) (n = 8).</td>
</tr>
<tr>
<td>Table 6:</td>
<td>Effect of crude TSP on DNA synthesis ($[^{3}\text{H}]$–thymidine incorporation) and RNA synthesis ($[^{3}\text{H}]$–uridine incorporation) in matched pairs of <em>H. virescens</em> testes (dpm / testis ± SE) (3 LE TSP / testis) (n = 20).</td>
</tr>
<tr>
<td>Table 7:</td>
<td>Effect of TSP (3–30 kDa fraction) on protein synthesis initiated by 2 µg mRNA from different sources from <em>H. virescens</em> (dpm ± SE). Student’s T–test performed on control vs. TSP treatments of the same mRNA source. <em>P</em>-value less than 0.05 in paired Student’s T-test (n = 10).</td>
</tr>
<tr>
<td>Table 8:</td>
<td>Effect of TSP (3 – 30 kDa fraction) on protein synthesis initiated by 2 µg mRNA from different sources from <em>M. sexta</em> (dpm ± SE). Student’s T–test performed on control vs. TSP treatments of the same mRNA source. <em>P</em>-value less than 0.05 in paired Student’s T-test (n = 10).</td>
</tr>
<tr>
<td>Table 9:</td>
<td>Effect of TSP (3 – 30 kDa fraction) + &lt;3kDa fraction on protein synthesis initiated by 2µg mRNA from different sources of <em>H. virescens</em> (dpm ± SE). Student’s T–test performed on control vs. TSP + cofactor treatments of the same mRNA source. <em>P</em>-value less than 0.05 in paired Student’s T–test (n = 10).</td>
</tr>
<tr>
<td>Table 10:</td>
<td>Effect of TSP (3 – 30 kDa fraction) + &lt;3kDa fraction on protein synthesis initiated by 2µg mRNA from different sources of <em>M. sexta</em> (dpm ± SE). Student’s T–test performed on control vs. TSP + cofactor treatments of the same mRNA source. <em>P</em>-value less than 0.05 in paired Student’s T–test (n = 10).</td>
</tr>
</tbody>
</table>
Table 11: Percent reduction of $[^{35}\text{S}]$–methionine incorporation into proteins translated from mRNA from various tissues of *H. virescens* after treatment with TSP or TSP + <3kDa fraction.
* P–value less than 0.05 in paired Student’s T-test (n = 10)...... 66

Table 12: Percent reduction of $[^{35}\text{S}]$–methionine incorporation into proteins translated from mRNA from various tissues of *M. sexta* after treatment with TSP or TSP + <3kDa fraction.
* P–value less than 0.05 in paired Student’s T-test (n = 10)...... 67

Table 13: Effects of TSP (3 – 30 kDa fraction) on protein synthesis initiated by 2 µg mRNA from different sources of *H. virescens* (dpm ± SE) using wheat germ extract assay. Student’s T–tests performed on control vs. TSP treatments of the same mRNA source.
* P–value less than 0.05 in paired Student’s T–test (n = 10)...... 73

Table 14: Effect of recombinant TSP14 on protein synthesis initiated by 2 µg mRNA from different sources of *H. virescens* (dpm ± SE). Student T–test performed on control vs. TSP14 treatments of the same RNA source. TSP14–treated groups received 0.8 µg/sample.
* P–value less than 0.05 in paired Student’s T–test (n = 10)...... 79

Table 15: Effect of recombinant TSP14 on protein synthesis initiated by 2µg mRNA from different sources of *M. sexta* (dpm ± SE). Student t–test performed on control vs. TSP14 treatments of the same mRNA source. TSP14–treated groups received 0.8 µg TSP14 (n = 10)......................................................... 80

Table 16: Dose response to recombinant TSP14 of *H. virescens* homogenate 2µg mRNA initiating protein synthesis in a rabbit reticulocyte lysate assay (dpm ± SE) (n = 15)............................................. 81

Table 17: Percent reduction of $[^{35}\text{S}]$–methionine incorporation in *in vitro* *H. virescens* testes assay and the percent of protein synthesis recuperation after washing the treated testes (n = 24).............. 86
LIST OF FIGURES

Figure 1: In vitro incorporation of $^{35}$S–methionine by matched pairs of H. virescens testes. TSP treatment received 3 LE of crude TSP for a 4 hr incubation period (n = 50). ................................. 47

Figure 2: Percent reduction of $^{35}$S–methionine incorporation by H. virescens testes in the in vitro testes assay using 3 LE of crude TSP treated left testis and the right as a control vs. crude TSP–treated right testis and the left as a control (n = 22) ................. 48

Figure 3: Effect of time on TSP inhibition of in vitro incorporation of $^{35}$S–methionine by matched pairs of H. virescens testes (3 LE crude TSP / testis) (n = 20) .......................................................... 52

Figure 4: Effect of time on the mean percent reduction $^{35}$S–methionine incorporation by crude TSP–treated H. virescens testes (3 LE crude TSP / testis) (n = 20) ......................................................... 53

Figure 5: Effect of crude TSP dose on the percent reduction of $^{35}$S–methionine incorporation on H. virescens testes (4 hr incubation time) (n = 15) ................................................................. 54

Figure 6: Effect of TSP dose on the percent reduction of $^{35}$S–methionine incorporation on H. virescens testes (1 hr incubation time) (n = 8) ................................................................. 58

Figure 7: Effect of crude TSP on DNA synthesis ($^{3}$H–thymidine incorporation) in matched pairs of H. virescens testes (dpm / testis ± SE) (3 LE TSP / testis) (n = 20) ........................................... 59

Figure 8: Effect of crude TSP on RNA synthesis ($^{3}$H–uridine incorporation) in matched pairs of H. virescens testes (dpm / testis ± SE) (3 LE TSP / testis). (n = 20) ........................................... 60

Figure 9: Mean percent reduction (± SE) of protein synthesis by TSP (3 – 30 kDa fraction) in the rabbit reticulocyte lysate assay using 2 $\mu$g of mRNA from various tissues of H. virescens (n = 10) ............... 68

Figure 10: Mean percent reduction (± SE) of protein synthesis by TSP (3 – 30 kDa fraction) in the rabbit reticulocyte lysate assay using mRNA from various tissues of M. sexta (n = 10) ......................... 69

Figure 11: Mean percent reduction (± SE) of protein synthesis by TSP (3 – 30 kDa fraction) and possible cofactor (< 3 kDa fraction) in the rabbit reticulocyte lysate assay using 2 $\mu$g of mRNA from various tissues of H. virescens (n = 10) ......................... 70

Figure 12: Mean percent reduction (± SE) of protein synthesis by TSP (3 – 30 kDa fraction) and possible cofactor (< 3 kDa fraction) in the rabbit reticulocyte lysate assay using 2 $\mu$g of mRNA from various tissues of M. sexta (n = 10) ......................... 71

Figure 13: Percent reduction (± SE) of protein synthesis by TSP (3 – 30 kDa fraction) in the wheat germ extract assay using 2 $\mu$g of mRNA from various tissues of H. virescens (n = 10) ......................... 74
Figure 14: Micrograms of recombinant TSP14 produced by a single 150 ml cell culture flask when the cells were infected with the recombinant baculovirus expression vector, pAd.CMV–Link.1 containing the TSP14/polyhistidine fusion insert. TSP14 was isolated using a Ni–NTA resin (n = 42)………………………………… 76

Figure 15: Micrograms of recombinant TSP14 produced by a single M. sexta larvae when inoculated with the recombinant baculovirus expression vector, pAd CMV–Link.1 containing the TSP14/polyhistidine fusion insert. TSP14 was isolated using a Ni–NTA resin (n = 24)…………………………………………. 77

Figure 16: Percent reduction (± SE) of protein synthesis by recombinant TSP14 in the rabbit reticulocyte lysate assay using mRNA from various tissues of H. virescens. TSP14 treated groups received 0.8 µg / sample (n = 10)……………………………………………… 82

Figure 17: Percent reduction (± SE) of protein synthesis by recombinant TSP14 in the rabbit reticulocyte lysate assay using 2µg of mRNA from various tissues of M. sexta. TSP14– treated groups received 0.8 µg / sample (n = 10)……………………………………………… 83

Figure 18: Dose response of H. virescens homogenates to recombinant TSP14 in rabbit reticulocyte lysate assay with 2 µg mRNA (n = 15)……… 84

Figure 19: Cross–section of a control H. virescens larva of the same age as six days post–parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT magnified 100X. Note cross-sections through Malpighian tubules (MT) in lower right quadrant; cross-section through gut (G) at 7 O’clock, and fat body (FB) occupying the upper center area, and darkly colored integument (I) visible at 3 and 9 O’clock………………. 87

Figure 20: Cross–section of a H. virescens larva six days post–parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT magnified 100X. Note cross–section of the gut (G) and contents located in the lower middle portion, surrounded with smaller circles of cross-sections of Malpighian tubules (MT). This section also contains fat bodies (FB) (10 O’clock) and more lightly staining strips of muscle (M) (8-9 O’clock and 11 O’clock). Note that the overall appearance of the tissues is stained darker than the control tissues in Figure 19…………….. 88

Figure 21: Cross–section of a control H. virescens larva of the same age as six days post–parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (400X). Cross–sections through the Malpighian tubules (MT). Note relatively pale colors of all tissues…………………………………………………………… 90
Figure 22: Cross-section of a *H. virescens* larva six days post-parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (400X). Sections through Malpighian tubules (MT). Note relatively intense staining, presumed to be from reactions of the TSP14 antiserum to the presence of TSP in these tissues.

Figure 23: Cross-section of a control *H. virescens* larva of the same age as six days post-parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (400X). Note sections through fat body (FB) (1–2 O’clock), muscle (M) (large area extending from 11 O’clock to the right lower corner), and trachea (T) (lower left corner). Note the relatively pale color of all tissues.

Figure 24: Cross-section of *H. virescens* larva six days post-parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (400X). Note sections through muscle (M) running from 12 to 6 O’clock surrounded by fat body (FB). Sections through the Malpighian tubules (MT) are visible at 4–5 O’clock. Note relatively intense staining of fat body and Malpighian tubules in contrast of the control (Figure 23). Muscle stained somewhat darker than controls but less intensely than other tissues. The darker staining is presumed to be from reactions of the TSP 14 antiserum to the presence of TSP in these tissues.

Figure 25: Cross-section of a control *H. virescens* larva of the same age as six days post-parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT magnified (400X). Note the section through a trachea (T) (running from 12 to 6 O’clock) with muscle tissue (M) parallel on both sides. Note relatively pale staining of all tissue.

Figure 26: Cross-section of *H. virescens* larva six days post-parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (400X). Note section through trachea (T) (center to photo to 2 O’clock) and muscle (M) (cross-sections running from 5 to 11 O’clock). Note intense staining of these tissues compared to the control (Figure 25), presumably from the reaction of the TSP14 antiserum to the presence of TSP in these muscles.

Figure 27: Cross-section of a control *H. virescens* testis (Te) of the same age as seven days post-parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (100X). Note the relatively unstained condition of this tissue.
Figure 28: Cross-section of a *H. virescens* testis (Te) seven days post–parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (100X). Note the much smaller size and the intense staining (compared to the control in Figure 27), presumably from the reaction of the TSP14 antiserum to the presence of TSP in this tissue.

Figure 29: Cross-section of a control *H. virescens* testis (Te) of the same age as seven days post–parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (400X). Note the relatively unstained condition of both the cell membranes and cytoplasm.

Figure 30: Cross-section of a *H. virescens* testis (Te) seven days post–parasitization of the experimental group probed with antibodies against TSP14 and developed with BCIP/MBT magnified 400X. Note the relatively intense staining of both the cell membranes and cytoplasm, presumably from the reaction of the TSP antiserum to the presence of TSP in this tissue.
## LIST OF FILES

<table>
<thead>
<tr>
<th>File Name</th>
<th>Format</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>FADTHES.pdf</td>
<td>Portable Document Format</td>
<td>2930 Kb</td>
</tr>
</tbody>
</table>
LITERATURE REVIEW

Host-Parasite System

*Heliothis virescens*

*Heliothis virescens*, whose larva is commonly called the tobacco budworm, is a moth in the Family Noctuidae. It can readily be found in the eastern and southwestern parts of the United States. As immatures, they develop through five instars. During this time they are significant pests on such crops as tobacco, alfalfa, cotton and clover where they bore into the buds and blossoms as well as feed on the leaves. Along with their relative *Helicoverpa zea*, the corn earworm, *H. virescens* is estimated to cause upwards of $1 billion annually in crop loss (Fitt, 1989).

The traditional method of controlling *H. virescens* in field crops is to treat the crops with foliar insecticides. This method, however, has actually proven more likely to cause increased *H. virescens* damage because of the lethal effects on beneficial organisms and *H. virescens’* ability to develop resistance to the insecticides (Jurat–Fuentes et al., 2002), especially with frequent exposure to pyrethroids (Kanga et al., 1995). As a result of these consequences with the use of insecticides for pest management, the agricultural community has tried alternative methods of crop protection. These include using the microbial insecticide *Bacillus thuringiensis* (Stone and Simms, 1993) and the nuclear polyhedrosis virus (Andrews et al., 1975) on field crops. Another alternative method of controlling *H. virescens* is to release larger than normal populations of their natural enemies – predators or parasitoids. One such natural enemy thought to have promise is the parasitoid *Microplitis croceipes*. The release of *M. croceipes* in the field can increase the parasitization rate by up to 76% (King and Coleman, 1989), leaving individuals unable to properly develop, mature, and reproduce. Nevertheless, the promise of *M. croceipes* does not stop there. In order to fully realize the potential of these organisms a closer examination of the host-parasite system should be considered with emphasis on the interactions taking place inside the *H. virescens* host.
Microplitis croceipes

*M. croceipes* is a member of the Order Hymenoptera and the Family Braconidae. *M. croceipes* is a solitary endoparasitoid of *H. virescens*. The female wasp oviposits between 30-600 eggs, with an average of 300 in her lifetime (Powell and Elzen, 1989). Only a single egg at a time is oviposited in the larval host’s hemocoel. The egg hatches approximately 40 hours following oviposition. The *M. croceipes* larva will pass through two complete instars while inside the host. The developing parasitoids do not feed directly on host tissues, but rather obtain nutrients from the host’s hemolymph. The mature second instar burrows through the host’s soft cuticle, ecdysing during the process, and then spinning a cocoon outside the host. Pupation occurs in the cocoon with 24 hours, unless the larva is in a diapause condition. After about nine days in the cocoon the adult *M. croceipes* will emerge. The adult wasp has a life expectancy of approximately ten days.

Once parasitized, *H. virescens* larvae feed significantly less than unparasitized larvae (Powell and Elzen, 1989). This reduced feeding leads to a hindered development in parasitized *H. virescens* larvae. The overall situation, however, is far more complicated. The parasitoid injects more than just an egg into its host during oviposition (Salt, 1968). Along with the egg, parasitoids also inject venom, calyx fluid, and polydnavirus (Vinson and Dahlman, 1989). It has been shown that polydnavirus and calyx fluid can alter host physiology (Stoltz, 1993), and the alteration can be enhanced by venom (Ferkovich and Dillard, 1990). Other factors that can alter host physiology are secretions from the larval parasitoid as well as secretions from cells associated with the hatching or developing parasitoid (Soldevila and Jones, 1993), such as teratocytes. The effects of these factors are similar and can often be redundant, leading some researchers to believe that certain effects are the result of cooperative interactions between these factors (Luckhart and Webb, 1996). The advances in *in vitro* teratocyte rearing (Greany, 1986; Zhang et al., 1994; Consoli et al., 2001), as well as further isolation of teratocyte secreted peptides (Okuda and Kadono-Akuda, 1995; Schepers et al., 1998), have allowed far more precise studies. These studies have made the physiological effects of teratocytes and their associated secreted proteins more evident (Dong et al., 1996; Schepers et al., 1998).
**Teratocytes**

Teratocytes have two roles in larval growth–trophic and immune suppressive–that facilitate successful parasitization of the host. During embryogenesis of *M. croceipes*, as well as other wasps in the Braconidae and Scelionidae families, the primary oocyte undergoes meiotic division, giving rise to cells called polar bodies (Tremblay and Caltagirone, 1973). These polar bodies are responsible for the development of an external membrane that surrounds the developing egg (Tremblay and Caltagirone, 1973). This extra-embryonic membrane is commonly called the serosa or serosal membrane (Dahlman and Vinson, 1993). Pennacchio et al. (1994a) reported that the serosal membrane originated from the anterior pole of the embryo and developed to join with cells in the posterior pole to form a complete membrane around the developing embryo. During eclosion, the serosal membrane is torn open and dissociated into individual cells. In *Cardiochiles nigriceps* the cells around the abdomen seem to lack definite boundaries and are quickly broken down upon dissociation, while the cells near the poles expand and disperse upon the larvae hatching (Pennacchio et al., 1994a). The cells that expand and disperse are termed teratocytes (Dahlman and Vinson, 1993).

Teratocytes increase in ploidy, however, they do not undergo cellular division (Strand and Wong, 1991). This means that the serosal membrane is the sole contributor of teratocytes for the parasitoid. There will never be more teratocytes than those initially dissociated from the serosal membrane. The number of teratocytes released from the serosa is species–specific. Vinson and Ivantsch (1980a) reported on a case of only eight teratocytes being released, where on the other end of the spectrum, Zhang et al. (1994) came to the conclusion that approximately 914 teratocytes were released per *M. croceipes* parasitoid larvae in vivo.

Physically, teratocytes have a diameter between 5 and 86 microns upon dissociation, and can experience a five to tenfold increase in diameter during their existence in the host (Vinson and Iwantsch, 1980a). Teratocyte size is dependent upon the species of both the parasitoid and the host (Alleyne et al., 2001). But their environment can also be manipulated to stimulate growth beyond the species–specific size. Teratocytes transferred into non–parasitized hosts have been reported to grow as large as 1 mm (Strand and Wong, 1991), as have teratocytes found in pupae of hosts injected with teratocytes as 5th instar larvae (Pennacchio et al, 1994b). Consoli et al. (2001) believe that it is the host-specific factors that are essential to teratocyte development.
and not parasite–derived factors. On the other hand, Schepers (1995) found that *M. croceipes* teratocytes reared *in vitro* had diameters on the order of one–half the size of those measured *in vivo* by Zhang (1989). The DNA contents of teratocytes increase until the teratocytes are 7 days old (Hotta et al., 2001). As teratocytes develop, they become covered with a thick dense blanket of microvilli (Dahlman, 1991). Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have revealed large extensively ramified nuclei, extensive reticula, swollen endoplasmic reticulum, sequestering mitochondria, organized Golgi bodies, mylen-like structures, putative autophagic vacuoles, and cellular outpocketings (Dahlman, 1991).

The presence of teratocytes is essential for parasitoid larvae survival (Barratt and Johnstone, 2001). Functionally, teratocytes can typically be characterized into three categories: trophic, immunosuppressive, or secretory (Vinson and Iwantsch, 1980b).

The trophic function of teratocytes is supported by the drastic increase in teratocyte volume (due to increased size), followed by a decrease in teratocyte number towards the latter stages of the parasitoids’ growth and development (Barratt and Sutherland, 2001). This function is further evidenced by the presence of either whole or parts of teratocytes in the digestive tracks of certain parasitoids (Sluss, 1968; Arakawa and Kitano, 1989; Strand and Wong, 1991). With the understanding that the teratocytes are a source of specific nutrients, it is hypothesized that the increased surface area due to the dense covering of microvilli functions to absorb nutrients from the host’s hemolymph (Dahlman and Vinson, 1993). Strand et al. (1986) showed that the teratocytes of the egg parasite, *Telenomus heliothidis*, secrete histolytic enzymes that digest the content of the egg host allowing for further consumption by the parasitoid.

The first to speculate on teratocytes’ immunosuppressive properties was Salt (1968). The basis of his argument was that if teratocytes had only a trophic function, all teratocytes should be consumed before the parasitoid larva exited the host. Since this often was not the case, those teratocytes that remained could be explained if their function was to protect the developing parasitoid. It has also been shown by Salt (1971), Vinson (1972) and Kitano (1974), that teratocytes helped prevent encapsulation of the parasitoid larva when they were introduced into the host’s hemocoel, even in the absence of the host’s venom or calyx fluid, thus removing the possibility that polydnavirus is solely responsible for the immunosuppressive response. This is
Teratocyte Secretory Products

The immunosuppressive properties of teratocytes can also be directly related to their proposed secretory function. *M. croceipes* teratocytes secrete proteins and possibly other substances. The secreted proteins are singularly and collectively referred to as TSP (teratocyte secreted proteins, or teratocyte secreted peptides). TSP has been shown to inhibit the synthesis of juvenile hormone esterase (JHE) and ecdysteroid (Zhang et al., 1992). It has also been shown that TSP inhibits the proper development of *H. virescens* host tissues as well as the production of arylphorins or storage proteins (Zhang et al., 1997). Christiansen–Weniger and Hardie (2000)...
propose that teratocytes from *Aphidius ervi* disrupt the endocrine system of *Acyrthosiphon pisum* due to the observed developmental arrest, especially in the formation of wings.

Vinson and Iwanstch (1980b) proved that teratocytes secreted proteins by showing that \(^{14}C\)-labeled amino acids were incorporated into teratocyte proteins as well as in new proteins existing in the medium. Later, Lawrence (1990) indicated that a novel protein appeared in the hemolymph of parasitized hosts after three days. She went on to describe the protein as being secreted by the serosal membrane of the parasitoid *Biosteres longicaudatus*. In addition, Dahlman (1991) reported that teratocytes secrete different proteins into the medium than are produced by the host.

Teratocyte secretory products as mentioned earlier, are primarily proteins secreted by the teratocytes during parasitism. Schepers et al. (1998) concluded that the teratocytes from one *M. croceipes* larva produce between 0.4 and 0.8 µg of protein per day for up to approximately five days before tapering off. Using a 5–20% gradient non-denaturing PAGE, he also identified 15 peptides between 3–400 kDa, the most abundant of which were 347, 308, 352, 252, 233, and 42 kDa. A 3–30 kDa TSP fraction separated with molecular weight cutoff filters contained peptides of 18, 16, 13, and 11 kDa. This fraction accounts for roughly 5% of the total amount of protein secreted by teratocytes, yet it appears to have the major effect on growth inhibition (Schepers, 1995). Falabella et al. (2000) obtained similar results by using teratocytes from the parasitoid *Aphidius ervi*. They found several secreted proteins, with two, 15 kDa and 45 kDa, being significantly more abundant.

Of these secreted proteins, it appears that one–14 kDa (Dahlman et al., 2002), is at least partially responsible for the teratocyte-mediated inhibition of host growth and development (Dahlman et al., 2003). *In vitro* production of this 14 kDa protein can be extended for up to twelve days *in vitro* using parasite larvae and media exchanges every third day (Hoy and Dahlman, 2002). A recombinant version of this protein can be produced using a yeast expression vector and has been shown to inhibit *in vitro* translation of *H. virescens* RNA (Rana et al., 2002). Antibiosis-type insect resistance has been demonstrated in transgenic tobacco plants expressing the teratocyte secretory protein (TSP14) gene (Maiti et al. 2003).
Eukaryotic Protein Synthesis

Eukaryotic protein synthesis, or the translation of the nucleotide sequence of an mRNA molecule into the corresponding polypeptide sequence, is an intricate process involving numerous factors. Merrick’s (1992) review equates explaining protein synthesis to describing football or baseball to someone who does not know the game. In that regard, the “players” will be introduced individually, and then how all the “players” together synthesize protein will be explained.

Eukaryotic Initiation Factor Type–1

Eukaryotic initiation factor type 1 was purified by Schreier et al. (1977). They showed eIF–1 to be a single polypeptide with a molecular weight of 15 kDa. Subsequent studies by Benne and Hershey (1978) and Voorma et al. (1979) demonstrated that eIF–1 has approximately a 20% effect on stimulating protein synthesis. They also showed that eIF–1 had this small effect on several steps of translation instead of just one. Because of its small size and its fairly minimal effects on protein synthesis (Lang et al., 2002) eLF-1 is one of the least studied of the known eukaryotic initiation factors.

Eukaryotic Initiation Factor Type–2

Eukaryotic initiation factor type 2 has been purified by numerous labs. It is widely accepted that it consists of three subunits of 52 kDa (γ), 38 kDa(β), and 35 kDa(α). The DNA sequence of three subunits has been identified (Pathak et al., 1988; Cigan et al., 1989). The primary responsibility of eIF–2 is to bind with the initiating Met–tRNA in a GTP–dependent manner to form a tertiary complex. This tertiary complex provides an aminoacyl–tRNA in the p–site of the ribosome and identifies the AUG “start” codon in the mRNA. Studies have shown that the β subunit is the site of the GTP binding (Dholakia et al., 1989; Anthony et al., 1990). Current evidence using chemical cross–linking suggests that both the β and the γ subunits are responsible for the connection of the initiating tRNA, with the β subunit seeming to have a much stronger role (Westerman et al., 1981). It has also been shown, however, that the γ subunit is the
only subunit capable of carrying out the essential function(s) of eIF–2 in the absence of the other two subunits (Erickson et al., 2002).

**Eukaryotic Initiation Factor Type–2B**

Guanine nucleotide exchange factor (GEF), also known as eIF–2B, has been well characterized by several labs (Panniers and Henshaw, 1983; Dholakia and Wahba, 1988). It contains five polypeptides with molecular weights of 82, 67, 58, 39 and 26 kDa. As its name indicates, it functions to facilitate the exchange of nucleotides. During initiation, eIF–2 and GTP form a complex. But eIF–2 has a 100-fold preference for GDP over GTP, its necessary substrate. Thus, for protein synthesis to proceed at a reasonable rate, GEF facilitates the removal of GDP from the eIF–2-GDP complex allowing reutilization of eIF–2.

**Eukaryotic Initiation Factor Type–3**

Eukaryotic initiation factor type–3 is the largest of all the known initiation factors. It has a molecular weight of between 600 and 650 kDa depending upon the species (Schreier et al., 1977; Seal et al., 1983a). It can be comprised of up to eleven non-identical subunits (Browning et al., 2001). Its main function is to facilitate the binding of the 5’ end of the mRNA to the 40S subunit of the ribosomes. Lutsch et al. (1985) used a hydrodynamic analysis along with electron microscopy to show that eIF–3 is shaped like a flat triangular prism approximately 7 nm thick and having sides approximately 17, 17 and 14 nm. Interferon can inhibit translation in a dose–dependent manner by binding to eIF–3, and can be reversed by the addition of purified eIF–3 (Guo et al., 2000).

**Eukaryotic Initiation Factor Type–4A**

Eukaryotic initiation factor type 4A is a single polypeptide chain with a molecular weight of about 45 kDa (Das et al., 1982; Grifo et al., 1983). Eukaryotic IF-4A functions as a single stranded RNA–dependent ATPase. Its major responsibility is to unwind the 5’ end of the mRNA being translated (Ray et al., 1985; Abramson et al., 1987). Several other proteins have been identified with homologous amino acid sequences to eIF–4A. Within this homologous amino acid sequence lies a D-E-A-D (aspartic acid–glutamic acid–alanine–aspartic acid) box motif.
Using biochemical characterization of eIF–4A and other D-E-A-D box proteins, the group are considered putative RNA helicases (Linder et al., 1989; Wasserman and Steitz, 1991).

**Eukaryotic Initiation Factor Type–4B**

Eukaryotic initiation factor type 4B has been characterized as a dimer with identical subunits of about 69 kDa each. The molecular mass was determined using the molecular mass of the cDNA clone (Milburn et al., 1990). When the cDNA was sequenced, two regions (AFLGNL and KGFGYAEF) were identified as indicators of RNA recognition motifs (Milburn et al., 1990). Eukaryotic IF–4B functions to assist in cross-linking of eIF–4A to mRNA (Abramson et al., 1987), and to stimulate the RNA–dependent ATPase and helicase activities of eIF–4A (Lawson et al.; 1989; Rozen et al., 1990).

**Eukaryotic Initiation Factor Type–4C**

Eukaryotic initiation factor type 4C is a low molecular weight peptide of approximately 17 kDa (Benne and Hershey, 1978; Dever et al., 1989). Though eIF–4C has been isolated in an active form as a monomer, it has also been isolated as a high molecular weight complex with eIF–5 (Schreier et al., 1977). A unique quality of eIF–4C is that it appears to be the only heat stable initiation factor. It has the ability to maintain 85% of its activity after being heated to 90°C for 5 minutes (Seal et al., 1983b). The main function of eIF–4C is to facilitate the binding of the 60 S ribosomal subunit.

**Eukaryotic Initiation Factor Type–4D**

Eukaryotic initiation factor type 4D is also a low molecular weight peptide slightly smaller than eIF–4C (~16 kDa). Its function is not completely understood, but it appears to be active late in the initiation process. (Kemper et al., 1976; Benne and Hershey, 1978)

**Eukaryotic Initiation Factor Type–4E**

Eukaryotic initiation factor type 4E is a peptide with a molecular weight of approximately 24 kDa. It has been cloned in humans and extensive amino acid sequencing has been done from the rabbit protein (Rychlik et al., 1987). Eukaryotic IF–4E is responsible for the recognition of the
m7G cap on the 5’ end of eukaryotic mRNA’s (Webb et al., 1984). It is for this reason that eIF–4E is often referred to as the cap binding protein, or CBP.

**Eukaryotic Initiation Factor Type–4F**

Eukaryotic initiation factor type 4F is a complex peptide. It is composed of subunits having molecular weights of 24, 45, and 220 kDa that Grifo et al. (1983b) determined in themselves are other eukaryotic initiation factor types. For example, 24 kDa peptide is the cap binding protein (eIF–4E) (Sonenberg et al., 1979). The 45 kDa subunit is eIF–4A (Conroy et al., 1990). The 220 kDa peptide is important because the proteolitic cleavage of this peptide renders eIF–4F inactive and subsequently can cause the inhibition of translation of mRNA’s. Members of the Picornaviridae family of viruses are known to inhibit protein synthesis by eIF–4F cleavage (Ventoso et al., 1998).

**Eukaryotic Initiation Factor Type–5**

Eukaryotic initiation factor type 5 is a single polypeptide with a molecular weight of about 125 kDa (Merrick et al., 1975). This peptide functions as a ribosome–dependent GTPase that facilitates the joining of the two ribosomal subunits (Benne and Hershey, 1978). It does this by interacting with the 40S initiation complex, causing hydrolysis of the ribosome–bound GTP. This hydrolysis causes the release of eIF–2 allowing the 60S ribosome subunit to bind and form the 80S ribosomal initiation complex (Ribera et al., 1997; Ribera and Puigdomenech, 1999).

**Eukaryotic Initiation Factor Type–6**

Eukaryotic initiation factor type 6 is also a single polypeptide chain. It has a molecular weight of about 25 kDa (Raychaudhuri et al., 1984). Since eIF-6 was initially purified and characterized, little more has been published about this initiation factor.

**Elongation Factor Type–1α**

Elongation factor type 1α is one of the most abundant cytoplasmic proteins, consisting of about three to ten percent of the soluble proteins in the cytoplasm (Merrick et al., 1990). EF–1α functions as a signal transducing GTP–binding protein (Bourne et al., 1991). It has also been
shown to have glutathione–S–transferase activity (Kobayashi et al., 2001). Most of what is known about EF–1α comes from the extensive studies on its prokaryotic equivalent EF–Tu (Jurnack, 1985; Kjeldgaard and Nyborg, 1992).

Elongation Factor Type –1βγ

Elongation factor type 1βγ is a complex of two polypeptide chains. The β subunit is about 48 kDa (Riis et al., 1990) and the γ subunit is about 35 kDa (Riis et al., 1990). EF–1βγ has been isolated in huge aggregates with molecular weights in excess of 2000 kDa (Carvalho et al., 1984; Lauer et al., 1984). EF–1βγ functions to facilitate nucleotide exchange (Ryazanov et al., 1991). EF–1βγ’s prokaryotic counterpart is EF–Ts, however, unlike EF–1α and its counterpart, EF–Tu, there is very little amino acid sequence homology between EF–1βγ and EF–Ts (Janssen et al., 1991; Sanders et al., 1991).

Elongation Factor Type –2

Elongation factor type 2 is a single polypeptide with a molecular weight of about 95 kDa (Rapp et al., 1989). EF–2 is equivalent to the prokaryotic protein EF–G and is responsible for the GTP–dependent translocation step of elongation (Rapp et al., 1989). One unique aspect of EF–2 is that a histidine residue is post–translationally modified to diphthamide (van Ness et al., 1978). This transformation is not required for normal cell function (Omura et al., 1988) but it is necessary for Diphtheria Toxin to deactivate EF-2 by ADP-rybosylation (van Ness et al., 1978).

Release Factor

Eukaryotes have only one release factor. Release factor (RF) is believed to contain approximately 475 amino acids (Lee et al., 1990). RF exists as a dimer (Konecki et al., 1977) and functions in a GTP-dependent manner (Tate and Caskey, 1974). RF releases the newly translated protein by hydrolyzing the aminoacyl linkage.

Now that the “players”, have been introduced along with an overview of their roles, it is time to see how they all must interact to translate a strand of mRNA into a protein.
Initiation

The designated starting point of protein synthesis is called initiation. In order to begin synthesizing protein, the cell must produce a substantial amount of free 40S ribosomal subunits needed to form initiation complexes. Though normal physiological conditions favor the formation of inactive 80S ribosomes, there are small amounts of free 40S and 60S ribosomal subunits. At this point, eIF-6 binds to the 60S ribosomal subunits (Raychaudhuri et al., 1984) and provides most of the activity to keep the ribosomal subunits separate (Russell and Spremulli, 1979). Trachsel et al. (1977) demonstrated that eIF–3 binds exclusively to the 40S ribosomal subunit. By so binding, eIF–3 functions to keep the 80S ribosome from forming (Peterson et al., 1979b). Immediately after the formation of the 40S ribosomal subunit ·eIF–3 complex, eIF–4C binds to the complex. The binding of eIF–4C to the complex happens in such short proximity to its formation, that it is speculated that it may play a role in or assist in the formation of the complex (Goumans et al., 1980).

Simultaneously, but separately, eIF–2, GTP, and Met–tRNA are binding together to form a ternary complex. This ternary complex must then bind to the 40S ribosomal subunit-eIF–3-eIF4C complex. This whole complex is referred to as the 43S complex. The ternary complex can bind to free 40S ribosomal subunits, however, the yield and stability of the overall complex is greatly reduced when eIF–3 and eIF–4C are not present (Peterson et al., 1979a). The two aforementioned complex formations happen in the absence of any mRNA. The next step, however, includes the binding of the protein template, mRNA.

For mRNA to attach correctly to the 43S complex, three initiation factors, as well as ATP, are required. This binding is started by eIF–4F, which recognizes the m7G cap that can be found on the 5’ ends of eukaryotic mRNA (Bannerjee, 1980). This single step is of extreme importance to translational regulation. The recognition of the 5’ cap by eIF–4F is due to eIF–4F’s 24 kDa subunit (Rhoads et al., 1985; Blum et al., 1989). It has been shown that the availability of the 5’ cap for interaction with the 24 kDa subunit correlates with the efficiency of translation of mRNA (Lawson et al., 1986).

Once eIF–4F has bound to the 5’ cap, eIF–4B immediately, if not simultaneously, binds with eIF–4F (Grifo et al., 1983b). Next, the mRNA “in front” of the 5’ cap begins to unwind (Lawson et al., 1988). It is believed that additional molecules of eIF–4A and eIF–4B are
required for the additional ATP–dependent unwinding of the mRNA further downstream (Jackson, 1991).

Now that the mRNA is unwound, the 43S complex containing the ternary complex (eIF–2, GTP, and Met–tRNA) and EIF–3 must bind to the mRNA · initiation factor complex. Once this takes place, the complex moves 5’ to 3’ down the mRNA until it locates the initiator AUG codon by matching up the anticodon with the initiator tRNA in the complex (Cigan et al., 1988). On occasion, the first AUG codon is passed by to allow access to another AUG sequence (Kozak, 1999). Then eIF–5 triggers the eIF–2 dependent hydrolysis of the bound GTP (Gosh et al., 1989). Once this hydrolysis takes place, the initiation factors are released from the surface of the 40S ribosomal subunit allowing the 60S ribosomal subunit to bind forming the full 80S ribosome (Merrick, 1979).

**Elongation**

Once the initiation of protein synthesis is complete, the initiator tRNA is bound to the P–site (peptidyl–tRNA site) of the 80S ribosome, and the process of elongation may begin. Elongation is a cyclical process where codons determine the addition of specific aminoacyl–tRNAs. This is accomplished by EF–1α directing the binding of the appropriate aminoacyl–tRNA and GTP to the anticodon. Once the appropriate aminoacyl–tRNA has been bound, GTP is hydrolysed. This causes the release of the EF–1α · GDP complex and the binding of the aminoacyl–tRNA to the A site (the site for binding incoming aminoacyl–tRNAs) of the 80S ribosome (Moazed and Noller, 1986).

While the first aminoacyl–tRNA enters in the A site, a new tRNA is being put in the recently vacated P site. The occupation of both the A site and the P site triggers the activation of the peptidyl transferase site in the 60S ribosomal subunit, thus forming the peptide bond. This happens through nucleophilic attack by the α–amino group of the aminoacyl–tRNA in the A site on the carbonyl of the ester linkage of the tRNA in the P site (now called peptidyl tRNA) (Herrera et al., 1991).

Next the 3’ end of the new peptidyl–tRNA is switched to the P site while the anticodon remains in the A site. The 3’ end of the deaclyated–tRNA is changed to the E site (exit site), while the anticodon does not move. This is how the polypeptide chain being synthesized is able
to grow, but does not move (Odom et al., 1990). In order for the new polypeptide to grow appropriately, EF–2 mediated translocation must occur. In this process, EF–2 causes the GTP dependent movement of the mRNA in three nucleotide intervals to get a new codon into the A site. All of the other sites also shift accordingly (unacylated–tRNA in the E site and peptidyl–tRNA in the P site).

**Termination**

As the ribosome moves down the mRNA it will eventually come across a stop codon (UAA, UGA, or UAG). At this point, RF recognizes the stop codon and the nucleotide immediately following (Brown et al., 1990). It binds to GTP and induces the hydrolysis of the aminoacyl linkage along with the GTP, thus causing the release of the newly synthesized peptide and the RF · GDP complex. This efficient termination of protein synthesis by RF allows for the recycling of mRNA and ribosomes for subsequent translation.

**Regulation**

The regulation of protein synthesis, whether it be stimulatory or inhibitory, is usually associated with the regulation of the translation factors. Since the majority of translation factors are initiation factors, the major site of regulation occurs during initiation.

Currently, the main mechanism of protein synthesis regulation is the phosphorylation or de–phosphorylation of certain initiation factors that can affect the formation of the ternary complex. It is currently believed that there are in excess of 40 phosphorylation sites involved with the regulation of translation. It is also thought that each site contributes slightly to this regulation, which allows for the ability to “finely tune” the regulation of translation with the use of specific kinases and phosphatases (Duncan and Hershey, 1987; Tuazon et al., 1989).

One target of regulation is eIF–2. It has a strong affinity for GDP and requires eIF–2B to function as an exchange factor in order to be removed from the GDP (Rowlands et al., 1988). Regulation takes place by the activation of kinases. Certain kinases, such as HCR and dsI (Chen et al., 1991) can phosphorylate the serine residues in the α–subunit of eIF–2. It is the phosphorylation of functional serine residues at 48 and 51 that cause this regulation (Kramer, 1990). If eIF–2 is phosphorylated, the eIF2 · GDP complex that is released during initiation will
bind with eIF–2B. This eIF–2 · GDP · eIF–2B complex is stable, however, and will not exchange the GDP for GTP. Since this complex is stable, the eIF–2B is not being recycled, leading to a depletion of usable eIF–2B, thus causing an accumulation of eIF–2 · GDP complexes (Kramer, 1990). This same effect can be achieved by the selective inactivation of eIF–2α phosphatase activity (Kimball et al., 1991).

Another site of regulation derives from the mRNA specific initiation factors, and more precisely, eIF–4B and eIF–4F. Both of these proteins are phosphorylated (Hershey, 1989). It has been shown that the phosphorylation of both of these initiation factors can lead to an increase in protein synthesis (Morley and Traugh, 1990; Morley and Traugh, 1991; Morley et al., 1991).

There are also some examples in the synthesis of new proteins, and more specifically, in the control of translation of specific mRNAs, that suggest that the translation of mRNA may be auto–regulated. One example is β–tubulin, which is a major precursor of microtubules. Cellular mechanisms regulate the equilibrium of α tubulin, β tubulin, and polymerized tubulins in microtubules. The cells have the ability to sense the concentration of free heterodimers and regulate the stability of the mRNA (Gay et al., 1989). The entity responsible for this ability is the first four amino acids translated (MREI) (Yen et al., 1988). This series seems to be unique to β–tubulin. It is thought that as the newly synthesized β–tubulin peptide is extended from the ribosome, it can sense the concentration of heterodimers via a mechanism of direct binding. If this binding occurs, it triggers the activation of a ribosomal nuclease, which subsequently degrades the mRNA being translated. But if the concentration of heterodimers is low, and direct binding does not occur, the mRNA remains intact and translation continues normally to synthesize new β–tubulin.

Another example of auto–regulation is the production of GCN4 protein in S. cerevisiae. The mRNA for GCN4 protein includes a 590 nucleotide region located upstream (5’) of the GCN4 region. This region contains four small ORF’s that are at about 230, 300, 415, and 440 nucleotides. As mentioned earlier, translation begins at the 5’ end in a cap–dependent manner. Then the ribosome begins scanning 5’ to 3’ until it gets to the first AUG start codon. When it comes to the start codon for ORF–1 it begins to translate the four or five amino acid peptide. Following termination of ORF–1, the 40S ribosomal subunit continues scanning 5’ to 3’ looking
for the next start codon. For the next start codon to be recognized, however, the ternary complex (eIF–2 · GTP · Met–tRNA) must be present. When amino acid levels are high in the cell, the ternary complex is bound quickly and a second round of translation begins at either ORF–3 or ORF–4. The consequence of the translation of these latter ORFs is that adequate recovery of the ternary complex in time for a third round of translation of the GCN4 region is not allowed, thus no GCN4 protein is synthesized. But if the cell is in a state of amino acid deficiency, after ORF–1 is translated, the ternary complex is re–acquired much less efficiently. When this happens the 40S ribosomal subunit ends up scanning right by ORF–3 and ORF–4 before the ternary complex is bound. This allows for the translation of the GCN4 region (Abastado et al., 1991). Though this scheme may not seem to make sense at first, it occurs because GCN4 protein is a transcription activator for amino acid biosynthetic enzymes.

Though these are two examples of mRNA specific regulation of protein synthesis, they are by no means the only examples. They may be isolated instances that are unique to their particular protein in their particular organism. The more likely scenario, however, (especially in the case of the β–tubulin, example) is that these mechanisms may be prototypical, or at least a skeletal backbone for other systems of regulation. Examples of such regulation systems are: GM–CSF (Shaw and Kamen, 1986); histones (Baumbach et al., 1987); transferrin (Müllner et al., 1989), and MAT α1 (Parker and Jacobson, 1990).

**Toxic Inhibition of Protein Synthesis**

Protein synthesis is a very complicated process that involves several factors allowing for precise regulation, but at the same time, makes the process susceptible to unintended inhibition. There are several compounds, with several different properties, that will inhibit protein synthesis differently.
**Diphtheria Toxin**

One compound that inhibits protein synthesis is diphtheria toxin. It is a single chain polypeptide of 62 kDa with a secondary structure containing two intra-chain disulfide bonds. Activation of the toxin can be achieved by mild proteolytic cleavage and reduction of the disulfide bonds. This activation results in diphtheria toxin splitting into two molecular fragments. The first fragment, called A, has a molecular weight of 22 kDa, is hydrophilic and corresponds to the amino terminal end of the diphtheria toxin peptide. The second segment, called B, has a molecular weight of 40 kDa, is hydrophobic, and corresponds to the carboxyl terminal of the diphtheria toxin peptide.

Fragment A is the catalytically active portion and can inhibit protein synthesis in a cell free system, like the rabbit reticulocyte lysate or wheat germ extract. Fragment A, however, is not toxic to animals or even cell cultures because it does not bind to or cross the cell membrane. Fragment B also has been shown to have absolutely no catalytic or toxic activity. Fragment B functions to facilitate the transport of fragment A across the cell membrane in order to enter the cytosol. Fragment B does this by recognizing and binding to a glycoprotein receptor on the cell surface. Being lipophilic (or hydrophobic), fragment B can then cross the cell membrane and bring fragment A along with it (Uchida, 1982).

Antibodies raised against fragment B can neutralize the toxic effects of diphtheria toxin by binding to fragment B and therefore disabling it’s ability to bind to the cell surface receptor. But antibodies raised against fragment A have no neutralizing effect against the toxic effects of diphtheria toxin in either animals or cultured cells. It is this data that supports the idea that fragment A is internally located with fragment B wrapped around it in the intact diphtheria toxin peptide.

Once in the cytosol, fragment A inhibits protein synthesis by acting on EF-2. If EF-2 is not bound to a ribosome, fragment A can ADP–ribosylate it. Fragment A catalyzes the reaction $\text{EF–2} + \text{NAD}^+ \rightarrow \text{ADP–ribose–EF–2} + \text{Nicotinamide} + \text{H}^+$. In this reaction, fragment A catalyzes the ADP–ribose moiety of NAD to bind with the modified amino acid, diphthamide, found in eEF–2. Though the ribosylated EF–2 can form a complex with the ribosomes and GTP; it cannot catalyze the hydrolysis of GTP, which in turn causes the cessation of translocation and therefore protein synthesis.
Ricin

Another well–known protein that inhibits protein synthesis, albeit in a completely different manner, is ricin. Since its isolation and characterization, many structurally and functionally related proteins have been identified from a variety of higher plants. All these proteins interact with eukaryotic ribosomes causing them to be unable to participate in protein synthesis. These proteins, collectively known as Type I ribosomes–inactivating proteins (I RIP), are for the most part, monomers of about 30kDa and have a tendency to be N–glycosylated (Lord et al., 1991). Even though they have the ability to shut down protein synthesis, type I RIP’s are not considered cytotoxic because they have no means of entering the cytosol in order to come in contact with the ribosome substrate.

In certain cases, however, the ribosome inactivating protein is covalently attached to a second peptide by a disulfide bond. This second peptide is always a galactose binding lectin, also with a molecular weight of about 30kDa. These heterodimers are called type II ribosome–inactivating proteins (II RIP). These toxins enter the cell by first binding to the cell surface galactosides, where they can then cross the cell membrane. Once in the cytosol, they can attach to the ribosome, shutting down protein synthesis, and ultimately causing the cell to die. Ricin is by far the most extensively studied type II RIP.

Ricin is a heterodimer consisting of a ribosome inactivating chain of 32kDa (A chain), and a galactose/N–acetyl galactosamine binding lectin with a molecular weight of 34kDa (B chain). The two chains are connected by a single disulfide bond (Montfort et al., 1987).

The A chain consists of eight alpha helices named A to H and eight beta sheets, named a–h. The 117 residues which comprise the amino terminal form a compact folding unit. Beta sheets b to f then form an extended mixed sheet and combine with alpha helices A and B to make up the “bottom” of the molecule. Helices C to G then pack in a group and sit on top of the extended mixed sheet structure. In this group lies the five turns in length through the center of the molecule. Alpha helix E is largely nonpolar, but contains two very important chemically active sites located near the carboxyl terminus and lying approximately one helical turn apart, facing outward to form an active site. The rest of the A chain folds up as a compact unit, but it is activated to alpha helix A. One side of the unit helps to form the active site cleft, while the other
half of the unit makes strong links with the B chain to form the ricin heterodimer (Katzin et al., 1991).

The B chain consists of two homologous domains. Domain 1 contains the amino terminus, while domain 2 contains the carboxyl terminus. Each of the two domains is comprised of four subdomains. One subdomain is a 17 amino acid linking peptide called lambda, while the other three subdomains are homologous peptides consisting of 40 amino acids called alpha, beta and gamma. These homologous subunits seem to come from multiplication and fusion of DNA encoding for a galactose–binding sites that are formed, only two are functional in ricin today (1 alpha and 2 gamma). This “modern” galactose–binding pocket is shallow and can interact with only about half of the galactose molecule. The bottom of the pocket is formed by a three amino acid kink in the polypeptide. The top consists of an aromatic side–chain with Trp37 from 1 alpha and Try248 from 2 gamma. These two residues contact the hydrophobic face of the galactose. Hydrogen bonds form between the sugar and these two residues and account for the epimeric specificity of binding (Rutenber and Robertus, 1991).

Ricin, as a type II ribosome–inactivating protein, is an N–glycosidase depurinating adenine within a specific RNA sequence. Determining the exact mechanism for this process has been a multidimensional process. Structurally, it is believed that ricin’s target in the 28S RNA is a hairpin loop containing the tetranucleotide loop, GAGA. The structure of these hairpin loops has been determined by nuclear magnetic resonance spectroscopy (Heus and Pardi, 1991). Also, the catalytic importance by X–ray analysis has been investigated using a specific technique, site–directed mutagenesis. It was shown that the conversion of Arg 180 to Gln(R180Q) reduces activity 2000x. The conversion of Glu 177 to Gln, however, reduces activity 200x. Further kinetic analysis on these two sites indicates that mutations cause changes in $k_{cat}$ while leaving the $k_m$ fairly unaffected. This indicates that Arg 180 and Glu 177 are not involved in substrate binding, but instead are important in stabilizing the transition state of the catalyzed reaction. When Tyr80 and 130 are converted to Phe, activity is decreased about 10x, where conversions to Ser decrease activity about 100x (Ready et al., 1991).

Using both the structural analysis and the kinetics studies, a mechanism has been proposed for the depurination of adenine by the A chain of ricin. In this mechanism, the substrate binds in the cleft and adenine is stuck between Tyr80 and 123 in a stacking interaction.
The leaving group adenine is protonated by Arg 180, thus facilitating the breaking of the C1’–N9 bond causing the formation of an oxycarbonium in the ribose. Glu 177 ion pairs with this transition state structure to stabilize it. A water molecule, lying on the opposite side of the sugar ring from adenine, is polarized by Arg 180 to a hydroxide. This reactive hydroxide will rapidly attack the carbonium in the ribose to complete the reaction.

Although the A chain is considered the enzymatically active portion of ricin, the B chain also has an important role to allow ricin to enter the cell. Ricin primarily enters the cell by endocytosis via coated pits after the B chain binds to surface receptors containing surface galactose residues. After the surface bound toxin is endocytized, the B chain also facilitates the entrance of the toxic A chain into the cytosol where it can find its substrate. The same galactose–binding function that allows the surface binding also facilitates entrance of the A chain into the cytosol. The reason why the galactose–binding function of the B chain is necessary intracellularly is not completely known. It has been shown that a portion of endocytized ricin is transported by the endosomes to the trans–Golgi network, suggesting that translocation of the A chain takes place in the trans–Golgi network or at some post–network compartment. Experiments have also shown that ricin, and other plant and bacterial toxins, must go through the Golgi before entering the cytosol. In eukaryotic cells, there is a retrograde vesicular membrane flow through the trans–Golgi network to the endoplasmic reticulum. This flow allows the retrieval of endoplasmic reticulum proteins that have drifted from their normal place. This retrieval uses a receptor that recycles between the endoplasmic reticulum and the Golgi that recognizes a tetrapeptide near the carboxyl terminus of the proteins. In mammals, this peptide is called KDEL (Sandvig et al., 1991). Certain bacterial toxins have a KDEL–like sequence allowing translocation through the trans–Golgi network and into the cytosol via the endoplasmic reticulum lumen. Ricin, however, does not have a KDEL–like sequence. It is believed that the ricin B chain uses its galactose–binding ability to bind to galactose present on a recycling glycoprotein. Attached to the glycoprotein, ricin can then go through the trans–Golgi network to the endoplasmic reticulum. They use the KDEL sequence on the glycoprotein to enter the cytosol via the endoplasmic reticulum in hepatocytes (Pelham et al., 1992).
HYPOTHESES

*H. virescens* larvae parasitized by *M. croceipes* express a variety of physiological and behavioral characteristics that ultimately result in the death of the host. The unusual thing is that the host’s tissues are not consumed by the parasitoid larva. Instead, the parasitoid derives its nutrients from the hemolymph of the host. Further, the extra-embryonic cells released from the parasitoid egg at the time of the hatch, termed teratocytes, make a significant, although not an exclusive, contribution to the observed changes in the host. It is also certain that at least one secretory product of the teratocytes (TSP14) causes significant changes in the growth and development of the host, particularly in the reduction of the synthesis of selected host proteins. Finally, it has been demonstrated recently that the expression of the TSP14 gene in transgenic tobacco plants results in significant death and repressed development of *H. virescens* larvae reared on these plants, when compared to transgenic tobacco plants containing a control gene (Maiti et al., 2003). Therefore, it is useful to learn more about the mechanisms and characteristics of TSP14 that might be involved in the observed reduction of protein synthesis in both *in vivo* and *in vitro* systems. The work described in the following sections is an attempt to do just this.

Initial studies tested the feasibility of using *H. virescens*’ testes as a protein-synthesizing organ in *in vitro* tissue assays. Prior to this point, Zhang et al. (1997) showed that crude TSP inhibited production of storage proteins, and Miller et al. (1990) concluded that *H. virescens* testes synthesize and transport storage proteins. Accordingly, it was hypothesized that crude TSP would inhibit protein synthesis when using testes in the *in vitro* tissue assay. It was further hypothesized that since there was no physiological difference between the two testes in an individual, there would be no difference in how crude TSP affected the two testes of a single individual. It was also thought that crude TSP would exhibit a dose response and be affected by exposure time much like other inhibitory proteins (Lord et al., 1994).

A review of toxic proteins revealed that the process of translation was a popular target for inhibition (Uchida, 1982; Lord et al., 1994; Sandvig et al., 2002). Thus, it was hypothesized that crude TSP would have no effect on nucleic acid synthesis (replication or transcription). Also,
due to the known modes of action of other toxic proteins such as Diphtheria toxin (Uchida, 1982), ricin (L<cit>ord et al., 1994) and Shiga toxin (Sandvig et al, 2002), it was hypothesized that crude TSP would inhibit protein synthesis in the cell free systems (rabbit reticulocyte lysate and wheat germ extract assays). Also, this inhibition should be identical for all mRNA, independent of the tissue or the insect species, since there are no cell surface receptors or extracellular substances, such as proteases, to interfere with the crude TSP. The caveat raised by Rana et al. (2002), however, still exists. This is that the translation of mRNAs from different sources and their inhibition could be affected by the nature of the 5’ or 3’ untranslated regions, or the nature of the initiation codon and its nucleotide environment.

With respect to the localization of TSP, since the above-mentioned protein toxins all use some sort of cell surface receptor, be it a glycoprotein receptor or a galactose-binding pocket, it was hypothesized that TSP would employ a similar method. This would lead to TSP being bound to the external surface of the cell membrane, as well as being in the cytosol where it would inhibit protein synthesis.

Schepers (1995) alluded to the possibility of a small (< 3kDa) protein that seemed to have some biological activity. Since the 3 – 30 kDa fraction of TSP seemed to have nearly the same biological activity as crude TSP and the > 3kDa fraction of TSP showed little biological activity, it was hypothesized that the addition of the cofactor would have little effect on the overall biological activity of TSP.

Expressing a recombinant protein in a baculovirus system using insect cell lines is a fairly common procedure (Summers and Smith, 1988). Also, recombinant proteins have been expressed in Bombyx mori (Choudary et al., 1995) via injection as well as in M. sexta via ingestion (Cha et al., 1997). It was hypothesized that recombinant TSP14 could be produced using a baculovirus system.

Since the cDNA-His tag fusion that was inserted into the baculovirus to make the TSP14 was designed from the 14 kDa protein Schepers (1995) found to be biologically active, it seemed reasonable that the recombinant TSP14 should have the same biological properties as the natural protein. Thus, it was hypothesized that recombinant TSP14 would inhibit protein synthesis in the same dose dependent manner as the natural TSP.
MATERIALS AND METHODS

General

Maintenance and Care of Heliothis virescens

*H. virescens* larvae were reared in a laboratory colony in the insect physiology laboratory at the University of Kentucky. This colony originated from a colony at Texas A & M University in 1976. First through pharate fourth instar larvae were reared on approximately 25 ml of “Heliothis premix” artificial diet from Arthro Feeds (Stonefly Industries, Bryan, Texas) in Solo 5.5 oz. cups and held at 27±2°C in long–day conditions (16L: 8D). Individual larvae were transferred as pharate fourth instars into 5/8 oz. plastic cups containing approximately 5 ml of Vanderzant diet (Vanderzant et al., 1962). The cups were then kept in an incubator at 27°C and in long–day conditions. Various, yet specific developmental stages were used for experimentation, thus making it important to initiate each experiment with homologous developmental stages. The larvae were separated according to specific morphological features described by Webb and Dahlman (1985).

Upon pupation, pupae were collected, washed with a 0.03% NaClO solution, rinsed with water and allowed to air dry on the bench top. Between 30-40 pupae were placed in a one gallon glass jar with a sugar water (10% sucrose)/auromycin (0.07%) (chlorotetracycline hydrochloride) (American Cyanamid Company, Agricultural Division, Wayne, NJ) wick and the jar was topped with a laboratory paper towel held in place over the jar with rubber bands. Auromycin was added for systemic bacterial control on the wick. The jars were kept in an incubator at 27°C and in long–day conditions. When the adult moths emerged, most would lay eggs on the paper towel lid. The eggs were collected daily by replacing the paper towel lids with new ones. The eggs attached to the paper towel were washed for 30 sec. with a 0.03% NaClO solution and rinsed twice with water. The paper towels were then set flat on the bench top to air dry. Once dry, the paper towels were cut into pieces and placed into a solo 5.5 oz. cup with approximately 25 ml of “Heliothis premix” artificial diet.
Maintenance and Care of *Microplitis croceipes*

*M. croceipes* were reared in the insect physiology laboratory at the University of Kentucky. Our laboratory colony was maintained by parasitization of pharate fourth instar *H. virescens* by *M. croceipes* at a ratio of 6:1 in a 19 x 1.5 cm Petri dish for one hour. Pharate fourth instar larvae were used because *M. croceipes* prefer to parasitize this stage and the parasitoid larvae develop faster (Powell, 1988). Also, with the use of a pharate larva, due to the head capsule slippage, the *H. virescens* cannot defend themselves with their mandibles and damage the *M. croceipes*. Parasitized *H. virescens* were held in individual polystyrene 5/8 oz. cups containing approximately 5 ml of Vanderzant diet and kept at 27°C and long–day conditions. The parasites emerged and spun cocoons eight to ten days after oviposition. Cocoons were removed from the cups, washed with a 0.7% bleach solution, and rinsed with water. The cocoons were allowed to air dry on a laboratory paper towel and then placed in a 10 x 2 cm Petri dish in an incubator at 27°C and long–day conditions.

Adult *M. croceipes* were monitored for emergence each day. Upon emergence, they were placed in a 27.5 x 20 x 11 cm plastic box at ratio of 1:1, male to female. Antennae length was used as the distinguishing morphologic characteristic because male antennae are visibly longer than female antennae. A new box was set up each day with separate supplies of water and a 1:3 honey: water solution. The box had eight, half–inch holes, two on each side, to allow access for an aspirator to place and remove the parasitoids from the box. The holes were plugged with rubber stoppers. The plastic boxes were kept in an incubator at 27°C and long–day conditions. Parasitoids were mainly used for parasitization between 24 and 96 hours after emergence. This reduced the possibility of host illness and mortality due to infection by microbes from a contaminated wasp ovipositor. Between uses, the boxes were soaked in a 0.7% bleach solution for at least two hours for decontamination, rinsed with water and allowed to air dry.
Maintenance and Care of _Manduca sexta_

_M. sexta_ were reared in the insect physiology laboratory at the University of Kentucky. Adult _M. sexta_ moths were kept in the greenhouse in an approximately three cubic foot cage with a single tobacco plant approximately one ft. tall and a 10% sucrose solution food source. The female moths oviposited on the tobacco plant. The tobacco plant was taken out of the cage daily and the eggs were removed and placed into a 10 x 2 cm Petri dish. It was important to remove all the eggs daily because doing so allowed for the relative homology of developmental stages with time. In addition, if eggs hatched on the plant the emerged larvae would feed on the plant. The eggs were then kept in the laboratory at room temperature and long–day conditions.

Upon hatching, an entire day’s eggs were placed on folded 11 cm circle of filter paper, which in turn was placed on a wire mesh rack. The rack itself was inserted in a 27.5 x 20 x 11 cm plastic box with a plastic lid. The boxes were kept at room temperature and long–day conditions. The larvae were fed slabs of artificial diet (Bell and Joachim, 1976), with the amount depending on the size and number of larvae. The boxes were cleaned and new paper towels and diet added daily. When the larvae reached the wandering stage, they were removed from the box and placed in individual 10 x 2 cm Petri dishes with several pieces of paper towel approximately 6.5 cm\(^2\). Petri dishes containing each day’s wandering larvae were stacked and taped together. Approximately two weeks following pupation the pupae were removed from the Petri dishes and placed into small paper bags, six pupae per bag. The bags were stored on a shelf at room temperature until the moths emerged. The moths were then removed to the cage in the greenhouse.

Production of Teratocytes

Teratocytes were obtained from an _in vitro_ culture of _M. croceipes_ eggs dissected from superparasitized _H. virescens_. Pharate fourth instar _H. virescens_ larvae were removed from the colony for superparasitization. Ten _H. virescens_ larvae were placed in a 19 x 1.5 cm plastic Petri dish. Ten female _M. croceipes_ were added to the Petri dish to create a 1:1 ratio of hosts to wasps. The Petri dish was put in a lighted incubator at 27ºC for two hours. Superparasitized _H. virescens_ larvae were removed and placed into individual 5/8 oz. plastic cups containing approximately 5 ml of Vanderzant diet. The cups were held in an incubator at 27ºC for 28 hours.
**M. croceipes** eggs were obtained by sterile dissection of the superparasitized *H. virescens* larvae 28 hours post parasitization in Ex–cell 400 medium (JRH Biosciences, Lexana, KS). All dissecting instruments, water, and pipette tips were sterilized by autoclaving at 121°C for 20 min. The horizontal laminar flow hood was sterilized with a germicidal ultraviolet light, and surface sterilized with 70% ethanol. The Ex-cell medium was stored in 50 ml sterile bottles capped with sterile rubber septums at 4°C. The medium was removed with a sterile disposable 5cc syringe and needle and placed a sterile 6.0 x 1.5 cm plastic Petri dish. Gentamicin sulfate from Sigma was added from a gentamicin base stock of 20 mg/ml in sterile deionized water to the medium for a final concentration of 20 µg/ml.

Superparasitized *H. virescens* larvae were surface sterilized for approximately five seconds by emersion in 95% ethanol, for five minutes in 70% ethanol and then placed in sterile water. One larva was dissected per well in a nine well depression plate. Each well contained 100 µl of Ex-cell 400 medium. A larva was placed in the media. Using two fine forceps, with their points touching each other, the integument was gently pinched at a location near the middle of the larvae. The forceps were then slowly and gently pulled apart causing a small tear in the integument. Next the forceps were released and moved to the anterior and posterior of the larva. The integument was then gently pulled apart about half the length of the larva, exposing, but taking care not to rupture, the midgut. The exposed midgut was dipped into the medium and the integument massaged with forceps from the ends towards the torn middle, forcing the *M. croceipes* eggs out into the medium (Scheppers et al, 1998).

Eggs from individual *H. virescens* larvae were drawn from the well with a micropipette and washed seven times with transfers between fresh 100 µl drops of sterile Ex-cell 400 medium. Three 100 µl drops of Ex-cell 400 medium were placed in a Falcon 5.0 x 0.9 cm seal-tite Petri dish. Then ten washed eggs were placed in each drop and the Petri dish was closed and placed in a Billups–Rothenburg modular incubator chamber in an incubator at 27°C and long-day conditions. *H. virescens* were dissected in groups of three or four. The size of the group depended on the number of eggs harvested from each individual so that there was no melanization of the trace haemolymph. For each drop containing eggs, there was an empty drop of Ex-cell 400 medium for use as a control in experiments and to provide additional fluid for humidity control.
Production and Collection of TSP

*M. croceipes* eggs all hatched approximately 14 hours after dissection, and teratocytes were dissociated from the chorion as a result of the movement of the hatching larva. The teratocytes, designated as 0 hours old at this time, began to increase in size and secrete proteinaceous material. When the teratocytes were three days old, the teratocytes, parasitoid larvae, and medium were placed into 1.5 ml micro-centrifuge tubes. The larvae settled to the bottom of the tube via gravity, and the medium and teratocytes were removed with a pipette, and put in a new micro-centrifuge tube. The tubes containing the teratocytes and medium were centrifuged at 800 g for four minutes to gently pellet the teratocytes. The supernatant containing the proteinaceous secretions of the teratocytes (TSP) was put in a separate micro-centrifuge tube and labeled with the number of larvae that had hatched, volume of the medium, age of TSP and current date. The TSP and teratocytes were either immediately put on ice, if they were going to be used within 24 hours; otherwise they were stored at -70°.

Effects and Properties of TSP on *H. virescens* Testes

General Procedures for Protein Inhibition Tissue Assay

The appropriate tissue was removed, washed, and weighed by a Cahn electro-balance if necessary. Calculations were performed to determine the appropriate amount of TSP or TSP14, and that amount was added to individual 500µl micro-centrifuge tubes labeled “experimental.” An equal volume of Ex–cell medium or imidizole buffer was used in the tubes labeled “control.” The dose of TSP obtained from cell cultures was expressed in terms of larval equivalents (LE), which is defined as the amount of TSP released by the number teratocytes derived from one *M. croceipes* larva. The tubes were brought to a volume of 45 µl with Graces medium without methionine. The tissues were placed in the tubes containing media. Five µl [35S]-methionine (≈4.5 µCi) were added to each tube (bringing the total volume to 50 µl). The tubes were incubated for an appropriate amount of time (usually four hours) at 27°C on an orbital shaker at 150 rpm.

After the incubation, 200 µl 28% trichloroacetic acid (TCA) was added to each tube to stop the reaction by precipitating the protein. The tissues were washed twice, for ten minutes each time, in 250 µl 20% TCA. Then the tissues were washed two times in 250 µl 10% TCA;
two times in 500 µl 100% ethanol; one time in 500 µl 50% ethanol: 50% diethyl ether, and one
time in 500 µl 100% diethyl ether. The samples were air–dried.

When the samples were dry 250 µl 1% lauryl sulfate (SDS) in 0.1 N NaOH was added to
dissolve the protein. Fifty µl of the dissolved samples were added to 3 ml scintillation cocktail
and radioactive emissions were quantitated by scintillation spectroscopy. Counts were converted
to dpm/µg of tissue when appropriate, and the effects of TSP were expressed as the percent
decrease from the control.

**Inhibition of Protein Synthesis**

The testes were weighed using a Cahn electrobalance, and the weights recorded. The
testes were then put individually into 500 µl micro-centrifuge tubes with five µl [³⁵S]-methionine
(~4.5µCi), and three larval equivalents of TSP in experimental tubes. An equal volume of Ex-
Cell® 400 medium was put in control tubes. Next tubes were brought to a total volume of 50 µl
with Ex-Cell® 401 medium without methionine. The testes were then incubated at 27°C on the
orbital shaker at 150 rpm for four hours, followed by the above described extraction procedures.

**Left vs. Right Testes**

These experiments utilized the same methods as above. The testes were designated left or right
with respect to the dissector with the insects’ head capsule being the furthest away from the
dissector and the ventral side up.

**Incubation Times**

The same procedures as above were used. Instead of incubating for four hours, however,
the testes were incubated on the orbital shaker for either, 0.5, one, two, four, six, or eight hours.
Also, due to the results from the previous studies, the testes were not weighed.

**Dose Response**

The same procedure as above was used except the dose of TSP was 0, 0.1, 0.2, 0.3, 0.4,
0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, or 4.0 larval equivalents. In the assays where a one–hour
incubation time was used we held the individual testes in a well of a round-bottomed 96-well
plate. Then the appropriate amount of TSP (3 – 30 kDa fraction) was added (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8, 3.0, 3.2, 3.4, 3.6, 3.8, or 4.0 LE) followed by the 5µl \[^{35}\text{S}^{-}\text{methionine}\] (~4.5µCi), beginning the incubation time.

**Nucleic Acids**

Once again the same procedure as above were followed except that Ex–Cell® 400 medium was used instead of Ex–Cell® 401 medium without methionine. \[^{3}\text{H}^{-}\text{Uridine}\] was used for RNA analysis, and \[^{3}\text{H}^{-}\text{Thymidine}\] was used for DNA analysis.

**Insect Injections**

Premolt fourth instar *H. virescens* of roughly the same size were removed from the colony and isolated without food for approximately 12 hours. This gave the larvae time to develop into new fifth instars as well as to purge some gut contents. The larvae were weighed and the weights recorded. The TSP was concentrated using dialysis tubing or microcon 3 kDa cutoff filters (Amicon, Beverly, MA). The TSP was then aliquoted to the appropriate dosage and brought up to a volume of 10 µl per injection. A sterile disposable insulin syringe was loaded with enough TSP to inject all the replicates of a particular dose and loaded into an automatic microinjector. The larvae were individually placed in a carbon dioxide chamber for approximately 30 seconds to make them flaccid. The needle was inserted longitudinally into the posterior of the second to last proleg, and the injection made. The wound was sealed with a small drop of bee’s wax. The larvae were placed in individual 5/8 oz. plastic cups with approximately 2 ml of Vanderzant diet. The larvae were monitored and weighed every 24 hours until death or pupation.

**Statistics**

The percent reduction in protein RNA and DNA synthesis in response to TSP exposure was obtained by subtracting the experimental dmp’s from the control dpm’s, then dividing by the control dpm’s and multiplying by one hundred. Student’s T-tests were used to compare experimental and controls values.
Effects of TSP on Translation of mRNA in Cell Free Systems

Dissection to Collect Tissues

Second day digging stage fifth instar (D2) *H. virescens* were visually sexed by locating the testes, which look like two kidney–shaped yellow shadows on the dorsal side of the 5th abdominal segment. The males (individuals with testes) were removed from the laboratory colony for dissection. First they were surface sterilized for approximately five seconds in 95% ethanol, next for approximately three minutes in 70%, and then they were kept in sterile water. Hemolymph was collected by folding the larva in half so the prolegs were exposed, then a proleg was snipped off and hemolymph collected in labeled capillary tubes and kept on ice. Following this step, the larva was pinned to a paraffin dissection dish ventral side up. A longitudinal incision was made along the right leg–line, and the integument pinned back to the dish. The midgut, located down the middle of the larva, was carefully snipped anteriorly by the foregut and posteriorly by the hindgut. The midgut was removed, the contents were squeezed out, and the tissue was washed gently in sterile saline. The removal of the midgut revealed a pair of yellow kidney–shaped testes in the 5th abdominal segment of the larva. They were teased free of the tracheal connections and transferred to a drop of saline where tubules and fat body were removed. The fat body, found throughout the hemocoel, was picked out, and quickly put in a drop of saline to remove hemolymph. The ventral nerve cord, located longitudinally between the leg-lines, was teased out around the ganglia and removed. Labial glands are translucent glandular tubes that run from the head, longitudinally down the sides of the larva. They were most easily removed by grasping them with forceps and gently pulling them out. After collection, all tissues were washed briefly in sterile saline and stored on ice.

Isolation of Total RNA

RNA isolation methods are adaptations of Chomczynski and Sacchi (1989). All aqueous solutions were treated with 0.05% diethyl pyrocarbonate (DEPC) overnight at room temperature and autoclaved for 30 minutes to remove any DEPC in the solution. All non–disposable glass and plastics were rinsed with 0.1 N NaOH, 1mM EDTA and then thoroughly rinsed with RNase–free (DEPC–treated) water. All glassware was then baked at 200°C overnight. Special care was taken to use sterile technique in order to prevent RNase contamination of the samples.
Approximately 250 mg of tissue was placed in a 1.5 ml micro-centrifuge tube along with 300 µl ice cold 4M guanidine isothiocyanate, 20 mM sodium acetate, 0.5% sarkosyl, 0.1 mM dithiothreitol and homogenized for 15–30 seconds. Then 300 µl of 49:49:2 water saturated phenol: chloroform: isoamyl alcohol was added to the mixture. The tubes were then vortexed for 15 seconds and chilled on ice for 15 minutes. The tubes were then centrifuged at 10,000 X g for 20 minutes at 4°C. The top aqueous phase containing the RNA was removed using special care not to disrupt the interface, and placed in a new DEPC–treated micro–centrifuge tube. The organic phase was saved until all the RNA was isolated and analyzed. An equal volume (usually about 400 µl) of isopropanol was added to the aqueous phase. The tubes were briefly vortexed and incubated at –20°C overnight. The RNA was centrifuged at 10,000 X g for 15 minutes at 4°C causing a pellet to form at the bottom of the tube. The isopropanol was decanted, and the RNA pellet was resuspended using 150 µl 4M guanidine isothiocyanate, 20 mM sodium acetate, 0.5% sarkosyl, and 0.1 mM dithiothreitol. Another 200 µl isopropanol was added to the RNA and incubated for 2 hours at –20°C. The RNA was again centrifuged at 10,000 X g for 15 minutes at 4°C. The isopropanol was decanted and the pellet washed with 500 µl 75% ethanol. The RNA was again centrifuged at 10,000 X g for 15 minutes at 4°C. The ethanol was decanted and the pellet dried in a vacuum desiccator for about 15 minutes. The RNA pellet was then resuspended in 50 µl of RNase–free water. RNA quantity was determined by ultraviolet absorption spectrophotometry at 260 nm with the concentration of RNA in µg/µl being ten times the percent absorbance. RNA purity was determined by ultraviolet absorption spectrophotometry at 260 nm and 280 nm. The ratio of Ab\text{260}/Ab\text{280} should be around 2 if the sample is pure nucleic acid. Most RNA was used immediately after isolation, but some was stored at –20°C for later use.

Isolation of Messenger RNA

Messenger RNA (mRNA) was isolated using the PolyATtract® mRNA Isolation System (Promega Corporation, Madison, WI). Sterile, RNase–free tubes containing 100 – 1,000 µg total RNA suspended in 500 µl RNase–free water placed in a heating block at 65°C for 20 minutes. Then 13 µl of 20X SSC and 3 µl biotinylated–oligo(dT) probe were added, and the mixture allowed to cool for 10 minutes. While the mixture was cooling, the streptavidin–paramagnetic
particles (SA–PMPs) were washed. This was done by placing the tube containing the SA–PMPs in the magnetic stand until the SA–PMPs collected at the side of the tube (about 10 seconds), and decanting the supernatant. The SA–PMPs were then washed three times with 300 µl 0.5X SSC, each time, collecting the SA–PMPs with the magnetic stand and decanting the supernatant. After the SA–PMPs were washed, they were re–suspended in 100 µl 0.5X SSC. The cooled RNA mixture was then added to the tube containing the SA–PMPs and incubated at room temperature for 10 minutes. The SA–PMPs were then captured using the magnetic stand and the supernatant decanted and saved in a separate tube. The SA–PMPs were then washed four times with 300 µl 0.1 X SSC, each time, collecting the SA-PMPs with the magnetic stand and decanting the supernatant. The mRNA was then resuspended by adding 100 µl RNase-free water to the SA-PMPs and gently flicking the tube. The SA-PMPs were then captured by the magnetic stand, and the aqueous mRNA was transferred to a new sterile RNase–free tube. The elution was repeated using 150 µl RNase–free water and the supernatants pooled for a total volume of 250 µl. Quantity and quality of the mRNA was determined by ultraviolet absorption spectrophotometry identical to the total RNA procedure.

Agarose–Formaldehyde Electrophoresis for RNA

The 1% agarose 2.2 M formaldehyde gel was cast by dissolving 1 g agarose in 72 ml of water. It was then cooled to 60°C in a water bath. When the agarose solution cooled to 60°C, 10 ml of 10x 0.2 M MOPS buffer and 18 ml of 12.3 M formaldehyde were added. The gel was placed in a tank with 1x MOPS running buffer. Equal amounts of RNA were allocated out for each sample (usually 10 µg, but could vary depending on availability and quality of sample), and the samples were brought to 11 µl with ribonuclease–free water. Each sample was prepared for electrophoresis by adding 5 µl of 10x 0.2 M MOPS running buffer, 9 µl 12.3M formaldehyde and 25 µl of formamide. The samples were mixed and incubated at 55°C for 15 minutes. Ten µl of formaldehyde loading buffer and 2 µl of ethidium bromide were added to each sample. The samples were loaded onto the gel by pipetting 2.5 µg (approximately ¼ of the sample) of RNA into each well, and the gel was run at 50 V until the bromophenol blue dye front migrated half to two thirds of the way down the gel.
Rabbit Reticulocyte Lysate Assay

Rabbit reticulocyte lysate protein synthesis assay, first described by Pelham and Jackson (1976) has since been extensively used to study mechanisms of protein synthesis inhibitors such as phosphorylation by protein kinase (Farrell et al., 1977) and activation of specific endonucleases (Clemens and Williams, 1978). Thus, rabbit reticulocyte lysates are used in the current study to remove the cellular components of the previous experiments, allowing TSP direct access to the translational "machinery". In this study, we utilized a nuclease treated rabbit reticulocyte lysate system (Promega Corporation, Madison, WI). The standard protocol was used, and modified to accommodate the individual needs of the experiments.

Messenger RNA (mRNA) from various sources dissolved in water (luciferase RNA was used as a control) were heated at 67°C for 10 minutes and immediately cooled on ice in order to increase efficiency of translation by destroying localized regions of secondary structures. Two µg of mRNA, measured by absorbance spectroscopy, were added to 35 µl rabbit reticulocyte lysate (Promega Corporation, Madison, WI) along with one µl 1mM amino acid mixture minus methionine. Two µl [\textsuperscript{35}S]-methionine (1,200 Ci/mmol at 10 mCi/ml), 1 µl Rnasin ribonuclease inhibitor (Promega Corporation, Madison, WI) and the appropriate amount of TSP or TSP14 (usually three LE of TSP or 0.8 µg TSP14), brought to five µl with Ex-cell medium or imidizole buffer respectively (five µl Ex-cell medium or imidizole buffer were used in controls) were added next. The reactions were brought to a total of 50 µl with ribonuclease-free water. The 500 µl microfuge reaction vessels were placed in a 30°C water bath for one hour. The tubes were removed from the water bath and 50 µg RNase was added to each tube to digest aminoacyl tRNA’s. Two µl samples were removed from each reaction vessel and added to 98 µl 1 M NaOH/2% H\textsubscript{2}O\textsubscript{2}. The 100 µl sample was placed on a 22 mm Ø Whatman® GF/C glass microfiber filter (Cat. No.1822 021). The filter papers were identified by cutting small notches around the edges. Filter papers were washed in 10% TCA for ten minutes and then in 5% TCA for ten minutes. Next, they were rinsed in 95% ethanol for five minutes followed by being rinsed briefly in 100% ethanol. After these processes, they were allowed to air dry. Each piece of filter paper was added to three ml scintillation cocktail. Methionine incorporation into newly synthesized proteins was measured by scintillation spectroscopy.
Wheat Germ Extract Assay

The wheat germ extract assay was similar to the rabbit reticulocyte lysate assay, as far as it was a cell free *in vitro* translation assay. The wheat germ assay has been used extensively to determine the functional activity of mRNA’s (Krieg and Melton, 1984). Its purpose in this study was as an alternative to the rabbit reticulocyte lysate assay to determine whether TSP acted differently in cell free *in vitro* translation systems of plant and animal origin. As with the rabbit reticulocyte lysates, we used a nuclease treated wheat germ extract kit (Promega Corporation, Madison, WI). The standard protocol was modified to accommodate the needs of the experiments.

Messenger RNA (mRNA) from the various sources dissolved in water (Brome Mosaic Virus was used as a control) were heated at 67°C for ten minutes and immediately cooled on ice. Two µg of mRNA were added to a 500 µl micro–centrifuge tube along with 25 µl wheat germ extract (Promega, Madison, WI) and four µl 1mM amino acid mixture minus methionine, 2.5 µl \[^{35}\text{S}]\)-methionine (1,200 Ci/mm at 10 mCi/ml), 3 µl 1M potassium acetate, 1 µl Rnasin ribonuclease inhibitor (Promega Corporation, Madison, WI) and the appropriate amount of TSP or TSP14. This amount usually was 3 LE of TSP or 0.8 µg TSP14), brought to 5 µl with Ex–cell medium or imidizole buffer respectively (5 µl Ex–cell medium or imidizole buffer were used in controls). The total volume was adjusted to 50 µl using ribonuclease–free water. The reaction tubes were put in a 25°C water bath for one hour, after which time they were removed from the water bath and placed on ice to stop the reaction. Two µl samples were removed from each vial and added to 98 µl 1 M NaOH/ 2% H₂O₂. The sample was placed on a 22 mm Ø Whatman® GF/C glass microfiber filter. The filter papers were identified by cutting small notches around the edges. Filter papers were washed in 10% TCA for ten minutes and then in 5% TCA for ten minutes. First, they were rinsed in 95% ethanol for five minutes, next they were rinsed briefly in 100% ethanol, and then they were allowed to air dry. Each piece of filter paper was added to 3 ml scintillation cocktail. Methionine incorporation into newly synthesized proteins was measured by scintillation spectroscopy.
TSP14 Production

**Sf–9 and Tn–5 Cell Culture Maintenance**

Cultures of both *Sf–9* and *Tn–5* cells came from cell lines maintained by the laboratory of Dr. Bruce Webb at the University of Kentucky and were maintained in the insect physiology laboratory at the University of Kentucky. All cell culture methods were adapted from Summers and Smith (1988). All cell culture work was done in a laminar flow hood. All instruments, flasks, 10 ml pipettes, and pipette tips were sterile and disposable. The hood was sterilized with a germicidal ultraviolet light, and surface sterilized with 70% ethanol. *Sf–9* cells were grown with *Sf–900* medium, and *Tn–5* cell were grown with *Tn–50* medium in a monolayer culture in 75 ml plastic cell culture flasks. Since the doubling time for both these cell lines is 18 – 24 hours, they were sub–cultured every other day. To sub–culture the cells, they were first re–suspended by rapidly running medium over them with a pipette. During this procedure, the cells could be seen falling off the surface of the flask. The number of cells in the culture was counted by applying a small drop of medium and cells to a hemocytometer. Then two – 2.5 x 10^6 cells (0.5 – 1.0 ml) were transferred to a new 75 ml flask containing four ml of fresh medium. The flask was rocked gently to wet the growth surface of the flask, and to distribute the cells evenly. Then the cells were incubated at 27°C.

On occasion, cells were frozen for later use. This was of particular value if something had happened to the cell line. Freezing the cells was accomplished by re–suspending the cells in the medium, as described above. The cells and the medium were transferred to sterile lightweight centrifuge tubes and centrifuged at 1000g for ten minutes. The supernatant was removed and the cells were re–suspended in fresh medium at a concentration of 4.0 x 10^6 cells/500 µL in a micro–centrifuge tube. The suspension was then diluted with an equal volume medium containing 20% DMSO. The cells were then frozen slowly by putting the tube in a Styrofoam micro–centrifuge tube rack, kept in -20°C for one hour, and then put in -80°C overnight. The micro–centrifuge tubes were then transferred to liquid nitrogen for storage.
**In vitro Production of Recombinant TSP14**

The TSP14 cDNA has been expressed as a fusion to a poly–histidine tag by Bruce Webb’s lab to simplify purification of the expressed protein. Baculovirus methods were once again adapted from Summers and Smith (1988). The cDNA-His tag fusion was inserted into pAd.CMV–Link.1, a baculovirus expression vector (BaculoGold, PharMingen). From this a viral stock solution was made containing $4 \times 10^7$ plaques per ml. Sf–9 or Tn–5 cells were removed from culture and placed in 150 ml sterile plastic cell culture flasks containing 8 ml of appropriate medium. Approximately 1 x $10^6$ cells were put in each flask. The cells were incubated at $27^\circ$C for 24 hours. This is sufficient time for the cells to double in number for a total of 2 x $10^6$ cells per flask. Two ml of viral stock solution were then added to the flasks. The flasks were placed under aluminum foil and rocked slowly for 90 minutes. After 90 minutes, the medium containing the virus was carefully poured off to prevent losing the cells and replaced with 10 ml fresh medium that did not contain virus. The flasks were incubated for 64 – 72 hours at $27^\circ$C. During this time, the cells infected with the virus would transcribe the viral genome and synthesize the viral proteins, including the TSP14. The viral infection would also cause cell lysis releasing the TSP14 protein into the supernatant. The contents of the flasks were poured into a sterile 50 ml tube and centrifuged at 1000g for five minutes to pellet the cells. The supernatant was removed and concentrated with Centriprep 3kDa molecular weight cut off filters (Amicon, Beverly, MA). This concentrated medium contained TSP14.

TSP14 from the recombinant baculovirus was isolated from the medium based on adhesion of the fusion-protein to a Ni–NTA resin (Qiagen). A sterile 15 ml plastic tube with a cap was washed with approximately five ml 50mM NaCl in 20 mM phosphate buffer at pH 7.8. Approximately five ml of Ni–NTA resin was added to the tube. The resin was washed three times with 50mM NaCl in 20 mM phosphate buffer at pH 7.8, for two minutes per wash and centrifuged gently for one minute between washes. This separated the resin and allowed us to pour off the buffer. The resin was then washed three times with 50mM NaCl in 20 mM phosphate buffer at pH 6.0 in the same manner. The concentrated medium containing TSP14 was added in five ml increments. The concentrated medium was added to the tube and rocked for five minutes. The tube was then gently centrifuged for one minute and the supernatant medium was poured off into a new plastic tube. This was repeated until all the concentrated
media was put through the Ni–NTA resin. The resin was then washed three more times with 50mM NaCl in 20 mM phosphate buffer at pH 7.8, and three more times with 50mM NaCl in 20 mM phosphate buffer at pH 6.0. Five ml of 50 mM imidizole buffer was added to the tube. The tube was rocked for five minutes and gently centrifuged for one minute. The imidizole was poured off into a labeled tube. This procedure was repeated with 200mM, 350mM, and 500mM imidizole. The TSP14 would usually elute in the 200mM and 350mM samples.

**In vivo Production of Recombinant TSP14**

*M. sexta* larvae were chosen to produce recombinant TSP14 for a number of reasons. They are large insects, which makes handling them, especially giving injections, fairly easy. They are unaffected by *M. croceipes* TSP (Zhang et al., 1997), which should prevent problems due to feedback inhibition of protein synthesis. *M. sexta* were also readily available in our laboratory.

To inoculate the larvae via injection, the methods of Maeda (1988) were modified. *M. sexta* were reared in the colony as described above. The larvae were removed from the colony as newly molted fourth instars, weighed, and put in a separate 27.5 X 20 X 11 cm plastic box with a wire mesh (10 – 15 larvae per box). The next day the larvae were sedated in a CO$_2$ chamber for approximately 30 sec. The larvae were injected with 20 µL of viral stock containing about 8 X 10$^5$ plaque forming units using a 0.5 ml insulin syringe and an automatic microinjection device. The injection was made by inserting the needle longitudinally into the posterior of the second to last (or penultimate) proleg. The wound was sealed with a small drop of bee’s wax to prevent excess bleeding and loss of injected virus. Following the injection, the larvae were returned to the plastic box and allowed to feed. Two days after the first injection, the larvae received another viral injection in the same manner as the first one.

Six days after the initial injection, the hemolymph of the larvae was collected. The larvae were sedated with CO$_2$ and folded in half with their prolegs exposed. Using dissecting scissors, one of the first set of prolegs was snipped off and gentle pressure was applied by hand to allow the hemolymph to flow out of the larva to be collected in a sterile micro–centrifuge tube. The hemolymph was immediately placed on ice to prevent melanization and diluted with two volumes of phosphate buffered saline (10 mM sodium phosphate, 0.9% NaCl) pH 7.4. The
sample was then passed through an Amicon 100 and microcon 30 kDa molecular weight cutoff filters to remove the higher molecular weight proteins, then concentrated using a microcon 3kDa cutoff filter. The recombinant TSP14 was in the retentate of the 3kDa filter and was isolated using a Ni–NTA resin (Qiagen) as described above.

The second method of infection was an adaptation from Cha, et al. (1997). Artificial diet was prepared as above. Approximately two cm of diet was poured into a 15 X 25 cm plastic box. When the diet cooled, and just began to congeal, ten ml of viral stock containing about 4 X 10^7 pfu was spread over the top and gently mixed throughout the diet. New fourth instar M. sexta larvae were removed from the colony and put on a mesh wire in a 27.5 X 20 X 11 cm plastic box and allowed to feed on the diet containing the recombinant virus for one week. After the week, the larvae were homogenized in phosphate buffered saline (10 mM sodium phosphate, 0.9% NaCl) at pH 7.4. The homogenates were centrifuged at about 250g at 4°C to pellet the large debris. The supernatant was poured off and put through a 0.22 µm filter to further remove debris. The sample was then concentrated using a Centriprep 3 kDa molecular weight cutoff filter. The TSP14 was then isolated from the concentrated retentate using a Ni–NTA resin as described above. The presence of TSP14 was verified by SDS-PAGE and Western blotting and then quantified by Bradford assay.

**Localization of TSP**

**Synthesis of a Radio–labeled Probe**

TSP14 was amplified via polymerase chain reaction (PCR) using TSP16 5’ (CTAGGTACCATGCCATCCAAAATTTTAATTTC) and TSP16 3’-REV (GAACATCTAATGATGATGATGATGATG) as primers and the cloning vector as the template. Approximately 100 ng of a TSP14 PCR amplimer was added to ten µl of Klenow buffer (Gibco). The volume was brought to 42 µl with sterile water, and the tube was heated at 95°C in a block heater for ten minutes. Then, two µl bovine serum albumin, two µl nTP’s (without ATP), two µl of α[^32P]-dATP and one µl of large fragment DNA polymerase (Klenow) were added to the tube.

The tube was incubated for three to four hours at room temperature. After this period 200 µl of stop buffer (5 mM EDTA/ 0.1% SDS) was added to stop the reaction. Verification of the probe synthesis was achieved by placing ten µl of the probe on two or three 22 mm Ø
Whatman® GF/C glass microfiber filters. One filter paper was washed three times with 10% trichloroacetic acid (TCA). All filters were put in 70% ethanol and allowed to dry. The filter papers were added to 3 ml of scintillation and analyzed with scintillation spectroscopy.

**Northern Blot**

The agarose-formaldehyde gel containing RNA was removed from the gel tank and washed with ribonuclease-free water to remove the formaldehyde. For each gel, three pieces of blotter paper and one piece of nylon membrane were cut to the size of the gel. The transfer stack was formed by stacking plastic wrap, the gel, the nylon membrane, three pieces of blotter paper, and about 6 inches of laboratory paper towels from bottom to top. A full one liter bottle was placed on top of the stack and the RNA was transferred from the gel to the membrane over night. The membrane was removed from the stack and rinsed with 2x SSC buffer (0.15M NaCl, 0.015M trisodium citrate 2H2O, pH 7.0), and allowed to air dry. The membrane was irradiated with ultraviolet light to cross-link the RNA to the membrane, then was rinsed with 6x SSC. The membrane was put in a hybridization tube with 10 ml formamide prehybridization solution with salmon sperm DNA and incubated in a hybridization oven with rotation at 42°C for three hours. The DNA probes with suitably specific activity (~10⁸ dpm/µg) were heated to 100º for ten minute and put on ice. The probe was added to the hybridization tube at 10 ng /ml and incubated with rotation at 42°C overnight. The hybridization solution was poured into radioactive waste containers, and the membrane was washed two times with 2x SSC/ 0.1% SDS by rotating for five minutes at 42°C. The membrane was then washed with 0.2x SSC/ 0.1% SDS by rotating for ten minutes at 42°C until no radioactivity was detected with a Geiger counter in the discarded wash solution. The membrane was put in UV-transparent plastic wrap, with hybridization, visualized by auto–radiography.
SDS–Polyacrylamide Gel Electrophoresis for Proteins

SDS–PAGE was performed using adaptations of the Laemmli method (Laemmli, 1970). Gels 8.0 cm wide x 7.3 cm long x 0.75 cm thick, were 12% acrylamide/bis unless otherwise stated. They were cast and run with the Bio Rad Mini–Protein II Electrophoresis Cell System. The gels were run at a constant voltage of 200 V until the bromophenol dye front was near the bottom of the gel.

Western Blot

The stacking gel was removed from the SDS–PAGE gel and discarded. The running gel was equilibrated for ten minutes in Towbin Transfer Buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 20% MeOH). For each gel to be transferred, four pieces of blotter paper and one piece of Immobilon P (Amicon, Beverly, MA) membrane were cut to the size of the gel. The membrane was wet in 100% methanol, then the membrane and blotter paper were soaked for approximately five minutes in transfer buffer. The transfer stack was assembled as two pieces of blotter paper, membrane, gel, and another two pieces of blotter paper, bottom to top, and placed in between transfer plates. The proteins were transferred from the gel to the membrane by applying 20 V for 30 minutes Towbin et al., 1979). The membrane was blocked by incubating with gentle shaking for one to 12 hours in 5% dried milk in tris–buffered saline, 0.5% Tween–80 (TBS–Tween). Rabbit antibodies produced against TSP14 were added at a ratio of 1:5,000 and incubated with gentle shaking for two to 12 hours. The antibody/milk solution was poured off and the membrane was washed three times for ten minutes each with TBS–Tween with gentle shaking. The secondary antibody, goat anti–rabbit, was added with TBS–Tween at a ratio of 1:2,000 and incubated with gentle shaking for two to 12 hours. The secondary antibody/TBS–Tween solution was discarded. The membrane was washed three times for ten minutes each with TBS–Tween, and two times with alkaline phosphatase buffer. The blot is developed by adding 66µl nitroblue tetrazolium (NBT) and 33µl 5–bromo–4–chloro–3–indolyl phosphate (BCIP) to 10 ml alkaline phosphatase buffer. The BCIP/NBT solution was added to the membrane and gently shaken by hand. When the blot developed to a desired degree, the reaction was stopped by flushing the blot with deionized water.
In an attempt to increase the sensitivity of the western blots, the SuperSignal® (Pierce, Rockford, IL) chemiluminescent substrate was used to develop the blot instead of BCIP/NBT. When the chemiluminescent substrate was going to be used some alterations to the western blot protocol were made. Instead of Immobilon P, nitrocellulose was used as the transfer membrane. After the transfer, the membrane was blocked with 3% bovine serum albumin (BSA) in tris–buffered saline, 0.5% Tween–80 (TBS–TWEEN) instead of 5% dried milk. The primary antibody (rabbit–anti–TSP14) was added at a ratio of 1:50,000 instead of 1:5,000 and the blots were washed six times instead of three times after incubation with the antibodies.

To detect the proteins on the blot, equal amounts (four ml of each for a 60 cm² blot) of SuperSignal® Stable Peroxide Solution and SuperSignal® West Luminol/Enhancer Solution were combined and added to the blot in a clean tray. The membrane was incubated in the solution for five minutes with gentle shaking. The membrane was removed from the solution and placed in a plastic exposure envelope, making sure to remove all bubbles between the membrane and the surface of the envelope, and sealed. In the darkroom, the blot was placed against Kodak X–Omat film (protein side down), and exposed for approximately 30 seconds. The film was immediately developed and examined because the blot could be re–exposed as long as it remained wet. Shorter or longer exposure times were sometimes necessary depending on the strength of the signal.

**Dehydration and Infiltration of *H. virescens* for Histology**

The heads and last posterior segments were removed from third, fourth, and fifth instars of both parasitized and non–parasitized *H. virescens* larvae. The larvae were placed in Bouin’s fixative (1: 5: 15 glacial acetic acid: formaldehyde: saturated picric acid) for 30 minutes. The samples were dehydrated in a series of increasing concentrations of ethanol as follows: 70% for 30 minute; 85% for 15 minute; 85% for ten minute; 95% for 30 minute; and absolute ethanol, three times for ten minute each. The larvae were then transferred to methyl benzoate for six hours. During this time, the jars were put in a vacuum desiccator and a vacuum of 5–10 mm of Hg was applied to remove air trapped in the tracheal system or the hemocoel. The samples were transferred twice to fresh methyl benzoate for four to six hours each. The samples were transferred to a 3:1 methyl benzoate: benzene solution for 15 minutes, a 1:1 methyl benzoate:
benzene solution for ten minutes, and a 1:3 methyl benzoate: benzene solution for five minutes. The samples were put in benzene three times for five minutes each. The samples were transferred to a solution of 1:1 benzene: paraffin for one hour at 60°C. Next, they were transferred through three changes of paraffin for 12 hours each at 60°C. Finally, the samples were placed in fresh molten paraffin and oriented before the paraffin solidified so they could be easily cut out of the block and prepared for sectioning.

**Immunohistochemistry**

The immunohistochemistry methods were adapted from Hayashi et al. (1988). Six to 10 µm sections the paraffin imbedded samples were sectioned into by a microtome, adhered to glass slides in a solution of 1:1:1 of egg white: glycerol: water, then dried over night on a slide warmer at 52°C. The sections were placed in a slide rack and dewaxed with a Hemo-D® (Fisher), a xylene substitute, for 20 minutes. After this dewaxing, the sections were transferred to fresh Hemo-D® for an additional three minutes. The sections were re-hydrated with two, three minute baths of 100% ethanol, one three minute bath of 95% ethanol, one three minute bath of 85% ethanol, one three minute bath of 70% ethanol, and one three minute bath of deionized water. The slides were then rinsed briefly (one minute) in phosphate buffered saline (10 mM sodium phosphate, 0.9% NaCl)/ 0.05% Tween-80 (PBS-Tween) to help prevent nonspecific binding.

The sections were put in 15% glacial acetic acid for 15 minutes to block endogenous alkaline phosphatase activity, and rinsed for three minutes in PBS–Tween. The slides were then placed horizontally in a humid chamber and the sections were flooded with PBS containing 1% bovine serum albumin (BSA) and 3% fetal bovine serum (FBS) to block nonspecific binding sites. After a 30 minute incubation the solution was removed from the sections with special attention given to carefully remove the excess solution from around the edges of the slide. Still in the horizontal position, the sections were flooded with rabbit antibodies produced against TSP14 diluted 1:200 in PBS–1%BSA, 3%FBS and incubated for 2 hours. Following incubation, the slides were rinsed three times for two minutes each time in PBS–Tween, then placed back in the humid chamber and incubated for one hr with a goat ant–rabbit alkaline phosphatase antibody at a ratio of 1:100 in PBS–BSA–FBS. Following this incubation, the slides were rinsed three times for two minutes each in PBS–Tween. In order to detect the antibodies, the sections were flooded
with BCIP/NBT solution (30 µl of BCIP and 45 µl NBT were added to 10 ml of Tris buffer, pH 8.2) and incubated for 15 minute, followed by a three minute wash in PBS–Tween. The slides were dried around the edges and a coverslip mounted with Apathy’s mountant (20 g gum arabic, 20 g cane sugar, 20 g potassium acetate, 40 ml distilled water 40 ml, 0.02 g Thymol 0.02) or a 1:1 mixture of glycerol and water. Acrylic nail polish was used to seal the edges of the coverslip to prevent drying and contamination.
RESULTS

Effects of TSP on Protein Synthesis in *H. virescens* Testes

Initial studies were conducted to test the feasibility of using *H. virescens*’ testes as a protein–synthesizing organ in *in vitro* tissue assays. Prior to this point, Schepers (1995) had shown that TSP reduced the uptake of methionine in *in vitro* fat body tissue assays. In order to prove the appropriateness of testes as an alternative to fat body, testes would have to show results similar to those of fat body in the *in vitro* tissue assay. It was found that individual testes incubated in the presence of three larval equivalents of crude TSP showed a significant decrease in the incorporation of methionine (*t*=8.79, *p*=0.0) with TSP treated testes averaging 0.81 ± 0.04 dpm/µg fresh tissue compared to the control testes averaging 4.39 ± 0.42. The TSP treated testes showed an overall average reduction in methionine uptake of 76.6 ± 1.6% (Table 1) (Figure 1).

Once it was determined that the testes were a suitable alternative to fat body, attention was turned to the relationship between a pair of testes from the same larva. If it could be shown that methionine was incorporated into each testis at similar rates, and that both the left and right testis responded to TSP in a similar manner, we could then confidently use a pair of testes as a unit in the *in vitro* tissue assay, with one being the experimental subject, and the other acting as its control. This study found no significant differences in methionine incorporation between left and right testes treated with three larval equivalents of crude TSP (0.75 ± 0.06 vs. 1.06 ± 0.15 dpm/µg fresh weight) (*t*=1.93, *p*=0.068) and when not treated with TSP (5.39 ± 1.36 vs. 5.16 ± 1.20 dpm/µg fresh weight) (*t*= 0.132, *p*= 0.896).

Also, there were no significant differences in the mean percent reduction of methionine incorporation (76.1 ± 5.1 vs. 69.0 ± 6.7) (*t*= 0.83, *p*=0.41). (Table 2) (Figure 2) Once it was determined that both testes from one individual responded similarly to either TSP or control treatments in the *in vitro* tissue assay, efforts were directed to optimizing the specific conditions of the assay.

Prior to these tests the standard incubation time for the assay was four hours. When we used various incubation times in the *in vitro* assay, we found that incorporation of methionine in
Table 1:  *In vitro* incorporation of $[^{35}\text{S}]$–methionine by matched pairs of *H. virescens* testes (dpm / µg fresh weight tissue ± standard error) (t = 8.79; p = 0.0; n = 50). TSP treatment received 3 LE of crude TSP for 4 hr incubation period.

<table>
<thead>
<tr>
<th></th>
<th>TSP</th>
<th>Control</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.81 ± 0.04</td>
<td>4.39 ± 0.42</td>
<td>76.6 ± 1.6</td>
</tr>
</tbody>
</table>
Table 2: A comparison of matched pairs of *H. virescens* testes *in vitro* incorporation of $[^{35}\text{S}]$-methionine (mean dpm/µg tissue ± SE) when one of each pair was incubated with 3 larval equivalents of crude TSP for 4 hours (n = 22).

<table>
<thead>
<tr>
<th>Treated Testis</th>
<th>TSP</th>
<th>Control</th>
<th>Mean % Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left</td>
<td>0.75 ± 0.06</td>
<td>5.39 ± 1.36</td>
<td>76.1 ± 5.1</td>
</tr>
<tr>
<td>Right</td>
<td>1.06 ± 0.15</td>
<td>5.16 ± 1.20</td>
<td>69.0 ± 6.7</td>
</tr>
</tbody>
</table>
In vitro incorporation of $[^{35}\text{S}]$–methionine by matched pairs of *H. virescens* testes. TSP treatment received 3 LE of crude TSP for a 4 hr incubation period (n = 50).
Figure 2: Percent reduction of $[^{35}\text{S}]$–methionine incorporation by *H. virescens* testes in the *in vitro* testes assay using 3 LE of crude TSP treated left testis and the right as a control vs. crude TSP–treated right testis and the left as a control (n = 22).
control testes was approximately linear for eight hours (Table 3), (Figure 3), while incorporation in testes treated with crude TSP occurred at a significantly reduced rate compared to the control only during the first hour and then at only a significantly reduced rate compared to the control (Table 3) (Figure 3). There was no significant additional incorporation of methionine during the remaining seven hours of incubation in the presence of TSP. When the data were viewed from the perspective of percent reduction of methionine incorporation compared to controls, a significant reduction was observed during the first hour of incubation. Subsequently, the rate increased gradually as the result of continued incorporation by the controls, but no additional incorporation by the TSP–treated testes was observed (Table 3) (Figure 4).

Once we had the information on the effect of incubation time, we turned our attention to the amount of crude TSP added to the assay using a four–hour incubation time. We hoped to determine the magnitude of the tissues’ observed response to TSP on protein synthesis. In short, we wanted to establish whether the tissues would respond to TSP in a graded or quantal manner. A graded response is one where the magnitude of the response increases continuously with greater concentration of the agent causing the response. A quantal response is one where a minimum dose is required to obtain a response in a given number of subjects.

Crude TSP reduced \[^{35}\text{S}\]–methionine incorporation by testes of *H. virescens* in a dose dependant manner. The most profound effect was observed between 0.0 (0 % reduction) and 0.3 (53 ± 0.45% reduction) larval equivalents (Table 4) (Figure 5). The percent reduction continued to increase with higher doses peaking at 77.4 ± 1.2% at 4.0 larval equivalents (Table 4) (Figure 5). Due to the dramatic increase at the lower concentrations, and the subsequent leveling off in the higher concentrations, as well as the fact that the most dramatic reduction of protein synthesis came in the first hour of the incubation, we conducted a second dosage experiment using a one-hour incubation time. This experiment showed slightly different results than the four-hour incubation. The synthesis of new proteins was inhibited in a linear manner over a range of four larval equivalents, with 0.2 larval equivalents showing the least reduction of protein synthesis (3.8% ± 1.6%) and 4.0 larval equivalents showing the greatest reduction of protein synthesis (93.6% ± 3.0%) (Table 5), (Figure 6). Hence, it could be concluded that testes respond to TSP in a graded manner.
Table 3: Effect of time on TSP inhibition and percent reduction of *in vitro* incorporation of $[^{35}\text{S}]$-methionine by matched pairs of *H. virescens* testes.
(3 LE crude TSP / testis) (dpm / testis ± SE) (n = 20).

<table>
<thead>
<tr>
<th>Incubation Time (hrs)</th>
<th>Control</th>
<th>TSP</th>
<th>Mean % Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>5,567 ± 97</td>
<td>2,854 ± 73</td>
<td>48.4 ± 1.5</td>
</tr>
<tr>
<td>1.0</td>
<td>18,912 ± 312</td>
<td>4,079 ± 106</td>
<td>78.3 ± 0.7</td>
</tr>
<tr>
<td>2.0</td>
<td>25,425 ± 717</td>
<td>4,370 ± 340</td>
<td>82.4 ± 1.6</td>
</tr>
<tr>
<td>4.0</td>
<td>44,295 ± 1,725</td>
<td>4,763 ± 244</td>
<td>89.2 ± 0.4</td>
</tr>
<tr>
<td>6.0</td>
<td>72,824 ± 734</td>
<td>4,605 ± 100</td>
<td>93.6 ± 0.2</td>
</tr>
<tr>
<td>8.0</td>
<td>98,071 ± 1,412</td>
<td>4,531 ± 198</td>
<td>95.3 ± 0.2</td>
</tr>
</tbody>
</table>
Table 4: Effect of crude TSP dose on the percent reduction of $[^{35}\text{S}]$-methionine incorporation on *H. virescens* testes (4 hour incubation) (n = 15).

<table>
<thead>
<tr>
<th>Larval Equivalents (TSP)</th>
<th>Percent Reduction</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>35.2</td>
<td>1.55</td>
</tr>
<tr>
<td>0.2</td>
<td>43.0</td>
<td>0.58</td>
</tr>
<tr>
<td>0.3</td>
<td>53.2</td>
<td>0.45</td>
</tr>
<tr>
<td>0.4</td>
<td>53.3</td>
<td>0.73</td>
</tr>
<tr>
<td>0.5</td>
<td>50.9</td>
<td>1.09</td>
</tr>
<tr>
<td>1.0</td>
<td>59.4</td>
<td>1.07</td>
</tr>
<tr>
<td>1.5</td>
<td>61.0</td>
<td>1.20</td>
</tr>
<tr>
<td>2.0</td>
<td>58.5</td>
<td>1.41</td>
</tr>
<tr>
<td>2.5</td>
<td>65.5</td>
<td>1.00</td>
</tr>
<tr>
<td>3.0</td>
<td>68.2</td>
<td>1.08</td>
</tr>
<tr>
<td>3.5</td>
<td>76.0</td>
<td>1.72</td>
</tr>
<tr>
<td>4.0</td>
<td>77.4</td>
<td>1.18</td>
</tr>
<tr>
<td>4.5</td>
<td>72.4</td>
<td>0.90</td>
</tr>
</tbody>
</table>
Figure 3: Effect of time on TSP inhibition of \textit{in vitro} incorporation of $[^{35}\text{S}]$-methionine by matched pairs of \textit{H. virescens} testes (3 LE crude TSP / testis) (n = 20).
Figure 4: Effect of time on the mean percent reduction $[^{35}\text{S}]$–methionine incorporation by crude TSP-treated *H. virescens* testes (3 LE crude TSP / testis) (n = 20).
Figure 5: Effect of crude TSP dose on the percent reduction of $[^{35}\text{S}]$–methionine incorporation on *H. virescens* testes (4 hr incubation time) (n = 15).
Effect of TSP on Nucleic Acid Synthesis in *H. virescens* Testes

Protein synthesis is a relatively complex process that is ultimately controlled by information contained in cellular DNA and RNA. We were interested in learning whether the inhibition of protein synthesis we observed in testes exposed to TSP was a result of an inhibition of DNA replication. To test this, we conducted *in vitro* testes assays with $[^3]H$–thymidine to measure production of new DNA. The results of these assays showed that TSP had no significant effect on the incorporation of $[^3]H$–thymidine into new deoxyribonucleic acids ($t=0.809$, $p=0.423$). There was an average of $8,098 \pm 265$ dpm for the control and $8,473 \pm 381$ dpm for the TSP treated group. (Table 6) (Figure 7)

Once we established that the production of DNA was not inhibited by TSP, we focused our attention towards transcription of RNA. The inhibition of transcription by TSP was another process that could manifest itself as a reduction in protein synthesis. To investigate the inhibition of transcription we conducted the *in vitro* testes assay using $[^3]H$–uridine to measure the production of new RNA. The results from these experiments showed that TSP had no significant effect in the incorporation of $[^3]H$–uridine into new ribonucleic acids ($t=0.169$, $p=0.866$). An average of $18,740 \pm 1,097$ dpm per testis was observed in the controls compared to $19,020 \pm 1,236$ dpm per testis in the TSP treated samples. (Table 6) (Figure 8).

Effects of TSP on Translation of mRNA in Cell Free Systems

The direct effect of TSP on the synthesis of proteins was investigated with the use of cell free systems. The rabbit reticulocyte lysates contain all the cellular components necessary for protein synthesis but lack mRNA. Lysates are treated with micrococcal nuclease to destroy endogenous mRNA that could cause unwanted background translation. The use of different mRNAs from different tissues, as well as different species, allowed us to investigate whether the source of mRNA played a role in TSP–mediated inhibition of protein synthesis. When the rabbit reticulocyte lysate assay was conducted using $2 \mu g$ of mRNA from various tissues from both *H. virescens* and *M. sexta*, TSP significantly inhibited protein synthesis with every source of mRNA tested from both species. When the percent reduction in protein synthesis was calculated from values of all individual tissues from both *H. virescens* and *M. sexta*, the mean value was $43 \pm 1\%$. The mean percent reduction in protein synthesis from mRNA from *M. sexta* tissues was 18
Table 5: Effect of TSP dose on the percent reduction of $[^{35}\text{S}]$–methionine incorporation on *H. virescens* testes (1 hr incubation) ($n = 8$).

<table>
<thead>
<tr>
<th>Larval Equivalents (TSP)</th>
<th>Percent Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>3.8 ± 1.6</td>
</tr>
<tr>
<td>0.4</td>
<td>8.2 ± 2.3</td>
</tr>
<tr>
<td>0.6</td>
<td>11.9 ± 1.7</td>
</tr>
<tr>
<td>0.8</td>
<td>16.0 ± 2.8</td>
</tr>
<tr>
<td>1.0</td>
<td>19.6 ± 2.6</td>
</tr>
<tr>
<td>1.2</td>
<td>20.4 ± 2.4</td>
</tr>
<tr>
<td>1.4</td>
<td>22.7 ± 2.5</td>
</tr>
<tr>
<td>1.6</td>
<td>26.9 ± 2.7</td>
</tr>
<tr>
<td>1.8</td>
<td>31.6 ± 2.7</td>
</tr>
<tr>
<td>2.0</td>
<td>34.3 ± 2.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Larval Equivalents (TSP)</th>
<th>Percent Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2</td>
<td>37.3 ± 3.1</td>
</tr>
<tr>
<td>2.4</td>
<td>45.2 ± 1.6</td>
</tr>
<tr>
<td>2.6</td>
<td>51.5 ± 2.7</td>
</tr>
<tr>
<td>2.8</td>
<td>56.7 ± 2.2</td>
</tr>
<tr>
<td>3.0</td>
<td>63.1 ± 1.8</td>
</tr>
<tr>
<td>3.2</td>
<td>68.2 ± 1.6</td>
</tr>
<tr>
<td>3.4</td>
<td>76.6 ± 2.4</td>
</tr>
<tr>
<td>3.6</td>
<td>82.4 ± 3.0</td>
</tr>
<tr>
<td>3.8</td>
<td>89.9 ± 2.4</td>
</tr>
<tr>
<td>4.0</td>
<td>93.6 ± 3.0</td>
</tr>
</tbody>
</table>
**Table 6:** Effect of crude TSP on DNA synthesis ([³H]–thymidine incorporation) and RNA synthesis ([³H]–uridine incorporation) in matched pairs of *H. virescens* testes (dpm / testis ± SE) (3 LE TSP / testis) (n = 20).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>TSP Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>8,098 ± 265</td>
<td>8,473 ± 381</td>
</tr>
<tr>
<td>RNA</td>
<td>18,740 ± 1,097</td>
<td>19,020 ± 1236</td>
</tr>
</tbody>
</table>
Figure 6: Effect of TSP dose on the percent reduction of $[^{35}\text{S}]$–methionine incorporation on *H. virescens* testes (1 hr incubation time) (*n* = 8).
Figure 7: Effect of crude TSP on DNA synthesis ([³H]-thymidine incorporation) in matched pairs of *H. virescens* testes (dpm / testis ± SE) (3 LE TSP / testis) (n = 20).
Figure 8: Effect of crude TSP on RNA synthesis ([\(^3\)H]–uridine incorporation) in matched pairs of *H. virescens* testes (dpm / testis ± SE) (3 LE TSP / testis). (n = 20)
percentage points greater than the mean from all *H. virescens* tissues (53 ± 1% vs. 35 ± 2% respectively). This was primarily due to the relatively low percent reduction of protein synthesis from mRNA sources of *H. virescens* midgut and hemolymph tissues (14 and 18% respectively) (Table 7) (Table 8) (Figure 9) (Figure 10).

There was a significant difference in the percent reduction of protein synthesis in the mRNA from *H. virescens* tissues (14 – 52%). This was due to the low values from midgut and hemolymph (Table 7) (Figure 9). There was no significant difference in the percent reduction of protein synthesis between *M. sexta* tissue sources of mRNA (41 – 64%) (Table 8, Figure 10).

Schepers (1995), alluded to a cofactor, possibly a small protein (< 3kDa), secreted by developing teratocytes. He did not believe this protein was the principle inhibitory factor, but thought it might play a companion role in the TSP-mediated inhibition of protein synthesis in fat body assays. We thought that the rabbit reticulocyte lysate assay would be an appropriate tool to investigate the merits of this cofactor. When the cofactor derived from the 3 kDa molecular weight cut–off sieve filtrates was added to the rabbit reticulocyte lysate assay along with TSP, the resulting comparison of protein synthesis inhibition was erratic, or had no effect, when compared to protein synthesis inhibition by TSP alone (3 – 30 kDa fraction) (Table 9) (Table 10) (Figure 11) (Figure 12).

When *H. virescens* was used, the mRNA from whole insect homogenates showed no significant reduction in protein synthesis (41 % reduction with TSP and 47% with the added cofactor). In two tissues, the cofactor added to TSP exhibited less protein synthesis inhibition. The reduced inhibition of protein synthesis was 23% using mRNA isolated from testes and 26% using mRNA isolated from labial glands. The mRNA from the remaining four tissues, hemolymph, gut, fat body and nerve cord, all showed a significant increase in protein synthesis inhibition. These tissues showed increases in protein synthesis inhibition of 17%, 31%, 13%, and 28% respectively (Table 11) (Table 12).
Table 7: Effect of TSP (3–30 kDa fraction) on protein synthesis initiated by 2 µg mRNA from different sources from *H. virescens* (dpm ± SE). Student’s T–test performed on control vs. TSP treatments of the same mRNA source.

* P-value less than 0.05 in paired Student’s T-test (n = 10).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>TSP</th>
<th>Mean % Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luciferase Control</td>
<td>78,156 ± 3,502</td>
<td>45,311 ± 1,595</td>
<td>41 ± 3%*</td>
</tr>
<tr>
<td>Homogenate</td>
<td>35,244 ± 1,896</td>
<td>20,639 ± 832</td>
<td>41 ± 3%*</td>
</tr>
<tr>
<td>Hemolymph</td>
<td>56,556 ± 1,498</td>
<td>48,668 ± 937</td>
<td>14 ± 2%*</td>
</tr>
<tr>
<td>Gut</td>
<td>49,070 ± 1,114</td>
<td>40,054 ± 790</td>
<td>18 ± 3%*</td>
</tr>
<tr>
<td>Fat Body</td>
<td>42,942 ± 854</td>
<td>20,720 ± 486</td>
<td>52 ± 2%*</td>
</tr>
<tr>
<td>Testes</td>
<td>69,511 ± 1,505</td>
<td>34,200 ± 576</td>
<td>51 ± 1%*</td>
</tr>
<tr>
<td>Labial Glands</td>
<td>63,405 ± 2,214</td>
<td>37,526 ± 882</td>
<td>40 ± 2%*</td>
</tr>
<tr>
<td>Nerve Cord</td>
<td>53,964 ± 1,141</td>
<td>32,315 ± 614</td>
<td>40 ± 2%*</td>
</tr>
</tbody>
</table>

62
**Table 8:** Effect of TSP (3 – 30 kDa fraction) on protein synthesis initiated by 2μg mRNA from different sources from *M. sexta* (dpm ± SE). Student’s T–test performed on control vs. TSP treatments of the same mRNA source.

* P–value less than 0.05 in paired Student’s T–test (n = 10).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>TSP</th>
<th>Mean % Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luciferase Control</td>
<td>78,156 ± 3,502</td>
<td>45,311 ± 1,595</td>
<td>41 ± 3%*</td>
</tr>
<tr>
<td>Homogenate</td>
<td>63,877 ± 3,081</td>
<td>31,589 ± 853</td>
<td>49 ± 4%*</td>
</tr>
<tr>
<td>Hemolymph</td>
<td>50,778 ± 1,040</td>
<td>29,228 ± 701</td>
<td>42 ± 1%*</td>
</tr>
<tr>
<td>Gut</td>
<td>43,687 ± 1,122</td>
<td>23,593 ± 585</td>
<td>45 ± 2%*</td>
</tr>
<tr>
<td>Fat Body</td>
<td>49,069 ± 1,003</td>
<td>18,823 ± 391</td>
<td>62 ± 1%*</td>
</tr>
<tr>
<td>Labial Glands</td>
<td>49,373 ± 898</td>
<td>17,674 ± 391</td>
<td>64 ± 1%*</td>
</tr>
<tr>
<td>Nerve Cord</td>
<td>46,110 ± 1,259</td>
<td>19,921 ± 614</td>
<td>56 ± 3%*</td>
</tr>
</tbody>
</table>
Table 9: Effect of TSP (3 – 30 kDa fraction) + <3kDa fraction on protein synthesis initiated by 2µg mRNA from different sources of H. virescens (dpm ± SE). Student’s T–test performed on control vs. TSP + cofactor treatments of the same mRNA source.
* P–value less than 0.05 in paired Student’s T–test (n = 10).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>TSP + cofactor</th>
<th>Mean % Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luciferase Control</td>
<td>78,156 ± 3,502</td>
<td>32,509 ± 899</td>
<td>58 ± 2%*</td>
</tr>
<tr>
<td>Homogenate</td>
<td>35,244 ± 1,896</td>
<td>18,001 ± 522</td>
<td>47 ± 4%*</td>
</tr>
<tr>
<td>Hemolymph</td>
<td>56,556 ± 1,498</td>
<td>39,153 ± 1,531</td>
<td>31 ± 2%*</td>
</tr>
<tr>
<td>Gut</td>
<td>49,070 ± 1,114</td>
<td>24,816 ± 539</td>
<td>49 ± 1%*</td>
</tr>
<tr>
<td>Fat Body</td>
<td>42,942 ± 854</td>
<td>14,858 ± 497</td>
<td>65 ± 2%*</td>
</tr>
<tr>
<td>Testes</td>
<td>69,511 ± 1,505</td>
<td>49,580 ± 869</td>
<td>28 ± 2%*</td>
</tr>
<tr>
<td>Labial Glands</td>
<td>63,405 ± 2,214</td>
<td>53,762 ± 1,253</td>
<td>14 ± 4%*</td>
</tr>
<tr>
<td>Nerve Cord</td>
<td>53,964 ± 1,141</td>
<td>17,391 ± 573</td>
<td>68 ± 1%*</td>
</tr>
</tbody>
</table>
Table 10: Effect of TSP (3 – 30 kDa fraction) + <3kDa fraction on proteinsynthesis initiated by 2µg mRNA from different sources of *M. sexta* (dpm ± SE). Student’s T–test performed on control vs. TSP + cofactor treatments of the same mRNA source.

* P–value less than 0.05 in paired Student’s T-test (n = 10).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>TSP + cofactor</th>
<th>Mean % Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luciferase Control</td>
<td>78,156 ± 3,502</td>
<td>32509±899</td>
<td>58 ± 2%*</td>
</tr>
<tr>
<td>Homogenate</td>
<td>63,877 ± 3,081</td>
<td>30,317 ± 747</td>
<td>52 ± 2%*</td>
</tr>
<tr>
<td>Hemolymph</td>
<td>50,778 ± 1,040</td>
<td>23,824 ± 753</td>
<td>53 ± 2%*</td>
</tr>
<tr>
<td>Gut</td>
<td>43,687 ± 1,122</td>
<td>39,350 ± 909</td>
<td>9 ± 4%*</td>
</tr>
<tr>
<td>Fat Body</td>
<td>49,069 ± 1,003</td>
<td>15,886 ± 218</td>
<td>67 ± 1%*</td>
</tr>
<tr>
<td>Labial Glands</td>
<td>49,373 ± 898</td>
<td>20,588 ± 319</td>
<td>58 ± 1%*</td>
</tr>
<tr>
<td>Nerve Cord</td>
<td>46,110 ± 1,259</td>
<td>18,290 ± 322</td>
<td>60 ± 1%*</td>
</tr>
</tbody>
</table>
**Table 11:** Percent reduction of $[^{35}\text{S}]$–methionine incorporation into proteins translated from mRNA from various tissues of *H. virescens* after treatment with TSP or TSP + <3kDa fraction.

* P-value less than 0.05 in paired Student’s T-test (n = 10).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TSP</th>
<th>TSP w/ cofactor</th>
<th>TSP w/ cofactor – TSP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luciferase</td>
<td>41 %</td>
<td>58 %</td>
<td>+ 17 %*</td>
</tr>
<tr>
<td>Homogenate</td>
<td>41 %</td>
<td>47 %</td>
<td>+ 6 %</td>
</tr>
<tr>
<td>Hemolymph</td>
<td>14 %</td>
<td>31 %</td>
<td>+ 17 %*</td>
</tr>
<tr>
<td>Gut</td>
<td>18 %</td>
<td>49 %</td>
<td>+ 31 %*</td>
</tr>
<tr>
<td>Fat Body</td>
<td>52 %</td>
<td>65 %</td>
<td>+ 13 %*</td>
</tr>
<tr>
<td>Testes</td>
<td>51 %</td>
<td>28 %</td>
<td>- 23 %*</td>
</tr>
<tr>
<td>Labial Glands</td>
<td>40 %</td>
<td>14 %</td>
<td>- 26 %*</td>
</tr>
<tr>
<td>Nerve Cord</td>
<td>40 %</td>
<td>68 %</td>
<td>+ 28 %*</td>
</tr>
</tbody>
</table>
Table 12: Percent reduction of $[^{35}\text{S}]$–methionine incorporation into proteins translated from mRNA from various tissues of *M. sexta* after treatment with TSP or TSP + <3kDa fraction.

* P–value less than 0.05 in paired Student’s T-test (n = 10).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TSP</th>
<th>TSP w/ cofactor</th>
<th>TSP w/ cofactor – TSP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luciferase</td>
<td>41 %</td>
<td>58 %</td>
<td>+ 17 %*</td>
</tr>
<tr>
<td>Homogenate</td>
<td>49 %</td>
<td>52 %</td>
<td>+ 3 %</td>
</tr>
<tr>
<td>Hemolymph</td>
<td>42 %</td>
<td>53 %</td>
<td>+11 %*</td>
</tr>
<tr>
<td>Gut</td>
<td>45 %</td>
<td>9 %</td>
<td>-36 %*</td>
</tr>
<tr>
<td>Fat Body</td>
<td>62 %</td>
<td>67 %</td>
<td>+ 5 %*</td>
</tr>
<tr>
<td>Labial Glands</td>
<td>64 %</td>
<td>58 %</td>
<td>- 6 %*</td>
</tr>
<tr>
<td>Nerve Cord</td>
<td>56 %</td>
<td>60 %</td>
<td>+ 4 %</td>
</tr>
</tbody>
</table>
Figure 9: Mean percent reduction (± SE) of protein synthesis by TSP (3 – 30 kDa fraction) in the rabbit reticulocyte lysate assay using 2 µg of mRNA from various tissues of *H. virescens* (n = 10).
Figure 10: Mean percent reduction (± SE) of protein synthesis by TSP (3 – 30 kDa fraction) in the rabbit reticulocyte lysate assay using mRNA from various tissues of *M. sexta* (n = 10).
Figure 11: Mean percent reduction (± SE) of protein synthesis by TSP (3 – 30 kDa fraction) and possible cofactor (< 3 kDa fraction) in the rabbit reticulocyte lysate assay using 2 µg of mRNA from various tissues of *H. virescens* (n = 10).
Figure 12: Mean percent reduction (± SE) of protein synthesis by TSP (3 – 30 kDa fraction) and possible cofactor (< 3 kDa fraction) in the rabbit reticulocyte lysate assay using 2µ of mRNA from various tissues of *M. sexta* (n = 10).
When mRNA from *M. sexta* was used, the whole body homogenate and the nerve cord showed no significant reduction in protein synthesis (49% with TSP, 52% with TSP and the added cofactor, 56% with TSP, and 60% with TSP and the added cofactor respectively). When mRNA from gut tissue and labial glands was used, there was less inhibition of protein synthesis when the cofactor was added with the TSP. Inhibition of protein synthesis dropped 36% in the samples from gut tissue and 6% in samples from labial glands. The remaining two samples, hemolymph and fat body, showed a significant increase in protein synthesis inhibition (Table 12).

These results are consistent with Schepers’ (1995) findings that protein synthesis inhibition was significantly increased in the fat body of not only *H. virescens*, but *M. sexta*, as well. The fact that other tissues did not react in the same manner, however, could indicate the possible existence of some small proteins secreted by teratocytes that have tissue specific inhibitory properties, but are unrelated to those exhibited by TSP.

The rabbit reticulocyte lysate assay is the classic cell free animal system, but we also conducted preliminary studies on the effect of TSP on the classic cell free plant assay (wheat germ extract assay). The wheat germ extract assay was very similar to the rabbit reticulocyte lysate assay with the obvious exception of that the translational machinery was of wheat germ (plant) origin as opposed to rabbit reticulocytes. When we performed the wheat germ extract assay using mRNA from various *H. virescens* tissues, TSP significantly inhibited protein synthesis from all sources of mRNA tested. Overall, TSP inhibited the synthesis of new proteins by 47±1%. There was no significant difference in the percent reduction among the sources of mRNA from the *H. virescens* tissues (37-57%). (Table 13), (Figure 13). TSP produced similar inhibitory effects in the rabbit reticulocyte lysate assay and the wheat germ extract assay, yielding 35 ± 2% and 47±1% reduction of new protein synthesis respectively with respect to *H. virescens* tissue mRNA.


**Table 13:** Effects of TSP (3 – 30 kDa fraction) on protein synthesis initiated by 2 µg mRNA from different sources of *H. virescens* (dpm ± SE) using wheat germ extract assay. Student’s T–tests performed on control vs. TSP treatments of the same mRNA source. * P–value less than 0.05 in paired Student’s T–test (n = 10).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>TSP</th>
<th>Mean % Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brome Mosaic</td>
<td>64,224 ± 997</td>
<td>27,683 ± 755</td>
<td>57 ± 1%*</td>
</tr>
<tr>
<td>Homogenate</td>
<td>61,497 ± 1,027</td>
<td>26,414 ± 1,259</td>
<td>57 ± 2%*</td>
</tr>
<tr>
<td>Hemolymph</td>
<td>56,553 ± 767</td>
<td>34,926 ± 1,572</td>
<td>38 ± 3%*</td>
</tr>
<tr>
<td>Gut</td>
<td>66,270 ± 1,127</td>
<td>33,269 ± 1,439</td>
<td>50 ± 2%*</td>
</tr>
<tr>
<td>Fat Body</td>
<td>57,038 ± 859</td>
<td>27,372 ± 1,045</td>
<td>52 ± 1%*</td>
</tr>
<tr>
<td>Testes</td>
<td>62,040 ± 1,389</td>
<td>33,486 ± 1,289</td>
<td>46 ± 2%*</td>
</tr>
<tr>
<td>Labial Glands</td>
<td>66,698 ± 1,213</td>
<td>31,665 ± 969</td>
<td>52 ± 2%*</td>
</tr>
<tr>
<td>Nerve Cord</td>
<td>60,911 ± 2,126</td>
<td>37,971 ± 958</td>
<td>37 ± 3%*</td>
</tr>
</tbody>
</table>
Figure 13: Percent reduction (± SE) of protein synthesis by TSP (3 – 30 kDa fraction) in the wheat germ extract assay using 2 µg of mRNA from various tissues of *H. virescens* (n = 10).
TSP14 Production

As mentioned earlier, the ability to produce large quantities of TSP14 would make a significant contribution towards future studies and possible applications of TSP14. Recombinant expression of TSP14 in a cell culture was one possibility for this mass production. At the suggestion of Dr. Bruce Webb, we used the pAd. CMV–Link: 1 baculovirus expression vector containing the TSP14–polyhistidine fusion insert for this work. The Sf–9 and Tn–5 insect cell cultures were chosen because they were readily available to our laboratory and were standard cultures for baculovirus infection. Initial attempts at in vitro production of TSP14 using Sf–9 and Tn–5 cells infected with recombinant baculovirus produced an average of 26.5±0.7 µg TSP14 per 150 ml cell culture flask, far less than expected. The Sf–9 and Tn–5 cell cultures produced an average of 26.6±0.8 µg and 26.3±1.2 µg respectively (t= 0.22, p=0.83) (Figure 14). When the effectiveness of the two cell lines was compared, we found no significant difference between the Sf-9 and the Tn-5 cells.

Because our attempts to produce TSP14 in cell cultures yielded disappointing amounts of biologically active protein, we turned our attention to alternative methods. We tried to produce TSP14 in vivo in an organism that had shown to be resistant to TSP. Zhang (unpublished data) showed that TSP injected into M. sexta larvae had no significant developmental effects. Thus, M. sexta was chosen due to its in vivo resistance to TSP as well as the fact it was a fairly large larva that could be easily handled and manipulated. Two separate methods were investigated, one involved the larva ingesting recombinant baculovirus added to their food, and the other involved injecting the larva with recombinant baculovirus. An average of 9.7±0.4 µg TSP14 per larva was produced via the injection method while the ingestion method was slightly less productive, yielding only 8.7±0.5 µg per larva (t= 1.66, p=0.10) (Figure 15).
Figure 14: Micrograms of recombinant TSP14 produced by a single 150 ml cell culture flask when the cells were infected with the recombinant baculovirus expression vector, pAd.CMV–Link.1 containing the TSP14/polyhistidine fusion insert. TSP14 was isolated using a Ni–NTA resin (n = 42).
**Figure 15:** Micrograms of recombinant TSP14 produced by a single *M. sexta* larvae when inoculated with the recombinant baculovirus expression vector, pAd CMV–Link.1 containing the TSP14/polyhistidine fusion insert. TSP14 was isolated using a Ni–NTA resin (n = 24).
Efficacy and Stability of TSP14

Although production of TSP14 was not as successful as had been anticipated, we were able to produce enough to study its biological inhibitory capacity. Because much less TSP14 could be used in the rabbit reticulocyte lysate assay as compared to the \textit{in vitro} testes assay, we chose to study the effectiveness of TSP14 in the rabbit reticulocyte lysate assay. When TSP14 was introduced to the rabbit reticulocyte lysate assay with the mRNA from the various tissues and two different species, the synthesis of new proteins was significantly reduced with every source of mRNA tested from both species with remarkable uniformity (Table 14) (Table 15) (Figure 16) (Figure 17). Overall, TSP14 inhibited the synthesis of new proteins by 66.2 ± 0.4%. Protein synthesis inhibition was almost identical between both species with translation of the mRNA from \textit{H. virescens} tissues being inhibited 65.8 ± 0.6% and the translation of mRNA from \textit{M. sexta} tissues was inhibited 66.0 ± 0.7%. When we compared the different tissues from the same species, there was no significant difference in the percent reduction of protein synthesis. When the mRNA from the same organ in different species was compared, the organs reacted in similar manners regardless of the species (Table 14) (Table 15) (Figure 16) (Figure 17).

We had already shown that crude TSP inhibited protein synthesis in a dose dependent manner, so we investigated the dose response to TSP14 with the rabbit reticulocyte lysate assay. When various doses of TSP14 were introduced to the rabbit reticulocyte lysate assay, the synthesis of new proteins was also reduced in a dose dependant manner. The dose response expressed as percent reduction of protein synthesis appears to be linear between 0.0 (0 % reduction) and 0.8 (76 ± 1%) micrograms of TSP14. Beyond 0.8 μg/sample, the inhibition plateaued with only a 4% increase in percent reduction between 0.8 and 1.0 μg of TSP14 (Table 16) (Figure 18).
**Table 14:** Effect of recombinant TSP14 on protein synthesis initiated by 2 µg mRNA from different sources of *H. virescens* (dpm ± SE). Student T–test performed on control vs. TSP14 treatments of the same RNA source. TSP14–treated groups received 0.8 µg/sample.

* P–value less than 0.05 in paired Student’s T–test (n = 10).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>TSP14</th>
<th>Mean %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luciferase</td>
<td>78,719 ± 2,450</td>
<td>23,771 ± 1,218</td>
<td>70 ± 1 %</td>
</tr>
<tr>
<td>Homogenate</td>
<td>78,769 ± 1,123</td>
<td>26,448 ± 1,349</td>
<td>66 ± 2 %</td>
</tr>
<tr>
<td>Hemolymph</td>
<td>78,132 ± 1,031</td>
<td>26,012 ± 1,074</td>
<td>67 ± 1 %</td>
</tr>
<tr>
<td>Gut</td>
<td>72,623 ± 654</td>
<td>25,981 ± 967</td>
<td>64 ± 2 %</td>
</tr>
<tr>
<td>Fat Body</td>
<td>79,236 ± 1,209</td>
<td>22,585 ± 514</td>
<td>71 ± 1 %</td>
</tr>
<tr>
<td>Testes</td>
<td>74,362 ± 1,443</td>
<td>27,652 ± 939</td>
<td>63 ± 2 %</td>
</tr>
<tr>
<td>Labial Glands</td>
<td>72,075 ± 927</td>
<td>25,356 ± 692</td>
<td>65 ± 1 %</td>
</tr>
<tr>
<td>Nerve Cord</td>
<td>76,423 ± 845</td>
<td>27,027 ± 899</td>
<td>65 ± 1 %</td>
</tr>
</tbody>
</table>
Table 15: Effect of recombinant TSP14 on protein synthesis initiated by 2 µg mRNA from different sources of *M. sexta* (dpm ± SE). Student t-test performed on control vs. TSP14 treatments of the same mRNA source. TSP14–treated groups received 0.8 µg TSP14 (n = 10).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>TSP14</th>
<th>Mean % Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luciferase Control</td>
<td>78,719 ± 2,450</td>
<td>23,771 ± 1,218</td>
<td>70 ± 1 %</td>
</tr>
<tr>
<td>Homogenate</td>
<td>76,712 ± 1,432</td>
<td>23,697 ± 816</td>
<td>69 ± 1 %</td>
</tr>
<tr>
<td>Hemolymph</td>
<td>75,738 ± 12,667</td>
<td>24,506 ± 1,065</td>
<td>68 ± 1 %</td>
</tr>
<tr>
<td>Gut</td>
<td>72,370 ± 1,149</td>
<td>27,830 ± 967</td>
<td>61 ± 2 %</td>
</tr>
<tr>
<td>Fat Body</td>
<td>77,977 ± 962</td>
<td>26,542 ± 1,081</td>
<td>66 ± 2 %</td>
</tr>
<tr>
<td>Labial Glands</td>
<td>78,002 ± 1,578</td>
<td>26,432 ± 1,245</td>
<td>66 ± 2 %</td>
</tr>
<tr>
<td>Nerve Cord</td>
<td>78,651 ± 1,024</td>
<td>26,685 ± 1,057</td>
<td>66 ± 1 %</td>
</tr>
</tbody>
</table>
Table 16: Dose response to recombinant TSP14 of *H. virescens* homogenate 2µg mRNA initiating protein synthesis in a rabbit reticulocyte lysate assay (dpm ± SE) 

(n = 15).

<table>
<thead>
<tr>
<th>Micrograms of TSP14</th>
<th>$[^{35}S]$-methionine incorporation (dpm/sample)</th>
<th>Mean % Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>76,681 ± 987</td>
<td>N/A</td>
</tr>
<tr>
<td>0.1</td>
<td>64,266 ± 990</td>
<td>16 ± 2 %</td>
</tr>
<tr>
<td>0.2</td>
<td>58,282 ± 824</td>
<td>24 ± 2 %</td>
</tr>
<tr>
<td>0.3</td>
<td>55,857 ± 727</td>
<td>27 ± 1 %</td>
</tr>
<tr>
<td>0.4</td>
<td>52,432 ± 642</td>
<td>31 ± 1 %</td>
</tr>
<tr>
<td>0.5</td>
<td>47,139 ± 1,086</td>
<td>38 ± 2 %</td>
</tr>
<tr>
<td>0.6</td>
<td>36,884 ± 828</td>
<td>52 ± 1 %</td>
</tr>
<tr>
<td>0.7</td>
<td>29,158 ± 857</td>
<td>62 ± 1 %</td>
</tr>
<tr>
<td>0.8</td>
<td>18,415 ± 719</td>
<td>76 ± 1 %</td>
</tr>
<tr>
<td>0.9</td>
<td>17,542 ± 593</td>
<td>77 ± 1 %</td>
</tr>
<tr>
<td>1.0</td>
<td>15,370 ± 558</td>
<td>80 ± 1 %</td>
</tr>
</tbody>
</table>
Figure 16: Percent reduction (± SE) of protein synthesis by recombinant TSP14 in the rabbit reticulocyte lysate assay using mRNA from various tissues of *H. virescens*. TSP14 treated groups received 0.8 µg / sample (n = 10).
Figure 17: Percent reduction (± SE) of protein synthesis by recombinant TSP14 in the rabbit reticulocyte lysate assay using 2µg of mRNA from various tissues of *M. sexta*. TSP14–treated groups received 0.8 µg / sample (n = 10).
Figure 18: Dose response of *H. virescens* homogenates to recombinant TSP14 in rabbit reticulocyte lysate assay with 2 µg mRNA (n = 15).
Localization of TSP

Since it was determined that in cell free systems, TSP acts directly on translation, it must enter the cells of these tissues (testes, fat body) known to be affected by incubation with TSP. Preliminary studies were conducted to test TSP’s ability to gain access to the hosts’ cytosol by utilizing the in vitro testes assay. The testes were incubated in the presence of TSP for either 4, 5, 8, 12, 16 or 30 minutes. The testes were then washed and allowed to continue their 4 hr incubation in the absence of TSP. The testes that remained in TSP for 4 hr showed an average reduction in protein synthesis of 70±1 %, while the washed testes showed an average reduction of protein synthesis of about 28±3 %. After being washed, there was an average recovery of protein synthesis of 60±3 % (Table 17).

Ascertaining that there was no significant difference in the inhibition of protein synthesis with the various wash times might indicate that TSP initially enters the cell, then once inside the cytosol, can actively inhibit protein synthesis for up to 30 minutes. This inhibition, however, does not last for the remainder of the assay. The fact that protein synthesis partially recovers after the testes were washed leads us to believe one or both of two options. First, that TSP entered the cytosol, but is also was expelled, thus facilitating the intake of new TSP from the external cellular environment. Second, that after a certain amount of time, greater than 30 minutes and less than four hours, TSP was metabolized, dissociated, or some other way rendered biologically inactive.

Dahlman et al. (2003) showed that purified TSP antiserum only reacted with TSP on Western blots of hemolymph from parasitized H. virescens from three days following parasitization (one day after egg hatch) through the time of parasite egression. The TSP signal declined rapidly once the parasite larvae exited from the host. Therefore, we chose to use H. virescens larvae six days or more days post–parasitization (and appropriate non–parasitized controls to study the immunohistochemistry of their tissues. Paraffin sections of larvae were probed with TSP antiserum and developed using BCIP/NBT solution. Overall sections from non–parasitized larvae showed very little reaction between the BCIP/NBT and alkaline phosphatase attached to the TSP antibody (dark blue/brown), when compared to tissue from larvae six days post–parasitization (Figures 19 and 20). It was assumed that the darker color indicated a higher concentration of the presumed presence of TSP in the tissue. Cellular differentiation was difficult because no other dyes were used.
Table 17: Percent reduction of $[^{35}\text{S}]$–methionine incorporation in *in vitro* *H. virescens* testes assay and the percent of protein synthesis recuperation after washing the treated testes (n = 24).

<table>
<thead>
<tr>
<th></th>
<th>TSP Control 4hr incubation</th>
<th>Wash</th>
<th>Recovery of protein synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 min wash</td>
<td>69.8±1.6 %</td>
<td>27.7±2.9 %</td>
<td>60.0±4.1 %</td>
</tr>
<tr>
<td>5 min wash</td>
<td>70.4±1.8 %</td>
<td>24.6±3.2 %</td>
<td>65.4±4.4 %</td>
</tr>
<tr>
<td>8 min wash</td>
<td>70.2±1.8 %</td>
<td>34.9±2.3 %</td>
<td>49.6±3.6 %</td>
</tr>
<tr>
<td>12 min wash</td>
<td>70.0±1.6 %</td>
<td>26.7±3.3 %</td>
<td>61.6±4.6 %</td>
</tr>
<tr>
<td>16 min wash</td>
<td>72.1±1.2 %</td>
<td>29.4±2.7 %</td>
<td>58.7±3.9 %</td>
</tr>
<tr>
<td>30 min wash</td>
<td>70.4±2.0 %</td>
<td>25.4±2.7 %</td>
<td>64.1±3.4 %</td>
</tr>
</tbody>
</table>
Figure 19: Cross–section of a control *H. virescens* larva of the same age as six days post–parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT magnified 100X. Note cross-sections through Malpighian tubules (MT) in lower right quadrant; cross-section through gut (G) at 7 O’clock, and fat body (FB) occupying the upper center area, and darkly colored integument (I) visible at 3 and 9 O’clock.
Figure 20: Cross-section of a *H. virescens* larva six days post-parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT magnified 100X. Note cross-section of the gut (G) and contents located in the lower middle portion, surrounded with smaller circles of cross-sections of Malpighian tubules (MT). This section also contains fat bodies (FB) (10 O’clock) and more lightly staining strips of muscle (M) (8-9 O’clock and 11 O’clock). Note that the overall appearance of the tissues is stained darker than the control tissues in Figure 19.
The Malpighian tubules in parasitized larvae responded very intensely to the antiserum (Figure 21 vs. Figure 22). Fat body also was darkly stained in parasitized larvae (Figure 23 vs. Figure 24). In contrast, muscle did not show as intense a response as either Malpighian tubules and fat body (Figure 23 vs. Figure 24 compared to Figure 25 vs. Figure 26).

Sections were also made of testes removed from insects seven days after parasitization. The testes from parasitized larvae showed similar results to the whole insect cross–sections (Figure 27 vs. Figure 28), with much of the reaction occurring on the cell membrane, but also seen on the cytoplasm (Figure 29 vs. Figure 30). The other very noticeable result of this work was the substantial developmental retardation of the testes from parasitized individuals when compared to the testes of non–parasitized larvae. Testes from parasitized larvae were significantly smaller and seemed to lack the cellular organization of the non–parasitized counterparts.
Figure 21: Cross-section of a control *H. virescens* larva of the same age as six days post-parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (400X). Cross-sections through the Malpighian tubules (MT). Note relatively pale colors of all tissues.
Figure 22: Cross-section of a *H. virescens* larva six days post-parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (400X). Sections through Malpighian tubules (MT). Note relatively intense staining, presumed to be from reactions of the TSP14 antiserum to the presence of TSP in these tissues.
Figure 23: Cross-section of a control *H. virescens* larva of the same age as six days post-parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (400X). Note sections through fat body (FB) (1–2 O’clock), muscle (M) (large area extending from 11 O’clock to the right lower corner), and trachea (T) (lower left corner). Note the relatively pale color of all tissues.
Figure 24: Cross-section of *H. virescens* larva six days post-parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (400X). Note sections through muscle (M) running from 12 to 6 O’clock surrounded by fat body (FB). Sections through the Malpighian tubules (MT) are visible at 4–5 O’clock. Note relatively intense staining of fat body and Malpighian tubules in contrast of the control (Figure 23). Muscle stained somewhat darker than controls but less intensely than other tissues. The darker staining is presumed to be from reactions of the TSP 14 antiserum to the presence of TSP in these tissues.
Figure 25: Cross-section of a control *H. virescens* larva of the same age as six days post-parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT magnified (400X). Note the section through a trachea (T) (running from 12 to 6 O’clock) with muscle tissue (M) parallel on both sides. Note relatively pale staining of all tissue.
**Figure 26:** Cross-section of *H. virescens* larva six days post–parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (400X). Note section through trachea (T) (center to photo to 2 O’clock) and muscle (M) (cross–sections running from 5 to 11 O’clock). Note intense staining of these tissues compared to the control (Figure 25), presumably from the reaction of the TSP14 antiserum to the presence of TSP in these muscles.
Figure 27: Cross-section of a control *H. virescens* testis (Te) of the same age as seven days post–parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (100X). Note the relatively unstained condition of this tissue.
Figure 28: Cross-section of a *H. virescens* testis (Te) seven days post–parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (100X). Note the much smaller size and the intense staining (compared to the control in Figure 27), presumably from the reaction of the TSP14 antiserum to the presence of TSP in this tissue.
Figure 29: Cross-section of a control *H. virescens* testis (Te) of the same age as seven days post–parasitization. Probed with antibodies against TSP14 and developed with BCIP/MBT (400X). Note the relatively unstained condition of both the cell membranes and cytoplasm.
Figure 30: Cross-section of a *H. virescens* testis (Te) seven days post-parasitization of the experimental group probed with antibodies against TSP14 and developed with BCIP/MBT magnified 400X. Note the relatively intense staining of both the cell membranes and cytoplasm, presumably from the reaction of the TSP antiserum to the presence of TSP in this tissue.
DISCUSSION

It is widely accepted that endoparasitic wasps have the ability to manipulate their hosts’ physiology for the purpose of survival. Physical symptoms of this manipulation in the *M. croceipes*–*H. virescens* system are a significant inhibition of host growth as well as an inability to progress past the wandering pre–pupal stage of development. Internally, there is a disruption of the host’s immune system as well as reduced titers of such proteins as arylphorins and juvenile hormone esterase as well as ecdysteroids. *M. croceipes* teratocytes and their secreted proteins (TSP) are active participants in the physiological and developmental arrest of the *H. virescens* host (Dahlman and Vinson, 1993) (Schepers et al., 1998).

Prior to this study, the *in vitro* tissue assay of choice to study the effects of teratocytes and TSP was the fat body assay. This assay was utilized because the fat body produces an abundance of three arylphorins (storage proteins) of 74, 76, and 82 kDa respectively. The presence and relative quantities of these proteins can easily be verified by SDS–PAGE, making them an excellent tool for monitoring protein production. In the fat body assay, the fat body had to be carefully removed from the larvae and then washed four or five times. This was a somewhat labor intensive process, and quite variable because of the amorphous nature of this tissue. Miller et al. (1990), showed that the 76 kDa and 82 kDa arylphorins were also produced by the testes sheath of *H. virescens*. This led us to try testes as a possible replacement tissue instead of fat body. Preliminary studies showed that $^{35}$S–methionine incorporation was reduced when testes were incubated with TSP.

Testes proved to be a better tissue for our purposes for several reasons. Testes were a nicely packaged tissue, relatively easy to locate and remove, leading to much less variation in tissue mass collected per larva. Testes required much less cleaning than fat body. The size and sensitivity of the testes allowed us to reduce the volume of the assay, thus reducing the required amount of TSP and radioisotopes.

Further studies showing that the two testes from each individual were nearly the same size and they reacted in an identical manner to TSP. This allowed us to use one testes from an individual as a control and the other as the experimental subject. This not only removed the variability between tissues from different individuals, but also negated the need to weigh each
sample. The use of this assay, and the small amount of material required, further facilitated some of the following studies that required large number of samples.

Also, to this point, the standard testes assay followed a rigid protocol derived from the fat body assay. The assay used three larval equivalents of TSP and incubated them for four hours. Though these studies showed that the percent reduction of protein synthesis increased somewhat linearly with respect to time, it also showed that the rate of reduction seemed to tail off after one hour. This information allowed the assay to be done in considerably less time than the previous fat body assay.

As mentioned earlier, the fat body assay was conducted using three larval equivalents of TSP. We asked the question, however, whether TSP was acting in a graded or a quantal manner. If TSP had a graded effect, the magnitude of the response would be proportional to the amount of TSP present in the system. If the response was quantal, the amount of TSP would not matter—as long as it was present at a threshold level that would achieve a certain effect on a designated number of individuals.

We found that TSP acts in a graded manner—it is dose dependent. We found that TSP's effectiveness increased with dose. The greatest dose–dependent relationship occurred between 0.1 and 1.0 larval equivalents when a four–hour incubation time was used. This allowed for less TSP to be used. We found, moreover, that this graded response could be illustrated even more effectively if we reduced the incubation time of the assay to one hour. This assay showed an almost linear relationship between the amount of TSP and the magnitude of the inhibitory response. This information about both testes and TSP gave us significant advances over the previous fat body assay, allowing us to reduce the amount of materials, especially the TSP and the radioactive isotopes, and also reducing the amount of time required for each assay.

Once we concluded that TSP reduced the amount of proteins produced in an in vitro system, we questioned where in the process of protein synthesis did this inhibition occur. A reduction in protein production can be attributed to a disruption anywhere between DNA replication to translation. For example, aflatoxins are considered genotoxins because they cause mutations in the DNA (Cleveland et al., 2003), thus resulting in improper transcription. Amanitin is a protein from a toadstool that inhibits the synthesis of RNA by hindering RNA polymerase activity (Vetter, 1998). Diphtheria toxin (Ratts et al., 2003) and ricin (Sandvig et al., 2002) are toxins that directly affect protein synthesis that have been mentioned earlier.
We chose to address the issue of where along the process of protein synthesis did the inhibition occur by first conducting thymidine and uridine incorporation assays. If the inhibition occurred in DNA replication, we would see a reduction in thymidine incorporation. If the inhibition occurred in RNA synthesis, then there would be a reduction in uridine incorporation. Since TSP did not cause any significant reduction in the incorporation of either thymidine or uridine, it could be concluded that TSP acts directly on the mechanism of translation or the assembly of amino acids in protein synthesis.

To further study the process of protein synthesis, we chose to eliminate the possibility of inhibition via a cell surface receptor mechanism by using cell free systems. We used primarily the rabbit reticulocyte lysates for this purpose, although the wheat germ extract assay also was used to determine whether there was a difference between an animal based and a plant based assay. Once it was determined that there was no difference in the responsiveness between the two assays, we exclusively used the rabbit reticulocyte lysate assay.

In his 1995 thesis, Schepers identified the active component of TSP in the 3–30 kDa fraction when filtered through molecular weight cutoff filters. He noticed, however, that some portion of the active protein was getting through, or that there was a protein in the low molecular fraction that also was biologically active. While performing the rabbit reticulocyte lysate assay we investigated this phenomenon.

We found that, as we expected, the 3–30 kDa fraction inhibited protein synthesis. The 3kDa filtrate had no biological activity on its own, however, when the two fractions were combined, there seemed to be an enhancement of the protein synthesis inhibition. This lead us to believe that components in the 3 kDa filtrate somehow assist the biologically active fraction, but cannot inhibit protein synthesis on their own. They may do this by acting in an enzyme–like manner, facilitating binding to ribosomes or an initiation or elongation factor, but only affecting the rate of binding, not allowing the binding to occur.

At approximately this time, Dr. Bruce Webb cloned a cDNA from the biologically active (3–30 kDa) fraction of TSP to encode for a 14kDa protein. This cDNA was modified by the addition of a poly–histidine tag to facilitate the purification of the target protein. That cDNA, when expressed in a recombinant baculovirus system and purified with a nickel ion column, yielded a 14 kDa protein, which Webb and Dahlman have termed TSP14. Since TSP14 is encoded by a cDNA clone of a biologically active protein in M. croceipes TSP, it should have
similar inhibitory properties as its counterpart secreted from live teratocytes. Due to its ability to show observable results with small amounts of TSP14 used, the rabbit reticulocyte lysate assay was chosen to study the effects of TSP14.

The recombinant TSP14 behaved exactly as expected in the rabbit reticulocyte lysate assay. Just as TSP had done, it inhibited protein synthesis from all sources of mRNA. It also inhibited protein synthesis in a graded manner, much like TSP. Protein synthesis was inhibited in a linear dose dependent manner starting at 0.1 µg, and the inhibitory effects seem to plateau at approximately 0.8 µg. with, however, a slight increase up through 1.0 µg with TSP14. The remarkable aspect is that TSP14 has an inhibitory effect at the miniscule concentration of 0.14 µM.

The results of the above studies demonstrated a need for production of TSP14. As mentioned earlier, the poly–histidine TSP14 was expressed in a baculovirus system. We originally produced the TSP14 by infecting cell cultures of Sf–9 and Tn–5 cells, then insolating the TSP14. Using this method yielded an average of 27 µg of TSP14 per preparation. But Webb (personal communication) stated that similar preparations usually should yield 1–3 mg of the target protein. We believe that the difference stems from the fact that the TSP14, after being secreted by the baculovirus infected cells, binds to the receptors on the cell membrane and is retaken up by the cell – thus inhibiting its own further translation – a kind of feedback inhibition.

Although impaired, this system had the ability to produce enough TSP14 for our studies. It would be beneficial, however, to be able to make fairly large quantities of TSP14 (mg quantities). Such quantities could facilitate more in–depth and diverse research on its inhibitory properties.

In our efforts to be able to make more TSP14 we explored Maeda’s (1988) methods. Maeda expressed baculovirus–coded proteins in vivo in the larvae of the silkworm Bobmyx mori. We chose to substitute M. sexta for B. mori. We did this because M. sexta had many of the same favorable characteristics as B. mori –for example, it is large and easy to handle, and also because our lab was familiar with M. sexta and already had a colony. Further, in previous unpublished studies from our lab, it was shown that injected TSP and teratocytes had no development effects on M. sexta larvae, and that TSP had no effect on tissues from M. sexta in in vitro tissue assays. Gretch et al. (1991) showed that M. sexta could be used to make proteins expressed in the AcMNPV baculovirus.
The results were not quite what we had expected. The larvae produced an average of less than 10 µg of TSP14 per larva. While we were working with Maeda's method, I read the findings of Cha et al. (1997), who inoculated the hornworms via oral ingestion as opposed to injection. Cha reported a yield of 1.58 mg of a Green Fluorescent Protein per larvae. Our results were not nearly that fruitful; with yields still averaging less than 10 µg of TSP14 per larva. Our results with this method were almost identical to those from the injection method. It was not a surprise the ingestion method did not work well because our TSP16 gene was located in the polyhedrin locus, thus leaving the DNA susceptible to degradation in the acidic environment of the insect’s digestive track. Though I am not sure why the injection method did not work well, there still may be the possibility of using constructs with different parameters, or the possibility of constructing a recombinant adenovirus to be expressed in mammalian cells. Moreover, since the completion of these experiments, Rana et al. (2002) has successfully produced the TSP14 protein in a yeast expression system. This protein is biologically active and can be produced in quite large quantities, thus allowing for the more extensive studies on the inhibitory mechanism of TSP14.

The localization of TSP, as it inhibited protein synthesis, was first brought to our attention when we noticed that washing testes incubated with TSP allowed for approximately a 60% recovery of protein synthesis, but could not completely restore it. This indicated that TSP was entering the cells to act on the translation process. The fact that the time elapsing from when the TSP was added to when it was washed off did not seem to effect the recovery of protein synthesis was difficult to explain. This could result, however, from the relatively rapid entry of TSP into the cell (in less than four minutes), compared with the relatively slow passage of time while TSP is active inside the cell, probably greater than 30 minutes, before it dissociates (or is enzymatically rendered inactive). TSP’s ability to enter the cell was further verified by the inhibition in the cell free systems like the rabbit reticulocyte lysate and the wheat germ extract. Neither of the above, however, was considered definitive proof. This proof came with the ability to conduct immunohistochemistry studies using an antibody made by a rabbit against TSP14. We were then able to show TSP inside the cells of parasitized H. virescens larva. Though the majority of the signal appears to be on the cell surface, this is completely understandable. It is believed recTSP14, a recombinant TSP14 protein produced by a yeast expression system (Rana et al., 2002) may require a specific factor, such as a membrane-bound cell surface receptor, to
facilitate entry into target cells. The researchers also believed that this factor was only found in certain cell types. This corresponds to why certain tissues develop a much greater signal than others.

Also, while examining the immunohistochemical slides, it was evident that parasitization caused significant developmental retardation, especially noticeable in the testes. The testes themselves were approximately ¼ the size of their unparasitized counterparts of the same chronological age. Moreover, the testes of unparasitized individuals seem to have a very definite, highly organized structure, while the testes of parasitized individuals seemed to possess a less defined internal structure. By means of observation while making the paraffin sections, the testes of parasitized individuals were found to be much more fragile than those from the unparasitized. The irreversible destruction of reproductive tissue of immature male insects in a host–parasitoid system, or parasitic castration, is a well–documented phenomenon (Reed and Beckage, 1997). It is believed that this is just one more way that parasitoids manipulate the host physiology in order to ensure survival. The intense positive signal for TSP indicates that TSP may play a significant role in parasitic castration.
REFERENCES


VITA

Francis Anthony DiLuna
Born January 2, 1971 in Winchester, Massachusetts

Education
Bachelor of Science with Honors in Biology 1993
Bates College Lewiston, Maine
Thesis: Secondary effects of azadirachtin, a botanical insecticide, in the food chain.

Professional Positions
Science Teacher 2001 – present
Woburn High School Woburn, Massachusetts

Senior Scientist 1999 – 2001
Serono Reproductive Biology Institute Randolph, Massachusetts

Scholastic and Professional Honors
Sigma Xi 1993
Ohio Valley Entomology Association Student Paper Competition
Second Place 1995
Third Place 1997

Professional Memberships
Society of Toxicology
Network of Educators in Science and Technology