



128
536
THS

THESIS

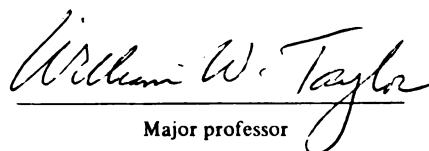
1



This is to certify that the
thesis entitled
An Immunological Approach to Identify Larval
Rainbow Smelt Remains in the Stomach
Contents of Yearling Rainbow Smelt

presented by
Ted J. Sledge

has been accepted towards fulfillment
of the requirements for
Master of Science degree in Fish. & Wildl.


Major professor

Date January 4, 1995

**LIBRARY
Michigan State
University**

PLACE IN RETURN BOX to remove this checkout from your record.
TO AVOID FINES return on or before date due.

DATE DUE	DATE DUE	DATE DUE
NOV 03 2005	NOV 02 2007	

AN IMMUNOLOGICAL APPROACH TO IDENTIFY LARVAL RAINBOW SMELT
REMAINS IN THE STOMACH CONTENTS OF YEARLING RAINBOW SMELT

By

Ted J. Sledge

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Fisheries and Wildlife

1995

ABSTRACT

AN IMMUNOLOGICAL APPROACH TO IDENTIFY LARVAL RAINBOW SMELT REMAINS IN THE STOMACH CONTENTS OF YEARLING RAINBOW SMELT

By

Ted J. Sledge

Taxonomic identification of larval fish in stomach contents of fish predators is difficult because larval fish are rapidly digested and become quickly unidentifiable. To investigate an alternative method to visual identification for detecting larval fish in the stomach contents of predator fish, an immunoblot technique was utilized to detect larval rainbow smelt (*Osmerus mordax*) soluble proteins in the stomach contents of yearling rainbow smelt. An antiserum was produced from soluble proteins of larval rainbow smelt and was evaluated to determine its ability to distinguish larval smelt proteins from heterologous proteins. The antiserum immunoreacted with other fish species; however, the antiserum did not cross react with invertebrate prey items. A "key band" was identified in the immunoreactive banding sequence of larval rainbow smelt protein extract and was useful in differentiating larval rainbow smelt proteins from other fish species. Thus immunoblots have potential to identify larval rainbow smelt proteins in the stomachs of yearlings by utilizing the "key band" as evidence of larval smelt.

ACKNOWLEDGEMENTS

This research was sponsored by the Michigan Sea Grant College Program, project numbers R/GLF-35 and R/GLF-42, under grant number NA89AA-D-SG083 from the Office of Sea Grant, National Oceanic and Atmospheric Administration (NOAA), U.S. Department of Commerce, and funds from the State of Michigan. Additional support was provided by the Michigan Polar-Equator Club.

I would first like to thank Dr. William Taylor for providing me the opportunity for graduate studies in the fisheries field and his following support and guidance during my graduate career at Michigan State. I also thank the other members of my thesis guidance committee, Dr. Niles Kevern and Dr. John Wilson for their support and advice. A special thanks goes to Dr. John Wilson and his students in the biochemistry laboratory for their assistance and patience.

I especially thank the my fellow graduate students, Russ Brown, Paola Ferreri, Salvador Becerra, Ed Roseman, and Shawn Sitar for their support, suggestions, and friendship. I thank Russ Brown, Ed Roseman, Shawn Sitar, Salvador Becerra; and student interns Randy Claramunt, Mike Winters, and Eric Grundemann for their assistance during field

sampling. In addition, I thank Jeremy Wilder, Daniel Weisenreder, and Eric Grundemann for their assistance in processing samples in the laboratory.

Finally, I would like to thank my family for their love and encouragement, especially my wife, Heather, who insisted I follow my dreams.

TABLE OF CONTENTS

LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF APPENDICES	xi
INTRODUCTION	1
MATERIALS AND METHODS	5
Immunological Methodology Overview	5
Rainbow Smelt, Fish, and Prey Item Collection	10
Soluble Protein Extract Preparation	12
Smelt Stomach Content Sample Preparation	12
Antigen Preparation	14
Antiserum Production	15
Titre Evaluation	15
Cross Reactivity	15
RESULTS	18
Titre of Antisera	18
Competitive Elisa	21
Immunoblot	21
Larval Smelt Key Band Utility	27
Larval Smelt Key Band Limitations	34
DISCUSSION	39
APPENDICES	45
LIST OF REFERENCES	60

LIST OF TABLES

Table 1.	Extracts and concentrations used in competitive ELISA and immunoblots.....	13
Table 2.	Immunoreactivity with antisera raised to rainbow smelt larvae.....	22
Table 3.	Mean absorbance values (490 nm) for competitive ELISA experiment using 100 ug of each extract...	23

LIST OF FIGURES

Figure 1.	Flow diagram illustrating the steps in production of the larval smelt antiserum....	6
Figure 2.	Flow diagram illustrating the steps in evaluating the larval smelt antiserum.....	8
Figure 3.	Illustration of an (A) enzyme linked immunosorbent assay (ELISA) and a (B) competitive ELISA.....	9
Figure 4.	Illustration of an immunoblot.....	11
Figure 5.	Form of raw data output generated by the gel documentation software.....	17
Figure 6.	Pre-immune and first bleed titre evaluation.....	19
Figure 7.	First bleed and second bleed titre evaluation.....	20
Figure 8.	Immunoblot experiment using larval smelt protein probed with larval rainbow smelt antiserum.....	24
Figure 9.	Molecular weight determination of the larval smelt key band.....	25
Figure 10.	Larval smelt key band immunoreactivity of pure larval smelt extract.....	26
Figure 11.	Immunoblot for single-prey experiment probed with larval rainbow smelt antiserum.....	28
Figure 12.	Immunoblot for single-fish species experiment probed with larval rainbow smelt antiserum.....	29
Figure 13.	Larval smelt key band immunoreactivity of larval smelt extract compared to other fish extracts.....	30

Figure 14.	Immunoblot experiment illustrating the utility of the larval smelt antiserum in detecting the larval smelt key band when in combination with an abundance of heterologous smelt stomach lining proteins.	31
Figure 15.	Larval smelt key band immunoreactivity of pure larval smelt extract compared to larval smelt and stomach lining extracts combined.....	32
Figure 16.	Immunoblot experiment illustrating the utility of the larval smelt antiserum in detecting the larval smelt key band when in combination with an abundance of heterologous prey extracts.....	33
Figure 17.	Larval smelt key band immunoreactivity of pure larval smelt extract compared to larval smelt and other prey extracts combined.....	34
Figure 18.	Minimum detectable amount of pure larval smelt extract.....	35
Figure 19.	Immunoblot experiment illustrating the utility of the larval smelt antiserum in detecting the larval smelt key band in various life stages of smelt.....	36
Figure 20.	Larval smelt key band immunoreactivity for different life stages.....	37
Figure 21.	Immunoblot for yearling stomach content experiment probed with larval smelt antiserum.....	38

LIST OF APPENDICES

Appendix 1.	Antisera production.	45
Appendix 2.	Rabbit immunization and bleed protocol according to University Laboratory Animal Resources (ULAR), Michigan State University	47
Appendix 3.	Rabbit immunization and bleed schedule. . .	49
Appendix 4.	Titre evaluation protocol.	50
Appendix 5.	Competitive ELISA protocol.	52
Appendix 6.	Immunoblot protocol.	53
Appendix 7.	SDS gradient acrylamide gel protocol. . .	55
Appendix 8.	List of SDS acrylamide gel reagents and Laemmli loading dye reagents.	57
Appendix 9.	Protein blotting protocol.	58
Appendix 10.	NBT (p-nitro-blue tetrazolium chloride) stain protocol.	59

INTRODUCTION

Rainbow smelt (*Osmerus mordax*) were introduced in Lake Michigan in 1923 as the result of a single stocking in Crystal Lake, Michigan in 1912 (Van Oosten 1937). From the eastern shore of Lake Michigan, rainbow smelt dispersed rapidly throughout the upper Great Lakes and have become a significant component of the Great Lakes ecosystem. Rainbow smelt provide forage for lake trout (*Salvelinus namaycush*) and introduced salmonids including coho salmon (*Oncorhynchus kisutch*), chinook salmon (*O. tshawytscha*), rainbow trout (*O. mykiss*) and brown trout (*Salmo trutta*) (Stewart et al 1981). In addition, rainbow smelt are considered to be a competitor and predator of indigenous coregonines in the upper Great Lakes (Crowder 1980).

Historical records show that rainbow smelt abundance in the Great Lakes has been unstable with periods of marked increases and decreases (Baldwin et al. 1979). These fluctuations have been attributed to parasitism (Nepszy et al. 1978), disease (Van Oosten 1947), climatic factors during spawning such as water temperature and wave action (Rothschild 1961, Rupp 1965), and cannibalism (Henderson and Nepszy 1989).

Cannibalism has long been identified as a potential

regulatory mechanism in fish populations (Ricker 1954). This phenomenon occurs in many freshwater species (Dominey and Blumer 1984); however, its importance in natural populations has seldom been demonstrated (Alm 1952, Grimm 1981, Holst 1992, He and LaBar 1994). Generally, correlations between age class abundances (Alm 1952, Regier 1969, Grimm 1981) and/or direct numerical examinations of stomach content have been used to infer the importance of cannibalism in a population (Holst 1992, He and LaBar 1994).

Although, evidence of cannibalism through numerical analysis of stomach contents has been used to determine the importance of cannibalism in wild populations, it is limited because some prey items become visually unidentifiable soon after ingestion and thus may be under represented in diet analysis (Calver 1984). This is especially true for small soft tissue prey such as larval fish in which much of the key taxonomic identifying features, including fin rays, myomeres, and pigmentation are quickly lost after ingestion. Consequently, the occurrence of larval smelt in stomach content of older smelt is likely underestimated (Henderson and Nepszy 1989), and therefore, the significance of cannibalism as a population regulatory mechanism may be undervalued.

Historically, alternative methods to taxonomically identifying fish remains included examining backbone and

stomach, and scale morphology of ingested prey (Garman 1982, Knight et al. 1984). Other methods for the identification of partially digested piscine prey include serological and immunological techniques (Giles and Phillips 1985, Hartman and Garton 1992). These techniques have been useful because identification does not rely on visual descriptive taxonomic features of fish larvae. Immunological techniques for stomach content analysis first began in the field of entomology in an effort to determine blood meals of blood sucking insects (Weitz 1956). Since then, immunological methods have been employed to examine predator prey relationships and food web dynamics in a number of studies (Feller et al. 1979, Walter et al. 1986, Pierce et al. 1990).

An immunoassay is a technique for assessing the presence of a substance with an immunological reaction. The basis behind this reaction involves the recognition and binding characteristics of antibodies to antigens. For diet analysis immunoassays, an antiserum is produced to recognize prey specific proteins in the stomachs of predators. Recent advances to visualize the reaction between antibody and antigen for diet analysis include enzyme linked immunosorbent assay (ELISA) (Engvall and Perlmann 1972) based techniques such as ELISPOT immunoassays and immunoblots (westernblot) (Theilacker et al. 1986, Zagursky

and Feller 1988, Bailey et al 1993). ELISA based assays are useful for diet analysis when compared to other immunoassays because of their greater sensitivity and quantitative qualities (Theilacker et al. 1986, Zagursky and Feller 1988).

The most important aspect in determining the utility of an immunoassay for stomach content analysis is the quality of the antiserum produced (Feller 1991). Quality is a measure of how effective the antiserum is in detecting prey specific proteins and the degree that the antiserum cross reacts with the proteins from non-target prey. In cases where cross reactions occur, the antiserum is considered polyspecific. In most cases, cross reactions are the result of shared antigenetic determinants (epitopes) among phylogenetically related taxa (Feller et al. 1979; Feller and Gallagher 1982). To minimize the problems associated with cross reactivity, attempts can be made to create a monospecific antiserum. However, the development of a monospecific antiserum is time consuming, and obtaining sufficient quantities of pure antigen to develop an antiserum is difficult (Gallagher et al. 1988). Alternatively, if an antiserum is determined to be polyspecific, a number of immunoelectrophoretic methods, including crossed immunoelectrophoresis (Grisley and Boyle 1984) and immunoblots (Zagursky and Feller 1988), can be

employed to determine specific cross reactive components. Thus, individual cross reactive proteins can be identified to ascertain the potential utility of the antiserum to answer the desired questions.

The primary goal of this study was to evaluate the utility of an immunological technique to recognize larval rainbow smelt proteins from other known prey proteins. The underlying intent was to provide a stomach content analysis alternative to evaluate the degree of cannibalism in rainbow smelt. The specific objectives of this study were to:

- 1) develop an antiserum to larval rainbow smelt proteins;
- 2) determine the degree of cross reactivity with non-target prey proteins and
- 3) identify monospecific proteins which can serve as positive evidence of larval rainbow smelt in mixed stomach contents of yearlings and older smelt.

MATERIALS AND METHODS

Immunological Methodology Overview

Rainbow smelt larvae were homogenized in order to obtain a larval smelt soluble protein extract (Figure 1). This whole body extract was then introduced into a rabbit whose immune system produced antibodies to fight this newly introduced antigen. The rabbit was bled to collect these

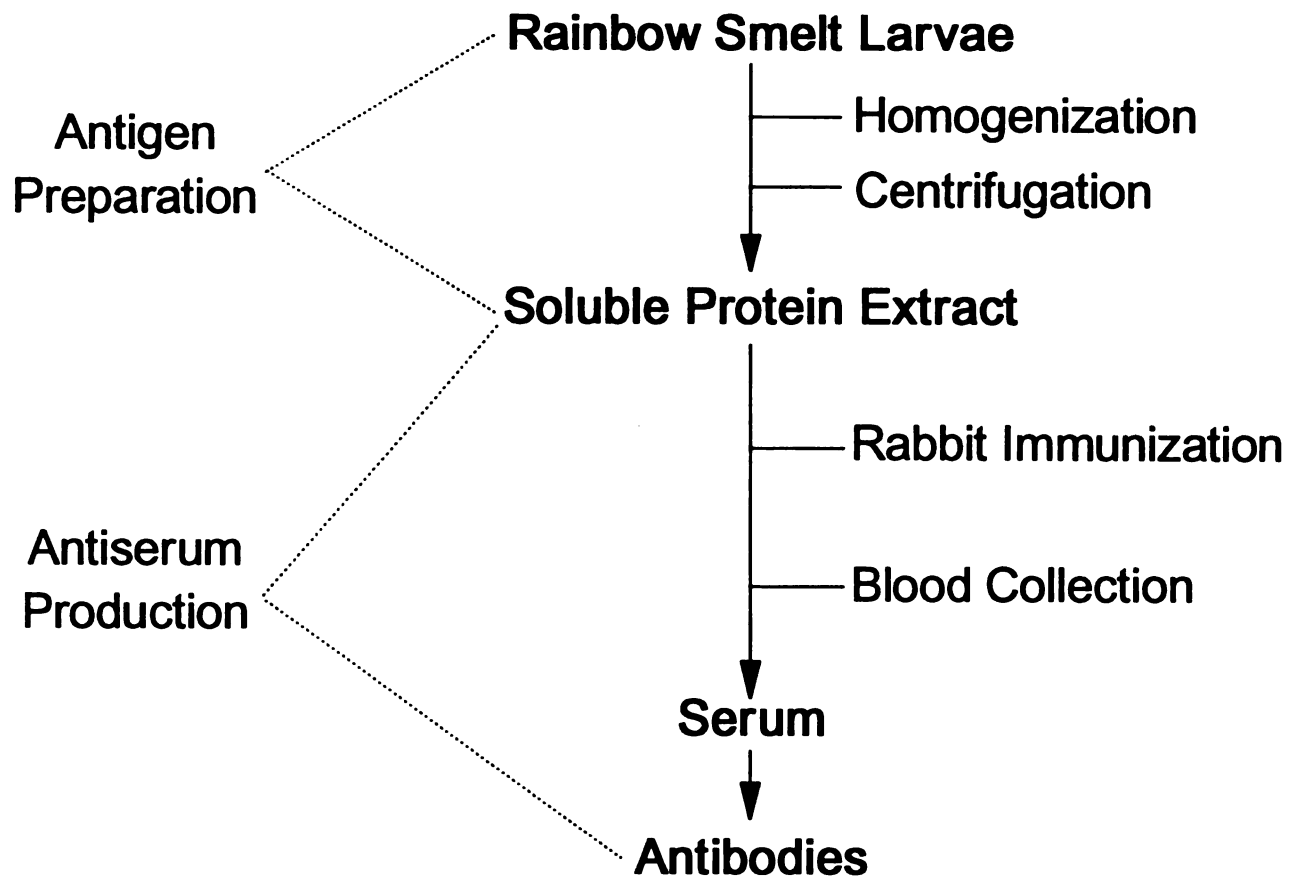


Figure 1. Flow diagram illustrating the steps in production of the larval smelt antiserum.

antibodies which would recognize and bind to larval rainbow smelt proteins. In order to determine the applicability of an immunological technique to recognize larval smelt proteins in mixed stomach contents of yearlings and older smelt, rabbit serum was tested for antibody concentration (titre) and evaluated for cross reactivity with invertebrate prey, other fish muscle tissue, and adult smelt stomach lining protein extracts using competitive ELISA and immunoblot techniques (Figure 2).

An indirect or sandwich ELISA technique was employed to test for titre (Figure 3). In principle, an antigen (larval smelt extract) is immobilized and incubated with a primary antibody (rabbit anti-larval smelt proteins). When the antibody recognizes and binds to a target antigen (larval smelt proteins), the formation of the antigen-antibody complex is not visible. Thus, a secondary antibody (goat anti-rabbit) coupled with an enzyme (horseradish peroxidase) is utilized to recognize and bind to the primary antibody portion of the complex. A substrate is added and reacts with the enzyme causing the formation of color. Peroxidase activity is measured with the aid of a colorimeter or spectrophotometer.

A competitive ELISA method was used to determine the degree of cross reactivity with non-target protein extracts (Figure 3). The primary antibody was incubated with a known

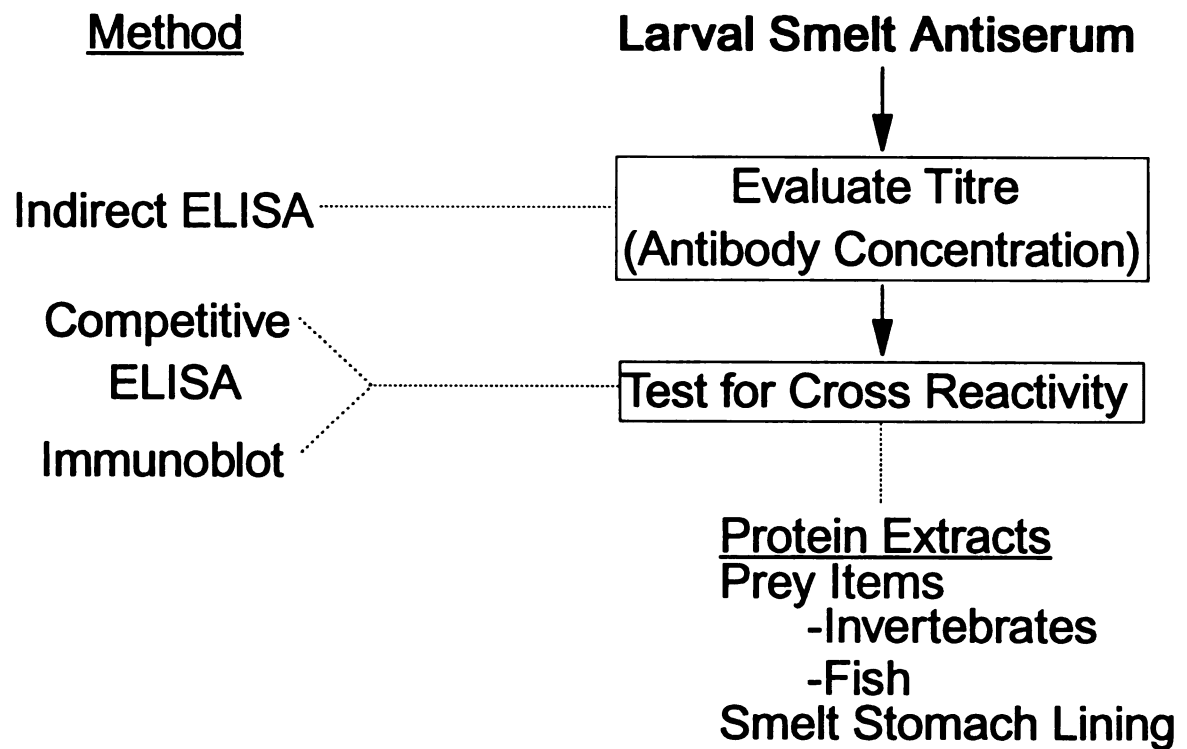


Figure 2. Flow diagram illustrating the steps in evaluating the larval smelt antiserum.

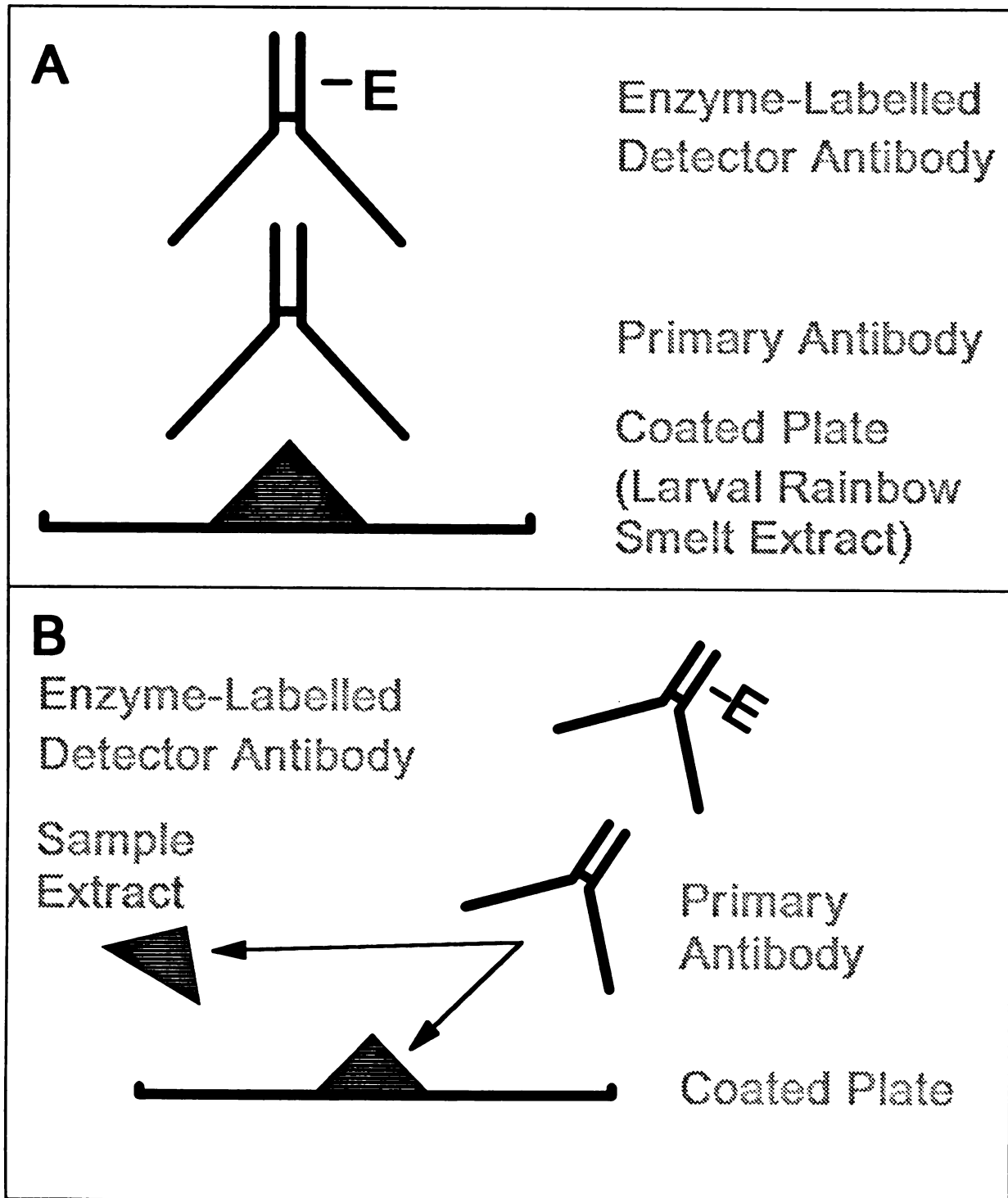


Figure 3. Illustrations of an (A) enzyme linked immunosorbent assay (ELISA) and a (B) competitive ELISA. Adapted from Kemeny (1991).

quantity of a non-target protein extract before it was allowed to react with larval rainbow smelt proteins. The sample extract essentially competes for binding sites with larval rainbow smelt proteins. Thus, if cross reactivity occurs, there would be a reduction in color intensity when compared to a control in which the primary antibody was not incubated with a non-target protein extract.

Immunoblots (western) were also used to determine the degree of cross reactivity with non-target protein extracts. In this technique, protein extracts were separated by electrophoresis and transferred to nitrocellulose before antibody incubation (Figure 4). This was advantageous because specific protein immunoreactivity could be evaluated by measuring color intensity at particular banding locations. Thus, by employing a immunoblot technique, individual cross reactive non target proteins could be identified. For additional information describing immunological methods and ELISA designs used in this study, see Kemeny (1991).

Rainbow Smelt, Fish, and Prey Item Collection

Rainbow smelt, other fish, and invertebrate prey species were collected for immunological analysis in St. Martin Bay, Lake Huron in 1993 (Brown 1994). Additional sampling for yearling and older smelt, and other fish species was conducted in 1994 during larval smelt out

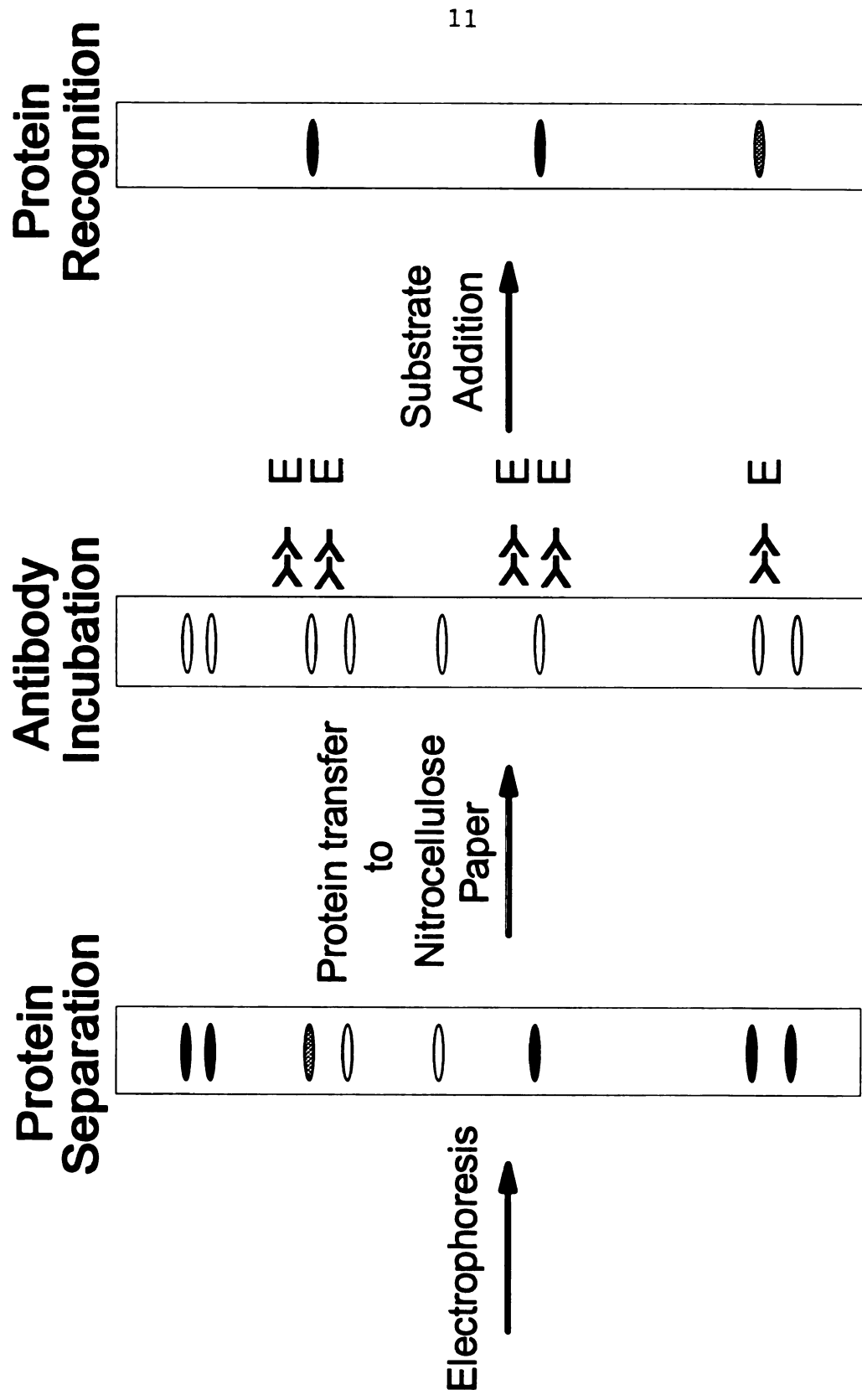


Figure 4. Illustration of an immunoblot. Adapted from Kemeny (1991).

migrations from tributaries entering the bay using a 45.72 m X 1.22 m beach seine with 6 mm diameter mesh (15.24 m X 1.22 m bag with 3 mm diameter mesh). Specimens were separated and identified to the lowest possible taxa (Auer 1982; Merritt and Cummings 1988; Balcer 1984; Pennak 1978). The samples were frozen at -10 to -20 C and kept frozen until laboratory processing.

Soluble Protein Extract Preparation

Invertebrate prey items, smelt stomach lining, and fish (smelt and others), were homogenized in phosphate buffered saline (PBS) (10 mM Na phosphate, 154 mM NaCl (pH 7.0)). Samples were placed in a 1.5 ml micro centrifuge tube and homogenized with a pellet pestle. The homogenates were then placed in a micro centrifuge and spun for 15 minutes (15,000 X g). The supernatants were collected and protein concentrations were determined (BCA Protein Assay, Pierce Chemical Co, Rockford, IL.) (Table 1). The protein extracts were frozen at -20 C until immunological analysis.

Smelt Stomach Content Sample Preparation

Rainbow smelt for stomach content analysis were immediately placed on ice and transferred to a field processing location. The stomach contents of individual fish were removed and placed in a 1.5 ml micro centrifuge tube with 0.1 ml phosphate buffered saline (PBS) (10 mM Na phosphate, 154 mM NaCl (pH 7.0)). The samples were frozen

Table 1. Whole body extracts and concentrations used in competitive ELISA and immunoblots. (M)=muscle tissue

Protein Extract	Protein Concentration (mg/ml)
Larval Smelt (antigen)(<u>Osmerus mordax</u>)	1.00
Smelt Eggs	31.57
Larval Smelt (5 mm-7 mm)	1.64
Larval Smelt (5 mm-20 mm)	1.14
Yearling Smelt (60 mm)(M)	12.23
Adult Smelt (120 mm)(M)	5.91
Adult Smelt Stomach Lining	2.89
Sucker spp. Larvae (Catastomidae)	15.24
Alewife (<u>Alosa pseudoharengus</u>)(M)	5.77
3-Spine Stickleback(<u>Gasterosteusaculeatus</u>)(M)	4.14
Brook Stickleback (<u>Culaea icostans</u>)(M)	10.73
Johnny Darter (<u>Etheostoma nigrum</u>)(M)	9.86
Longnose Dace (<u>Rvinichthys cataractae</u>)(M)	10.83
Mottled Sculpin (<u>Cottus bairdi</u>) (M)	11.09
Spottail Shiner (<u>Notropis hudsonius</u>)(M)	8.71
Lake Herring Larvae (<u>Coregonus artedii</u>)(M)	18.57
Whitefish (<u>Coregonus clupeaformis</u>)(M)	9.27
Zooplankton (Copepoda, Cladocera)	8.12
Opossum shrimp (<u>Mysis relicta</u>)	4.42
Amphipods (Gammaridae)	2.52
Mayfly Adult (Ephemeridae)	5.16
Mayfly Nymph (Ephemeridae)	2.56
Black Fly Larvae (Simulidae)	3.85
Midge Larvae (Chironomidae)	4.19
Midge Larvae (Ceratopogonidae)	11.16

at -10 to -20 C until immunological analysis was performed. In the laboratory, rainbow smelt stomach contents were individually homogenized as explained above, and soluble protein extracts were collected and used in subsequent immunoblot analysis.

Field collected fish specimens for immunological stomach content analysis should be treated with care. The overall goal is to stop the digestive process immediately and inhibit protease activity while in storage. The best way to do this is to place the collected fish on dry ice immediately to stop the digestive process and preserve the specimens in a freezer at -20 C to -80 C. In this study, collected smelt were immediately placed on ice and dissected as soon as possible before freezing the stomach content only. Even though this was probably not as effective in initially ceasing digestive processes, it was necessary because the removal of stomach content of small fish is difficult after a fish is frozen.

Antigen Preparation

To obtain soluble larval smelt proteins for the antigen, smelt larvae were homogenized in phosphate buffered saline (PBS) (10 mM Na phosphate, 154 mM NaCl (pH 7.0)). The digestive tracts were removed to minimize non-target protein contamination from ingested prey. Larval smelt ranged from 5 mm to 20 mm in length. The homogenate was then placed in

an ultra centrifuge and spun for 1 hour (165,480 X g at 4 C). The supernatant was collected and determined to contain 1.0 mg/ml protein (BCA Protein Assay, Pierce Chemical Co, Rockford, IL.). The soluble protein extract was frozen at -20 C. Prior to rabbit immunization, the extract was filtered (0.2 um).

Antiserum Production

Antisera to rainbow smelt larvae were produced through the Rabbit Antibody Production Service (RAPS) Program, University Laboratory Animal Resources (ULAR), Michigan State University (Appendix 1). All technical services including rabbit immunizations and blood collections were performed by ULAR personnel.

Titre Evaluation

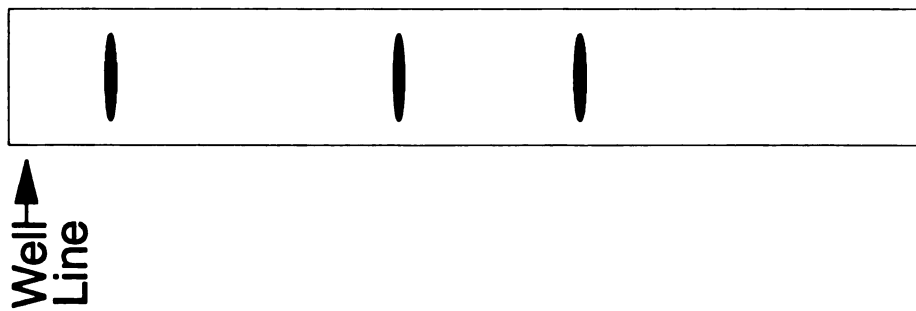
Rabbit sera were tested to evaluate the strength of the produced antibodies. Serial dilutions of the antiserum (rabbit-anti smelt larvae proteins) were tested for antibody activity using an indirect ELISA technique (Appendix 4).

Cross Reactivity

A competitive ELISA method (Appendix 5) was used to determine the degree of cross reactivity with protein extracts from invertebrate prey items, smelt stomach lining, and fish (smelt and others) muscle tissues. If cross reactivity was observed, immunoblots were then utilized to determine specific cross reactive proteins (Appendix 6).

All immunoblots were examined for non target cross reactivity by noting color development. Non target cross reactive banding location and intensity were compared to the larval smelt protein banding sequence in an effort to discover monospecific proteins associated with larval smelt. Differences in color intensity at specific banding locations were compared visually and by computer gel documentation software (Ultra Violet Products LTD, Cambridge U.K.). The computer software read the intensity of a particular band in the form of pixels per area. Distinct bands on an immunoblot were displayed as a peak in the raw data output (Figure 5). Relative intensity (pixels) at individual immunoreactive banding locations was determined by subtracting base line height from peak height. Although a base line height was subjectively determined, this measure of immunoreactive intensity appeared to be a suitable method to compare individual band intensities between larval smelt and non-target species.

Immunoblot



Well→
Line

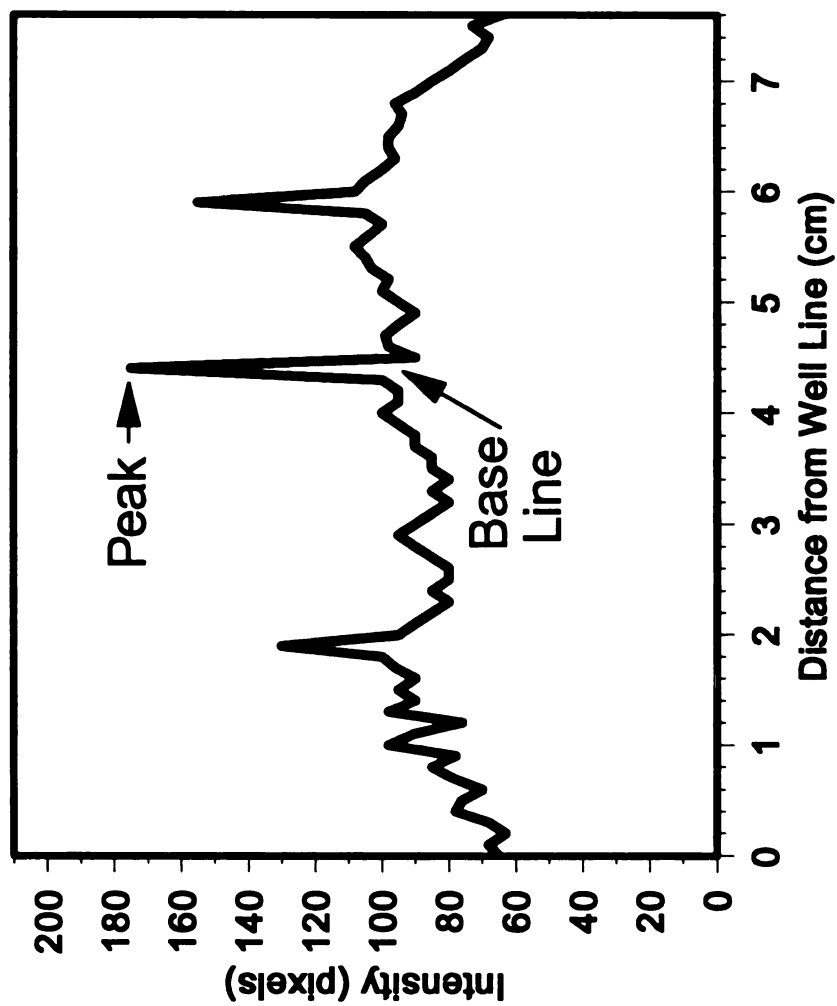


Figure 5. Form of raw data output generated by the gel documentation software.

RESULTS

Titre of Antisera

Dilutions of rabbit sera were evaluated to determine an suitable dilution of the raised antisera to larval rainbow smelt to be used in subsequent competitive ELISA and immunoblot experiments. A suitable dilution should be in a range that shows a significant change in absorbance per change in serum dilution. Analysis of the sera collected 42 days after initial immunization showed that both rabbits produced antibodies to larval smelt proteins (Figure 6). Rabbit #1 had slightly higher absorbance values, indicating stronger recognition and binding capabilities to smelt larvae. Both rabbits preimmune sera showed negligible antibody activity.

Sera collected 56 days after initial immunization showed a slight decrease for both rabbits in antibody strength when compared to the sera collected 42 days after initial immunization (Figure 7). At very low concentrations (<1:50000), absorbance levels were near 0 A°, and therefore were too dilute to be useful in later competitive ELISA and immunoblot experiments.

A dilution ratio of 1 part serum to 1000 parts dilute appeared to be suitable and was utilized in subsequent

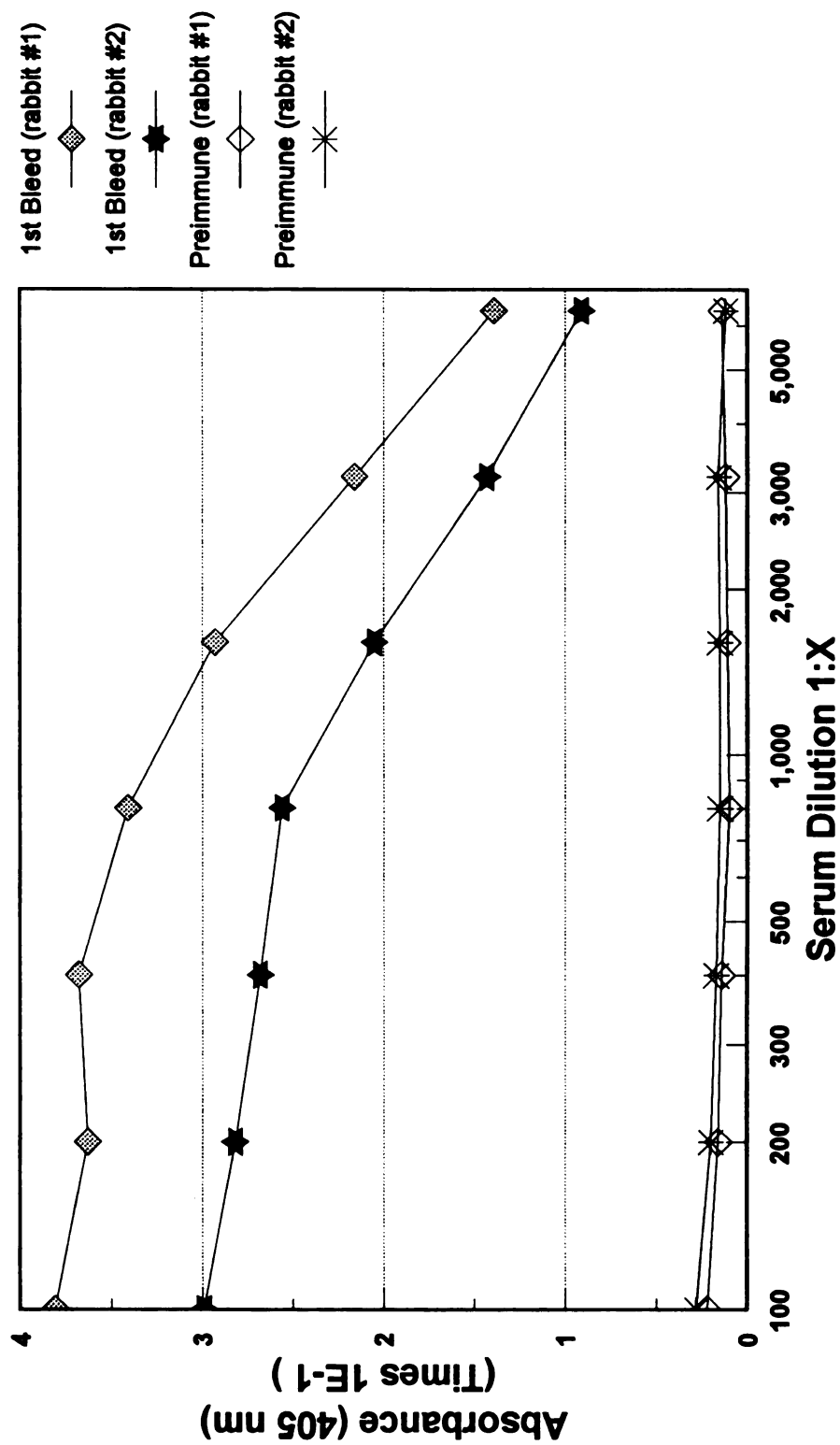


Figure 6. Pre-immune and first bleed (42 days after initial immunization) titre evaluation.

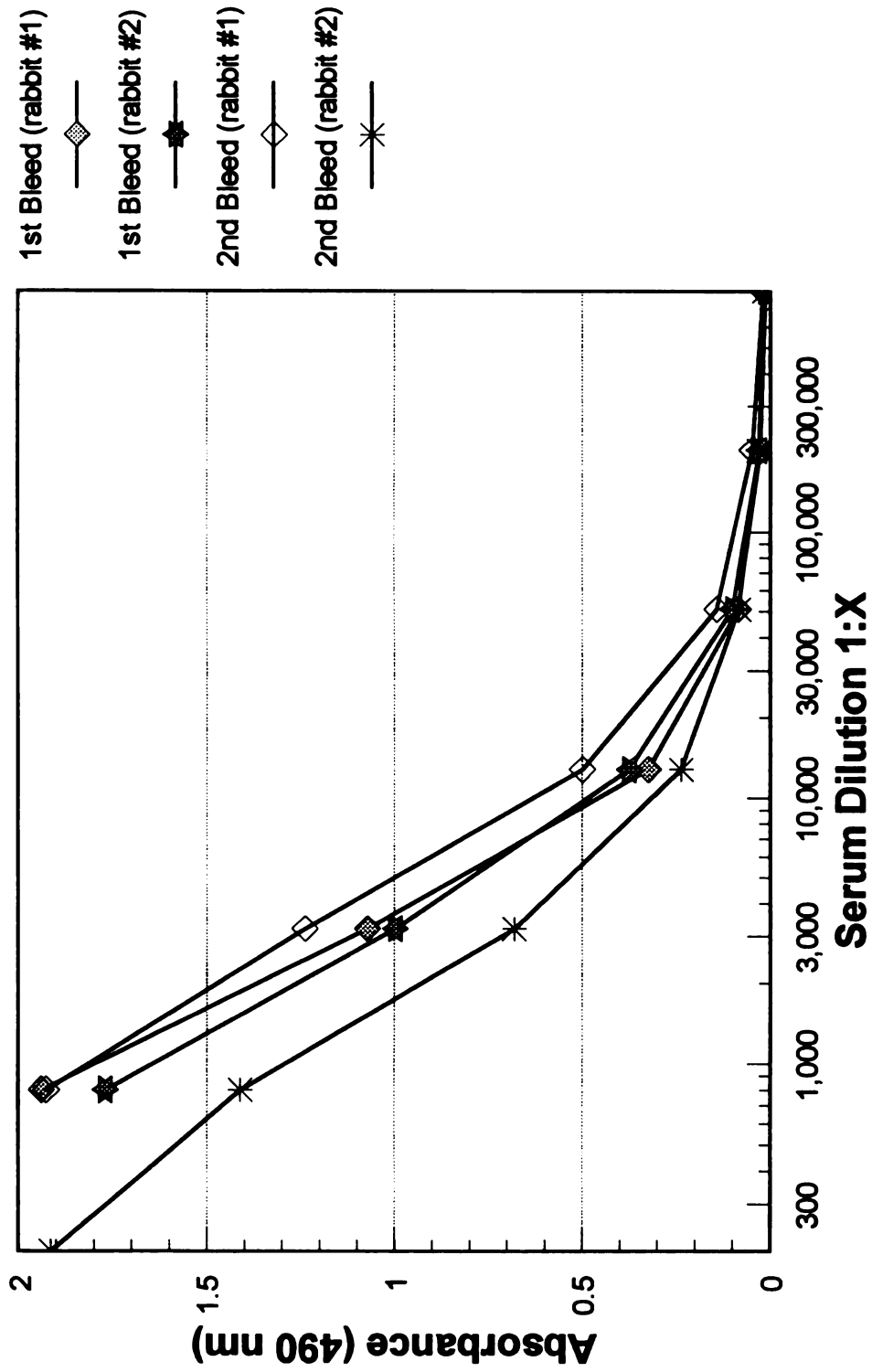


Figure 7. First bleed (42 days after initial immunization) and 2nd bleed (56 days) titre evaluation.

immunological tests. Because rabbit #1 sera had greater antibody reactivity when compared to rabbit #2, all competitive ELISA and immunoblot experiments utilized rabbit #1 sera (a blend of sera collected days 42 and 56 after initial immunization).

Competitive Elisa

The antiserum produced to larval rainbow smelt proteins was determined to be polyspecific, reacting to some degree with all protein extracts that were examined (Table 2). The lowest absorbance level, thus the most immunoreactive, was observed for homologous (self) proteins of rainbow smelt larvae (antigen) (Table 3). Adult rainbow smelt muscle tissue and mayfly larvae extracts also displayed high reactivity to the produced antiserum, respectively expressing 78% and 88% of the immunoreactivity of homologous larval rainbow smelt proteins. All other protein extracts expressed less than 43% of the immunoreactivity of larval rainbow smelt proteins.

Immunoblot

A band of markedly increased intensity was observed in the immunoreactive banding sequence of the homologous larval rainbow smelt protein (antigen) extract (Figure 8). The molecular weight of the protein at this band location was determined to be approximately 63,500 daltons using a biotinylated standard kit (Bio-Rad Laboratories, Richmond,

Table 2. Immunoreactivity with antisera raised to rainbow smelt larvae.

Protein Extract	Competitive ELISA	Immunoblot	Immunoblot (Key Smelt Band)
Larval Smelt (antigen)	+	+ (7)	+
Smelt Eggs	-	+ (2)	o
Larval Smelt (5-7mm)	-	+ (2)	o
Larval Smelt (5-20mm)	-	+ (2)	+
Yearling Smelt	-	+ (5)	+
Adult Smelt	+	+ (3)	+
Adult Smelt Stomach Lining	-	+ (3)	o
Sucker spp. Larvae	-	+ (3)	o
Alewife	+	+ (3)	+
Threespine Stickleback	+	+ (3)	+
Brook Stickleback	-	+ (2)	+
Johnny Darter	-	+ (2)	o
Longnose Dace	-	+ (2)	+
Mottled Sculpin	-	+ (2)	o
Spottail Shiner	-	+ (2)	o
Lake Herring Larvae	-	+ (2)	o
Lake Whitefish	-	+ (2)	+
Zooplankton (copepoda, cladocera)	+	o (4)	o
Opossum Shrimp (Mysis)	+	o (3)	o
Amphipoda	+	o (3)	o
Mayfly Adult	+	o (4)	o
Mayfly Nymph	+	o (3)	o
Black Fly Larvae	-	o (2)	o
Midge Larvae (chironomidae)	+	o (2)	o
Midge Larvae (ceratopogonidae)	-	o (2)	o

+ =positive reaction, o =no reaction, - =not tested, ()=trials

Table 3. Mean absorbance values (490 nm) (Competitive ELISA, 100 ug of each extract tested). (M)=Muscle tissue

Protein Extract	Mean	SE
Control	1.497	0.025
Larval Smelt (antigen)(<u>Osmerus mordax</u>)	0.283	0.005
Adult Smelt (M)	0.550	0.009
Alewife (<u>Alosa pseudoharengus</u>)(M)	1.037	0.016
Threespine Stickleback (<u>Gasterosteus aculeatus</u>)	1.070	0.008
Opossum shrimp (<u>Mysis relicta</u>)	1.286	0.033
Amphipods (Gammaridae)	0.987	0.026
Mayfly Adult (Ephemeraidae)	1.223	0.017
Mayfly Larvae (Ephemeraidae)	0.421	0.032
Zooplankton (Copepoda, Cladocera)	0.969	0.009
Midge Larvae (Chironomidae)	1.172	0.003

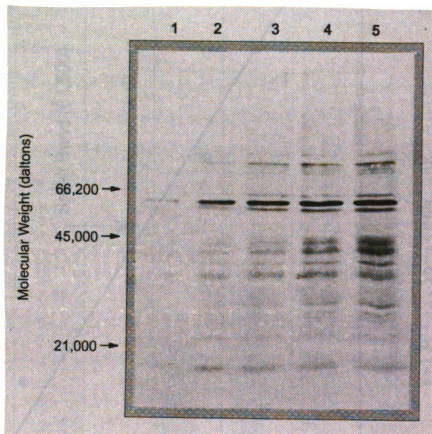


Figure 8. Immunoblot experiment using larval smelt proteins probed with larval rainbow smelt antiserum. Lanes 1-5 contain 7.5, 15, 22.5, 30, and 37.5 ug of pure larval smelt protein, respectively.

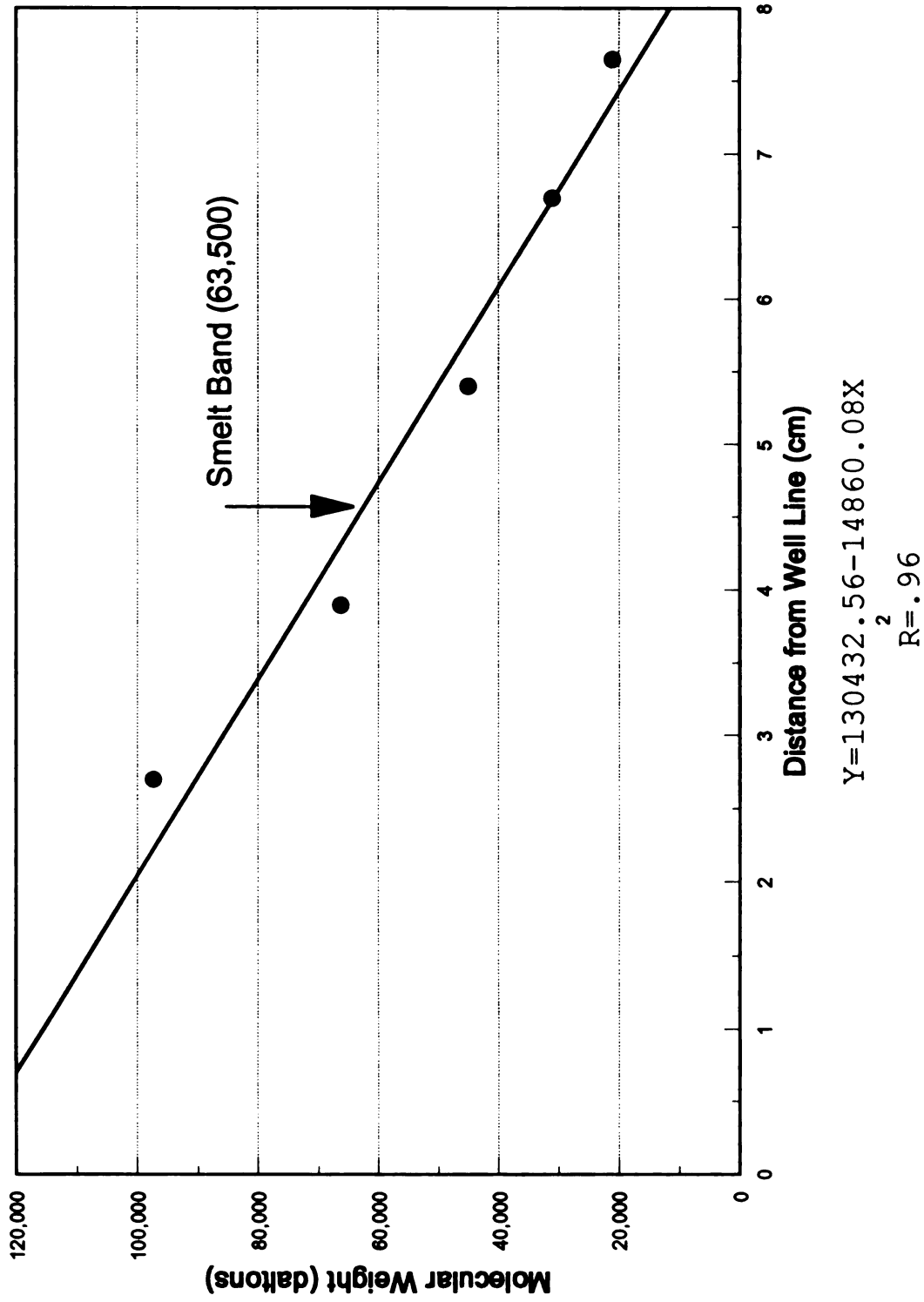


Figure 9. Molecular weight determination of the larval smelt key band.

CA) (Figure 9). The relative intensity of the band increased with an increase in larval smelt protein (ug) and appeared to level off at the higher protein concentrations (37.5 ug) (Figure 10). Because of its greater immunoreactivity when compared to other immunogenic larval smelt proteins, this banding location was evaluated further in subsequent experiments in an effort to determine its utility as positive evidence of smelt in stomach content samples of yearling smelt. In the following experiments, this band was referred to as the "key band".

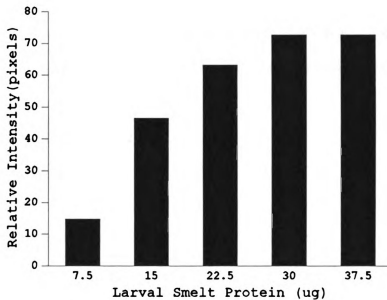


Figure 10. Larval smelt key band immunoreactivity of pure larval smelt extract.

In general, the antiserum to larval smelt proteins cross reacted with smelt stomach lining extract and all non-smelt muscle tissue extracts; however, no immunoreactivity

was observed for invertebrate prey extracts (Table 1; Figure 11). Even though all non-smelt muscle tissue extracts reacted with the antiserum raised to larval rainbow smelt proteins, only longnose dace (*Rhynchithys cataractae*), brook stickleback (*Culaea iconstans*), threespine stickleback (*Gasterosteus aculeatus*), lake whitefish (*Coregonus clupeaformis*), and alewife (*Alosa pseudoharengus*) showed immunoreactivity at the key band location (Figures 12 and 13). However, at equal quantities of total protein, the relative intensity at the key band location for these protein extracts on average expressed only 11.6% of the relative intensity of the larval smelt extract. Smelt stomach lining extract did not show immunoreactivity at the key band location.

Larval Smelt Key Band Utility

Immunoblot experiments demonstrated that the larval smelt key band was recognizable when in combination with an abundance of heterologous (non-self) proteins. Controls which contained pure larval smelt (antigen) extract (5-50 ug) were compared to samples which contained a mixture of larval smelt proteins and stomach lining proteins (250 ug) (Figure 14). In all cases, the larval smelt key band was recognized; however, the relative intensity of the key band decreased when larval smelt protein was in combination with

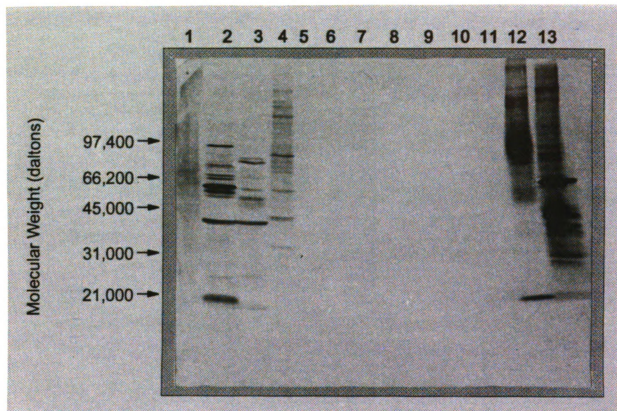


Figure 11. Immunoblot for single-prey experiment probed with larval rainbow smelt antiserum. Lane 1) contains lake whitefish extract; 2) adult smelt; 3) alewife; 4) threespine stickleback; 5) amphipoda; 6) mayfly nymph; 7) opossum shrimp; 8) midge larvae (ceratopogonidae); 9) zooplankton; 10) mayfly adult; 11) midge larvae (chironomidae); 12) smelt stomach lining; 13) larval smelt. Each lane contains 50 ug protein.

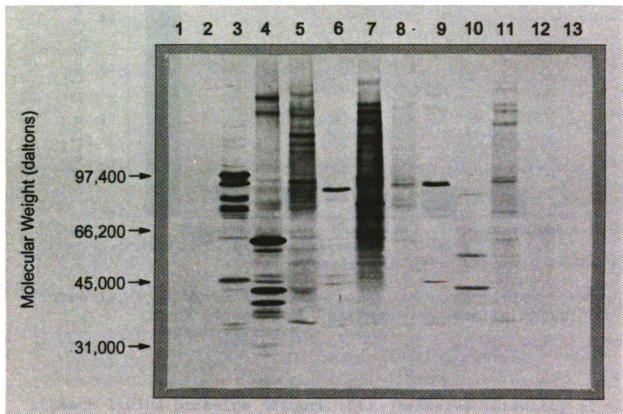


Figure 12. Immunoblot for single-fish species experiment probed with larval rainbow smelt antiserum. Lanes 1 and 13 are blanks. Lane 2) contains sucker spp. extract; 3) spottail shiner; 4) rainbow smelt; 5) johnny darter; 6) longnose dace; 7) brook stickleback; 8) lake whitefish; 9) mottled sculpin; 10) alewife; 11) threespine stickleback; 12) lake herring. Each lane contains 50 ug protein.

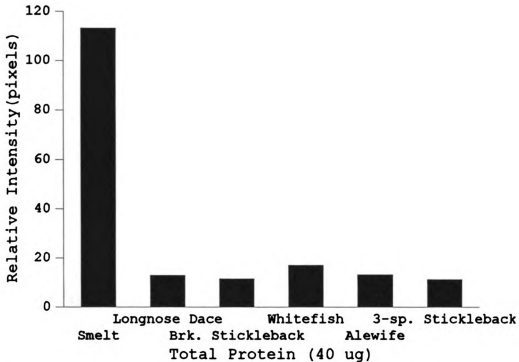


Figure 13. Larval smelt key band immunoreactivity of larval smelt extract compared to other fish extracts.

stomach lining proteins (Figure 15). Relative intensity at the key band location was reduced by 87% when 5 ug of larval smelt proteins was in combination with 250 ug of smelt stomach lining proteins. There was a 41% reduction in relative intensity when 50 ug of larval smelt proteins was in combination with 250 ug of smelt stomach lining proteins.

Similarly, an immunoblot experiment was conducted to determine if the larval smelt key band was recognizable when larval smelt (antigen) proteins were in combination with a mixture of non-target prey proteins (Figure 16). Midge

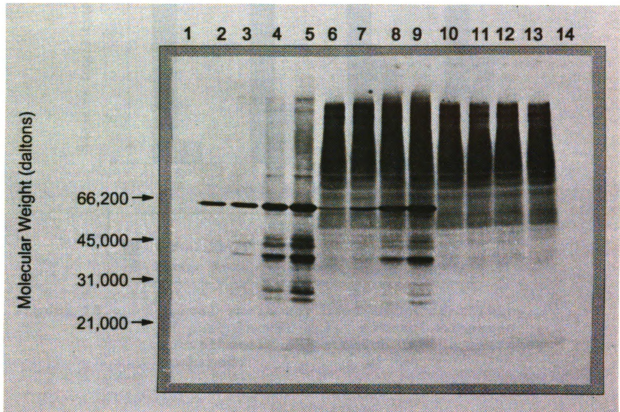


Figure 14. Immunoblot experiment illustrating the utility of the larval smelt antiserum in detecting the larval smelt key band when in combination with an abundance of heterologous smelt stomach lining proteins. Lanes 1 and 14 are blanks. Lanes 2-5 contain 5, 10, 25, and 50 ug pure larval smelt protein, respectively. Lanes 6-9 contain 5, 10, 25, and 50 ug of pure larval smelt protein; each are in combination with 250 ug of smelt stomach lining protein. Lanes 10-13 all contain 250 ug smelt stomach lining only.

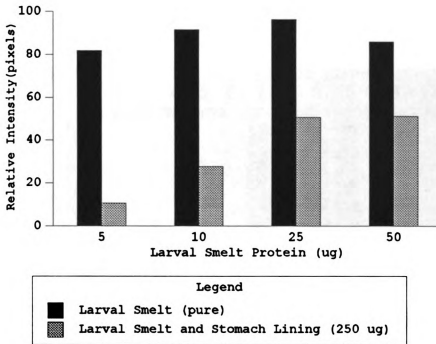


Figure 15. Larval smelt key band immunoreactivity of pure larval smelt extract compared to larval smelt and stomach lining extracts combined.

larvae (ceratopogonidae), adult mayfly (ephemeridae), sucker larvae (catostomidae), and adult smelt stomach lining protein extracts were chosen to simulate yearling smelt stomach contents. The prey items were selected because they appeared to be important food items through direct numerical examinations of yearling smelt stomach contents. Controls which contain (10-30 ug) larval smelt protein were compared to samples which contained a mixture of larval smelt proteins (10-30 ug) and the non-target proteins (130 ug).

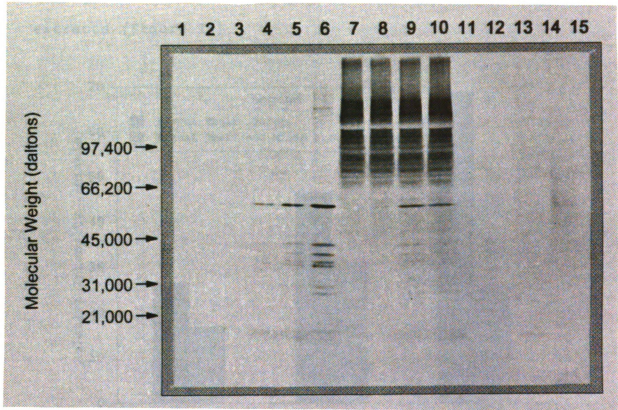


Figure 16.

Immunoblot experiment illustrating the utility of the larval smelt antiserum in detecting the larval smelt key band when in combination with an abundance of heterologous prey extracts. Lane 1-3 and 11-15 are blanks. Lanes 4-6 contain 10, 20 and 30 ug pure larval smelt protein, respectively. Lane 7 contains 130 ug mixed prey proteins (40 ug midge larvae, 40 ug mayfly adult, 20 ug smelt stomach lining and 30 ug sucker spp. larvae). Lanes 8-10 contain 10, 20, and 30 ug larval smelt protein, respectively, each in combination with 130 ug mixed proteins.

The larval smelt key band was visible in all cases, although the relative intensity of the key smelt band decreased an average of 43% when in combination with non-target protein extracts (Figure 17).

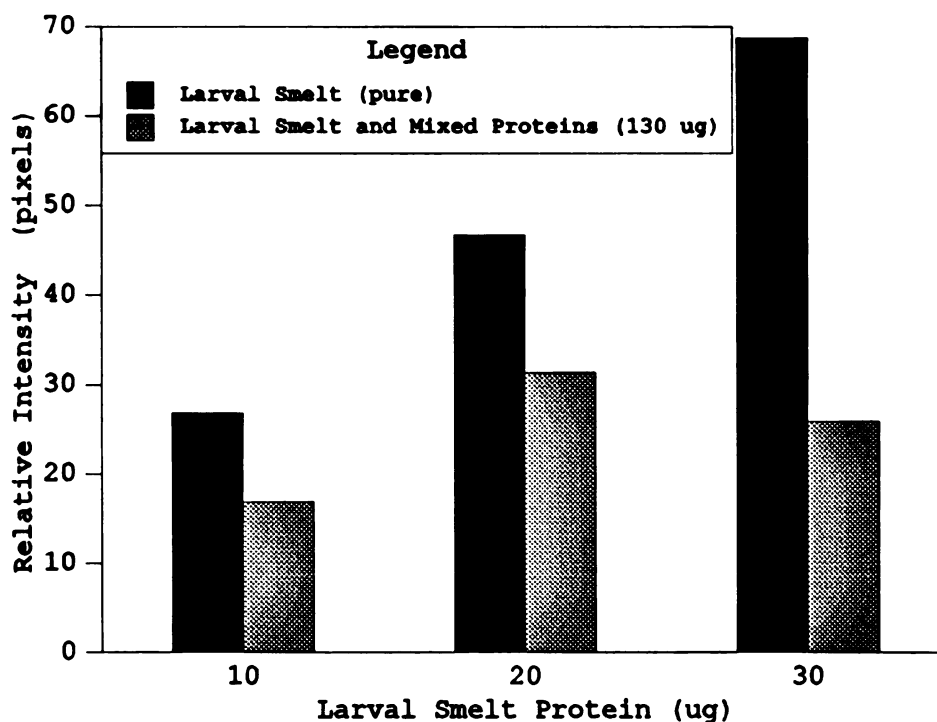


Figure 17. Larval smelt key band immunoreactivity of pure larval smelt extract compared to larval smelt and other prey protein extracts combined.

Larval Smelt Key Band Limitations

To determine the minimum amount of larval smelt (antigen) proteins detectable by the smelt larvae antiserum, pure larval smelt protein extract ranging from 0.1 to 10 ug total protein was tested for immunoreactivity.

Immunoreactivity which included reactivity at the key band location was evident in samples which contained greater than 1 ug total larval smelt protein (Figure 18). Notably, the key band was the only detectable immunoreactive band in the 1 ug total larval smelt sample. No visible immunoreactivity was observed in samples containing 0.1 or 0.5 ug total larval smelt (antigen) protein.

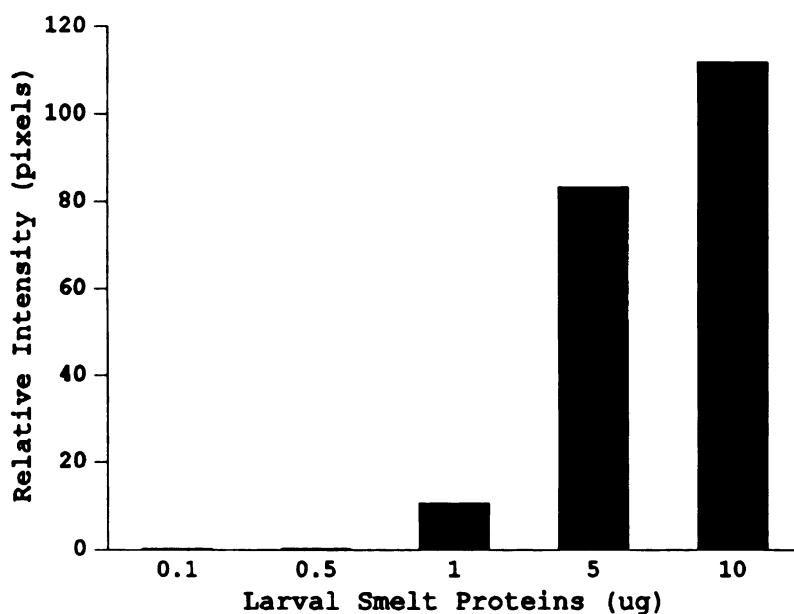


Figure 18. Minimum detectable amount of pure larval smelt extract.

Smelt life stages including the eggs, larvae (5-7 mm), larvae (5-20 mm), yearlings (>50 mm), and adults (<120 mm) were also examined for immunoreactivity (Figure 19). Experiments demonstrated that all extracts immunoreacted with the smelt larvae antiserum; however, the key band was evident only in larvae (5-20 mm), yearling, and

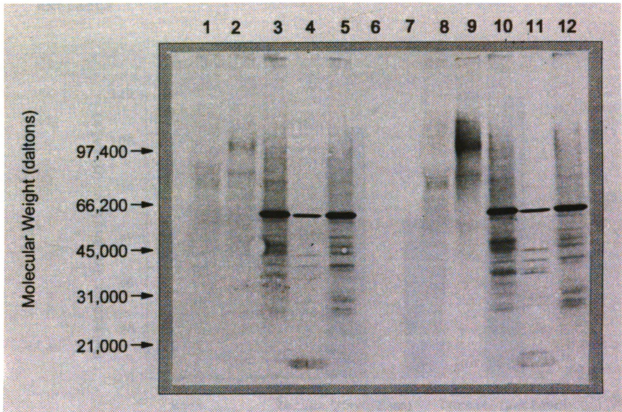


Figure 19.

Immunoblot experiment illustrating the utility of the larval smelt antiserum in detecting the larval smelt key band in various life stages of smelt. Lanes 1-5 contain 20 ug protein from egg, larvae (5-7mm), larvae (5-20mm), yearling, and larvae (antigen), respectively (adults not shown). Lanes 6 and 7 are blanks. Lanes 8-12 are replicates of lanes 1-5 containing 40 ug of each protein.

adult extracts at approximately equal to the relative intensity of the larval smelt (antigen) extract (Figure 20). The key band was not observed in eggs or larvae (5-7 mm) extracts.

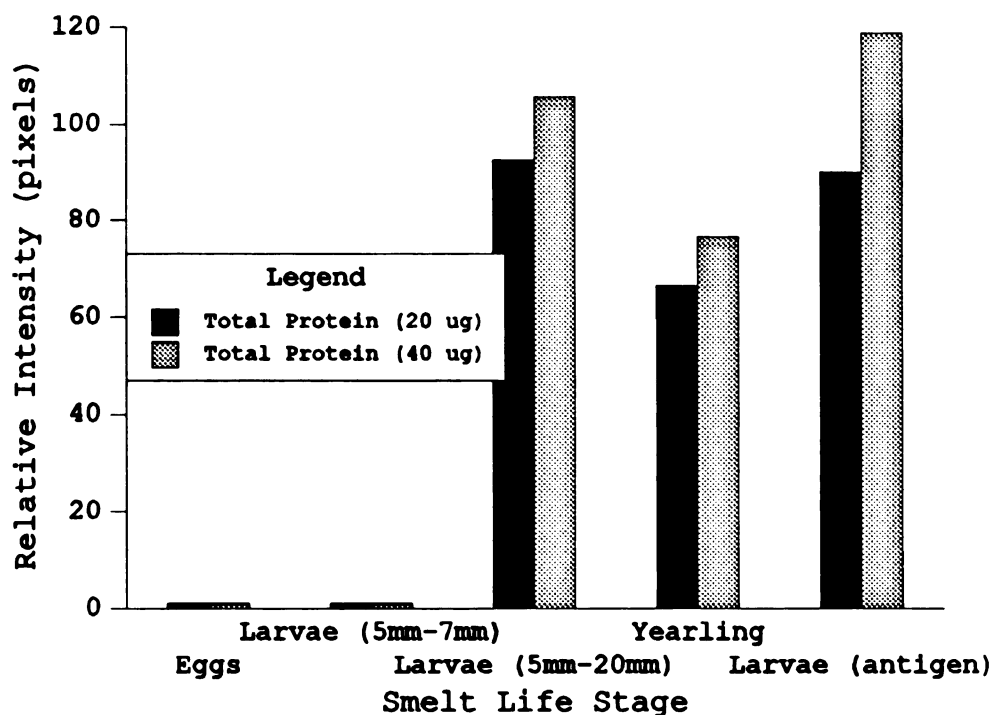


Figure 20. Larval smelt key band immunoreactivity for different life stages of smelt.

Stomach contents of individual yearling smelt were analyzed with immunoblots (Figure 21). Yearlings used for immunoblot analyses had an average length of 69.7 mm and average wet weight of 1.87 g. The average wet weight of stomach contents was 23.9 mg. Direct visual evaluation of the stomach contents showed that 62.5% of the smelt

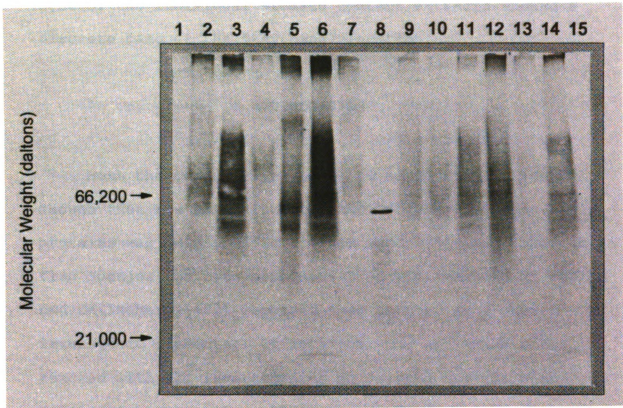


Figure 21. Immunoblot for yearling stomach content experiment probed with larval smelt antiserum. Lanes 1 and 15 are blanks. Lanes 2-7 and 9-14 contain yearling smelt stomach content extracts. Lane 8 contains 20 ug larval rainbow smelt protein.

contained larval fish, presumably sucker spp. (catostomidae) and 100% contained zooplankton. A total of 48 individual stomach content extracts were probed with the larval smelt antiserum for immunoreactivity at the "key band" location. None of the individual stomach content extracts showed a discrete band at the "key band" location.

DISCUSSION

Both the competitive ELISA and immunoblot analyses showed that the antiserum produced to larval rainbow smelt proteins was polyspecific. Cross reactivity was observed in fish species for both methods. This was expected as Feller and Gallagher (1982) reported that related taxa share immunogenic elements. Of interest, the antiserum cross reacted with all invertebrates to varying degrees when tested with competitive ELISA but no cross reactivity with invertebrates was apparent when an immunoblot technique was utilized. This discord between methods can be attributed to differences in sensitivity or epitope determination. Immunoblots are inherently less sensitive, and the fact that proteins must be denatured before analyses can cause changes in epitope recognition between the two methods. Under denatured conditions, a protein is essentially straightened

from its normal folded configuration. Thus, if the antiserum is recognizing discontinuous epitopes (amino acid residues brought into spatial proximity as a result of folding) under competitive ELISA conditions, it is plausible, that the degree of immunoreactivity would be reduced or lost when the protein is unfolded for immunoblot analyses (Wilson 1991).

Attempts were made to identify a monospecific protein of larval rainbow smelt to be used as a positive indicator of smelt larvae in mixed protein samples. Visual evaluation of immunoblots showed no apparent protein unique to larval smelt when compared with other fish species. However, a "larval smelt key band" was evaluated because of noticeably greater immunoreactivity. This band was useful because it was recognizable at very low total protein amounts (5 ug) when in the presence of heterologous proteins. Assuming a conversion to soluble protein is roughly 0.4 % of larval smelt wet weight, 5 ug of soluble rainbow smelt protein is approximately equivalent to 1.25 mg wet weight.

Because larval stages of non-smelt species were difficult to collect, muscle tissues from older size classes of individual fish species were used in immunoblot experiments. In doing so, the main assumption was that the muscle tissue was representative of the larval stages of these species. The key band was observed in the

immunoreactive banding sequences of five fish species: longnose dace, brook stickleback, threespine stickleback, lake whitefish, and alewife. Even though the immunoreactivity at the band was faint for all five species, the utility of the key band would be limited for stomach content analysis when these species are suspected prey because the band is not specific to larval rainbow smelt. However, if the larval stages of these cross reactive fish species are absent during the sampling period, as in the case of this study, the key band as an indicator of smelt in stomach contents could prove useful.

The antiserum was unable to detect the key band in the smelt egg protein extract or small larval smelt extract (5-7 mm); however the key band was detectable in larvae (5-20 mm) and larger rainbow smelt muscle tissue extracts. Presumably, the protein at the key band location is a muscle protein. Eggs obviously have no muscle development and newly emergent larvae (5-7 mm) have very little muscle. Thus, the recognition capabilities of the key band is dependent on the amount of muscle tissue present.

Interpretation of individual yearling smelt stomach content samples was difficult because immunoblots showed little or no discrete banding. This in part could have been a result of protease activity in the samples or an excess amount of protein loaded to the polyacrylamide gel.

Although interpretation of each blot is subjective, it is believed that if larval smelt were a portion of the stomach contents, the "key band" would have been apparent in the immunoreactive banding sequence as long as some larval smelt remains have not been affected by digestive processes. The lack of immunologically identifying larval smelt in the stomachs of 48 individual yearling smelt may also be attributed to the fact that during the sampling period of yearling smelt, only rainbow smelt larvae between approximately 5-7 mm in length were potential prey. Thus the "key band" as an indicator of cannibalism may be unrecognizable due to the lack of muscle development of the newly emergent rainbow smelt larvae. However, visual numerical stomach content analysis of 200 other yearling smelt collected during the same period showed no occurrence of cannibalism.

Immunoblots have the potential to determine if a predator consumed larval smelt by noting the occurrence of the key band, but the amount of larval smelt consumed is difficult to assess. Although, ELISA based techniques are quantitative, it is almost impossible to accurately determine the amount of smelt proteins present in a mixed sample of heterologous proteins by evaluating immunoblot banding intensities. The main reason for this is that the amount and variety of heterologous proteins in the stomach

contents of predators are different for each fish examined, thus the degree of cross reactivity and the effects on the intensity of the key band are unknown.

The effects of digestion on the recognition capabilities of the antiserum produced to larval rainbow smelt is unknown. Protease activity in a predator's stomach can essentially reduce the recognition capabilities of the antiserum by altering target-prey proteins. The utility of an antiserum to recognize target prey proteins depends on the digestion rate of the predator, the size of the ingested prey, and the amount of time elapsed since ingestion.

Assuming that there is at least some material not affected by the digestive process or a target prey contains digestion resistant proteins, an immunoblot is potentially a useful tool to assess stomach contents of field collected specimens.

The costs and benefits of an immunological approach should be considered before implementation of such methods in fisheries research. Although immunological assays have great potential, they are time consuming to develop and perfect. The underlying success of any immunological assay depends chiefly on the specificity of the utilized antiserum. Much time and effort is involved in producing an antiserum and testing the antiserum for cross reactivity with all possible prey to eliminate the potential false positive results. In some cases, the desired specificity

level of an antiserum may never be reached to be effective in an immunoassay. Although there are no guarantees, once an immunoassay is developed with antiserum specificity at a desire level, results should prove rewarding.

Although initial cost may be high, immunological approaches in fisheries research are invaluable in situations where the inability to identify egg or larval stages of fish prey in the stomachs of suspected predators is impeding our full understanding of species interactions. As in this study, immunoblots are useful in early life history studies where predation on the larval stage is thought to be an important mechanism affecting first year survival and recruitment.

APPENDICES

Appendix 1. Antisera production.

Two Female New Zealand White rabbits were used, each one weighed between 2.27 to 2.72 kg. Daily maintenance, feeding schedule, and technical services including injections and bleeds were performed by ULAR personnel. Each rabbit was bled prior to immunization (Appendix 2). Approximately 20 ml of blood were collected from each rabbit and individually stored in large centrifuge tubes. Pre-immune and subsequent bleeds were prepared by placing the tubes in a 37 C water bath for 1/2 hour to form a clot. The sides of the tube were scraped with a wooden stir rod and then placed in a refrigerator overnight at 4 C. The blood was centrifuged (400 X g) for 10 minutes. The serum was collected and centrifuged (400 X g) again for 10 minutes. The serum was then frozen at -20 C.

Each rabbit was initially immunized (Appendix 2) with a 1:1 mixture of filtered larval smelt protein extract (see antigen prep), 125 ug and 500 ug respectively, and Freund's Complete Adjuvant. Rabbits were individually injected with different quantities of protein to insure antibody titre production at desired levels. The antigen-adjuvant mixture was placed in an ice bath and homogenized using a ultrasonic pulsator. An emulsion was formed and then tested for separation in water. If the emulsion did not separate when

floated on the water, it was acceptable for injection. A second injection was performed 28 days after the initial immunization following the same protocol described above, however, Freund's Incomplete Adjuvant was used. Blood collections began 42 days after the initial immunization and were tested for titre. Final bleeds were performed 64 days after the initial injections (Appendix 3).

Appendix 2. Rabbit immunization and bleed protocol according to University Laboratory Animal Resources (ULAR), Michigan State University.

A 1:1 mixture of previously filter antigen and Freund's Complete Adjuvant was drawn up in a sterile syringe through a sterile 22 or 25 gauge needle. The skin at the injection sites was swabbed with disinfectant (i.e. 70% alcohol, or dilute Nolvasan solution). The total volume injected was 1 ml. The volume at any one subcutaneous site was no more than 0.1 ml. A fold of skin was picked up by pinching the skin between the thumb and fingers. The needle was inserted in the subcutaneous space. After removal of the needle, the injected area was massaged to dispense the injected material. A subsequent boost injection was made 28 days post first injection with Freund's Incomplete Adjuvant following the same procedure.

Blood Collections

Thirty minutes prior to blood collection the rabbit was injected subcutaneously with acepromazine to reduce stress to the animal and to keep it calm. The rabbit was restrained in a cloth towel. Hair covering the ear vessel was plucked and the surface of the ear was cleaned with warm soapy water or dilute Nolvasan solution. A topical anesthetic, Xylocaine, was applied to the ear to reduce discomfort to the animal during vena puncture. For blood

collection from the marginal ear vein, an approved intravenous catheter was used. The 24 gauge 3/4 inch catheter was advanced into the vein by sliding it off the stylet. Blood for pre-immune serum was drawn through the catheter into a sterile syringe. The amount of blood collected was no more than the amount calculated by the following formula:

$$\text{ml blood collected} = \text{rabbit weight in lbs.} \times 5.4$$

After collection, gauze was held with pressure on the puncture site until bleeding stopped. The ear was cleaned with warm soapy water and Vaseline was applied to the area. The rabbit was checked periodically for 30 minutes for any problems.

Final Bleed

Rabbits received 20 mg Rompun and 10 mg Ketamine subcutaneously prior to cardiac puncture. The animal was then exsanguinated.

Appendix 3. Rabbit immunization and bleed schedule.

Day 1	-Pre-immune bleed
	-First immunization injection (Freund's Complete)
Day 28	-First immunization boost (Freund's Incomplete)
Day 42	-Test bleed (evaluate titre level)
Day 56	-Test bleed (evaluate titre level)
Day 64	-Final Bleed

Appendix 4. Titre evaluation protocol.

A known amount of larval smelt proteins was adsorbed to a plastic surface. The surface was rinsed to remove excess proteins, and the adsorbed proteins were then allowed to react the primary antibody, rabbit-anti smelt larvae proteins. Excess primary antibody was washed away, and the secondary antibody, horseradish peroxidase-labelled goat anti-rabbit was added to bind with the primary antibody which previously reacted with the adsorbed smelt larvae proteins. After the removal of the excess secondary antibody, a solution containing H_2O_2 and o-phenylenediamine was added. The presence of peroxidase activity was detected by the formation of orange color. Absorbance values were used as measure of immunoreactivity.

Reagents

- 1) Filtered rainbow smelt larval proteins, diluted to 0.0125 mg/ml with 50 mM Na borate (pH 8.5).
- 2) PBS/Tween - 10 mM Na phosphate, 154 mM NaCl, 0.05% Tween-20 (pH 7.0).
- 3) Rabbit anti-smelt larvae proteins diluted 1 in 100 in PBS/Tween containing 0.2% BSA. Dilutions ranged from 1:100 to 1:6400.
- 4) Horseradish labelled goat anti-rabbit (Bio Rad Laboratories, Richmond, CA) diluted 1 in 1000 in PBS/Tween containing 0.2% BSA.
- 5) Staining Solution - Dissolve o-phenylenediamine (0.4 mg/ml stain) in 50 mM Na Citrate, pH 4.0. Add 30% H_2O_2 (1 ul/ml stain) to give final concentration of 2.2 mM

o-phenylenediamine and 0.03% H_2O_2 . Prepare immediately before use.

- 6) 4 N H_2SO_4 .

Procedure

- 1) Dispense 0.1 ml portions of the smelt larval protein solution into wells of a 96 well microtitre plate. Let stand overnight (12 hours) at room temperature.
- 2) Rinse plates with PBS/Tween. Add 0.2 ml to each well, then remove by aspiration. Repeat cycle 3 times.
- 3) Add 0.1 ml of rabbit anti-smelt larvae at varying dilutions per well and incubate for 1 hour at room temperature. 3 replicates for each dilution ratio.
- 4) Rinse as in (2)
- 5) Add 0.1 ml of the HRP-labelled anti-rabbit solution per well and incubate for 1 hour.
- 6) Rinse as in (2)
- 7) Add 0.1 ml of staining solution per well. After color development (approximately 5 minutes), add 0.1 ml H_2SO_4 to each well to stop the reaction and stabilize color.
- 8) Read color intensity on a plate reader using a 405 nm or 490 nm filter (preferred).

Appendix 5. Competitive ELISA protocol.

Cross reactivity with non target soluble protein extracts was examined following the same methodology described in the titre protocol; however, known amounts of non target protein extracts were incubated in a 1:1000 dilution of rabbit anti-smelt larvae in phosphate buffered saline (PBS) (10 mM Na phosphate, 154 mM NaCl, (pH 7.0)) for 30 minutes (Appendix 4, reagent step #3). This mixture of larval smelt antiserum and non target protein extracts was then used to incubate the adsorbed larval smelt proteins (Appendix 4, procedure step #3). The presence of peroxidase activity was detected by the formation of orange color. The degree of immunoreactivity with non target protein extracts was measured by comparing antigen (larval smelt) absorbance values with the various non target protein extract absorbance values.

Appendix 6. Immunoblot protocol.

The desired soluble protein extracts of known concentrations (BCA Protein Assay, Pierce Chemical Co, Rockford, IL) (Table 1) were separated using gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (Appendix 7). The proteins were allowed to separate overnight (16 hours) at 50 volts D.C.

The protein banding sequences were then transferred to 0.45 um nitrocellulose paper (Appendix 9) at 24 volts D.C. for 30-45 minutes. The nitrocellulose paper was blocked with 5% dry milk in TBS (tris buffered saline) (20 mM Tris, 500 mM NaCl, pH 7.5) for 30 minutes. The blocking solution was removed and the nitrocellulose paper was then incubated for 1 hour in a 1:1000 dilution (see titre results) of the primary antibody, rabbit-anti-smelt larvae proteins and 1% gelatin/TBS. The solution was removed, and the blot was rinsed with TTBS (0.1% Tween 20/TBS) for 10 minutes. The rinsing cycle was repeated two times. The blot was then incubated for 1 hour in a 1:1000 dilution of the secondary antibody, goat anti-rabbit HRP (horseradish peroxidase) (Bio Rad Laboratories, Richmond, CA) and 1% gelatin/TBS. The blot was rinsed as above and a final rinse was made with TBS. The blot was then placed in a NBT (p-nitro-blue tetrazolium chloride) based stain (Appendix 10) to visualize

peroxidase activity. The blot was allowed to develop for approximately 5 minutes at which time 2% Na azide was added to cease the reaction. A positive reaction was a purple color.

Appendix 7. SDS gradient acrylamide gel protocol.

A vertical slab unit (The Sturdier SE 400, Hoefer Scientific Instruments, San Francisco, CA) was used to produced a 14 cm X 16 cm X 1.5 cm gel. A SDS acrylamide gel (gradient 6.5%-20%) with a 5% stacking gel was used for all protein separation procedures.

Reagents

- 1) Acrylamide gel reagents (Appendix 8).
- 2) Water saturated butanol.
- 3) 5X-SDS-PAGE running buffer - .25 M Tris, 1.92 M glycine.
- 4) Protein extracts diluted in PBS - 10 mM Na phosphate, 154 mM NaCl.
- 5) 4X loading dye (Laemmli) (Appendix 8).

Procedure

- 1) Set up vertical slab unit according to directions provided by Hoefer Scientific Instruments.
- 2) Attach a two well gel maker to a stand positioned above the slab unit. Place a mixing device in the outer well.
- 3) Prepare 20% acrylamide solution, omitting the TEMED for the time being. Pour in the outer well of the gel maker. Make sure stop cock positioned between the wells of the gel maker is closed.
- 4) Prepare 6.5% acrylamide solution and add to the inner well of the gel maker. Immediately add the proper amount of TEMED to the outer well. Activate the mixer, open the stop cock between the wells, and gravity feed the mixing gel solutions to the slab

unit. Overlay with 1-2 ml water saturated butanol and allow to set for 30 minutes.

- 5) Decant the butanol and rinse the gel with distilled water several times.
- 6) Place a 15 lane comb between the glass plates of the slab unit.
- 7) Prepare 5% stacking gel and add to the slab unit. Allow to set 30 minutes.
- 8) Gently, remove the comb and rinse the gel with distilled water.
- 9) Add 250 ml running buffer to lower reservoir on the slab unit.
- 10) Add 250 ml running buffer and 1.25 ml 20% SDS to the top reservoir.
- 11) Prepare protein extracts for loading by adding 3 parts extract to 1 part 4x Laemmli dye. Place in a 100 C water bath for 3 minutes.
- 12) Add desired quantity (ul) of protein extract and loading dye mixture to the gel.
- 13) Connect slab unit to a power supply at 50 volts D.C. and allow proteins to separated overnight (16 hours).

Appendix 8. List of SDS acrylamide gel reagents and Laemmli loading dye reagents.

SDS Acrylamide Gel

<u>Solution</u>	<u>5% (ml)</u>	<u>6.5% (ml)</u>	<u>20% (ml)</u>
35.4% acrylamide 0.62% Bis (w/v)	2.11	2.42	7.45
20% SDS	0.75	.066	.066
1 M Tris pH 6.8	1.88	-----	-----
1 M Tris pH 8.8	-----	4.9	4.9
H ₂ O	10.8	5.82	.78
10% Am. persulfate	.075	.016	.031
TEMED	.015	.016	.005

Laemmli Loading Dye

<u>Reagent</u>	<u>Quantity</u>
1 M Tris Cl (pH 6.8)	2.5 ml
SDS	0.8 g
Glycerol	1.6 ml
15M 2-mercaptoethanol	1.8 ml
0.5% Bromphenol Blue	0.2 ml

Add distilled water to 10 ml total volume.

Appendix 9. Protein blotting protocol.

After protein extracts were separated using SDS polyacrylamide gel electrophoresis, the protein banding sequences were transferred to nitrocellulose paper by electrophoresing perpendicular to the banding direction on the gel using a Genie Electrophoretic Blotter (Idea Scientific Co. Minneapolis, MN.).

Procedure

- 1) Set up Genie Blotter according to instructions provided by Idea Scientific Co. Minneapolis, MN.
- 2) Connect the Genie Blotter to a 24 volt D.C. power supply for 30-45 minutes.

Reagents/supplies

- 1) 3 mm filter paper.
- 2) Nitrocellulose paper (0.45 um) 14 cm X 16 cm.
- 3) Running Buffer - 200 ml absolute methanol, 800 ml water, 3.03 g Tris, 14.4 g Glycine.

Appendix 10. NBT (p-nitro-blue tetrazolium chloride) stain protocol.

Reagents

- 1) 50 mM Na phosphate (pH 7.0)
- 2) Nitro-blue tetrazolium chloride (NBT)
- 3) NADH
- 4) H_2O_2
- 5) Phenol

Procedure

- 1) Warm 60 ml Na phosphate in a 37 C water bath.
- 2) Add 15 mg NBT and stir until dissolved.
- 3) Add 100 mg NADH and stir until dissolved.
- 4) Add 33 ul H_2O_2 and stir.
- 5) Add 100 ul of liquid phenol warmed in 65 C water bath and stir.
- 6) Use as soon as possible.

LIST OF REFERENCES

- Alm, G. 1952. Year-class fluctuations and span of life of perch. Inst. Fresh. Res. Drott. 33:17-38.
- Auer, N.A. (ed). 1982 Identification of larval fishes of the great Lakes basin with emphasis of the Lake Michigan drainage. Great Lakes Fishery Commission Special Publication 82-3:744p.
- Bailey, K.M., R.D. Brodeur, N. Merati, and M.M. Yoklavich. 1993. Predation on walleye pollock (*Theragra chalcogramma*) eggs and yolk-sac larvae by pelagic crustacean invertebrates in the western Gulf of Alaska. Fish. Oceanogr. 2(1):30-39.
- Balcer, M.D., N.L. Korda and S.L. Dodson. 1984. Zooplankton of the Great Lakes: a guide to the identification and ecology of the common crustacean species. The University of Wisconsin Press, Madison, WI. 174 p.
- Baldwin, N.S., R.W. Saalfeld, M.A. Ross and H.J. Buettner. 1979. Commercial fish production in the Great Lakes 1867-1977. Great Lakes Fishery Commission Technical Report No. 3.
- Brown, R.W. 1994. Reproduction, early life history, and recruitment of rainbow smelt in St. Martin Bay, Lake Huron. Ph.D. Dissertation, Michigan State University, East Lansing, MI.
- Calver, M.C. 1984. A review of ecological applications of immunological techniques for diet analysis. Australian Journal of Ecology 9:19-25.
- Crowder, L.B. 1980. Alewife, rainbow smelt and native fishes in Lake Michigan: competition or predation? Environmental Biology of Fishes 5:225-233.
- Dominey W.J. and L.S. Blumer. 1984. Cannibalism of early life stages in fishes. (In) Infanticide (G. Hausfater and S.B.Hrdy, eds) pp. 43-64. Aldine Publishing Co. New York, NY.

- Feller, R.J., G.L. Taghon, E.D. Gallagher, G. E. Kenny, and P. A. Jumars. 1979. Immunological methods for food web analysis in a soft-bottom benthic community. *Mar. Bio.* 54:64-75.
- Feller, R.J. and E.D. Gallagher. 1982. Antigenic similarities among estuarine soft-bottom benthic taxa. *Oecologia* 52:305-310.
- Feller, R.J. 1991. Dietary analysis of penaeid shrimp: the immunoassay approach. (In) *Frontiers in Shrimp Research* (Dougherty W.J. and M.A. Davidson, eds). Elsevier Science Publishers. Amsterdam, Netherlands
- Envall, E. and P. Perlmann. 1972. Enzyme-linked immunosorbent assay, ELISA. *J. Immunol.* 109:129-135
- Gallagher, E.D. P.A. Jumars and G.L. Taghon. 1988. The production of monospecific antisera to soft-bottom benthic taxa. (In) *Immuno-chemical Approaches to Coastal, Estuarine and Oceanographic Questions* (Yentsch C.M., F.C. Mague and P.K. Horan, eds). Springer-Verlag. New York, NY
- Garman, G.C. 1982. Identification of ingested prey of fish based on scale characteristics. *North American J. Fish. Man.* 2:201-203.
- Giles, N. and R.S. Phillips. 1985. A note on the production and use of antisera for the detection of part-digested sticklebacks in predator stomach-content samples. *J. Fish. Biol.* 27:827-829.
- Grimm, M.P. 1981. The composition of northern pike (*Esox lucius*) population in four shallow waters in the Netherlands, with special reference to factors influencing 0+ biomass. *Fish. Mgmt.* 12:61-79.
- Grisley, M.S. and P.R. Boyle. 1985. A new application of serological techniques to gut content analysis. *J. Exp. Mar. Biol. Ecol.* 90:1-9.
- Hartman, K.J. and D.W. Garton. 1992. Electrophoretic identification of partially digested prey of piscivorous fish. *North American J. Fish. Man.* 12:260-263.

- He, X. and G.W. LaBar. 1994. Interactive effects of cannibalism, recruitment, and predation on rainbow smelt in Lake Champlain: a modeling synthesis. J. Great Lakes Res. 20(1):289-298.
- Henderson, B.A. and S.J. Nepszy. 1989. Factors affecting recruitment and mortality rates of rainbow smelt (*Osmerus mordax*) in Lake Erie, 1963-1985. Journal of Great Lakes Research 15:357-366.
- Holst, J.C. 1992. Cannibalism as a factor regulating year class strength in the Norwegian spring-spawning herring stock. International Council for the Exploration of the Sea. Pelagic Fish Committee H:14.
- Kemeny, D. M. 1991. A Practical Guide to Elisa. Pergamon Press. Oxford, England.
- Knight, R.L., F.J. Margraf and R.F. Carline. 1984. Piscivory by walleyes and yellow perch in western Lake Erie. Transactions of the American Fisheries Society 113:677-693.
- Merritt, R.W. and K.W. Cummins (eds). 1988. An Introduction to the Aquatic Insects of North America (2nd ed). Kendall/Hunt Publishing Co. Dubuque, IA.
- Nepszy, S.J., J. Budd and A.O. Dechtiar. 1978. Mortality of young-of-the-year rainbow smelt (*Osmerus mordax*) in Lake Erie associated with the occurrence of *Glugea hertwigii*. Journal of Wildlife Diseases 14:233-239.
- Pennak, R.W. 1978. Fresh-Water Invertebrates of the United States. John Wiley and Sons, Inc. New York, NY.
- Pierce, G.J., J.S.W. Diack and P.R. Boyle. 1990. Application of serological methods to identification of fish prey in diets of seals and dolphins. J. Exp. Mar. Biol. Ecol. 137:123-140.
- Regier, H. A., V. C. Applegate and R. A. Ryder. 1969. The ecology and management of walleye in western Lake Erie. Great Lakes Fisheries Commission Technical Report 15.
- Ricker, W. E. 1954. Stock and Recruitment. Journal of the Fisheries Research Board of Canada 11:559-623.

- Rothschild, B.J. 1961. Production and survival of eggs of the American smelt, *Osmerus mordax* (Mitchell), in Maine. Transactions of the American Fisheries Society 90:42-48.
- Rupp, R.S. 1965. Shore-spawning and survival of eggs of the American smelt. Transactions of the American Fisheries Society 94:160-168.
- Stewart, D.J., J.F. Kitchell and L.B. Crowder. 1981. Forage fishes and their salmonid predators in Lake Michigan. Transactions of the American Fisheries Society 110(6):751-763.
- Theilacker, G.H., A.S. Kimball and J.S. Trimmer. 1986. Use of an ELISPOT immunoassay to detect euphausiid predation on larval anchovy. Mar. Ecol. Prog. Ser. 30:127-131.
- Van Oosten, J. 1937. Dispersal of smelt, *Osmerus mordax* (Mitchell), in the Great Lakes Region. Transactions of the American Fisheries Society 66:160-171.
- Van Oosten, J. 1947. Mortality of smelt, *Osmerus mordax* (Mitchell), in Lakes Huron and Michigan during the fall and winter of 1942-43. Transactions of the American Fisheries Society 74:310-337.
- Walter, C.B., E. O'Neill and R. Kirby. 1986. "ELISA" as an aid in the identification of fish and molluscan prey of birds in marine ecosystems. J. Exp. Mar. Biol. Ecol. 96:97-102.
- Weitz, B. 1956. Identification of blood-sucking arthropods. Bull. Wld. Hlth Org. 15:473-490.
- Wilson, J.E. 1991. Monoclonal antibodies and proteolysis. (In) Methods of Biochemical Analysis, Protein Structure Determination. Vol. 35. (Suelter, C.E., eds). John Wiley and Sons, Inc. New York, NY.
- Zagursky, G. and R.J. Feller. 1988. Application of immunoblotting for dietary analysis. (In) Immunochemical approaches to coastal, estuarine and oceanographic questions (Yentsch C.M., F.C. Mague and P.K. Horan, eds). Springer-Verlag, New York, NY.

MICHIGAN STATE UNIV. LIBRARIES



31293013992171