DRAFT FINAL REPORT

on

COMPARATIVE EVALUATION OF VITELLOGENIN METHODS

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1.0 INTRODUCTION

The U.S. Environmental Protection Agency (EPA) is implementing an Endocrine Disruptor Screening Program (EDSP) comprised of a battery of Tier 1 screening assays and Tier 2 tests. An international effort is also underway to develop and coordinate screens and tests appropriate for use in investigating potential endocrine disrupting chemicals. The Organization for Economic Cooperation and Development (OECD) has established an Endocrine Disruptor Testing and Assessment (EDTA) task force to oversee the coordination of this effort. One of the Tier 1 assays under development is a short-term screening assay designed to detect substances that interact with the estrogen and androgen systems of fish. It is thought that the inclusion of the fish screening assay in Tier 1 is important, because estrogenic and androgenic controls on reproduction and development in fish may differ significantly from that of higher vertebrates, such that mammalian screening methods may not identify potential endocrine disruptor chemicals (EDCs) in this important class of animals. The measurement of a biochemical marker, vitellogenin (VTG) in oviparous vertebrates is generally agreed to be a good indicator for estrogenic and anti-estrogenic effects and is proposed as one of several endpoints in the fish screening assay. VTG is a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually active female oviparous fishes, but can be induced to occur in males in response to estrogenic substances. Different methods are available to assess VTG induction in fishes including measurement of the VTG protein with enzyme-linked immunosorbant assays (ELISA) or gas chromatography-mass spectrometry (GC-MS), and mRNA detection. Both plasma and whole body measurements have been proposed.

The purpose of this study was to conduct a survey of existing VTG analytical methods for suitability in a routine screening program. This comparison was not intended to be a validation of a given method, but an evaluation across methods to ascertain the qualitative and/or quantitative comparability of the variety of methods currently available.

The specific objectives of the study included:

1) The preparation of a standard evaluation series of fish plasma and tissue taken from fathead minnow (*Pimephales promelas*) (e.g. whole body homogenate) to provide a range of VTG and mRNA concentrations produced in male and female fish (exposed or not exposed to an estrogen compound). The series was produced with 1) uninduced male, 2) uninduced female, 3) induced male and 4) induced female fathead minnows. In addition to the standard series, a set of control VTG samples were prepared.

2) The identification of laboratories to participate in the analysis of the standard evaluation series and the coordinated transfer of the samples to the participating laboratories and the collection of analytical results. Each laboratory employed the specific analytical technique routinely used by the laboratory to measure VTG or mRNA. This resulted in a variety of analytical methods applied to the standard series. Each laboratory reported the results of the analysis and provided a detailed analytical protocol.

2.0 SAMPLE PREPARATION AND HANDLING

The VTG standard evaluation series was prepared from fathead minnow (*Pimephales promelas*) plasma and whole body homogenates. Fish were acquired, exposed, and used to prepare plasma and whole body homogenates under an animal care protocol reviewed and approved by the Battelle's Animal Care Committee (AAALAC accreditation). To generate samples for the series from uninduced male, induced male, uninduced female, and induced female fish, sets of fish were exposed to estrogen in the laboratory, or remained unexposed. The unexposed group of adult male and female fish were used to generate uninduced background concentrations for male and female fish. The production of VTG was induced in adult male and female fathead minnows via exposure to17β-estradiol in the laboratory (Korte et al. 2000). After a 1-week exposure, when maximal VTG protein levels in plasma were anticipated, the fish were sacrificed, plasma was collected, and whole body homogenates were prepared. Several steps were employed to aid in preserving the integrity of the samples, including the use of an inhibitor, cold processing, and quick freezing to stabilize the VTG samples. To generate a sufficient amount of material for the study, a large number of samples were collected and frozen upon collection. Following the processing of the individual fish, the group of plasma or tissue samples were quickly thawed and combined to prepare pooled samples for the series. The exposed fish samples provided the plasma and tissue samples for the induced male and induced female for the standard series. Samples composited from individual unexposed fish provided the uninduced male and uninduced female sample in the standard series. This approach resulted in four samples within the series.

In addition to the samples representing four VTG levels found in plasma and whole body homogenate from uninduced and induced male and female fish for the standard series, two control samples were generated to complete the plasma analytical series. These additional samples included a positive and negative control, prepared with unexposed male plasma. To prepare the negative control, anti-VTG antibodies were used to remove the protein from the unexposed male plasma resulting in a sample with minimal levels of protein for the analysis. For the positive control, VTG was purified from plasma from exposed male fathead minnows (Denslow et al. 1999) and negative control plasma was spiked with a known amount of the protein. This resulted in six samples in the standard series, covering a range of VTG concentrations from minimal to high levels of protein in male and female plasma.

With the goal to provide fully replicate samples to the analytical laboratories, multiple aliquots of each sample were prepared and stored in a repository until coordinated shipment to the participating laboratories. Each aliquot from pooled samples represented an amount of material (plasma or tissue) equivalent to that obtained from a single fish. The samples were shipped on dry ice with appropriate Chain-of-Custody documentation and with instructions for sample receipt, storage, analysis, and data reporting. The laboratories verified that the samples remained frozen during shipping and were advised to store the samples under conditions similar to those found in the repository and to avoid exposing the samples to freeze-thaw cycles. This activity was thoroughly documented to ensure sample integrity was not compromised. As a result , 2 of 11 shipments for ELISA analysis were determined to have been compromised during shipping (thawed), and replacement sample sets were provided for analysis following the same procedure.

A similar approach was applied to the preparation of an mRNA standard evaluation series. Adult male and female fathead minnows were exposed to 17β-estradiol, and liver tissue samples were collected from fish after a 2-day static renewal exposure. This provided samples representing uninduced male, induced male, uninduced female, and induced female, respectively. The shipping of mRNA were analogous to those previously described for ELISA samples. All shipments were received by testing laboratories were un-compromised and received in good condition.

3.0 SAMPLE METHODS

Standard Series

One hundred adult male fathead minnows and 190 adult females were exposed to a nominal concentration of 300 nG/L of 17ß-estradiol in a 7-day static renewal exposure, while an additional 400 (210 male and 190 female) fish remained unexposed. On day 2, 240 exposed and unexposed fish (80 male exposed / 80 male unexposed and 160 female exposed and 160 unexposed) were sacrificed and the liver tissue was harvested for the mRNA standard series. The liver tissue was collected under cold conditions as rapidly as possible and placed in RNA stabilizing buffer prior to being placed in frozen storage at approximately -20°C.

On day 7 of the exposure, the remaining exposed fish were anesthetized with tricaine methane sulfonate (MS-222) and blood was collected from the caudal vessels into heparinized hematocrit tubes. The hematocrit tubes were centrifuged and the plasma was transferred to tubes containing the inhibitor aprotinin, quick frozen in liquid nitrogen, and stored at -80 °C. Plasma was collected from the unexposed fish in a similar manner. A subset of fish from the exposed and unexposed groups was used to prepare whole body homogenates for analysis. Whole body tissue homogenate was prepared by placing the fish into ice-cold ELISA assay buffer in a 1:1 ratio. The samples were homogenized on ice and following homogenization, the samples were centrifuged and the supernatant was harvested and frozen at -80°C. Care was taken in each of the steps to collect and process the samples in a timely manner under cold conditions followed by a quick freeze, to limit the time from collection to storage. The stability of the protein vitellogenin was addressed in the study through controlled sample collection, processing, storage, and shipping. In each of the steps used to collect and process the VTG samples, care was given to performing the steps 1) in a timely manner, 2) under cold conditions, 3) with the use of protease inhibitors, 4) followed by a quick freeze, to limit the time from collection to storage. Following collection, plasma and tissue homogenate samples were pooled to create the composite samples for the series. From this pooled material multiple samples were created for each analytical method and given a unique code , and entered into a repository management system.

Sample sets of the plasma and homogenate standard series were provided to 11 participating laboratories for VTG analysis by ELISA. Each participating laboratory was provided three samples to be analyzed in triplicate, packaged to limit the need to freeze-thaw during analysis. Each sample represented an individual fish equivalent for each of the fish conditions being considered (i.e., exposed female, exposed male, unexposed female, and unexposed male). Because the samples were analyzed fully blind, each vial contained enough

plasma to be analyzed in triplicate, while representing the volume of plasma obtained from an individual fish. Messenger RNA analysis was conducted on the liver tissue by three participating laboratories who agreed to analyze five replicate samples of each sample type (e.g., unexposed males) in triplicate analysis. As for the ELISA samples, the integrity of the samples during transfer to the participating laboratories was carefully monitored and documented, and proper storage conditions were maintained prior to analysis.

VTG Purification

For purification of VTG, adult male fish were exposed to 300 ng/L of 17β-estradiol in a 7-day static renewal exposure. On day 7, plasma was collected as described for the standard series and pooled for purification. The purified VTG was used to create a positive control sample within the range of the standard series and to provide as a standard to the analytical laboratories. The VTG was purified from the estrogenized plasma using anion exchange chromatography methods developed by Denslow et al. (1999) (Appendix B). VTG was separated from other plasma proteins using the BIOCAD Perfusion TM Chromatography System and anion exchange media (POROS 20HQ). The plasma was pre-equilibrated in running buffer (20 mM Bis-tris propane, 150 mM NaCl, pH 9.0), loaded onto the column, rinsed with running buffer to elute non-binding proteins, and the VTG released using a linear gradient of NaCl (150-800 mM). VTG was the last protein to elute (500-600 mM NaCl) the column. To verify the identity of the peak, the elution profile was compared to a run using male plasma. After pooling the fractions containing VTG, the pH was adjusted to 7.0 using 500 mM Bis-tris propane, the following reagents were added: protease inhibitor Aprotinin (10KIU/ml), azide (0.02%), and cryoprotectant (50% glycerol). Aliquots were stored at -80ºC until needed and to prevent freeze/thaw effects, once the aliquot was thawed, it was be stored at -20ºC (where it remains a liquid), with stability of up to one year (Kroll & Denslow, unpublished technique).

To ensure that the purified VTG was pure and of high quality, a number of analyses were conducted. Total protein on the purified VTG was determined first by Bradford (Coomassie plus TM, Pierce) using bovine serum albumin as a standard and then the concentration revalidated by amino acid analysis. Purity was determined by SDS-PAGE and yielded two high-molecular-weight proteins (180 & 200 KDa).

Vitellogenin is sensitive to freeze/thaw events that can fracture the protein and affect ELISA results (Kroll & Denslow, unpublished results). To control for this variation, the purified vitellogenin was frozen only once after purification. After thawing an aliquot, vitellogenin is stable for 1 year at –20ºC, and remains in liquid form since it contains 50% glycerol. Stability of the VTG at –20ºC has been verified using positive controls and determining VTG concentration by ELISA over a period of 1 year.

4.0 ANALYTICAL METHODS OF THE PARTICIPATING LABORATORIES

A number of methods have been developed for the quantification of VTG in blood plasma, liver tissue, or whole-body homogenates. The various methods differ in sensitivity, specificity, and technical difficulty. Currently, the most popular approach to measure VTG is

some form of an ELISA. The ELISA employs enzyme-linked antibodies and an adsorbent surface to detect specific antigens in solution. The ELISA has been widely used to quantify VTG in teleosts due to the ease in use and unlike the radioimmunoassay (RIA), ELISA does not require the use of radioactive isotopes. There are a variety of ELISA designs that typically fall into three general assay formats including competitive, sandwich, and direct ELISAs. Competitive ELISAs incorporate a step in which the samples and antibody (antibody-capture) or labelled antigen (antigen-capture) are incubated together prior to adding the sample on the test plate. This non-equilibrium design is often used to enhance sensitivity and counteracts potential preferential binding (Edmunds et al. 2000). Sandwich ELISAs employ two antibody preparations to detect the antigen. The antigens can recognize different epitopes on the target analyte, thereby providing a large degree of specificity and sensitivity. In a direct antibody-capture ELISA, the sample and standards are adsorbed directly on the surface of the microwell plate. After incubation, the wells are blocked and anti-VTG antibody is added to bind to the VTG attached to the well. As with other ELISAs, subsequent steps culminate in the development of color reflective of the amount of antigen present in the sample. The ELISA protocols included in this study are presented in Appendix A).

Mass spectrometry (MS) offers future possibilities for becoming a reference method for VTG and for combining multiple protein analysis from a single tissue sample. In general, MS approaches to protein quantification attempt to measure the protein largely in its intact form or rely on digestion procedures (chemical or enzymatic) to reduce the size of the protein into smaller fragments. The MS technique allows both the direct measurement of the VTG mass and generation of peptide-fingerprinting data for further identification (Wunschel and Wahl, 2002).

An alternative to measuring the VTG protein is to quantify the messenger ribonucleic acid (mRNA) for VTG that codes for the protein. Two methods for quantifying fish VTG mRNA have emerged, the ribonuclease protection assay (RPA) and the quantitative reverse transcription-polymerase chain reaction (QRT-PCR), although other methods exist (e.g., Northern blot, slot-blot) that have drawbacks relative to sample throughput or sensitivity. All methods can be used for absolute or relative quantification of mRNA.

Specific protocols employed by the participating laboratories (Appendix A) were applied to the samples in this study. The analysis can be grouped into the general categories of VTG and mRNA as the target analyte. Within these categories, multiple methods were applied to the sample series. The participating labs received multiple aliquots of the standard series as a contingency to prevent the need for sample freeze-thaw cycles and to limit the number of shipments to each laboratory. Each laboratory was asked to analyze the samples three times within approximately 4 weeks, with a minimum of three replicates.

5.0 PARTICIPATING LABORATORIES

The laboratories participating in this study were selected based upon their previous experience in the measurement of the VTG protein or mRNA. The laboratories had established protocols in routine use and were willing to commit to analysis during the study period. Two laboratories were provided with sample sets but were unable to complete the analysis within the

timeline of the study. The laboratories that analyzed the samples provided are presented in Table 1. The laboratories that conduct ELISA VTG measurements analyzed the plasma and whole body homogenate standard series. The laboratories that measured mRNA analyzed the liver tissue standard series. Two of these laboratories, Oregon State University and the Finnish Environmental Institute applied their ELISA assay to the samples, however, their antibodies (carp monoclonal and trout) did not interact with the samples. The results from 8 ELISA laboratories and 3 mRNA laboratories are summarized in this report.

6.0 DATA ANALYSIS

Data analysis was intended to provide descriptive statistics and plots that allow a general assessment of the objectives of the study. Statistically, the first objective was to determine if an increasing concentration of VTG was produced by the standard series. This series was represented, in order, by 1) uninduced male, 2) uninduced female, 3) induced male, and 4) induced female fathead minnows. The second statistical objective was to determine the analytical results and variation for the set of control and spiked VTG samples. The third statistical objective was to compare the analytical results and variation of each lab's analytical method including the antibody, standard, and assay used.

Analysis of the data yielded descriptive statistics including the number of samples, means, standard deviations, medians, first and third quartiles, and the coefficient of variation (CV). Simple linear regression of the ranked average VTG concentration (mean of the with-in run analyses) and plots of the analytical results against the concentration series were used to assess the strength of the VTG concentration trend (ignoring the positive control). Tukey's Honestly Significant Difference (HSD) multiple comparison test was conducted on the ranked average VTG concentrations to specifically determine if neighboring means in the series were significantly different (i.e., the blank mean compared to the uninduced male mean, the uninduced male mean compared to the uninduced female mean, and so on). Linear regression for each laboratory was also conducted on the average VTG concentrations observed for the blank and the uninduced male data. The regression results allow a test of the null hypothesis that the slope equals 0 and provides an measure of the strength of the trend. The multiple comparison testing (which is less powerful than the regression analysis due to the smaller degrees of freedom for testing) provides a test of how quickly differences can be detected in the series. Excel spreadsheet software (Microsoft Excel) and Minitab statistical software (Minitab Inc.) were used for this analysis.

Table 1. Summary of Reporting Laboratories

Lab ID #	Participating Laboratory	Method(s) applied to the standard series
1	The University of Florida Protein Chemistry and Biomarkers Res. Lab. Gainesville, Florida, USA	mRNA - RT-PCR ELISA - Fathead minnow based, monoclonal antibody, direct ELISA Biosense ELISA kit
2	The University of Idaho Department of Biological Sciences Moscow, Idaho, USA	mRNA - qRT-PCR TaqMan
15	Molecular Light Technology Research Ltd. Cardiff, UK	mRNA - HPA (hybridization protection assay)
14	Battelle Pacific Northwest National Laboratory Richland, WA, USA	GC-MS
3	Oregon State University Environmental and Molecular Toxicology Corvallis, Oregon, USA	ELISA - Trout based polyclonal antibodies in a competitive ELISA
4	US EPA Duluth, Michigan, USA	ELISA - Fathead minnow based polyclonal antibodies, competitive, antibody-capture
56	University of Exeter Environmental and Molecular Fish Biology Exeter, United Kingdom Brixham Environmental Laboratory AstraZeneca , United Kingdom	ELISA - Carp based polyclonal antibodies, competitive ELISA
713	Biosense Laboratories Bergen, Norway Battelle Pacific Northwest National Laboratory Sequin, WA, USA	ELISA - Carp based polyclonal and monoclonal antibodies, sandwich ELISA
8	INERIS (National Institute of Industrial Environment and Risks - France) Verneuil en Halatte, France	ELISA - Zebrafish based polyclonal antibodies, competitive ELISA
11	University of Southern Denmark Institute of Biology Odense, Denmark	ELISA - Zebrafish based anti-lipovitellin direct non-competitive sandwich ELISA
12	The Finnish Environmental Institute Helsinki, Finland	Carp based monoclonal antibody indirect ELISA

7.0 RESULTS

As noted in Section 3.0, Sample Methods, sample sets of the plasma and homogenate standard series were provided to 11 participating laboratories for VTG analysis by ELISA. It should be noted that all laboratories provided their services without compensation, and that every attempt was made to assist the laboratories in performing this complex task. Three samples were provided to each lab to be analyzed in triplicate, each vial containing enough plasma to represent an individual fish. One of the participating laboratories performed the analysis of the standard series once, rather than in triplicate, and the results reflect this singular value accordingly.

This study included several techniques for the detection of the induction of VTG in fathead minnows. These techniques include ELISA (enzyme-linked immunosorbent assay), RT-PCR (reverse transcription-polymerase chain reaction) and GC-MS (gas chromatography - mass spectrometry). The advantages and disadvantages of these techniques include those of sensitivity, reproducibility, and cost. Two of the techniques, ELISA and GC-MS, measured levels of the protein VTG, and RT-PCR measured the up-regulation of messenger RNA. The advantage of measuring mRNA compared with measuring the expression of the protein VTG include very fast response upon exposure and detection that can be very sensitive. However, the increased levels of mRNA are less persistent after exposure, and the technique requires expensive specialized equipment. Alternatively, ELISA and GC-MS measure the VTG protein that persists longer post-exposure compared with mRNA. The immunologically based ELISA relies on antigen-antibody interactions, with associated antibody specificity questions for quantification. GC-MS does not have the problems of specificity associated with immunoassay and offers the potential to measure multiple proteins in a single sample. Although GC-MS requires very expensive, advanced equipment, the technique can provide critical performance evaluations by providing a secondary means to measure the level of protein in standards and other QC criteria required for a screening assay.

A comprehensive survey of the literature and experts in the field of induction of VTG in fish (Battelle 2002) revealed that the technique of ELISA is currently the most widely developed and applied technique, with multiple methods that can be applied to fathead minnows. The ELISA technique is represented in this study by assays developed in several species of fish that can be applied to fathead minnows. These various assays rely upon different types of antibodies (e.g. monoclonal and polyclonal) and are performed with multiple approaches (e.g. competitive, sandwich ELISA). A summary of the ELISA methods by general type that were employed by the laboratories participating in this study are presented in Table 2, with the specifics of the ELISA presented in Table 1. It should be noted that one laboratory applied two ELISA methods to its set of samples, and that this allowed the comparison of three laboratories employing a commercially available kit.

Table 2. Summary of Antibody Methods Used by Participating Laboratories

Lab ID	Lab Name	ELISA Method Antibody
1	University of Florida	Fathead Minnow
1	University of Florida	Carp-sandwich
4	US EPA Duluth	Fathead Minnow
5	University of Exeter	Carp-competitive
6	Brixham Environmental	Carp-competitive
7	Biosense	Carp-sandwich
8	INERIS	Zebrafish
11	University of Southern Denmark	Zebrafish
13	Battelle Sequin	Carp-sandwich

The more specialized technique of RT-PCR to measure fathead minnow mRNA is conducted in a very limited number of laboratories. However, three laboratories agreed to participate in this study, thereby allowing an assessment of the variability of this method. These laboratories included Lab1; University of Florida, Lab 2; University of Idaho, Lab 15;MLT Research. The technique of GC-MS requires highly advanced, expensive equipment and the new application of this method to the measurement of fathead minnow VTG was assessed by one participating laboratory (Lab 14; Battelle Richland) in this study.

The ELISA methods that will be applied to the plasma and whole body homogenate standard series are immunologically based. ELISA relies on antigen-antibody interactions and the associated antibody specificity must be controlled for quantification. A variety of antibodies are used by the various methods. In addition to the use of the standard homologous to each method, the participating laboratories were supplied with purified fathead minnow VTG for use as a standard. As a result, six of the laboratories used the VTG purified in this study to analyze the samples in addition to their homologous standard (Table 3). In addition to the samples within the series from exposed and unexposed fish, each laboratory received a sample spiked with purified fathead minnow VTG. A summary of the concentration, standards, antibody, and assay codes are presented in Table 4. The concentration codes are based upon the exposure history and sex of the fish used to generate the samples in the series. The standard codes identify the results based on the use of the standard routinely employed by eh individual laboratory, or based upon the fathead minnow standard provided to each of the participating laboratories as a part of this study. The antibody codes group the assays into 4 general categories of antibody type used in the various assays. The assay code defines the use of a commercial kit (Carp sandwich ELISA) vs the assay unique to the individual laboratories. These groupings were used to analyze the variability of the reported results.

Table 3. Summary of Standards Employed by Participating Laboratories

Lab #	Lab Name	Homologous Std	Purified fathead minnow Std
1	University of Florida	X	X
4	US EPA Duluth	X	X
5	University of Exeter	X	-
6	Brixham Environmental	X	-
7	Biosense	X	X
8	INERIS	X	X
11	University of Southern Denmark	X	X
13	Battelle	X	X

Table 4. Summary of the Concentration, Standards, Antibody, and Assay Codes

Concentration	Conc. Code	STD	STD Code	Antibody	Antibody Code	Assay	Assay Code
Blank	0	homologous	1	Carp sandwich	1	kit	1
Unexposed Male	1	Purified fathead minnow	2	Fathead Minnow	2	Unique	0
Unexposed Female	2	--	--	Carp competitive	3	--	--
Exposed Male	3	--	--	Zebrafish	4	--	--
Exposed Female	4	--	--	--	--	--	--
Positive Control	5	--	--	--	--	--	-

To assess the overall variability of analysis by all of the various methods, the reported ELISA results were analyzed irrespective of method or the standard employed. This reflects the variability encountered when a number of methods for use in the measurement of VTG in fathead minnow samples are applied to standard samples spanning a wide range of concentrations. When conducting the analysis, each of the participating laboratories evaluated three analytical replicates for each of three sample replicates (Appendix C). The three analytical replicates are a measure of the within-run variability. When all of the reported results were used, the within-run variability for plasma had a wide range of coefficients of variation (CVs), ranging from 0% to 173% with a mean of 13% (Table 5; Appendix D). The within-run variability for homogenate was similar with CVs ranging from 0% to 162%. Indeed, most of the large CV's were associated with samples that had one or two observations less than detected. If all undetected values are ignored the maximum CV for plasma and homogenate become 83% and 141%, respectively. The quartiles of the CV distribution remain the same with or without the

less than detected values. For both sample types, 75% of the within-run CVs were less than 16%. The CVs for both the plasma and homogenate samples tended to be less than 30% for all concentrations except for those derived from the blank and unexposed males (Figures 1 and 2). With this assessment of the within-run variability, all the remaining analyses were conducted on the mean result of the analytical replicates.

Table 5. Descriptive Statistics of the CVs of the Within-run Analytical Results where Q1 and Q3 are the 1st and 3rd Quartiles, Respectively

Series	Concentration	Mean	Minimum	Maximum	Q1	Q3	N
Plasma	Blank	18%	0%	142%	0%	26%	40
Plasma	Uninduced Male	23%	0%	173%	0%	23%	45
Plasma	Uninduced Female	10%	2%	83%	2%	9%	39
Plasma	Induced Male	9%	0%	74%	3%	10%	40
Plasma	Induced Female	8%	2%	20%	4%	14%	36
Plasma	Positive Control	9%	1%	24%	4%	11%	42
Plasma	All	13%	0%	173%	3%	13%	242
Homogenate	Uninduced Male	26%	0%	162%	0%	39%	45
Homogenate	Uninduced Female	21%	0%	94%	1%	20%	44
Homogenate	Induced Male	19%	0%	102%	3%	16%	40
Homogenate	Induced Female	7%	1%	22%	3%	11%	40
Homogenate	Positive Control	15%	0%	141%	2%	9%	42
Homogenate	All	18%	0%	162%	2%	15%	211

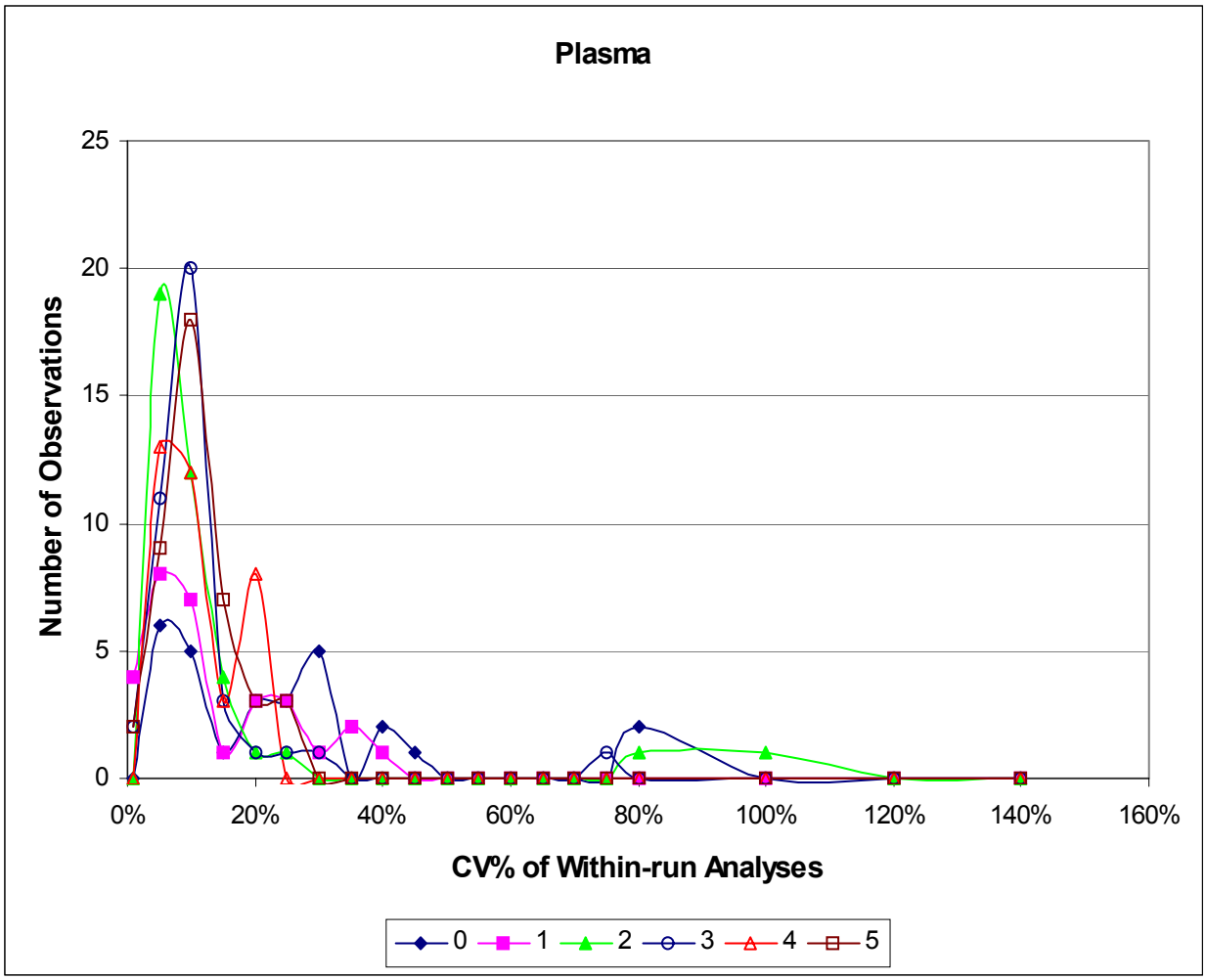


Figure 1. Distribution of the Within-run Coefficient of Variation (CV) of the VTG Analytical Result on Plasma Samples for Each Concentration Code (0 - 5)

Code 0 = Blank; Code 1 = Uninduced Male; Code 2 = Uninduced Female;
Code 3 = Induced male; Code 4 = Induced Female; Code 5 = Positive Control

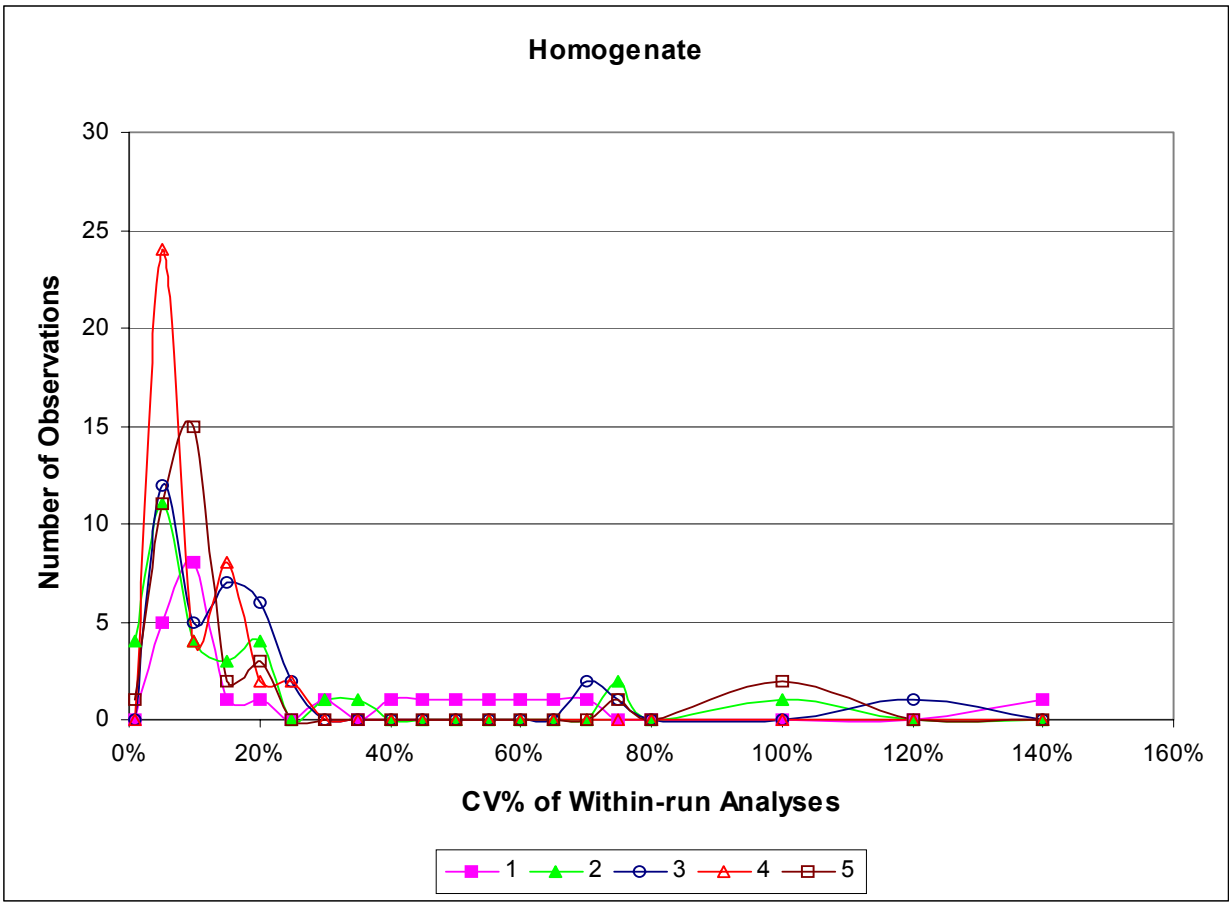


Figure 2. Distribution of the Within-run Coefficient of Variation (CV) of the VTG Analyt-ical Result on Homogenate Samples for Each Concentration Code (1 - 5)

Code 1 = Uninduced Male; Code 2 = Uninduced Female;
Code 3 = Induced male; Code 4 = Induced Female; Code 5 = Positive Control

In addition to assessing the within run variability from the analytical replicates, the three sample replicates provided a measure of intra-assay variability. The intra-assay variability for plasma and homogenate had CVs ranging from 0% to 173% (Table 6; Appendix E). For both sample types 75% of the intra-assay CVs were less than 51%. Again, many of the large CVS were due to means calculated with one or more values less than the detection limit. If all such means are ignored, the maximum CV for plasma and homogenate become 124% and 125%, respectively.

Table 6. Descriptive Statistics of the CVs of the Intra-Assay Analytical Results where Q1 and Q3 are the 1st and 3rd Quartiles Respectively

Series	Concentration	Mean	Minimum	Maximum	Q1	Q3	N
Plasma	Blank	27%	0%	99%	0%	54%	14
Plasma	Uninduced Male	43%	0%	173%	12%	63%	14
Plasma	Uninduced Female	36%	2%	111%	12%	57%	14
Plasma	Induced Male	21%	1%	105%	7%	21%	14
Plasma	Induced Female	37%	2%	124%	12%	40%	12
Plasma	Positive Control	27%	6%	92%	12%	36%	14
Plasma	All	32%	0%	173%	9%	44%	82
Homogenate	Uninduced Male	46%	0%	173%	6%	63%	16
Homogenate	Uninduced Female	29%	0%	72%	11%	53%	16
Homogenate	Induced Male	45%	3%	165%	10%	42%	14
Homogenate	Induced Female	30%	1%	145%	4%	47%	14
Homogenate	Positive Control	21%	0%	87%	4%	29%	14
Homogenate	All	34%	0%	173%	7%	51%	74

One goal of this study was to create a series of analytical samples that included a wide range of VTG concentrations in male and female plasma and whole body homogenates. It was anticipated that male fish unexposed to estrogenic compounds would provide minimal levels of VTG in plasma and tissues, with unexposed female fish, exposed male, and exposed female fish generating increasing levels of VTG in their respective systems. Inclusive of all reported results, the general trend for the plasma samples observed for each laboratory averaged over antibodies, standards, and assays was the expected increase based on the series (i.e., uninduced male < uninduced female < induced male < induced female fathead minnows; Figure 3). However, for several of the average laboratory results, the uninduced male (Code 1) results were equal or only slightly greater than the prepared blank (Code 0). For half of the average laboratory homogenate results the uninduced male (Code 1) VTG concentrations were approximately equal to or greater than that of uninduced female results (Code 2; Figure 4). All but Laboratory 13 showed an increase in average VTG concentration between uninduced female results and induced males (Code 3). Further, two of the average laboratory results (laboratories 5 and 6) showed a decrease in average VTG concentration between the induced male samples and the induced female samples (Code 4). Descriptive Statistics for the Mean VTG Results Averaged Over Laboratory, Antibody, Standard, and Assay are provided Table 7.

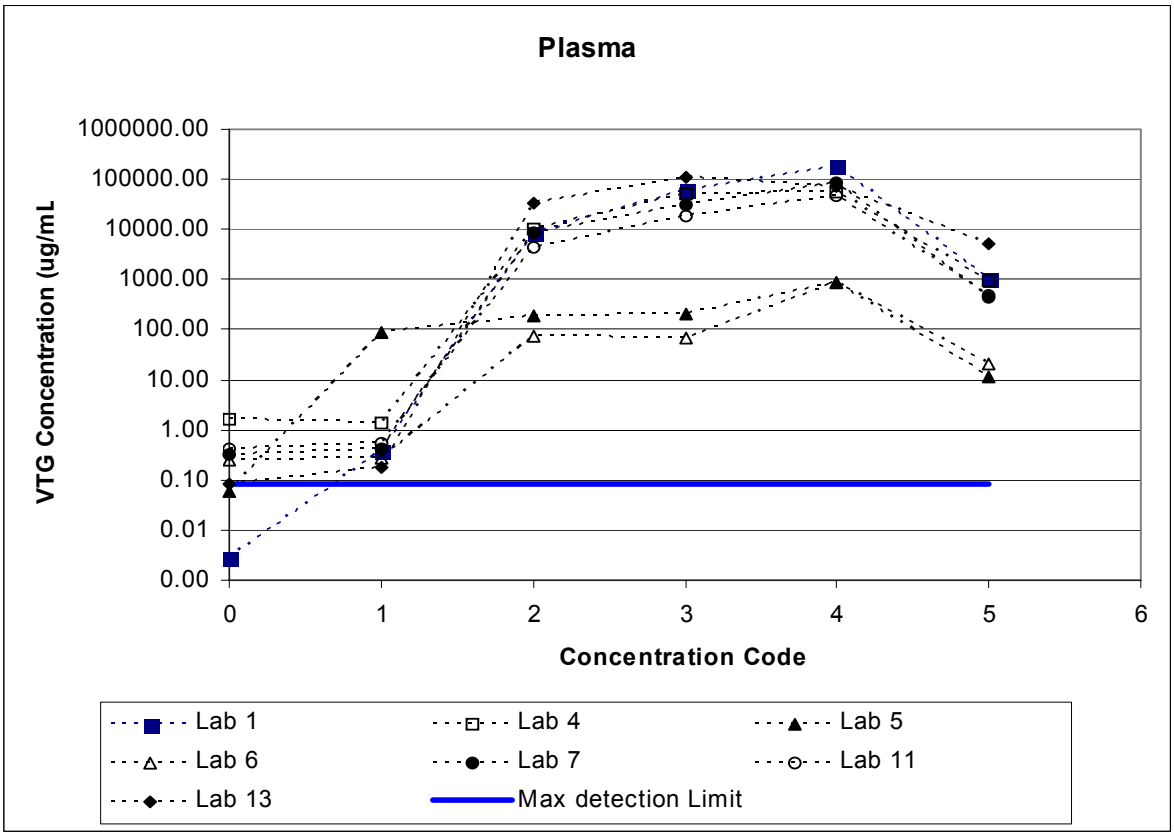


Figure 3. Each Laboratories VTG Concentration for A Given Concentration Code Averaged Over Antibody, Standard, and Assay for Plasma Samples

Code 0 = Blank; Code 1 = Uninduced Male; Code 2 = Uninduced Female;
Code 3 = Induced male; Code 4 = Induced Female; Code 5 = Positive Control

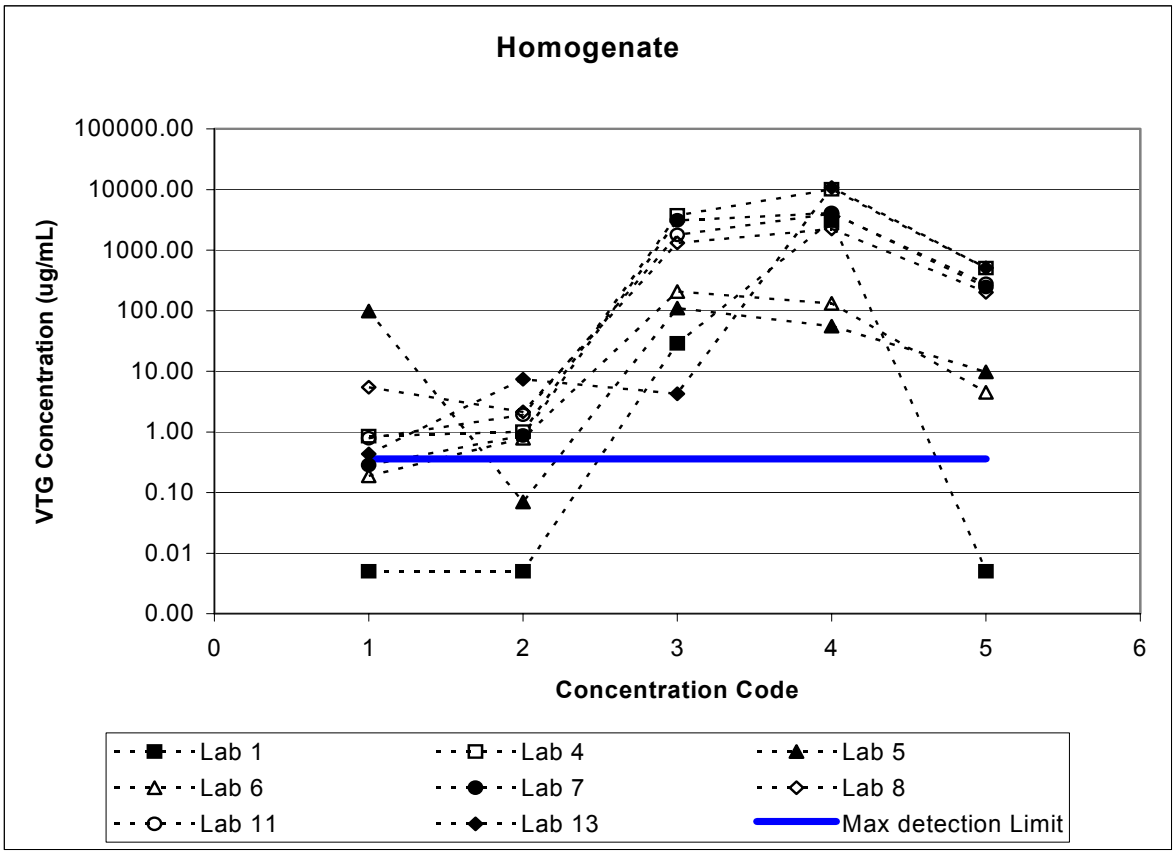


Figure 4. Each Laboratories VTG Concentration for A Given Concentration Code Averaged Over Antibody, Standard, and Assay for Homogenate Samples

Code 1 = Uninduced Male; Code 2 = Uninduced Female;
Code 3 = Induced male; Code 4 = Induced Female; Code 5 = Positive Control

Table 7. Descriptive Statistics for the Mean VTG Results Averaged Over Laboratory, Antibody, Standard, and Assay

Series	Concentration Code	N	Average	Standard Deviation	CV%
Plasma	0	43	0.4	0.7	161%
	1	48	6	38.3	635%
	2	41	10560	19574	185%
	3	43	43847	37612	86%
	4	38	74071	60909	82%
	5	45	1258	2113	168%
Homogenate	0	0	-	-	-
	1	48	7.2	42.6	588%
	2	47	2	3.1	160%
	3	43	1452	1588	109%
	4	43	4881	4492	92%
	5	45	251	237	95%

A number of the participating laboratories were able to perform their analysis using both their homologous standard (i.e. unique carp, zebrafish or fathead minnow) and the fathead minnow standard purified for this study. This offers the comparison of method specificity and standardization in a screening assay. To examine the results based upon the standard used within the assay, the percentage difference between the average replicate VTG concentrations obtained with the homologous standard (H) and Purified fathead minnow standard (B) was calculated as (H-B)/B (100%). Thus, negative values represent greater VTG concentrations obtained with the Purified fathead minnow standard. The results of this analysis are presented in Figure 5 (Appendix E) by analytical laboratory. Note, for small concentrations, small absolute differences between the results of each standard may still be a large proportion of the Purified fathead minnow standard result. The intent in highlighting these small differences is due to the need for great precision at small doses.

When the type of assay is examined, the Carp sandwich ELISA followed by the zebra fish assay produced the greatest variation between standard results for plasma (-82% and -75% respectively), and the fathead minnow followed by the zebra fish antibody based assays produced the greatest variation between standards for the homogenate samples (200% and 99% respectively; Figure 5). The large difference observed with the homogenate results is due to averaging small numbers associated with three less than detected values in the homologous standard and seven less than detected values with the Purified fathead minnow standard. In contrast to the plasma samples, nearly all of the fathead minnow antibody homogenate data had greater VTG concentrations with the homologous standard.

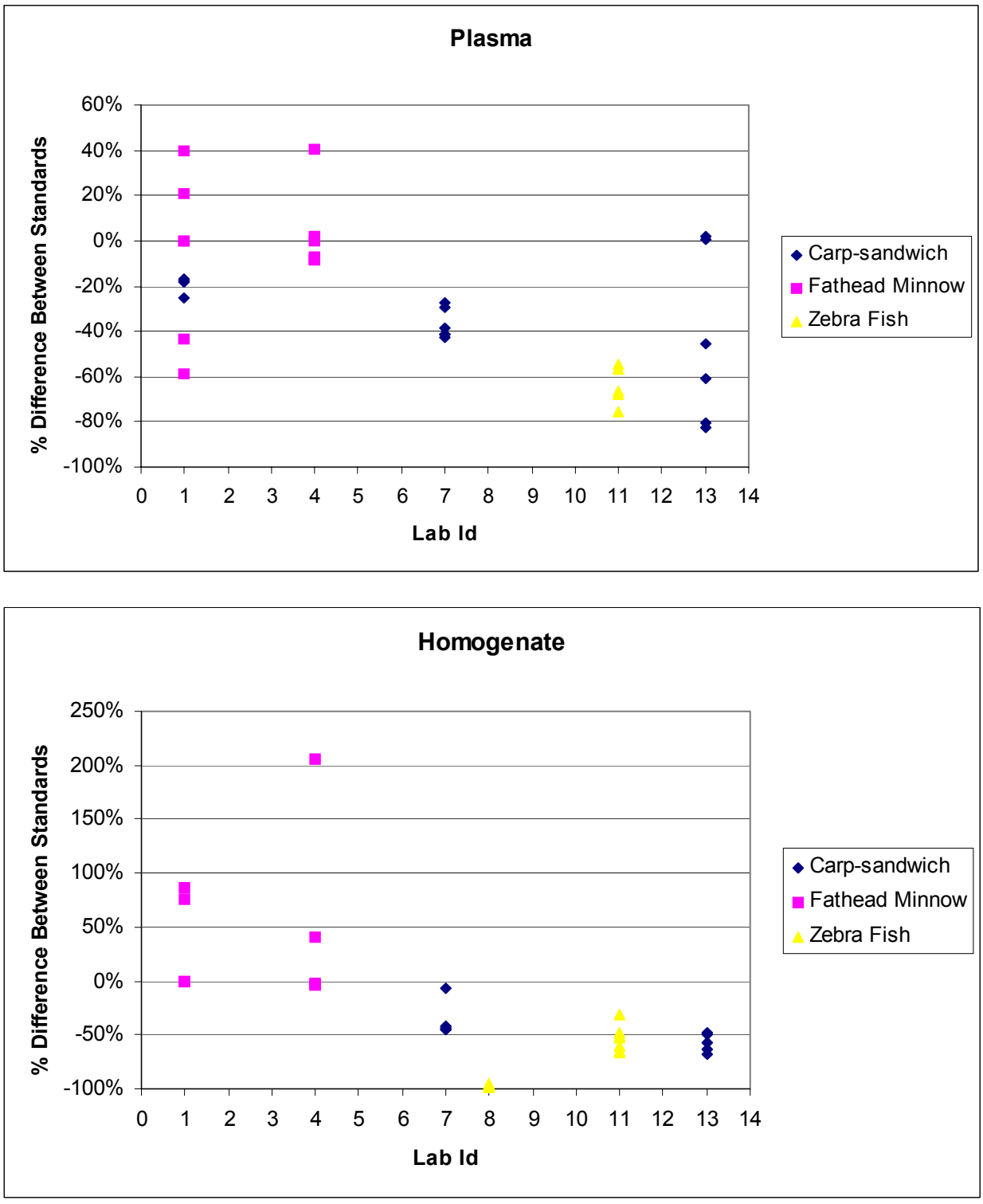
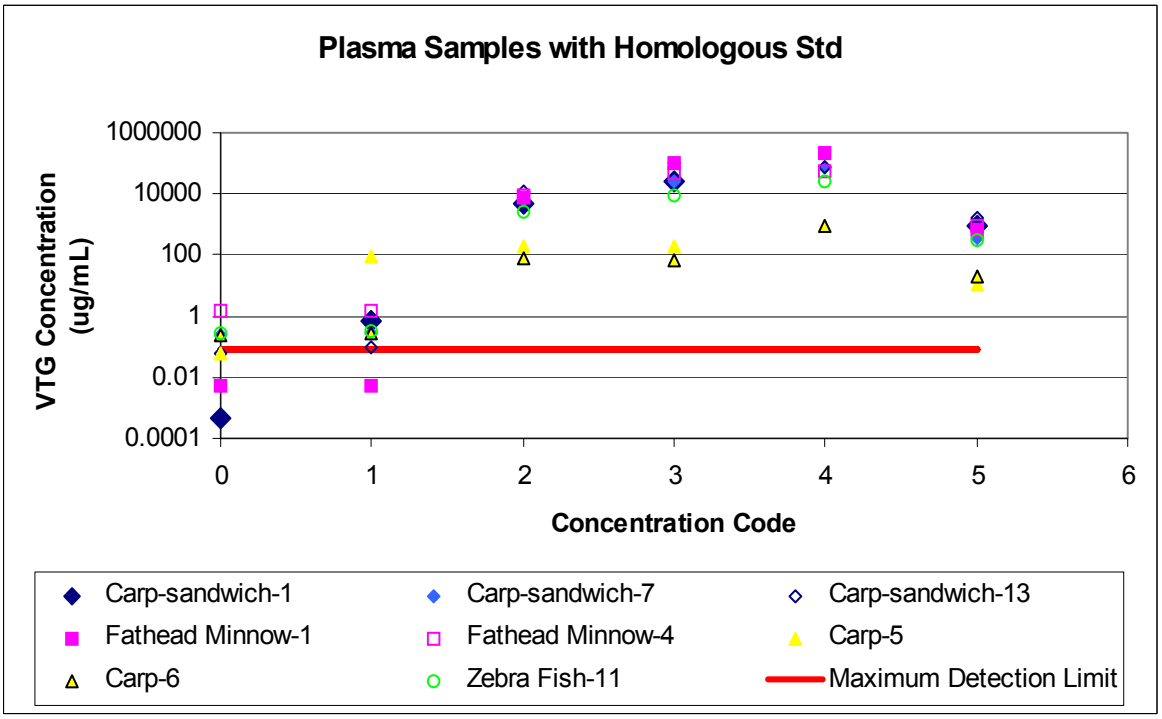


Figure 5. The Percentage Difference in VTG Concentrations Averaged Over Replicates Between the Homologous and Purified fathead minnow Standard Data in Plasma and Homogenate Samples

In the remaining types of assays (zebrafish and the carp-based sandwich), the concentrations based upon the purified fathead minnow standard were equal to or lower than the homologous standard. This reflects a reduced specificity of the zebrafish and carp-based antibodies to the fathead minnow vitellogenin, resulting in lower values reported with the homologous standard. Because of the large variation in the results between the two standards, data based on each standard was evaluated separately for all remaining analyses.

When the concentration of VTG in plasma and homogenate samples is examined with the assays performed in the manner routine to the participating laboratories (i.e. homologous standard), the carp antibody based competitive ELISAs tended to produce the lowest VTG concentrations in the plasma standard samples (Figure 6). Further, these ELISAs did not distinguish between the exposed male and unexposed female samples (concentration codes 2 and 3 respectively). It should be noted that the carp antibody competitive ELISA were not reported with the Purified fathead minnow standard. The carp antibody based competitive ELISAs with homogenate samples still produced low VTG concentrations, but not the lowest reported values (Figure 7). There was slightly less variation in VTG concentrations with the Purified fathead minnow standard for both sample types. For the data produced using the purified fathead minnow standard, the carp sandwich ELISA and the fathead minnow antibody tended to produce greater variation between laboratories for the blank and uninduced male plasma samples (Figure 6). The fathead minnow antibody produce greater variation between laboratories for the uninduced male and female samples for homogenate samples (Figure 7).



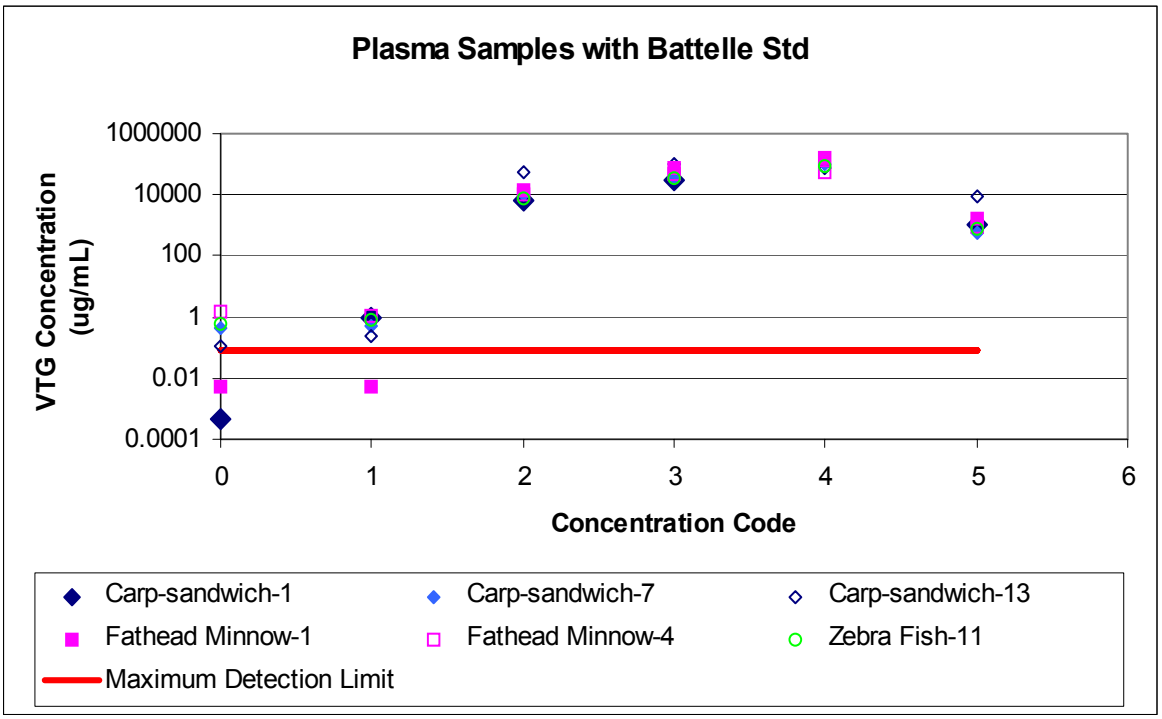
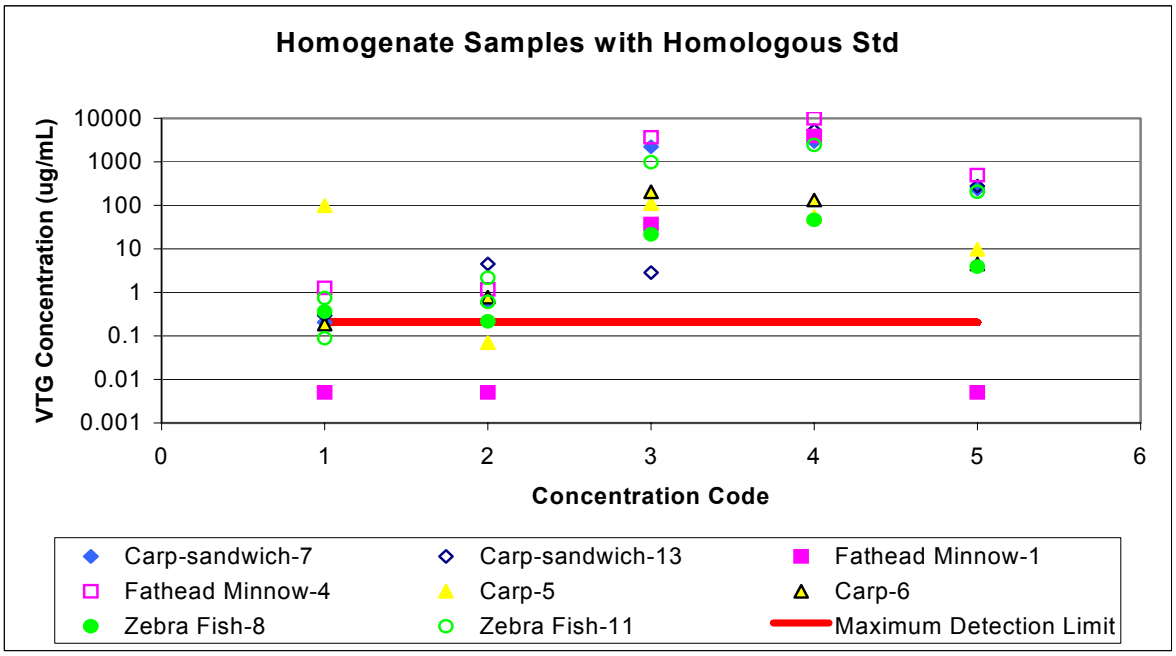


Figure 6. The VTG Concentration in Plasma Samples for Each Standard, Laboratory, Antibody, and Concentration Code

Code 1 = Uninduced Male; Code 2 = Uninduced Female;
Code 3 = Induced male; Code 4 = Induced Female; Code 5 = Positive Control



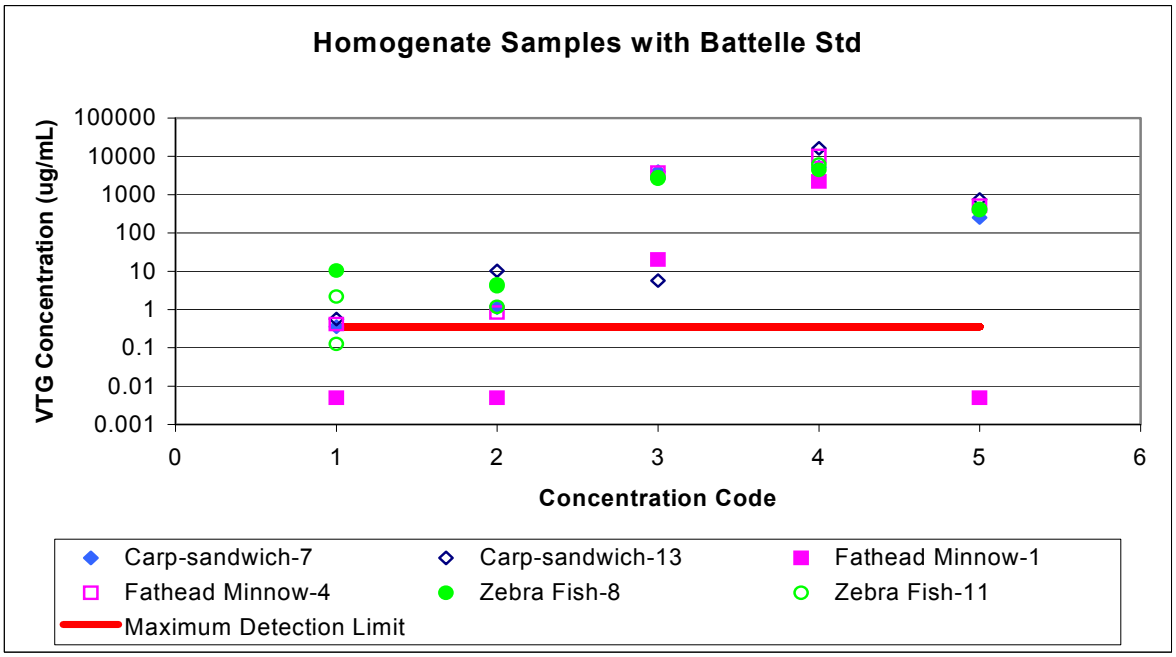


Figure 7. The VTG Concentration in Homogenate Samples for Each Standard, Laboratory, Antibody, and Concentration Code

Code 1 = Uninduced Male; Code 2 = Uninduced Female;
Code 3 = Induced male; Code 4 = Induced Female; Code 5 = Positive Control

Simple linear regression analysis of the ranked VTG concentration within sample type, laboratory, standard, and antibody confirmed significant positive slopes ($p <= 0.003$) across the concentration codes 0 - 4 (blank through induced female series) for all combinations except for Laboratory 5 using the homogenate samples, homologous standard, and carp antibody data ($p = 0.128$, $n = 12$; Table 9). The lack of significance for this set of observations was due to a greater average concentration of VTG observed in the uninduced males than in the uninduced females (Figure 7). All regressions had a minimum of 8 degrees of freedom for error. The positive control (concentration code 5) was not used for this analysis. With the one exception for homogenate samples, this demonstrates the successful measurement of the increasing concentrations of VTG in the samples among multiple assays, sample type, standard, antibody and laboratory.

Tuckey's HSD was used to compare the mean ranked concentrations between successive pairs in the series using laboratories as replicates (Table 10). For this comparison, observations were ranked within sample type, standard, and antibody. There were only 2 to 3 replicates for each concentration and 4 to 9 degrees of freedom for error. None of the successive concentrations were found to be significantly different for any of the sample type, standard, and antibody combinations (family-wise error rate = 0.05). However, most comparisons separated by two concentrations were significantly different for plasma samples used with the Carp sandwich ELISA antibody. The remainder of the comparisons had too little replication and too large of a variation within classes to achieve significance other than between the lowest and highest concentrations. Homogenate samples analyzed with homologous standards and fathead minnow and carp antibodies had no detected significant differences between any of the concentrations. Therefore, even though the regressions across the series from blank to induced female were significant, the variability between laboratories to reproduce the ranks for a given concentration code was too large to detect differences between successive concentration pairs in the series.

When the variability of results by laboratory and standard were examined, the C'S associated with the plasma zebrafish antibody results were lower with the Purified fathead minnow standard than with the homologous standard (Figure 8). The Carp sandwich ELISA CVs tended to be equal or greater with the Purified fathead minnow standard than with the homologous standard. This trend was repeated in the homogenate samples (Figure 9). The fathead minnow CVs tended to be greater in the homogenate samples than in the plasma samples for both the homologous and Purified fathead minnow standard results. Greater than 50% of the CVs obtained with the carp antibody were greater than 30%.

Three laboratories applied the same commercial carp sandwich ELISA to the sample series, including the low level samples and positive control. This allows for some measure of the application of one of the methods in this study by multiple laboratories; however, similar assessments of the other methods in this study would be required to draw general conclusions based on this very limited data set. For those laboratories that used this method in this study, a simple linear regression of the average VTG concentration (within-run average) between the blank and the uninduced male concentrations was conducted to determine whether a significant slope was obtained. For the plasma samples (Laboratories 1, 7, and 13), only Laboratory 13 produced a significant slope between these two concentrations using both standards ($p = 0.006$, 4

d.f. for error). For the homogenate samples (Laboratories 7 and 13), both Laboratories 7 and 13 produced significant slopes using the homologous standard ($p < 0.05$, 4 d.f. for error), and Laboratory 7 produced a significant slope using the purified fathead minnow standard ($p = 0.005$, 4 d.f. for error). The means and standard deviations for each of the concentrations is presented in Table 11. Although these results indicate that the detection of background or low levels of VTG in fathead minnow samples can be achieved with the application of this ELISA, the lack of consistent concentrations and significant differences between samples among laboratories requires additional study for method assessment.

The positive control samples for both the plasma and homogenate were spiked to 500 µg/ml VTG with the fathead minnow VTG purified for this study. With three laboratories conducting the carp sandwich ELISA, these results can be examined for statistical significance. The positive control concentrations was not achieved with the carp sandwich ELISA using laboratories as replicates (Table 12). The results of the positive control were again highly variable both within and between laboratories. The concentrations detected in plasma ranged from 337 to 8834 µg/ml VTG in plasma and from 235 to 745 µg/ml VTG in the homogenate control sample. In addition to the variation found in the Carp sandwich ELISA method, a similar wide range of concentrations for the control samples were detected among all of the assays (Table 8; Figure 6 and 7)

Table 8. Results of the Linear Regression on Within-Laboratory, Sample Type, Standard, and Antibody Ranked VTG Concentrations Observed in the Series Codes 0 Through 4

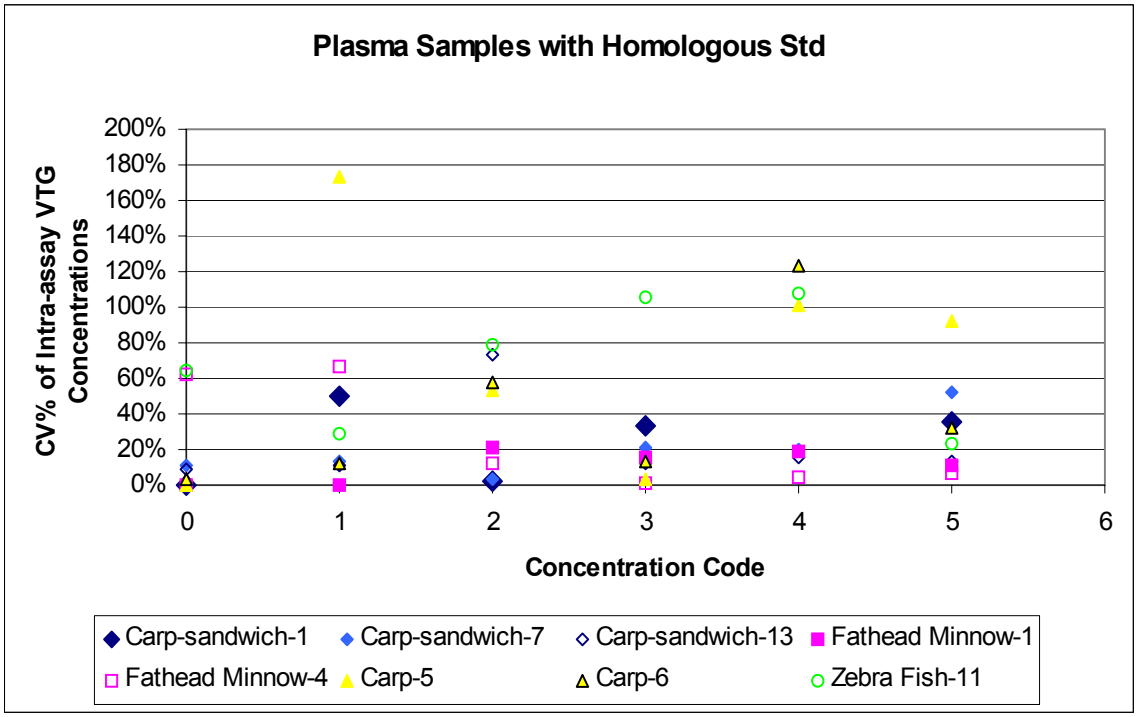
Series	Standard	Antibody	Lab	Slope	Std Error	d.f.	p-value
Plasma	1	1	1	2.629	0.2175	8	< 0.001
Plasma	1	1	7	2.9333	0.2368	13	< 0.001
Plasma	1	1	13	2.7	0.3965	13	< 0.001
Plasma	1	2	1	3.4	0.2736	12	< 0.001
Plasma	1	2	4	2.6099	0.2962	12	< 0.001
Plasma	1	3	5	2.7944	0.5344	12	< 0.001
Plasma	1	3	6	2.485	0.3272	11	< 0.001
Plasma	1	4	11	4.7	0.6595	27	< 0.001
Plasma	2	1	1	2.629	0.2175	8	< 0.001
Plasma	2	1	7	2.9333	0.2368	13	< 0.001
Plasma	2	1	13	2.4	0.5243	13	0.001
Plasma	2	2	1	3.4	0.2736	12	< 0.001
Plasma	2	2	4	2.6074	0.3509	12	< 0.001
Plasma	2	4	11	4.0104	0.3722	19	< 0.001
Homogenate	1	1	7	3.2333	0.2993	10	< 0.001
Homogenate	1	1	13	2.5333	0.5582	10	0.001
Homogenate	1	2	1	3.5	0.6455	10	< 0.001
Homogenate	1	2	4	2.6667	0.4922	10	< 0.001
Homogenate	1	3	5	1.5667	0.9432	10	0.128
Homogenate	1	3	6	2.8	0.4115	10	< 0.001
Homogenate	1	4	8	2.4	0.6143	10	0.003
Homogenate	1	4	11	4.7521	0.5216	18	< 0.001
Homogenate	2	1	7	3.2333	0.2993	10	< 0.001
Homogenate	2	1	13	2.5333	0.5582	10	0.001
Homogenate	2	2	1	3.5	0.6455	10	< 0.001
Homogenate	2	2	4	2.8667	0.3627	10	< 0.001
Homogenate	2	4	8	2.4	0.6143	10	0.003
Homogenate	2	4	11	4.0057	0.5089	15	< 0.001

Table 9. Results of Tukey's HSD Multiple Comparison Test On the Ranked VTG Concentrations Using Laboratories as Replicates

Series	Standard	Antibody	0 to 1	0 to 2	0 to 3	0 to 4	1 to 2	1 to 3	1 to 4	2 to 3	2 to 4	3 to 4	Error d.f.
Plasma	1	1	NS	*	*	*	NS	*	*	NS	NS	NS	9
Plasma	1	2	NS	NS	NS	*	NS	*	*	NS	NS	NS	5
Plasma	1	3	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	5
Plasma	1	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
Plasma	2	1	NS	*	*	*	NS	*	*	NS	NS	NS	9
Plasma	2	2	NS	NS	NS	*	NS	NS	*	NS	NS	NS	5
Plasma	2	4	-	-	-	-	NA	NA	NA	NA	NA	NA	0
Homogenate	1	1	-	-	-	-	NS	NS	*	NS	NS	NS	4
Homogenate	1	2	-	-	-	-	NS	NS	NS	NS	NS	NS	4
Homogenate	1	3	-	-	-	-	NS	NS	NS	NS	NS	NS	4
Homogenate	1	4	-	-	-	-	NS	NS	*	NS	NS	NS	6
Homogenate	2	1	-	-	-	-	NS	NS	*	NS	NS	NS	4
Homogenate	2	2	-	-	-	-	NS	NS	NS	NS	NS	NS	4
Homogenate	2	4	-	-	-	-	NS	NS	*	NS	*	NS	6

NS = Not Significant
* = Significant at a Family-Wise Error Rate of 0.05
NA = Not Applicable

- = No Data



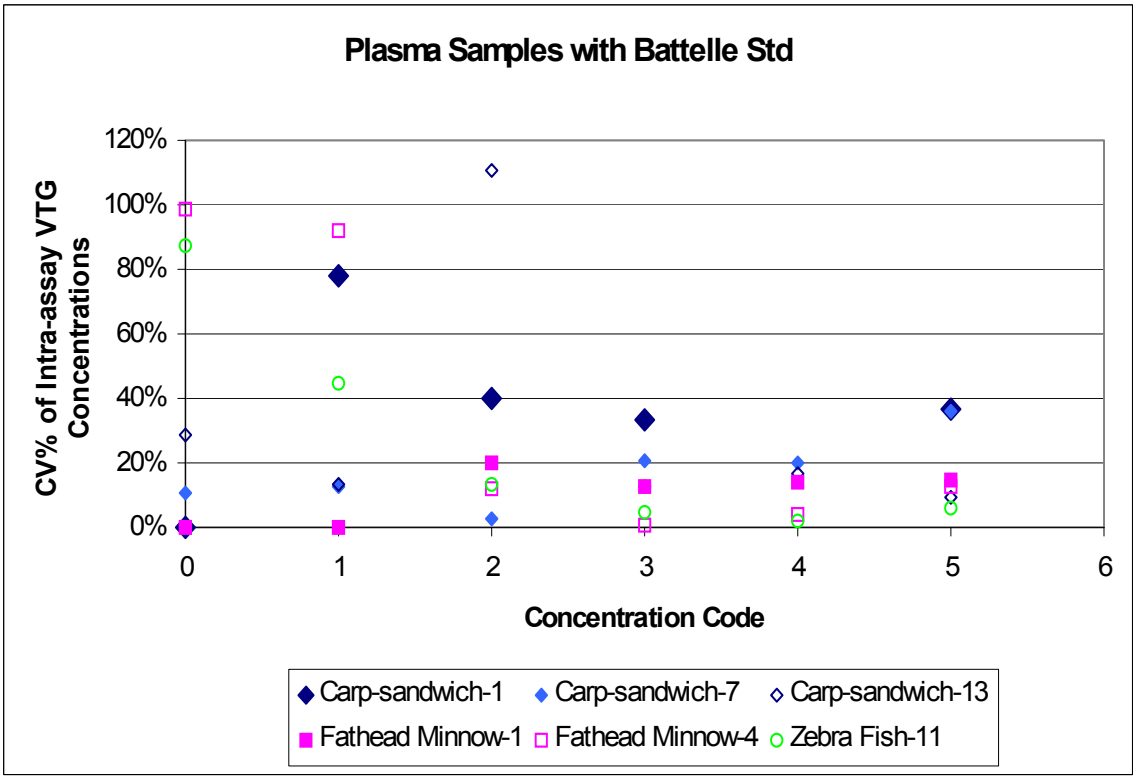
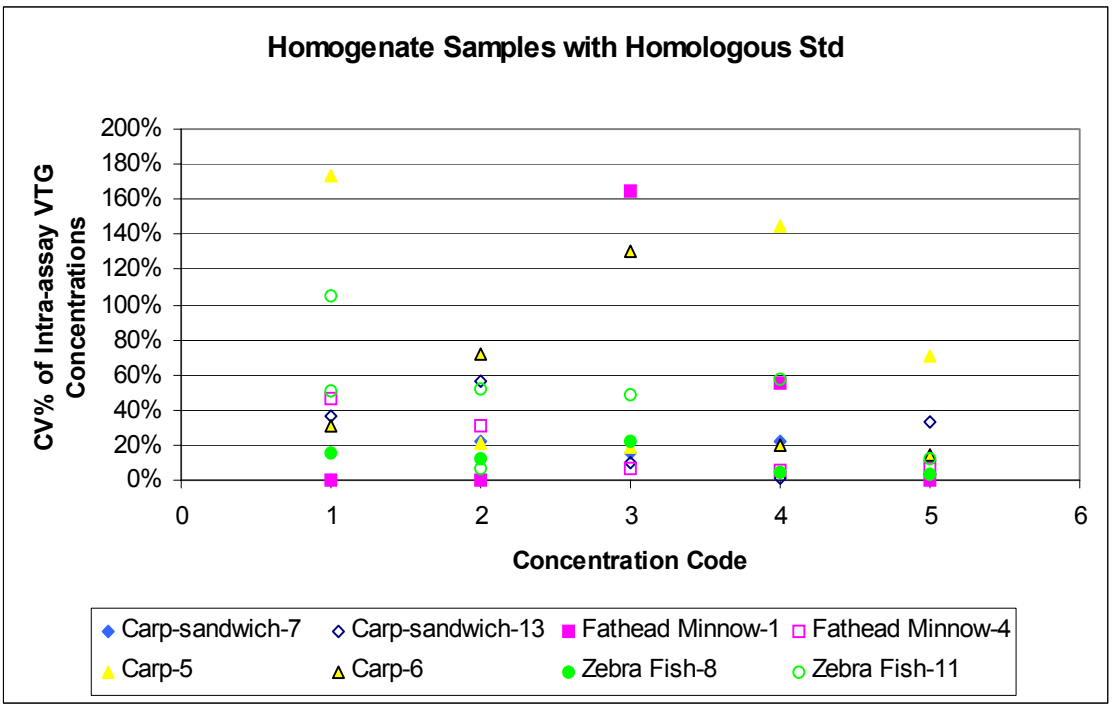


Figure 8. The CVs of the Average VTG Concentration for Each Laboratory, Antibody, and Concentration Code for Plasma Samples

Code 1 = Uninduced Male; Code 2 = Uninduced Female;Code 3 = Induced male; Code 4 = Induced Female; Code 5 = Positive Control



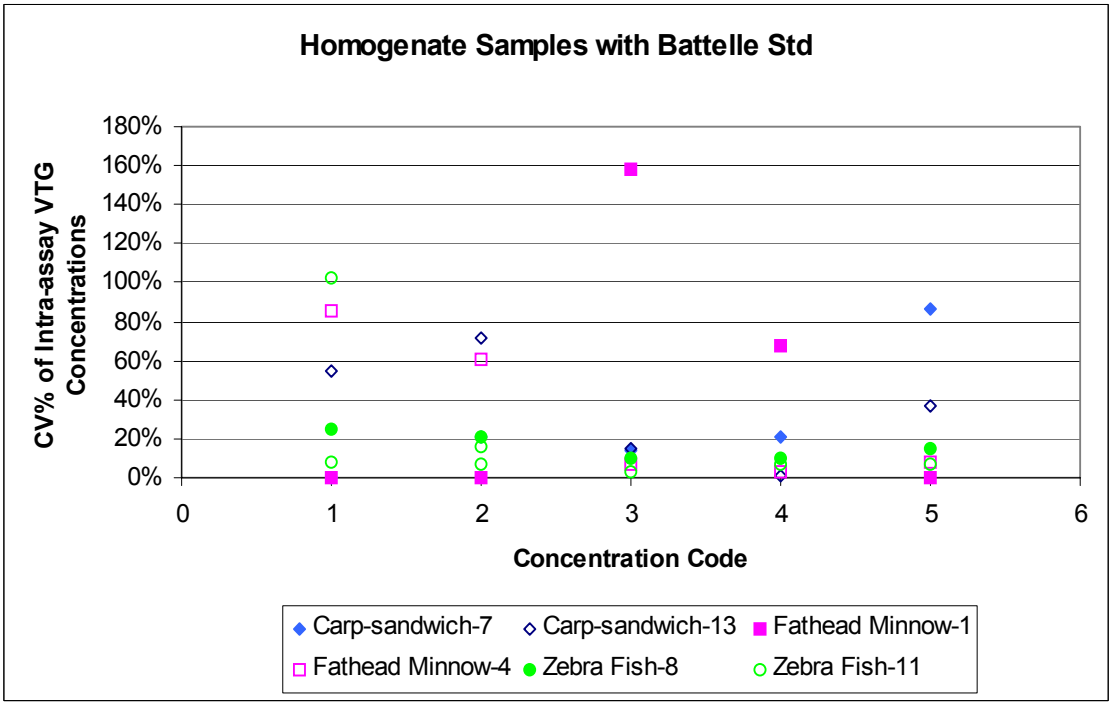


Figure 9. The CVs of the Average VTG Concentration for Each Laboratory, Antibody, and Concentration Code for Homogenate Samples

Code 1 = Uninduced Male; Code 2 = Uninduced Female;
Code 3 = Induced male; Code 4 = Induced Female; Code 5 = Positive Control

Table 10. Descriptive Statistics for the VTG Concentrations Obtained from the Carp sandwich ELISA Each Sample Type, Standard, Laboratory, and Concentration Code

Series	Std	Lab Id	Concentration Code	Mean	Qualifier	Stdev
Plasma	Homologous	1	0	0.0005	U	0
Plasma	Homologous	7	0	0.24		0.03
Plasma	Homologous	13	0	0.06		0.01
Plasma	Homologous	1	1	0.67		0.33
Plasma	Homologous	7	1	0.30		0.04
Plasma	Homologous	13	1	0.10		0.01
Plasma	Homologous	1	2	5056		96
Plasma	Homologous	7	2	6611		194
Plasma	Homologous	13	2	11197		8227
Plasma	Homologous	1	3	25584		8410
Plasma	Homologous	7	3	25252		5227
Plasma	Homologous	13	3	105878		13282
Plasma	Homologous	1	4	NA		NA
Plasma	Homologous	7	4	70513		14022
Plasma	Homologous	13	4	71344		11469
Plasma	Homologous	1	5	930		335
Plasma	Homologous	7	5	337		177
Plasma	Homologous	13	5	1573		207
Plasma	Purified fathead minnow	1	0	0.0005	U	0
Plasma	Purified fathead minnow	7	0	0.42		0.04
Plasma	Purified fathead minnow	13	0	0.11		0.03
Plasma	Purified fathead minnow	1	1	0.89		0.69
Plasma	Purified fathead minnow	7	1	0.50		0.06
Plasma	Purified fathead minnow	13	1	0.25		0.03
Plasma	Purified fathead minnow	1	2	6130		2459
Plasma	Purified fathead minnow	7	2	10727		296
Plasma	Purified fathead minnow	13	2	56311		62311
Plasma	Purified fathead minnow	1	3	31277		10442
Plasma	Purified fathead minnow	7	3	34673		7224
Plasma	Purified fathead minnow	13	3	104978		13163
Plasma	Purified fathead minnow	1	4	NA		NA
Plasma	Purified fathead minnow	7	4	99859		19822
Plasma	Purified fathead minnow	13	4	70144		11469
Plasma	Purified fathead minnow	1	5	1117		411
Plasma	Purified fathead minnow	7	5	572		204

Series	Std	Lab Id	Concentration Code	Mean	Qualifier	Stdev
Plasma	Purified fathead minnow	13	5	8834		804
Homogenate	Homologous	7	1	0.21	U	0
Homogenate	Homologous	13	1	0.3		0.11
Homogenate	Homologous	7	2	0.61		0.13
Homogenate	Homologous	13	2	4.48		2.51
Homogenate	Homologous	7	3	2212		334
Homogenate	Homologous	13	3	2.87		0.28
Homogenate	Homologous	7	4	2946		649
Homogenate	Homologous	13	4	5281		60.1
Homogenate	Homologous	7	5	235		8.34
Homogenate	Homologous	13	5	275		91.4
Homogenate	Purified fathead minnow	7	1	0.36	U	0
Homogenate	Purified fathead minnow	13	1	0.57		0.31
Homogenate	Purified fathead minnow	7	2	1.13		0.23
Homogenate	Purified fathead minnow	13	2	10.3		7.36
Homogenate	Purified fathead minnow	7	3	3987		566
Homogenate	Purified fathead minnow	13	3	5.67		0.86
Homogenate	Purified fathead minnow	7	4	5219		1079
Homogenate	Purified fathead minnow	13	4	16222		201
Homogenate	Purified fathead minnow	7	5	252		218
Homogenate	Purified fathead minnow	13	5	745		278

U = Value less than detected

Table 11. Upper and Lower 95% Confidence Limits for the Mean VTG Concentrations Obtained from the Carp Sandwich ELISA Using Laboratories as Replicates

Series	Std	Lab Id	Concentration Code	Mean	Average	Stdev	LCL	UCL
Plasma	Homologous	1	5	929.8889	946.6546	618.4238	-589.596	2482.905
Plasma	Homologous	7	5	336.7841				
Plasma	Homologous	13	5	1573.291				
Plasma	Purified fathead minnow	1	5	1117.333	3508.06	4620.824	-7970.71	14986.83
Plasma	Purified fathead minnow	7	5	572.4025				
Plasma	Purified fathead minnow	13	5	8834.444				
Homogenate	Homologous	7	5	235.2678	255.0291	27.94667	3.939306	506.1188
Homogenate	Homologous	13	5	274.7903				
Homogenate	Purified fathead minnow	7	5	252.0114	498.6467	348.7949	-2635.14	3632.431
Homogenate	Purified fathead minnow	13	5	745.2819				

mRNA Results

Laboratory 1 and 2 Protocols; Taqman theory. The Taqman real-time PCR technique provides a gene specific assay for the measurement of VTG mRNA. The Taqman is very similar in action to regular PCR, except that in addition to amplifying target DNA, it also has the ability to calculate the amount of material present at each cycle by fluorescent signal allowing for relative comparison of starting mRNA material. The fluorescent signal is attached to a probe that binds between the forward and reverse primers of the target sequence. The signal is quenched when intact and is only released when the target sequence is amplified creating a direct correlation between amount of fluorescent signal and amount of amplified material. To calculate the amount of mRNA in samples, a standard curve is used with known amounts of mRNA. Based on amplification data relative to the standard curve, it is possible to calculate the amount of starting material in unknown samples. It is necessary to know a portion of the sequence of the gene to be measured in order to design Taqman specific PCR primers and probe. The specific sequence for FHM VTG has been published in the database and is available.

Laboratory 1. The FHM VTG specific sequences were used to develop Taqman specific primers and probe using Primer Express software (Applied Biosystems). Gene specific plasmids containing the VTG fragment were used to make the standards for the standard curve. To develop the standard curve, plasmid containing the cloned gene fragment, was diluted to 1ng/ul ($2.56x10^8$ ERα copies and $2.68x10^8$ VTG copies respectively). Performing serial dilutions, a standard curve range of 268 to $2.68x10^7$ copies was obtained for VTG. 18S rRNA was used as a normalizing gene to accommodate for error in preparation. Once a sample was run for ERα and/or VTG mRNA and 18S rRNA, mRNA amounts could be calculated. Raw data was

normalized based on average vs. individual 18S rRNA values and then calculated to determine amount of mRNA in copies per ug of total RNA.

Laboratory 2. Total RNA was extracted from a portion of the liver tissue sample supplied using a commercially available kit (QIAGEN). Total RNA was used in a real-time quantitative reverse transcription- polymerase chain reaction (qRT-PCR) on an Applied Biosystems (ABI) Model 7900 real-time PCR machine. The ABI TaqMan reagent system was used with primers and probe designed to a specific region of the fathead minnow VTG DNA sequence. Primers and probe sequence were selected using ABI Primer Express software, and sufficient reagents were synthesized by ABI. RT reactions were performed with oligo-dT, followed by PCR using TaqMan Universal PCR Master Mix and the VTG specific probe (fluorescent-labeled) and primers. As an internal standard the fathead minnow 12S ribosomal gene was used to allow post-PCR normalization of starting RNA amounts. Specific primers and probe for the 12S ribosomal gene were designed and synthesized similar to the VTG reagents described above. A standard curve developed by serially diluting a known reference sample was used in each assay, to which unknown samples were compared.

Laboratory 15. A hybridization protection assay (HPA) was applied to RNA extracted from the liver tissue of the fathead minnows. An oligonucleotide probe, labeled with a chemiluminescent acridinium ester, was introduced into a sample of the extracted RNA to hybridize with any complimentary target present. Hybridization is followed by a selection step, in which label attached to free probe is hydrolyzed to a non-chemiluminescent derivative, while label attached to hybridized probe is protected from such hydrolysis. Following this selection step, chemiluminescence is measured using a luminometer. The light intensity is proportional to the concentration of VTG mRNA in the sample.

The reported results from the three laboratories that applied their method to the detection of mRNA in the fathead minnow samples are summarized in Table 12. As described previously, Laboratory 1 and 2 applied RT-PCR and the third Laboratory (Lab15) applied a hybridization protection assay (HPA) to the samples. All three laboratories were provided with liver tissue and performed all analytical steps, including isolation of RNA from the tissue, as would be applied to analytical samples collected as a part of a screening assay, representing individual fish units. Although not directly comparable due to method differences and approach, all three methods distinguished between exposed and unexposed fish. In the unexposed fish, the levels were variable with each lab reporting generally higher, similar, or lower levels in the unexposed males vs. the levels found in unexposed females.

Table 12. Summary of the reported mRNA results for the liver samples from unexposed and exposed male and female fathead minnows.

Sample Code	Sample Type	Lab 1	Lab 2	Lab 15
1	unexposed male	3380	0.81	<0.1
1	unexposed male	430	0.61	<0.1
1	unexposed male	500	0.75	<0.1
1	unexposed male	43	0.48	<0.1
1	unexposed male	36	0.47	<0.1
1	unexposed male	**875** +/-1415*	**0.62** +/-0.20†	**<0.1** +/-0‡
2	unexposed female	26	1.56	<0.1
2	unexposed female	23	1.22	<0.1
2	unexposed female	700	0.32	0.22
2	unexposed female	100	0.27	<0.1
2	unexposed female	15	0.50	NA
2	unexposed female	**180** +/-307*	**0.78** +/-.60†	**0.22** +/-0‡
3	exposed male	216000	1470	19.9
3	exposed male	186000	1300	13.8
3	exposed male	145000	851	3.38
3	exposed male	134000	878	13.2
3	exposed male	198109.4	2150	NA
3	exposed male	**176000** +/-35000*	**1330** +/-533†	**13** +/-7.0‡
4	exposed female	180000	1950	16.6
4	exposed female	179000	3260	18.8
4	exposed female	154000	3040	13.3
4	exposed female	160000	2170	6.36
4	exposed female	176000	2500	NA
4	exposed female	**170000** +/-12000*	**2600** +/-560	**14** +/-5.0‡

* pg VTG mRNA / µg total RNA; † mRNA VTG / total RNA ; ‡ fmol VTG mRNA / µg total RNA

GC-MS Results

Mass spectrometric (MS) analysis of VTG and other large glycosylated biomolecules has allowed for a general approach to be utilized for specifically identifying proteins both in purified forms and from within mixtures. A common type of ionization technique for MS that allows analysis of large biomolecules without fragmentation is matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS).

The technique of peptide mapping using MALDI-MS has been used for nearly a decade to identify proteins. This technique relies on the mass measurement of peptides produced by proteolytic digestion and comparing them with the predicted peptide masses from each protein in the database. Algorithms are then used to compare and determine a probability score to match the experimental data with candidates in the database. The mathematical tools for identifying proteins using this approach are available publicly at <http://prowl.rockefeller.edu/cgi-bin/ProFound> through an easily navigable web interface. Protein identification using the

peptide mapping approach is limited to a small (mixtures of four or fewer) number of proteins, so pre-separation of proteins from complex mixtures is often required.

This approach was used to directly analyze VTG from fathead minnow plasma using a combination of liquid chromatography and mass spectrometry. A simple membrane filtration pre-purification step was coupled to an analytical scale anion exchange separation. This approach to MALDI-MS analysis requires a relatively small plasma sample (< 10 µl) and is suitable for use with plasma volumes typically obtained from individual fathead minnows. Correction for incomplete recovery of the VTG was achieved through the use of an internal standard. Peak identity as VTG was confirmed through automated fraction collection, trypsin digestion and MALDI-MS analysis.

VTG was relatively easy to purify although performing analytical separation on it proved challenging. VTG is well known to degrade quickly and so precautions to add protease inhibitors (PMSF) and keep the sample on ice need to be made. Even with these precautions, analysis of the sample needs to occur immediately after purification, as VTG apparently degrades in a matter of hours after the microcon purification. Both the large size and dimeric nature can lead to peak broadening, so urea was added as a denaturant. Once unfolded, large proteins are able to take on different conformations and so the pore size of the resin used in the separation must be consid-ered to avoid loses. For this reason, a non-porous resin was selected for this work. A smaller inner diameter (ID) column was also used to minimize dead volume, flow rate and increase sensitivity for smaller quantities of material. The drawback to using a small ID column with a non-porous resin is the decreased sample capacity. Precautions must be taken to not overload the column and reduce the resolution of the separation. If a large amount of protein is apparent (often from a small precipitate visible on the microcon 100 filter) then a larger volume of buffer can be used when retrieving the sample from the membrane. A detailed protocol is presented in Appendix F.

Figure 10 contains the UV trace of equal amounts of BSA and VTG after going through the microcon 100 cleanup step and directly injection on the AX column. Twenty µg of each protein was used in the cleanup (the BSA added just prior to retrieval) and 10 µg of each injected. The peaks are well resolved and of equal area. This sample represents roughly the detection limit of VTG that can be *injected,* with a concentration of 250 ng/ul producing a peak visible in the chromatogram. Figure 11 shows the chromatogram of a sample similar to shown in figure ten, however in this sample the 20 µg of VTG has been added to 10 µl of plasma taken from an untreated fish 219PR33. In figure 12, the chromatogram for plasma from an untreated fish (219PK18) has been cleaned up in a similar fashion without the addition of VTG. Only the peak for BSA is apparent with no peak appearing at the retention time of VTG. This indicates that there is a nearly linear relationship between the peak areas for BSA and the VTG standard using 10 or 20 µg of BSA as an internal standard when compared to 5 µg to 40 µg of VTG injected. 50 µg is nearing the upper limit of column capacity and so amounts of VTG beyond were not investigated in this study.

10 ug each VTG and BSA injected

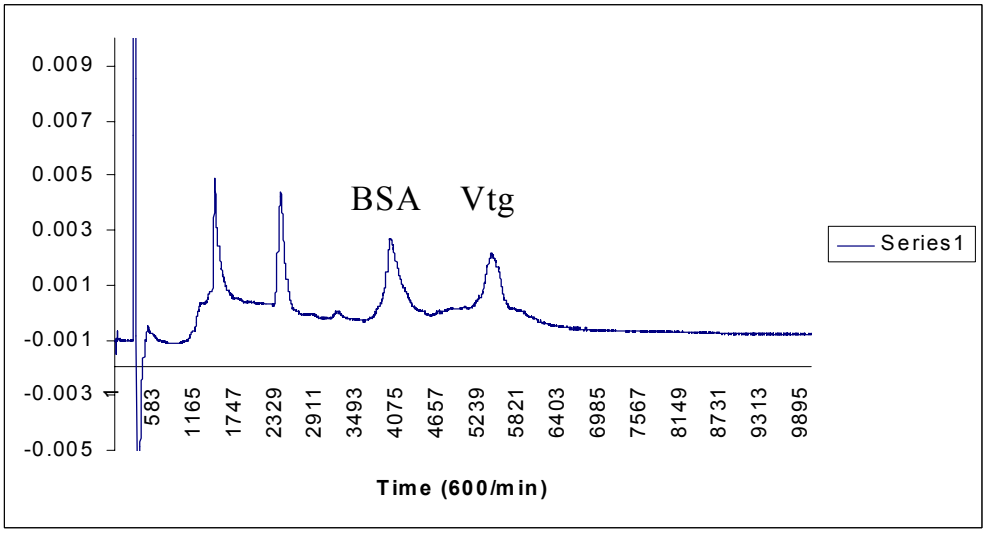


Figure 10. UV trace of equal amounts of BSA and VTG after purification for GC-MS.

10 ug VTG injected after "spike"into 219PR33 control plasma:
Purified using microcon 100 (BSA added [10ug injected])

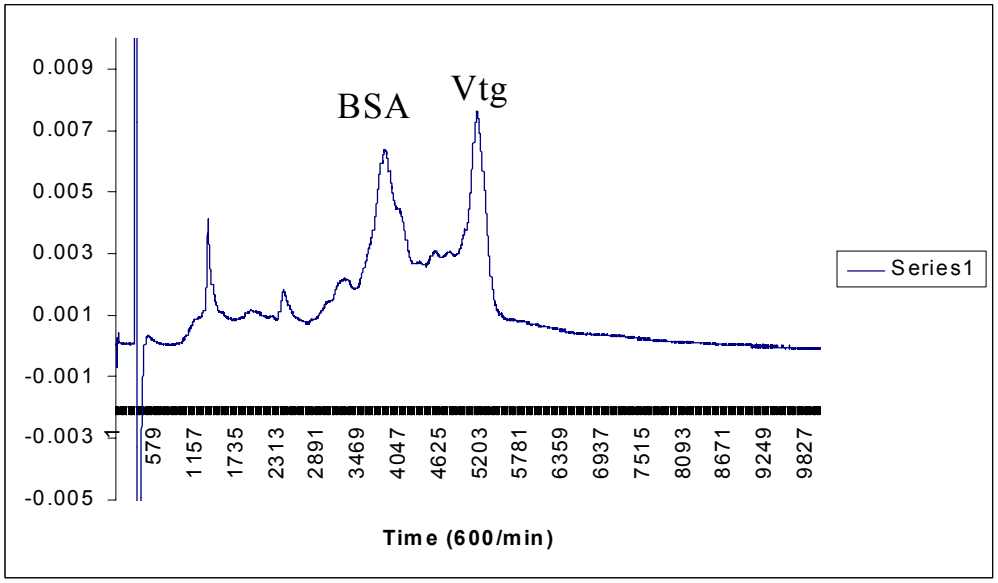


Figure 11. 10 ug VTG injected after "spike"into 219PR33 control plasma: Purified using microcon 100 (BSA added [10ug injected])

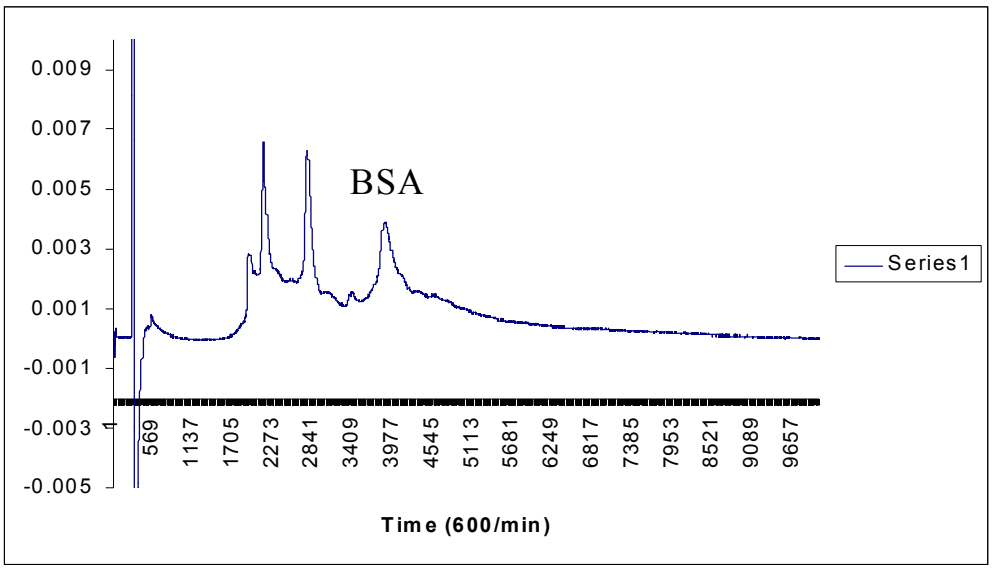


Figure 12. No VTG "Spiked" into 219Pk18 Control Plasma: Purified using microcon 100 with BSA added (10ug injected)

The exposed plasma samples had a large amount of protein remaining on top of the microcon membranes in some cases, and so the volume of buffer added before the sample was collected from the membrane was larger (100 µl). Figure 13 is the chromatogram for treated plasma sample 219PU18 where 10 µl of the 100 µl total volume was injected. The six µg of BSA is barely visible in comparison to the VTG peak. Note the scale difference between this figure and previous figures making the BSA peak appear almost non-existent. A one to five dilution of the same sample was run with additional BSA added (for a final of 20 µg injected) and the chromatogram is shown in Figure 14.

219PU18 Exposed Plasma:
Diluted 1 to 5 with additional BSA (20 ug BSA injected)

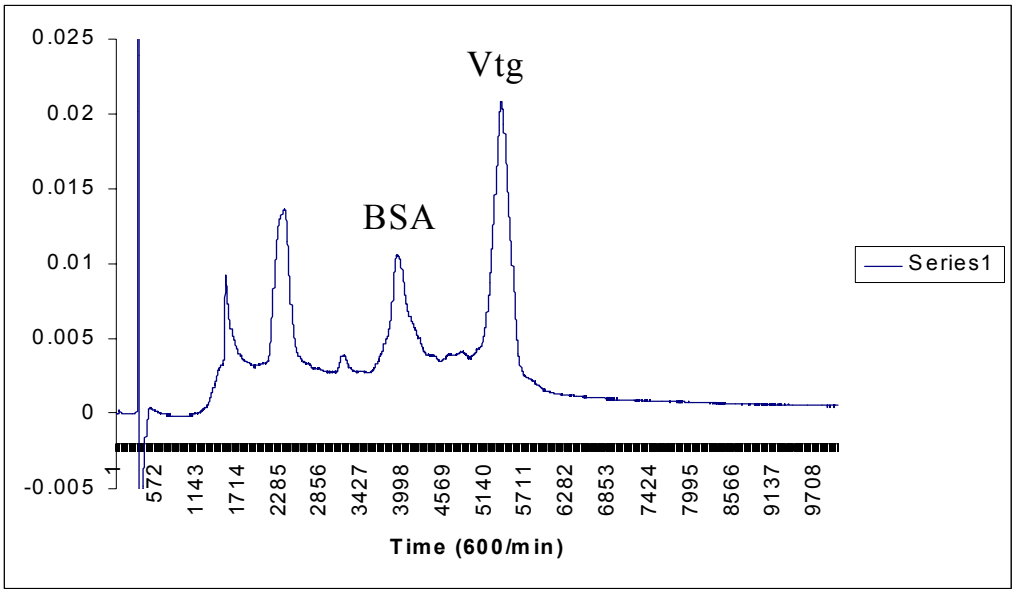


Figure 13. 219PU18 Exposed Plasma: Purified using microcon 100 with 60 ug BSA (6 ug injected)

219PU18 Exposed Plasma:
Purified using microcon 100 with 60 ug BSA (6 ug injected)

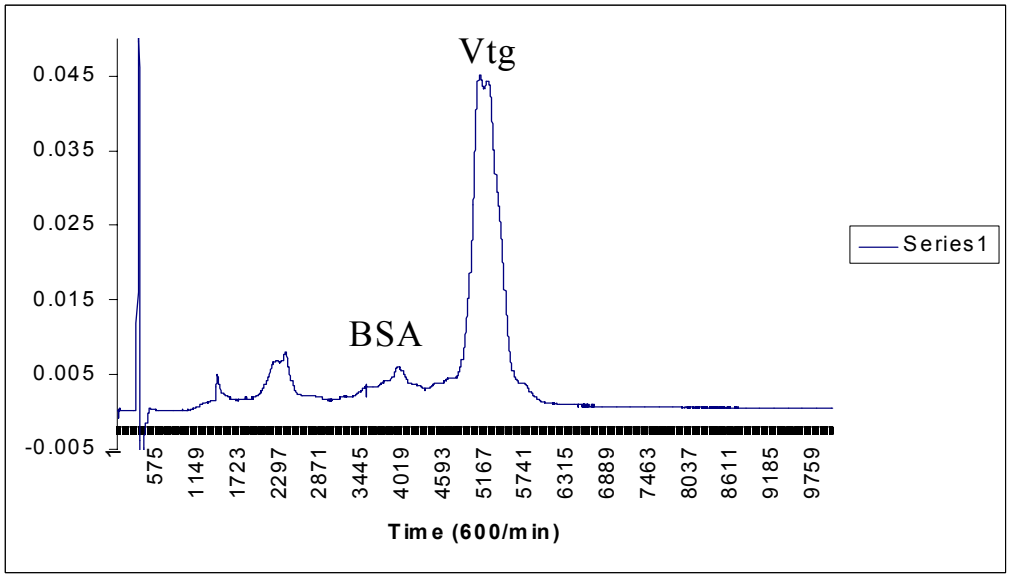


Figure 14. 219PU18 Exposed Plasma: Diluted 1 to 5 with additional BSA (20 ug BSA injected)

Figure 15 is a comparison of the peptide digest mass spectra from VTG fractions collected from two "treated" plasma samples and from the VTG standard. The associated statistics from the Profound search engine for the protein hits for each data set are given. The search was conducted against all chordate proteins in the "non redundant" database (combination of swissprot, NCBI, OWL and others) using a 1.5 Da mass error for the average peptide mass ($M+H^+$) and a protein size range of 100 to 300 kDa. The output provides what rank each database hit received (top 50 listed), the probability score associated with it (with 1.0 being a perfect match). Also included is a percentage of the protein sequence represented as well as a "Z score" for the top match which is a measure of distance between the top match and nearest matches (> 1.2 = 90th percentile of correct matches if compared to a random population of sequences, >1.6 = 95th percentile and >2.0 = 99th percentile respectively). Table 13 is a list of the top sequence matches for the VTG peak from treated plasma sample 219PT18. The top two hits receiving perfect probability scores are the VTG sequences for both fathead minnow and the common carp. A match with the zebra fish was also made at a much lower rank and probability.

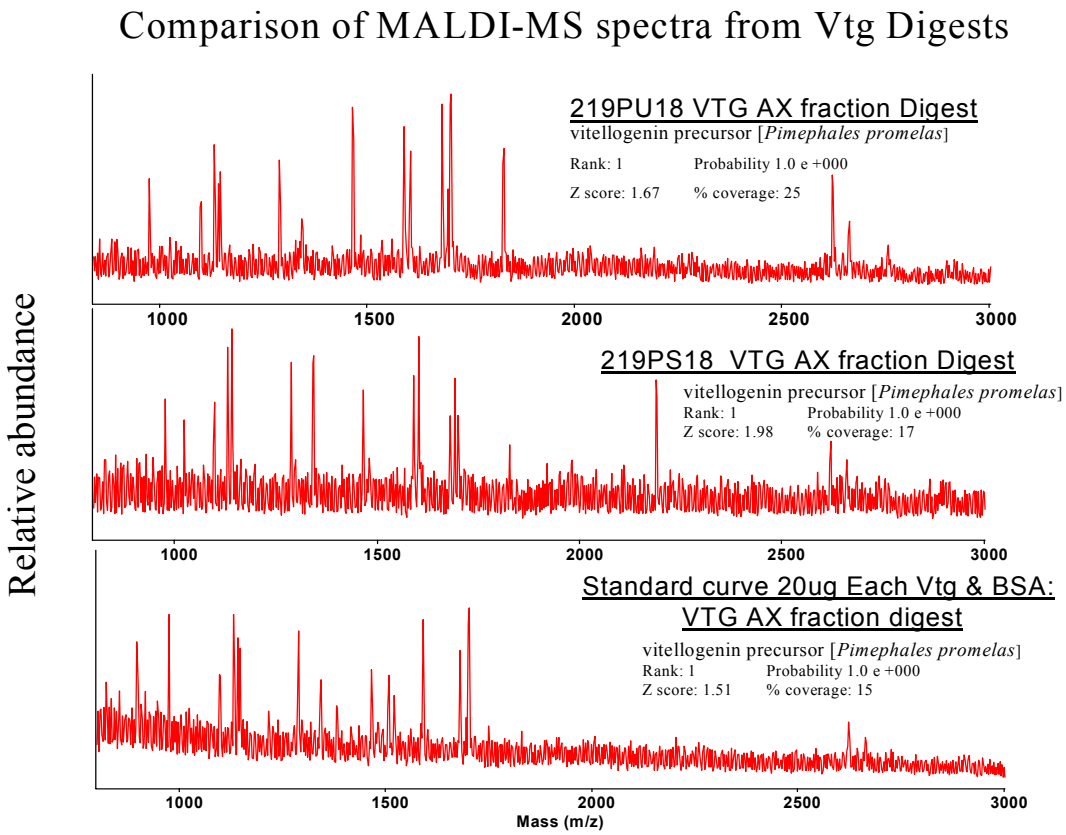


Figure 15. Comparison of the peptide digest mass spectra from VTG fractions collected from two exposed female plasma samples and from the VTG standard.

Table 13. The top sequence matches for the VTG peak from exposed female plasma sample 219PT18.

Vtg Anion Exchange Fraction from treated plasma 219PT18: database search with peptide masses

Rank	Probability	Z score	Protein Information and Sequence Analyse Tools (T)	%	*pI*	kDa
+1	**1.0e+000**	2.08	**gi|4572552|gb|AAD23878.1|AF130354_1 vitellogenin precursor [Pimephales promelas]**	17	9.0	146.26
	-	-	**gi|15778562|gb|AAL07472.1|AF414432_1 vitellogenin [Cyprinus carpio]**	13	9.1	148.24
+2	***2.8e-005***	0.14	gi|6006011|ref|NP_005492.1| (NM_005501) integrin alpha 3, isoform b, [Homo sapiens]	14	6.5	118.74
+3	6.4e-007	-	gi|21362287|ref|NP_653099.1| RIKEN cDNA 2210402G22 [Mus musculus]	25	9.1	66.13
4	2.3e-007	-	T gi|20344336|ref|XP_111772.1| similar to put. gag and pol gene (aa 1-814) [Mus musculus]	19	9.6	85.00
+5	9.8e-008	-	T gi|16550881|dbj|BAB71072.1| (AK056006) unnamed protein product [Homo sapiens]	27	9.8	64.01
6	1.4e-008	-	T gi|13385164|ref|NP_079982.1| RIKEN cDNA 4432405K22 [Mus musculus]	17	5.2	76.51
+7	1.1e-008	-	**T** gi|14735371|ref|XP_027054.1| (XM_027054) KIAA0674 protein [Homo sapiens]	19	5.0	92.49
+8	7.6e-009	-	**T** gi|6005944|ref|NP_009058.1| (NM_007127) villin 1; Villin-1 [Homo sapiens]	15	6.0	92.68
9	6.8e-009	-	**T gi|21391472|gb|AAK58480.1| vitellogenin 1 [Danio rerio]**	12	8.9	128.02

Table 14 lists the results from all of the treated plasma samples including whether any VTG could be detected, if so what was the estimated amount present. All VTG concentrations are calculated for the 10 µl total Plasma volume received. Two of the samples (219PS18 and 219 PG18 unexposed female) were analyzed early in the refinement of the method were initially diluted too much. The undiluted samples were analyzed later after some degradation had occurred due to the presence of many peaks eluting earlier than expected. Therefore, no estimates of VTG concentration were calculated for those samples although a definite peak appeared at the correct retention time and a fraction for 219PS18 was collected, digested and analyzed by MALDI-MS. The results for that are given in Table 14. Two samples had no detectable VTG in them, 219PI18 and 219PM18 (unexposed male). Sample 219PD18 (unexposed male) did appear to have a very small peak at the correct retention time, but it is very small and is difficult to definitively say that VTG is present. Therefore it is given a tentative assignment. Not all samples containing VTG had the fractions collected for MALDI-MS analysis. For those fractions analyzed by MALDI-MS fingerprinting, the rank, probability score and Z score are given for each. Z score values above 1.6 are considered to be a confident match in the 95th percentile (or 95% chance that it is a correct hit).

Table 14. Summary of the results of GC-MS analysis of unexposed and exposed male and female fathead minnow plasma samples.

Sample ID	Sample type	VTG detected	Probability	Z score	% coverage	Original Concentration
219PU18	exposed female	Yes	1	1.67	25	100 ug/ul
219PT18	exposed female	Yes	1	2.08	17	75 ug/ul
219PS18	unexposed female	Yes	1	1.98	17	NC
219PG18	unexposed female	Yes	NC	NC	NC	NC
219PI18	unexposed male	No	##	##	##	##
219PM18	unexposed male	No	##	##	##	##
219PD18	unexposed male	Yes**	NC	NC	NC	< 0.5 ug/ul ‡

** = Tentative assignment
NC = not calculated
= Not applicable
‡ = estimate of original concentration, not concentration injected.

8.0 DISCUSSION

The purpose of this study was to survey the existing ELISA methods that are currently available for use in detecting the protein vitellogenin in fathead minnow plasma and whole body homogenate samples. A variety of methods routinely performed by the participating laboratories were applied to a standard series of samples. All of the samples were provided blind coded and each laboratory received three replicates of each sample within the coded sample set, and each sample was assayed in triplicate (3 wells per sample). For example, the standard series consisted of 4 samples derived from induced and uninduced male and female fish, and a positive and negative control. This resulted in 6 samples within the plasma series, spanning a range of concentrations. Each of the participating laboratories were provided with these six samples in triplicate, for a total of 18 samples for analysis (blind coded). Upon analysis, each laboratory analyzed each of the 18 samples in triplicate wells (with the exception of one laboratory, as noted in the results, which provided results for one analysis rather than in triplicate). Because of the blind nature of the samples and the wide range of concentrations present in the samples, this required multiple dilutions of the sample to ensure a response within the working range of the assays. This required a significant investment of time and resources that was donated by the participating laboratories to aid in reaching the goals of this study, which is gratefully acknowledged. In addition the intent of study was not to validate a given method, protocol, system, or technique but rather a survey of methods that are currently used in attempt to discern the relative variability among those methods. Also, the use of trade names, identification of

laboratories, and methods described in this do not constitute endorsement by the U.S. Environmental Protection Agency or Battelle Memorial Institute.

To generate the standard series of samples, wet laboratory exposures to estrogen were conducted with male and female fathead minnows, and the plasma and whole body homogenates were prepared from pooled fish. This resulted in four sample types within the series (unexposed male and female, and exposed male and female). In addition to the 4 samples in the series, antibodies were used to remove the VTG signal from unexposed male plasma to create a blank sample. A known amount of purified fathead minnow VTG was added to a portion of this plasma to create a positive control sample, resulting in six samples in the plasma series. Multiple aliquots of each sample in the series were created and entered into a repository system. The samples were processed with care to limit the degradation of the protein during collection and processing and were maintained at -80°C in the repository. Within a two week period, the samples were sent to all of the participating laboratories, while maintaining the integrity of the samples (samples remained frozen throughout the transfer and were stored at -80°C by the participating laboratories).

Twelve laboratories received sets of the homogenate and plasma sample series for analysis. Two laboratories were unable to analyze the samples within the time frame of the study, and two laboratories conducted the analysis but found that their antibodies did not react with the fathead minnow samples (carp monoclonal and trout). Eight laboratories reported results of the plasma and homogenate analysis, and six of these also used the fathead minnow VTG that was purified for this study (and used to create the positive control sample in the series) in their assay (Table 2). Of the eight laboratories reporting results, 2 applied a fathead minnow ELISA (one monoclonal antibody-based and one polyclonal based), two applied a carp-based competitive ELISA, two applied zebrafish-based ELISAs and three applied a commercially available carp-based sandwich ELISA kit (Biosense) to the samples (note: one laboratory applied both a fathead ELISA and the commercial kit).It should be noted that all of the participating laboratories have significant experience in conducting ELISAs and their method was applied to the samples in the routine manner employed by each laboratory.

Please note that the major goal of this study was to conduct a survey of existing ELISA methods that might be applied to fathead minnow samples. In the process of conducting the study, three laboratories used the same method during the study, thereby allowing for some statistical comparison of the analysis of the samples in the series by one method. This was not an attempt to validate a particular method, and the results obtained from the use of this method, by circumstance, by a statistically valid number of laboratories should not be used to assess the strength or weakness of this method compared to other methods. Rather, it should be assumed that the encountered variability in results would be found with the application of any one of the methods in this study by multiple laboratories.

All of the reported results in this study can be pooled to examine the inter- and intra-assay variation in the analysis, and the trend of concentration in the standard series. The results of the study can also be examined based upon the type of ELISA (e.g. fathead minnow), the standard used (the one provided (purified fathead minnow VTG) or the one typically used in the assay (homologous)) and the laboratory performing the analysis (e.g. Lab 1). These categories

can be used to assess the trend in concentration of the series, the ability to measure the concentration in the positive control, and the ability to distinguish between the concentration of VTG in individual samples (e.g. blank from unexposed male). Each of these factors has direct implications to the application of an ELISA method for use in a routine fish screening assay.

The analyses in this report address the within-run variability, the intra-assay variability (based on the mean triplicate result), and the general trend of the ELISA VTG results associated with the standard evaluation series of fish plasma and tissue (e.g., whole body homogenate). This series was represented by 1) uninduced male, 2) uninduced female, 3) induced male, and 4) induced female fathead minnows, respectively. In addition to the standard series, a set of positive and negative control VTG results are summarized. The distribution of CVs of the resulting triplicate mean VTG concentrations are summarized for a given concentration, laboratory, antibody, and standard; across laboratories, antibodies, standards, and assays for a given concentration; and by antibody for a given laboratory, standard, and concentration.

All reported results:

Within-run variability - Each laboratory analysed each sample in three replicate wells (within-run) during analysis. When all of the reported results are examined, the within-run variability for plasma had CVs ranging from 0% to 173% with a mean of 13% (Table 6; Appendix D). The within-run variability for homogenate was similar, with CVs ranging from 0% to 162%. The distribution of these CVs can be seen in Figure 1 and Figure 2, revealing that the results from replicate wells per sample within the ELISA assays resulted in CVs less than 16% for 75% of the samples (when all of the reported results for a sample, in both the homogenate and plasma series (1-5), among all laboratories are pooled). The CVs for both the plasma and homogenate samples tended to be less than 30% for all concentrations except for those derived from the blank and unexposed males (Figure 1) which are near the detection limit of the assays. The range of CV's for replicate ELISA wells per samples indicates that when all of the methods are applied to the range of samples, low variation is typically achieved except when measuring the background to low level response (pre-absorbed plasma blank and unexposed male assumed to be devoid of VTG). The detection of low-level VTG is a critical component of a method for use in a screening assay to detect the induction of the VTG protein. It should be noted that several laboratories reported the results of triplicate analysis noting that one of the value in the series of three was an outlier that they would normally not include in their analytical reporting, but that were included for the purposes of this study.

Intra-assay - Each of the six samples in the series (e.g. unexposed male, positive control) were provided to the laboratories in triplicate (blind-coded). These triplicate samples for analysis provided a measure of intra-assay variability. The intra-assay variability for plasma and homogenate had CVs ranging from 0% to 173% (Table 7; Appendix D). For both sample types, 75% of the intra-assay CVs were less than 51%. This high level of intra-assay variability indicates that when a sample is provided to multiple laboratories employing a variety of methods (the results using multiple standards are also included in this sample set) the methods provide a high degree of variability when replicate samples are analyzed. To further examine this variability that is critical to the application of ELISA to a screening assay, the data was further examined by individual laboratory.

Trend of standard series - A goal of the study was to generate a range of concentrations of VTG in male and female plasma and whole body homogenates. Continuing the examination of all of the reported data, the general trend for the plasma samples observed for each laboratory averaged over antibodies, standards, and assays was an increasing concentration of VTG in the series (i.e., uninduced male < uninduced female < induced male < induced female fathead minnows; Figure 3). The homogenate samples, however, tended to suggest that uninduced male VTG concentration was approximately equal or slightly greater than that of uninduced females, which were both less than that of the induced males (Figure 4). Induced female fathead minnows produced the greatest concentrations of VTG in both the plasma and the homogenate. The CVs for each concentration are all greater than 80%; thus the variability between laboratories, antibodies, and standards is large. This high degree of variability indicates that there are significant differences in the results among reporting laboratories, indicating that the methods currently in use provide a variety of results and that for the application of these methods to fathead minnow samples must be further examined. This analysis included all reported results and additional analysis was conducted based upon laboratory and type of method to examine this variability.

Difference in Standards

A critical aspect of the performance of an analytical method is the use of standard and controls in an assay. For this study we purified fathead minnow VTG and used it to create a positive control sample and for use as a standard in the assay. Six of the participating laboratories reported results based on this standard, in addition to their own standard. The percentage difference in results between standards calculated as (H-B)/B (100%) was large ranging from -99% to 200%. This is a significant difference, although anticipated based upon the nature of the antibody-based ELISA to VTG from various species and from various purifications. Thus, standards were analyzed separately in the subsequent analysis in this report.

Ranking of VTG concentrations in the series. When the results from each of the laboratories is analyzed by standard employed in the assay, all produced a significant regression of the ranked VTG concentrations against the series code 0 though 4, with the exception of Laboratory 5 using the homogenate samples, homologous standard, and carp antibodies. However, none of the successive pairs in the series were found to be significantly different using Tuckey's HSD when using laboratories as replicates. Therefore, even though the regressions across the series from blank to induced female were significant, the variability between laboratories to reproduce the ranks for a given concentration code was too large to detect differences between successive concentration pairs in the series. This indicates that with or without the inclusion of a standard for use in the analysis, the survey of methods in this study shows that the variation in results among laboratories and methods does not allow for the determination of the concentration of samples within the standard series of samples, although the general trend of the samples can be observed when all results are examined.

Standard homologous to method. Eight laboratories reported results based upon their homologous standard routinely run in their assay. When examined by type of antibody and form of ELISA, the carp antibody based competitive ELISAs tended to produce the lowest VTG concentrations in the plasma homologous standard samples (Figure 6). (As previously noted, the

Biosense kit and the "carp-based" ELISA use antibodies generated in carp. However, the Biosense kit uses multiple antibodies (monoclonal and polyclonal) in a form of ELISA known as a sandwich ELISA, which differs from the carp-based competitive from of the ELISA employed by two of the participating laboratories) Further, in contrast to the other methods, the carp-based competitive ELISAs did not distinguish between the exposed male and unexposed female samples (concentration codes 2 and 3 respectively). The overall low measured concentrations and the inability to distinguish between exposed and unexposed fish limit the application of the carp-based competitive ELISA to the fathead minnow samples for use in a routine screening assay. The Biosense kit, fathead minnow and zebrafish methods distinguish among the samples in the plasma series with similar reported concentrations. The Biosense and fathead minnow antibodies generally produced the greatest plasma concentrations, the zebra fish antibody the next greatest, and the carp the least (Figure 6). This pattern was not maintained for the homogenate samples which had the zebra fish antibodies with the least VTG concentrations in the induced male and female samples. There was no consistent pattern for the CVs (Figures 8 and 9), indicating that the among the methods in the study greater variation can not be attributed to a type of antibody-based assay. Large CVS are present in the both the plasma and the homogenate series, showing analytical variation irrespective of sample type.

Battelle standard. For the data produced using the Battelle provided standard, the zebrafish antibody tended to produce lower plasma results than the Biosense and fathead minnow antibodies (Figure 6). This is consistent with the use of the homologous standard. In general, the Battelle standard resulted in higher concentrations derived for the samples. This is consistent with the reduced specificity of the various antibodies to fathead minnow VTG. However, as for the homologous standard, the CVS are still generally large for both the homogenate and the plasma samples limiting the interpretation of the results. The CVS associated with the plasma zebrafish antibody results were lower for higher concentration codes (Figure 8). This trend is not repeated in the homogenate samples (Figure 9).

Multiple Laboratories Applying Same Method

The design of this study does not permit the validation of any of the methods employed to measure VTG or mRNA. Rather this study is designed to be a comparative survey of the existing methods and evaluation of how similar or dissimilar results obtained among a variety of methods vary. Although the assessment of the application of one method by multiple laboratories was not a major goal of this study, in the process of conducting the study, three laboratories applied the same carp-based sandwich....to the sample series, including the low level samples and positive control. This is a very limited data set, and the authors of this report urge readers to please note that the results of this study should not be used as a validation of this method.

The carp-based sandwich ELISA which was utilized by multiple laboratories in this study during the process of sample preparation and analysis. As presented previously, the CVs of the results of the application of the various methods to standard series limit the interpretation of the results of this study. The triplicate analysis of the series by one method allows for some measure of the application of one of the methods in this study by multiple laboratories, however, similar assessments of the other methods in this study would be required to draw general conclusions.

Blank and unexposed male samples. For those laboratories that used the Biosense kit in this study, a simple linear regression of the average VTG concentration (within-run average) between the blank and the uninduced male concentrations was conducted to determine if a significant slope was obtained. For the plasma samples, only Laboratory 13 produced a significant slope between these two concentrations using both standards ($p = 0.006$, 4 d.f. for error). For the homogenate samples, both laboratories 7 and 13 produced significant slopes using the homologous standard ($p < 0.05$, 4 d.f. for error), and Laboratory 7 produced a significant slope using the Battelle standard ($p = 0.005$, 4 d.f. for error) (Table 11). With the desire to measure the induction of VTG in male fish in a screening assay, the variable results of the application of an established assay by several skilled laboratories when applied to the same set of samples is significant. Although these results indicate that the detection of background or low levels of VTG in fathead minnow samples can be achieved with the application of an ELISA, the lack of consistent concentrations and significant differences between samples among laboratories requires additional study for method assessment.

Positive control. One additional analysis was conducted on the results from the commercial ELISA kit as applied to fathead minnow samples. The positive control samples for both the plasma and homogenate were spiked to 500 µg/ml VTG with the fathead minnow VTG purified for this study. With three laboratories conducting the Biosense ELISA, these results can be examined for statistical significance. It should first be noted that the results were not unique to the use of the Biosense kit, and similar results were found with the less replicated methods (e.g. fathead minnow). The positive control concentrations was not achieved with the Biosense kit using laboratories as replicates (Table 12). The results of the positive control were again highly variable both within and between laboratories. The concentrations detected in plasma ranged from 337 to 8834 µg/ml VTG in plasma and from 235 to 745 µg/ml VTG in the homogenate control sample. As previously noted, In addition to the variation found in the Biosense assay, a similar wide range of concentrations for the control samples were detected among all of the assays (Table 8; Figure 6 and 7)

9.0 SUMMARY

A standard series of plasma and homogenate samples representing a range of VTG concentrations in male and female fathead minnows was generated for this study. In addition to the samples in the series, a set of positive and negative controls were generated for the study. Fathead minnow VTG was purified for use in the positive control and as a standard for use by the participating laboratories in their method. A repository of samples was created and maintained, and 12 laboratories agreed to participate in the study. Sets of the homogenate and plasma series (blind coded) were shipped in coordination to the participating laboratories while maintaining the integrity of the samples. Two of the participating laboratories were unable to complete their analysis in the time frame of the study, and two additional laboratories reported that their antibodies did not react with the fathead minnow samples. Of the eight remaining laboratories, six completed the analysis of their sample sets with their homologous standard and with the standard provided in the study. Two laboratories employed fathead minnow-based ELISA, two were zebrafish based, two were competitive carp based. Three of these used a commercially available kit.

There was significant variation in the reported ELISA results in this study. When all of the reported results were analyzed, the within-run variability ranged from 0% to 173%, with 75% of the CVs less than 16%. This indicates a wide range of intra-assay variability among the methods in this study. The CVs for both the plasma and homogenate samples tended to be less than 30% for all concentrations except for those derived from the blank and unexposed males (Figure 1) which are near the detection limit of the assays. With the need to measure the induction of VTG in male fish in a screening assay, the ability to detect VTG in unexposed males was examined further in the results from three laboratories conducting the same assay. The ability to detect VTG and to discern between the blank and the unexposed male samples was present, but inconsistent among laboratories.

When the replicate samples were examined for all reported results, the intra-assay variability for plasma and homogenate had CVs ranging from 0% to 173% (Table 7; Appendix D). For both sample types, 75% of the intra-assay CVs were less than 51%. This demonstrates that for a sample provided to multiple laboratories employing a variety of methods, the results will be subject to a high degree of variability among replicates, limiting the usefulness of the results. A similar high degree of variability was shown when the control samples were analyzed by one method by multiple laboratories. Determining the source of this variability will be critical in the application of ELISA in a screening assay.

Although affected by large variations in reported results by the various methods, the trend of the standard series revealed increasing levels of VTG (uninduced male < uninduced female < induced male < induced female fathead minnows; Figure 3). This trend was demonstrated irrespective of the standard used in the assay. However, the carp-based competitive ELISAs tended to produce the lowest VTG concentrations in the plasma homologous standard samples (Figure 6) and the carp-based competitive ELISAs did not distinguish between the exposed male and unexposed female samples. The significantly lower reported concentrations and the inability to distinguish between exposed and unexposed fish may limit the application of the carp-based competitive ELISA to the fathead minnow samples for use in a routine screening assay.

The use of the provided fathead minnow VTG standard in the assays typically resulted in higher concentration reported for the samples. This is generally reflective of the differences in specificity of the various antibodies in the methods. The use of the standard did not result in a normalization of reported concentrations, nor in achieving the concentration in the positive control (spiked with the same VTG as in the standard). The variation in the reported concentration for the positive control varied significantly among laboratories and among assays.

The application of mRNA analysis to the detection of mRNA in the fathead minnow samples was performed by three laboratories in this study (Table 12). Two laboratories (Lab 1 and Lab 2) applied RT-PCR and the third Laboratory (Lab15) applied a hybridization protection assay (HPA) to the samples. All three laboratories performed all analytical steps, including isolation of RNA from the liver tissue, as would be applied to analytical samples resulting from a fish screening assay. All three methods distinguished between exposed and unexposed fish, but direct comparison is limited due to differences in methods and analytical approach. The range of mRNA levels encompassed a wider range in the RT-PCR analysis than in those found in the

series through the application of the HPA method. In the unexposed fish, the detection of mRNA levels were variable, with the labs reporting generally higher, similar, or lower levels in the unexposed males vs. the levels found in unexposed females.

For GC-MS analysis, a purification and separation step was implemented prior to MALDI-MS analysis that allowed detection of VTG on an analytical scale suitable for smaller sample sizes. MALDI-MS provided a general means of confidently identifying VTG by matching experimental data with sequences in protein databases. However, problems with sample degradation after thawing prevented quantitative estimates of VTG in plasma samples harvested from estradiol exposed and unexposed minnows. Further insight into the true detection limits and reproducibility of quantitation for this method are required.

10.0 REFERENCES

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