

AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR LIPOVITELLIN
QUANTIFICATION IN COPEPODS: A SCREENING TOOL FOR ENDOCRINE TOXICITY

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Abstract—Vitellogenin (VTG) has been widely used as a biomarker of estrogenic exposure in fish, leading to the development of standardized assays for VTG quantification. However, standardized quantitative assays for invertebrate, particularly crustacean, lipovitellin (also known as vitellin [VTN]) are lacking. In this study, a fluorescence-based VTN enzyme-linked immunosorbent assay (ELISA) was developed to quantify microquantities of VTN in the estuarine, sediment-dwelling copepod *Amphiascus tenuiremis*. This ELISA utilizes a VTN-specific polyclonal antibody developed against amphipod (*Leptocheirus plumulosus*) embryo VTN and exhibits specificity toward female copepod proteins. In routine assays, the working range of the ELISA was 31.25 to 1,000 ng/ml (75–25% specific binding/maximum antibody binding [B/B₀]) with a 50% B/B₀ intra- and interassay variation of 3.9% ($n = 9$) and 12.5% ($n = 26$), respectively. This ELISA is capable of detecting VTN as low as 2 ng/ml, and can accurately detect VTN in as few as four copepods. The ELISA significantly discriminated positive (gravid female) and negative (male) samples, and was suitable for screening endocrine toxicity in copepods. Stage-I juvenile copepods were individually reared to adults in aqueous microvolumes of the phenylpyrazole insecticide, fipronil, and whole-body homogenate extracts were assayed for VTN levels. Fipronil-exposed virgin adult females, but not males, exhibited significantly higher levels of VTN relative to control males and females. This crustacean VTN ELISA is likely useful for evaluating endocrine activity of environmental toxicants in copepods and other crustacean species.

Keywords—Lipovitellin Enzyme-linked immunosorbent assays Copepod Endocrine toxicity Fipronil

INTRODUCTION

In the majority of the world's estuaries, persistent contaminants of potential endocrine toxicity reside in sediments almost continuously at nonlethal exposure concentrations. As a result, sediment infauna, particularly meiofauna, are chronically exposed to a wide range of chemicals such as herbicides, insecticides, metals, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons. A number of environmental toxicants have been screened for invertebrate hormonal activity but generally possess a weak ability to disrupt hormone action via receptor binding [1]. Indeed, observed endocrine effects more likely are triggered indirectly through cell-signaling pathways [2] because a number of contaminants—particularly insecticides—interact with neurotransmitter-mediated processes. In addition, environmental factors (e.g., ultraviolet light) alone can have significant effects on endocrine processes in estuarine invertebrates [3], further driving the need for adequate assessments of environmental endocrine disruption. Although estrogenic upregulation of yolk proteins has been thoroughly studied in male fish models over the past 10 years [4–7], little research exists regarding toxicant-triggered downstream effects on crustacean female vitellogenesis and/or male feminization [8]. Furthermore, the effects of female or male yolk expression on reproductive maturation and success is not well understood.

In crustaceans, once oogenesis commences, the ovaries of reproductively mature females synthesize and secrete the lipid-rich yolk protein vitellogenin (VTG) into the hemolymph [9,10]. Vitellogenin and lipovitellin (also known as vitellin

[VTN]) have been characterized in a number of crustaceans [11] and recently were immunolabeled in embryos of the amphipod *Leptocheirus plumulosus* by using VTN-specific antibodies and confocal laser-scanning microscopy [12]. Vitellogenin is taken up by developing embryos as VTN, and provides free amino acids, lipids, carbohydrates, and ecdysteroids for normal development before hatching [13]. Although 17 β -estradiol is known to specifically regulate vitellogenesis in female fish [14], many different species-specific vitellogenic pathways likely exist for Crustacea. Generally, vitellogenesis in females is hormonally regulated by complex positive and negative feedback loops of steroids, juvenoids, and neuropeptide hormones [9,10]. Neurotransmitters also regulate ovarian development through the induction or suppression of neuropeptides [10]. For example, the neurotransmitter 5-hydroxytryptamine, or serotonin, indirectly regulates ovarian development by inducing the release of gonad-stimulating hormone [10,15–17], the neuropeptide responsible for triggering vitellogenesis in reproductively mature females [18]. Accordingly, because multiple vitellogenic-related endocrine cascades potentially are responsive to toxicant exposure, crustacean VTN synthesis and production has emerged as a useful and ecologically important endpoint in ecotoxicological studies [8]. However, considering the differences between vertebrate and invertebrate endocrine systems, the mechanisms of vertebrate endocrine disruption (e.g., estrogenic or anti-estrogenic) are almost certainly not parallel to those in invertebrates.

A limited number of nonradiometric enzyme-linked immunosorbent assays (ELISAs) have been developed to quantify VTG or VTN in freshwater and marine crustaceans. These include direct [19–21], sandwich [22], and indirect competitive [23–25] ELISAs, all designed primarily for yolk detection in

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larger decapod species (e.g., crabs, shrimp, and lobsters). To accurately quantify VTN in smaller (<200- μ m length) crustaceans (e.g., copepods), an ELISA needs to be exquisitely sensitive. Overall, direct (noncompetitive) and indirect (competitive) ELISAs yield moderate (80–200 ng/ml) to high (>1 ng/ml) sensitivity, respectively. Moreover, the use of fluorescent substrates (e.g., 4-methylumbelliferyl phosphate) can increase assay sensitivity, and should be utilized in ELISA development. To this end, amphipod (*L. plumulosus*)-specific VTN polyclonal antibodies were used to develop an indirect, competitive ELISA to detect VTN in the estuarine, sediment-dwelling harpacticoid copepod *Amphiascus tenuiremis* (Copepoda: Harpacticoida). This copepod VTN ELISA was validated as a screen for endocrine toxicity of environmental toxicants by using the γ -aminobutyric acid (GABA)-disrupting phenylpyrazole insecticide fipronil as a model. *Amphiascus tenuiremis* has been used as an ecotoxicological model for 10 years to evaluate acute, chronic, and reproductive toxicity of environmental toxicants [26,27]. Infaunal copepods are the second most abundant metazoan group in estuarine and shallow-marine sediments—after nematodes—and are extremely important in food webs and carbon–nutrient cycling [28]. We have previously characterized VTN of *A. tenuiremis* and found that it is a phospholipoglycoprotein with an amino acid composition nearly identical to VTN of *L. plumulosus* (D.C. Volz, unpublished data).

MATERIALS AND METHODS

Assay animals

Embryos from gravid grass shrimp (*Palaemonetes pugio*) were collected from laboratory culture (National Oceanic and Atmospheric Administration, National Ocean Service, Center for Coastal Environmental Health and Biomolecular Research, Charleston, SC, USA) for VTN purification and held at -20°C until extraction. We used VTN from grass shrimp eggs as the ELISA standard because grass shrimp VTN reacts well with *L. plumulosus* anti-VTN antibodies, and these easily collected eggs yield a large quantity of VTN suitable for ELISA procedures. Freshly extruded eggs were collected because these contained the highest quantity (80–90%) of yolk.

Amphiascus tenuiremis (Mielke) is an amphiatlantic sediment-ingesting harpacticoid copepod found in estuarine habitats ranging from the North Sea–Baltic intertidal to the southern Gulf of Mexico [29]. It completes a life cycle in only 15 d at 25°C [27]. *Amphiascus tenuiremis* is easily cultured through multiple generations in sediments under flow, and it has well-characterized life-table and life-history parameters [30].

Antibody preparation

Rabbit primary antisera was raised against VTN purified from fresh eggs of the estuarine amphipod *L. plumulosus*, reared in laboratory sediments [12]. Anti-*L. plumulosus* VTN immunoglobulins G (IgGs) (~ 4 mg/ml) were purified from rabbit serum by protein A affinity chromatography (1.0 ml HiTrap Protein A immunoaffinity column, Amersham Biosciences, Piscataway, NJ, USA) and used for immunoaffinity VTN purification, Western blot, and ELISA procedures. Alkaline phosphatase-labeled goat anti-rabbit IgG (BIO-RAD, Hercules, CA, USA) was used as the secondary antibody in Western blot and ELISA procedures.

Immunoaffinity purification of the standard protein

Purified anti-*L. plumulosus* VTN IgGs were dialysis-exchanged to coupling buffer (0.1 M NaHCO_3 , pH 8.3; 0.5 M NaCl) and conjugated to CNBr-activated Sepharose[®] 4B (Amersham Biosciences). Briefly, CNBr-activated Sepharose 4B (1 g) was suspended in 1 mM HCl washed with 200 ml of 1 mM HCl over a sintered glass filter. The activated and swelled Sepharose gel (~ 3.5 ml) was incubated with approximately 4 to 6 mg of anti-*L. plumulosus* VTN IgG in coupling buffer for 2 h while shaking at room temperature. Excess IgG was washed with 50 ml of 0.45- μ m-filtered coupling buffer over a sintered glass filter. The remaining active groups on the coupled gel were then blocked with 0.1 M Tris-HCl (pH 8.8) for 2 h at room temperature while shaking. The coupled and blocked gel was then washed, in alternation, three times with 50 ml of 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl and three times with 50 ml of 0.1 M Tris-HCl (pH 8.8) containing 0.5 M NaCl. The gel was then transferred to an amber vial containing 0.05% sodium azide–phosphate-buffered saline (PBS) (10 mM phosphate, pH 7.5; 137 mM NaCl; 2.7 mM KCl) and stored at 4°C until chromatographic application.

A VTN standard for ELISA development was purified from the embryos of the estuarine grass shrimp *P. pugio* by immunoaffinity chromatography. Eggs of *P. pugio* (~ 300 fresh eggs) were homogenized three times in cold (4°C) PBS and filtered through a 0.45- μ m HT Tuffryn[®] Membrane (Gelman Laboratory, Ann Arbor, MI, USA) to eliminate free-standing lipids. The PBS-equilibrated IgG-conjugated Sepharose was incubated with egg homogenate from *P. pugio* in PBS overnight at room temperature while shaking. An empty chromatography column (C10/10 empty column, Amersham Biosciences) was packed with VTN-bound Sepharose and washed with 10 ml of 0.45- μ m-filtered PBS and 10 ml of 0.45- μ m-filtered 0.05% Tween-20–PBS. All 1-ml wash fractions were collected in 1.5-ml centrifuge tubes. Lipovitellin was eluted off of the immunoaffinity matrix with 30 ml of 0.1 M citric acid (pH ~ 3 –4) and collected in 1-ml fractions containing 200 μ l of 0.45- μ m-filtered 1 M Tris-HCl (pH ~ 9). Absorbance (280-nm) readings were read in a quartz cuvet by using an Ultraviolet-2401 PC UV/Vis spectrophotometer (Shimadzu, Columbia, MD, USA). The gel was regenerated by washing, in alternation, three times with 20 ml of 0.1 M Tris-HCl (pH 8.0) containing 0.5 M NaCl and 20 ml of 0.1 M sodium acetate (pH 4.0) containing 0.5 M NaCl. The regenerated gel was returned to an amber vial containing 0.05% sodium azide–PBS and stored at 4°C . We have found that the IgG-conjugated Sepharose can be reused for approximately 10 immunoaffinity applications if washed and stored properly.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots were performed to verify the purification of the VTN standard from eggs of *P. pugio* after immunoaffinity chromatographic application. Additionally, Western blots were performed to verify the cross-reactivity of polyclonal anti-*L. plumulosus* (estuarine amphipod) VTN IgG [12] to female-specific VTN in gravid female *A. tenuiremis*. Pre- and post-affinity purification egg homogenate of *P. pugio* or proteins of *A. tenuiremis* (female and male) from 100 individuals were diluted in sample buffer (62.5 mM Tris-HCl, pH 6.8; 25% glycerol; 2% SDS; 0.01% bromophenol blue) and subjected to 8% or 14% SDS-PAGE, respectively.

Table 1. Comparison of three lipovitellin (VTN) enzyme-linked immunosorbent assays

	Assay 1 ^a	Assay 2 ^b	Assay 3 ^c
r^2 ^d	0.9967	0.9580	0.9514
Sensitivity (ng/ml)	1.9	7.8	15.6

^a Anti-VTN immunoglobulin G (IgG), alkaline phosphatase-labeled goat anti-rabbit (GAR) IgG, and 4-methylumbelliferyl phosphate (4-MUP) substrate.

^b Anti-VTN IgG, horseradish peroxidase-labeled goat anti-rabbit and QuantaBlu[®] peroxidase substrate (Pierce, Rockford, IL, USA).

^c Biotinylated anti-VTN IgG, alkaline phosphatase-labeled streptavidin, and 4-MUP substrate.

^d Calculated from a four-parameter standard curve model.

Proteins were transferred to a nitrocellulose membrane (BIO-RAD) for 3 h at 50 V in transfer buffer (25 mM Tris; 192 mM glycine) at 4°C. Membranes were then blocked with 2% gelatin-Tris-buffered saline (TBS) (20 mM Tris, pH 7.5; 500 mM NaCl) overnight. After rinsing two times with 0.05% Tween-20-TBS (TTBS) for 10 min each, membranes were incubated with anti-*L. plumulosus* VTN IgG diluted 1:2,000 in 1% gelatin-TTBS for 1 h. Membranes were rinsed two times, 5 min each, with TTBS and incubated with a 1:3,000 dilution of alkaline phosphatase-labeled goat anti-rabbit IgG (BIO-RAD) in 1% gelatin-TTBS for 1 h. After two rinses with TTBS (5 min each) and 1 rinse with TBS (5 min), membranes were developed in a 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium color development solution (BIO-RAD) until purple bands were visible. All incubations were done at room temperature while shaking.

Indirect, competitive ELISA development

Standard curve development. Three different ELISA approaches were initially tested for precision and sensitivity. The third approach involved a biotin-antibody conjugate where primary VTN-specific IgGs were covalently coupled with biotin via two aminohexanoic chains and purified from excess biotinylation reagents by using a FluoReporter[®] Mini-Biotin-XX Protein Labeling Kit (Molecular Probes, Eugene, OR, USA). An indirect, competitive ELISA approach incorporating an alkaline phosphatase-labeled goat anti-rabbit secondary IgG and the fluorescent substrate 4-methylumbelliferyl phosphate yielded the highest precision and sensitivity (Table 1). Therefore, details of this ELISA approach are described.

Black 96-well ELISA plates with clear, flat bottoms (Corning Costar, Corning, NY, USA) were used for all ELISA procedures to eliminate cross-talk from the fluorescent substrate. All blanks, negative controls, and standards were run in quadruplicate. Blank and nonspecific binding (NSB) wells were coated at 100 μ l/well with coating buffer (50 mM sodium carbonate, pH 9.6) containing bovine serum albumin (BSA) at 2.5 μ g/ml. All standard wells were coated at 100 μ l/well with coating buffer (50 mM sodium carbonate, pH 9.6) containing partially purified VTN from *P. pugio* at 2.5 μ g/ml. All wells were incubated overnight at room temperature while shaking. Coating buffer was discarded and blocking buffer (0.1% BSA-TBS) was added to each well (300 μ l/well) and incubated for 1 h at room temperature while shaking. After three washes with TTBS, 0.1% BSA-TBS was added to blank (150 μ l/well) and NSB (75 μ l/well) wells and VTN from *P. pugio* (0.9–1,000 ng/ml) diluted in 0.1% BSA-TBS was added (75 μ l/well) to wells designated as standards. Zero standard

(B₀) wells received 0.1% BSA-TBS (75 μ l/well) and represented the maximum antibody binding in the absence of VTN competitor. Anti-*L. plumulosus* VTN IgG was diluted 1:2,000 in 0.1% BSA-TBS and added to wells (75 μ l/well) designated as NSB or standards, thus yielding a final 1:4,000 antibody dilution in each well. The competition reaction was incubated with shaking for 5 min at room temperature and then overnight at 4°C. After three washes with TTBS, 0.1% BSA-TBS was added to blank (150 μ l/well) wells and a 1:2,000 dilution of alkaline phosphatase-labeled goat anti-rabbit IgG (GAR-AP) in 0.1% BSA-TBS was added to standard and NSB wells (150 μ l/well). All wells were incubated for 2 h at room temperature while shaking. After two washes with TTBS and one wash with TBS, the fluorescent substrate 4-methylumbelliferyl phosphate (4-methylumbelliferyl phosphate liquid substrate system, Sigma-Aldrich, St. Louis, MO, USA), was added to all wells (100 μ l/well), including blanks, and developed for 1.5 h in the dark at room temperature while shaking. We found that fluorescence for this substrate reached equilibrium after 1.5 h. After development, standards were immediately read with a BIO-TEK FL₈₀₀ fluorescence microplate reader (360/460 nm filters, BIO-TEK Instruments, Winooski, VT, USA). The specific binding ($B = [\text{fluorescence of standard}] - [\text{fluorescence of NSB}]$) of each standard and maximum binding ($B_0 = [\text{fluorescence of 0 ng/ml standard}] - [\text{fluorescence of NSB}]$) of the anti-VTN antibody were calculated and all data were logit transformed based on specific to maximal binding ratios [31]. The VTN ELISA standard curves were then plotted as logit-log and linearly regressed to determine ELISA sensitivity and working range. Lipovitellin unknowns were calculated based on the logit-log curve.

Precision and accuracy. To test the precision and accuracy of the ELISA, three standard curves were generated as mentioned above, and each standard curve was started on a different plate and day. All standard curves were logit-log transformed as stated above. Lipovitellin populations at approximately 25% (1,000 ng/L; $n = 24$), 50% (250 ng/L; $n = 26$), and 75% (31.25 ng/L; $n = 21$) inhibition of maximum binding (B_0) were assayed to determine intra-assay (within-run) and interassay (between-run) variation (coefficient of variation) within the working range of the curve. Specific binding (B) fluorescence values were used to calculate all coefficients of variation.

Sensitivity and specificity. Sensitivity is defined as the least detectable sample concentration that is significantly different from the 0 standard [31], whereas specificity points to the degree of discrimination of the ELISA between positive and negative samples [32]. To determine the assay sensitivity for copepod VTN quantification, gravid female *A. tenuiremis* were collected from sediment monoculture and aliquotted as one, two, or four individuals into 1.5-ml centrifuge tubes. To test the specificity of the assay, males (VTN-poor) and gravid females (VTN-rich) were collected from sediment monoculture and aliquotted (five individuals per replicate) into 1.5-ml centrifuge tubes. After transferring copepods, residual seawater was aspirated to 5 μ l by using 0.03-mm-diameter borosilicate glass pasteur pipets. Seawater controls (0.2- μ m-filtered 5- μ l aliquots) were assayed to account for any salt interference in the ELISA. All samples were kept at -20°C until homogenization and VTN analysis. Samples and controls were assayed for VTN and logit-log transformed as above. The resulting VTN concentration data were log-transformed when normality failed and tested for significance with one-way analysis of

variance and Tukey multiple comparison procedures ($\alpha = 0.05$). Statistical procedures were performed with SigmaStat® statistical software (Ver 2.0, SPSS Science, Chicago, IL, USA).

Preparation of copepod homogenate for ELISA

Immediately before ELISA analysis, samples of *A. tenuiremis* were homogenized in 100 μ l of TBS for 30 s and centrifuged at 12,000 rpm for 5 min. Copepod supernatant (80 μ l) was transferred to a 1.5-ml centrifuge tube and stored at -20°C until VTN analysis. The VTN ELISA was performed as stated above with the following exceptions: 75 μ l of the copepod homogenate in TBS was added to the coated and blocked well, and anti-VTN IgG diluted 1:2,000 in 0.2% BSA-TBS was added to each copepod sample well yielding a final 1:4,000 antibody dilution and 0.1% BSA concentration. Data were reduced by logit-log transformation as stated above. Because copepod size prohibits the ability to quantify VTN and total protein from the same copepod homogenate, all VTN concentrations were normalized as picograms of VTN per copepod.

Effects of the phenylpyrazole insecticide, fipronil, on VTN

To test the validity of the VTN ELISA for screening compounds with potential endocrine toxicity, Stage-I copepodite juveniles of *A. tenuiremis* were reared individually in isolation to sexual (reproductive) maturity (≤ 12 d) in 96-well microplate volumes (200 μ l) of the GABA-disrupting phenylpyrazole insecticide fipronil (0 and 0.6 $\mu\text{g/L}$ nominal). Control and treatment solutions were 90% renewed every 3 d, and developing copepods were fed a 1:1 mixed algal cell suspension of *Isochrysis galbana* and *Dunaliella tertiolecta* at 10^7 cells/ml every 6 d. After developing copepods reached the adult stage, control and fipronil-exposed males or females were gently transferred with glass micropipettes to respective glass crystallizing dishes containing the appropriate treatment solution. Copepods were then transferred to a 5% glutaraldehyde seawater (30‰) solution, fixed for 30 min, washed four times with clean, filtered seawater, and aliquotted (five individuals per replicate) to 1.5-ml centrifuge tubes. Adult copepods were then homogenized and assayed for VTN as stated above. The resulting VTN concentration data were tested for significance with one-way analysis of variance and Tukey multiple comparison procedures ($\alpha = 0.05$). Statistical procedures were performed using SigmaStat statistical software (Ver 2.0).

RESULTS

Purification of a VTN standard

Elution profiles of egg homogenate from *P. pugio* after immunoaffinity chromatography are shown in Figure 1. Immediately after addition of 0.1 M citric acid (pH ~ 3), VTN isoforms were eluted off the immunoaffinity column and collected as a purified VTN fraction (fraction 25). After purification and dialysis, this fraction and the prepurification egg homogenate of *P. pugio* were resolved on an 8% SDS-PAGE gel and immunoblotted with polyclonal anti-VTN antibodies (Fig. 2). In both the complex egg homogenate and the purified VTN fraction, the antibodies specifically probed only VTN isoforms (Fig. 2). Thus, the VTN fractions were deemed specific and suitable for ELISA development and validation.

Development and validation of the ELISA

Three different approaches were initially screened to test ELISA sensitivity and precision (Fig. 3). An ELISA incor-

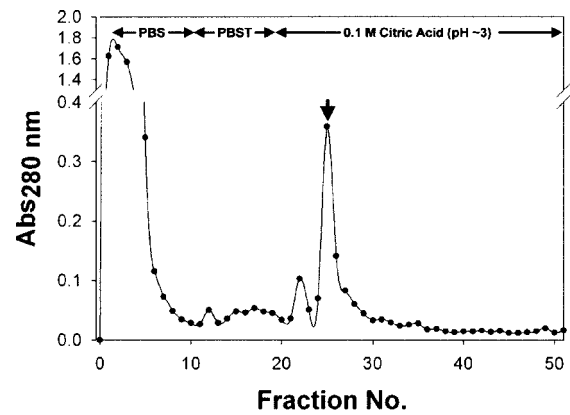


Fig. 1. Purification of lipovitellin (VTN) from fresh, newly extruded eggs of *Palaemonetes pugio*. Several VTN proteins were purified (arrow) via immunoaffinity column chromatography with VTN antibody-conjugated Sepharose. Nonbound VTN was washed with phosphate-buffered saline (PBS) and 0.05% Tween-20-PBS (PBST), and eluted with 0.1 M citric acid (pH ~ 3). All fractions were collected in 1 ml and absorbance (Abs) was measured at 280 nm.

porating an unlabeled *L. plumulosus* primary antibody (anti-VTN IgG), an alkaline phosphatase-labeled secondary antibody (goat anti-rabbit IgG), and a fluorescent substrate (4-methylumbelliferyl phosphate) yielded the highest precision ($r^2 = 0.9967$) and sensitivity (VTN at 1.9 ng/ml; Table 1). This ELISA design was then optimized by performing a two-dimensional dilution of both the coating VTN and primary antibody solution (Fig. 4). An optimization of coating VTN concentration (2.5 $\mu\text{g/ml}$) with primary antibody (1:2,000) dilution yielded optimal fluorescence (50% competition = 16,079 fluorescence units). The optimal secondary antibody solution was previously optimized to a 1:2,000 dilution (data

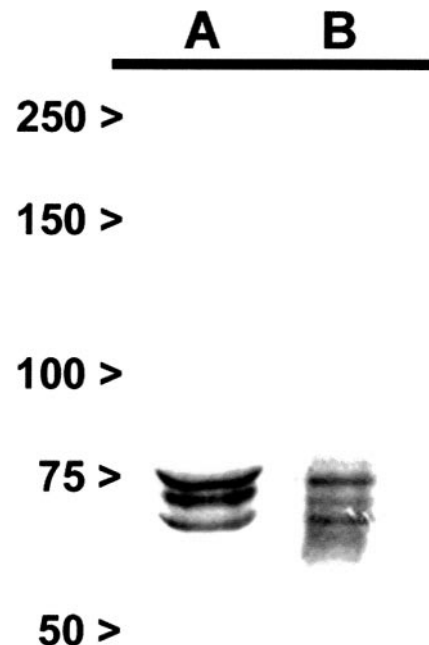


Fig. 2. Crude egg protein extract of *Palaemonetes pugio* (lane A) and purified lipovitellin(s) (VTNs) (lane B) were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Lipovitellin(s) in both lanes were identified by using amphipod (*Leptocheirus plumulosus*) VTN-specific polyclonal antibodies. Note that the antibodies identify only VTN in both the crude egg protein extract and pure VTN solution.

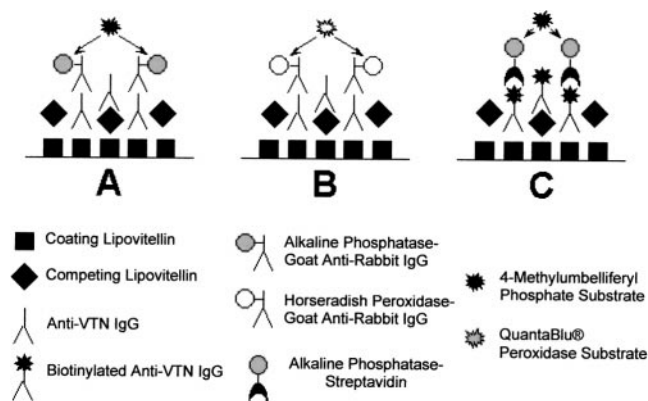


Fig. 3. Illustration demonstrating three approaches initially screened for lipovitellin (VTN) detection in an enzyme-linked immunosorbent assay format. All three formats involved competing reactions between VTN-specific antibodies and either bound or free VTN. Sensitivity and precision were tested with the following combinations: (A) anti-VTN immunoglobulin G (IgG), alkaline phosphatase-labeled goat anti-rabbit IgG, and 4-methylumbelliferyl phosphate substrate; (B) anti-VTN IgG, horseradish peroxidase-labeled goat anti-rabbit IgG, and QuantaBlu[®] peroxidase substrate; and (C) biotinylated anti-VTN IgG, alkaline phosphatase-labeled streptavidin, and 4-methylumbelliferyl phosphate substrate. Incubation and development times were identical in all three approaches.

not shown). Standard curves utilizing the optimal coating concentration and antibody dilutions were then generated to evaluate the performance of the VTN ELISA. Figure 5 shows a representative logit-log transformed standard curve for this ELISA. The VTN ELISA exhibited high precision ($r^2 > 0.95$), a low detection limit (1.9 ng/ml), and a broad working range (B/B₀: 25–75%, 31.25–1,000 ng/ml). The reproducibility of the ELISA was tested by assaying VTN at 25% (1,000 ng/ml), 50% (250 ng/ml), and 75% (31.25 ng/ml) antibody binding. The intra-assay and interassay coefficient of variations ranged from 3.9 to 16.8% within the working range of the standard curves (Table 2), and are considered acceptable for an ELISA assay [33].

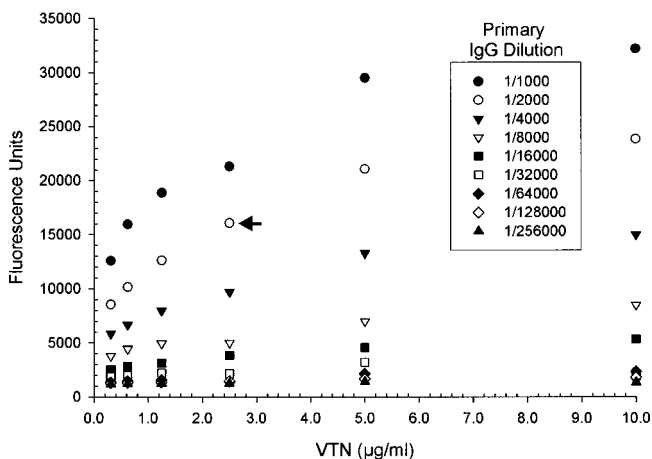


Fig. 4. Determination of optimal concentration of coating lipovitellin (VTN) and primary VTN immunoglobulin G (IgG) for the development of the VTN enzyme-linked immunosorbent assay. The optimal concentrations of coating VTN and anti-VTN antibody were 2.5 µg/ml and 1:2,000, respectively, at 50% antibody binding (arrow) based on the maximum fluorescence generated from the two-dimensional titration.

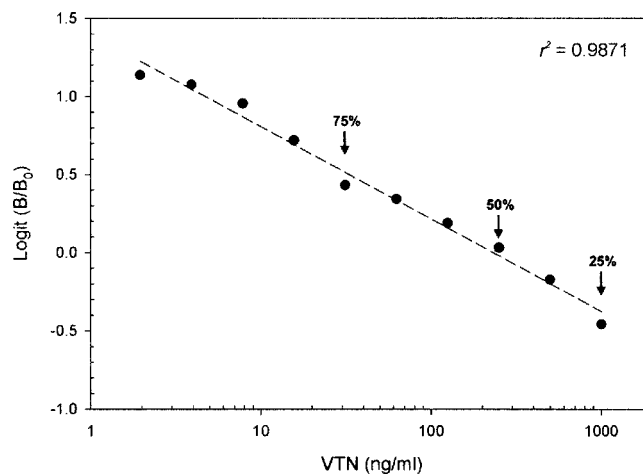


Fig. 5. Representative standard curve for the lipovitellin (VTN) enzyme-linked immunosorbent assay (ELISA). Data were logit transformed based on the ratio of specific to maximum antibody binding (B/B₀). Lipovitellin concentrations of 1,000, 250, and 31.25 ng/ml exhibit 25, 50, and 75% antibody binding, respectively. These concentrations were tested for reproducibility and represent the working range of the VTN ELISA.

Sensitivity and specificity to copepods

The anti-*L. plumulosus* VTN antibodies developed previously [12] cross-reacted with only one VTN isoform (~90 kDa) in protein homogenate from gravid female *A. tenuiremis*, and did not cross-react with proteins from male *A. tenuiremis* resolved by 14% SDS-PAGE (Fig. 6). By using the ELISA system described above, we significantly ($p < 0.001$) detected VTN (relative to seawater controls) in one gravid female copepod (Fig. 7). However, mean VTN concentrations in aliquots of one (14.5 ng/ml) and two (12.9 ng/ml) gravid female copepods were not significantly different ($p = 0.818$) from one another, and they did not fall within the working range (31.25–1,000 ng/ml) of the VTN ELISA standard curve. In contrast, VTN concentrations (46.5 ng/ml) in aliquots of four gravid female copepods were significantly higher ($p < 0.001$) than VTN titers from both one and two gravid females, and did fall within the working range of the standard curve. Therefore, this ELISA was able to detect VTN with confidence in as few as four gravid copepods. To test the specificity of the VTN ELISA within the working range of the standard curve, gravid females or males (five copepods per replicate) were collected from sediment monocultures and assayed for VTN. The ELISA was able to significantly discriminate positive (gravid female) and negative (male) samples (Fig. 7). Gravid females exhibited >10-fold higher VTN concentrations (3,590 pg/copepod) than did males (340 pg/copepod).

Table 2. Characteristics of the lipovitellin (VTN) enzyme-linked immunosorbent assay

	Binding		
	75%	50%	25%
VTN (ng/ml)	31.25	250	1,000
Intra-assay CV ^a (%)	5.1	3.9	10.5
Interassay CV (%)	11.4	12.5	16.8

^a CV = coefficient of variation.

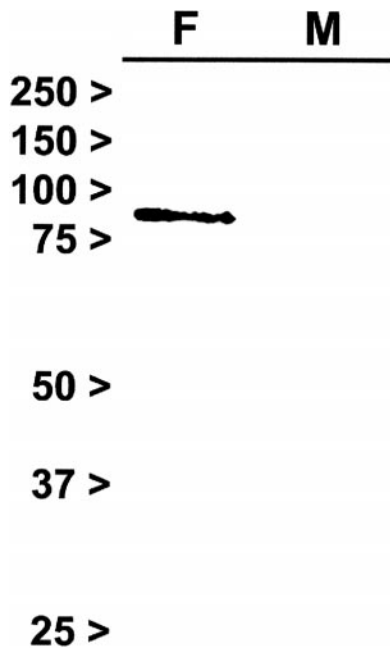


Fig. 6. Cross-reactivity of amphipod (*Leptocheirus plumulosus*) lipovitellin (VTN)-specific antibodies to VTN in female (F) and male (M) copepods (*Amphiascus tenuiremis*). Crude proteins from *A. tenuiremis* were separated by 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and identified with anti-VTN antibodies. Lipovitellin proteins were not detected in extract from male copepods via Western blot procedures.

Exposure to the GABA-disrupting insecticide fipronil

Stage-I copepodite juveniles *A. tenuiremis* reared individually to adulthood in aqueous fipronil were assayed for survival and VTN concentrations. A 12-d exposure to fipronil was not significantly toxic, with >90% survival in the control and 0.6- $\mu\text{g/L}$ treatments. Chronic exposure to fipronil did not significantly induce VTN in virgin male copepods (VTN at 141.2 pg/copepod), but it did strongly ($p < 0.01$) induce VTN in virgin females (VTN at 263.3 pg/copepod) relative to control virgin females (VTN at 83.5 pg/copepod) and virgin males (VTN at 78.3 pg/copepod) (Fig. 8).

DISCUSSION

We report here the development and validation of a polyclonal antibody ELISA to quantify VTN in the marine meiobenthic harpacticoid copepod *A. tenuiremis* after exposure to toxicants with suspected endocrine activity. Because the diminutive size of amphipod and copepod embryos prohibits the ability to purify suitable quantities of standard VTN for ELISA applications, VTN isoforms were purified from grass shrimp (*P. pugio*) embryos by using immunoaffinity chromatography. The sensitivity of the ELISA was an important parameter during method development because our objective was to quantify VTN in microcrustaceans without the typical need to pool large numbers of individuals (i.e., compromising statistical power) after toxicant exposure. We tested three different indirect, competitive ELISA approaches incorporating a variety of primary and secondary antibody conjugates and fluorescent substrates, and found that an unconjugated *L. plumulosus*-specific primary antibody, an alkaline phosphatase-labeled secondary antibody, and a 4-methylumbelliferyl phosphate substrate yielded the greatest sensitivity and precision (Table 1). The final VTN ELISA assay exhibited a broad working range of 31.25 to

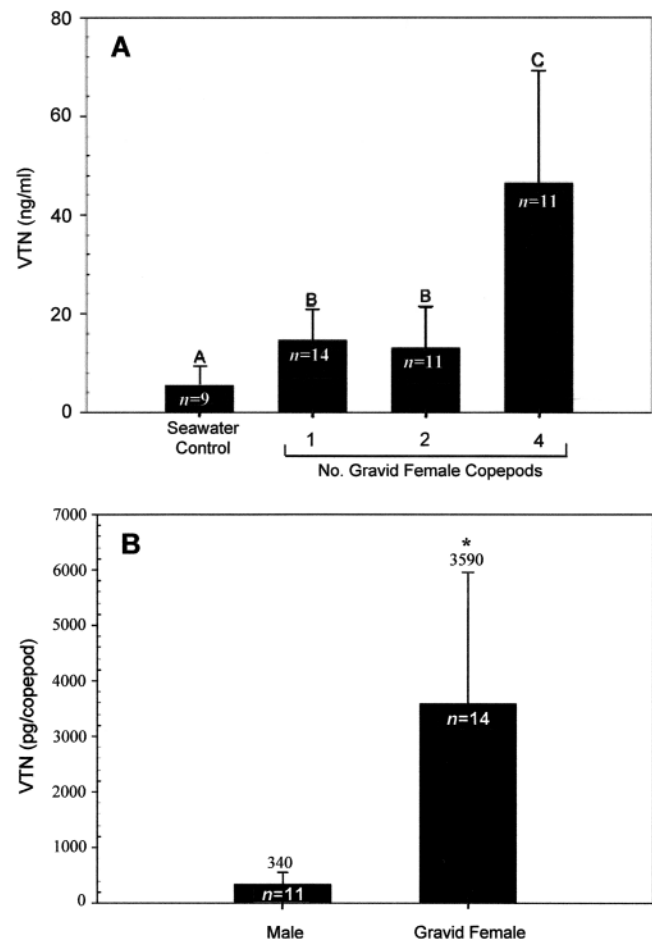


Fig. 7. Sensitivity (A) and specificity (B) of the lipovitellin (VTN) enzyme-linked immunosorbent assay (ELISA) to the sediment-dwelling marine copepod *Amphiascus tenuiremis*. Although this ELISA can detect VTN in one individual gravid female copepod, VTN quantification from a pooled sample of four gravid females yields the highest confidence and precision. For the specificity of the ELISA, each replicate represented a pooled sample of five copepods and VTN concentrations were normalized as pg/copepod. Error bar = standard deviation. Bars with dissimilar letters are significantly different ($p < 0.01$). An asterisk (*) indicates a significant difference from males ($p < 0.001$).

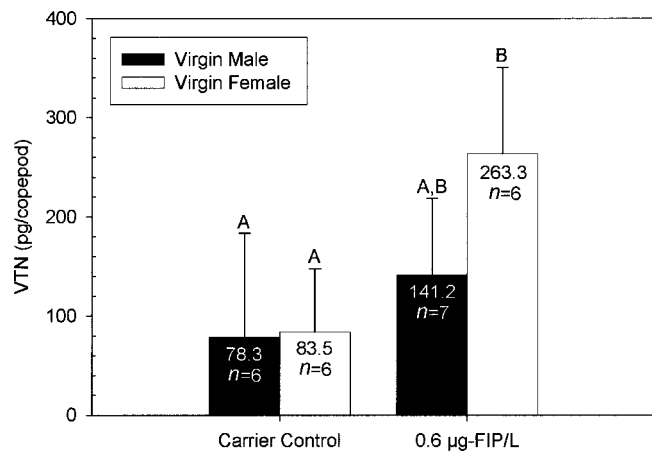


Fig. 8. Mean lipovitellin (VTN) concentrations in adult male and female *Amphiascus tenuiremis* reared individually for 12 d in micro-well volumes of aqueous fipronil (FIP). Each replicate represents a pooled sample of five copepods. Error bar = standard deviation. Bars with dissimilar letters are significantly different ($p < 0.01$).

1,000 ng/ml (Fig. 5), reproducibility over time, with intra-assay and interassay coefficients of variation ranging from 3.9 to 16.8% (Table 2), detection limits of 2 ng/ml, and the ability to quantify VTN in small numbers (four or more) of copepods. We have used these same antibodies for VTN quantification in grass shrimp [3] and these antibodies would likely be useful for other species of macro- or microcrustaceans.

Although this ELISA is capable of significantly detecting VTN in single copepods, quantification from a pooled sample of four or more individuals yields the greatest statistical confidence and precision. As such, this ELISA was able to discriminate between pooled positive (gravid females) and negative (males) from sediment monocultures, where vitellogenic females exhibit >10-fold higher VTN concentrations than males. Although VTN was not detected in sediment-cultured males by Western blotting (Fig. 6), it was detected in these populations of males by ELISA at levels comparable to virgin adult females exposed to fipronil (Figs. 7 and 8). The increased sensitivity of the ELISA system (ng/ml) likely produced the better detection of VTN from males relative to Western analysis ($\mu\text{g/ml}$) [34]. Interestingly, copepod males assayed from sediment culture had higher VTN titers than males reared in microwell volumes of seawater. The food diversity and quantity in laboratory sediment cultures is higher than in experimental microwell chambers, and may contribute a greater source of cholesterol and phytoecdysteroids potentially responsible for hormonally regulated vitellogenic pathways. In comparison, trace quantities of VTN often are detected in male fish by ELISA [34], possibly attributed to low levels of circulating estrogens or to dietary-induced phytoestrogen exposure [35].

Although 17β -estradiol frequently is used to validate fish VTG ELISAs for environmental endocrine-disruptor screening [34,36,37], crustaceans do not possess vertebrate steroids and likely are not responsive to steroidal estrogens [38]. For example, the injection of 17β -estradiol, progesterone, or 17α -hydroxyprogesterone had no significant effect on yolk protein levels in the hemolymph of the ridgeback shrimp (*Sicyonia ingentis*) [24]. Unfortunately, for invertebrates generally, a reliable VTG-inducing positive control chemical has not been found. To this end, and to test the validity of the developed crustacean VTN ELISA for screening environmental toxicants, we exposed developing *A. tenuiremis* to the modern GABA-disrupting insecticide, fipronil [39,40], and screened virgin adult males and females for VTN concentrations. Fipronil is a phenylpyrazole insecticide that targets GABA-regulated chloride channels and is rapidly gaining use throughout the United States for residential termite and fire ant control, turf grass management, and rice cultivation. In this study, fipronil at 0.6 $\mu\text{g/L}$ significantly induced VTN in virgin females, but not virgin males, possibly because of direct or indirect neurohormonal signaling (Fig. 8). Elevated female VTN levels may keep fipronil biologically unavailable before and after mating and reproduction. Similarly, pyrene-exposed female grass shrimp (*P. pugio*) have been seen to exhibit significantly higher levels of VTN than pyrene-exposed males [41]. Lipovitellin induction in female crustaceans may be a resistance mechanism for reducing the toxicity of lipophilic environmental contaminants via sequestration in lipoproteins.

In summary, a polyclonal anti-crustacean VTN ELISA was developed and validated for endocrine toxicity screening in the meiobenthic copepod *A. tenuiremis*, and may potentially be used for other crustacean species. The ELISA allowed ac-

curate quantification of VTN from whole-body homogenates of four or more male or female copepods. Moreover, this ELISA can be used to quantify VTN in copepods exposed to suspected endocrine-active chemicals. In conjunction with rapid copepod life-cycle bioassay techniques [26,27], this VTN ELISA can be used as a screening tool for endocrine toxicity. In this study, we found that VTN was induced in female, but not male, copepods when exposed to sublethal concentrations of fipronil, and possibly acts a mechanism for reducing toxicant bioavailability.

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