Development of a quantitative enzyme-linked immunosorbent assay for vitellin in the mysid *Neomysis integer* (Crustacea: Mysidacea)

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Abstract

Mysid crustaceans have been put forward by several regulatory bodies as suitable test organisms to screen and test the potential effects of environmental endocrine disruptors. Despite the well-established use of mysid reproductive endpoints such as fecundity, egg development time, and time to first brood release in standard toxicity testing, little information exists on the hormonal regulation of these processes. Control of vitellogenesis is being studied intensively because yolk is an excellent model for studying mechanisms of hormonal control, and vitellogenesis can be chemically disrupted. Yolk protein or vitellin is a major source of nourishment during embryonic development of ovigorous egg-laying invertebrates. The accumulation of vitellin during oocyte development is vital for the production of viable offspring. In this context, we developed a competitive enzyme-linked immunosorbent assay (ELISA) for vitellin of the estuarine mysid *Neomysis integer*. Mysid vitellin was isolated using gel filtration, and the purified vitellin was used to raise polyclonal antibodies. The ELISA was sensitive within a working range of 4 to 500 ng vitellin/mL. Serial dilutions of whole body homogenates from female *N. integer* and the vitellin standard showed parallel binding curves, validating the specificity of the ELISA. The intra- and interassay coefficients of variation were 8.2% and 13.8%, respectively. Mysid vitellin concentrations were determined from ovigorous females and eggs at different developmental stages. The availability of a quantitative mysid vitellin ELISA should stimulate further studies on the basic biology of this process in mysids. Furthermore, it could provide a means to better understand and predict chemically induced reproductive effects in mysids.
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1. Introduction

Most of the current knowledge of crustacean endocrinology is based on studies with decapods such as crabs, lobsters, crayfish and shrimp (Carlisle and Knowles, 1959; Chang, 1997; Charmantier et al., 1997; DeFur et al., 1999; Fingermann, 1987; Lafont, 2000; Quackenbush, 1986). We recently published a comprehensive review on the use of mysid shrimp as potential models to study hormonal regulation and its disruption by chemicals (Verslycke et al., 2004). In addition, mysids have been put forward as suitable test organisms by several other researchers (DeFur et al., 1999; LeBlanc, 1999) and regulatory authorities (CSTEE, 1999; US-EPA, 2002) for the evaluation of endocrine disruptors. Despite the well-established use of mysid reproductive endpoints such as fecundity, egg development time, and time to first brood release in standard toxicity testing, little information exists on the hormonal regulation and basic biology of these processes (Verslycke et al., 2004). Few studies have examined the effects of contaminants on gonadal maturation in crustaceans, and the lack of knowledge of invertebrate endocrinology in general, is one of the main reasons for the very limited progress that has been made regarding endocrine disruption research in invertebrates (Oetken et al., 2004). Much attention has recently been given to vitellogenin, the

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precursor to the yolk protein vitellin in egg-laying vertebrates and invertebrates, as an indicator of exposure to endocrine disruptors (Billinghurst et al., 2000; Fenske et al., 2001; Tsukimura, 2001; Versonnen and Janssen, 2004). Vitellogenesis involves the production of yolk proteins that act as nutrient sources for developing embryos. Consequently, any event that affects the synthesis of the yolk precursor vitellogenin will also modify reproductive success. Studies on the hormonal regulation of vitellogenesis in mysids at this point are nonexistent because assays to measure the relevant hormones are not available. In a recent study, we purified and characterized vitellin from the mysid Neomysis integer (Ghekiere et al., 2004). N. integer is the dominant hyper-benthic mysid in the upper reaches of European estuaries. It is sensitive to many toxicants at environmentally relevant concentrations, and has been suggested as a more ecologically relevant alternative to high-latitude and low-saline systems than the standard toxicity test species Americamysis bahia (Emson and Crane, 1994; Mees et al., 1995; Mees and Jones, 1997; Roast et al., 1999, 2001; Verslycke et al., 2003; Wildgust and Jones, 1998).

Hormonal control of vitellogenesis in crustaceans is closely linked with the molt cycle (Fig. 1). Molting, a well-studied hormonally regulated process, is critical in the development and maturation of every arthropod (DeFur, 2004). There are several feedback mechanisms for the control of molting hormones (e.g., ecdysone) and a number of peptide hormones that regulate vitellogenesis and molting in crustaceans (Chang, 1993; DeFur et al., 1999; Meusy and Payen, 1988; Oberdörster and Cheek, 2000). The production of vitellin is under direct control of peptide hormones like the 'vitellogenesis-inhibiting hormone' (VIH) produced by the X-organ located in the eye-stalk, and the 'vitellogenesis-stimulating hormone' (VSH). The complex hormonal regulation of vitellogenesis makes it an excellent model for studying mechanisms of hormone signalling at the cellular and molecular level (Billinghurst et al., 2000; Tuberty et al., 2002).

Vitellogenesis involves two phases, primary and secondary vitellogenesis. Primary vitellogenesis is continuous and primary follicles have endogenous vitellin. The secondary vitellogenesis takes place during the reproductive season. The prominent feature of secondary vitellogenesis is the uptake of exogenous vitellogenin in the oocytes (Charniaux-Cotton, 1985). In mysids, juveniles are released from the marsupium immediately before ecdysis of the mother, shortly after which she lays a new batch of eggs in the marsupium. A secondary vitellogenic cycle starts for a new batch of oocytes on the second day of the molt cycle, offering an example of the type 2 pattern for the regulation of simultaneous gonadal and somatic growth as seen in Decapoda, Amphipoda and Isopoda (Adiyodi and Subramoniam, 1983; Charniaux-Cotton, 1985).

In this study, we developed a competitive ELISA to measure vitellin concentrations in N. integer, which will allow for future investigations into hormonal regulation of mysid vitellogenesis and its potential disruption by chemicals.

2. Materials and methods

2.1. Experimental animals

N. integer were collected from the shore by hand net in the Galgenweel, a shallow brackish water near the river Scheldt
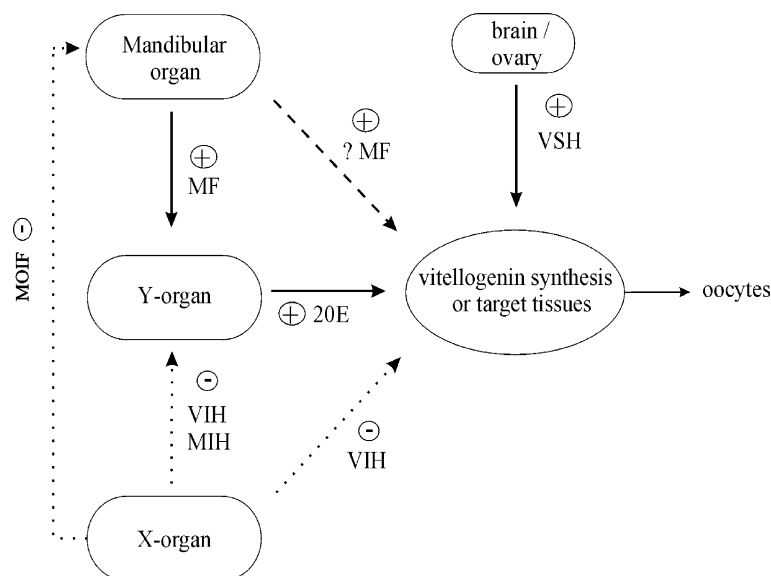

Fig. 1. Simplified scheme of the hormonal control of the crustacean molt cycle and vitellogenesis. Adapted from DeFur et al., 1999; Meusy and Payen, 1988; Oberdörster and Cheek, 2000. Interrupted arrows (−) represent inhibition and full arrows (+) stimulation. The following hormones play an important role in regulating crustacean molting and vitellogenesis: 20 E, 20-hydroxyecdysone, the active molting hormone; MF, methyl farnesoate; MOIF, mandibular organ-inhibiting factor; VIH, vitellogenesis-inhibiting hormone; VSH, vitellogenesis-stimulating hormone.

in Antwerp (Belgium) in summer of 2004. After a 24-h acclimation period to the maintenance temperature, the organisms were transferred to 200-L glass aquaria. Culture medium was artificial seawater (Instant Ocean®, Aquarium Systems, France), diluted with aerated deionized tap water to a final salinity of 5 psu. A 14-h light : 10-h dark photoperiod was used during culturing and water temperature was maintained at 15 °C. Cultures were fed daily with 24–48-h-old *Artemia* ssp. nauplii ad libitum. Hatching of the *Artemia* cysts was performed in 1-L cylinder-conical vessels under vigorous aeration and continuous illumination at 25 °C.

2.2. Vitellin purification

Egg masses were taken from ovigorous females, weighed (335 mg, the total wet mass of eggs used for purification) and homogenized on ice in 1.8 mL buffer (136 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, 2% glycine ethyl ester, 0.03% EDTA, 1 mM PMSF, 0.2% aprotinin, 2 mM leupeptin, and 0.02% sodium azide; pH 7.3). Homogenates were centrifuged at 4 °C for 15 min at 11,000 g. The supernatant was centrifuged a second time (15 min, 11,000 g). Crude homogenates were stored at 4 °C until further purification.

The supernatant (1.8 mL) was subjected to gel filtration using an Äkta system (Amersham Biosciences, Uppsala, Sweden) by injecting the sample from a 2 mL sample loop onto a HiLoad 16/60 200 prep grade Superdex column (Amersham). The column was equilibrated with 20 mM TBS, 5 mM EDTA, pH 7.6, during two column volumes. Elution of the sample was performed with 1 column volume at a flow rate of 1 mL/min. Eluates were monitored at 280 and 474 nm for concomitant maximum protein and carotenoid pigment absorption, respectively. Fractions of 1.5 mL were automatically collected. The diluted fractions that eluted with concomitant peaks at both 280 and 474 nm were pooled and concentrated in an ultrafiltration device with a 10,000 molecular weight cut-off membrane (Vivascience, Hannover, Germany).

2.3. Production of polyclonal vitellin antibodies

Polyclonal antibodies against vitellin were produced in New Zealand white rabbits by Eurogentec (Seraing, Belgium). The antiserum was stored in aliquots at −80 °C.

2.4. Development of a homologous competitive ELISA for N. integer vitellin

The assay is based on a competition for the vitellin antibody between vitellin coated on the wells of a microtiter plate and free vitellin molecules in the sample solution. The antigen–antibody complex bound to the plate is detected by a secondary antibody directed against the primary vitellin antibody. This secondary antibody is conjugated with the enzyme horseradish peroxidase. The enzyme activity is revealed by adding a suitable substrate and hydrogen peroxide, and is measured colorimetrically.

2.5. General ELISA protocol

2.5.1. Coating the plates

Purified vitellin was thawed on ice and diluted in coating buffer (0.05 M sodium carbonate buffer, pH 9.6). The wells of 96-well microtiter plates (Nunc F96 Maxisorp™ Immuno Plate) were coated with 100 μL of vitellin solution (100 ng vitellin/mL coating buffer), sealed and incubated overnight at 4 °C. For determination of non-specific binding (NSB) effects, three wells per plate were treated with coating buffer only.

2.5.2. Preincubation of samples/standards

For the standards, purified vitellin was diluted in PBS-T blocking buffer (0.01 M phosphate-buffered physiological saline solution with 0.05% Tween 20 and 1% fatty acid-free bovine serum albumin (BSA) to a concentration of 2000 ng vitellin/mL. From this stock solution, serial dilutions were prepared in PBS-T blocking buffer. In parallel, samples with an unknown vitellin content were diluted in PBS-T blocking buffer. The vitellin standards and unknown samples (60 μL/well) were incubated in non-coated 96-well microtiter plates with vitellin antibody (60 μL/well, 1:10,000 in PBS-T blocking buffer). Our vitellin standard was quantified using the Bradford method with BSA as reference protein. For the NSB, 60 μL/well of blocking buffer was mixed with 60 μL of the antibody solution only. The incubates were mixed on a rotary shaker, and the plates were sealed and incubated overnight at 4 °C.

2.5.3. Antibody incubation

The coated plates were washed three times with 100 μL PBS-T washing buffer (0.01 M phosphate-buffered physiological saline solution with 0.05% Tween 20, pH 7.4). To reduce background, the plates were blocked with 150 μL of PBS-T blocking buffer/well for 30 min at 37 °C. After this blocking step, the plates were washed another three times with PBS-T, before 100 μL of the sample/antibody or standard/antibody incubates were pipetted into the wells. The plates were sealed and incubated for 120 min at 37 °C. The first antibody incubates were then removed and the plates were washed three times with PBS-T. Second antibody (125 μL) against rabbit IgG (goat anti-rabbit IgG, whole molecule, peroxidase conjugate; Sigma) was added to each well at a dilution of 1:2000 in PBS-T blocking buffer and the plates were sealed and incubated at 37 °C for 60 min.

2.5.4. Detection

The plates were washed three times with PBS-T and then 125 μL of the enzyme substrate solution was added to each well. This solution was prepared by dissolving 0.5 mg/mL of *o*-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich) in 0.05 M phosphate-citrate buffer, pH 5.0 (51 mM dibasic sodium phosphate, 24 mM citric acid). After addition of 0.5 μL/mL of H_2O_2 (30%; Merck), the substrate solution was immediately pipetted into the plates (125 μL/well). The enzyme reaction was allowed to proceed for 10

min in the dark, at which point the color reaction was stopped by the addition of 30 μL of 3 N H_2SO_4. The absorbance of the reaction product was read at 490 nm using a microtiter plate reader. The absorbance values obtained in the ELISA were inversely proportional to the amount of vitellin present in the sample. Vitellin content in samples was quantified from the log-transformed standard curve.

2.6. Quantification of vitellin in eggs and whole body homogenates

Eggs in the marsupium of gravid females were staged under a microscope. Mauchline (1980) gives a description of the different developmental stages of the embryos. After decapitation of the gravid females, mysid embryos were removed with a fine spatula while submerged in Tris–HCl pH 7.2. Individual eggs were placed in 60 μL Tris–HCl pH 7.2, and further diluted 100 times using the same buffer to quantify vitellin. Vitellin was quantified in 10 replicate eggs of each developmental stage. Gravid females with embryos of stage I were homogenized in 200 μL Tris–HCl pH 7.2 and diluted 10,000 times to quantify vitellin. Ten replicates were used.

3. Results

3.1. Purification and characterization of vitellin

We purified and characterized vitellin of *N. integer* using gel filtration and polyacrylamide gel electrophoresis (PAGE) with different stains. Polyclonal antibodies were produced and Western blotting demonstrated that these were specific against vitellin of *N. integer*. Details are given in Ghekiere et al. (2004).

3.2. Development and validation of a competitive ELISA

Dilutions of the homologous antiserum between 1:5000 and 1:30,000 (data not shown), together with a second antibody titer of 1:2000, produced the best and most reproducible assay conditions. The secondary antiserum dilution was chosen on the basis of other vitellin/vitellogenin
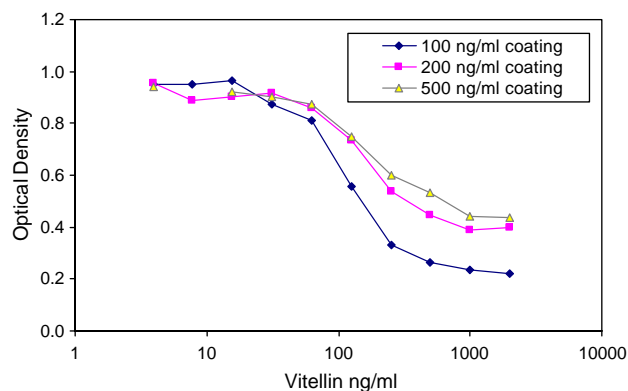

Fig. 2. The effect of different coating concentrations (100, 200 and 500 ng/mL) on the standard curve of *N. integer* vitellin with a primary antibody dilution of 1:10,000 and a secondary antibody dilution of 1:2000.

ELISAs which use dilutions between 1:3000 and 1:1000 (Fenske et al., 2001; Sagi et al., 1999; Vazquez Boucard et al., 2002). The effect of different coating concentrations (100, 200 and 500 ng/mL) on the standard curve is shown in Fig. 2. The standard curve with a coating concentration of 100 ng/mL showed the largest working range. For routine applications of the assay, a primary antibody titer of 1:10,000, a secondary antibody dilution of 1:2000 and a vitellin coating concentration of 100 ng vitellin/mL were chosen. The working range for the assay was between 4 and 500 ng vitellin/mL (Fig. 3A). Serial dilutions of whole body homogenate of female *N. integer* showed a good parallelism or similar curves with the standard within the working range of the assay (Fig. 3B).

The reproducibility of the assay was evaluated. Egg samples with low to high vitellin levels were analyzed multiple (4–5) times in the same and in separate assays. The intra- and interassay coefficients of variation were 8.2% and 13.8%, respectively.

3.3. Vitellin levels in eggs and whole body homogenates

The quantitative ELISA allowed us to measure vitellin levels in a single egg of *N. integer*. Fig. 4 shows vitellin levels of eggs at different developmental stages. The development of the eggs within the marsupium can be divided into three
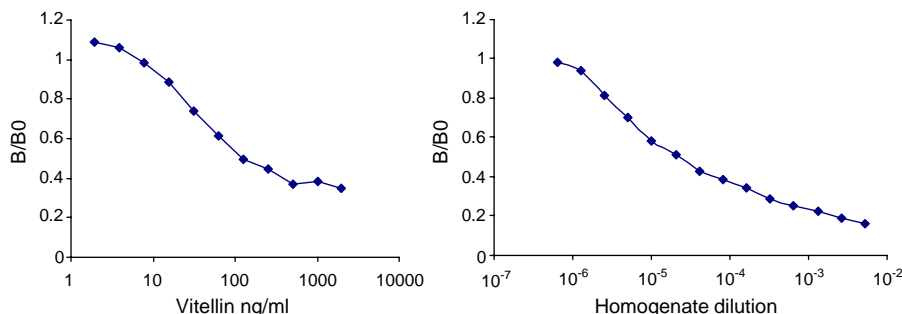

Fig. 3. A: ELISA standard curve of vitellin from *N. integer* with serial dilutions from 2000 to 1.95 ng/mL. B: Serial dilutions of whole body homogenate from female *N. integer*. B/B_0, is the optical density of the sample divided by optical density of the saturated well.
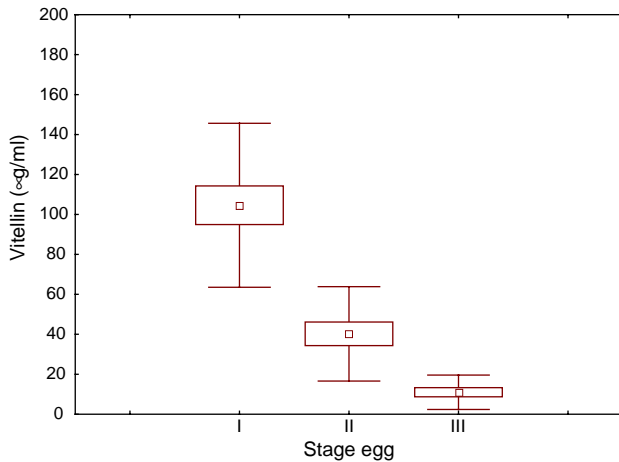
Fig. 4. Vitellin levels in eggs at different developmental stages. Box-plot shows the mean (small square), standard error (box) and the standard deviation (whisker) of 10 replicate measurements.

stages, which correspond with the stages described by Mauchline (1980) as eggs (stage I), eyeless larvae (stage II) and eyed larvae (stage III). Eggs of stage I, II and III have vitellin levels of 104.6 (±41.0), 40.2 (±23.6) and 11 (±8.6) 1 g/mL respectively. Vitellin levels are expressed in µg/mL since we had to dilute one egg to measure vitellin concentrations, therefore these results are µg/mL from a single egg. The results shown in Fig. 4 are from ten replicates. Vitellin levels were also quantified vitellin levels in gravid female animals. Females with eggs of stage I in their marsupium have vitellin levels of 542 (±120.3) µg/mL. Concentrations were higher in gravid animals than those in individual stage I eggs indicating the possible presence of extra-ovo vitellin in the gravid animals.

4. Discussion

Accurate methods to quantify vitellogenin and vitellin in crustaceans can contribute to elucidate crustacean reproduction and its potential disruption by endocrine disrupting chemicals. Previous studies often relied on oocyte size or ovarian weight (Anilkumar and Adiyodi, 1980, 1985; Eastman-Reks

and Fingerman, 1984). While such measurements have provided important insights into vitellogenesis, they are time-consuming and indirect. Specific immunoassays are generally rapid, precise, reproducible, and therefore provide distinct advantages over other bioassays (Chard, 1987; Lee and Watson, 1994). Vitellin has been measured mostly in crustacean hemolymph and ovaries (Table 1). Ovarian vitellin and protein concentrations are closely correlated with the ovarian stage, and the accumulation of vitellin is linked with an increase in ovarian weight (Lee and Chang, 1997). Vitellin levels in the ovaries of white prawn *Fenneropenaeus indicus* increased from 5.9 to 372.3 mg per ovary during ovarian development (Vazquez Boucard et al., 2002). Tsukimura (2001) reports that vitellogenin levels in crustacean hemolymph ranges from 0.03–10 mg/mL.

The present study is the first to report vitellin concentrations in eggs of a well-established crustacean test species, the mysid *N. integer*. Vitellin levels decreased with the development of the egg, in accordance with the role of vitellin as a major source of nourishment for the developing embryo. It has been shown that vitellin concentrations in eggs are an important and useful indicator of egg quality, and vitellin levels can be used to predict female reproductive performance (Arcos et al., 2003). The ELISA developed in this study was capable of detecting vitellin levels in *N. integer* from 4 to 500 ng/mL. This range is comparable to the ELISA developed for ridgeback shrimp *Sicyonia ingentis* vitellin (0.3–300 ng/mL; Tsukimura et al., 2000) and for Chinese mitten-handed crab *Eriocheir sinensis* vitellin (7.8–500 ng/mL; Chen et al., 2004). Our ELISA is slightly more sensitive than the one developed for blue crab *Callinectes sapidus* (62–1500 ng/mL; Lee and Watson, 1994) and for the copepod *Amphiascus tenuiremis* (31–1000 ng/mL; Volz and Chandler, 2004). In the ELISA standard curve (Fig. 3A) the value of B/B_0 generally exceeds 0.3. This observation could be due to unspecificity of the secondary antibody, the ratio between primary to secondary antibody, and/or the ratio between coating and primary antibody, i.e., antibody titre too high and/or high coating concentration could encourage unwanted binding of antibody to plate despite high concentration of free vitellin in plate. However, our results indicate that our ELISA was sufficiently sensitive and similar to previously published

Table 1
Overview of available ELISAs to quantify vitellogenin or vitellin in freshwater and marine crustaceans

Species	Tissue	References
Blue crab, *Callinectes sapidus*	Ovary, hemolymph	Lee and Watson, 1994
Freshwater prawn, *Macrobrachium rosenbergii*	Hemolymph, ovary, hepatopancreas	Lee and Chang, 1997
Crayfish, *Cherax quadricarinatus*	Hemolymph	Sagi et al., 1999
Tiger shrimp, *Penaeus monodon*	Hemolymph	Vincent et al., 2001
Ridgeback shrimp, *Sicyonia ingentis*	Hemolymph	Tsukimura et al., 2000
Lobster, *Homarus americanus*	Hemolymph	Tsukimura et al., 2002
Penaeid prawn, *Fenneropenaeus indicus*	Hemolymph, ovary, hepatopancreas	Vazquez Boucard et al., 2002
Copepod, *Amphiascus tenuiremis*	Whole body homogenate	Volz and Chandler, 2004
Crab, *Eriocheir sinensis*	Ovary	Chen et al., 2004
Mysid shrimp, *Neomysis integer*	Whole body homogenate, eggs	Present study

ELISAs to quantify vitellin have similar results (Fenske et al., 2001; Lee and Watson, 1994; Tsukimura et al., 2000).

The induction of vitellogenin in male fish has been used extensively as a biomarker of estrogen exposure (Fenske et al., 2001; Heppel et al., 1995; Tyler et al., 1996; Versonnen and Janssen, 2004). Several researchers have looked at using vitellogenesis in egg-laying invertebrates in a similar way. However, standardized quantitative assays to study vitellogenesis in invertebrates are largely unavailable. Vitellin or vitellogenin has been isolated or partially characterized in several crustacean species (for an overview refer to Tuberty et al., 2002). Still, only a limited number of enzyme-linked immunosorbent assays (ELISAs) to quantify vitellogenin or vitellin in freshwater and marine crustaceans have been developed (Table 1). The complex and still poorly understood regulation of vitellogenesis in many crustaceans, limits its use as a biomarker of endocrine disruption at this time. Future laboratory and field studies with crustaceans, using assays like the one developed in this study, will help to unravel the hormonal regulation of crustacean vitellogenesis and will also allow for the assessment of the potential impact of endocrine disruptors on the reproduction of crustaceans.

In this study, a competitive ELISA to quantify vitellin in the estuarine mysid *N. integer* was successfully developed and allows accurate quantification of vitellin from whole-body homogenates as well as single eggs. The availability of a mysid vitellin ELISA is of particular importance for two reasons: (1) as a research tool to study the hormonal control of vitellogenesis in a non-decapod crustacean (2) as a potential assay to study chemically induced disruptions in mysid vitellogenesis and how this relates to effects on well-established reproductive endpoints. The development of suitable standard invertebrate test methods for endocrine disrupting compounds remains an urgent need and mysid crustaceans could provide unique opportunities through their routine use in regulatory screening and testing programs world-wide.

Acknowledgements

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