Comparative Biochemistry and Physiology, Part C 166 (2014) 88–95

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Evaluation of yolk protein levels as estrogenic biomarker in bivalves; comparison of the alkali-labile phosphate method (ALP) and a species-specific immunoassay (ELISA)

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ARTICLE INFO

Article history:
Received 12 June 2014
Received in revised form 12 June 2014
Accepted 17 July 2014
Available online 25 July 2014

Keywords:
Vitellogenin
Bivalvia
Mollusk
Yolk protein
Endocrine disruption
ELISA
ALP
Unio

ABSTRACT

Altered concentration of the vertebrate yolk protein precursor vitellogenin is a recognized biomarker for endocrine disruption in fish, and within recent years yolk protein alteration has also been associated with endocrine disruption in bivalves. Species-specific, direct and sensitive methods for quantification of vitellogenin in fish have been available for years whereas bivalve yolk protein levels have been estimated indirectly by alkali-labile phosphate (ALP) liberated from high molecular weight proteins because the sequence and biochemical structure of most bivalve yolk proteins are unknown. By applying a species-specific enzyme-linked immunosorbent assay (ELISA) for accurate determination of yolk protein level the impact of 17β-estradiol (57, 164 and 512 ng/L) on the freshwater bivalve *Unio tumidus* was investigated and compared with ALP estimations. Seven weeks of exposure during the pre-spawning and spawning period had no consistent effect on yolk protein concentration in hemolymph, and ALP levels in hemolymph also remained unchanged in both males and females. Further, basal male and female ALP levels were indistinguishable whereas the ELISA demonstrated that yolk protein levels of females exceeded male levels at the time of sampling, although male basal levels were high compared to fish. Altogether it is shown that individual ALP levels do not reflect yolk protein levels and hence hemolymph ALP levels cannot serve as biomarker for estrogenic exposure during the pre-spawning and spawning period in *U. tumidus*. The necessity of sensitive and validated biomarkers for reliable interpretation of data and the utility of ALP and yolk protein levels as biomarkers in bivalves are discussed.

1. Introduction

Bivalves are important for the ecological balance in aquatic ecosystems as they remove phytoplankton by filtration. High filtration capacity, sessile lifestyle and longevity make bivalves suitable as biomonitoring organisms in which effects of chemicals like endocrine disrupting compounds (EDCs) can be detected; however, standardized and validated endocrine endpoints for bivalves are scarce. A commonly used, sensitive and reliable biomarker for exposure to estrogenic EDCs in fish is altered levels of vitellogenin (Vtg), the precursor of the egg-yolk proteins vitellin and phosvitin (Jones et al., 2000). Vtg is synthesized in the liver of oviparous vertebrates and transported via the blood to the ovaries and the process is controlled by the estrogen receptor pathway and hence regulated by estrogen levels (Pakdel et al., 1991). Juvenile and male Vtg levels are generally low compared to sexually mature females but exposure to estrogens or xenoestrogens induces Vtg synthesis manifold. Knowledge on yolk protein structure and endocrine control of yolk protein synthesis in mollusks is limited and hence interpretation of mollusk experimental data is often based on vertebrate physiology and endocrinology. Even knowledge on basic biology and reproduction of most freshwater bivalves is scarce. The Swollen river mussel (*Unio tumidus*) is a common freshwater bivalve in Northern Europe. Gravid females were observed in the United Kingdom from late April to late July (Aldridge, 1999) and in Finland from May to June (Pekkarinen, 1993) but detailed information on health, seasonal conditions and reproduction in Danish populations is to our knowledge not available.

Fish Vtg's are highly phosphorylated proteins (Yao and Crim, 1996) but the biochemical structure of freshwater bivalve yolk protein has to our knowledge not been published. Due to the phosphate richness of fish Vtg their plasma Vtg levels were earlier indirectly estimated by the alkali-labile phosphate (ALP) method that detects inorganic phosphate liberated from phosphorylated proteins like Vtg. Later antibody based enzyme assays that directly quantify Vtg concentrations in plasma or tissue homogenates were developed (Jones et al., 2000), and today these enzyme assays (enzyme-linked immunosorbent assay, ELISA) involving species-specific antibodies for Vtg are commonly used for direct quantification of Vtg levels in fish (Jones et al., 2000) due to higher sensitivity and specificity compared to the ALP method

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http://dx.doi.org/10.1016/j.cbpc.2014.07.008
1532-0456/

(Pottinger et al., 2005). Estrogens and xenoestrogens have been reported to induce ALP levels in bivalve mollusks in the laboratory (Matozzo and Marin, 2008; Ricciardi et al., 2008) and in field studies (Gagne et al., 2001b; Pampanin et al., 2005; Long et al., 2014); however, unresponsiveness has also been reported (Matozzo and Marin, 2008). Only few ELISAs for bivalve yolk proteins have been developed (Kang et al., 2003; Park and Choi, 2004) and even though ALP induction has been used as a biomarker for estrogenic impact in bivalves for 15 years (Blaise et al., 1999; Gagne et al., 2001b; Matozzo et al., 2008; Long et al., 2014) a standardized ALP method with regard to target tissue, extraction or precipitation method etc. could surprisingly not be retrieved in the peer-reviewed literature. The development of biomarkers for endocrine disruption in bivalves is largely based on vertebrate endocrinology and development and validation of specific and sensitive methods is at present missing (Scott, 2013), which could explain the above-mentioned inconclusive ALP results.

Vtg induction is a recognized biomarker in fish partly due to the pronounced difference in unexposed male and female levels (reviewed by (Scott, 2013)). When estimated by ALP, several studies report similar basal levels in bivalve males and females (Gagne et al., 2001b; Quinn et al., 2004; Ricciardi et al., 2008) suggesting that bivalve ALP levels might not reflect yolk protein levels accurately. Recently, the applied methodology, argumentation and interpretation of data in the field of EDCs and their possible effects on invertebrates including bivalves have been critically discussed (Jubeaux et al., 2012; Scott, 2013). In this manuscript we draw attention to some issues that should be considered when using ALP levels as biomarker for estrogenic exposure in bivalves.

As our lab is experienced in development of ELISAs for fish Vtg (Holbech et al., 2001; Bjerregaard et al., 2006) we were encouraged to develop a species-specific ELISA for yolk protein in the common freshwater bivalve *U. tumidus* and compare the obtained results to the results obtained by the ALP method. The utility of yolk protein induction and ALP levels as reliable estrogenic biomarkers in bivalves was investigated by exposing *U. tumidus* to 17β-estradiol (E2).

2. Materials and methods

2.1. Animals and husbandry

U. tumidus with a shell length ranging from 48 to 75 mm (median 58 mm) were collected from Lake Thorsø (Denmark) in mid April 2011 and acclimated for approximately three weeks. In this lake *U. tumidus* coexist with another member of the *Unionidae* the painter's mussel (*Unio pictorum*). Those two *Unio* species can be distinguished from each other by looking at the hinge teeth but that requires the animals to be killed. A non-lethal method to differentiate between the species is to make a ratio between shell height and shell length (Aldridge, 1999). Based on a batch of *Unios* (n = 86) collected in Lake Thorsø in 2010 and 2011 we found the ratio for *U. tumidus* to be between 0.47 and 0.58 and for *U. pictorum* it was between 0.40 and 0.46 (Supplementary material). Only individuals with a ratio above 0.47 were selected for the experiment. At the end of the experiment the species identification of each individual was verified by examination of the teeth. It is important to separate the species as their reproductive biology differs e.g. their timing of gamete release differ.

Stainless steel tanks (48 L) were used for the exposure and the entire setup was placed outdoors at ambient temperature and light conditions. Tanks were aerated and supplied with thoroughly washed sand (4 cm deep layer) and ground water was used as water supply. Peristaltic pumps (Ole Dich) were used for the flow-through supply of water and stock solutions. The water flow was 96 L/day, oxygen saturation remained above 70% and temperature was within the range 10.1–16.3 °C (median 15.4 °C). Copper concentrations in the water remained below 5 ppb. The mussels were fed daily with dried *Spirulina* powder dissolved in water (12 mg/animal/day).

2.2. Exposure

17β-Estradiol (Sigma-Aldrich, Denmark, Cas no. 50-28-2) was dissolved in acetone and the final acetone concentration in the exposure tanks remained below 0.01‰. The exposure was started on May 4 2011 and continued until June 28–30. The exposure groups (in duplicate tanks) were: control, acetone control, 57, 164, and 512 ng E2/L (Table 1) and each tank contained 15 mussels (30 per exposure). Water samples from all exposure tanks were collected twice per week for determination of actual E2 concentrations.

2.3. Sampling and sample preparation

Hemolymph (0.5–1 mL) was collected from the anterior adductor muscle as described by Gustafson et al. (2005) placed on ice and stored in duplicate samples at −80 °C for later analysis by ELISA and ALP. Sampling took place over two consecutive days due to the high number of animals. The soft parts were removed from the shells, blot dried with paper towel, weighed and snap frozen in liquid nitrogen and stored at −80 °C or fixed with Bouin's fixative for 24 h followed by dehydration in 70% ethanol. Animals were sexed based on microscopic examination of the gonads. For the ELISA on tissue homogenates, a whole body homogenate (WBH) was prepared by thawing mussels on ice, adding the tissue weight of homogenization buffer (50 mM Tris–HCl, pH 7,4 and 1% protease inhibitor cocktail (P 8340; (Sigma-Aldrich, MO, USA))) and homogenizing the mixture on ice with an IKA T-10 Ultra Turrax homogenizer (S10N-10G dispersing element attached). The homogenate was centrifuged for 1 h at 50,000 ×*g*, and the supernatant was collected and stored at −80 °C until further analyses could be performed. Prior to ELISA both hemolymph and WBH supernatant samples were diluted in dilution buffer (PBS pH 7.3, 0.3% BSA, 0.1% Tween-20).

2.4. Preparation of yolk protein as antigen

Mature ovarian tissue containing visible eggs was dissected from female *U. tumidus* during the breeding season and eggs newly transferred to gill tissue were collected from females during the late breeding season. Both tissue and eggs were immediately frozen in liquid nitrogen and stored at −80 °C. The main yolk protein from the tissue and the eggs was purified by gel filtration chromatography and anion exchange chromatography according to the method described by Holbech et al. (2001), dialyzed, concentrated by freeze drying and verified by SDS-PAGE.

2.5. Gel filtration

Frozen ovarian tissue or eggs from gills were crushed with a pestle in a mortar filled with liquid nitrogen. The powder obtained was weighed to nearest mg and 3 times the weight of ice cold homogenization buffer (50 mM Tris–HCl, pH 7.4 and 1% protease inhibitor cocktail (P 8340; (Sigma-Aldrich, MO, USA))) was added. The solution was mixed and sonicated 2 × 15 s on ice and centrifuged for 2 h at 50,000 ×*g*. The supernatant was removed from the pellet with a pipette. Samples of 60 μL supernatant were collected for SDS-PAGE and stored at −80 °C. The remaining supernatant was applied to a gel filtration column (Sephacryl S-300 HR, XK 26/70) and calibrated with 50 mM TRIS

Table 1
Nominal and actual E2 concentrations calculated as mean values (±SEM). Values are based on all water samples from both replicates in each exposure group. The total number of water samples per exposure tank was 12 or 13.

Nominal concentration, ng/L	Actual concentration, ng/L	±SEM
100	57	4.0
320	164	8.9
1000	512	36.1