Comparative Biochemistry and Physiology, Part C 192 (2017) 16–22

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

A field study of hemolymph yolk protein levels in a bivalve (*Unio tumidus*) and future considerations for bivalve yolk protein as endocrine biomarker

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ARTICLE INFO

Article history:
Received 9 August 2016
Received in revised form 16 November 2016
Accepted 22 November 2016
Available online 24 November 2016

Keywords:
Biomarker
yolk protein
bivalve
mollusc
endocrine disrupting compounds
ELISA
background levels

ABSTRACT

Induction of yolk protein in male fish is a recognized biomarker for estrogenic exposure because the estrogen-dependent induction mechanism is well investigated and there is a clear difference in yolk protein levels of unexposed males and females. Attempts have been made to use induction of bivalve yolk protein as biomarker for estrogenic exposure. However, several biomarker validation criteria have not yet been investigated e.g. an in-depth understanding of the induction mechanism and background variability is needed and reliable detection assays are yet to be developed.

To obtain background knowledge about yolk protein levels freshwater bivalves (*Unio tumidus*) were collected in an uncontaminated Danish lake over the course of a year (33 collection dates). The hemolymph yolk protein concentration of 569 individuals was determined by a species specific enzyme-linked immunosorbent assay (ELISA) and male and female gonadal development cycles were established. The average yolk protein levels of males and females collected at each sampling date overlapped in some periods; the male and female range was 66,946 - 169,692 ng/mL and 88,731 - 681,667 ng/mL, respectively. Because male and female hemolymph yolk protein levels overlap, great care should be taken if yolk protein induction in bivalve hemolymph is considered as endocrine biomarker.

1. Introduction

Effects of endocrine disrupting chemicals (EDCs) have mainly been studied in vertebrate species such as fish. Vertebrate endocrinology is highly conserved across classes and is generally well understood. Little is known about the possible effects of EDCs on invertebrates such as molluscs but a well documented example is the masculinizing effects of tributyltin (TBT) on female marine snails (Gibbs and Bryan, 1986). However, the underlying mechanism has not yet been fully understood. To understand better the effects of EDCs on molluscs the development of valid biomarkers is requested but unfortunately, detailed knowledge about regulation of bivalve reproductive endocrinology is scarce (Porte et al., 2006; Hutchinson, 2007). Consequently, scientific data from mollusc studies have been evaluated by vertebrate-based knowledge despite significant differences in endocrinology and reproduction. For example the hypothalamic-pituitary-gonad (HPG) axis, which plays a key role in hormonal regulation of vertebrate reproduction, is absent in invertebrates (Kawada et al., 2013).

Vertebrate vitellogenin is a high density phospho-lipo-glycoprotein and it is the yolk protein precursor in oviparous vertebrates. It is synthesized in the liver as a response to circulating estrogens binding to the estrogen receptor (ER), and the protein is transported in the blood to the ovary. Induction of vitellogenin is a sensitive and validated biomarker for exposure to exogenous estrogens or xenoestrogens in fish and it is implemented in an OECD test guideline (OECD, 2011). If the biochemical structure of bivalve yolk protein resembles fish vitellogenin is yet unknown as bivalve yolk protein has not been sequenced yet. Induction of yolk protein in the hemolymph of bivalves has been used as a potential biomarker for estrogenic exposure in both laboratory experiments and field surveys (Blaise et al., 1999; Matozzo and Marin, 2008; Long et al., 2014; Leonard et al., 2016). However, recent research shows that yolk protein synthesis and the induction mechanism in fish and bivalves cannot be directly compared (Morthorst et al., 2014), and several critical preconditions for using yolk protein as an estrogenic biomarker in bivalves have not been investigated. The hemolymph of invertebrates is the equivalent to vertebrate blood but bivalve yolk protein seems to be directly synthesized in the ovary and consequently not transported in the hemolymph (Agnese et al., 2013), and estrogenic exposure of the freshwater bivalve *Unio tumidus* did not induce yolk protein levels in the hemolymph (Morthorst et al., 2014).

U. tumidus (*Unionoidae* family) is a common species in clean Danish lakes and streams and reach a size of 20 mm within their first year and become sexually mature during their second year (Aldridge, 1999). Their reproductive strategy is characterized by a period of female gravidity. The eggs are transported to the outer gill demibranchs in spring

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http://dx.doi.org/10.1016/j.cbpc.2016.11.003
1532-0456/

and here fertilization takes place when water containing male sperm is filtered by the female. The females nourish the fertilized eggs in the gills and the embryos develop into a larval stage called glochidia. The glochidia are released to the water and begin their parasitic juvenile stage by attaching to the gills or finns of fish. The juvenile mussels metamorphize while attached to the fish and finally detach from the host and settle in the sediment. *U. tumidus* is a dioecious species with a sex ratio of 1:1 and hermaphroditism is rare among adults however, hermaphroditism has been observed in young individuals (Pekkarinen, 1993; Aldridge, 1999).

Natural annual variations in background levels of yolk protein have never been investigated in bivalves. Here male and female yolk protein concentrations were determined in the hemolymph of *U. tumidus* in order to establish an annual overview of the natural male and female background concentrations of yolk protein and to clarify if the hemolymph of *U. tumidus* is a suitable compartment for detection of yolk protein levels.

2. Materials and methods

2.1. Collection of mussels and sampling

Between April 2013 and May 2014 a total number of 855 mussels (*U. tumidus*) were collected in Lake Thorsø (Denmark), which is considered clean and has a sandy sediment. The mussels were collected at a water depth of 0.5-1 m by dragging a rake gently through the sediment. The animals were transported to the laboratory in plastic bags and sampling was performed immediately or the following day.

Briefly, shell length and height was noted and the gills were investigated macroscopically for the presence of eggs or glochidia. Samples of hemolymph were collected with a syringe from the anterior adductor muscle and the sex of each individual was determined by microscopical investigtion of a gonad smear. Sex determination was possible in 180 females and 389 males and hence a total number of 569 individuals makes up the basis of the present data set (Fig. 4). Those 569 individuals had a shell length ranging from 36 to 81 mm (median 56 mm). The softparts were removed and frozen at -80°C for future ELISA on whole-body homogenate or preserved in Bouin's fixative (Sigma-Aldrich, Denmark) or paraformaldehyde in PBS (4%) for future standard hematoxylin-eosin histology or immunohistology. Detailed sampling procedures are described in Morthorst et al. (2014).

In Lake Thorsø *U. tumidus* coexist with *Unio pictorum* and both species belong to the *Unionidae* family. The shell morphology of both species is very similar but they can be distinguished from each other by calculating the shell height:length ratio; the ratios for *U. pictorum* and *U. tumidus* are 0.40-0.46 and 0.47-0.58, respectively (Aldridge, 1999; Morthorst et al., 2014). Only individuals with a ratio ≥0.47 were included in the present dataset.

2.2. ELISA

A recently developed direct non-competitive sandwich ELISA (enzyme-linked immunosorbent assay) against the main yolk protein of *U. tumidus* was used to determine yolk protein levels in hemolymph of individual mussels (Morthorst et al., 2014). Procedures for homogenisation, sample preparation, ELISA etc. were performed as described in Morthorst et al. (2014). Briefly, 150 μl/well of each sample (in triplicate) was added to 96-well microtiter plates. After washing with buffer the plates were incubated with Biotin coupled anti-yolk protein IgG (150 μl/well) and later Poly-HRP streptavidin (150 μl/well). Finally, the substrate (150 μl/well of TMB plus) was added and the reaction was stopped by adding 0.2 M H_2SO_4 (150 μl/well). The plates were read at 450 nm on a plate reader. Hemolymph samples were diluted 1000 times.

2.3. Histology

A total number of 115 randomly selected individuals were preserved for standard hematoxylin-eosin histology. Following preservation in Bouin's fixative for 24 hours the softparts were dehydrated in series of ethanol and embedded in paraffin, cut on a Microtome in sections of 5 μm and stained with eosin and hematoxcylin. Sections of gill and gonad tissue were investigted by light microscopy and sex and the stage of gonadal development were noted. Classification of the gonadal development stages is based on the four developmental stages described by Duinker et al. (2008) but stage 1 (early filling) and stage 2 (filling) are grouped into one stage (stage 1). Male stages (Fig. 2A-C): The gonad consists primarily of storage tissue and very few acini with immature sperm cells only (Stage 0), the gonad consists of storage tissue and acini containing few mature sperm cells - the gonad could be in a developing or resorption/ degradation stage (Stage 1) and the gonad consists of some storage tissue but primarily acini filled with mature sperm cells (Stage 2).

Female stages (Fig. 2D-F): The gonad consists primarily of storage tissue and very few acini containing single primary oocytes only (Stage 0), the gonad consists of storage tissue and acini with oocytes in different developmental stages - the gonad could be in a developing or resorption/ degradation stage (Stage 1) and the gonad consists of some storage tissue but primarily acini containing oocytes in different developmental stages (Stage 2).

2.4. Handling of data and statistics

Datasets were tested for normality and homogeneity of variance. The average male and female yolk protein concentrations for each sampling date were compared by a t-test and non-normally distributed data were compared by a Mann-Whitney test on ranks. Differences between average male or female levels throughout the year were analysed by a Kruskal Wallis comparison on ranks followed by an all pairwise multiple comparison (Dunn's method) because the data were not normally distributed. Statistical tests were performed in Sigma Plot 12.0 and significance was considered at $p \leq 0.05$.

3. Results

Male yolk protein levels are constant througout the year except in the winter period from November 22 to January 7 (5 subsequent samplings). In this period male levels are significantly lower compared to the majority of dates ($p \leq 0.05$). Also the female yolk protein levels are low during this period however, a rapid increase is observed after January 7 and the increase is significant from most other dates on February 12 and March 12 ($p \leq 0.05$). The female yolk protein concentrations remain at this level until the end of March where the concentration decrease to about half but the decrease is not significant. Also a small but not significant increase is observed in the period June 26 to the end of October.

The average female yolk protein level is significantly higher than the corresponding male average level on 27 of 33 sampling dates. The exceptions are: June 11 2013, July 3 2013, August 28 2013, December 16 2013, January 1 2014 and May 19 2014 (Fig. 1). The average female yolk protein levels are higher than the highest male average on 26 of 33 sampling dates.

From April to October male gonads are poorly developed and contain no or only few mature sperm cells (Stage 0 and 1). In October some males have gonads containing more acini and from November until March all male gonads contain many acini and the acini lumina are filled with mature sperm cells (stage 2) (Fig. 3A).

In July and August most female gonads are very poorly developed - mainly storage tissue and only few acini containing single primary oocytes (stage 0) but from September until April the majority of females have gonads containing acini with oocytes in different developmental