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Vitellogenin-like protein measurement in caged *Gammarus fossarum* males as a biomarker of endocrine disruptor exposure: Inconclusive experience

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ABSTRACT

A vitellogenin (Vg) mass spectrometry-based assay was recently developed to actively biomonitor and assess the exposure of the amphipod *Gammarus fossarum* to endocrine-disrupting chemicals in freshwater hydrosystems. This paper focuses on the appropriate use of this biomarker, which requires good knowledge of its basal level in males and its natural variability related to intrinsic biotic and environmental abiotic factors. To obtain the lowest biomarker variability, we first studied some of these confounding factors. We observed that the spermatogenesis stage did not have an impact on the Vg level, allowing flexibility in the choice of transplanted gammarids. In the second part of the study, males were transplanted in two clean stations for 21 days, with results indicating a spatial and temporal variability of Vg levels. These Vg changes could not be correlated to environmental factors (*e.g.*, temperature, pH and hardness of waters). Vg induction was then assessed in 21 stations having various levels of contamination. Inductions were observed for only two of the impacted stations studied. Under reference and contaminated conditions, a high interindividual variability of Vg levels was observed in caged organisms, severely limiting the sensitivity of the biomarker and its ability to detect a significant endocrine-disruptor effect. This may be explained by unidentified environmental factors that should later be determined to improved the use of Vg as a biomarker in male *G. fossarum*. Moreover, as discussed in this paper, recent advancements regarding the pleiotropic functions of the Vg gene in some species may complicate the application of this biomarker in males of invertebrate species.

1. Introduction

In oviparous species, vitellogenins (Vg) or other related proteins from the large lipid transfer protein superfamily (vitellogenin-like proteins) are precursors of the egg-yolk proteins that ensure normal embryonic development and consequently play a key role in reproduction (Hayward et al., 2010; Smolenaars et al., 2007). In female vertebrates, Vg production is under the control of the estrogen receptor pathway, and the quality and quantity of eggs are directly related to the amount of this protein in organisms (Brooks et al., 1997; Tyler and Sumpter, 1996). Conversely, the Vg gene is silent or poorly expressed in males, and Vg induction is a well-known effect of exposure to endocrine disruptors (ED; *e.g.*, estrogens and mimetic-estrogens) (Sumpter and Jobling, 1995). Consequently, measurement of Vg mRNA or protein level in male fish is currently proposed as a relevant tool for diagnosing ED effects. Indeed, clear relationships have been established between abnormal Vg inductions and testosterone level changes (Folmar et al., 1996), testicular abnormalities (Lye et al., 1997), and intersex incidence (Jobling et al., 2002). On the contrary, despite their obvious ecological importance, this issue has received far less attention in crustaceans currently used in aquatic ecotoxicology, such as branchiopods, copepods, isopods, amphipods, and mysids, and therefore few tools are available to diagnose ED exposure and/or ED effects in these organisms (deFur et al., 1999; deFur, 2004; Oetken et al., 2004). However, several field observations have suggested the occurrence of ED within wild crustacean populations. One of the most convincing and well documented of these observations is the occurrence of intersex individuals among a wide range of taxa (LeBlanc, 2007). Hormonal regulation of reproduction in crustaceans is mainly based on ecdysteroid and terpenoid hormones in females and on the androgenic gland hormone (AGH) in males (Charniaux-Cotton, 1954). The AGH positively controls the differentiation and maintenance of male sexual characteristics, such as masculinization of pleopods and cheliped morphology, development of male gonopore complex, and conversion of ovarian tissue

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to testicular tissue, in particular by inhibiting the synthesis and production of Vg (Ford, 2008; Ventura et al., 2011). Consequently and by analogy to the method applied in aquatic vertebrates, the measurement of Vg in crustaceans has been proposed as a biomarker of AGH pathway disruption (LeBlanc, 2007).

A review of the literature showed that the relevance and reliability of Vg measurement as a specific biomarker of ED exposure in crustaceans is unclear. In their review, Matozzo et al. (2008) indicated that Vg synthesis and induction in crustaceans can be used as a reliable biomarker of exposure to xenoestrogenic contaminants. However, several studies in marine crustaceans showed that vertebrate-estrogenic compounds have few effects on survival, development, and reproduction (Andersen et al., 2001; Lye et al., 2008; Tatarazako et al., 2002). Finally, Hannas et al. (2011) clearly demonstrated that the measurement of Vg mRNA levels in *Daphnia magna* can be used in biomonitoring the exposure of daphnids to some environmental chemicals but it is not useful as an indicator of exposure to estrogenic chemicals. These *a priori* contradictory observations can be partly explained by various drawbacks that limit a robust and reliable deployment of Vg measurements in invertebrates.

In crustaceans and in an ecotoxicological context, the use of specific methods for Vg assessment has been limited. To our knowledge, measurement of the expression of the gene encoding this protein was only applied to a few species, such as *D. magna* (Tokishita et al., 2006), the copepod *Tigriopus japonicus* (Lee et al., 2008), and the amphipod *Gammarus fossarum* (Xuereb et al., 2011). Vg production was more commonly assessed at the protein level, using indirect methods such as enzyme-linked immunosorbent assay (ELISA) (Ghekiere et al., 2006) and alkali-labile phosphate (ALP) measurement (Gagné et al., 2005). However, this latter method, largely used in ecotoxicology approaches, can lead to surprising observations, such as similar basal levels in control male and female organisms (Aarab et al., 2004; Quinn et al., 2004, 2006).

As underlined by Ford (2008), nearly all the studies related to Vg measurements in crustaceans following chemical exposure in the field and the laboratory have focused mainly on females. However, females display natural fluctuations of Vg levels during their reproductive-molt cycle (*e.g.*, Jubeaux et al., 2012; Okumura and Aida, 2000; Xuereb et al., 2011). Therefore, accurate use and reliable interpretation of data require detailed knowledge of the reproductive cycle of crustaceans to discriminate between natural changes and chemical-induced variations (*e.g.*, Jubeaux et al., 2012; Xuereb et al., 2011). However, such knowledge is rarely reported in the literature (*e.g.*, Gagné et al., 2005; Lee and Noone, 1995). Moreover, Vg is a protein naturally produced in females and its modulation cannot be directly related to a specific exposure to ED compounds, indeed it could be the result of indirect effects related, for example, to energy depletion, impacting the molt or reproductive cycle. To our knowledge, very few studies have developed and proposed Vg measurement as a specific ED biomarker in male crustaceans as done, for example, by Xuereb et al. (2011) in *G. fossarum* and Sanders et al. (2005) in *Palaemon elegans*.

The diversification and nonconservation of functions between orthologous proteins during animal evolution (*e.g.*, neofunctionalisation, subfunctionalisation) is another source of misuse or misinterpretation of Vg-annotated genes or proteins as ED biomarkers in invertebrates. Many other biological functions not related to reproduction have been discovered during the last decade for Vg-like proteins in several metazoan lineages (see Zhang et al., 2011). Such pleiotropy can even be accompanied by loss or gain of function as exemplified by the protein ortholog of Vg, which plays a role in clotting in decapods, while egg-yolk protein is produced by a Vg paralog gene belonging to the subfamily of apolipoproteins (Avarre et al., 2007), highlighting the relevance of functional controls in the development of biomarkers (Nikinmaa and Rytkönen, 2011; Xuereb et al., 2011).

Among crustaceans, gammarids are recognized as a relevant sentinel species in ecotoxicology (review by Kunz et al., 2010), because they are widespread, can be easily used under laboratory and field (caging) conditions, are known to be sensitive to contaminants, and methods are available to measure certain life traits (Coulaud et al., 2011; Felten et al., 2008; Geffard et al., 2010) and molecular biomarkers (Dedourge-Geffard et al., 2009; Jubeaux et al., 2012; Lacaze et al., 2010; Xuereb et al., 2007). Recently, a method based on liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) was developed allowing the absolute quantification of this protein in *G. fossarum* (Simon et al., 2010). At the same time, the function of this protein in relation to the reproduction processes of this species (oogenesis and embryonic development) and its potential use in males as a specific ED biomarker under laboratory conditions has been investigated (Jubeaux et al., 2012).

The present study aimed to assess the potential use of Vg measurement in male *G. fossarum* as a relevant, reliable, and sensitive ED biomarker in biomonitoring programs. In this context and as clearly shown by Coulaud et al. (2011) and Lacaze et al. (2011a), *in situ* bio-assays (caging of control gammarids) constitute a powerful experimental design and allow a reduction in variability compared to field monitoring of indigenous organisms. Because biomarkers could be influenced by some biotic (*e.g.*, age, life stages, sex, reproductive stage) and environmental abiotic factors (*e.g.*, temperature, hardness) (Handy et al., 2003), the impact of these potential confounding factors should be characterized to improve the use of Vg as a biomarker in male *G. fossarum* for *in situ* approaches. For this, our experimental design comprised three steps. (1) The impact of the reproductive status (spermatogenesis) on Vg levels in males was studied in order to determine its influence on basal Vg levels in males. (2) The impact of environmental abiotic factors (seasonal and spatial variability) on the Vg levels in males was assessed by caging organisms in reference stations characterized by high annual temperature changes and differences in water hardness levels. (3) The relevance of Vg measurement in caged *G. fossarum* males as a biomonitoring tool was examined, by performing a large-scale field survey (Rhône watershed) of 21 chemical stations, 5 as reference (uncontaminated) and 16 as contaminated by organic and inorganic compounds.

2. Materials and methods

2.1. Reagents

Acetonitrile, methanol, and water (LC–MS grade) were obtained from Fisher Scientific (Strasbourg, France). Dithiothreitol (DTT), iodoacetamide (IAM), formic acid >95% (FA) (LC–MS grade), nitric acid (LC–MS grade), trypsin (type IX-S from Porcine Pancreas), ammonium bicarbonate (AMBIC), TRIS, EDTA, Triton X, sodium chloride, leupeptin, and aprotinin were purchased from Sigma–Aldrich (St Quentin-Fallavier, France). Absolute ethanol and ether were obtained from Carlo Erba (Val de Reuil, France). Isotopically labeled peptide ILIPGV*(^{13}C5^{15}N)K was purchased from Millegen (Labège, France).

2.2. Gammarid collection and maintenance

Gammarids were collected upstream of the Bourbre River (La Tour du Pin, France; 287 mg L^{-1} of $CaCO_3$) and quickly transported to the laboratory (Irstea, Lyon). This station displayed good water quality according to RNB data records (Réseau National de Bassin, French Watershed Biomonitoring Network) and high