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Vitellogenin is not an appropriate biomarker of feminisation in a Crustacean

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ABSTRACT

The expression of the yolk protein vitellogenin (Vtg) has been used as a biomarker of feminisation in multiple fish species throughout the world. Since the late 1990s, researchers have attempted to develop similar biomarkers to address whether reproductive endocrine disruption also occurs in the males of invertebrate groups such as the Crustacea. To date, the vast majority of studies investigating *Vtg* induction in male Crustacea have resulted in negative or inconclusive results, leading researchers to question the utility of *Vtg* expression as a biomarker in this taxon. This study measured the expression of *Vtg* genes in two intersex phenotypes (termed internal and external) found in the male amphipod, *Echinogammarus marinus*, and compared them with those of normal males and females. Males presenting the external intersex phenotype are infected with known feminising parasites and display a variety of feminised traits including oviduct structures on their testes and external female brood plates (oostegites). The internal intersex male phenotype, that displays a pronounced oviduct structure on the testes without the external intersex characteristics, is not parasite infected and it is thought to be a result of environmental contamination. Given their morphology, these phenotypes might be considered highly 'feminised' or 'de-masculinised' and can be utilised to test the suitability of feminisation biomarkers. The *E. marinus* transcriptome was searched for genes resembling *Vtg* and two sequences were revealed, that we subsequently refer to as *Vtg1* and *Vtg2*. Results from a high-throughput transcriptomic sequencing screen of gonadal cDNA libraries suggested that very low expression (in this manuscript gene transcription is taken to represent gene expression, although it is acknowledged that in addition to transcription, translation, transcript processing, mRNA stability and protein stability can regulate gene expression) of *Vtg1* and *Vtg2* in normal males (ESTs = 1 and 0 for *Vtg1* and *Vtg2*, respectively), internal intersex males (ESTs = 0 for both *Vtg* sequences) and external intersex males (ESTs = 5 and 0 for *Vtg1* and *Vtg2*, respectively). In contrast, the sequencing suggested notable levels of expression of both *Vtg* genes in females (ESTs = 1133 and 84 for *Vtg1* and *Vtg2*, respectively). Subsequent qPCR analysis validates these expression levels, with the signal for *Vtg1* and *Vtg2* transcripts in all male phenotypes being indistinguishable from that caused by contamination of trace levels of genomic DNA or the low-level amplification non-target sequences. These findings suggest that *Vtg* expression is not notably induced in highly feminised amphipods and is therefore not an appropriate biomarker of feminisation/de-masculination in crustaceans. We discuss our findings in the context of previous attempts to measure Vtg in male crustaceans and suggest a requirement for more appropriate taxon-specific biomarkers to monitor feminisation in these groups.

1. Introduction

There is now strong evidence that oestrogenic contaminants (including natural, synthetic and oestrogen mimics) have caused feminisation and intersexuality in fish on an international scale (Jobling and Tyler, 2003; Jobling et al., 2006). The yolk protein vitellogenin (Vtg) has long been used as a biomarker of feminisation in fish exposed to oestrogenic compounds (Sumpter and Jobling, 1995) and is now used extensively as a reliable indicator of long-term reproductive disruption in a wide range of fish species (e.g. Kirby et al., 2004; Kidd et al., 2007; Scott et al., 2007; Bosker et al., 2010).

Although not as comprehensively studied as those of vertebrates, several *Vtg* genes have been identified within Crustacea (e.g. Mak et al., 2005; Tsang et al., 2003). The primary site of *Vtg* transcription appears to be the ovary, with several studies having highlighted extraovarian synthesis of Vtg in the hepatopancreas of some species (Fainzilber et al., 1992; Tsutsui et al., 2000; Mak et al.,

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2005; Raviv et al., 2006; Tsang et al., 2003). Scientists have also developed and utilised Vtg as a biomarker to determine whether feminisation and/or reproductive endocrine disruption is occurring in invertebrates (Matozzo et al., 2008; Simon et al., 2010; Jubeaux et al., 2012a,b). Such studies have included a diverse range of crustaceans, such as *Daphnia*, mysids, amphipods, crabs, crayfish, lobsters and various shrimps and prawns (Fainzilber et al., 1992; Lee and Noone, 1995; Sagi et al., 1999; Tsutsui et al., 2000; Allen et al., 2002; Tsang et al., 2003; Ghekiere et al., 2004; Volz and Chandler, 2004; Ghekiere et al., 2005; Mak et al., 2005; Sanders et al., 2005; Zapata-Perez et al., 2005; Ghekiere et al., 2006; Raviv et al., 2006; Simon et al., 2010; Hannas et al., 2011; Xuereb et al., 2011; García and Heras, 2012; Jubeaux et al., 2012b,c). Many of these studies measure Vtg or Vtg-like protein levels or the induction of *Vtg* genes in crustaceans following exposure to environmental contaminants in both the field and laboratory.

Although it has been demonstrated that chemical exposure has the ability to interfere with the normal process of vitellogenesis, the utility of Vtg as a biomarker for feminisation in crustaceans is controversial and has recently been brought into question (Ford, 2012). For example, Ford (2008) noted that the vast majority of studies have demonstrated changes in Vtg concentration in juveniles and/or females, and very few studies have shown expression of Vtg in males. Hannas et al. (2011) recently found that while Vtg is not induced by oestrogens, it was induced (~>10 fold) by exposure to a range of industrial chemicals (notably, piperonyl butoxide, chlordane and nonylphenol) in female *Daphnia*. In addition, Hannas et al. (2011) also found that Vtg was suppressed by ecdysteroids (crustacean moulting hormones) suggesting that Vtg induction by certain chemicals maybe due to their anti-ecdysteroid activity. Furthermore, the UK Department of Environment, Food and Rural Affairs (DEFRA) lead a study entitled 'Endocrine disruption in the marine environment (EDMAR)' and observed no induction of Vtg in male shrimp (*Crangon crangon*) or crabs (*Carcinus maenus*) from contaminated sites or exposed to oestrogens known to induce Vtg in fish (Allen et al., 2002). The report also points out that Vtg could not be detected in crabs infected with *Sacuculina carcini*, a parasite known to cause signs of feminisation in its host. These observations have cast doubt on the utility of Vtg as a biomarker of oestrogen exposure in these crustacean species (Matthiessen et al., 2002).

Due to their high population densities, widespread distribution and key ecological position (e.g. Thomas, 1993; Beare and Moore, 1997; Bocher et al., 2001; Kunz et al., 2010), amphipods are well suited to the ecotoxicological study of environmental contaminants. For these reasons, considerable efforts have been made to develop Vtg as a biomarker in this group (Simon et al., 2010; Xuereb et al., 2011). Studies have utilised mass spectrometry and q-RT-PCR assays to measure *Vtg* transcripts and proteins in the freshwater amphipod *Gammarus fossarum* following laboratory exposures and caged field studies downstream of urban wastewater treatment plants (Xuereb et al., 2011; Jubeaux et al., 2012b,c). However, the authors of the *G. fossarum* studies have also uncovered concerns regarding the utility of Vtg as a biomarker of endrocrine disruption in crustaceans. In a study that measured the Vtg levels in caged *G. fossarum* at 16 contaminated sites with a wide range of contamination profiles, significant inductions were only observed at two locations (Jubeaux et al., 2012c). Furthermore, even when significant Vtg induction was observed (in field studies or laboratory exposures), the induction factor was no greater than 15 (Xuereb et al., 2011; Jubeaux et al., 2012b,c), a considerably lower level than that observed in similarly exposed fish. Also, gammarids with significantly higher Vtg levels present notable levels of inter-individual variability (Xuereb et al., 2011; Jubeaux et al., 2012b,c). The authors suggest this variability may be due to unequal sensitivities to the compounds or inter-individual variation in the moult stages (and interactions with moulting hormones). It has also been suggested that unknown environmental factors may greatly influence Vtg levels in males, and the authors also commented that the observed inductions may be the consequence of Vtg fulfilling a poorly understood and non-reproductive functional role in immune response (e.g. Nakamura et al., 1999; Zhang et al., 2005; Seehuus et al., 2006; Shi et al., 2006; Rono et al., 2010; Zhang et al., 2011). Therefore, despite the significant induction of Vtg proteins and transcripts in some field studies and exposure experiments, the robustness and sensitivity of the Vtg as a marker of endocrine disruption and feminisation in amphipods is questionable.

The amphipod *Echinogammarus marinus* (Leach, 1815) lives in the intertidal zone and is widely distributed throughout northwest coasts of Europe. Surveys of *E. marinus* populations recorded an increased incidence of intersexuality (an abnormal sexual phenotype that results in both male and female secondary sex characteristics occurring on the same individual) at industrially contaminated sites (Ford et al., 2004a, 2006). Intersexuality and the associated reproductive costs can occur in amphipods of both sexes (Ford et al., 2003; Ford et al., 2004b; Yang et al., 2008). In addition, it is known that *E. marinus* males present two morphologically and transcriptomically distinct intersex phenotypes, termed external and internal intersexuality (Ford et al., 2005a; Yang et al., 2008; Short et al., 2012a). External intersex males possess rudimental brood plates (oostegites), a feature normally associated with females, and sometimes exhibit an ovotestis (consisting of a pronounced oviduct-like structure on the testes as described in Ford and Fernandes, 2005). Internal intersex males possess an ovotestis but never present brood plates (Yang et al., 2008, 2011). External intersexuality has been linked to infection by feminising microsporidian and paramyxean parasites (Ginsburger-Vogel, 1991; Ironside et al., 2003; Ford et al., 2006; Yang et al., 2011; Short et al., 2012b), while Short et al. (2012a) found that the internal intersex phenotype is not associated with infection by any known feminising parasite and may result directly from the influence of endocrine disrupting contaminants. Given the extent of feminisation or 'de-masculinisation' displayed by both the *E. marinus* male intersex phenotypes, Ford et al. (2004a) suggested they may serve as useful models for crustaceans with endocrine disruption and could be utilised to test the suitability of feminisation biomarkers.

Recently, the transcriptomes of *E. marinus* specimens presenting various sexual phenotypes have been sequenced using a high throughput sequencing platform (unpublished data) enabling both the search for *Vtg* transcript sequences and the comparison of expression patterns of *Vtg* (in this manuscript gene transcription is taken to represent gene expression, although it is acknowledged that in addition to transcription, translation, transcript processing, mRNA stability and protein stability can regulate gene expression) in males, females and the male intersex phenotypes. This study aimed to identify *Vtg* and *Vtg*-like sequences within the *E. marinus* transcriptome and quantify the expression of these genes in the gonads and hepatopancreas of males, females and the two male intersex phenotypes. In the light of these results and that of the published literature, the utility of Vtg as a biomarker of feminisation in crustaceans is discussed.

2. Materials and methods

2.1. Sampling

Echinogammarus marinus were collected from beneath seaweed and stones in the intertidal zone of Inverkeithing, Scotland (56° 1′ 38″ N, 3° 23′ 37″ W). Animals were anaesthetised for 20–30 s using a mixture of clove oil and seawater (0.2 μl/ml) and categorised using a stereo-microscope into the following phenotypes: normal male, external intersex males (possessing brood plates), internal intersex