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 we refer to as *Vtg1* and *Vtg2*. Results from a high-throughput transcriptomic sequencing screen of gonadal cDNA libraries suggested very low 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.

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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 (eg 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 (eg 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., 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; Jubeaux et al., 2012b). 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, 1994; 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; Jubeaux et al., 2012c). 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 (eg 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; Jubeaux et al., 2012c). 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; Jubeaux et al., 2012c), 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; Jubeaux et al., 2012c). 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 (eg Nakamura et al., 1999; Zhang et al., 2005; Seehus 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 et al., 2005b). Internal intersex males possess an ovotestis but never present brood plates (Yang et al., 2008; Yang et al., 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., 2010, 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 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 seconds 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 male (only possessing an ovotestis) and females (Ford et al., 2005a; Yang et al., 2008; 2011).

2.2. Animal dissection, parasite screening and RNA isolation

Tissues were expeditiously dissected from animals and snap frozen in liquid nitrogen and stored in RNAlater-ICE solution (Ambion, UK) according to the manufacturer instructions. A sample of muscle tissue was taken from each animal and genomic DNA was purified using the DNeasy Blood and Tissue Kit (Qiagen, UK) following the manufacturer’s protocol, and then quantified using spectrophotometry (NanoDrop ND-1000). The genomic DNA was then screened using PCR (Yang et al., 2011; Short et al., 2012b) for the presence of ribosomal DNA from the feminising intracellular paramyxean and microsporidian parasites known to infect *E. marinus*. Following determination of parasite infection, RNA was extracted from the stored tissue samples using Tri-Reagent, according to the manufacturer protocol (Ambion, UK). The RNA was cleaned using RNA clean and concentrator 5 columns (Zymo Research, Orange, California, USA) according to the optional manufacturer protocol that includes the addition of 10 U of DNase I to digest genomic DNA (DNase I (RNase free), New England Biolabs, UK). RNA purity was assessed using a spectrophotometer (NanoDrop ND-100) to assess suitable 260/280 and 260/230 ratios and RNA integrity was established using agarose gel electrophoresis.

2.3. Identification of Vtg *sequences*

Isolated RNA (see Section 2.2) from a range of tissues (muscle, head, hepatopancreas and gonad from parasitised, unparasitised, male, female, and juveniles) was pooled and 1.5 μg was reverse transcribed into double stranded cDNA (ds cDNA) using the MINT cDNA synthesis kit (Evrogen, Moscow, Russia). The ds cDNA library

was then normalised using the Trimmer normalisation kit (Evrogen, Moscow, Russia) and subsequently sequenced using the 454 GS-Flx titanium sequencing platform to generate expressed sequence tags (ESTs) that were assembled into contiguous (contig) sequences to create an *E. marinus* transcriptome database (unpublished data). This database was mapped (e-value cut-off 1e-5) against non-redundant sequences in GenBank using BLASTX. Any contig sequences annotated as 'vitellogenin' or 'vitellogenin-like' were extracted and aligned using Clustal Omega (Goujon et al., 2010; Sievers et al., 2011) against published crustacean *Vtg* sequences available in GenBank.

2.4. Determination of Vtg *expression using high-throughput sequencing*

Total RNA was isolated as previously described (see Section 2.2) from the gonads of animals presenting a range of phenotypes (normal uninfected males and females, external intersex males and internal intersex males). Messenger RNA (mRNA) from each phenotype group was purified using the Poly(A) Purist kit (Ambion, UK) from total RNA isolated from the gonadal tissues (for each phenotype, RNA was isolated from 27 animals except for the internal intersex male group, where 12 animals were used). The quantity and integrity of the isolated mRNA was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, California, USA) and used to generate barcoded SOLiD fragment libraries (at the Centre for Genomic Research, University of Liverpool) suitable for high-throughput sequencing (ABI-SOLiD system v4) following ABI-SOLiD protocols (SOLiD™ 4 System Library Preparation Guide, Life Technologies). The libraries were sequenced on one lane of a single flow-chip and the resulting 75 bp ESTs (20.5 million for normal uninfected males, 17.3 million for external intersex males, 14.1 million for internal intersex males and 22.1 million for normal uninfected females) were trimmed to remove adapter sequences and mapped to the previously assembled 'transcriptome atlas' (CLC Genomics Workbench v4.9) to give a digital gene expression profile for the contig sequences annotated as *Vtg* or *Vtg-like*. The ESTs attributed to each putative *Vtg* sequence were normalised for each phenotype library using the kilobase of exon model per million mapped reads (RPKM) method (Mortazavi et al., 2008).

2.5. Primer design and reference gene choice for qPCR

Forward and reverse primers predicted to produce a suitably sized product were designed against the two putative *Vtg* sequences (*Vtg1* and *Vtg2*) using primer3 software

(Rozen and Skaletsky, 2000). The resulting oligonucleotide primers for *Vtg1* (*Vtg1F* 5'-ACA CCA CCT CAC CAG TCT CC-3' and *Vtg1R* 5'-TCC TAT TCT GGG TCG AGT GG-3') and *Vtg2* (*Vtg2F* 5'-TGT GCA TCC ACG GAG TAG AG-3' and *Vtg2R* 5'-CTG GGG CAC GAC TGA TAG AT-3') were synthesised (MWG Eurofins, Germany) and used to perform qPCR validation of *Vtg* gene expression. To allow an accurate comparison of *Vtg1* expression relative to *Vtg2*, a standard curve was created to determine the amplification efficiencies of both *Vtg* primer sets. Primer set efficiencies differed by 3.8% and fell within the accepted range (90-110%). These efficiencies were accounted for when determining differences in *Vtg1* expression relative to *Vtg2*. The qPCR validation of *Vtg* gene expression was performed in conjunction with the reference gene T-complex protein 1 subunit theta (*TcompF* 5'-GTG ACG CCA CTA CCA GGA AT-3' and *TcompR* 5'-GCC CTT CGT GGT ACA CTC AT-3').

2.6. Construction of cDNA libraries and qPCR

The mRNAs contained within 500 ng of total RNA isolated from gonadal and hepatopancratic tissue of animals presenting a range of phenotypes was reverse transcribed using the GoScript Reverse Transcription System (Promega, Germany) following the manufacturer specifications and using $OligoDT_{15}$ primers and RNasin Ribonuclease Inhibitor. The resulting 20 µl solutions containing single stranded cDNA were stored at -80°C. The qPCR reations were carried out using a real-time PCR cycler (Eco Illumina) using GoTaq qPCR Master Mix (Promega, Germany) in a 15 µl volume containing 5.7 µl ultra-pure water, 1 µl of cDNA, 0.4 µl of the forward and reverse primer (from a 10 µM stock) and 7.5 µl of 2X GoTaq qPCR Master Mix. Each 15 µl PCR reaction was performed in triplicate with minus Reverse Transcriptase controls. All reactions were initially incubated at 95°C for 2 minutes before undergoing 45 cycles of 95°C for 5 seconds and 60°C for 30 seconds. Following the final cycle, the reactions underwent a 15 second 95°C denaturing step followed by a 15 second 55°C hybridisation step before PCR product melt curves where determined during a further temperature increase to 95°C. Melt curve analysis was performed to confirm the specificity of the PCR product in each reaction. Upon completion of the melt curve analysis, the amplification data were analyzed by plotting the fluorescence signal ΔRn against the cycle number. An arbitrary threshold was selected within the linear phase of the log ΔRn

against cycle number plot. The quantification cycle (or Cq value) was defined as the cycle number at which ΔRn crossed the threshold. The relative expression of *Vtg1* and *Vtg2* genes between normal males, females and the intersex male phenotypes was determined using the ΔΔCq method with normalization to the expression to the T-complex protein 1 subunit theta reference gene using the dedicated software package (Eco Software v3.0). To confirm the amplification of the of correct target sequence of *Vtg1*, *Vtg2* and the T-complex protein 1 subunit theta, representative PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, UK) and sequenced using the Sanger method (Source BioScience UK Ltd).

3.0 Results

3.1. Identification of two E. marinus Vtg sequences

The *Echinogammarus marinus* transcriptome database (unpublished) was searched for any contiguous sequences reasonably annotated (e-value below 1e-5) as 'vitellogenin' or 'vitellogenin-like'. This revealed the existence of two distinct putative *E. marinus* vitellogenin partial coding sequences (submitted to GenBank) that we have termed *Vtg1* and *Vtg2*. The 2115 bp *Vtg1* sequence contains a 1827 bp open reading frame predicted to encode 609 amino acids. A BLAST analysis (tblastx) against non-redundant sequences in GenBank reveals the sequence is most similar to multiple unannotated 'transcribed RNA sequences' (eg GAKD01006973.1 and GAJQ01003147.1) belonging to freshwater amphipods *Melita plumulosa* and *Hyalella azteca*. The annotated sequence with highest level of similarity (e-value 2e-20) to *Vtg1* is a partial coding sequence described as 'vitellogenin-like protein precursor' (GU985184.1) isolated from the freshwater amphipod *Gammarus fossarum*, a sequence that has been characterised for its potential application in studies of endocrine disruption (Simon et al., 2010; Xuereb et al., 2011; Jubeaux et al., 2012a; Jubeaux et al., 2012b). The *Vtg2* sequence consists of a 643 bp open reading frame predicted to encode a 214 amino acid sequence. Similar to *Vtg1*, a BLAST analysis reveals the *Vtg2* sequence is highly similar to multiple unannotated *Melita plumulosa* 'transcribed RNA sequences' (eg GAKD01007467.1). The sequence showing the highest degree of similarity (e-value 6e-26) with annotation was the complete coding sequence for the vitellogenin protein of the decapod *Cherax quadricarinatus* (AF306784.1) (Abdu et al., 2002)

3.2. Determination of gonadal Vtg *expression using high-throughput sequencing*

RNA isolated from the gonads of animals presenting a range of phenotypes and infection statuses (normal males uninfected by feminising parasites, n=27, females uninfected by feminising parasites, n=27, external intersex males infected by feminising parasites n=27 and internal intersex males uninfected by feminising parasites, n=12) was used to make cDNA libraries for sequencing using a high-throughput sequencing platform. The resulting ESTs were mapped to the already assembled 'transcriptome atlas'

(unpublished data) to give a digital gene expression profile for *Vtg1* and *Vtg2* (**Table 1**). The results suggest expression of both *Vtg* genes in female ovaries, with notably higher levels of *Vtg1*. In contrast, the results suggest low or negligible expression of both *Vtg1* and *Vtg2* in the testes of normal males as well as both external and internal intersex males.

3.3. Determination of Vtg *expression using qPCR*

To expand our understanding of *Vtg* expression and validate the levels suggested by the high-throughput sequencing, qPCR experiments were performed to determine the relative expression of *Vtg1* and *Vtg2*. The T-complex protein 1 subunit theta gene was chosen as a reference gene for these experiments on the basis of similar levels of expression in male and female gonads as suggested by the high-throughput transcriptomic screen (an average RPKM of 45.88 in male gonadal libraries and an RPKM of 48.11 in the female library). In addition, qPCR reactions performed using eight biologically independent cDNA libraries constructed using the same amount of male gonadal RNA presented similar Cq values (the cycle number at which the fluorescent signal (ΔRn) crossed an identical arbitrary threshold set within the linear phase of amplification), with a maximum 0.91 Cq value difference for the eight independent male libraries and a maximum 0.65, 0.23 and 0.27 difference between three independent external intersex, normal male and two internal intersex male libraries respectively. A similar maximum Cq value difference of 0.27 was observed between three independent female libraries (data not shown). A comparison of the average Cq value for all eight male libraries with the average for the three female libraries revealed a difference in Cq value of 2.3 between male and female gonadal libraries, with a similar maximum difference in Cq value being observed between the gonads and hepatopancreas (data not shown).

A comparison of *Vtg1* expression in ovaries and female hepatopancreas suggests expression in both organs, with appreciably more *Vtg1* transcripts in the ovaries suggesting it is the main site of *Vtg1* expression (**Figure 1A**). The signal representing *Vtg2* expression in the hepatopancreas cannot clearly be distinguished from the low-level amplification of non-target sequences (data not shown). A further comparison of *Vtg1* and *Vtg2* expression in ovaries reveals a greater quantity of *Vtg1* transcripts (**Figure 1A**)

and suggests a difference that is in broad agreement with the value revealed by the high-throughput sequencing (**Table 1**). To determine whether the two male intersex phenotypes present an elevated level of *Vtg2* expression, qPCR was performed using gonadal cDNA libraries made from RNA pooled from 27 animals for the normal male, female and external intersex male library and 12 animals for the internal intersex male library. The findings suggest that while *Vtg2* expression in females is high relative to normal males, no discernable difference is observed in the levels of *Vtg2* expression in both male intersex phenotypes relative to normal males (**Figure 1B**). Although the internal intersex phenotype presents an apparent 1.5 fold increase in comparison to normal males, the expression range within the error bars reveals this value does not represent meaningful upregulation of *Vtg2*.

Further qPCR experiments were performed to determine whether the two male intersex phenotypes present an elevated level of *Vtg1* expression. Three biologically independent gonadal cDNA libraries were made for the normal male, female and external intersex male phenotype (nine animals contributing to each library). Two biologically independent cDNA libraries were constructed to represent the internal intersex male phenotype (six animals contributing to each library). As for *Vtg2*, high *Vtg1* expression is seen in females, however, none of the three external intersex or two internal intersex male samples present a higher level of *Vtg1* expression than is seen in normal males (**Figure 1C**). A qPCR experiment using cDNA libraries constructed using RNA isolated from the hepatopancreas of normal and intersex animals (27 animals contributing to each library) also revealed no increase in *Vtg1* expression in males presenting an intersex phenotype (**Figure 1C**).

It is important to note that the contribution of *Vtg1* and *Vtg2* transcripts to the fluorescent signal observed in any of the male phenotypes is questionable. Although analysis of the PCR product obtained from the female libraries revealed unambiguously that the intended target sequence had been amplified, the male libraries revealed a mixed sequencing trace (data not shown), suggesting the amplification of non-target sequences. This is not completely unexpected given the low, and perhaps effectively non-existent, expression (evidenced by a high Cq value) of *Vtg1* and *Vtg2* in all male phenotypes. Given this finding, it can be said that both the *Vtg1* and *Vtg2* qPCR assays reveal that the

observed fluorescent signal in the male intersex libraries is indistinguishable from that observed in normal males, whether that signal results from the amplification of weakly expressed *Vtg*, low-level amplification of non-target sequences, or some combination of both.

4.0 Discussion

4.1. E. marinus Vtg *genes*

A search of the *Echinogammarus marinus* transcriptome database revealed two distinct *Vtg* sequences (termed *Vtg1* and *Vtg2*) that are similar to sequences previously isolated from other crustacean species and annotated as *Vtg*. The *E. marinus Vtg1* sequence is clearly orthologous to the *Vtg*-like sequence of the amphipod *G. fossarum* (Xuereb et al., 2011) and several unannotated 'transcribed RNA sequences' belonging to the amphipods *Melita plumulosa* and *Hyalella azteca* (see Section 3.1). Interestingly, the closest matches after the amphipod sequences are for predicted *Vtg*-like and *Vtg* sequences from the honeybees *Apis Florea* (XM_003689645.1) and *Apis mellifera* (NM_001011578.1) respectively, followed by a *Vtg* sequence isolated from the branchiopod *Daphnia magna* (AB114859.1). A search for matches outside the Amphipoda but still within the Malacostraca do not reveal a clear *Vtg1* orthologue. The closest match is the result of a relatively weak similarity (24.2%) between a 327 amino acid region of the predicted *E. marinus* sequence and a sequence isolated from the signal crayfish *Pacifastacus leniusculus* (AF102268.1) annotated as a clotting protein precursor; a protein that is expressed in both sexes (Hall et al., 1999) and plays a role in the crustacean immune system (reviewed in Vazquez et al., 2009). Given the considerable number of decapod *Vtg* sequences available, the lack of a clear decapod orthologue in GenBank may suggest that the *E. marinus Vtg1* sequence and its amphipod orthologues represent a type of *Vtg* that is only present in some crustacean clades, a possibility that requires further investigation. In contrast to *Vtg1*, the *E. marinus Vtg2* sequence has clear amphipod and decapod orthologues, with strong similarities being observed between *Vtg2* and apolipoprotein sequences isolated from a wide range of arthropod species.

4.2. Vtg *expression in normal male and female tissues*

The high-throughput sequencing screen and qPCR experiments both suggest that *Vtg1* is more abundant than *Vtg2* in the ovary (using a cDNA library constructed with the pooled RNA from 27 animals). Furthermore, although lower than observed for the ovary, qPCR analysis reveals *Vtg1* expression is clearly evident in the hepatopancreas, while the expression signal corresponding to *Vtg2* cannot clearly be distinguished from the low-

level amplification of non-target sequences. These findings are consistent with studies that show the ovary and hepatopancreas are major sites of Vtg synthesis in crustaceans (eg Fainzilber et al., 1992; Chen et al., 1999; Tsutsui et al., 2000; Tseng et al., 2001; Abdu et al., 2002; Tseng et al., 2002; Tsang et al., 2003; Serrano-Pinto et al., 2004; Tsutsui et al., 2004; Mak et al., 2005; Kang et al., 2008; Raviv et al., 2006; Phiriyangkul and Utarabhand, 2006; Tiu et al., 2006; Xie et al., 2009; Ara and Damrongphol, 2012) and suggest that the ovary is the primary site of Vtg synthesis in amphipods, as has been established for other crustaceans (Fainzilber et al., 1992; Raviv et al., 2006). In many crustacean species, some *Vtg* genes are expressed predominantly or even exclusively in the hepatopancreas (eg Chen et al., 1999; Abdu et al., 2002; Tsang et al., 2003; Tsutsui et al., 2004; Mak et al., 2005; Ara and Damrongphol, 2012), however, the qPCR carried out for this study suggests that neither of the two *E. marinus Vtg* sequences present a bias towards hepatopancreatic expression. However, whether the expression patterns of the two *Vtg* genes change throughout the reproductive and moult cycles would require further investigation.

Comparison of expression levels suggest that the *Vtg* genes are ~140-500 fold more highly expressed in *E. marinus* females than males. This figure is in broad agreement with previous comparisons conducted on *G. fossarum* samples using both mass spectrometry and qPCR (Xuereb et al., 2011; Jubeaux et al., 2012b). However, sequencing of the PCR product following amplification of *Vtg* transcripts in male cDNA libraries revealed a mixed sequencing trace, meaning that the fluorescent signal recorded for males included contributions from non-target sequences (very likely amplified as a result of the low number of specific target sequences in those libraries). This indicates that the extent of differential expression observed between males and females suggested by this study can only represent a minimum estimate. The levels of *Vtg1* expression between the three biologically independent samples (using cDNA constructed with RNA pooled from nine animals for each sample) suggest that transcript levels can fluctuate considerably in *E. marinus* females. The mRNA levels of *Vtg* were also observed to vary significantly in female *G. fossarum* during the moult cycle, with higher levels recorded during inter-moult and first pre-moult stages (Xuereb et al., 2011). As no consideration was given to the moult stages of animals used in this study, it is possible that the female

sample displaying a notably higher level of *Vtg1* transcripts contained more inter-moult and first pre-moult stage animals. These findings suggest that in order to reliably distinguish between naturally and chemically induced changes of gene expression in females, a fuller understanding of the moult and reproductive cycles in *E. marinus* is needed.

4.3. Expression of the Vtg gene in feminised males

This study has used both high-throughput sequencing and qPCR assays to determine that the level of *Vtg* expression in *E. marinus* males presenting distinct intersex phenotypes is very low and indistinguishable from that observed in normal males. The extent of feminisation or 'de-masculinisation' presented by both the male intersex phenotypes has made *E. marinus* a potential model for the study of sexual dysfunction in crustaceans, from the molecular to the population level (eg Ford et al., 2008; Short et al., 2012a; Ford et al., 2007). Furthermore, the very low level of *Vtg* expression in normal male amphipods (Xuerub et al., 2011), combined with findings showing that this expression remains effectively unchanged throughout all stages of spermatogenesis (Jubeaux et al., 2012c), makes an amphipod species presenting male intersex phenotypes ideal for testing the suitability of *Vtg* genes as biomarkers of feminisation. Therefore, we suggest that the failure to observe any discernable increase in *Vtg* levels in males presenting intersex phenotypes reflects poorly on the possibility of reliably detecting *Vtg* expression in a phenotypically normal male crustacean that has undergone subtle feminisation. The reasons why such feminised, or 'de-masculinised', male amphipods do not present an increased level of *Vtg* are not known. However, Ford (2008) has suggested that it might be quite difficult to feminise a male crustacean without some prior 'de-masculinisation' (due to the over-riding influence of the androgenic gland) and concluded 'in determining whether endocrine disrupting chemicals maybe impacting the sexual chemistry of male crustaceans it might be more fruitful, especially when attempting to design early-warning biomarkers of exposure (and effect), to address the question of de-masculinisation, rather than feminisation'.

4.4. Implications for the use of Vtg as a biomarker

The expression of *Vtg* in crustaceans has been shown to change following exposure to hydrocarbons, fungicides and heavy metals (Soetaert et al 2006; Soetaert et al

2007; Vandenbrouck et al., 2009; Vandegehuchte et al., 2010a Vandegehuchte et al., 2010b; Vandenbrouck et al., 2010). Therefore, if an adequate account is taken of natural fluctuations associated with the normal female reproductive cycle, *Vtg* expression may have applications as a marker of general stress or reproductive disruption in females. However, given the importance of *Vtg* in monitoring the feminisation of male vertebrates (e.g. Kidd et al., 2007; Bosker et al., 2010), the aberrant expression of the *Vtg* gene in male crustaceans is of great interest. The exposure of crustaceans to oestrogen and other vertebrate oestrogen receptor agonists results in a diverse range of effects and phenotypes (e.g. Billinghurst et al., 1998; Brown et al., 1999; Olmstead and LeBlanc, 2000; Atienzar et al 2002; Watts et al., 2002; Vandenbergh et al 2003; Lye et al 2008). Further investigations into the influence that such exposure has on the expression of crustacean Vtg (and Vtg-like) proteins reveals that the induction of Vtg is not universal (Billinghurst et al., 2000; Lye et al 2005; Clubbs and Brooks, 2007; Lye et al 2008). Furthermore, A DEFRA lead survey of marine organisms observed no induction of Vtg in crabs (*Carcinus maenus*) or the male shrimp (*Crangon crangon*) from contaminated sites or when exposed to oestrogens known to induce Vtg in fish (Allen et al., 2002). The same study also revealed that Vtg could not be detected in crabs infected with *Sacuculina carcini*, a parasite known to cause signs of feminisation in its host. In summarising the survey, Matthiessen et al. (2002) concluded 'it is therefore clear that vitellin is not a suitable biomarker of oestrogen exposure in these species'. Efforts to develop Vtg as a biomarker in the amphipod *G. fossarum* have also been undertaken (Simon et al., 2010; Xuereb et al., 2011) but the suitability of Vtg was questioned following its subsequent application. Studies measuring the induction of Vtg proteins in caged animals located downstream of urban wastewater treatment plants only found significant inductions at two out of 16 locations (Jubeaux et al., 2012c). Furthermore, even when significant Vtg inductions were seen (in field studies or laboratory exposures), a Vtg induction factor of no more than 15 is observed, with the animals that do show a significantly greater Vtg level presenting high levels of inter-individual variability (Xuereb et al., 2011; Jubeaux et al., 2012b; Jubeaux et al., 2012c). The authors suggest that the inter-individual variability may be due to unequal sensitivities to the compounds, inter-individual variation in the moult stages or a consequence of Vtg fulfilling a poorly understood and non-reproductive

functional role in the immune system. Indeed, the authors reporting on the recently published field study noted 'that it is difficult to propose Vg measurement in *G. fossarum* as a specific endocrine disruption biomarker' (Jubeaux et al., 2012c).

The varied and inconclusive results obtained following examination of *Vtg* expression in male crustaceans is likely to be indicative of the complicated, and relatively poorly understood, relationship between the steroid receptors of various crustacean groups and compounds known to influence the vertebrate endocrine system. Although the relationship between oestrogenic chemicals and crustacean feminisation is still uncertain, other (non-oestrogenic) chemicals in the environment may cause unambiguous crustacean feminisation. Indeed, chemical contaminants (or mixtures of contaminants) have been implicated as a cause of the intersex phenotypes observed in multiple crustacean groups (Moore and Stevenson, 1994; Jungmann et al., 2004; Ayaki et al., 2005; Short et al 2012a; Stentiford, 2012). However, the finding that obviously female sexual characteristics can appear on males in the absence of any *Vtg* expression suggests that the aberrant expression of *Vtg* in males is not suitable as an 'early warning' biomarker, even if there are chemicals that unambiguously feminise crustaceans.

5.0 Conclusion

The aim of this study was to assess the suitability of Vtg as a biomarker of feminisation in crustaceans by identifying *Vtg* and *Vtg*-like sequences within the *E. marinus* transcriptome and quantifying their expression in males, females and two male intersex phenotypes. This study found no discernable increase in the levels of either of the *E. marinus Vtg* genes in the intersex males. Therefore, on the basis of our findings and those in the literature, we propose that Vtg is not an appropriate biomarker for feminisation in a crustacean and suggest that to fully assess whether endocrine disruption is a problem in these ecologically and economically important taxa, crustacean-specific biomarkers of feminisation and de-masculinisation need to be developed.

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Fig. 1. Levels of *Echinogammarus marinus Vtg1* and *Vtg2* expression in the gonads and hepatopancreas of animals presenting a range of sexual phenotypes (error bars on all graphs represent standard deviations of qPCR triplicates). (**A**) A comparison of *Vtg1* expression in ovaries (Ov) and the female hepatopancreas (Hp) suggest a greater level of *Vtg1* expression in the ovary (left column, *Vtg1* Ov v Hp). Comparison of *Vtg1* and *Vtg2* expression (right column, *Vtg1* v *Vtg2* Ovary) in ovaries reveals a greater quantity of *Vtg1* transcripts (note: the fold change values take account of differences in *Vtg1* and *Vtg2* primer efficiencies). (**B**) Comparison of *Vtg2* expression in the ovaries of females and the testes of normal males (left column, *Vtg2* FvM) reveals substantial *Vtg2* expression in females relative to males. Comparison of expression in the testes of external intersex males and the testes of internal intersex males relative to the testes of normal males (*Vtg2* EIMvM and *Vtg2* IIMvM, middle and right columns respectively) reveals no discernable induction of *Vtg2* expression in the testes of males presenting either intersex phenotype. (**C**) Analysis of *Vtg1* expression in three biologically independent external intersex male libraries and two independent internal intersex male libraries relative to normal male libraries (columns labelled $EIM_{1\text{-}3}vM_{1\text{-}3}$ and $IIM_{1\text{-}2}vM_{1\text{-}2}$ respectively) reveals no discernable induction of *Vtg1* in the testes of males presenting either intersex phenotype relative to normal males. Comparisons of *Vtg1* expression in the ovaries of females and the testes of normal males (columns labelled $F_{1\text{-}3}vM_{1\text{-}3}$) reveal substantial *Vtg1* expression in females relative to males. Comparisons of *Vtg1* expression in the hepatopancreas of females and the hepatopancreas of normal males (column labelled FvM Hp) reveal a greater level *Vtg1* expression in females. Furthermore, no induction of *Vtg1* was observed in the hepatopancreas of males presenting either intersex phenotype relative to normal males (columns labelled EIMvM Hp and IIMvM Hp).

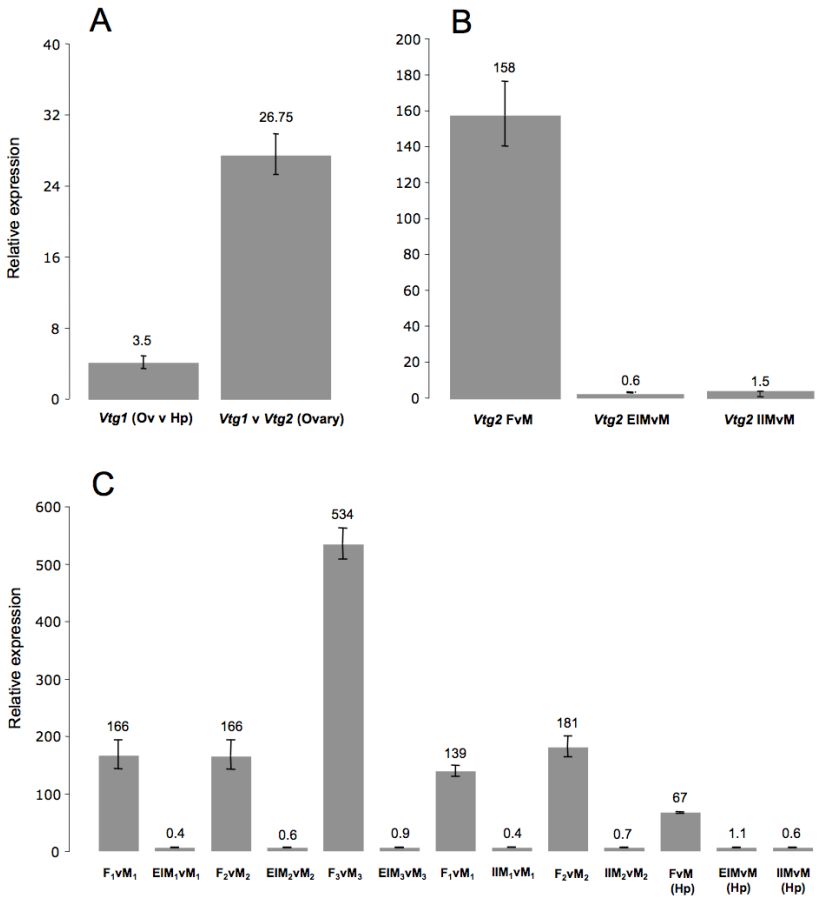
A
Relative expression
40
32
24
16
8
0
3.5
26.75
Vtg1 (Ov v Hp)
Vtg1 v Vtg2 (Ovary)
B
200
180
160
140
120
100
80
60
40
20
0
158
0.6
1.5
Vtg2 FvM
Vtg2 EIMvM
Vtg2 IIMvM
C
Relative expression
600
500
400
300
200
100
0
166
0.4
166
0.6
534
0.9
139
0.4
181
0.7
67
1.1
0.6
F1vM1
EIM1vM1
F2vM2
EIM2vM2
F3vM3
EIM3vM3
F1vM1
IIM1vM1
F2vM2
IIM2vM2
FvM (Hp)
EIMvM (Hp)
IIMvM (Hp)

Table 1. *E.marinus* gonadal expression profile for *Vtg1* and *Vtg2* genes following high-throughput transcriptomic sequencing of cDNA libraries representing a range of sexual phenotypes. Male = Normal Males (uninfected with feminising parasites); Fe = Females; EIM = External Intersex Males (infected); IIM = Internal Intersex Males (uninfected).

Vtg transcript	Male ESTs (RPKM)	Fe ESTs (RPKM)	EIM ESTs (RPKM)	IIM ESTs (RPKM)
E.marinus Vtg1	1 (0.04)	1133 (51.76)	5 (0.39)	0 (0.0)
E.marinus Vtg2	0 (0.0)	84 (12.62)	0 (0.0)	0 (0.0)