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Crustacean Intersexuality Is Feminization without Demasculinization: Implications for Environmental Toxicology

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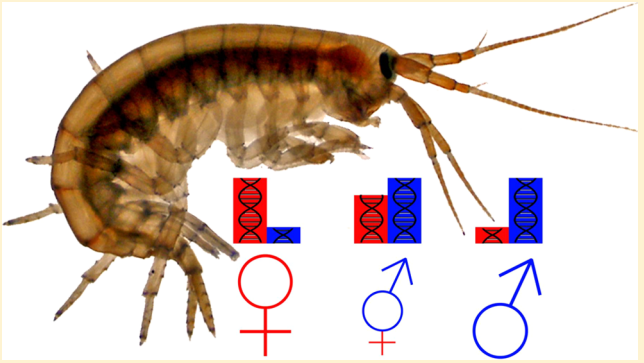
Supporting Information

ABSTRACT: The dysfunction associated with intersexuality in vertebrates and molluscs is often a serious threat to ecosystems. Although poorly understood, crustacean intersexuality is associated with contamination and includes forms linked to increased sex-ratio distorting parasites at polluted sites. Despite the importance of crustaceans for monitoring vulnerable aquatic habitats, little is known about the molecular basis of this abnormal sexual differentiation and any associated sexual dysfunction. To increase the relevance of crustaceans to environmental toxicologists, we comprehensively analyzed gene expression in amphipods presenting parasite- and nonparasite-associated intersexuality. Our findings reveal existing vertebrate biomarkers of feminization should not be applied to crustaceans, as orthologous genes are not induced in feminized amphipods. Furthermore, in contrast to vertebrates, where feminization and intersexuality is often associated with deleterious demasculinization, we find males maintain masculinity even when unambiguously feminized. This reveals a considerable regulatory separation of the gene pathways responsible for male and female characteristics and demonstrates that evidence of feminization (even if detected with appropriate biomarkers) is not a proxy for demasculinization in crustaceans. This study has also produced a comprehensive spectrum of potential molecular biomarkers that, when combined with our new molecular understanding, will greatly facilitate the use of crustaceans to monitor aquatic habitats.



INTRODUCTION

The study of vertebrate and mollusc intersexuality and its associated sexual dysfunction has increased our knowledge of reproductive biology and revealed threats to ecosystem health.[1−3] Intersexuality has also been found in many crustacean groups[4−11] and evidence indicates crustacean reproduction is vulnerable to anthropogenic pollutants, environmental change, and parasitic manipulation.[12−17] Investigations of vertebrate intersexuality are greatly facilitated by a molecular understanding of sexual differentiation and reproductive dysfunction.[18−22] However, despite its ecological and economic relevance, we are comparatively ignorant of the molecular biology underlying crustacean reproductive processes and only have a partial understanding of the nature and implications of crustacean intersexuality.[23] This means we lack appropriate reproduction-linked biomarkers, a situation that seriously impedes the use of crustaceans by ecotoxicologists wanting to monitor intertidal, estuarine and freshwater environments.[24]

Crustaceans present parasite-induced and nonparasite induced forms of intersexuality, and both forms have been associated with environmental contamination.[7−9,11,24−28] Parasite-induced intersexuality in crustaceans is linked with feminizing parasites that transmit vertically by infecting the eggs of their host.[16] Such parasites can often enhance their transmission by converting males into females.[16] A feat reportedly accomplished by preventing differentiation of the male androgenic gland (AG),[29,30] a specialized organ controlling male sexual differentiation.[31,32] Intersexuality occurs in cases of incomplete conversion, possibly due to a low parasite burden or suboptimal environmental conditions.[30,33−35] Although some parasite-induced intersexuality will exist naturally, a proportion may be indirectly caused by anthropogenic contamination, as contaminant-exposed crustaceans present significant increases in infection by microsporidia,[36,37] a phylum of parasites containing species capable of crustacean sexual conversion.[38,39] Furthermore, population

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analyses have revealed significant increases in infection by feminizing microsporidian species at contaminated sites.[11]

The consequences of both parasite and nonparasite associated crustacean intersexuality are the subject of debate.[23] The reproductive deficiencies associated with female intersexuality are statistically significant but relatively subtle[35,40,41] and might represent the acceptable cost of adaptive environmental sex determination mechanisms.[13,17,42] Furthermore, female intersexuality is not always associated with significant reductions in fecundity, suggesting it may not be maladaptive in some species.[43−45] The implications of parasite and nonparasite induced male intersexuality are less well understood.[23,28] However, its high frequency,[7] association with lower sperm counts,[28] pronounced morphological changes,[10,46,47] altered hormone levels[10] and behavioral changes[48] have led to the suggestion that intersexuality in males is of serious concern.[28] High levels of intersexuality may indicate a population in which a large proportion of all males are to some extent compromised, albeit less conspicuously. The scenario in crustaceans may be analogous to vertebrates, where male intersexuality can represent a normal condition,[49] as well as an abnormality indicative of sexual dysfunction with varying reproductive consequences.[19−21]

Molecular markers capable of monitoring feminization and demasculinization in vertebrates are continually being developed and applied.[18,19,50] Attempts to monitor sexual dysfunction in male crustaceans using genes orthologous to those mis-regulated in vertebrates has produced inconsistent results[51−54] and established vertebrate markers may not be suitable for monitoring crustacean populations.[52,55] Although the current lack of relevant genomic resources is hindering investigations,[24,27,56] the application of high-throughput sequencing technology to a suitable species could markedly facilitate the use of crustaceans to monitor aquatic habitats and rapidly create a model for the study of crustacean sexual dysfunction. *Echinogammarus marinus* (Leach, 1815) is one such species, it is a widespread intertidal amphipod and a population found at an environmentally impacted site presents significant levels of female bias and intersexuality typical of many impacted amphipod populations.[11,28,46] Furthermore, females and intersex animals in this population have a significantly higher prevalence of infection with two species of vertically transmitted microscopic feminizing parasite.[11,57] These have been identified as the microsporidian *Dictyocoela duebenum*, a species classified as an amphipod feminizer,[11,12,39,57] and a parasite belonging to the phylum Paramyxea,[57] a group also linked to female bias and intersexuality.[58] The majority of parasite-infected males from this population are classed "external intersex males" (EIM) on the basis that they possess rudimental brood plates externally, in addition to sometimes exhibiting an ovotestis.[10,27] This population also harbors uninfected males possessing an ovotestis but no brood plates.[28,59] These "internal intersex males" (IIM) are not associated with parasites and may be a result of anthropogenic contamination.[27] This *E. marinus* population represents a potential resource for revealing the molecular basis of parasite and nonparasite associated intersexuality and crustacean sexual differentiation.

Transcriptomic profiling of intersexuality in *E. marinus* will give insights into the processes of feminization and demasculinization in an unambiguously feminized crustacean. This is particularly relevant as the sexual dysfunction of vertebrate males exposed to feminizing agents largely results from a process of demasculinization that occurs concurrently with molecular feminization.[3,18,20−22,60] Furthermore, the expression patterns of sex-biased genes in parasite-infected specimens presenting incomplete sexual conversion will also shed light into the conversion mechanism (and its failure) employed by feminizing parasites. This study uses a transcriptomic screening approach to explore the molecular basis of crustacean intersexuality. Gene expression levels in normal and intersex phenotypes are compared by sequencing gonadal libraries and mapping these profiles to a newly created *E. marinus* transcriptome atlas. The gene expression profiles are validated and analyzed to reveal the feminization and demasculinization underling parasite and nonparasite associated crustacean intersexuality. This information will give ecotoxicologists a deeper understanding of abnormal sexual development at contaminated sites and uncover a wealth of molecular biomarkers for monitoring crustacean reproductive health in threatened habitats.

MATERIALS AND METHODS

Sampling. *E. marinus* were collected from beneath seaweed in the intertidal zone of Inverkeithing, Scotland (Latitude 56.027313, Longitude −3.393745) and Portsmouth, southern England (Latitude 50.791233, Longitude −1.042242). Animals were categorized as normal males (NM), normal females, external intersex males (EIM) [possessing brood plates], internal intersex males (IIM) [only possessing an ovotestis]) and screened for microsporidian and paramyxean parasites.[57,59]

Production of an *E. marinus* Transcriptome Atlas. RNA was extracted (Tri-Reagent. Ambion) from muscle, head, hepatopancreas, and gonadal tissues dissected from parasitised, unparasitised, male, female, and juveniles (n = 14 adults and 10 juveniles at various stages of development) and 1.5 μg was used to make double stranded (ds) cDNA (MINT cDNA synthesis kit, Evrogen). The ds cDNA was normalized (Trimmer normalization kit, Evrogen) and sequenced using 1.5 plates of the 454 GS FLX Titanium platform (Centre for Genomic Research, University of Liverpool). The expressed sequence tags (ESTs) were assembled using Newbler (v2.6) and Mira (3.4.1.1) software and combined with the CAP3 assembly program[61,62] to create a set of contiguous sequences termed the "transcriptome atlas". The atlas was annotated by comparison to nonredundant sequences in UniProt and FlyBase (BLASTX, E-value cut off $\leq 1 \times 10^{-05}$).

Roche 454 GS FLX Sequencing of *E. marinus* Gonadal cDNA Libraries. Total RNA was isolated (Tri-Reagent. Ambion) from animals characterized as NM (n = 9) and females (n = 9)[59] and 4 μg was used to make ds cDNA (MessageAmp aRNA Amplification Kit, Ambion) for sequencing using the Roche 454 GS FLX platform (Centre for Genomic Research, University of Liverpool). The resulting ESTs were mapped to the "transcriptome atlas" (CLC Genomics Workbench v4.9) to give digital gene expression profiles for the two phenotypes. Counting correction was applied to read counts uniquely mapping to each "atlas" contig using the kilobase of exon model per million mapped read (RPKM) method.[63]

ABI SOLiD 4 Sequencing of *E. marinus* Gonadal cDNA Libraries. Total RNA was extracted (Tri-Reagent. Ambion) from animals presenting a range of parasite infection statuses[57,59] and sexual phenotypes (uninfected normal males [NM] n = 24, external intersex males [EIM] n = 24, internal intersex males [IIM] n = 12 and normal females n = 24). The

mRNA was isolated from the total RNA (Poly(A) Purist kit, Ambion) and converted into a library for sequencing by the ABI SOLiD 4 System (SOLiD 4 System Library Preparation Guide, Life Technologies). The libraries were sequenced on one lane of a flow-chip and the 75 bp ESTs were mapped to the "transcriptome atlas" (CLC Genomics Workbench v4.9) to give digital gene expression profiles. Counting correction was applied to read counts uniquely mapping to each "atlas" contig using the RPKM method.[63]

Determination of Candidate Differentially Expressed Genes. The read counts mapping to contigs within the transcriptome atlas for each phenotype library were compared to identify candidate differentially expressed genes. The read counts mapping to each contig for any two libraries were made comparable by reference to the RPKM values. Significant differences in the read counts mapping to any contig for any two libraries were determined using a Chi-Squared Test, with the expected number of read counts mapping to any contig being half the sum of all reads from both libraries. Any contig with more than twice the number of mapped reads and an associated *P*-value of $<1 \times 10^{-08}$ was considered to potentially represent a differentially expressed gene.

Assignment of Annotated Contigs to Functional Groups and Enrichment of Ontology Terms. The distribution of molecular functions and biological processes associated with annotated contigs (BLASTX UniProt nonredundant database, *E*-value cutoff 1×10^{-5}) in the transcriptome atlas and the *Drosophila melanogaster* genome were analyzed and compared using Panther (version 8.1).[64] The Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7) was used to compare the unique UniProt accession numbers associated with the transcriptome atlas with the unique UniProt accession numbers associated with over-represented contigs to reveal enriched Gene Ontology (GO) terms (see Supporting Information, SI, for details).

The methodology for primer design, qPCR validation and reference gene choice are detailed in SI Material and Methods, along with further details of all methodologies.

RESULTS

***E. marinus* Transcriptome Atlas.** To create a set of reference sequences upon which gonadal transcriptome profiles could be mapped, an *E. marinus* transcriptome atlas was produced. All 43 590 atlas contig sequences (SI Table S3) have been deposited in the publicly accessible afterParty sequence database.[65] Analysis of the annotated (*E*-value $\leq 1 \times 10^{-05}$) portion of the transcriptome atlas (Panther Classification System, version 8.1)[64] reveals the *E. marinus* contigs fall into 17 broad (high level) biological process terms (SI Figure S1A). A similar distribution of terms was observed when all annotated *Drosophila melanogaster* genes were analyzed (SI Figure S1B). Nearly identical patterns of distributions were also found when the molecular functions associated with the *E. marinus* contigs were compared with those of all *D. melanogaster* genes (SI Figure S2A,B).

Determining *E. marinus* Genes with Sex-Biased Expression. To investigate the extent of any potential reproductive dysfunction that occurs in intersexes, it is necessary to have knowledge of genes linked to reproductive processes. Therefore, lists of *E. marinus* genes presenting sex-biased expression were created by comparing the normal male (NM) (uninfected) and normal female (uninfected) SOLiD generated libraries (SI Table S4). This revealed 1318 and 8485 contigs presenting over-representation in males and females, respectively. Of the 1318 "male" contigs, 198 (15.0%) could be annotated (BLASTX UniProt nonredundant database *E*-value cutoff $\leq 1 \times 10^{-05}$). Likewise, 2979 (35.1%) of the 8485 "female" contigs could be annotated using same cutoff (the numbers and sequences of all "male" and "female" contigs are available in the SI and the afterParty sequence database, respectively).

The SOLiD generated "male and female" contig lists were validated by several methods: sequencing independently prepared gonadal libraries with the Roche 454 GS FLX platform, determining the expression of 34 putative differentially expressed genes using RT-qPCR and analyzing the putative molecular function and expression of validated *E. marinus* sequences by comparison to *Drosophila* genes (SI, Results and Discussion for details of the validation sequencing, qPCR [SI Figure S3A,B] and comparison to *Drosophila* orthologues [SI Table S5]). The clear validation of the SOLiD generated "male" and "female" contig lists by these other methods suggests the approach and *P*-value threshold used to generate the putative lists of differentially expressed genes is justified.

Intersexes Present Common Changes to Gene Expression. To determine differences between NM and the two intersex phenotypes, the SOLiD generated reads counts obtained for NM (uninfected) and both the external intersex males (EIM) (infected) and internal intersex males (IIM) (uninfected) were compared. This reveals that 4847 and 3909 contigs are over-represented (i.e., have significantly more associated count corrected reads relative to the normal male) in EIM and IIM respectively, with ~70% of the over-represented contigs occurring in both IIM and EIM. By comparison, 70 and 44 contigs are under-represented in EIM and IIM respectively. The extent of commonality in the over-represented contigs is greater than would be expected by chance (Chi-square $X^2 = 7660$, df = 1, two-tailed *P*-value <0.0001, see SI for further details).

Clear Signature of Feminization but Not Demaculinization in Intersexes. The contigs over-represented in intersex males relative to NM were compared to all "female" and "male" contigs to reveal 2196 and 1814 "female" contigs are over-represented in the EIM and IIM respectively, with ~75% of the "female" contigs appearing on the lists for both intersex male phenotypes (Figure 1). By comparison, 10 and 2 "female" contigs are under-represented in EIM and IIM respectively, and

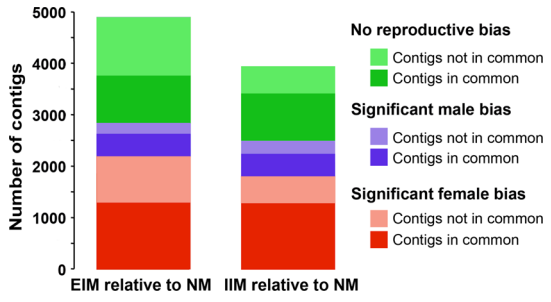

Figure 1. Reproductive identity and proportions of common contigs associated with significantly different numbers of associated reads in the *E. marinus* male intersex phenotypes relative to normal males. NM = normal males, EIM = external intersex males, and IIM = internal intersex males.

no contigs are common to both. Surprisingly, a notable number of "male" contigs were over-represented in the male intersex phenotypes (558 and 607 in EIM and IIM respectively) and ~84% of these "male" contigs present over-representation in both intersex phenotypes (Figure 1). By comparison, 21 and 29 "male" contigs are under-represented in EIM and IIM respectively, with 8 contigs common between lists (all over- and under-represented "reproductive" contigs and sequences associated with the intersex phenotypes are available in the SI and on the afterParty database respectively). The predicted fold changes associated with the over- and under-represented "female" and "male" contigs in intersex males relative to NM were examined (Figure 2) and suggest the majority of over-represented genes display a 2- to 8-fold increase in expression. However, a notable minority of over-represented "female" contigs are predicted to represent genes displaying <32-fold increase in intersexes relative to NM.

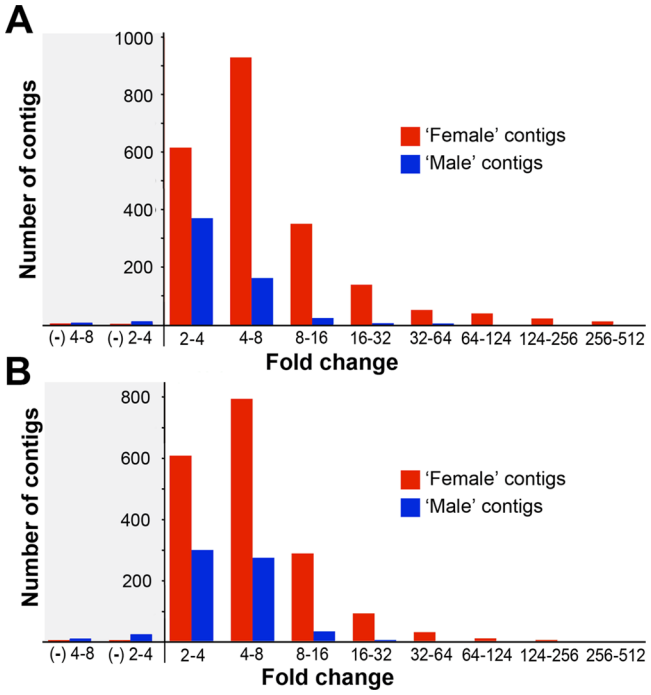

Figure 2. Predicted gene expression fold changes on the basis of over and under-represented "reproductive" contigs associated with both *E. marinus* male intersex phenotypes relative to normal males. A. Fold change associated with reproductive contigs presenting significantly altered representation in external intersex males relative to normal males. B. Fold change associated with reproductive contigs presenting significantly altered representation in internal intersex males relative to normal males.

The predicted expression changes (associated with a range of *P*-values) of "reproductive" contigs in intersex males were validated by RT-qPCR (SI Results and Discussion for details, primer sets available in SI Table S1). The results (SI Figures S4 and S5) support the SOLiD sequencing data, providing evidence for both molecular feminization and that "male" gene expression in intersexes is unchanged or is even moderately up-regulated.

Molecular Fingerprint of Feminization. On the basis that both the reproductive gene lists and the expression changes of reproductive genes in intersex males could be validated, the molecular functions of all "female" contigs as well as those "female" contigs over-represented in both intersex phenotypes were investigated. Comparison (using DAVID v6.7) of unique UniProt accession numbers associated with the transcriptome atlas (BLASTX, *E*-value cutoff 1×10^{-5}) with those associated with the "female" contig list reveals that, relative to the entire atlas, the "female" list contains 28 enriched GO terms (Figure 3A). Furthermore, relative to the entire atlas, there are 14 enriched GO terms associated with the "female" contigs over-represented in both intersex phenotypes (Figure 3B), suggesting a broad-scale molecular feminization of intersex males. Although there was a lower level of annotation for "male" contigs and far fewer "male" contigs (relative to female) presenting over-representation in intersexes, the same analysis was performed (SI Figure S6) and revealed enrichment of several GO term categories (SI Figure S6A). Subsets of these terms are also somewhat enriched in the annotated "male" contigs over-represented in both intersex phenotypes (SI Figure S6B).

DISCUSSION

***E. marinus* Transcriptome Atlas.** The *E. marinus* transcriptomic atlas adds to the much-needed collection of crustacean sequences[66−72] and complements the published amphipod transcriptomes enriched in maternal and zygotic transcripts.[66,72] This resource (available on the afterParty database) has been isolated from a widespread amphipod species and will contain many gene sequences of potential relevance to ecotoxicology (such as those associated with immune response, metal exposure and general stress). Furthermore, given the extent of validation, the "male" and "female" contigs represent reliable lists of sex-biased genes that will aid the study of crustacean reproduction. See SI, Results and Discussion for further reflections on the *E. marinus* transcriptome atlas and comparison of the *E. marinus* transcriptome with previously published crustacean sequences.

***E. marinus* Orthologues of Vertebrate Feminization Markers.** Attempts to apply biomarkers of vertebrate feminization, such as the yolk protein vitellogenin (Vtg), to crustacean species[73] have produced conflicting results[52,55] and, on the basis that no *Vtg* expression is observed in intersex males, it seems that crustacean *Vtg* orthogues would not be an effective "early warning" marker of sexual dysfunction.[55] In addition to *Vtg*, investigations have revealed other unambiguous markers of early ovarian differentiation in vertebrates[74] and evidence from multiple species has led to the suggestion that cytochrome P450 family 19 genes (also known as aromatase or *cyp19*), the forkhead transcription factor l2 (*foxl2a*) and follisatatin (*fst*) act in a concerted fashion to induce the female pathway in vertebrates.[74−80] Analysis of the *E. marinus* transcriptome atlas does reveal an amphipod orthologue (contig1105) to vertebrate follistatin-related proteins (e.g., XP_005639705), but this sequence is unlikely to act as a marker of crustacean feminization, as it is not over-represented in *E. marinus* females (or males). A further search also reveals the presence of a sequence predicted to encode a forkhead transcription factor (contig2412), and the SOLiD sequencing suggests this contig represents a gene with strong female biased expression. However, as for *Vtg*, its suitability as a biomarker of crustacean feminization is highly questionable as no increased expression is observed in either of the intersex male phenotypes relative to normal males. Like *Vtg*, the *cyp19* genes are widely used to monitor the feminization of male vertebrates.[50,81] However, the *cyp19* gene is thought to have first appeared in an

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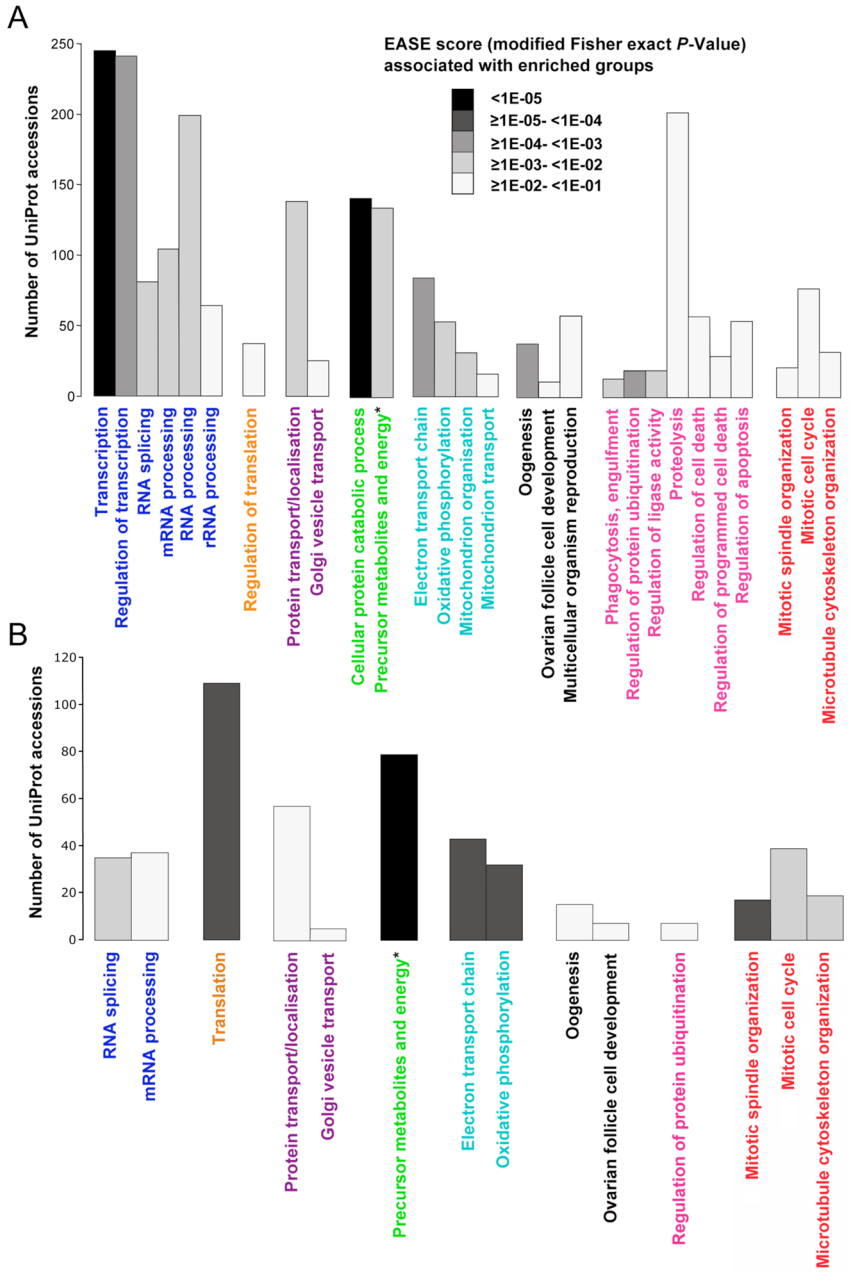

Figure 3. Gene Ontology (GO) terms enriched in all annotated "female contigs" and the "female contigs" over-represented in both intersex phenotypes relative to the distribution of functions associated with the annotated portion of the transcriptome atlas. A. Enriched GO terms in the "female contig" list. B. Enriched GO terms associated with "female contigs" over-represented in both intersex phenotypes. Related GO terms are the same color. The more highly enriched a category, the darker the shading of the representative bar. The extent of enrichment was determined using the EASE Score (a modified Fisher exact *P*-value, for gene-enrichment analysis) that ranges from 0 to 1. A score of 0 would represent perfect enrichment and a probability $<1 \times 10^{-01}$ was selected as a cutoff. As recommended (DAVID v6.7), a *P*-value of $\leq 5 \times 10^{-02}$ is considered to represent strong enrichment. *full GO term: Generation of precursor metabolites and energy.

early chordate[82] and analysis of the *Daphnia pulex* genome revealed no crustacean *cyp19* orthologue.[83] Likewise, no *cyp19* sequence was found in the *E. marinus* transcriptome, supporting the nonapplicability of this commonly utilized gene to crustaceans. The failure of genes, that are reliably mis-regulated in feminized vertebrates, to transfer to a feminized crustacean likely reflects the fundamental differences in the molecular pathways underlying the sexual development of these groups and is clear evidence that existing biomarkers of vertebrate feminization should not be applied to crustaceans.

Potential Markers of Crustacean Sexual Dysfunction. The qPCR experiments validated the expression of sequences presenting a wide range of fold changes and included "female" genes predicted to show pronounced up regulation in the intersex male phenotypes. These genes represent ideal candidate biomarkers for the future monitoring of sexual dysfunction in crustacean populations. For example, experiments would suggest that the genes represented by contig7888 (*Drosophila* orthologue FBgn0011837), contig10503 (no annotation), contig11311 (*Drosophila* orthologue FBgn0001230), and contig11569 (*Drosophila* orthologue

FBgn0027334) represent plausible markers of crustacean feminization, even though their roles in crustacean reproduction are currently unclear. However, the list of potential candidate markers goes beyond the genes used for the qPCR validation experiments. For example, although *E. marinus* appears to have no orthologue to the vertebrate *cyp19* (aromatase) genes, the search did reveal (contig15934) a potential member of the *cyp* family that is closely related to a thromboxane A synthase sequence isolated from a cDNA library derived from the ovaries of the giant tiger prawn *Penaeus monodon* (AFJ11398).[84] The *E. marinus* transcriptome profiles suggest this contig not only represents a gene that presents female biased expression but one that is also up regulated in both intersex male phenotypes relative to normal males. Furthermore, although the exact function of this gene is still a matter for study, it does appear to play an important role in the reproductive system of female crustaceans.[84] The failure to observe demasculinization in both *E. marinus* intersex phenotypes suggests feminization, even when detected using appropriate biomarkers, is not necessarily a proxy for demasculinization in crustaceans. This means any contaminant-induced male sexual dysfunction would have to be detected by directly measuring the mis-regulation of "male" genes. The sequences representing male-biased genes can potentially be used to directly monitor levels of reproductive health and demasculinization in male crustaceans (files containing sequences of all "male" contigs are available in the SI). Given the clear need,[23,24,28,55] the sequences produced for this study can be used to develop a range of reliable biomarkers for monitoring sexual dysfunction in this neglected group.

Feminization or Demasculinization of Intersexes. The broad-scale molecular feminization found in intersex males is consistent with the phenotypes and suggests a process of continuous feminization, as opposed to the phenotypes being purely a legacy of influences acting during early development. Furthermore, analysis of mis-regulated genes in both intersex phenotypes suggests that a broad range of common gene pathways, linked to processes such as oogenesis and ovarian follicle cell development, underlie both parasite and nonparasite associated intersexuality. Surprisingly, the transcriptomic screening indicated a subtle up regulation of some "male" genes in intersexes and certainly no clear signature of demasculinization, with the up regulated genes linked to processes such as nucleosome assembly and the cytoskeleton. Overall, the evidence suggests that male intersexuality represents a degree of feminization superimposed onto a fundamentally normal male, a finding consistent with some interpretations of intersex morphology. For example, the females of gammaridean amphipods are generally significantly smaller than males, however, despite their feminization, EIM are significantly larger than NM.[28,41] In addition, although male intersexes present ovotestes, it can be argued that, apart from the abnormal oviduct tissue, the morphology of a normal and intersex testes are not strikingly different.[10] Furthermore, although sperm counts are lower in intersexes, the difference is not statistically significant.[28] Given the lack of molecular demasculinization, it is possible the lower sperm counts result from a subtle inhibition of spermatogenesis caused by the aberrant "female" gene expression, rather than active demasculinization. The subtle up regulation of "male" genes may even reflect a host response, via regulatory feedback mechanisms, to the inhibition of normal processes by the aberrant gene expression (SI for further discussion of transcriptome and phenotype comparisons).

Due to the over-riding role of the androgenic gland (AG) in male sexual differentiation,[29−31] it has been hypothesized that no feminization of crustaceans is possible without notable levels of prior demasculinization.[56] Our observation of feminization without demasculinization reveals this hypothesis is incorrect. However, given that feminizing parasites are intent on fully converting their male hosts into females,[12,16,39] the molecular signature of male intersexuality leaves open the hypothesis that, in general, crustaceans can only undergo limited feminization without demasculinization and impairment of AG function.

Does Intersexuality Represent Sexual Dysfunction? Although the implications of crustacean intersexuality are uncertain, the sexual dysfunction associated with vertebrate and mollusc intersexuality is of serious concern.[85,86] Crustacean intersexuality might also impact reproductive capacity,[10,35,87] or it may be of little consequence and common to species possessing environmental sex determination,[13,43,45] it may even be a feature of normal reproductive development.[6,44] Intersexuality may also represent all of these scenarios, as it does in vertebrates.[19−21,49] The association between pollutants and crustacean intersexuality[7−9,11,25−28] and evidence for increased parasite infection in pollutant-exposed amphipods[36,37] suggests some proportion of intersexuality is abnormal. It is tempting to use the molecular signature associated with the intersexes to gauge the severity of any reproductive consequences. In this regard, the interpretation of *E. marinus* male intersexuality as feminization imposed onto a normal male suggests widespread forms of crustacean intersexuality[46] do not necessarily indicate serious sexual dysfunction. This is assuming that male intersexuality is not commonly associated with abnormal mating behaviors that compromise reproductive success.[48]

Crustaceans are a relatively poorly understood group and our study is based on examining two forms of intersexuality presented by a single species. Although, as *E. marinus* intersexuality is typical of that found in amphipods[46] and morphologically analogous forms are also described in decapod crustaceans,[8] our findings are likely to be relevant to malacostracans and, more tentatively, copepods.[6,88] However, crustacean intersexuality can differ considerably from the typical forms. Extreme intersex phenotypes have been reported in the amphipods *Gammarus duebeni* and *Corophium volutator*[48,87,89] and it will be of interest to determine if such intersexuality is associated with demasculinzation or represents a more advanced case of feminization without demasculization. The data produced for this study will greatly enhance the capacity of ecotoxicologists to characterize the various forms of crustacean intersexuality and determine the extent to which abnormal sexual phenotypes represent sexual dysfunction.

Feminization and Demasculinization: A Parasitic Perspective. The *E. marinus* EIM phenotype is associated with coinfection by two vertically transmitted feminizing parasite species,[57] the microsporidian *Dictyocoela duebenum*,[11,12,39,57] and a paramyxean protist.[58] It is thought that these parasites, either independently or in combination, facilitate transmission by converting their male host into a functioning female.[57] Evidence suggests that feminizing microsporidians convert hosts by preventing differentiation of the male AG[29,30] and that intersexuality occurs when this process fails to complete.[30,34,38] Our observation of feminization without demasculinization is consistent with a hypothesis that

the parasites convert males by both increasing female gene expression and inhibiting AG differentiation, and that this inhibition is effectively binary. In this scenario, EIM represent an unequivocal parasitic failure to inhibit AG differentiation despite inducing female gene expression. The reasons for this failure are uncertain but may be due to insufficient parasite burden at a critical developmental stage, suboptimal environmental conditions,[30,34,38] an effective host response, or some combination of these influences.

Bacteria from the genus *Wolbachia* can also fully convert male crustaceans into females.[15,16] Intersexuality can be induced in isopods by artificially infecting normal males using the blood of infected females, and this intersexuality is associated with a hypertrophied AG.[90] It will be of interest to determine whether such individuals present both molecular feminization and demasculinization. If so, then it could indicate that while feminizing microsporidian and paramyxean parasites (either individually or in combination) are capable of feminization and preventing initial masculinization, *Wolbachia* may possess the ability to feminize and demasculinise a host with a functioning AG. If correct, then this would add weight to the suggestion[29] there are differences in the manipulative capabilities of these divergent parasites. Given the link between contamination and increased parasitism,[11,36,37] we suggest such observations are relevant to ecotoxicologists, as it will be critical to understand the specific functional consequences associated with infection by particular parasite groups.

Independence of Reproductive Gene Pathways. The susceptibility of organisms to endocrine disrupting chemicals is linked to their underlying biology. Exposure of vertebrate species to feminizing agents leads to intersexuality via feminization and demasculinization.[3,18,21,22] For example, exposure of male trout to estrogenic treatments induces expression of early ovarian differentiation genes and suppresses genes critical to Sertoli cells and androgen synthesis in Leydig cells.[18] Such demasculinization results in intersex fish producing less semen with a lower density of, deficiently motile, sperm.[20,21] The association of feminization and demasculinization likely results from interactions between gene networks underlying male and female sexual differentiation.[18,79] The lack of down regulated male genes in unambiguously feminized male amphipods reveals a relatively large degree of separation in the gene pathways controlling male and female sexual differentiation. This separation is also suggested by female intersexuality, as the subtle or nonexistent reductions in fecundity[41,44] indicate no profound defeminization is associated with the masculinzation in these cases. The extent of separation is called into question by the observation that female amphipods with an implanted AG are masculinized and slightly defeminized.[29] However, this defeminization may be due to AG-induced masculinization inhibiting female reproductive processes rather than interactions between gene networks. Evidence for separation can be found outside the amphipods, as histological analysis of male intersexuality in lobsters reveal previtellogenic oocytes alongside normal seminiferous tubules containing male reproductive cells.[8] Furthermore, functional hermaphroditism is common in some crustacean groups. Indeed, species of caridean shrimp[91] and barnacle[92] are protandric simultaneous hermaphrodites, in which juveniles initially become males before developing female characteristics and becoming functional hermaphrodites.[91,93] This demonstrates divergent crustaceans can undergo absolute feminization while remaining functionally male. The evolution of such hermaphroditic reproduction is arguably more likely if the gene pathways underlying male and female sexual differentiation were largely independent in the gonochoristic ancestor. Our finding that the differentiation pathways are, to some extent, separate in a gonochoristic amphipod suggests such independence may be a widespread feature of crustacean biology. This independence has implications for crustacean susceptibility to anthropogenic contamination. Whereas vertebrates are feminized and demasculinised by a single estrogenic contaminant, a crustacean likely requires both feminizing and demasculinising agents to cause sexual dysfunction equivalent to that of vertebrates.[19,21,50] This hypothesis is consistent with high levels of intersexuality in very stable crustacean populations.[27,41,44] Therefore, both population and molecular data indicate the underlying biology, specifically the regulatory separation of gene pathways responsible for male and female characteristics, may confer crustaceans with a level of protection against the harmful consequences of abnormal reproductive gene expression.

■ ASSOCIATED CONTENT

Supporting Information

Further methodology and results relating to sequencing and qPCR validation, plus further discussion on the *E. marinus* transcriptome. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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