

1 **Crustacean intersexuality is feminisation without de-masculinisation: implications**  
2 **for environmental toxicology**

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18

19 **Abstract**

20

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

## 39 **Introduction**

40

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

54 Crustaceans present parasite-induced and non-parasite induced forms of  
55 intersexuality, and both forms have been associated with environmental contamination  
56 [7-9, 11, 24-28]. Parasite-induced intersexuality in crustaceans is linked with feminising  
57 parasites that transmit vertically by infecting the eggs of their host [16]. Such parasites  
58 can often enhance their transmission by converting males into females [16]. A feat  
59 reportedly accomplished by preventing differentiation of the male androgenic gland (AG)  
60 [29, 30], a specialised organ controlling male sexual differentiation [31, 32].  
61 Intersexuality occurs in cases of incomplete conversion, possibly due to a low parasite  
62 burden or suboptimal environmental conditions [30, 33-35]. Although some parasite-  
63 induced intersexuality will exist naturally, a proportion may be indirectly caused by  
64 anthropogenic contamination, as contaminant-exposed crustaceans present significant

65 increases in infection by microsporidia [36, 37], a phylum of parasites containing species  
66 capable of crustacean sexual conversion [38, 39]. Furthermore, population analyses  
67 have revealed significant increases in infection by feminising microsporidian species at  
68 contaminated sites [11].

69         The consequences of both parasite and non-parasite associated crustacean  
70 intersexuality are the subject of debate [23]. The reproductive deficiencies associated  
71 with female intersexuality are statistically significant but relatively subtle [35, 40, 41] and  
72 might represent the acceptable cost of adaptive environmental sex determination  
73 mechanisms [13, 17, 42]. Furthermore, female intersexuality is not always associated  
74 with significant reductions in fecundity, suggesting it may not be maladaptive in some  
75 species [43-45]. The implications of parasite and non-parasite induced male  
76 intersexuality are less well understood [23, 28]. However, its high frequency [7],  
77 association with lower sperm counts [28], pronounced morphological changes [10, 46,  
78 47], altered hormone levels [10] and behavioural changes [48] have lead to the  
79 suggestion that intersexuality in males is of serious concern [28]. High levels of  
80 intersexuality may indicate a population in which a large proportion of all males are to  
81 some extent compromised, albeit less conspicuously. The scenario in crustaceans may  
82 be analogous to vertebrates, where male intersexuality can represent a normal condition  
83 [49] as well as an abnormality indicative of sexual dysfunction with varying reproductive  
84 consequences [19-21].

85         Molecular markers capable of monitoring feminisation and de-masculinisation in  
86 vertebrates are continually being developed and applied [18, 19, 50]. Attempts to  
87 monitor sexual dysfunction in male crustaceans using genes orthologous to those mis-  
88 regulated in vertebrates has produced inconsistent results [51-54] and established  
89 vertebrate markers may not be suitable for monitoring crustacean populations [52, 55].  
90 Although the current lack of relevant genomic resources is hindering investigations [24,

91 27, 56], the application of high-throughput sequencing technology to a suitable species  
92 could markedly facilitate the use of crustaceans to monitor aquatic habitats and rapidly  
93 create a model for the study of crustacean sexual dysfunction. *Echinogammarus*  
94 *marinus* (Leach, 1815) is one such species, it is a widespread intertidal amphipod and a  
95 population found at an environmentally impacted site presents significant levels of  
96 female bias and intersexuality typical of many impacted amphipod populations [11, 28,  
97 46]. Furthermore, females and intersex animals in this population have a significantly  
98 higher prevalence of infection with two species of vertically transmitted microscopic  
99 feminising parasite [11, 57]. These have been identified as the microsporidian  
100 *Dictyocoela duebenum*, a species classified as an amphipod feminiser [11, 12, 39, 57],  
101 and a parasite belonging to the phylum Paramyxea [57], a group also linked to female  
102 bias and intersexuality [58]. The majority of parasite-infected males from this population  
103 are classed 'external intersex males' (EIM) on the basis that they possess rudimental  
104 brood plates externally, in addition to sometimes exhibiting an ovotestis [10, 27]. This  
105 population also harbours uninfected males possessing an ovotestis but no brood plates  
106 [28, 59]. These 'internal intersex males' (IIM) are not associated with parasites and may  
107 be a result of anthropogenic contamination [27]. This *E. marinus* population represents a  
108 potential resource for revealing the molecular basis of parasite and non-parasite  
109 associated intersexuality and crustacean sexual differentiation.

110 Transcriptomic profiling of intersexuality in *E. marinus* will give insights into the  
111 processes of feminisation and de-masculinisation in an unambiguously feminised  
112 crustacean. This is particularly relevant as the sexual dysfunction of vertebrate males  
113 exposed to feminising agents largely results from a process of de-masculinisation that  
114 occurs concurrently with molecular feminisation [3, 18, 20-22, 60]. Furthermore, the  
115 expression patterns of sex-biased genes in parasite-infected specimens presenting  
116 incomplete sexual conversion will also shed light into the conversion mechanism (and its

117 failure) employed by feminising parasites. This study uses a transcriptomic screening  
118 approach to explore the molecular basis of crustacean intersexuality. Gene expression  
119 levels in normal and intersex phenotypes are compared by sequencing gonadal libraries  
120 and mapping these profiles to a newly created *E. marinus* transcriptome atlas. The gene  
121 expression profiles are validated and analysed to reveal the feminisation and de-  
122 masculinisation underling parasite and non-parasite associated crustacean  
123 intersexuality. This information will give ecotoxicologists a deeper understanding of  
124 abnormal sexual development at contaminated sites and uncover a wealth of molecular  
125 biomarkers for monitoring crustacean reproductive health in threatened habitats.

126

## 127 **Materials and Methods**

128

### 129 **Sampling**

130

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

137

### 138 **Production of an *E. marinus* transcriptome atlas**

139

140 RNA was extracted (Tri-Reagent. Ambion) from muscle, head, hepatopancreas and  
141 gonadal tissues dissected from parasitised, unparasitised, male, female, and juveniles  
142 (n=14 adults and 10 juveniles at various stages of development) and 1.5 µg was used to

143 make double stranded (ds) cDNA (MINT cDNA synthesis kit, Evrogen). The ds cDNA  
144 was normalised (Trimmer normalisation kit, Evrogen) and sequenced using 1.5 plates of  
145 the 454 GS FLX Titanium platform (Centre for Genomic Research, University of  
146 Liverpool). The expressed sequence tags (ESTs) were assembled using Newbler (v2.6)  
147 and Mira (3.4.1.1) software and combined with the CAP3 assembly program [61, 62] to  
148 create a set of contiguous sequences termed the 'transcriptome atlas'. The atlas was  
149 annotated by comparison to non-redundant sequences in UniProt and FlyBase  
150 (BLASTX, E-value cut off  $\leq 1E-05$ ).

151

#### 152 **Roche 454 GS FLX sequencing of *E. marinus* gonadal cDNA libraries**

153

154 Total RNA was isolated (Tri-Reagent. Ambion) from animals characterised as NM (n=9)  
155 and females (n=9) [59] and 4 $\mu$ g was used to make ds cDNA (MessageAmp aRNA  
156 Amplification Kit, Ambion) for sequencing using the Roche 454 GS FLX platform (Centre  
157 for Genomic Research, University of Liverpool). The resulting ESTs were mapped to the  
158 'transcriptome atlas' (CLC Genomics Workbench v4.9) to give digital gene expression  
159 profiles for the two phenotypes. Counting correction was applied to read counts uniquely  
160 mapping to each 'atlas' contig using the kilobase of exon model per million mapped read  
161 (RPKM) method [63].

162

#### 163 **ABI SOLiD™ 4 sequencing of *E. marinus* gonadal cDNA libraries**

164

165 Total RNA was extracted (Tri-Reagent. Ambion) from animals presenting a range of  
166 parasite infection statuses [57, 59] and sexual phenotypes (uninfected normal males  
167 [NM] n=24, external intersex males [EIM] n=24, internal intersex males [IIM] n=12 and  
168 normal females n=24). The mRNA was isolated from the total RNA (Poly(A) Purist kit,

169 Ambion) and converted into a library for sequencing by the ABI SOLiD™ 4 System  
170 (SOLiD™ 4 System Library Preparation Guide, Life Technologies). The libraries were  
171 sequenced on one lane of a flow-chip and the 75 bp ESTs were mapped to the  
172 'transcriptome atlas' (CLC Genomics Workbench v4.9) to give digital gene expression  
173 profiles. Counting correction was applied to read counts uniquely mapping to each 'atlas'  
174 contig using the RPKM method [63].

175

### 176 **Determination of candidate differentially expressed genes**

177

178 The read counts mapping to contigs within the transcriptome atlas for each phenotype  
179 library were compared to identify candidate differentially expressed genes. The read  
180 counts mapping to each contig for any two libraries were made comparable by reference  
181 to the RPKM values. Significant differences in the read counts mapping to any contig for  
182 any two libraries were determined using a Chi-Squared Test, with the expected number  
183 of read counts mapping to any contig being half the sum of all reads from both libraries.  
184 Any contig with more than twice the number of mapped reads and an associated *P*-value  
185 of <1E-08 was considered to potentially represent a differentially expressed gene.

186

### 187 **Assignment of annotated contigs to functional groups and enrichment of ontology** 188 **terms**

189

190 The distribution of molecular functions and biological processes associated with  
191 annotated contigs (BLASTX UniProt non-redundant database, E-value cut-off 1e-5) in  
192 the transcriptome atlas and the *Drosophila melanogaster* genome were analysed and  
193 compared using Panther (version 8.1) [64]. The Database for Annotation, Visualization  
194 and Integrated Discovery (DAVID v6.7) was used to compare the unique UniProt



195 accession numbers associated with the transcriptome atlas with the unique UniProt  
196 accession numbers associated with overrepresented contigs to reveal enriched Gene  
197 Ontology (GO) terms (see Supporting Information for details).

198

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

## 202 **Results**

203

### 204 ***E. marinus* transcriptome atlas**

205

206 To create a set of reference sequences upon which gonadal transcriptome profiles could  
207 be mapped, an *E. marinus* transcriptome atlas was produced. All 43,590 atlas contig  
208 sequences (Supporting Information, Table S3) have been deposited in the publicly  
209 accessible afterParty sequence database [65]. Analysis of the annotated (E-value  $\leq 1E-$   
210 05) portion of the transcriptome atlas (Panther Classification System, version 8.1) [64]  
211 reveals the *E. marinus* contigs fall into 17 broad (high level) biological process terms  
212 (Supporting information, Fig. S1A). A similar distribution of terms was observed when all  
213 annotated *Drosophila melanogaster* genes were analysed (Fig. S1B). Nearly identical  
214 patterns of distributions were also found when the molecular functions associated with  
215 the *E. marinus* contigs were compared with those of all *D. melanogaster* genes (Fig. S2A  
216 and S2B).

217

### 218 **Determining *E. marinus* genes with sex-biased expression**

219

220 To investigate the extent of any potential reproductive dysfunction that occurs in  
221 intersexes, it is necessary to have knowledge of genes linked to reproductive processes.  
222 Therefore, lists of *E. marinus* genes presenting sex biased expression were created by  
223 comparing the normal male (NM) (uninfected) and normal female (uninfected) SOLiD  
224 generated libraries (Supporting Information, Table S4). This revealed 1318 and 8485  
225 contigs presenting overrepresentation in males and females respectively. Of the 1318  
226 'male' contigs, 198 (15.0%) could be annotated (BLASTX UniProt non-redundant

227 database E-value cut-off  $\leq 1E-05$ ). Likewise, 2979 (35.1%) of the 8485 'female' contigs  
228 could be annotated using same cut-off (the numbers and sequences of all 'male' and  
229 'female' contigs are available in the Supporting Information and the afterParty sequence  
230 database respectively).

231 The SOLiD generated 'male' and 'female' contig lists were validated by several  
232 methods: sequencing independently prepared gonadal libraries with the Roche 454 GS  
233 FLX platform, determining the expression of 34 putative differentially expressed genes  
234 using RT-qPCR and analysing the putative molecular function and expression of  
235 validated *E. marinus* sequences by comparison to *Drosophila* genes (Supporting  
236 Information, Results and Discussion for details of the validation sequencing, qPCR [Fig  
237 S3A and S3B] and comparison to *Drosophila* orthologues [Table S5]). The clear  
238 validation of the SOLiD generated 'male' and 'female' contig lists by these other methods  
239 suggests the approach and *P*-value threshold used to generate the putative lists of  
240 differentially expressed genes is justified.

241

#### 242 **Intersexes present common changes to gene expression**

243

244 To determine differences between NM and the two intersex phenotypes, the SOLiD  
245 generated reads counts obtained for NM (uninfected) and both the external intersex  
246 males (EIM) (infected) and internal intersex males (IIM) (uninfected) were compared.  
247 This reveals that 4847 and 3909 contigs are overrepresented (i.e. have significantly  
248 more associated count corrected reads relative to the normal male) in EIM and IIM  
249 respectively, with ~70% of the overrepresented contigs occurring in both IIM and EIM.  
250 By comparison, 70 and 44 contigs are underrepresented in EIM and IIM respectively.  
251 The extent of commonality in the overrepresented contigs is greater than would be

252 expected by chance (Chi-square  $X^2 = 7660$ ,  $df = 1$ , two-tailed  $P$ -value  $< 0.0001$ , see  
253 Supporting Information for further details).

254

### 255 **Clear signature of feminisation but not de-maculinisation in intersexes**

256

257 The contigs overrepresented in intersex males relative to NM were compared to all  
258 'female' and 'male' contigs to reveal 2196 and 1814 'female' contigs are overrepresented  
259 in the EIM and IIM respectively, with ~75% of the 'female' contigs appearing on the lists  
260 for both intersex male phenotypes (Fig. 1). By comparison, 10 and 2 'female' contigs are  
261 underrepresented in EIM and IIM respectively, and no contigs are common to both.  
262 Surprisingly, a notable number of 'male' contigs were overrepresented in the male  
263 intersex phenotypes (558 and 607 in EIM and IIM respectively) and ~84% of these 'male'  
264 contigs present overrepresentation in both intersex phenotypes (Fig. 1). By comparison,  
265 21 and 29 'male' contigs are underrepresented in EIM and IIM respectively, with 8  
266 contigs common between lists (all over- and underrepresented 'reproductive' contigs and  
267 sequences associated with the intersex phenotypes are available in the Supporting  
268 Information and on the afterParty database respectively). The predicted fold changes  
269 associated with the over- and underrepresented 'female' and 'male' contigs in intersex  
270 males relative to NM were examined (Fig. 2) and suggest the majority of  
271 overrepresented genes display a 2 to 8-fold increase in expression. However, a notable  
272 minority of overrepresented 'female' contigs are predicted to represent genes displaying  
273  $<32$ -fold increase in intersexes relative to NM.

274 The predicted expression changes (associated with a range of  $P$ -values) of  
275 'reproductive' contigs in intersex males were validated by RT-qPCR (Supporting  
276 Information Results and Discussion for details, primer sets available in Table S1). The

277 results (Supporting Information, Fig. S4 and S5) support the SOLiD sequencing data,  
278 providing evidence for both molecular feminisation and that 'male' gene expression in  
279 intersexes is unchanged or is even moderately up regulated.

280

### 281 **Molecular fingerprint of feminisation**

282

283 On the basis that both the reproductive gene lists and the expression changes of  
284 reproductive genes in intersex males could be validated, the molecular functions of all  
285 'female' contigs as well as those 'female' contigs overrepresented in both intersex  
286 phenotypes were investigated. Comparison (using DAVID v6.7) of unique UniProt  
287 accession numbers associated with the transcriptome atlas (BLASTX, E-value cut-off 1e-  
288 5) with those associated with the 'female' contig list reveals that, relative to the entire  
289 atlas, the 'female' list contains 28 enriched GO terms (Fig. 3A). Furthermore, relative to  
290 the entire atlas, there are 14 enriched GO terms associated with the 'female' contigs  
291 overrepresented in both intersex phenotypes (Fig. 3B), suggesting a broad-scale  
292 molecular feminisation of intersex males. Although there was a lower level of annotation  
293 for 'male' contigs and far fewer 'male' contigs (relative to female) presenting  
294 overrepresentation in intersexes, the same analysis was performed (Supporting  
295 Information, Fig. S6) and revealed enrichment of several GO term categories (Fig. S6A).  
296 Subsets of these terms are also somewhat enriched in the annotated 'male' contigs  
297 overrepresented in both intersex phenotypes (Supporting Information, Fig. S6B).

298

299 **Discussion**

300

301 **The *E. marinus* transcriptome atlas**

302

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

314

315 ***E. marinus* orthologues of vertebrate feminisation markers**

316

317 Attempts to apply biomarkers of vertebrate feminisation, such as the yolk protein  
318 vitellogenin (Vtg), to crustacean species [73] have produced conflicting results [52, 55]  
319 and, on the basis that no *Vtg* expression is observed in intersex males, it seems that  
320 crustacean *Vtg* orthogues would not be an ineffective 'early-warning' marker of sexual  
321 dysfunction [55]. In addition to *Vtg*, investigations have revealed other unambiguous  
322 markers of early ovarian differentiation in vertebrates [74] and evidence from multiple  
323 species has lead to the suggestion that cytochrome P450 family 19 genes (also known  
324 as aromatase or *cyp19*), the forkhead transcription factor I2 (*foxl2a*) and follisatatin (*fst*)

325 act in a concerted fashion to induce the female pathway in vertebrates [74-80]. Analysis  
326 of the *E. marinus* transcriptome atlas does reveal an amphipod orthologue (contig1105)  
327 to vertebrate follistatin-related proteins (e.g. XP\_005639705) but this sequence is  
328 unlikely to act as a marker of crustacean feminisation as it is not overrepresented in *E.*  
329 *marinus* females (or males). A further search also reveals the presence of a sequence  
330 predicted to encode a forkhead transcription factor (contig2412) and the SOLiD  
331 sequencing suggests this contig represents a gene with strong female biased  
332 expression. However, as for *Vtg*, its suitability as a biomarker of crustacean feminisation  
333 is highly questionable as no increased expression is observed in either of the intersex  
334 male phenotypes relative to normal males. Like *Vtg*, the *cyp19* genes are widely used to  
335 monitor the feminisation of male vertebrates [50, 81]. However, the *cyp19* gene is  
336 thought to have first appeared in an early chordate [82] and analysis of the *Daphnia*  
337 *pulex* genome revealed no crustacean *cyp19* orthologue [83]. Likewise, no *cyp19*  
338 sequence was found in the *E. marinus* transcriptome, supporting the non-applicability of  
339 this commonly utilised gene to crustaceans. The failure of genes, that are reliably mis-  
340 regulated in feminised vertebrates, to transfer to a feminised crustacean likely reflects  
341 the fundamental differences in the molecular pathways underlying the sexual  
342 development of these groups and is clear evidence that existing biomarkers of vertebrate  
343 feminisation should not be applied to crustaceans.

344

#### 345 **Potential markers of crustacean sexual dysfunction**

346

347 The qPCR experiments validated the expression of sequences presenting a wide range  
348 of fold changes and included 'female' genes predicted to show pronounced up regulation  
349 in the intersex male phenotypes. These genes represent ideal candidate biomarkers for  
350 the future monitoring of sexual dysfunction in crustacean populations. For example,

351 experiments would suggest that the genes represented by contig7888 (*Drosophila*  
352 orthologue FBgn0011837), contig10503 (no annotation), contig11311 (*Drosophila*  
353 orthologue FBgn0001230) and contig11569 (*Drosophila* orthologue FBgn0027334)  
354 represent plausible markers of crustacean feminisation, even though their roles in  
355 crustacean reproduction are currently unclear. However, the list of potential candidate  
356 markers goes beyond the genes used for the qPCR validation experiments. For  
357 example, although *E. marinus* appears to have no orthologue to the vertebrate *cyp19*  
358 (aromatase) genes, the search did reveal (contig15934) a potential member of the *cyp*  
359 family that is closely related to a thromboxane A synthase sequence isolated from a  
360 cDNA library derived from the ovaries of the giant tiger prawn *Penaeus monodon*  
361 (AFJ11398) [84]. The *E. marinus* transcriptome profiles suggest this contig not only  
362 represents a gene that presents female biased expression but one that is also up  
363 regulated in both intersex male phenotypes relative to normal males. Furthermore,  
364 although the exact function of this gene is still a matter for study, it does appear to play  
365 an important role in the reproductive system of female crustaceans [84]. The failure to  
366 observe de-masculinisation in both *E. marinus* intersex phenotypes suggests  
367 feminisation, even when detected using appropriate biomarkers, is not necessarily a  
368 proxy for de-masculinisation in crustaceans. This means any contaminant-induced male  
369 sexual dysfunction would have to be detected by directly measuring the mis-regulation of  
370 'male' genes. The sequences representing male-biased genes can potentially be used to  
371 directly monitor levels of reproductive health and de-masculinisation in male crustaceans  
372 (files containing sequences of all 'male' contigs are available in the Supporting  
373 Information). Given the clear need [23, 24, 28, 55], the sequences produced for this  
374 study can be used to develop a range of reliable biomarkers for monitoring sexual  
375 dysfunction in this neglected group.

376



377 **Feminisation or de-masculinisation of intersexes**

378

379 The broad-scale molecular feminisation found in intersex males is consistent with the  
380 phenotypes and suggests a process of continuous feminisation, as opposed to the  
381 phenotypes being purely a legacy of influences acting during early development.  
382 Furthermore, analysis of mis-regulated genes in both intersex phenotypes suggests that  
383 a broad range of common gene pathways, linked to processes such as oogenesis and  
384 ovarian follicle cell development, underlie both parasite and non-parasite associated  
385 intersexuality. Surprisingly, the transcriptomic screening indicated a subtle up regulation  
386 of some 'male' genes in intersexes and certainly no clear signature of de-  
387 masculinisation, with the up regulated genes linked to processes such as nucleosome  
388 assembly and the cytoskeleton. Overall, the evidence suggests that male intersexuality  
389 represents a degree of feminisation superimposed onto a fundamentally normal male, a  
390 finding consistent with some interpretations of intersex morphology. For example, the  
391 females of gammaridean amphipods are generally significantly smaller than males,  
392 however, despite their feminisation, EIM are significantly larger than NM [28, 41]. In  
393 addition, although male intersexes present ovotestes, it can be argued that, apart from  
394 the abnormal oviduct tissue, the morphology of a normal and intersex testes are not  
395 strikingly different [10]. Furthermore, although sperm counts are lower in intersexes, the  
396 difference is not statistically significant [28]. Given the lack of molecular de-  
397 masculinisation, it is possible the lower sperm counts result from a subtle inhibition of  
398 spermatogenesis caused by the aberrant 'female' gene expression, rather than active  
399 de-masculinisation. The subtle up regulation of 'male' genes may even reflect a host  
400 response, via regulatory feedback mechanisms, to the inhibition of normal processes by  
401 the aberrant gene expression (Supporting Information for further discussion of  
402 transcriptome and phenotype comparisons).

403           Due to the overriding role of the androgenic gland (AG) in male sexual  
404 differentiation [29-31], it has been hypothesised that no feminisation of crustaceans is  
405 possible without notable levels of prior de-masculinisation [56]. Our observation of  
406 feminisation without de-masculinisation reveals this hypothesis is incorrect. However,  
407 given that feminising parasites are intent on fully converting their male hosts into females  
408 [12, 16, 39], the molecular signature of male intersexuality leaves open the hypothesis  
409 that, in general, crustaceans can only undergo limited feminisation without de-  
410 masculinisation and impairment of AG function.

411

#### 412 **Does intersexuality represent sexual dysfunction?**

413

414 Although the implications of crustacean intersexuality are uncertain, the sexual  
415 dysfunction associated with vertebrate and mollusc intersexuality is of serious concern  
416 [85, 86]. Crustacean intersexuality might also impact reproductive capacity [10, 35, 87],  
417 or it may be of little consequence and common to species possessing environmental sex  
418 determination [13, 43, 45], it may even be a feature of normal reproductive development  
419 [6, 44]. Intersexuality may also represent all of these scenarios, as it does in vertebrates  
420 [19-21, 49]. The association between pollutants and crustacean intersexuality [7-9, 11,  
421 25-28] and evidence for increased parasite infection in pollutant-exposed amphipods  
422 [36, 37] suggests some proportion of intersexuality is abnormal. It is tempting to use the  
423 molecular signature associated with the intersexes to gauge the severity of any  
424 reproductive consequences. In this regard, the interpretation of *E. marinus* male  
425 intersexuality as feminisation imposed onto a normal male suggests widespread forms of  
426 crustacean intersexuality [46] do not necessarily indicate serious sexual dysfunction.  
427 This is assuming that male intersexuality is not commonly associated with abnormal  
428 mating behaviours that compromise reproductive success [48].

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

442

#### 443 **Feminisation and de-masculinisation: a parasitic perspective**

444

445 The *E. marinus* EIM phenotype is associated with co-infection by two vertically  
446 transmitted feminising parasite species [57], the microsporidian *Dictyocoela duebenum*  
447 [11, 12, 39, 57], and a paramyxean protist [58]. It is thought that these parasites, either  
448 independently or in combination, facilitate transmission by converting their male host into  
449 a functioning female [57]. Evidence suggests that feminising microsporidians convert  
450 hosts by preventing differentiation of the male AG [29, 30] and that intersexuality occurs  
451 when this process fails to complete [30, 34, 38]. Our observation of feminisation without  
452 de-masculinisation is consistent with a hypothesis that the parasites convert males by  
453 both increasing female gene expression and inhibiting AG differentiation, and that this  
454 inhibition is effectively binary. In this scenario, EIM represent an unequivocal parasitic

455 failure to inhibit AG differentiation despite inducing female gene expression. The reasons  
456 for this failure are uncertain but may be due to insufficient parasite burden at a critical  
457 developmental stage, suboptimal environmental conditions [30, 34, 38], an effective host  
458 response, or some combination of these influences.

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

472

### 473 **The independence of reproductive gene pathways**

474

475 The susceptibility of organisms to endocrine disrupting chemicals is linked to their  
476 underlying biology. Exposure of vertebrate species to feminising agents leads to  
477 intersexuality via feminisation and de-masculinisation [3, 18, 21, 22]. For example,  
478 exposure of male trout to oestrogenic treatments induces expression of early ovarian  
479 differentiation genes and suppresses genes critical to Sertoli cells and androgen  
480 synthesis in Leydig cells [18]. Such de-masculinisation results in intersex fish producing

481 less semen with a lower density of, deficiently motile, sperm [20, 21]. The association of  
482 feminisation and de-masculinisation likely results from interactions between gene  
483 networks underlying male and female sexual differentiation [18, 79]. The lack of down  
484 regulated male genes in unambiguously feminised male amphipods reveals a relatively  
485 large degree of separation in the gene pathways controlling male and female sexual  
486 differentiation. This separation is also suggested by female intersexuality, as the subtle  
487 or nonexistent reductions in fecundity [41, 44] indicate no profound de-feminisation is  
488 associated with the masculinisation in these cases. The extent of separation is called into  
489 question by the observation that female amphipods with an implanted AG are  
490 masculinised and slightly de-feminised [29]. However, this de-feminisation may be due to  
491 AG-induced masculinisation inhibiting female reproductive processes rather than  
492 interactions between gene networks. Evidence for separation can be found outside the  
493 amphipods, as histological analysis of male intersexuality in lobsters reveal  
494 previtellogenic oocytes alongside normal seminiferous tubules containing male  
495 reproductive cells [8]. Furthermore, functional hermaphroditism is common in some  
496 crustacean groups. Indeed, species of caridean shrimp [91] and barnacle [92] are  
497 protandric simultaneous hermaphrodites, in which juveniles initially become males  
498 before developing female characteristics and becoming functional hermaphrodites [91,  
499 93]. This demonstrates divergent crustaceans can undergo absolute feminisation while  
500 remaining functionally male. The evolution of such hermaphroditic reproduction is  
501 arguably more likely if the gene pathways underlying male and female sexual  
502 differentiation were largely independent in the gonochoristic ancestor. Our finding that  
503 the differentiation pathways are, to some extent, separate in a gonochoristic amphipod  
504 suggests such independence may be a widespread feature of crustacean biology. This  
505 independence has implications for crustacean susceptibility to anthropogenic  
506 contamination. Whereas vertebrates are feminised and de-masculinised by a single

507 oestrogenic contaminant, a crustacean likely requires both feminising and de-  
508 masculinising agents to cause sexual dysfunction equivalent to that of vertebrates [19,  
509 21, 50]. This hypothesis is consistent with high levels of intersexuality in very stable  
510 crustacean populations [27, 41, 44]. Therefore, both population and molecular data  
511 indicate the underlying biology, specifically the regulatory separation of gene pathways  
512 responsible for male and female characteristics, may confer crustaceans with a level of  
513 protection against the harmful consequences of abnormal reproductive gene expression.

514

### 515 **Acknowledgements**

516

517 The authors would like to acknowledge the Natural Environmental Research Council  
518 (NERC) for funding this work. SS, GY and YG were supported by NE/G004587/1  
519 awarded to PK and ATF. This work was also supported by NERC MGF223, NBAF438 &  
520 479 and the staff at the NERC Biomolecular Analysis Facility based at the University of  
521 Liverpool.

522

### 523 **Supporting Information**

524 Supporting information contains further methodology and results relating to sequencing  
525 and qPCR validation, plus further discussion on the *E. marinus* transcriptome. This  
526 information is available free of charge via the Internet at <http://pubs.acs.org/>.

527

528

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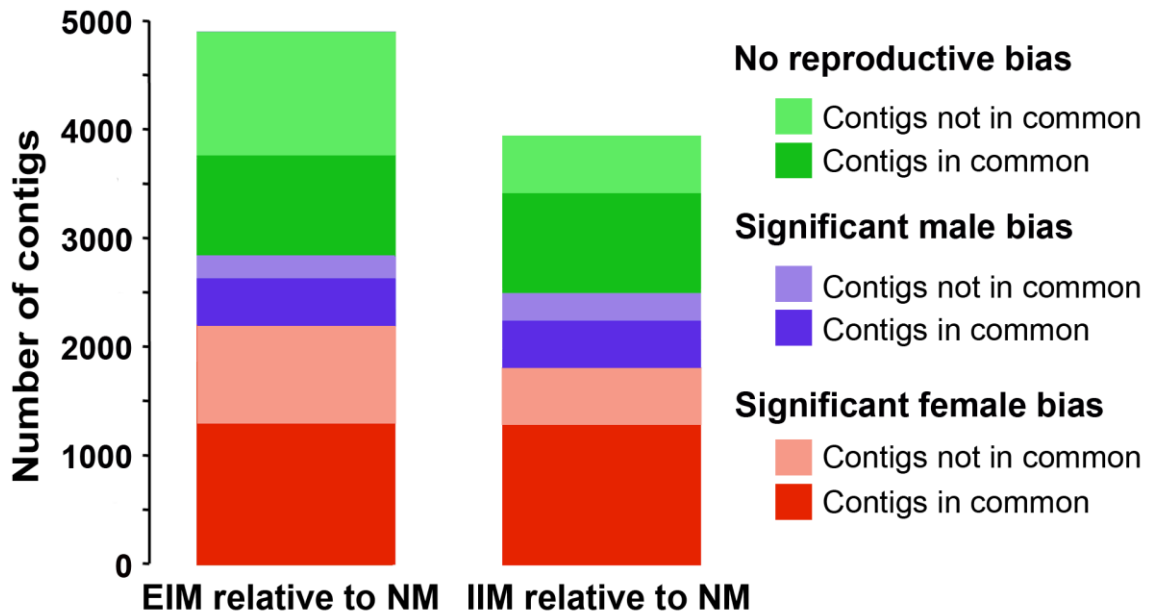
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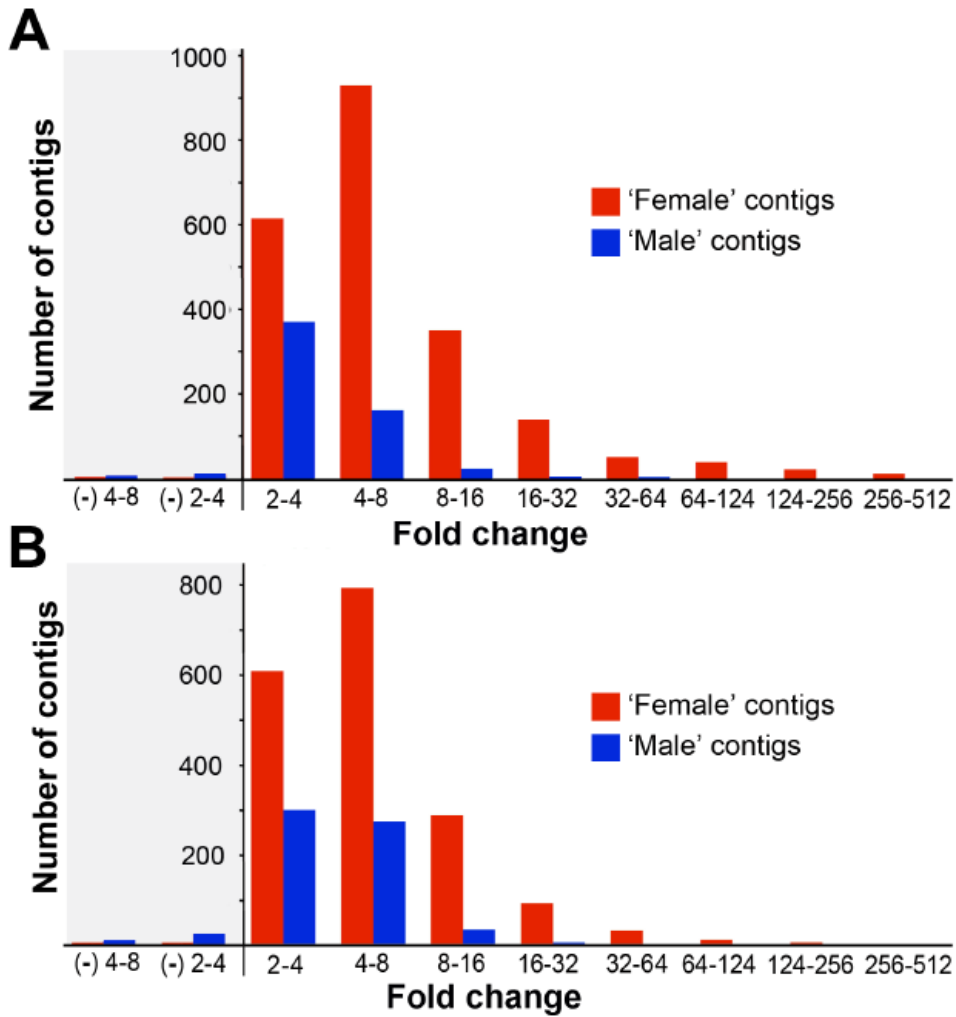
853 **Figure 1.** The reproductive identity and proportions of common contigs associated with  
854 significantly different numbers of associated reads in the *E. marinus* male intersex  
855 phenotypes relative to normal males. NM = normal males, EIM = external intersex  
856 males, IIM = internal intersex males.  
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860 **Figure 2.** Predicted gene expression fold changes on the basis of over and  
 861 underrepresented 'reproductive' contigs associated with both *E. marinus* male intersex  
 862 phenotypes relative to normal males. **A.** Fold change associated with reproductive  
 863 contigs presenting significantly altered representation in external intersex males relative  
 864 to normal males. **B.** Fold change associated with reproductive contigs presenting  
 865 significantly altered representation in internal intersex males relative to normal males.  
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869 **Figure 3.** Gene Ontology (GO) terms enriched in all annotated ‘female contigs’ and the  
 870 ‘female contigs’ overrepresented in both intersex phenotypes relative to the distribution  
 871 of functions associated with the annotated portion of the transcriptome atlas. **A.** Enriched  
 872 GO terms in the ‘female contig’ list. **B.** Enriched GO terms associated with ‘female  
 873 contigs’ overrepresented in both intersex phenotypes. Related GO terms are the same  
 874 colour. The more highly enriched a category, the darker the shading of the  
 875 representative bar. The extent of enrichment was determined using the EASE Score (a  
 876 modified Fisher Exact *P*-Value, for gene-enrichment analysis) that ranges from 0 to 1. A  
 877 score of 0 would represent perfect enrichment and a probability <1E-01 was selected as  
 878 a cut-off. As recommended (DAVID v6.7), a *P*-Value of ≤5E-02 is considered to  
 879 represent strong enrichment. \*full GO term: Generation of precursor metabolites and  
 880 energy.  
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