

# Identification of general patterns of sex-biased expression in *Daphnia*, a genus with environmental sex determination

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## 1 Abstract

2 *Daphnia* reproduce by cyclic-parthenogenesis, where phases of asexual reproduction are  
3 intermitted by sexual production of diapause stages. This life cycle, together with  
4 environmental sex determination, allow the comparison of gene expression between genetically  
5 identical males and females. We investigated gene expression differences between males and  
6 females in four genotypes of *Daphnia magna* and compared the results with published data on  
7 sex-biased gene expression in two other *Daphnia* species, each representing one of the major  
8 phylogenetic clades within the genus. We found that 42 % of all annotated genes showed sex-  
9 biased expression in *D. magna*. This proportion is similar both to estimates from other *Daphnia*  
10 species as well as from species with genetic sex determination, suggesting that sex-biased  
11 expression is not reduced under environmental sex determination. Among 7453 single copy,  
12 one-to-one orthologs in the three *Daphnia* species, 707 consistently showed sex-biased  
13 expression and 675 were biased in the same direction in all three species. Hence these genes  
14 represent a core-set of genes with consistent sex-differential expression in the genus. A  
15 functional analysis identified that several of them are involved in known sex determination  
16 pathways. Moreover, 75 % were overexpressed in females rather than males, a pattern that  
17 appears to be a general feature of sex-biased gene expression in *Daphnia*.

18

## 19 Short summary

20 In some species with environmental sex determination, gene expression can be compared  
21 between genetically identical males and females. Here, we investigated sex-biased expression  
22 in one such species, *D. magna*, and compared it with data from two congeners. We found that  
23 all three species have a common set of 675 genes with consistent differential expression and  
24 with a strong bias towards overexpression in females rather than males. Moreover, the  
25 proportion of sex-biased genes in each of the three *Daphnia* species was similar to *Drosophila*

26 species with genetic sex determination, suggesting that sex-biased expression is not necessarily  
27 reduced under environmental sex determination.

28

## 29 Introduction

30 Patterns of gene expression often differ strongly between male and female individuals  
31 of the same species (Ellegren & Parsch 2007). In part, this difference is driven by genes on sex  
32 chromosomes, which show a particularly strong tendency for sex-biased (or sex-differential)  
33 expression (Ellegren & Parsch 2007; Bergero & Charlesworth 2009; Grath & Parsch 2016).  
34 However, sex-biased expression also occurs in many autosomal genes, and their products also  
35 fundamentally contribute to differences between male and female phenotypes (Ellegren &  
36 Parsch 2007; Grath & Parsch 2016; Wright *et al.* 2017). A particularly interesting case is that  
37 of species with environmental sex determination (ESD), where the same genotype may develop  
38 into a male or female, depending on environmental cues. Pure ESD species do not have sex  
39 chromosomes, and sex differentiation entirely relies on autosomal genes (Bull 1985). In GSD  
40 species, on the other hand, sex chromosomes contain a particularly high number of sex-biased  
41 genes (Mank 2009; Grath & Parsch 2016), and some of the genetic differences between sexes  
42 (e.g., sex-specific genomic regions or allelic differences) cause further, downstream expression  
43 differences, including for autosomal genes (Yang *et al.* 2006; Wijchers & Festenstein 2011).  
44 Both these observations suggest that species with ESD may have a lower number of genes with  
45 sex-biased expression compared to species with GSD. Alternatively, however, species with  
46 ESD may show a higher number of genes with sex-specific expression than species with GSD,  
47 because no genetic differences exist between sexes, and their entire sex-specific phenotypes are  
48 by differential gene expression (Grath & Parsch 2016, Mank 2017).

49 Among the species with ESD that have so far been investigated for sex-biased  
50 expression (Torres Maldonado *et al.* 2002; Shoemaker *et al.* 2007; Yatsu *et al.* 2016;

51 Radhakrishnan *et al.* 2017), we find two species of the genus *Daphnia* (Colbourne *et al.* 2011;  
52 Huylmans *et al.* 2016). *Daphnia* reproduce by cyclical parthenogenesis: during the asexual  
53 phase of the life cycle, mothers clonally produce sons or daughters, but this asexual phase is  
54 intermitted by sexual reproduction, leading to the production of diapause eggs, which give rise  
55 to female hatchlings. Males and females are morphologically distinct (Scourfield & Harding  
56 1966), and the sex of the clonally produced offspring is determined by environmental factors  
57 such as shortened photoperiod and/or increased population density (Roulin *et al.* 2013;  
58 Korpelainen 1990; Hobaek & Larsson 1990). Specifically, the production of males is induced  
59 by a hormone emitted by the mother in response to environmental conditions (Olmstead &  
60 Leblanc 2002). Moreover, male production can also be induced experimentally by adding the  
61 hormone analogue methyl farnesoate (MF) to the culture medium at a precise moment of the  
62 ovarian cycle corresponding to 48 to 72h after moulting (Olmstead & Leblanc 2002).

63         Since the publication of the *D. pulex* genome (Colbourne *et al.* 2011), the amount of  
64 genomic and transcriptomic resources for the genus has markedly increased (Routtu *et al.* 2014;  
65 Xu *et al.* 2015; Dukić *et al.* 2016 ; Orsini *et al.* 2016; Huylmans *et al.* 2016; Giraudo *et al.*  
66 2017; Lynch *et al.* 2017; Spanier *et al.* 2017; Toyota *et al.* 2017; Ye *et al.* 2017; Herrmann *et*  
67 *al.* 2018). Previous studies have investigated sex-biased gene expression in two *Daphnia*  
68 species (Colbourne *et al.* 2011; Eads *et al.* 2007; Huylmans *et al.* 2016). The two species, *D.*  
69 *pulex* and *D. galeata* each belong to one of the two major phylogenetic groups within the  
70 subgenus *Daphnia* (Colbourne & Hebert 1997; Ishida, Kotov & Taylor 2006; Adamowicz,  
71 Petrussek & Colbourne 2009). These studies reported a high number of genes with sex-biased  
72 expression in both species and a preponderance of female-biased genes (i.e., genes  
73 overexpressed in females) compared to male-biased genes.

74         The genus *Daphnia* contains a second subgenus, Ctenodaphnia, which notably contains  
75 the species *D. magna*. This species is not only one of the major genomic model organisms of

76 the genus (Miner *et al.* 2012, GenBank accession number: LRGB00000000.1), but also for  
77 studies on sex differentiation under ESD and other sex-related traits, including local adaptation  
78 in male production, evolution of genetic sex determination (which occurs in some genotypes of  
79 *D. magna*, not investigated here), and uniparental reproduction (Kato *et al.* 2011; Galimov,  
80 Walser & Haag 2011; Svendsen *et al.* 2015; Reisser *et al.* 2016; Roulin *et al.* 2013). Here we  
81 present an analysis of sex-biased gene expression in *D. magna*, based on RNA-sequencing of  
82 males and females of four different genotypes (males and females of the same genotypes are  
83 members of the same clone). The four genotypes were used as biological replicates, that is, the  
84 expression of individual genes was classified as “sex-biased” only if the bias was consistent  
85 among all genotypes tested (i.e., if the overall pairwise test with four replicates was significant).  
86 While the primary aim was to study sex-biased gene expression in this important model  
87 organism, we also compare our results to those from the previous studies on *D. pulex* and *D.*  
88 *galeata*. The aims of this comparison were to identify general patterns of sex-biased gene  
89 expression in the genus, and to identify a core-set of genes with consistent sex-biased  
90 expression within the genus.

91

## 92 Material and Methods

### 93 Study design and origin of clones

94 We carried out RNA sequencing on adult males and females of *D. magna*, reared under  
95 standard culturing conditions (see below). We used four different genotypes which originated  
96 from a single population in Moscow, Russia (N55°45'48.65", E37°34'54.00"), as biological  
97 replicates. One library was prepared per genotype and sex, resulting in a total of eight libraries.  
98 Furthermore, each library consisted of eight technical (experimental) replicates, that is, eight  
99 clonal individuals of the same genotype and sex, pooled together into the library. Hence, a total  
100 of 64 individuals were raised for the experiment. Technical replicates were used to reduce

101 variation due to small differences in environmental conditions (light, temperature, food, etc.)  
102 on gene expression. Such small environmental differences may be caused, for instance, by  
103 different positions of individuals within the culture tubes in the culture chamber.

104

#### 105 Laboratory culturing

106 Gravid parthenogenetic females were transferred individually to standard culturing  
107 conditions: a single individual in a 50mL Falcon tube containing 20 mL of artificial medium  
108 for *Daphnia* (Klüttgen *et al.* 1994), fed with 150  $\mu$ L of algae solution (50 million of cells of  
109 *Scenedesmus* sp. per mL), and kept at 19°C under a 16:8 hour light-dark photoperiod. Each  
110 technical replicate was reared under these standard conditions during two pre-experimental  
111 clonal generations to remove potential maternal effects (Gorbi *et al.* 2011). To that end, one  
112 randomly selected offspring of the second clutch was transferred to a new tube to start the next  
113 clonal generation. Third-generation offspring were used for RNA sequencing.

114 Third generation males were produced by placing second generation females in a  
115 medium containing 400  $\mu$ M of methyl farnesoate (“MF”, Echelon Biosciences) just prior the  
116 production of their first clutch, as determined by well-swollen ovaries (Olmstead & Leblanc  
117 2002). This ensured that the sex of their second clutch offspring was determined in the presence  
118 of MF. Otherwise, males were treated in the same way as described for the females. In  
119 particular, the newborn males were transferred back to standard medium, just as the third-  
120 generation females, and, throughout, culture media were exchanged daily for both males and  
121 females.

122

#### 123 Sampling

124 Just before the third-generation females released their first clutch, all individuals (males  
125 and females) were transferred individually to a 1.5 mL well on a culture plate, where they were

126 kept for about three days before being sampled. Since RNA was extracted from whole  
127 individuals, no food was added during the last 12 h before sampling in order to minimize algal  
128 RNA contamination (most of which will be digested and hence degraded after 12 h). The period  
129 without food was kept relatively short to minimize starvation-dependent gene regulation.

130 To remove as much culture medium as possible, the individuals were blotted with  
131 absorbing paper (previously sterilized with UV radiation for 30 minutes), and then transferred  
132 to a 1.5 mL tube that was directly immersed in liquid nitrogen. Directly after the flash-freezing,  
133 three volumes of RNAlater ICE solution were added to preserve RNA, and samples were placed  
134 at -80°C.

135

#### 136 RNA extraction, library preparation, and RNA-sequencing

137 The eight technical replicates (individuals of the same genotype and sex) were pooled,  
138 resulting in two samples (one male, one female) per biological replicate. Total RNA extraction  
139 and purification was carried out for each of the eight samples following the protocol of the  
140 *Daphnia* genomic consortium (DGC; DGC, Indiana University October 11, 2007), using Trizol  
141 Reagents and Qiagen RNEasy Mini Kit. The extracted and purified RNA samples were  
142 transferred to -80°C and shipped on dry ice to the BSSE Genomic Sequencing Facilities,  
143 University of Basel, Switzerland.

144 Two lanes of cDNA corresponding to the eight biological replicates were constructed  
145 by the Department of Biosystems Science and Engineering (D-BSSE). The eight samples were  
146 individually labelled using TruSeq preparation kits. Each library (2 lanes) was sequenced on an  
147 Illumina NextSeq 500 sequencer with 76 cycles with the stranded paired-end protocol.

148

#### 149 Quality control and filtering

150 Read quality was assessed with FastQC v.0.10.1

151 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Paired-end sequences were  
152 subjected to adapter trimming and quality filtering using trimmomatic v.0.36 (Bolger, Lohse &  
153 Usadel 2014). After trimming of adapter sequences, terminal bases with a quality score below  
154 three were removed from both ends of each read. Then, using the sliding window function and  
155 again moving in from both sides, further 4 bp-fragments were removed while their average  
156 quality score was below 15.

157

### 158 Mapping and counting

159 Filtered reads were mapped to the publicly available *D. magna* genome assembly (NCBI  
160 database; Assembly name: daphmag2.4; GenBank assembly accession: GCA\_001632505.1,  
161 Bioprojects accession: PRJNA298946), consisting of 28'801 scaffolds, 38'559 contigs and a  
162 total sequence length of 129'543'483 bp, as well as a genome annotation with 26'646 genes,  
163 using the RNA-Seq aligner STAR (Dobin et al. 2013) using default settings. The raw counts  
164 (number of mapped reads per transcript per sample) were obtained with the software  
165 featureCounts (Liao, Smyth & Shi 2014), a fast tool for counting mapped paired-end reads.  
166 Counts were summarized at the gene level using the annotation file.

167

### 168 Differential gene expression

169 The analysis of differential gene expression was carried out with DESeq2 (version  
170 1.10.1, Love, Huber & Anders 2014) implemented in R (R Core Team 2017). Raw read counts  
171 were used as input data, and the subsequent analyses used the normalizations of read counts as  
172 performed by DESeq2, which is currently considered best practice for the analysis of RNA-  
173 sequencing data (Conesa *et al.* 2016; Schurch *et al.* 2016). The males vs. females comparison  
174 was carried out with a two-factor design taking into account clone identity and sex. All *p*-values  
175 were adjusted for multiple testing with the Benjamini-Hochberg method, as implemented in

176 DESeq2. Genes were considered differentially expressed (DE) if they had an adjusted  $p$ -value  
177  $< 0.05$  (False discovery rate, FDR = 5 %). The degree of sex bias was determined by the fold-  
178 change (abbreviated FC) difference between the treatments. DE genes were classified into five  
179 groups:  $<2$ -fold,  $>2$ -fold, 2- to 5-fold, 5- to 10-fold and  $>10$ -fold difference in expression  
180 (absolute changes rather than log-transformed changes). To obtain a broader overview of the  
181 expression profiles of the significantly DE genes, we performed a hierarchical clustering of the  
182 sex-DE genes, as implemented in DESeq2. The normalization in DESeq2 does not account for  
183 transcript length, hence it is possible that some differential exon usage (that could ultimately  
184 result in the existence of different isoforms) could be mistakenly interpreted as differential  
185 expression. However, because different transcripts of most genes differ only weakly to  
186 moderately in size (Chern *et al.* 2006), normalization by transcript length (which has been  
187 criticized for other reasons, Dillies *et al.* 2013) would not strongly affect the inferred levels of  
188 fold-change in expression levels. Therefore, inferences of differential expression should  
189 typically be robust, at least in the class of genes with a greater than two-fold change.

190

#### 191 Comparison of sex-biased gene expression with *D. galeata* and *D. pulex*

192 Protein sequences of *D. magna* (v2.4 GenBank: LRGB000000000), *D. pulex* (version  
193 JGI060905: [http://wfleabase.org/release1/current\\_release/fasta/](http://wfleabase.org/release1/current_release/fasta/)) and *D. galeata*  
194 ([http://server7.wfleabase.org/genome/Daphnia\\_galeata/](http://server7.wfleabase.org/genome/Daphnia_galeata/)) reference genomes were used as input  
195 in the software OrthoFinder (Emms & Kelly 2015), a fast method for inferring orthologous  
196 groups from protein sequences with enhanced accuracy. These correspond to 26'646, 30'940,  
197 and 33'555 protein sequences, respectively. We also used another software, OrthoMCL (Li,  
198 Stoeckert & Roos 2003) for inferring orthologs. Because the results were qualitatively and  
199 quantitatively similar, we present only the results of OrthoFinder here. For further analysis, we  
200 retained only those genes that were identified by OrthoFinder as single copy, one-to-one

201 orthologs in all three species. We then compared this list with our list of sex-DE genes (adjusted  
202  $p < 0.05$ ), as well as the lists of sex-DE genes in *D. galeata* and *D. pulex* (Colbourne *et al.* 2011;  
203 Huylmans *et al.* 2016). The R package VennDiagram (Chen & Boutros 2011) was used for  
204 visualization of sex-DE genes in the three species.

205 We then focused on the core subset of 675 orthologous genes that were found to be  
206 consistently sex-DE in all three species and used BLAST2GO (version 4.1.9, (Conesa *et al.*  
207 2016) to perform a functional annotation. The protein sequences of the *D. magna* genes in  
208 question were annotated using the NCBI nr database, allowing for 20 output alignments per  
209 query sequence with an e-value threshold of 0.001. The mapping and annotation steps  
210 implemented in BLAST2GO were run with default settings. Additionally, InterPro IDs from  
211 InterProScan were merged to the annotation to improve accuracy. Graphical representation of  
212 GO categories belonging to the 675 core-genes was obtained using the R package metacoder  
213 (Foster, Sharpton & Grünwald 2017). The BLAST2GO output files were searched for the terms  
214 “sex determination” (GO accession number 0007530), “sex differentiation” (0007548), “male  
215 sex differentiation” (0046661), “female gonad development” (0008585), “male gonad  
216 development” (0008584), “female sex determination” (0030237), “male sex determination”  
217 (0030238), and “female sex differentiation” (0046660). In addition, a universal list of genes  
218 involved in sex determination and sex differentiation pathways was established by searching  
219 the entire Genbank database for genes associated with the terms “sex  
220 determination/differentiation”. After removal of redundancies, we obtained a list of 541 genes  
221 (hereafter referred to as the “NCBI list of genes”). We compared the annotations of our 675  
222 core-genes (as determined by the BLAST2GO analysis) with this list to identify any shared  
223 genes. Finally, we performed an enrichment analysis of GO terms for the 675 core genes with  
224 consistent sex-biased expression in all three species, taking the 26’646 genes of *D. magna* as  
225 the reference GO composition. This was done using the GOatools Python script

226 (<https://github.com/tanghaibao/goatools>), which performs Fisher's exact tests for differences in  
227 frequencies of GO-terms between the two lists (with Bonferroni correction). Enriched GO  
228 categories were summarized by a reduction of the complexity and level of GO terms (medium;  
229 allowed similarity=0.7). UniprotKB was used to determine Gene Ontology Biological Process  
230 and Molecular Functions that were over-represented among genes DE between sexes and  
231 visualized with REVIGO (Supek *et al.* 2011).

232

## 233 Results

### 234 Data quality

235 The RNA sequencing of the eight libraries resulted in a total of 1.59 billion raw reads.  
236 An average of 99.01 % of raw reads passed the quality control. After end-trimming, an average  
237 of 93.03 % aligned to the reference genome, resulting in an average of 81 million aligned reads  
238 per library, which constitutes a robust data basis for differential gene expression analyses.  
239 **Table S1** shows the percentages of reads retained at each step in each of the samples.

240

### 241 Sex-biased gene expression in *Daphnia magna*

242 We found a high number of genes that were DE between males and females with a total  
243 of 11'197 out of 26'646 genes being DE (adjusted  $p < 0.05$ ), of which 8384 genes showed at  
244 least a two-fold change (**Table 1**). The strong sexually dimorphic expression patterns can be  
245 visualized in the expression heatmap of the 8384 DE genes with more than 2-fold expression  
246 difference between the sexes (**Fig. 1**). Overall, a slight, but significant ( $p < 0.0001$ ) majority of  
247 those genes were male-biased rather than female-biased (**Table1**). This male-bias was found  
248 for all categories, except for the genes with a weak (< 2-fold) sex bias (**Table 1**). The list of all  
249 sex-biased genes can be found in supplemental data (**Table S2**).

250

## 251 Comparisons of sex-biased gene expression among the three *Daphnia* species

252 The software OrthoFinder identified 7453 single copy, one-to-one orthologs present in  
253 all three species (**Table 2**). Among these, 5707 (76.5 %) were sex-DE in at least one species,  
254 and 707 genes (9.5 %) were sex-DE in all three species (**Fig. 2, Table2**). Only 32 of these 707  
255 genes (4.5 %) showed a different direction of bias in one of the species. The remaining 675  
256 genes were biased in the same direction in all three species, and we therefore refer to these  
257 genes as the core-set of sex-DE genes in *Daphnia*. Among the genes of the core-set, 75 % were  
258 female-biased (**Fig. 2**), and genes with a strong expression-bias between sexes were more likely  
259 to be included in this core-set (**Fig. 3**). Genes that showed significant sex-biased expression in  
260 only two out of the three species showed very similar patterns: a high proportion showed  
261 consistent bias (i.e., in the same direction in both species), and there was an excess of female-  
262 biased compared to male-biased genes (**Fig. 2**). The excess of female-biased genes was even  
263 observed among the genes with sex-biased expression in only one species (**Table S3**). This was  
264 not only the case in *D. pulex* and *D. galeata*, for which an excess of female-biased genes had  
265 been reported earlier (Huylmans *et al.* 2016), but also in *D. magna*, where this result contrasts  
266 with the slight excess of male-biased genes found when all 26'646 genes were considered (as  
267 opposed to only the 7453 genes, for which single-copy, one-to-one orthologs could be identified  
268 in the other two species). The list of the 7453 orthologs, as well as the data on sex-biased  
269 expression for the three species is given in the supplementary data (**Table S3**).

270

## 271 Functional analysis of the core-set of sex-DE genes

272 Among the core-set of 675 orthologous genes that were consistently sex-DE in all three  
273 species, 592 had an annotated function. The major GO categories of these genes are shown in  
274 **Fig. 4**. This figure highlights that the largest fraction of genes belongs to the categories “cellular  
275 process”, “metabolic process”, “single organism process” and “biological regulation”. The

276 results of the GO enrichment analysis are shown in **Fig. 5**. Enriched terms are linked to “RNA  
277 binding” processes, known to play a key role in post-transcriptional gene regulation (Glisovic  
278 *et al.* 2008; Cléry & Allain 2013) and, more generally, terms linked to “RNA”. Of the 592 genes  
279 only one gene has a GO term linked to sex determination or sex differentiation (which is neither  
280 more nor less than expected by chance): The gene “peptidyl-prolyl cis-trans isomerase FKBP4”  
281 has a female-biased expression in all three species and its GO term includes “male sex  
282 differentiation”. Moreover, among the same 592 core genes with a functional annotation, 14  
283 were listed in the NCBI list of genes known to be involved in sex determination or  
284 differentiation pathways in other species (**Table S4**).

285

## 286 Discussion

### 287 Sex-biased gene expression in *Daphnia magna*

288 We found a very high number of genes being DE between males and females in *D.*  
289 *magna*. This result is largely congruent with the previous studies on other *Daphnia* species  
290 (Colbourne *et al.* 2011; Huylmans *et al.* 2016), but the overall number and proportion of genes  
291 that show sex-biased expression is higher in *D. magna* than in the other two species. Indeed,  
292 the proportion of genes with sex-biased expression in *D. magna* is similar to that reported for  
293 species with genetic sex determination (GSD). For instance, two early, microarray-based  
294 studies on *Drosophila melanogaster* found two-fold or greater expression differences between  
295 sexes in 30 % to 40 % of all genes (Parisi *et al.* 2004, Innocenti & Morrow 2010). A more  
296 recent study based on RNA-sequencing found that about two-third of genes showed sex-biased  
297 expression in multiple *Drosophila* species (genes with a less than two-fold change included).  
298 In *D. magna*, the proportion among all genes is 42 % (31 % with a greater than two-fold  
299 change). However, considering only the set of single-copy, orthologous genes (which likely  
300 contain a lower proportion of annotation errors, see below), the proportion is 65% (41% with a

301 greater than two-fold change). Our results therefore suggest that these two systems, which are  
302 comparable in terms of body size and due to the fact that whole, adult animals were sampled,  
303 have similar proportions of sex-biased genes. More studies on comparable ESD-GSD species  
304 pairs will have to be investigated to determine the generality of this conclusion.

305 A recent study on *D. pulex* found that the annotation of the genome used here  
306 (Colbourne *et al.* 2011) likely contained a non-negligible fraction of pseudogenes or other false  
307 positives (Ye *et al.* 2017), and that the number of genes in the genome may be closer to 20'000  
308 than the initially estimated ~31'000. It is possible that the current estimates of the total number  
309 of genes in the *D. magna* (~27'000) and *D. galeata* (~34'000) genomes may also be  
310 overestimates. Using these genomes as reference in a differential gene expression analysis may  
311 have affected the estimate of the proportion of differentially expressed genes only if the  
312 proportion of falsely annotated genes is different among the DE genes than among the non-DE  
313 genes. It is unclear if any such bias exists. However, two independent lines of evidence suggest  
314 that, if anything, such a bias has led to an underestimation of the proportion of sex-DE genes:  
315 First, the proportion of sex-DE genes was higher among the single-copy orthologs (which less  
316 likely contain annotation error) than among all genes in two out of three *Daphnia* species.  
317 Second, *D. pulex* and *D. galeata*, which both have higher estimated number of genes than *D.*  
318 *magna*, have a lower estimated proportion (~20 %) of sex-DE genes. Yet, the differences in the  
319 proportions of sex-DE genes among the three *Daphnia* species may also be explained by  
320 differences in methodology and statistical power. The *D. pulex* data (Colbourne *et al.* 2011) are  
321 based on a microarray study, a methodology known to be less sensitive for lowly-expressed  
322 genes than RNA-sequencing (Harrison, Wright & Mank 2012). The *D. galeata* study  
323 (Huylmans *et al.* 2016) was based on RNA-sequencing but used only two clonal lines as  
324 biological replicates. As mentioned by Huylmans *et al.* (2016), this may have led to a rather low  
325 statistical power to detect sex-DE genes, especially among the considerable number of genes

326 that showed expression differences between the two clones.

327         When comparing the proportion of genes with sex-biased expression with other studies,  
328 it is important to remember that we performed RNA-sequencing on whole animals and hence  
329 included all tissues present at that the time of sampling in adult males and females. Patterns of  
330 sex-specific gene expression are known to be tissue-specific in many cases (Ellegren & Parsch  
331 2007; Toyota *et al.* 2017), with strongest differences being found in brains (at least in mammals)  
332 and, unsurprisingly, in gonad tissues (Mank 2009). Thus, it is difficult to compare our results  
333 with those in larger animals, where studies have mostly been carried out on specific tissues  
334 (e.g., 54.5 % of genes were found to be sex-DE in *Mus musculus* liver, (Yang *et al.* 2006)).  
335 Moreover, genes that do not show sex-biased expression may still differ in their expression  
336 patterns among tissues (Yang *et al.* 2006). Hence, when sampling whole animals, some of these  
337 genes may be identified as sex-biased because different tissues may occur in different  
338 proportions in males vs. females, for instance due to anatomical differences between sexes. It  
339 is possible that a part of the genes that were found to be sex-biased in our study and in other  
340 studies based on whole animals (e.g., *Drosophila*) are explained by such effects (i.e., sex-biased  
341 expression of these genes may be a consequence rather than a cause of the phenotypic  
342 differences between sexes (Mank 2017)).

343         Another factor that potentially contributes to an overestimation of the number of sex-  
344 DE genes is the fact that males were produced by artificially treating their mothers with the  
345 juvenile hormone analog MF (Huylmans *et al.* 2016). However, in a separate study we found  
346 that MF exposure changes expression levels of a much lower number of genes (only a few 100s)  
347 than were DE between sexes (Molinier *et al.*, in prep). Moreover, the males used in our  
348 experiment were exposed to MF only for three days when they were still oocytes inside the  
349 ovaries of their mothers up to one day after they were released from the brood pouch. It is thus  
350 highly unlikely that large proportions of the sex-DE genes are in fact explained by the effects

351 of early MF exposure and would not have shown up in naturally produced males (which also  
352 involves exposure to a natural juvenile hormone produced by their mother).

353 Contrary to previous findings in *D. galeata* and *D. pulex* (which both show a clear  
354 excess of female-biased genes over male-biased genes), we observed a slight excess of male-  
355 biased genes in *D. magna*. It is currently difficult to say whether this difference between studies  
356 reflects a biological reality (i.e., difference between species within the genus) or whether it may  
357 be explained by some methodological differences between the studies. Interestingly, however,  
358 a strong excess of female-biased genes was recovered also in *D. magna* when investigating the  
359 subset of single-copy genes for which one-to-one orthologs could be identified in the other  
360 species. The excess of female-biased genes might be a general feature in the genus *Daphnia*, at  
361 least for single-copy genes that are sufficiently conserved for orthologs to be identified across  
362 the major sub-clades of the genus. On the other hand, the strong excess of female-biased single-  
363 copy one-to-one orthologs, suggests that the remaining genes show a substantial excess of male-  
364 biased expression, at least in *D. magna*. The remaining genes likely include many paralogs and  
365 other less conserved genes, for which the identification of orthologs is difficult. Hence, different  
366 evolutionary rates of genes with male-biased vs. female-biased expression could drive the  
367 observed patterns. Genes with male-biased expression evolve faster than female-biased genes  
368 in *D. pulex* and *D. galeata*, as well as in *Drosophila*, *Caenorhabditis*, and several mammal  
369 species (see reviews by Ellegren & Parsch 2007 and Parsch & Ellegren 2013). Faster evolution  
370 of genes with male-biased expression might be explained by positive selection being more  
371 common in these genes (Ellegren & Parsch 2007), and may lead to ortholog identification being  
372 more difficult in these genes, which may explain or at least contribute to the observed difference  
373 between the two sets of genes in *D. magna*.

374

375 Comparisons of sex-biased gene expression among the three *Daphnia* species

376           The 7453 single-copy, one-to-one orthologs present in all three species represent less  
377 than 30 % of all genes used in the *D. magna* analysis. However, as pointed out above, the  
378 number of genes predicted by the current genome annotation used in this study may in fact be  
379 a rather strong overestimation of the true number of genes present in the species. Secondly, the  
380 software identified a considerably higher number of orthogroups, which, however, also include  
381 paralogs. We decided to restrict the analysis to single-copy, one-to-one orthologs because  
382 interpretation of expression patterns in paralogs is much less straightforward. For instance, if a  
383 sex-DE gene is single-copy in one species but has two paralogs in another species due to  
384 duplication after speciation, it is difficult to say which one of the two genes is more homologous  
385 in function, and hence should also be sex-DE if the gene belongs to the core-set of genes with  
386 sex-biased expression in the genus (Koonin 2005).

387           Only a low percentage of the genes found to be sex-biased in two and especially in three  
388 species were biased in different directions. Moreover, genes with a higher expression bias were  
389 more likely to be sex-DE in two or three species than just in one. While the latter observation  
390 may in part be explained by issues of statistical power (genes with a low degree of sex-bias  
391 have a lower probability to be detected), both observations nonetheless suggest that the 675  
392 orthologous genes that were found to be consistently sex-DE in all three species indeed  
393 represent a robust core-set of sex-biased genes in the genus *Daphnia*. It is likely that some genes  
394 that were DE between a pair of species but not in all species should also have been included in  
395 this core-set, as differential expression may have been non-significant in one species just due  
396 to a lack of statistical power. Indeed, the three studies differ in methodology (microarray vs.  
397 RNA-Sequencing), number of biological replicates, aspects of data analysis, etc (see above). In  
398 addition, the quality of the genome assemblies and annotations used to analyze these data may  
399 also differ between species. These differences may also explain some of the between-species  
400 differences in the number and proportion of sex-DE genes.

401

402 Functional analysis of the core-set of sex-DE genes

403 Our study identified a core-set of genes for which sex-biased expression is probably  
404 conserved in the genus *Daphnia*. Hence these genes may play a fundamental role in determining  
405 and maintaining male vs. female phenotypes in this genus with environmental sex  
406 determination. The functional analysis of these genes identified the gene “peptidyl-prolyl cis-  
407 trans isomerase FKBP4” with GO term “male sex differentiation”, an immunophilin protein  
408 with peptidylprolyl isomerase and co-chaperone activities. It is a component of steroid receptors  
409 heterocomplexes and may play a role in the intracellular trafficking of hetero-oligomeric forms  
410 of steroid hormone receptors between cytoplasm and nuclear compartments. Steroid receptors  
411 initiate the signal transduction for steroid hormones, including sexual hormones such as  
412 oestrogen and androgen (e.g., Voigt *et al.* 2009). Their role in sex dimorphism, also in species  
413 with environmental sex determination, thus makes sense, though the role of this particular gene  
414 has not yet been investigated in *Daphnia*. The 14 additional genes whose functional annotation  
415 matched of the descriptors of the genes on the NCBI list of genes involved in sex  
416 determination/differentiation pathways may represent further fundamental genes involved in  
417 sex determination or sex differentiation in *Daphnia*. They contain functions known to be  
418 implicated in sexual development in other species, such as the “Beta-catenin 1” which is a key  
419 transcriptional regulator of the canonical Wnt-signaling pathway, known to be implicated in  
420 female reproductive development in mammals (Bernard & Harley 2007, Liu, Bingham &  
421 Parker 2008). We also found the “fibroblast growth factor receptor”, receptor of Fgfs (fibroblast  
422 growth factors), whose function may be involved in sex determination and reproductive system  
423 development in many species and appears to be highly conserved (Colvin *et al.* 2001). Finally,  
424 the gene ‘ovarian tumor’ is also known to be involved and required in the determination of the  
425 sexual identity of female germ cells (Pauli, Oliver & Mahowald 1993). Our results suggest that

426 these genes are involved in maintaining phenotypic differences between sexes, also at the adult  
427 stage, at last in *Daphnia*. A large proportion of the identified core genes have unknown function.  
428 Therefore, we need more functional annotations, especially also on more closely related  
429 species, as well as sex-specific expression data on earlier developmental stages before we can  
430 obtain a clear mechanistic picture of sex determination and sex differentiation in *Daphnia* (Kato  
431 *et al.* 2011).

432 In conclusion, our study provides data on sex-biased gene expression for the model  
433 organism *D. magna* and for *Daphnia* in general, specifically by identifying a core-set of sex-  
434 DE genes in all three major subclades of the genus. More generally, our results suggest that the  
435 proportion of genes with sex-biased expression in ESD species is not lower than in species with  
436 GSD.

437

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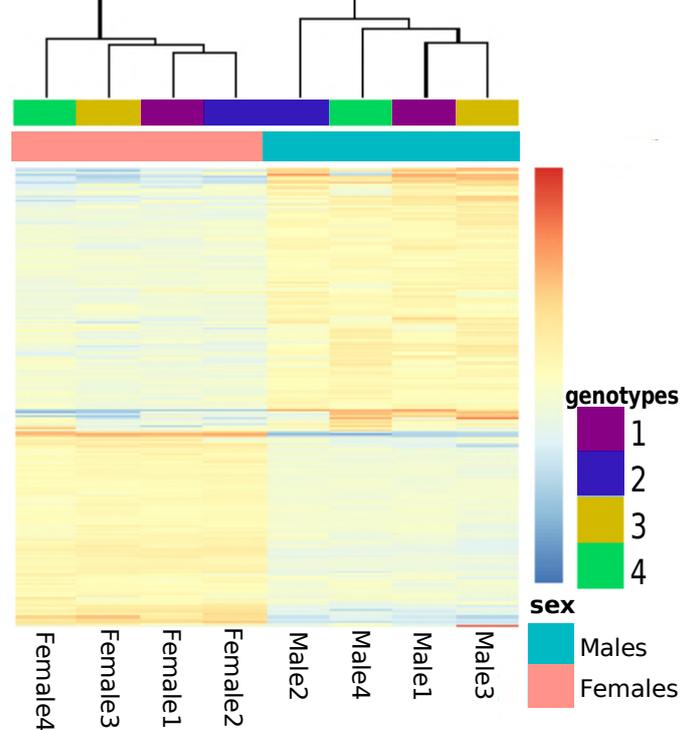
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**Figure 1:** Heatmap showing the normalized expression levels of the sex-DE genes ( $p < 0.05$ ) with at least a two-fold expression difference between males and females. Each line represents a gene and each column specific sample (genotype and sex), with relative expression levels indicated by colour (from highly overexpressed, red to highly underexpressed, blue, as indicated by the scale to the right). The dendrogram above the sample columns indicates clustering according to the Euclidean distance matrix implemented in the heatmap R

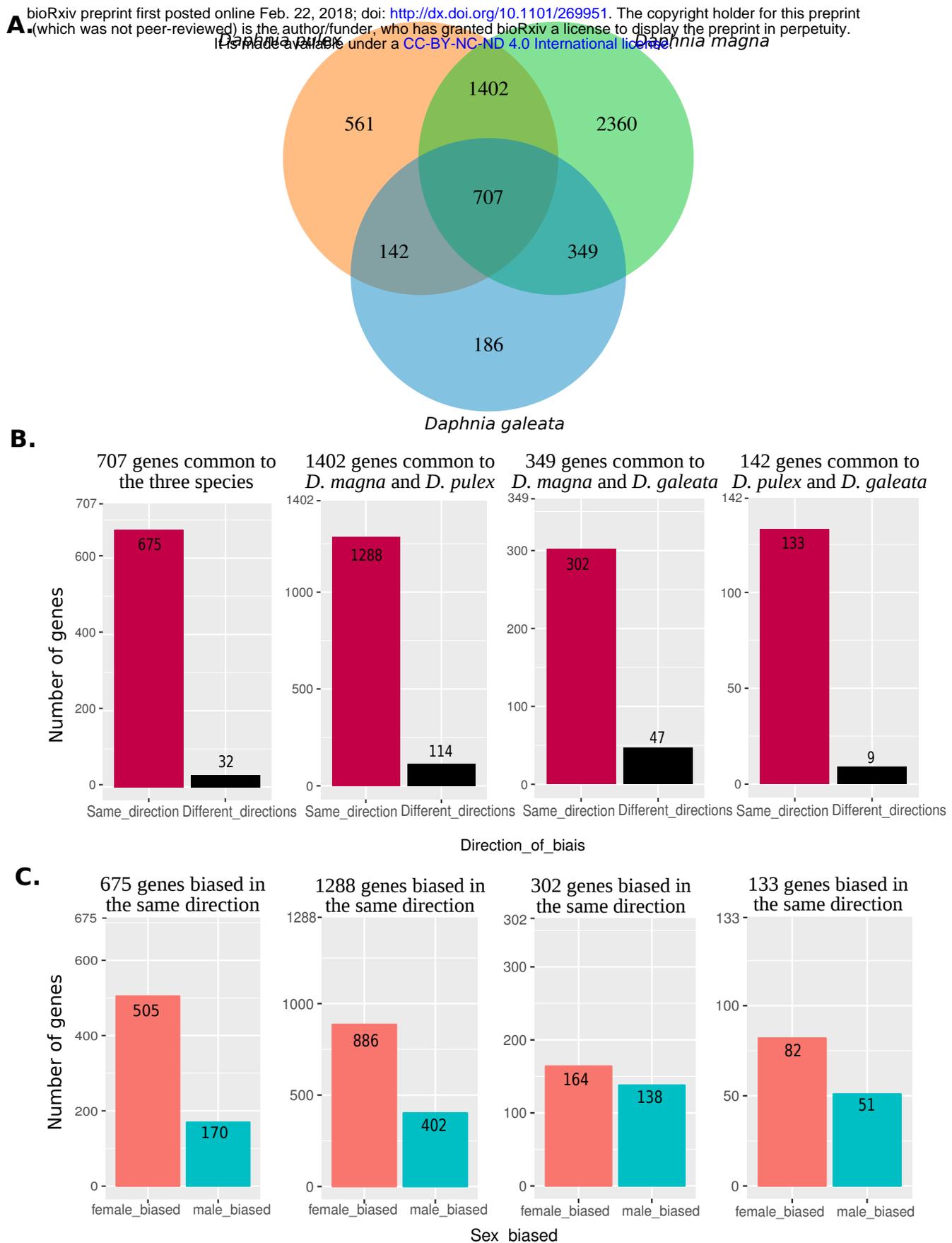
**Table 1:** Numbers of significantly (adjusted  $p < 0.05$ ) sex-DE genes in *Daphnia magna*, for different degrees of bias as well as percentage of genes with male-biased expression.

	All	< 2-fold	2- to 5-fold	5- to 10-fold	> 10-fold	> 2-fold
Number of sex-DE genes	11197	2813	6005	1409	970	8384
Percentage male-biased	53%	38%	57%	64%	53%	58%

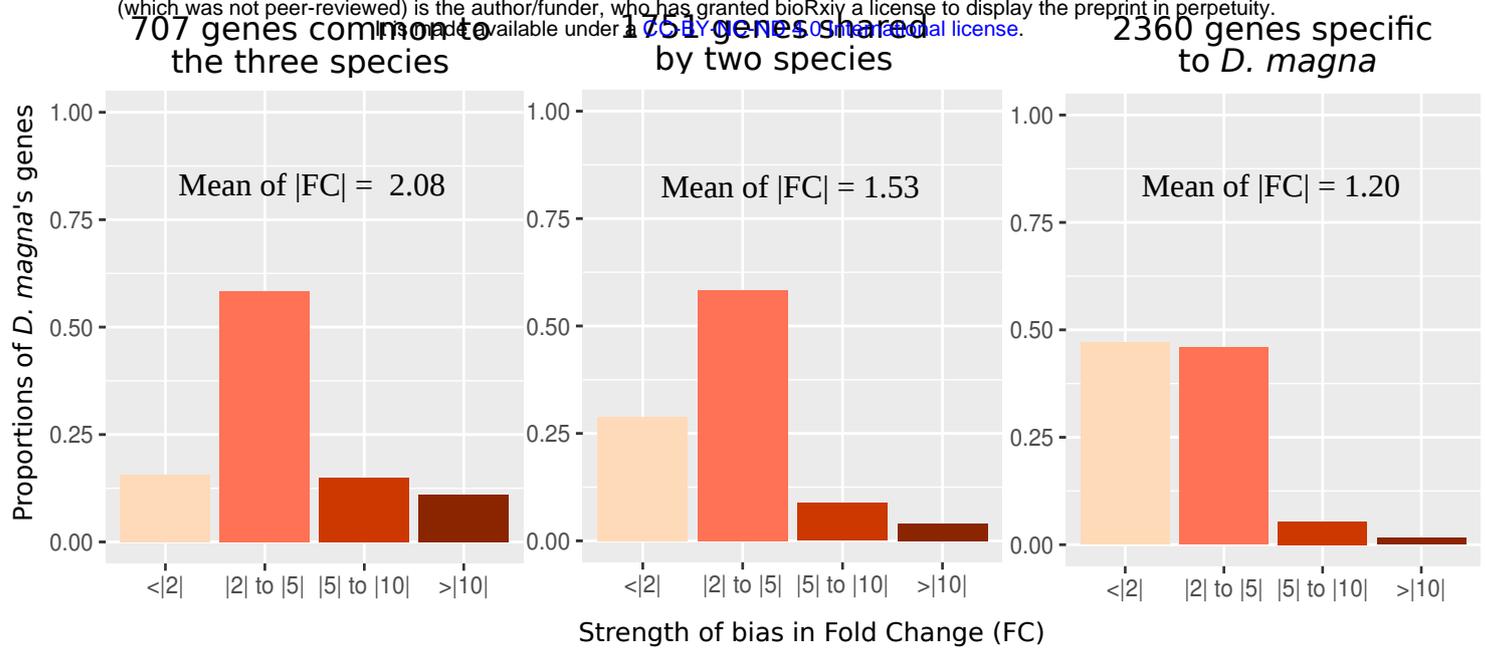
**Table 2:** Number of the one-to-one orthologous genes found by OrthoFinder in the three *Daphnia* species.

Species	Number of protein sequences in the genome	Number of Sex-biased genes <sup>1</sup>	Proportion of sex-biased genes	Number of 1-to-1 orthologues	Number of sex-biased 1-to-1 orthologues
<i>D. magna</i>	26646	11192	0.42	7453	4818
<i>D. pulex</i>	30940	6393	0.21	7453	2812
<i>D. galeata</i>	33555	5842	0.17	7453	1384

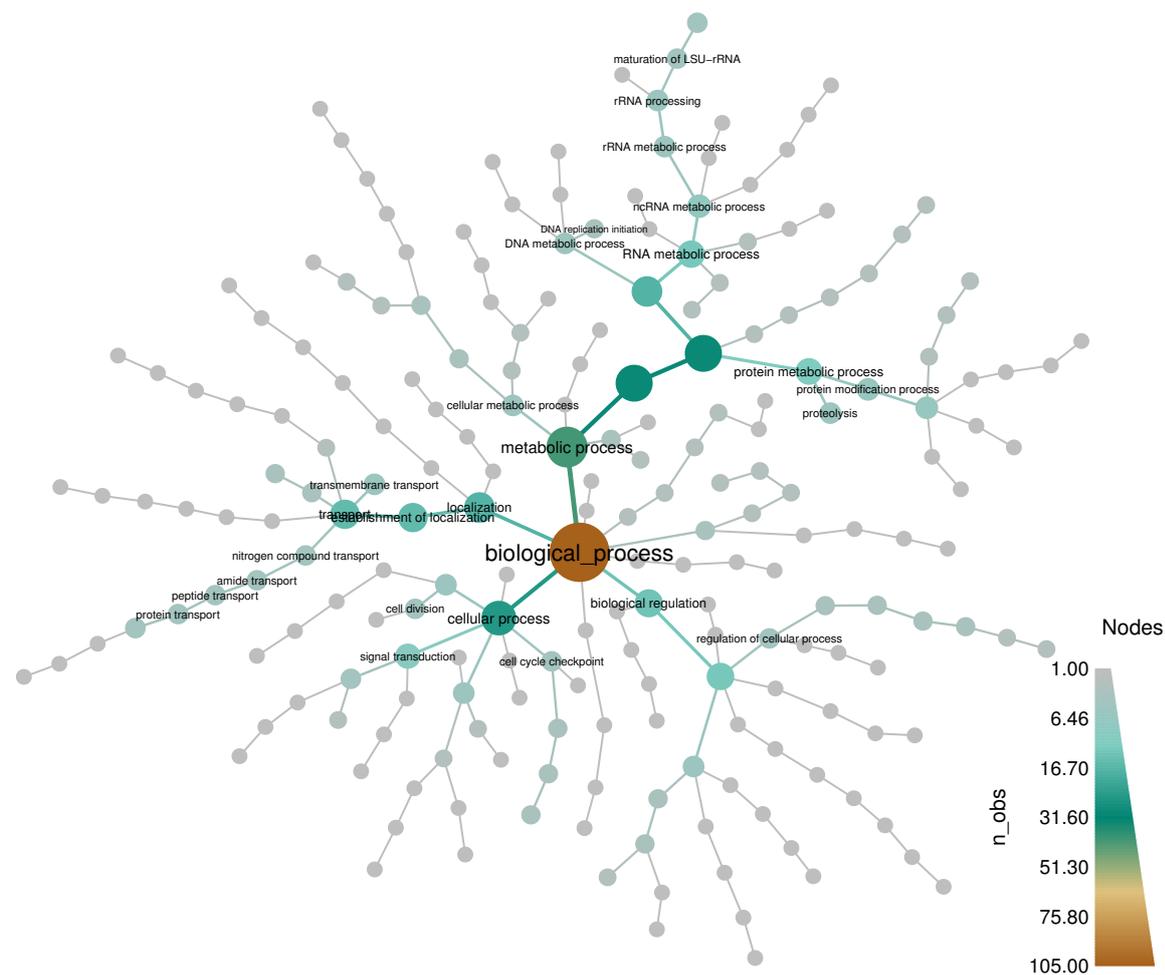
<sup>1</sup> as identified in the single species studies (current study, Colbourne et al 2011, Huyman



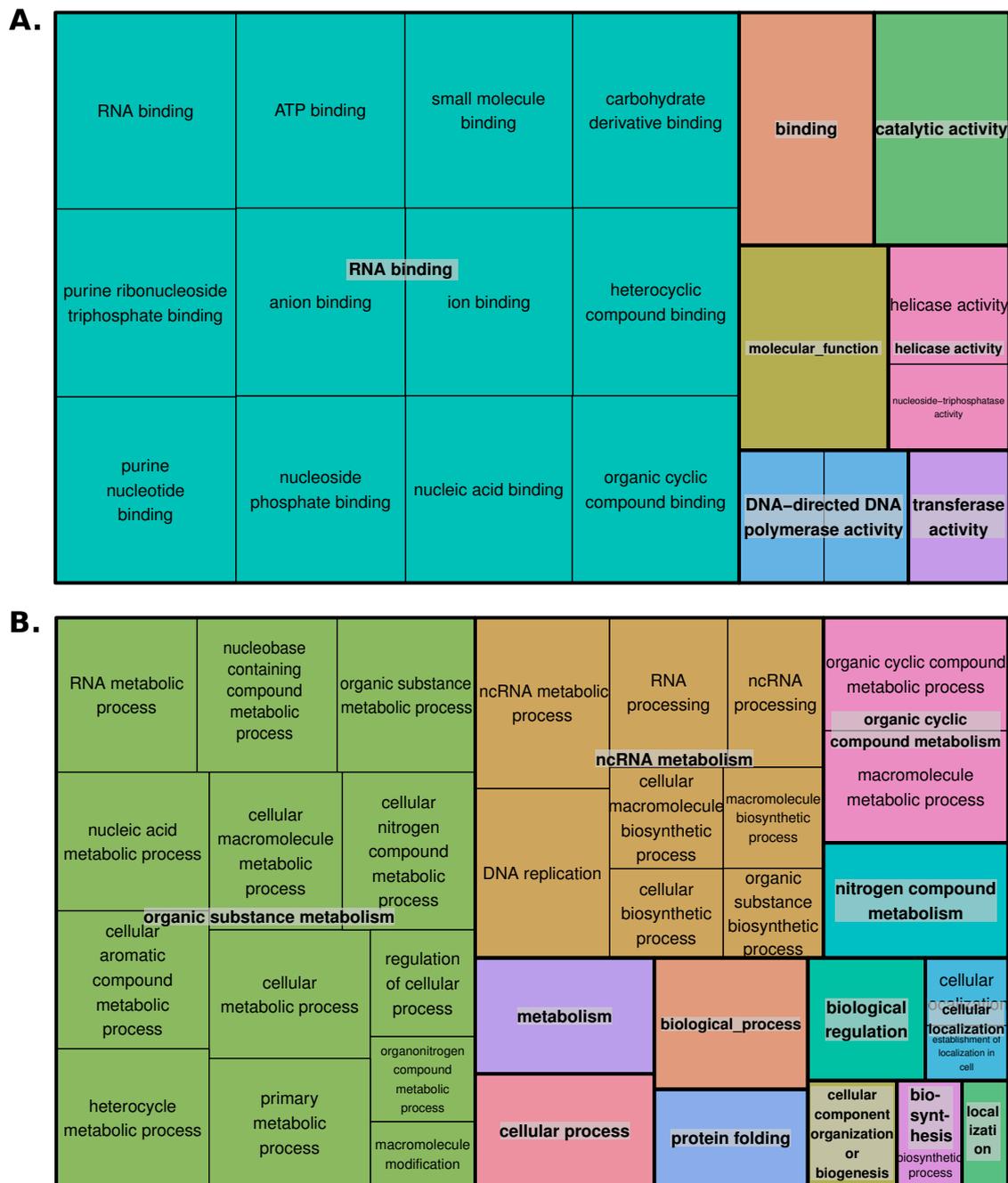
**Figure 2:** **A.** Venn diagram showing the number of sex-DE genes among the 7453 one-to one orthologs in each of the three species of *Daphnia*. **B.** Number of sex-DE genes being biased in the same vs. opposite directions. Panels from left to right: genes being sex-DE all three species (707 genes), genes being sex-DE only in *D. magna* and *D. pulex* (1402 genes), *D. magna* and *D. galeata*, (349 genes), and *D. pulex* and *D. galeata* (142 genes). **C.** Number of female-biased and male-biased genes in each of the four categories depicted in **Figure 2 B.** (only genes being biased in the same direction).



**Figure 3:** Proportion of genes with different degrees of sex-bias (the degree of sex-bias is summarized in four categories of fold change). Panels from left to right: genes being sex biased in all three species (707 genes), in two species (1751 genes), and only in *D. magna* (2360 genes). Only the 7453 genes, for which single-copy, one-to-one orthologs could be identified in all three species were considered for this analysis.



**Figure 4:** Composition and hierarchical organization of GO terms associated to the 675 genes with consistent sex-biased gene expression in all three species. n\_obs: number of genes in the given GO category.



**Figure 5:** Enrichment analysis of GO terms among the core-set of genes compared to the entire list of *D. magna* genes. Shown are over-represented (Bonferroni-corrected  $p < 0.05$ ) terms and functional categories (the latter distinguished by colour) for molecular functions (A) and biological processes (B). The size of each rectangle is proportional to the  $-\log(p\text{-value})$  for its category.