Reproduction associated gene expression under predation risk in *Daphnia* - a comparative transcriptomic approach

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Abstract

Organisms live in a dynamic and often challenging world. Phenotypic plastic responses allow organisms to rapidly adjust to a new environmental situation. Although phenotypic plastic responses to predation risk have been reported for the ecological and genomic model organism *Daphnia*, their genetic basis is not well understood. Here, we characterized the transcriptional profile of *Daphnia galeata* when exposed to fish kairomones. First, we investigated the differential gene expression, identifying candidate transcripts being involved in shifts of life history traits in *D. galeata*. A total of 125 differentially expressed transcripts (40 up- and 85 downregulated) were identified. The expression analysis revealed a surprisingly high variance between clonal lines, likely reflecting their different life history strategies. Second, we applied a gene co-expression network analysis to find clusters of tightly linked transcripts and reveal the genetic pathways underlying predator-induced responses. Our results showed that transcripts involved in remodeling of the cuticle, growth and digestion corresponded to life history shifts in *D. galeata*. Furthermore, we compared our results with previous studies on other *Daphnia* species to assess similarities in the predator-induced responses and *Daphnia* reproduction. Orthologs were identified for *D. magna* related to predator-induced responses and for *D. pulex* and *D. galeata* involved in reproduction. The unique combination of methods including the comparative approach allowed for the identification of candidate transcripts, their functions and orthologs associated with predator-induced responses and reproduction in *Daphnia*.

Keywords: RNA-seq, predator-induced response, gene co-expression, phenotypic plasticity, *Daphnia galeata*

Introduction

Organisms are challenged throughout their lives by environmental changes that have an impact on the health and fitness of each individual. Stress, an internal state initiated by an external factor (stressor) is relative and has to be considered with respect to the ecological niche of an individual (Van Straalen, 2003). A given phenotype that is advantageous in one environmental setup might become disadvantageous in another. In general, organisms have two possibilities to cope with environmental changes: return to the ecological niche by behavioral (i.e. migration) or physiological changes, or change the boundaries of their ecological niche by genetic adaptation (Van Straalen, 2003). The former is achieved at the phenotypic level describing phenotypic plastic responses, while the latter is a genetic

adaptation process, where genotypes with a higher fitness pass on their alleles to the next generation.

Predation is an important biotic factor structuring whole communities (e.g., Aldana et al., 2016; Boaden and Kingsford, 2015), maintaining species diversity (e.g., Estes et al., 2011; Fine, 2015) and driving natural selection in populations (e.g., Kuchta and Svensson, 2014; Morgans and Ord, 2013). Vertebrate as well as invertebrate aquatic predators, release kairomones into the surrounding water (Macháček, 1991; Schoeppner and Relyea, 2009; Stibor, 1992; Stibor and Lüning, 1994). In some instances, kairomones can be detected by their prey, inducing highly variable as well as predator-specific responses to reduce their vulnerability. These predator-induced responses are a textbook example of phenotypic plasticity and have been reported on in detail for a variety of *Daphnia* species (e.g., Boeing et al., 2006; Herzog et al., 2016; Weider and Pijanowska, 1993; Yin et al., 2011).

Daphnia are small branchiopod crustaceans and are an isogenic model organism widely used in ecology, evolution and ecotoxicology. Members of this family link trophic levels from primary producers to consumers in freshwater ecosystems and are, therefore, vulnerable to high predation risk (Lampert, 2011). Extensive shifts in behavior, morphology and life history traits have been observed in response to predation and predation risk. The responses induced by invertebrate predators include morphological changes such as the formation of helmets in *D. cucullata* (e.g., Agrawal et al., 1999) and *D. longispina* (e.g., Brett, 1992) and the formation of neck teeth in *D. pulex* (e.g., Tollrian, 1995). Vertebrate predators have been shown to induce behavioral changes linked to diel vertical migration (DVM: an avoidance strategy; Cousyn et al., 2001; Effertz and Von Elert, 2017) as well as changes in life history traits (Boersma et al., 1998; Effertz and Von Elert, 2017) in *D. magna.* The specificity of such predator-induced responses by vertebrate and invertebrate kairomones has been shown, e.g. for the *D. longispina* species complex from the Swiss lake Greifensee (Wolinska et al., 2007). The documented changes in life history traits included a decrease in size at maturity when exposed to fish kairomones and an increase when exposed to kairomones of the phantom midge larvae, a predatory invertebrate of the genus *Chaoborus*. The species *D. galeata* is somehow peculiar since individuals exposed to fish kairomones do not show a diel vertical migration behavior (Spaak and Boersma, 2001; Stich and Lampert, 1981), nor do they produce morphological changes like helmets or neck teeth (Tams et al., 2018). Long-term (14 days) exposure to fish kairomones in *D. galeata*

revealed substantial life-historical variation within and among populations, as well as trends congruent to previous studies such as a decrease in both age at first reproduction and somatic growth rate in the presence of fish kairomones (Boersma et al., 1998; Stibor and Lüning, 1994; Tams et al., 2018).

Although phenotypic plastic responses to predation risk have been extensively studied in the ecological and genomic model organism *Daphnia*, their genetic basis is not well understood. Combined approaches are necessary to understand the complexity of stress responses, such as those induced by predators. Gene expression profiling and gene co-expression analysis are current methods used to describe transcriptomes in different organisms, e.g. plants (reviewed by Serin et al., 2016), vertebrates (Ghazalpour et al., 2006), invertebrates (Zhao et al., 2016) and humans (reviewed by de la Fuente, 2010). Gene co-expression network analysis is used to infer gene functions through the modular structure of co-expressed genes and their functional relations (Bergmann et al., 2004). Genes within one co-expression module often share conserved biological functions (Subramanian et al., 2005). Hence, the transcriptional profile gains integrity when the modularity of the co-expressed transcripts is taken into account, revealing potential genetic pathways. The co-expression network of genes is constructed via an adjacency matrix (Langfelder and Horvath, 2008). Modules, cluster of highly interconnected genes, are identified using hierarchical clustering (Langfelder and Horvath, 2008). The benefit of the co-expression network analysis lies in the opportunity to correlate gene expression and phenotype data (Langfelder and Horvath, 2008) simplifying the process of candidate genes identification for further analysis or the design of future experiments.

Fish is a predator of all species and common response patterns are found, while the predator-induced responses mentioned above are known to vary across *Daphnia* species. One can therefore expect that the genetic basis of these responses is conserved response across species to a certain extent. Thus, a comparative transcriptomic approach will help reveal common transcripts involved in predator-induced responses across *Daphnia* species. The difficulty lies in finding homologous genes in species having diverged million years ago. OrthoMCL is a tool used to identify clusters of orthologous genes (orthologs) that are functionally conserved. The benefit of this tool is that known functions of orthologs in one species can be inferred to assign putative functions to orthologs in another species (Li et al., 2003). The comparative transcriptomics approach made use of the existing OrthoMCL information from the reference transcriptome of *D. galeata* (Huylmans et al., 2016) as well as

data/results of previous studies on *D. magna* (Orsini et al., 2016) and *D. pulex* (Asselman et al., 2018).

Our goal is to understand the underlying genetic basis of a predator-induced response in the freshwater grazer *D. galeata*. By using a transcriptomic approach (RNA-sequencing), we address the following questions: (i) Which transcripts are differentially expressed in *D. galeata* when exposed to fish kairomones?, (ii) which gene co-expression modules of the gene co-expression network correlate with predation risk and reproduction in *D. galeata*?, (iii) which Gene Ontology (GO) terms are enriched in transcript sets of interest?, (iv) do different *Daphnia* species share genes involved in predator-induced responses and reproduction? Since most of the predator-induced responses described earlier are related to *Daphnia* reproduction and growth, we expect to identify transcripts with GO terms relating to reproduction, growth, and/or kairomone perception. By doing so, we lay a valuable cornerstone in the understanding of the genetic basis of predator-induced responses in a freshwater keystone species, *Daphnia galeata*.

Materials and methods

Experimental organisms

This study was conducted on two *D. galeata* clonal lineages originally hatched from resting eggs collected from Müggelsee (northeast Germany) which differ in their life history responses in the presence of fish kairomones (Tams et al., 2018). In a large phenotypic experiment involving 24 clonal lines from four different lakes, Tams et al. (2018) revealed that within the Müggelsee population the variation for some life history traits increased when these clonal lines were exposed to fish kairomones, meaning a broader range of phenotypes were displayed for that life history trait. We chose the clonal lines M6 and M9 which differed in all of their life history traits and were at the contrasting ends of the phenotypic range of fish kairomone exposed *D. galeata* (Table S1).

Media preparation

ADaM (Klüttgen et al., 1994) was used as the basic medium for fish and *Daphnia* cultures. Two types of media, fish kairomone (FK) and control, were used for breeding and experimental conditions and their preparation is detailed in Tams et al. (2018). Briefly, fish kairomone medium was obtained by maintaining five ide (*Leuciscus idus*) in a 20L tank for 24 hours prior to medium use. All media were filtered (Whatman, membrane filters, ME28, Mixed cellulose-

ester, 1.2µm) prior to use and nourished with 1.0 mg C L-1, P rich *Acutodesmus obliquus*. Media was exchanged daily (1:2) to ensure a nutrient-rich environment and a constant fish kairomone concentration. The algae concentration was calculated from the photometric measurement of the absorbance rate at 800 nm. Cetyl alcohol was used to break the surface tension during breeding and the experiment to reduce juvenile mortality (Desmarais, 1997). Breeding and experimental phases were conducted at a temperature of 20°C and a 16h light / 8h dark cycle in a brood chamber with a light intensity of 30% (Rumed, Type 3201D).

Experimental design and procedures

Each clonal line was bred in kairomone-free water (control) and in kairomone water (fish) for two subsequent generations before the start of the experiment to minimize inter-individual variances. To this end, 20 egg-bearing females per clonal line were randomly selected from mass cultures. From these females of unknown age, neonates (<24h) were collected and raised under experimental conditions in 750 mL beakers at densities of <40 neonates per beaker. They served as grandmothers (F0) for the experimental animals (F2). Based upon previous work (Tams *et al.* 2018), we started the second (F1) generation after 16-20 days to ensure that offspring from the 3rd to 5th brood were used to start the next generation. The third generation of experimental individuals (F2) was started after 18 days. At the start of the experiment, a pair of neonates was introduced in the experimental vessels (50 mL glass tube) to compensate for juvenile mortality. Before the release of the first brood, at day 6, one of the individuals was randomly discarded (if necessary) so that only one individual remained in each vessel. During the 14 days of the experiment, neonates were removed every 24 hours and the number of broods of each experimental female was documented before media renewal. The adult females were pooled (n=20) and homogenized in RNAmagic (Bio-Budget technologies, Krefeld, Germany). Only experimental females bearing eggs were pooled, resulting in a minor difference in age and experimental time as some experimental females had been pooled a day later. The advantage of sampling females in their inter-molt stage (egg-bearing) is to ensure a stable gene expression (Altshuler et al., 2015). Five biological replicates were used per experimental condition (environment) and per clonal line, resulting in a total of 400 individuals (two clonal lines x two environments x 20 individuals x 5 biological replicates). The experiment lasted for 14 days for each experimental individual to assess the long-term effect of fish kairomones on gene expression level in *D. galeata* and to make correlations with previously collected data sets on life history possible.

Data collection and analysis

RNA isolation and preparation

Appropriate amounts of RNA were not available from single individuals, and hence we used pools of experimental individuals. Similar pooling approaches have been used in other *Daphnia* differential gene expression studies (Hales et al., 2017; Herrmann et al., 2018; Huylmans et al., 2016; Orsini et al., 2016; Rozenberg et al., 2015). Total RNA was extracted from pools of 20 egg-bearing adults after homogenizing with a disposable pestle and a battery-operated homogenizer in RNAmagic for 5 min. Samples were stored at –80°C until RNA isolation. Chloroform was added to the homogenate before centrifuging in Phasemaker tubes (Carlsbad, CA, USA) to separate the upper aqueous and lower phenol phase. The upper aqueous phase was transferred into a clean microcentrifuge tube and the RNA precipitated with absolute ethanol. RNA purification and DNAse treatment were conducted using a modified protocol of the Direct-zol™ RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA). Quality and quantity of purified RNA was checked by spectrophotometry using a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). The RNA integrity was confirmed with the Agilent TapeStation 4200 (Agilent Technologies, Santa Clara, CA USA). Only samples showing no degradation and RNA Integrity Numbers (RIN) > 7 were used for subsequent steps. Sequencing was performed for 12 samples (two clonal lines x two environments x three biological replicates).

RNA-seq library construction and sequencing

Library construction and sequencing was identical for all samples and was performed by the company Macrogen (Seoul, South Korea). mRNA-seq libraries were constructed using Illumina TruSeq library kits. Illumina HiSeq4000 (San Diego, CA, USA) platform was used for paired-end sequencing with 101-bp read length resulting in 48-79 million reads per library.

RNA-seq quality control and mapping

The quality of raw reads was checked using FastQC v.0.11.5 (Andrews, 2010). Adapter trimming and quality filtering were performed using Trimmomatic v.0.36 (Bolger et al., 2014) with the following parameters: ILLUMINACLIP: TruSeq3-PE.fa:2:30:10 TRAILING: 20 SLIDINGWINDOW: 4:15. After trimming, the read quality was checked again with FastQC to control for the successful removal of adapters. The cleaned reads were mapped to the reference transcriptome of *D. galeata* (Huylmans et al., 2016) using NextGenMap v.0.5.4 (Sedlazeck et al., 2013) with increased sensitivity (--kmer-skip 0 –s 0.0). All reads which had an identity < 0.8 and

mapped with a residue number < 25 were reported as unmapped. The option 'strata' was used to output only the highest mapping scores for any given read and thus the uniquely mapped reads. The quality of filtering and mapping reads was verified with QualiMap v.2.2.1 (Okonechnikov et al., 2016). Subsequently, the htseq-count python script implemented in HTSeq v.0.9.1 was used to quantify the number of reads mapped to each transcript (Anders et al., 2015).

Differential gene expression analysis

Differential gene expression analysis was performed in the R environment v.3.4.2 (R CoreTeam 2018) (R Core Team, 2017) with the R package *'DESeq2'* v.1.18.1 (Love et al., 2014) implemented in Bioconductor v.3.6 (Gentleman et al., 2004). The calculation was based on normalized read counts per environment (control & fish) using negative binomial generalized linear models. Prior to the analysis, all transcripts with a read count lower than 12 across all libraries were excluded to reduce multiple testing. Results were filtered post-hoc by an adjusted p-value (padj < 0.05) (Benjamini and Hochberg, 1995) to reduce the false discovery rate (FDR) and filtered for a log2 fold change =/> 1. Differentially expressed transcripts (DETs) were binned into four groups: <2-fold, 2- to 4-fold, 4- to 6-fold and >6-fold difference in expression. The three biological replicates were checked for homogeneity by principal component analysis (PCA). A differential expression analysis of genes between environments, between clonal lines and between environments within each clonal line was done. In addition, a two-factor analysis was applied to investigate a genotype-environment interaction (GxE). PCA plots were created in R with *'ggplot2'* v.2.2.1 (Wickham, 2009). The web tool jvenn (Bardou et al., 2014) was used to visualize the number of shared genes between groups.

Gene co-expression network analysis

Variance-stabilized read counts obtained from the previous '*DESeq2'*-analysis were used in the co-expression analysis. Subsequent analysis was performed in the R environment v.3.4.2 (R Core Team, 2017). First, an automatic, signed weighted, single gene co-expression network construction was performed with the R environment v.3.2.3 with the R package *'WGCNA'* v.1.61 (Langfelder and Horvath, 2008). Second, gene co-expression modules were identified using the Topological Overlap Matrices (TOM) with a soft cut-off threshold of 14 in '*WGCNA'*. Module eigengenes (ME), representing the average gene expression of their module, were calculated and used to infer correlation with life history traits following the resampling

procedure outlined below. Finally, the most interconnected genes per module, so called hub-genes were identified.

Module eigengene – trait correlation

Modules were related to external trait information that originated from a previous life history study by Tams et al. (2018) in which 24 clonal lines were exposed to fish kairomones. In the gene expression analysis, we had three biological replicates per clonal line, while we had one mean value for every life history trait measured per clonal line. Thus, in order to perform a correlation analysis, we had to assign the same mean trait value to all three biological replicates resulting in potential false or inflated correlations (pseudoreplication). To avoid this artifact, we randomly resampled the available individual trait values for each life history trait in every clonal line, to obtain one “unique” mean trait value per replicate per clonal line (supplementary script: 3_Resampling.Rmd). For example, we had trait values for 15 individuals for the trait ‘broods’ in the clonal line M6 exposed to fish kairomones (Table S1). In the first resampling step, we randomly picked the life history trait values of 75% of the individuals to calculate a mean. The process was then repeated twice to obtain 3 randomized mean values for this life history trait per clonal line. This step was repeated for every trait value in every clonal line. Finally, the correlation of module eigengenes and the resampled life history trait mean values was calculated. This whole procedure of resampling to calculate randomized means and their correlation to the module eigengenes was repeated 10,000 times to verify the robustness of the ME-trait correlation. We then counted the observations per ME-trait correlation where the correlation value was above a 0.5 absolute value. ME-trait correlations were considered as robust if they occurred in more than 95% of the iterations.

Gene set enrichment analysis (GSEA)

To identify the biological importance and the potential function of differentially expressed and co-expressed transcripts, we assigned Gene Ontology (GO) annotations using the reference transcriptome of *D. galeata* (Huylmans et al., 2016). We performed a gene set enrichment analysis in R with the package *’topGO’* v.2.30.0 (Alexa and Rahnenführer, 2016). The default algorithm 'weight01' was used taking the hierarchy of GO terms into account which results in fewer false positive results (Alexa and Rahnenführer, 2016). Given that, a multiple testing correction after the Fisher's exact test was not applied (Timmermans et al., 2009). GO terms of the three GO categories 'Molecular Function' (MF), 'Biological Process' (BP) and ‘Cellular Compounds’ (CC) with a p-value < 0.05 were considered significant.

A list of expected GO terms was created by using the AMIGO database (Carbon et al., 2009). Annotations, associations of genes or gene products, and GO terms were searched for in the database (http://amigo.geneontology.org/amigo/search/annotation). We chose to retain results for *Drosophila melanogaster* ('organism' filter) only, all other species being phylogenetically too distant. We expected candidate transcripts related to growth, reproduction and kairomone perception. Search terms were thus cell death, cell growth, chitin and molting; hatching, metabolism, reproduction, vitellogenesis, vitellogenin and yolk as well as external stimulus and sensory perception. Since this data mining approach did not focus on the direction of gene expression changes, we excluded GO terms containing positive and negative regulation to narrow down the list of expected GO terms. We excluded sex-specific terms like male, sex determination, etc. because only parthenogenetically reproducing females were used in this experiment. *Drosophila* specific terms, e.g. oviposition, were deleted from the list. Finally, a list of unique expected GO terms (hereafter, "expected_GO") remained with a total of 603 GO terms of which 340 belong to the search class growth, 59 to perception and 204 to reproduction (Table S2).

Comparative transcriptomics

OrthoMCL cluster information from the reference transcriptome of *D. galeata* was used (Huylmans et al., 2016) to conduct an interspecies comparison of candidate transcripts. These clusters contain orthologs of three *Daphnia* species (*D. galeata*, *D. pulex* and *D. magna*) and two arthropod species (*Drosophila melanogaster* and *Nasonia vitripennis*). A custom python script was used to parse the orthoMCL output and assign orthologous clusters to the transcripts of interest (supplementary script: OMCLFinal.py).

To reveal common predator-induced transcripts, we identified orthologs from candidate transcript sets related to predation risk for *D. galeata* and *D. magna*. The *D. galeata* transcript sets of interest derived from the above described gene co-expression network analysis (experimental design: long-term exposure to fish kairomones). The *D. magna* transcript sets of interest were generated by applying the same analysis workflow as described above for *D. galeata* to the published RNA-seq data by Orsini et al. (2016) (experimental design: short-term exposure to fish kairomones). RNA-seq data and reference *D. magn*a transcriptome were available from the International Nucleotide Sequence Database Collaboration BioProject PRJNA284518.

To identify common reproduction-related transcripts in *Daphnia* species, we identified orthologs from reproduction-related transcripts in *D. galeata* with a list of transcripts which was predicted to be involved in the reproduction of *D. pulex* (Asselman et al., 2018).

Although stressors and exposure durations varied between all three experiments of which the data sets for *D. galeata*, *D. magna* and *D. pulex* originate from, we expected to find shared transcripts involved in predator-induced responses and *Daphnia* reproduction, assuming a conserved predator-induced response at the molecular level.

Results

RNA-seq data quality

RNA samples passed all quality steps before RNA sequencing. All 12 samples were successfully sequenced, resulting in 48.2 to 79.2 million reads of 101-bp length. After trimming and quality control ~90% of trimmed reads were kept for further analysis. Of these trimmed reads 88-88.74% were uniquely mapped to the *D. galeata* reference transcriptome (Huylmans et al., 2016). A total of 32,903 transcripts remained after this process as the full data set.

Differential gene expression analysis

Before subsequent analysis all transcripts with a read count lower than 12 across all libraries were excluded, thus 23,982 transcripts remained for both clonal lines. Accordingly, 21,740 transcripts remained for clone M6 and 21,813 for clone M9.

A principal component analysis (PCA) was performed to visualize the grouping of read counts and identify batch effects. The first principal component (PC 1) explained 83% of the variance between clonal lines revealing no clear clustering of read counts per environment (Figure 1). PC 2 explained just 10% of the variance, which seems to be related to variance between replicates. To improve the visualization of replicate and environment differences, separate plots per clonal line were produced (not shown) resulting in no visible environmental effect.

The differential expression analysis revealed that there were no differentially expressed transcripts (DETs) between environmental groups, but a total of 5,283 DETs between clonal lines (2,228 up-regulated (42%), 3,055 down-regulated (58%)). Because of the strong clone effect, the clonal lines were analyzed separately in a one-factor analysis (Table 1A). Within clonal line M6, there were 30 DETs between environments of which 27 were down-regulated (90%) and 3 were up-regulated (10%). For clone M9 57 DETs were found between

environments of which 21 were up-regulated (37%) and 36 were down-regulated (63%). The expression fold-change (log2) of most of the DETs (53-63%) was above 2.

To account for the genotype-environment (GxE) interaction a two-factor analysis was applied (Table 1B). Between environments clone M6 had four DETs (up: 1 (25%); down: 3 (75%)), while clone M9 had 68 DETs (up: 29 (43%); down: 39 (57%)). The GxE resulted in 22 DETs (up: 7 (32%); down: 15 (68%)).

No DETs were shared between the two clonal lines (genotype) in regard to fish kairomone exposure (environment). Only a small number of DETs were shared within one clonal line for the one and two factor analysis (Figure 2). In total 125 transcripts were found to be differentially expressed between the two environments of which 40 were up- and 85 were downregulated when comparing fish environment (hereafter, 'FK') to control environment (Figure 2, Table S3). The differential expression of most of the FK-related DETs (~50%) was strong (fold change >2) (Table 1). No DETs were found for the reanalyzed *D. magna* data set. Further results for *D. magna* can be found in the supplementary section (Table S4, Figure S1).

<u>Gene co-expression network analysis</u>

The single network analysis revealed that the expressed transcripts clustered into 16 co-expression modules (CEM) (Figure 3, Table 2). Most transcripts were assigned to the modules 'turquoise', 'blue', 'brown' and 'yellow'. The 'grey' module includes all transcripts which could not be assigned to any module, representing 6% (n=1,525) of all transcripts. For each module, the hub-gene, or the most highly interconnected gene within a gene co-expression module, was identified (Table 2).

A total of five modules were robustly correlated to life history traits, fish kairomone exposure or clonal line with a correlation coefficient >0.5 or < -0.5. Three small gene co-expression modules 'salmon' (n= 107), 'red' (n= 519) and 'tan' (n= 116) were associated to fish kairomone exposure. The 'salmon' module correlated positively with fish kairomone exposure while the 'red' and the 'tan' module correlated negatively with fish kairomone exposure.

Two large gene co-expression modules, 'brown' (n= 4,760) and 'blue' (n= 4,868), were associated to reproduction-related traits. The 'brown' module was positively correlated with the life history trait total number of offspring of first brood ('brood1') and age at first reproduction ('AFR') as well as negatively correlated with total number of offspring of third

brood ('brood3') and total number of broods ('broods'). In contrast, the 'blue' module showed the exact opposite correlation pattern.

Three hub-genes of co-expression modules belonged to the previously identified FK-related DETs (Table 2), namely those for the co-expression modules 'midnightblue', 'salmon' and 'tan'. In total 49 of 125 FK-related transcripts identified through the differential gene expression analysis also belonged to a co-expression module of interest ('salmon' n=13 (~12%), 'tan' n=9 (~8%), 'red' n=3 (~0.6%), 'brown' n=17 (~0.3%), 'blue' n=7 (~0.1%)).

A total of 33 co-expression modules were found for the reanalyzed *D. magna* dataset including one module 'royalblue' being positively correlated to fish kairomone exposure. Detailed results can be found in the supplementary section (Table S5, Figure S2).

<u>GO annotation and gene set enrichment analysis (GSEA)</u>

The reference transcriptome of *D. galeata* had a total of 10,431 transcripts with Gene Ontology (GO) annotations (Huylmans et al., 2016). After the initial data filtering of low read counts, 8,173 (~34%) of the transcripts included in the analysis had a GO annotation and thus constituted the gene universe for the gene set enrichment analysis. Transcript sets of interest are either FK– or reproduction-related. FK-related transcripts of interest originated from the co-expression modules 'salmon', 'tan' and 'red' (total n= 742), and the differential gene expression analysis (one and two factor analysis; total n=125). Reproduction-related transcripts originated from the co-expression modules 'blue' and 'brown' (total n=9,628). Figure 4 presents an overview of the different transcript sets and the comparisons they were included in.

28 % of transcripts deriving from the co-expression modules of interest were annotated ('blue-brown' n= 2681; 'tan-red-salmon' n= 207). The lowest rate of annotation (23%) was for reproduction-related DETs (n=1,230) and the highest (33%) for the FK-related DETs (n=41).

Five out of the 15 hub-genes had a GO annotation; a total of 9 unique GO terms were assigned to all hub-genes (Table 2). The hub-gene of the 'green' module was involved in 'structural constituent of cuticle' and related to fish kairomone exposure. The hub-genes of the 'black' and the 'yellow' module were involved in 'protein binding' and were related to reproduction. The hub-gene of the 'red' module had several GO terms of which one, the 'oxidation-reduction process' related to reproduction. The hub-gene of the 'greenyellow' module had a fish kairomone exposure related annotation, 'calcium ion binding'.

We expected to find GO terms related to reproduction, growth and kairomone perception to be overrepresented in the gene set enrichment analysis. Lists of unique enriched GO terms were created to compare the list of "expected_GO" terms and the results of the GSEA with the two candidate transcripts sets, respectively. In total 29 unique GO terms were significantly enriched in the FK transcript set (hereafter, "uniqueGO_FK", Table 3A), and 46 unique GO terms were significantly enriched in the reproduction transcript set (hereafter, "uniqueGO_re", Table 3B). There was only a small overlap between "expected_GO" terms (Table S2), "uniqueGO_FK" (Table 3A) as well as "uniqueGO_re" (Table 3B). A total of five expected GO terms were identified (Figure 5): 'intracellular', 'growth factor activity' and 'calcium ion binding' in "uniqueGO_FK" as well as 'integral component of membrane' and 'carbohydrate metabolic process' in "uniqueGO_re". Five unique enriched GO terms were found related to fish kairomone exposure and reproduction: 'serine-type endopeptidase activity', 'extracellular matrix structural constituent', 'proteolysis', 'oxidation-reduction process' and 'collagen trimer'.

Looking at the total number of transcripts, a total of 2,465 transcripts (~30%) had at least one FK-related GO term, while 3,263 transcripts (~40%) had at least one reproduction-related GO term. While looking at differentially expressed transcripts, a total of 12 significantly enriched GO terms related to fish kairomone exposure were found as well as 15 significantly enriched GO terms related to reproduction.

Comparative transcriptomics

Interspecies comparison of short-term vs. long-term response to predation risk

We compared the results of a long-term exposure to fish kairomones in *D. galeata* to the results of the short-term exposure to fish kairomones in *D. magna* (Orsini et al., 2018). Since the 'salmon' *D. galeata* module and the 'royalblue' *D. magna* module correlated positively to fish kairomone exposure and had a similar size, we hypothesized that they had similar functions in both species and hence expected an overlap between the two sets of transcripts. In total, 9,461 orthoMCL clusters comprised at least one transcript for each of the three *Daphnia* species (Huylmans et al., 2016). No orthologous clusters were found between the 'salmon' *D. galeata* module and the 'royalblue' *D. magna* module. However, 34 orthologous clusters were identified which contained transcripts from the 'royalblue' *D. magna* module and the negatively correlated 'tan-red' *D. galeata* modules, resulting in 219 orthologs related to predator-induced responses in *Daphnia.* Two-thirds of the orthologous clusters were *Daphnia* specific, and the

remainder comprised sequences from the other two arthropod species, *Nasonia* and *Drosophila* (Table S6A).

Interspecies comparison of reproduction-linked transcripts in *Daphnia*

We compared the results of long-term exposure to fish kairomones in *D. galeata* to the predicted reproduction-related transcripts after the long-term exposure to cyanobacteria, insecticides and their combination in *D. pulex* (Asselman et al., 2018). The **intra**specific comparison within *D. galeata* revealed an overlap of 445 orthologous clusters that contain transcripts of both the co-expression modules 'blue' and 'brown' which related to reproduction (Figure 6). The **inter**specific comparison between *D. pulex* and *D. galeata* resulted in 42 orthologous clusters that contain transcripts related to reproduction (Figure 6). These 42 orthologous clusters comprised 221 *D. galeata* transcripts of which 140 had a GO term, and 300 *D. pulex* transcripts of which 50 belonged to the 258 predicted reproduction-related transcripts of Asselman et al. (2018). Altogether, *D. galeata* transcripts with a GO term were identified in 28 orthologous clusters and their GO terms (n=48) were extracted (Table S7). In total, these 42 orthologous clusters comprised 839 orthologs relating to reproduction in *Daphnia.* Two-thirds of the orthologs were conserved (i.e. contained *Drosophila* and *Nasonia* transcripts) while one third was *Daphnia* specific (Table S6B).

Discussion

Predator-induced responses in *Daphnia* have been long studied in depth, but few studies have addressed the link between such ecological traits and their underlying genetic pathways. To gain insights into the genetic basis of predator-induced responses, we performed gene expression profiling on two *D. galeata* clonal lines exposed to fish kairomones. We identified a number of transcripts correlated with shifts in life history and used gene co-expression network analysis to describe the potential functions of previously unknown biological pathways. By using an orthology approach, we were able to find common transcripts involved in predator-induced responses and in reproduction after exposure to stressors.

Interclonal variance

Stress responses at the organism level vary between genotypes as well as within genotypes. The latter is known as phenotypic plasticity (West-Eberhard, 1989) and is associated with variation in gene expression as a product of the genotype-environment interaction (GxE)

(Hodgins-Davis and Townsend, 2009). It is well-known that predator-induced responses vary across clonal lines in *Daphnia*, e.g. *D. pulex* clonal lines display different numbers of neck-teeth in response to *Chaoborus* kairomones (some clones even show no tooth production; Lüning, 1995), and *D. galeata* clones exhibit different life history strategies in response to fish kairomones, even within the same population (Tams et al., 2018). That is why using only one genotype (clonal line) of a species in a gene expression study makes it difficult to draw conclusions about the entire population or even species. Therefore, we used two clonal lines of one population to investigate the correlation between varying life histories and gene expression.

Overall, the gene expression analysis revealed surprisingly large differences between clonal lines of the same population in *D. galeata* (5,283 DETs). Since clonal lines were chosen for their antithetical life history response to fish kairomones, some interclonal variation was expected to occur. This difference could be explained by their opposing reproduction strategy: one clonal line (M9) matures early, produces more offspring and becomes larger, while the other (M6) matures later, produces less offspring and stays smaller.

A similar size of differentially expressed transcripts between clonal lines in *D. galeata* was found in a previous study which identified 5,492 DETs in clonal lines from Müggelsee and a clonal line from Jordán Reservoir in the Czech Republic (Huylmans et al., 2016). In brief, the variance between clonal lines from Müggelsee in this study was approximately the same as for clonal lines from Müggelsee and Jordán Reservoir (~400 km apart) (Huylmans et al., 2016). This rather large difference was attributed to the geographic distance between the two locations since it is known that the intrapopulation diversity of *Daphnia* varies across its distributional range (Walser and Haag, 2012), which makes it challenging to compare differential expression between genotypes (clonal lines) across species and populations. Being aware of this diversity, we chose two clonal lines from the same population. Nevertheless, the analysis revealed a high number of DETs, as if the clonal lines from one population stem from different populations.

In contrast, the re-analysis of the *D. magna* RNA-seq data of Orsini et al. (2016) revealed 2,929 DETs between the two clonal lines which were collected from Southern Germany and Southwest Finland (~2,000 km apart), representing the species distributional range (Orsini et al., 2016). In addition to the geographical distribution, they derived from very different habitats: a temporary rock pool system connected to the Baltic Sea and a fish-rearing pond. In summary, we hypothesize that geographical distance does not play a major role in intraspecific clonal variation in life history traits or gene expression in *Daphnia*.

The observed variance between the clonal lines of *D. galeata* in the differential gene expression analysis was also reflected in the gene co-expression network analysis. The majority of the transcripts correlated to clonal line were assigned to either the 'brown' or 'blue' module leading to the conclusion that this gene co-expression network is mostly driven by the clones' different reproduction strategies. The gene co-expression network constructed for *D. magna* also seems to be mainly driven by large clone-specific modules with very little effect of fish kairomone exposure. This is not surprising as the differential expression analysis for *D. magna* did not reveal differentially expressed transcripts for fish exposed individuals. Given that life history traits were not recorded for the *D. magna* clonal lines, we cannot infer whether the *D. magna* gene co-expression network correlates with life history traits or reproduction strategies.

Effect of fish kairomones on gene expression

Linking predator-induced responses to the underlying genome-wide expression patterns has rarely been attempted. One study looking at short-term exposure to fish kairomones (several hours) in *D. magna* revealed no gene expression response (Orsini et al., 2018). Other transcriptomic approaches have been used to link stress responses in *Daphnia* to environmental factors, such as food quality and anthropogenic stressors in *D. pulex*, which revealed 258 transcripts involved in *Daphnia* reproduction (Asselman et al., 2018). In another study over 200 differentially expressed genes were identified in *D. pulex* in response to invertebrate predation risk, of which the most prominent classes of upregulated genes included cuticle genes, zinc-metalloproteinases and vitellogenin genes (Rozenberg et al., 2015). Finally, a study on *D. ambigua* revealed ~50 responsive genes involved in reproduction, digestion and exoskeleton structure (Hales et al., 2017).

In contrast to the large differences between the two *D. galeata* clonal lines, the differential gene expression analysis revealed only a moderate number of transcripts differentially expressed between environments (control vs. fish) within each clonal line. We expected to find shared DETs between the two clonal lines from the same population, which were important in the response to kairomones regardless of clonal lines, but no shared DETs between environments were identified. Thus, a completely different set of transcripts seems to be linked to kairomone response within each clonal line. It is possible that any effect of fish kairomone exposure was obscured by the large clonal variation or by the antithetical reproduction strategies in the divergent set of transcripts. To clarify whether DETs are actually clone-specific

it would be necessary to generate RNA-seq data for more *D. galeata* clonal lines from the same and other populations, both with shared and divergent life histories.

As the chosen *D. galeata* clonal lines displayed strong shifts in life history after three generations of fish kairomone exposure we expected more pronounced changes in gene expression. Only a few transcripts were found to be significantly up-or downregulated in two *D. galeata* clonal lines. In comparison, Hales et al. (2017) observed 48 significantly differentially expressed genes after one generation of fish kairomone exposure and 223 and 170 genes differentially expressed in the second and third generation, respectively, without any predator kairomones. The clonal line used in the above-mentioned study showed strong transgenerational plasticity (phenotypic defenses maintained for multiple generations). It is unknown whether *D. galeata* clonal lines display this effect and pass on epigenetic modifications after exposure to fish kairomones for one generation. Further investigations are required to understand the epigenetic level of inheritance in *Daphnia*. However, the effect of kairomone exposure is expected to be cumulative and increase over the course of multiple generations, e.g. *D. pulex* displays the largest helmets when exposed to *Leptodora kindtii* kairomones for two generations compared to the first generation (Agrawal et al., 1999). For this reason, we expected the shifts in gene expression to be cumulative and to show the strongest changes in the third experimental generation.

A possible explanation for the weak changes in gene expression is that the response to kairomones is not only caused by changes in gene expression but additional posttranslational processes, such as miRNA-mediated regulation or increased degradation (Schwarzenberger et al., 2009). Another possibility is that life history changes are only marginally correlated with gene expression. The *D. galeata* clonal lines used here only displayed shifts in life history, whereas other *Daphnia* species show additional adaptations of morphology and behavior that could be caused by or correlated to much stronger differential gene expression, e.g. neck-teeth induction in *D. pulex* that was linked to 230 differentially expressed genes (Rozenberg et al., 2015).

The gene expression diverged between clonal lines. Fewer DETs were found in clonal line M6, and most were downregulated. The life history response of M6, decreasing body size to reduce the probability to be detected by vertebrate predators, seems to be the predominant strategy recorded in studies (e.g., Riessen, 1999). In contrast, about three times more DETs were found for clonal line M9 which were either up- or downregulated. *Daphnia* have the ability to rapidly adapt to local predator regimes (e.g., Declerck and Meester, 2003). The different life history

strategies of the clonal lines could be a result of clonal lines that are derived from a permanent lake with a fish population, therefore being locally adapted to predation. M6 might be better adapted for periods of high fish density, while M9 benefits during low fish density. This could also explain the large variation between clonal lines.

Gene pathways and functions linked to the predator-induced response

Overall our analysis revealed digestion- and growth-related enriched GO terms which are interesting because previous studies showed that predator-induced responses in *Daphnia* include changes in body size (e.g., Tams et al., 2018), morphological modifications (e.g., Laforsch and Tollrian, 2004) and the importance of peptidases for juvenile growth rate in *D. magna* (Schwarzenberger et al., 2012).

The 'salmon' gene co-expression module in *D. galeata* showed enrichment for terms summarized as 'serine-type endopeptidase activity', which is found in the gut of *D. magna* as the most important digestive protease (Agrawal et al., 2005). In *D. ambigua*, which is a species from the *D. pulex*-complex and more closely related to *D. galeata* than *D. magna* (Petrusek et al., 2005), the exposure to predator kairomones for one generation also lead to an up-regulation of genes related to digestive functions (Hales et al., 2017). Cyanobacterial protease inhibitors cause considerable damage to *Daphnia* populations by inhibiting the gut proteases and impairing digestion (Schwarzenberger et al., 2010). These studies concord with our results and suggests that an increase in serine-type endopeptidase activity leads to improved digestion and feeding efficiency that is necessary for the resource allocation that comes with shifts in life history, such as producing a greater number of offspring.

The GO term 'structural constituent of cuticle' was identified as biologically relevant in both clonal lines, M6 and M9, suggesting that even if there was no overlap in the affected transcripts, similar functions were affected. The structural constituent of cuticle was also found to be enriched in *D. pulex* exposed to *Chaoborus* kairomones (Rozenberg et al., 2015) and is related to remodeling of the cuticle. Furthermore, it was also enriched in the proteomic response of *D. magna* to *Triops cancriformis* (Otte et al., 2015) and is thought to be related to changes in carapace morphology as well as the formation of ultrastructural defenses of the cuticle (Rabus et al., 2013).

A gene co-expression network analysis also revealed that *D. magna* exposed to vertebrate and invertebrate predator kairomones are enriched for genes related to body remodeling and activation of cuticle proteins (Orsini et al., 2018). No pronounced morphological defenses have

been described for the investigated *D. galeata* clonal lines, but they displayed changes in body size and symmetry especially with regard to head shape (Tams et al., 2018). Furthermore, for *D. magna*, *D. pulex* and *D. cucullata*, not only visible morphology changes but also fortification of the carapace in the presences of predator kairomones has been recorded (Laforsch and Tollrian, 2004; Rabus et al., 2013). Our results indicated that ultrastructural defenses could also be present in *D. galeata*.

Altogether, cuticle-associated proteins seem to play an essential role in the response to vertebrate or invertebrate predators. DETs found in clonal line M6 showed the possible involvement of 'metallocarboxypeptidase activity', which is also known to be involved in the stress response to copper in *D. pulex* (Finlayson, 2016). Interestingly, 'chitin metabolic process', 'proteolysis', 'structural constituent of cuticle', 'chitin binding', 'serine-type endopeptidase' and 'metallopeptidase activity' were all found to be enriched in a gene expression analysis during the molt cycle in the marine copepod *Calanus finmarchicus* (Tarrant et al., 2014). Since *Daphnia* need to shed their rigid carapace in order to grow, molting is directly related to changes in body size. Another analysis of *D. magna* exposed to *Triops cancriformis* kairomones revealed the role of proteins related to the cuticle, muscular system, energy metabolism and regulatory proteins that may be involved in morphological carapace defenses and changes in resource allocation (Otte et al., 2014). In conclusion, a number of pathways that were hypothesized to be involved in kairomone response could be confirmed, such as transcripts related to body remodeling and growth.

Some biologically interesting gene functions were only found with the help of the gene co-expression network analysis and would have been overlooked with only a differential expression analysis. For example, the GO term 'growth factor activity' occurred in both 'red' and 'tan' modules, which correlated negatively with fish kairomone exposure and comprising transcripts not identified as DETs. Nevertheless, they could be extremely important for life history changes and might be directly related to changes in somatic growth rate and body length.

There were no hints found for the involvement of yolk protein genes or perception related genes. Only a small amount of expected GO terms were found in this analysis which could be explained by the small number of annotated transcripts (~34%). For a more comprehensive understanding of genetic links to phenotypic variation and their involved pathways, further annotations and therefore functional tests of candidate genes are needed. When GO

annotations progress, a re-analysis can reveal new insights to understand the genetic basis of predator-induced responses in phenotypes.

Interspecies comparisons of gene expression

The results of the long-term exposure experiment to fish kairomones (14 days) in *D. galeata* was put into the context of two recent transcriptomic studies in *Daphnia*. The first interspecies comparison revealed that the predator-induced response affected mostly transcripts (orthologs) which seem to be *Daphnia* specific (Table S6A). We compared the results of the long-term exposure (14 days) experiment to fish kairomones in *D. galeata* to the re-analyzed data set of Orsini et al. (2016) who investigated the short-term exposure (4 hours) to fish kairomones in *D. magna* to find common transcripts involved in predator-induced responses.

The gene expression analysis of the reanalyzed *D. magna* data set revealed no biologically relevant transcripts. This weak response could be due to the short exposure time to fish kairomones. Orsini et al. (2016) focused on characterizing the early transcriptional stress response to a total of 12 stressors. The biotic factor fish kairomone exposure had little impact on the differential expression, while the abiotic stressors caused stronger responses to *Daphnia* after a 24 h exposure (Orsini et al., 2018). Despite differences in research goals, we expected to find common transcripts involved in predator-induced responses because similar predator-induced responses have been observed in several studies across *Daphnia* species. Two-thirds of the discovered orthologs between *D. magna* and *D. galeata* seem to be *Daphnia* specific, suggesting that similar transcripts could be involved in the predator-induced responses in both species. It remains unclear whether and how the early stress response deviates from the long-time exposure.

The second interspecies comparison shed light on reproduction strategies of *Daphnia* after the exposure to stressors, revealing mostly transcripts (orthologs) which seem to be highly conserved within arthropods (Table S6B). We compared candidate transcripts of *D. galeata* to *D. pulex* transcripts predicted to be involved in reproduction (Asselman et al., 2018). In addition, we identified 29 orthologous groups which relate to reproduction and which contain at least one *D. galeata* transcript with a GO term (Huylmans et al., 2016) and one predicted *D. pulex* transcript. The GO terms of these transcripts were extracted and yielded 48 unique interspecies reproduction-related GO terms. Their functions can be summarized into enzymatic activities, metabolic processes, transport and binding. Five expected GO terms (Table S2) were found within the list of interspecies reproduction-related GO terms (Table S7): 'DNA binding',

‘carbohydrate metabolic process’, ‘signal transduction’, ‘zinc ion binding’ and ‘integral component of membrane’. Little to no information was found as to how these GO terms are involved in *Daphnia* reproduction, physiology or stress responses. An up-regulated transcript linked to ‘DNA binding’ was found in a gene expression study with *D. magna* which reduced their reproductive output when exposed to a certain amount of Bisphenol-A (Jeong et al., 2013). Hence, our results are a starting point for further investigations to understand the molecular mechanisms of reproduction in *Daphnia*.

In summary, the aim of this study was to characterize the genetic basis for the predator-induced response of the freshwater grazer *D. galeata*. The transcriptional profiling revealed differentially expressed transcripts and gene co-expression modules in connection to the presence of fish kairomones and *Daphnia* reproduction. The pathways discovered here represent a valuable starting point for future investigations addressing the functionality of certain transcripts per se or in respect to a stress response. For example, by providing detailed lists of candidate transcripts one can choose specific candidates to test their biological functions in knock-down experiments. Furthermore, we used an interspecies comparison to reveal conserved as well as *Daphnia* specific transcripts involved in predator-induced responses and *Daphnia* reproduction.

Authors’ contributions

The study was designed by VT, JHN and MC; laboratory work was carried out by JHN, AE and VT; gene expression and gene network analysis was done by JHN and VT; VT, JHN and MC wrote the manuscript, all authors gave final approval.

Conflict of interest

None declared.

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Data Accessibility

Raw RNA-seq reads for all 12 samples and the experimental set up for the analysis of DETs are available from ArrayExpress (accession E-MTAB-6234).

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Table 1. Number of differentially expressed transcripts (DETs) in *D. galeata* (p.adj=0.05, foldchange= log2). **(A)** Results of the one-factor analysis. 'Clone' = DETs between clonal lines (M6 over M9). 'M6' = DETs within clonal line M6 between treatments (fish over control). 'M9' = DETs within clonal line M9 between treatments (fish over control). **(B)** Results of the two-factor analysis. 'M6' = treatment effect for clonal line M6 (fish over control). 'M9' = treatment effect for clonal line M9 (fish over control). 'M6 vs M9': differences between the two clonal lines in control environment (M6 over M9). 'M6 vs M9 FK' = differences between clonal lines in in fish environment (FK) (M6 over M9). 'GxE' = genotype-environment interaction (clonal line – fish environment).

A

	All	<2-fold	2- to 4-fold	4- to 6-fold	< 6-fold
Clone	5283	1964	1486	927	906
up	2228	743	630	410	445
down	3055	1221	856	517	461
M6	30	11	11	6	2
up	3	3	0	0	0
down	27	8	11	6	2
M9	57	24	27	5	1
up	21	16	5	0	0
down	36	8	22	5	1

B

	All	<2-fold	2- to 4-fold	4- to 6-fold	< 6-fold
M6	4	1	2	0	1
up	1	0	0	0	1
down	3	1	2	0	0
M9	68	45	16	6	6
up	29	22	5	1	1
down	39	23	11	5	0
M6 vs M9	4687	1624	1204	899	960
up	1990	633	494	405	458
down	2697	991	710	494	502
M6 vs M9 FK	3820	1114	915	826	965
up	2016	611	478	428	499
down	1804	503	437	398	466
GxE	22	11	6	4	1
up	7	3	4	0	0
down	15	8	2	4	1

Table 2. Overview of gene co-expression modules in *D. galeata*. The table summarizes module color, total number of transcripts per module, the name of the most inter-connected gene (hub-gene), gene significances (GS) and its p-value for treatment (fish environment) and clonal line as well as differentially expressed transcripts (DETs) and Gene Ontology (GO) IDs and classes. The module 'grey' contains all co-expressed genes which were not assigned to a co-expression module. Gene significances describe the correlation of the gene to an external trait. The higher the absolute GS, the more biologically significant is the gene. Significant p-values (p<0.05) are highlighted in bold.

module	Total number of transcripts	hub-gene of co-expression module	GS.treatment	p.GStreatment	GS.clone	p.Gsclone	DETs	GO.ID	GO.class
turquoise	5154	abyss239	0.44	0.15	-0.51	0.09	no		
blue	4868	soapsoapd37687381411	-0.02	0.95	1.0	**0.00**	no		
brown	4760	soapsoap384083	0.00	0.99	-1.0	**0.00**	no		
yellow	4612	oasesvelvLoc2422d15233t1	-0.37	0.23	0.58	0.05	no	GO:0005515	protein binding
green	950	oasesvelvLoc7683t4	0.06	0.86	0.57	0.05	no	GO:0042302	structural constituent of cuticle
red	519	oasesvelvLoc2656t3	-0.54	0.07	-0.14	0.67	no	GO:0055114	Oxidation-reduction process
							no	GO:0004497	monooxygenase activity
							no	GO:0005057	copper ion binding
							no	GO:0016715	oxidoreductase activity,…..
black	491	oasesvelvLoc12661t5	-0.43	0.17	-0.12	0.64	no	GO:0005515	protein binding
pink	251	trinitytrinloc25528c0t5	0.19	0.55	0.24	0.46	no		
magenta	198	trinitytrinloc24643c0t2	0.38	0.22	0.41	0.19	no		
purple	181	oasesvelvLoc698d42270t2	0.02	0.95	0.50	0.10	no		
greenyellow	127	oasesvelvLoc21585d23838t2	0.04	0.89	0.61	**0.04**	no	GO:0005509	calcium ion binding
							no	GO:0054623	phospholipase A2 activity
							no	GO:0016042	lipid catabolic process
tan	116	trinitytrinloc6156c0t1	-0.55	0.06	0.13	0.69	**yes**		

salmon	107	abyssk84_f_262622	0.65	**0.02**	0.03	0.93	**yes**		
cyan	67	trinitytrinloc32639c0t1	-0.15	0.64	0.29	0.36	no		
midnightblue	56	abyssk80_j_452081	-0.43	0.16	-0.04	0.91	**yes**		
grey	1525	Genes not assigned to a module					no		

Table 3. List of unique Gene Ontology (GO) terms in gene expression datasets of *D. galeata*. Only significantly enriched GO terms are shown (classicFisher <0.05). **(A)** GO terms related to fish environment, including 29 unique GO terms ("uniqueGO_FK"). **(B)** GO terms related to reproduction, including 47 unique GO terms ("uniqueGO_re").

A

GO.ID	Term	Annotated	Significant	Expected	classicFisher	geneset	category
GO:0004252	serine-type endopeptidase activity	518	9	1.78	4.30E-05	salmon	MF
GO:0008762	UDP-N-acetylmuramate dehydrogenase activ...	5	1	0.02	0.017	salmon	MF
GO:0016887	ATPase activity	129	3	0.44	0.03	salmon	MF
GO:0016788	hydrolase activity, acting on ester bond...	219	3	0.75	0.039	salmon	MF
GO:0050660	flavin adenine dinucleotide binding	58	4	0.78	0.0076	red	MF
GO:0080019	fatty-acyl-CoA reductase (alcohol-formin...	13	2	0.17	0.0127	red	MF
GO:0016614	oxidoreductase activity, acting on CH-OH...	68	4	0.91	0.0131	red	MF
GO:0008083	growth factor activity	17	2	0.23	0.0214	red	MF
GO:0003824	catalytic activity	4470	71	60.11	0.025	red	MF
GO:0016972	thiol oxidase activity	2	1	0.03	0.0267	red	MF
GO:0003951	NAD+ kinase activity	2	1	0.03	0.0267	red	MF
GO:0005509	calcium ion binding	127	5	1.71	0.0281	red	MF
GO:0004930	G-protein coupled receptor activity	215	3	0.51	0.014	tan	MF
GO:0005201	extracellular matrix structural constitu...	100	2	0.24	0.023	tan	MF
GO:0008234	cysteine-type peptidase activity	147	2	0.35	0.047	tan	MF
GO:0042302	structural constituent of cuticle	209	4	0.16	8.20E-06	M6	MF
GO:0004181	metallocarboxypeptidase activity	47	1	0.04	0.035	M6	MF
GO:0004252	serine-type endopeptidase activity	518	7	1.67	0.0011	M9	MF
GO:0006508	proteolysis	929	9	3.76	0.008	salmon	BP
GO:0007156	homophilic cell adhesion via plasma memb...	22	3	0.3	0.0031	red	BP
GO:0006850	mitochondrial pyruvate transmembrane tra...	2	1	0.03	0.027	red	BP
GO:0006741	NADP biosynthetic process	2	1	0.03	0.027	red	BP

GO:0055114	oxidation-reduction process	427	11	5.81	0.0289	red	BP
GO:0007218	neuropeptide signaling pathway	4	1	0.01	0.011	tan	BP
GO:0007186	G-protein coupled receptor signaling pat...	250	4	0.69	0.024	tan	BP
GO:0006801	superoxide metabolic process	13	1	0.04	0.035	tan	BP
GO:0030131	clathrin adaptor complex	10	1	0.02	0.02	salmon	CC
GO:0005622	intracellular	1118	12	16.21	0.016	red	CC
GO:0005581	collagen trimer	100	3	0.13	0.00015	M9	CC
GO:0004867	serine-type endopeptidase inhibitor acti...	21	2	0.28	0.0319	red	MF
GO:0008083	growth factor activity	17	1	0.04	0.04	tan	MF
GO:0042302	structural constituent of cuticle	209	6	0.67	4.50E-05	M9	MF
GO:0005201	extracellular matrix structural constitu...	100	3	0.32	0.004	M9	MF
GO:0006508	proteolysis	929	9	3.16	0.002	M9	BP

B

GO.ID	**Term**	**Annotated**	**Significant**	**Expected**	**classicFisher**	**geneset**	**category**
GO:0004553	hydrolase activity, hydrolyzing O-glycos...	317	73	42.77	3.60E-06	blue	MF
GO:0016705	oxidoreductase activity, acting on paire...	143	34	19.29	0.00016	blue	MF
GO:0005506	iron ion binding	164	40	22.13	6.00E-04	blue	MF
GO:0004252	serine-type endopeptidase activity	518	93	69.88	0.0019	blue	MF
GO:0020037	heme binding	149	33	20.1	0.0024	blue	MF
GO:0008417	fucosyltransferase activity	125	28	16.86	0.00418	blue	MF
GO:0015299	solute:proton antiporter activity	10	5	1.35	0.0062	blue	MF

GO:0005201	extracellular matrix structural constitu...	100	23	13.49	0.00639	blue	MF
GO:0004672	protein kinase activity	763	123	102.94	0.00681	blue	MF
GO:0051033	RNA transmembrane transporter activity	4	3	0.54	0.00881	blue	MF
GO:0015116	sulfate transmembrane transporter activi...	11	5	1.48	0.01012	blue	MF
GO:0016805	dipeptidase activity	8	4	1.08	0.0147	blue	MF
GO:0008013	beta-catenin binding	2	2	0.27	0.01819	blue	MF
GO:0008199	ferric iron binding	9	4	1.21	0.02367	blue	MF
GO:0000062	fatty-acyl-CoA binding	10	4	1.35	0.03531	blue	MF
GO:0004013	adenosylhomocysteinase activity	3	2	0.4	0.04966	blue	MF
GO:0015930	glutamate synthase activity	3	2	0.4	0.04966	blue	MF
GO:0004842	ubiquitin-protein transferase activity	34	13	4.24	0.00027	brown	MF
GO:0004970	ionotropic glutamate receptor activity	56	16	6.98	0.00098	brown	MF
GO:0005515	protein binding	2135	292	266.21	0.00539	brown	MF
GO:0004677	DNA-dependent protein kinase activity	2	2	0.25	0.01554	brown	MF
GO:0004402	histone acetyltransferase activity	13	5	1.62	0.01624	brown	MF
GO:0004484	mRNA guanylyltransferase activity	18	6	2.24	0.01825	brown	MF
GO:000552	ATP binding	1009	147	125.81	0.01983	brown	MF

4							
GO:0005328	neurotransmitter:sodium symporter activi...	19	6	2.37	0.02393	brown	MF
GO:0009982	pseudouridine synthase activity	37	9	4.61	0.03447	brown	MF
GO:0004517	nitric-oxide synthase activity	3	2	0.37	0.04274	brown	MF
GO:0030151	molybdenum ion binding	3	2	0.37	0.04274	brown	MF
GO:0005975	carbohydrate metabolic process	385	77	52.42	2.30E-05	blue	BP
GO:0015074	DNA integration	56	18	7.62	0.00028	blue	BP
GO:0006508	proteolysis	929	145	126.48	0.00882	blue	BP
GO:0033227	dsRNA transport	4	3	0.54	0.00904	blue	BP
GO:0008272	sulfate transport	11	5	1.5	0.0105	blue	BP
GO:0055114	oxidation-reduction process	427	73	58.14	0.01863	blue	BP
GO:0006486	protein glycosylation	192	36	26.14	0.02624	blue	BP
GO:0006468	protein phosphorylation	759	121	103.34	0.02786	blue	BP
GO:0006812	cation transport	121	18	16.47	0.02918	blue	BP
GO:0016567	protein ubiquitination	19	8	2.36	0.00117	brown	BP
GO:0006303	double-strand break repair via nonhomolo...	5	3	0.62	0.01571	brown	BP
GO:0006836	neurotransmitter transport	19	6	2.36	0.02342	brown	BP

GO:0016192	vesicle-mediated transport	67	16	8.32	0.03683	brown	BP
GO:0006809	nitric oxide biosynthetic process	3	2	0.37	0.04239	brown	BP
GO:0001522	pseudouridine synthesis	39	9	4.84	0.04564	brown	BP
GO:0016020	membrane	1595	237	192.58	9.30E-07	blue	CC
GO:0005581	collagen trimer	100	23	12.07	0.0014	blue	CC
GO:0016021	integral component of membrane	748	111	90.32	0.0061	blue	CC
GO:0004252	serine-type endopeptidase activity	518	101	64.59	1.80E-06	brown	MF
GO:0004672	protein kinase activity	763	121	95.14	0.00266	brown	MF
GO:0020037	heme binding	149	27	18.58	0.02823	brown	MF
GO:0005506	iron ion binding	164	28	20.45	0.04395	brown	MF
GO:0006508	proteolysis	929	148	115.35	0.00012	brown	BP
GO:0006468	protein phosphorylation	759	120	94.25	0.00239	brown	BP
GO:0015074	DNA integration	56	15	6.95	0.00267	brown	BP

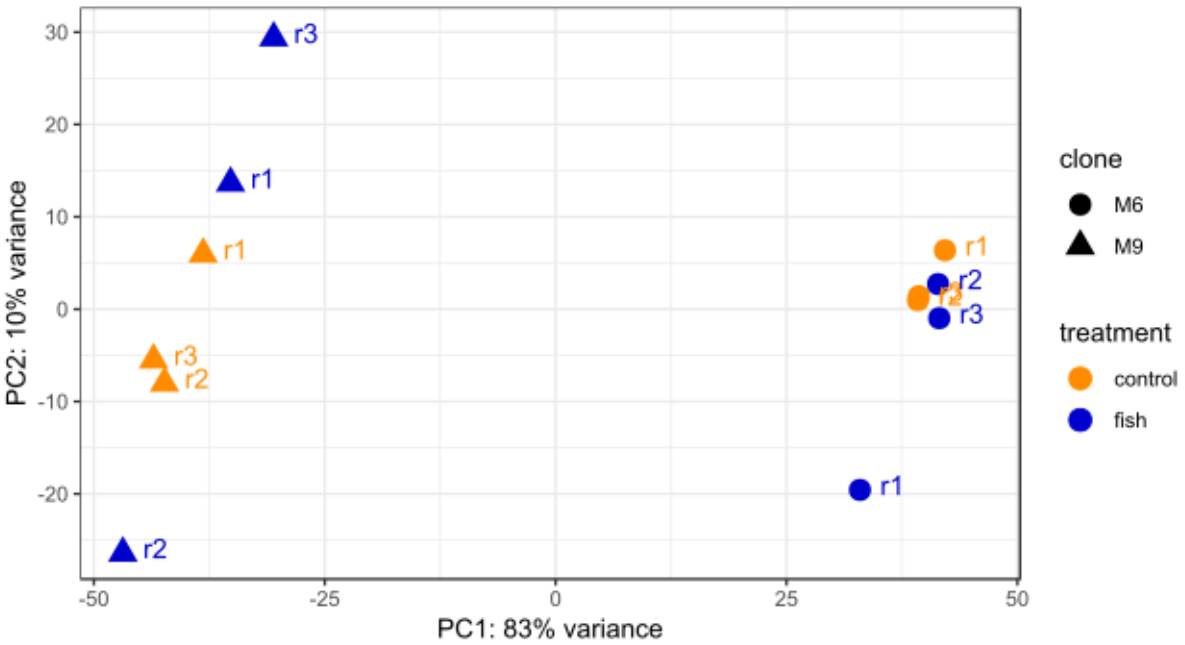

Figure 1. Principal component (PC) plot of the biological RNA-seq samples in *D. galeata*. Yellow: control environment. Blue: fish environment. Triangles: clonal line M9. Circles: clonal line M6.

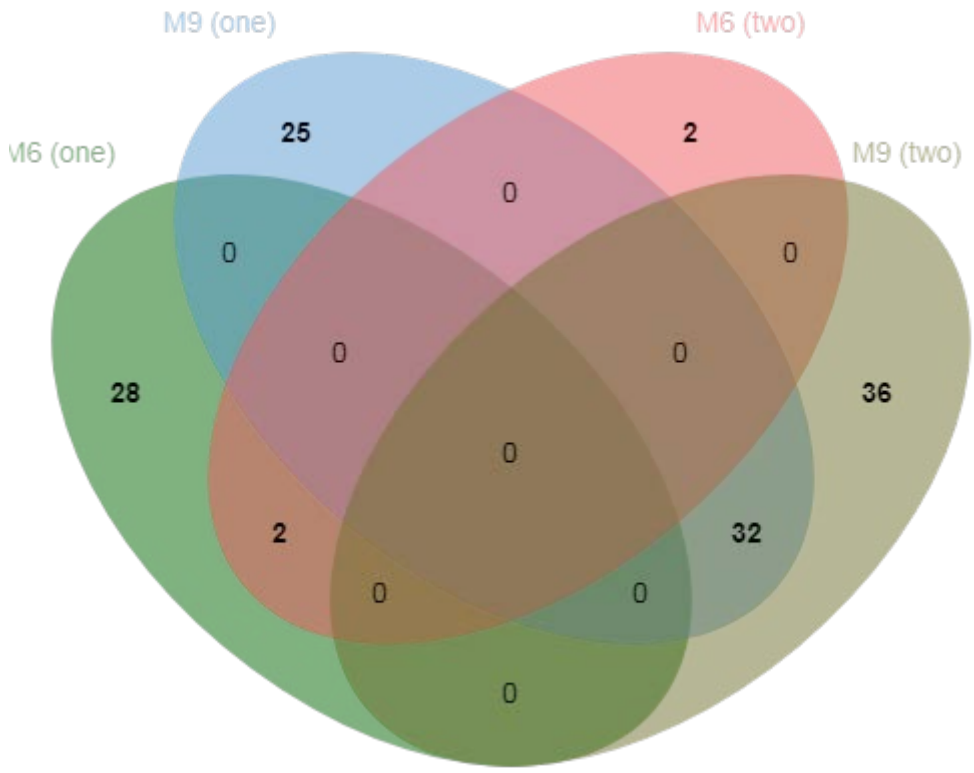

Figure 2. Venn diagram of the 125 differentially expressed transcripts (DETs) related to fish kairomone exposure (FK) in *D. galeata*. The set of FK-related DETs originates from the one and two factor analysis. 'M6 (one)' = DETs from the one factor analysis for the clonal line M6. 'M9 (one)' = DETs from the one factor analysis for the clonal line M9. 'M6 (two)' = DETs from the two factor analysis for the clonal line M6. 'M9 (two)' = DETs from the two factor analysis for the clonal line M9.

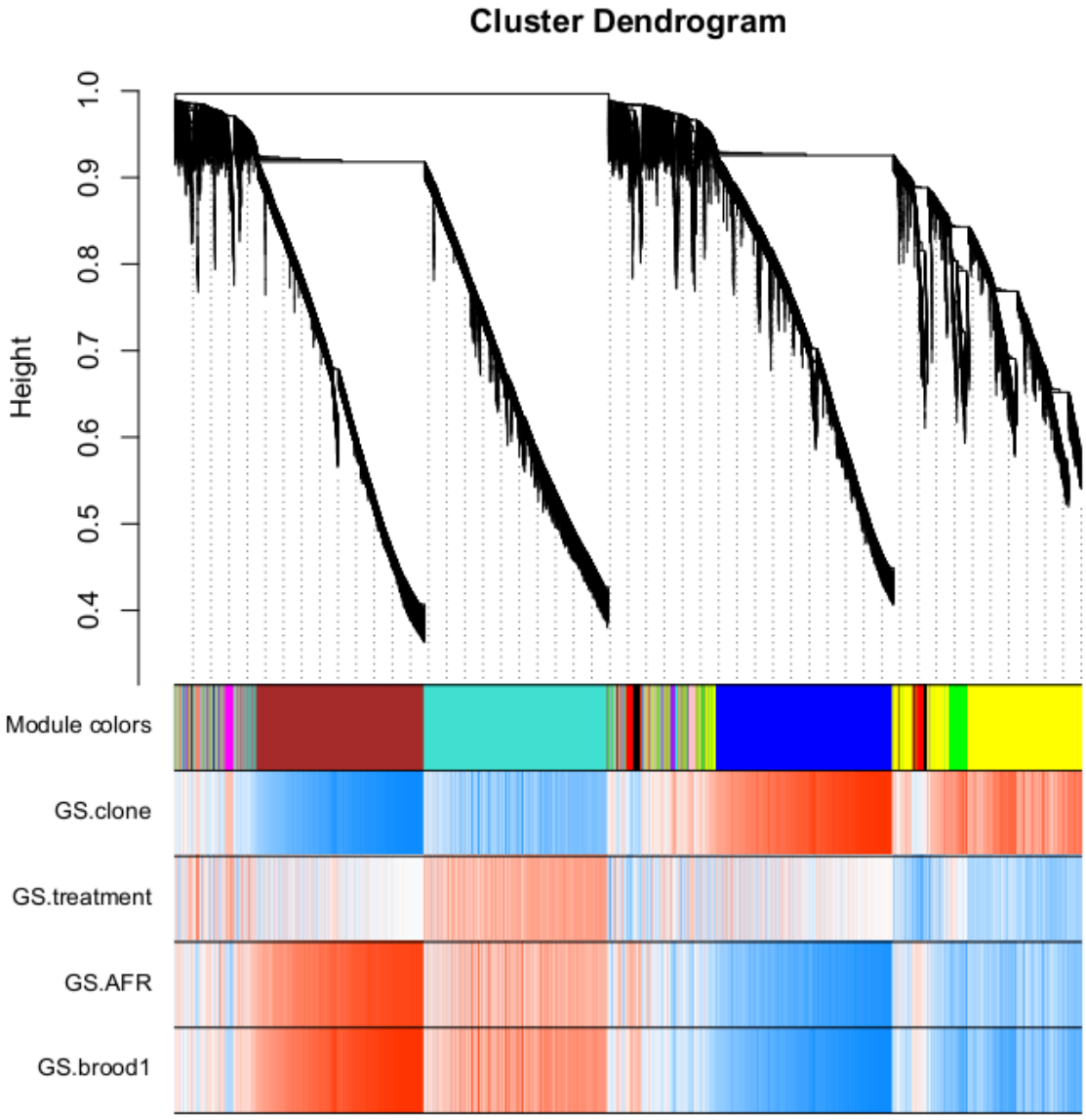

Figure 3. Cluster dendrogram of transcripts in *Daphnia galeata*, with dissimilarity based on the topological overlap matrices (TOM). Additional assignments are module colors, the gene significances (GS) for the trait clone (clonal line), treatment (fish kairomone exposure), age at first reproduction ('AFR') and numbers of offspring first brood ('brood1'). Red and blue indicate a positive and negative correlation of the module with the respective trait. Darker hues indicate higher correlation values.

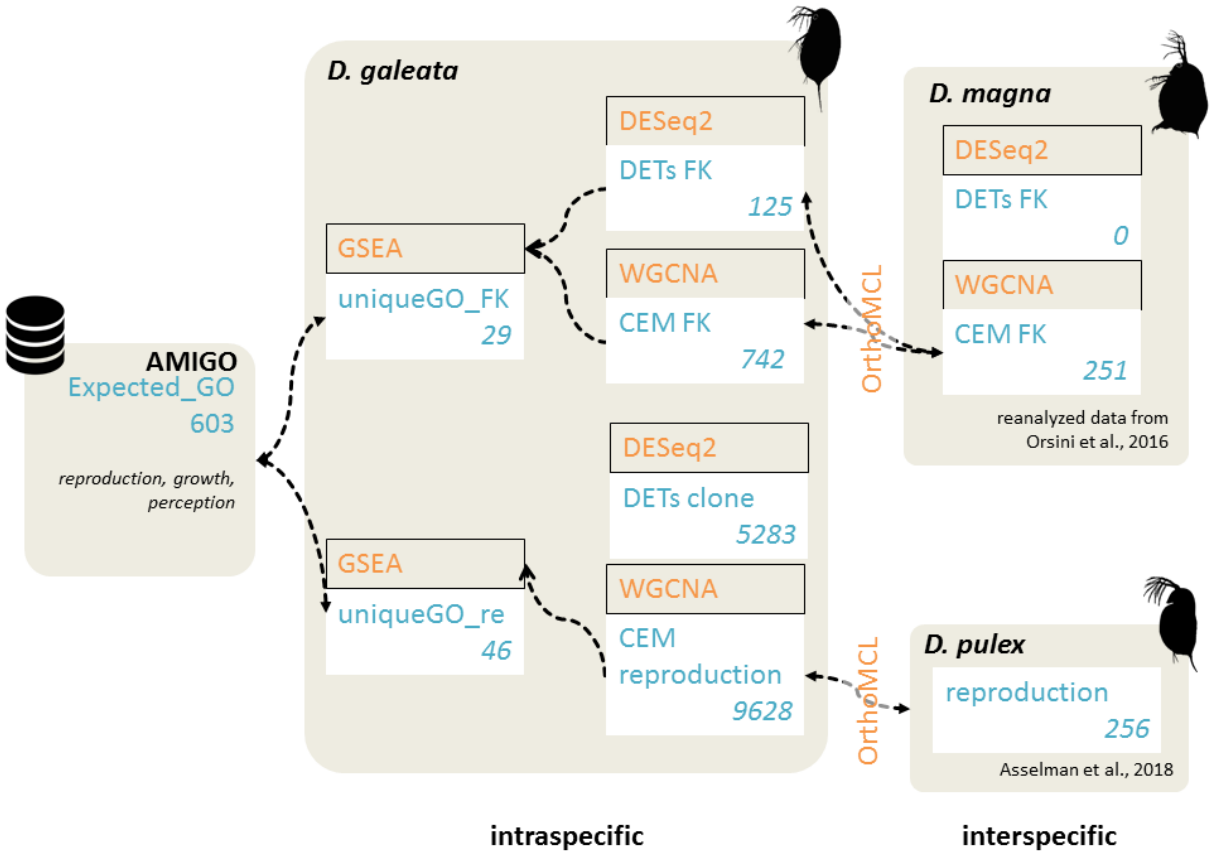

Figure 4. Overview of datasets created by gene expression and gene co-expression analysis and used for comparative transcriptomics. 'DESeq2' = gene expression analysis. 'WGCNA' = gene co-expression analysis. 'GSEA' = gene set enrichment analysis. 'OrthoMCL' = identification of orthologous clusters. 'DETs' = differentially expressed transcripts. 'CEM' = co-expression module. 'expected_GO' = expected GO terms (Table S2). 'uniqueGO_FK' = significantly enriched GO terms of FK-related transcripts (Table 3A). 'uniqueGO_re' = = significantly enriched GO terms of reproduction-related transcripts (Table 3B).

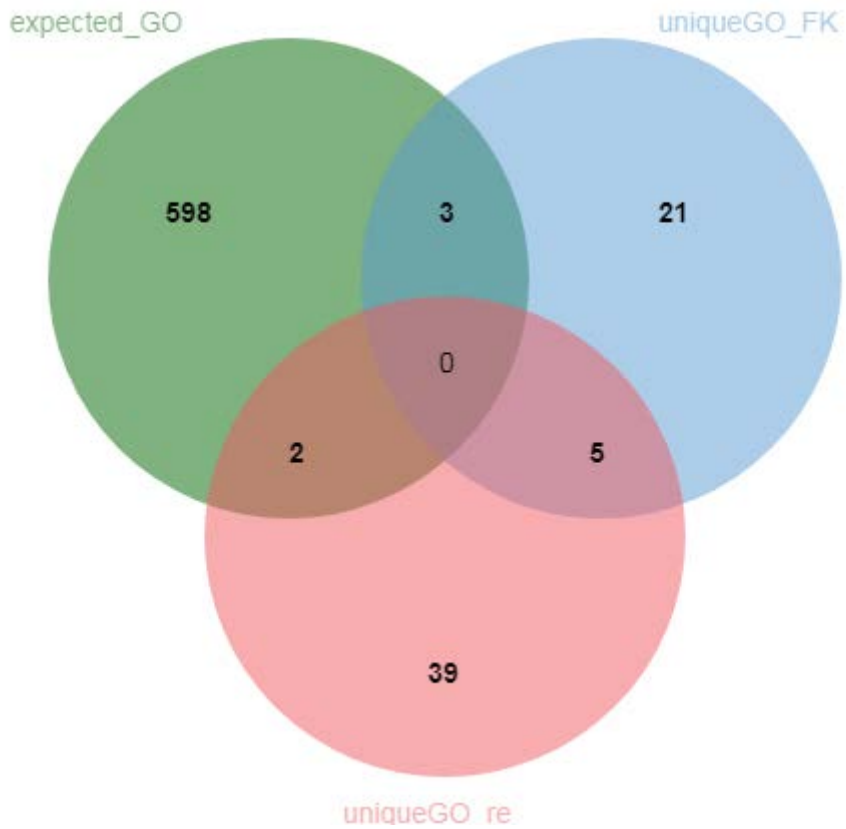

Figure 5. Venn diagram of Gene Ontology (GO) classes of *D. galeata* datasets. 'expected_GO' = classes derived from AMIGO database (Table S2). 'uniqueGO_FK' = significantly enriched GO classes related to fish environment (Table 3A). 'uniqueGO_re' = significantly enriched GO classes related to reproduction (Table 3B).

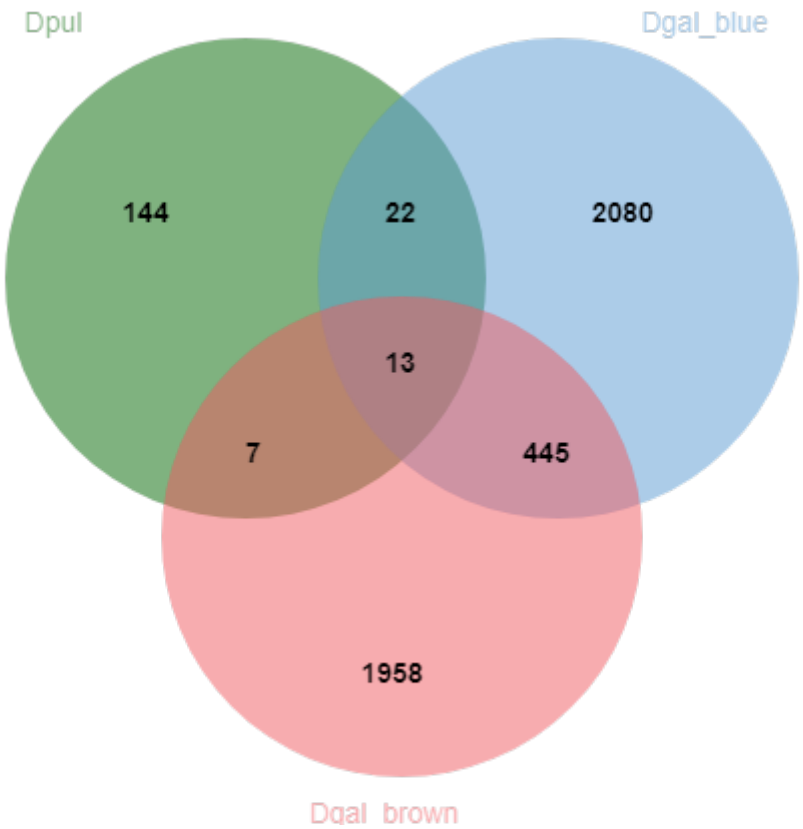

Figure 6. Venn diagram of orthologous clusters comprising reproduction-related transcripts. 'Dpul' = orthologous clusters for *D. pulex* (Asselman et al., 2018). 'Dgal_blue' & 'Dgal_brown' = orthologous clusters for *D. galeata* transcripts from the co-expression modules 'blue' and 'brown', respectively.