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# Effects of high rearing density on the transcriptome of *Drosophila simulans*

**Bachelor thesis**

submitted by
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Thank you!

# Abbreviations

| | |
|---|---|
| DE analysis | differential expression analysis |
| DE-genes | differentially expressed genes |
| cpm | Counts per million |
| lowD | group of flies reared in low population density |
| highD I | group of flies reared in high population density and collected on the 11$^{th}$ day after egg-lay |
| highD II | group of flies reared in high population density and collected on the 12$^{th}$ day after egg-lay |
| highD III | group of flies reared in high population density and collected on the 13$^{th}$ day after egg-lay |
| highD | all groups flies reared in high density together |
| CGE | common garden experiment |
| GO-term | gene ontology term defined by the GO consortium (The Gene Ontology Consortium, 2019) |
| Functional enrichment | Enrichment of information about biological functions |
| GO analysis | analysis which implements functional enrichment |
| GO-table | Result of the GO analysis, table with GO-terms, GO-IDs and more |
| PCA | Principal component analysis for the simplification of large data |
| PC1 | First principal component of the PCA |
| PC2 | following PC1 the second principal component |
| FC | Fold change, usually the level of gene expression between two groups |
| WGCN | weighted gene co-expression network |
| WGCNA | weighted gene co-expression network analysis |

# 1. Introduction

## 1.1. Population density

Population density is defined as the amount of living entities in a fixed volume or a certain area. The impact of diverse population densities on organisms have been studied in a large variety of species.

Several phenotypic changes due to high population density have been observed, including changed sexual selection in beetles (McCullough, Buzatto and Simmons, 2018), higher corticosterone levels in voles (Blondel *et al.*, 2016), less immune system function as well as a lower body condition in mammals, fish and birds (Newman *et al.*, 2015).

## 1.2. Population density and larval density in Drosophila

About 60% of the human genome is homologous to that of *Drosophila melanogaster* (Mirzoyan *et al.*, 2019). Moreover, the genome of these flies is less redundant and therefore contains homologs to 75 % of genes which cause human-diseases (Mirzoyan *et al.*, 2019). Hence, by studies in *Drosophila*, fundamental biological mechanisms could be revealed, which could also be relevant for humans.

A major reason *Drosophila* became such an important model organism is among many others that the flies are easy and cheap to rear and breed. Optimal population density varies in respect to the specific species and viability of the line (Greenspan, 2004). Unfortunately standardized conditions are not common, impeding comparisons between studies.

Many phenotypic changes due to high rearing density in *Drosophila* were revealed by observational studies. It is well known that such changes are usually the result of **phenotypic plasticity**[1] in the flies. In natural populations of *Drosophila* high larval densities or larval crowding is emerging as a result of an over availability of food (Atkinson, 1979). In this situation the fly experiences stressful conditions like scarcity of space, toxic waste products and a competition for food. This causes a complex and high selection pressure (Birch, 1955). Although this environment only emerges at times, it could be a major driver in the evolution and **adaptation**[2] of *Drosophila* (Horváth and Kalinka, 2016).

Intriguingly high larval density results in severer and often more detrimental phenotypic changes than high population density in adult flies (Morimoto *et al.*, 2017; Poças, Crosbie and Mirth, 2020). This is even more fascinating, considering that larvae undergo a

[1] **Phenotypic plasticity** is the change in the expressed phenotype of a genotype as a function of the environment (Scheiner, 1993). It describes the way an organism changes its phenotypes, due to different environments. Phenotypic plasticity is evolved by organisms, because it greatly enhances fitness in more diverse environments. Importantly it also shapes the direction of evolution. Although phenotypic plasticity is increasing the fitness of an organism, there is also an energy cost to it, according to Sultan and Spencer (2002). Interactions between gene loci and epigenetics are important mechanisms causing plasticity (Pigliucci and Pigliucci, 2001).

[2] **Evolutionary adaptation** is the alteration or adjustment of organisms in structure or habits which is inheritable. Nowadays it is well known that selection and genetic drift are the main drivers of evolution and therefore adaptation.

**metamorphosis**[3]. It shows that certain phenotypes are determined in the development of an organism (Pigliucci and Pigliucci, 2001)(p. 5).

The size and shape of a fly is related to various fitness associated traits. Under high larval density the body size of the adult fly decreases, as a result of the overall smaller cell number (Klepsatel *et al.*, 2014). Based on that, it has been shown that four morphological traits, thorax and wing length, sternopleural and abdominal bristle number vary under different larval crowding (Imasheva and Bubliy, 2003).

Interestingly mild larval crowding triggers an hormesis-like effect (Henry, Renault and Colinet, 2018; Lushchak *et al.*, 2019). To be specific it results in more thermal, starvation and toxic waste tolerance in *Drosophila*. It is shown that low concentrations of urea and uric acid also promotes cold tolerance, but not heat tolerance (Henry, Renault and Colinet, 2018). Moreover, mild larval crowding causes an increased longevity of the adult fly. The reason for this is not known, but putatively Hsp 70 or other heat shock proteins play a crucial role in this phenomenon (Buck *et al.*, 1993; Sørensen and Loeschcke, 2001). Other reasonable explanations for the longevity-phenomenon include changes in body size (Economos and Lints, 1984), subcellular organelle numbers, size and/or functional properties (Economos and Lints, 1985) or the adjustment of the composition of biomembranes (Moghadam *et al.*, 2015)**.**

Intriguingly, it has been found that the effect of longevity, seen in larval crowding, is at least partially caused by their diet (Klepsatel, Procházka and Gáliková, 2018). Since high population density enhances competition for limited food resources this is a crucial factor contributing to the phenotypic plasticity seen in this context. According to Klepsatel (Klepsatel, Procházka and Gáliková, 2018) by providing a yeast rich nutritional environment, the effects of high larval crowding are alleviated. A confirmation of this can also be seen in another study which proved that adult size is reduced by yeast restriction (Poças, Crosbie and Mirth, 2020). Moreover, it was found that the metabolome of the fly is also heavily

[3] At optimal rearing conditions **Drosophila's development**, meaning from egg to fully grown adult, takes about 9-10 days (Greenspan, 2004). Within one day the freshly laid eggs turn into an embryo, which starts to eat and grow. Then the organism undergoes three larval states (*Facts*, 2020). The last stage, before Drosophila is a fully-formed fly, is the pupal state, in which the organism performs its metamorphosis. Essentially the flies are reborn during metamorphosis (Bainbridge and Bownes, 1981). After the pupal state the fly ecloses as a fully grown adult.

affected by food competition in larval crowding (Henry, Renault and Colinet, 2018). It is further seen that the lipid content of the flies rises (Henry, Renault and Colinet, 2018), which is possibly also the reason for the increase in starvation tolerance (Zwaan, Bijlsma and Hoekstra, 1991).

Furthermore, it has been discovered that the behaviour of the flies is changing in high population density. Among other things mating behaviour is altered (Ribó, Ocaña and Prevosti, 1989), which is an important factor of the overall fitness of the flies. As well as male aggression is increased due to a higher level of a pheromone, namely cVA (Wang and Anderson, 2010).
Also *Drosophila's* synapses are differently regulated under high larval density (Stewart and McLean, 2004), which displays an enormous effort of the fly to **acclimate**[4] their behaviour and neural system to high larval density.
Flies develop more delayed and more distributed in time due to high larval density (Lints and Lints, 1971). It is suggested that the diet (Klepsatel, Procházka and Gáliková, 2018), toxic waste products (Botella *et al.*, 1985; Henry, Renault and Colinet, 2018), scarcity of space (Scheiring *et al.*, 1984) or a combination of many (Henry, Renault and Colinet, 2018) is causing the developmental delay. In addition, it has been found that in high larval densities fitness correlated negatively with development time (Horváth and Kalinka, 2016). This is suggesting that the larvae are competing as well for time.

## 1.3. Research question and goals

In natural populations of *Drosophila* sometimes high larval density arises due to the over availability of resources (Atkinson, 1979). Hence an immense amount of phenotypic plasticity has evolved. It is proven that high rearing density is a stressful environment owing to limitation of resources, habitat degradation and the spread of disease. Lots of changed phenotypes have been reported, for instance diverse morphological traits (Imasheva and Bubliy, 2003), development time (Horváth and Kalinka, 2016) and mating behaviour (Ribó,

[4] **Acclimation** is the process in which an organism undergoes especially physiological adjustments in response to environmental changes.

Ocaña and Prevosti, 1989) have been asserted to change in adult flies as a consequence of high larval density. Nevertheless, the field is missing the molecular explanation behind the observed plastic responses. This study aims to fill the gap with a high throughput transcriptomic resequencing approach and reveal not yet known altered traits and phenotypic plasticity in *D. simulans*[5].

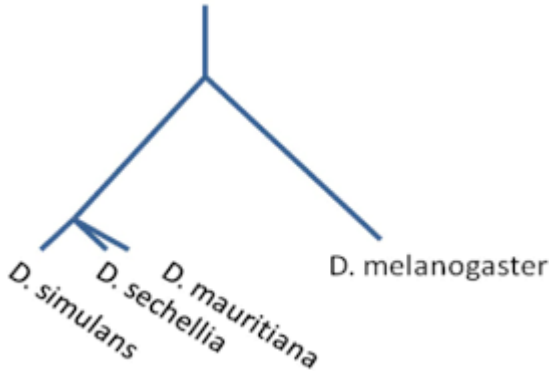


Fig. 1. Phylogenetic tree of Drosophila melanogaster species subgroup. (Phylogenetic tree of Drosophila melanogaster species gr | Open-i, 2020)

In order to structure this bachelor thesis, it was subdivided into three parts. Each of these three parts concentrates on finding the answer to the respective question, listed below:

1. How does rearing density affect gene expression of a *Drosophila* population?

2. Is there any difference in gene expression between the flies with different development rates in high density treatment?

3. Are gene expression changes by high rearing density advantageous or detrimental in the long run?

[5] *D. simulans* is a close relative of the more famous species *Drosophila melanogas*ter (greek drosos = the dew, philē = friend, melas = black, gastēr = stomach, belly; synonyme: *Sophophora melanogaster*). In Figure 1 a small part of the phylogenetic tree of the *Drosophilidae* family, containing the species *D. melanogaster* and *D. simulans,* can be seen.

All in all, the answers, which will be contained in this bachelor thesis, should contribute to the research goal of finding, asserting and measuring effects of rearing density in the transcriptome. I, as the creator of this bachelor thesis, implemented all the analyses on the computer as well as the comparison to literature.

# 2. Materials and Methods

## 2.1. Common Garden experiment

A *Drosophila simulans*, which was initially captured in Portugal, was maintained in a common garden experiment before being subjected to different rearing densities. The flies were reared at 28 °C during the day and 18 °C during the night for two generations. Afterwards, the layed eggs were used for the experiment. For the low rearing density, 400 eggs were put into the rearing bottles using a pipetting method (Nouhaud *et al.*, 2018) while four-fold of the number (1600) of eggs were used for high rearing density.

Because of the high rearing density the population undergoes a developmental delay. Unlike in the low density condition in which all flies eclose on the eleventh day after egg lay, the flies in high rearing density show varied developmental rates ranging from eleven to thirteen days. Flies which eclosed on different days were collected separately in order to investigate the cause of the variation. Thus, four groups of samples were collected (low rearing density on the $11^{th}$ day after egg lay (lowD), high rearing density collected on the $11^{th}$ day (highD I), high rearing density collected on the $12^{th}$ day (highD II) and high rearing density collected on the $13^{th}$ day (highD III)). Each sample comprised 50 males at the age of five days after eclosion. The flies were snap-frozen using liquid nitrogen and stored at -80 °C until the RNA extraction.

## 2.2. RNA-Seq library preparation

Flies taken from the -80°C storage were immediately immersed and homogenized in Qiazol (Qiagen, Hilden, Germany). Afterwards, Qiagen RNeasy Universal Plus Mini kit was used to extract the total RNA from the whole body of the flies. RNA-Seq libraries were prepared with the TruSeq stranded mRNA Library Prep Kit on a Neoprep device (software version 1.1.0.8 and protocol version 1.1.7.6, Illumina, San Diego, USA) starting with 100 ng of total RNA and using the default settings with an insert size of 200 bp and 15 PCR cycles. We avoided batch effects by randomising all libraries across library cards with identical lot numbers. 50 bp reads were sequenced on the Illumina HiSeq 2500 platform.

## 2.3. Data Preprocessing

Several quality check analyses were implemented to ensure reliability. Sequencing quality was assessed with FastQC (Wingett and Andrews, 2018). Due to a 3'-bias only four replicates of highD III with sufficient quality were usable and thereby utilized. The remaining two highD groups contained five replicates.

After sorting out the samples, which displayed poor quality, the low quality ends of the reads were trimmed. For this reason a modified Mott algorithm, implemented by the TrimReads function of the package 'readtools' (Gómez-Sánchez and Schlötterer, 2018) was applied. Afterwards the reads were mapped to the *Drosophila simulans* reference genome (Palmieri *et al.*, 2015) via gsnap (Wu *et al.*, 2016). A maximum of eight percent mismatch to the length of the read was allowed and further the lookout for novel splicing was enabled. Executing Rsubread (Liao, Smyth and Shi, 2019) the reads were counted on the basis of the *Drosophila simulans* reference genome (Palmieri *et al.*, 2015). To exclude samples, which contain degraded RNA, a gene body coverage analysis, using RSeQC (Wang, Wang and Li, 2012) was carried out. This ensured that consequently 3'-biased samples could be sorted out.

Subsequently the distribution of reads between replicates and groups was inspected, whereby the R-package edgeR (Robinson, McCarthy and Smyth, 2010) was utilized.

Initially, the data was cleaned from genes that had less than one count per million in at least one sample. Utilising the R package edgeR (Robinson, McCarthy and Smyth, 2010) estimated scaling factors (Robinson and Oshlack, 2010) were calculated and thereby the expression values were normalized. In the next step the dispersion between the samples was estimated (Chen, Lun and Smyth, 2014).

## 2.4. Differential expression analysis

In order to investigate the transcriptomic variation a principle component analysis (PCA) was carried out and visualised using the log transformed CPM of all genes. The PCA is a standard method used for high throughput RNA seq data for dimensional reduction (Wit *et al.*, 2012). Briefly, based on singular value decomposition (SVD), orthogonal linear combinations of the multiple variables are generated as principal components (PCs) to

summarize the variance among samples (Ringnér, 2008). Afterwards, we inspect the variance explained by the leading PCs and how the samples were separated.

To identify genes affected by the rearing density, a linear model-based approach implemented in edgeR (Robinson, McCarthy and Smyth, 2010) was used. Different contrasts between samples were tested based on a likelihood ratio test (Chen, Lun and Smyth, 2014). First of all, the differences between lowD and highD, further three contrasts, each between lowD and one highD data group were tested. Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) was used to account for multiple testing.
For the following functional enrichment the significantly differentially expressed genes were filtered, by a minimum of 1.25 fold change (FC) and furthermore subdivided into up-, and down-regulated genes.

## 2.5. Weighted gene co-expression network analysis

Genes do not operate independently. In order to understand the co-regulation of multiple genes, we performed a weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008). Briefly, pairwise correlation between the expression variation among all expression genes were estimated. Based on the correlation matrix, genes with similar expression (high correlation) would be clustered together into a co-expressed module. Using the function “blockwiseModules”, implemented in R package WGCNA (Langfelder and Horvath, 2008), a signed network with a minimal module size of 100 genes was constructed. A signed network takes the directionality of the correlation into account, separating up-, and down-regulated genes. Furthermore, the argument reassignThreshold was set to $10^{-4}$ for more precise module assignment. Default parameters were used if not specified. Co-expressed genes in each module were subjected to GO enrichment analysis for better understanding of their biological implication.

## 2.6. Functional enrichment analysis (GO analysis)

To obtain information about the biological processes of the different groups of genes, an analysis based on gene ontology terms (GO-terms) with the R package “TopGO” (*topGO*, 2020) was conducted.

GO-terms were defined by the gene ontology consortium (The Gene Ontology Consortium, 2019) in order to facilitate the analysis of gene functionality in high throughput data. The abundance of assayed genes in high throughput transcriptomic studies makes it impossible to investigate the functions of the genes of interest one by one. Therefore, genes are assigned to GO-terms reflecting biological processes, molecular functions or cellular components in a hierarchical system, containing specific and broader terms. The enrichment of specific GO terms by the candidate gene sets could provide more insights into the functional implication.

Using the “weight01” algorithm implemented in the topGO package which accounts for the potential bias introduced by the GO hierarchy, we tested for enrichment of biological processes among the genes of interest in this study. Only terms with more than five genes being annotated were considered. The sources of the GO annotation and gene ID mapping were based on the database for *Drosophila melanogaster* (namely, R objects “annFUN.org” and “org.DM.eg.db” in R/BioConductor V3.10).

## 2.7. Comparison of plasticity to evolutionary response

Overall, a *Drosophila* population exhibits substantial phenotypic plasticity in the face of high rearing density. However, it is interesting to learn about the fitness effect of the observed plasticity.

The *Drosophila* population used in the evolution-CGE was generated from 113 isofemale lines, which originally collected in Northern Portugal. Firstly, a reference base population was constituted (Nouhaud *et al.*, 2016), by keeping the isofemale lines at 18 °C for five generations at 50 flies/ vial (a small population size). Sequentially, ten independently replicated populations were created by pooling five females from each isofemale line. All of them were held under a 12h circadian cycle, consisting of alternating light and dark periods. Five replicates were reared at 28/18 °C (hot evolved) and five at 20/10 °C (cold evolved),

each temperature in the respective circadian period. Importantly, the flies were held at a population density (250 adult flies/bottle) of 1000 - 1250 adults per generation.

Summarizing, three data groups were created, each consisting of five replicates. Evolution occurred under high population density and thermal stress. Flies were harvested between the $77^{th}$ and $133^{th}$ generation.

Ultimately, like in the density CGE (Chapter 2.1.) the flies were frozen and prepared for the RNA-extraction. Afterwards, the creation of RNA-seq libraries and the data preprocessing was conducted using the exact same protocol as previously for highD and lowD (Chapter 2.2.; Chapter 2.3.).

The data analysis on the computer comprised a DE-analysis, in which the obtained genes were subdivided in up-, and down-regulated genes. DE genes from both directions of thermal stress were obtained. For the further comparison they were intersected, creating up-, and down- regulated genes from both thermal conditions together. In the next step, it was asserted if the DE-genes from both experiments were coinciding. This was done using Fisher's exact test. Four tests were done, which were corresponding to the respective comparison of down-, and up-regulated genes of both experiments.

Finally, a GO analysis was implemented, providing information about functional differences between the gene expressions.

## 2.8. List of used programs

Tab. 1. Programs used for the data preprocessing

| **programs** | **version** |
|---|---|
| R | 3.6.3 |
| Python | 3.8.4 |
| GMAP: Genomic Mapping and Alignment Program | 2019-09-12 |
| GSNAP: Genomic Short Nucleotide Alignment Program | 2019-09-12 |
| readtools | 1.5.2 |
| samtools | 1.10 |
| htslib | 1.10.2 |
| RSeQC | 2.6.4 |
| **R package** | |
| Rsubread | 2.2.6 |

Tab. 2. R packages used for the analyses.

| **program** | **version** |
|---|---|
| R | 3.6.3 |
| **R packages** | |
| edgeR | 3.28.1. |
| DESeq2 | 1.26.0 |
| ExpressionNormalizationWorkflow | 1.12.0 |
| Venndiagram | 1.6.20 |
| fastICA | 1.2-2 |
| WGCNA | 1.69 |
| TopGO | 2.38.1. |
| ggplot2 | 3.3.2 |
| EnhancedVolcano | 1.4.0 |

# 3. Results

## 3.1. Transcriptomic plasticity in response to high rearing density

In order to understand the general impact of high larval rearing density on the transcriptome of a *Drosophila* population, we contrasted the gene expression pattern of flies reared in high density, which includes 14 samples from three sequential time points, to five replicates of flies reared in low density.

### 3.1.1. How big is the impact on gene expression?

To summarize the transcriptomic variation of the samples in this study, a PCA was conducted (Fig. 2.). The data points of lowD and highD in Figure 2 are clearly differentiable via the first principal component (PC1), which explains 35.86% of variance in the data set. Such strong impact suggests that rearing density is an important factor that alters the gene expression of flies. Within the cluster of highD, samples collected on different days are also distinguishable by PC1. Furthermore, it seems that highD III data points are located between highD I data points and highD II data points.

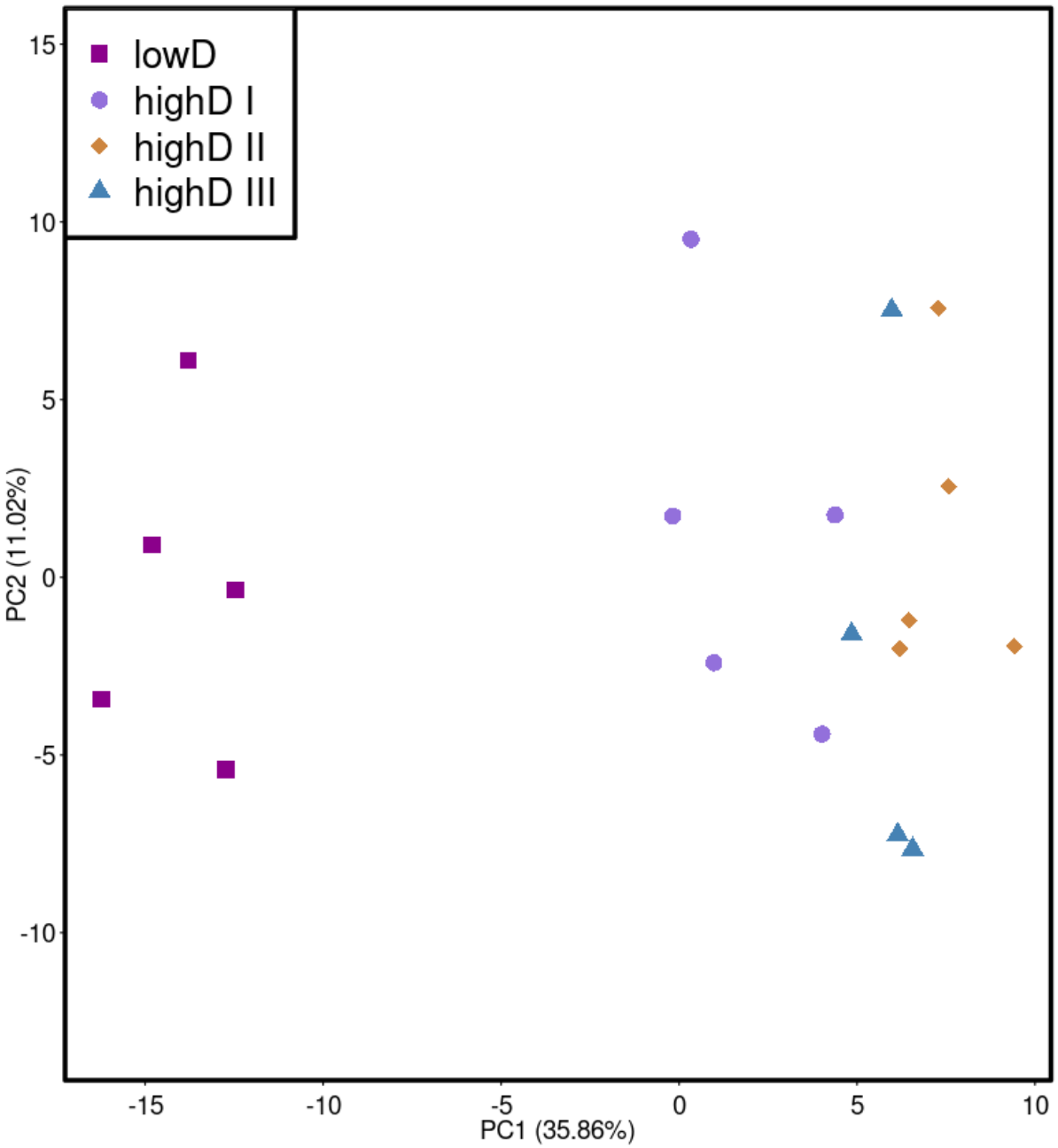


Fig. 2. Principal Component Analysis. PC1 explains 35.86% and PC2 explains 11.02% of the overall variance.

Although it has been already shown in the PCA that there is a relatively big difference of gene expression between highD and lowD, a further more thorough investigation of the quantity of differentially expressed genes had to be done.

Among all 10,821 expressed genes in *D. simulans*, 22.5 % of them are significantly differentially expressed (Fig. 3.). Furthermore, 453 genes are significantly differentially expressed and have an absolute fold change (FC) of more than 1.25 between lowD and highD (Fig. 3.). Depicting the differentially expressed genes in a Volcano plot (Fig. 3.), highD against lowD was contrasted. This means that genes which show a positive FC are highly expressed in highD and lowly expressed in lowD.

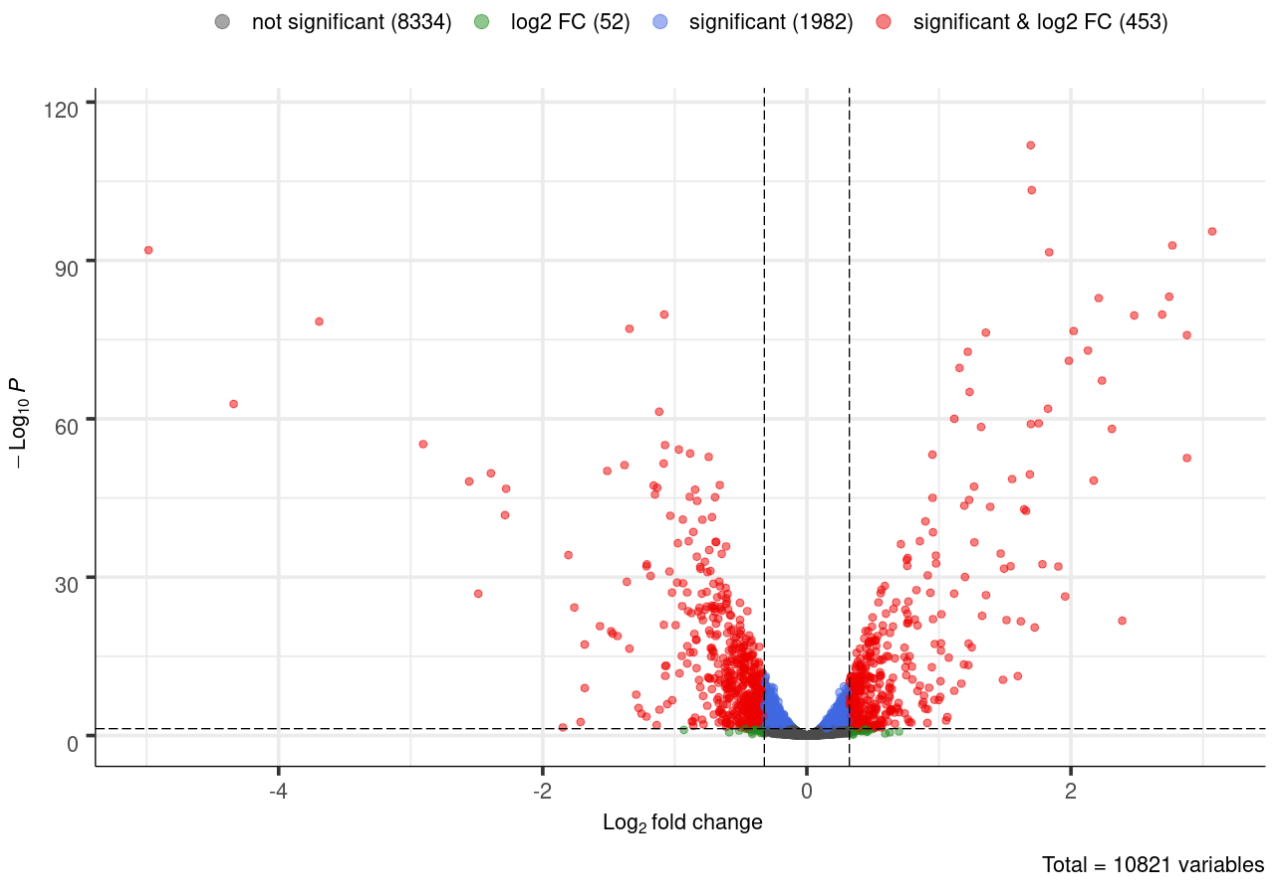


Fig. 3. Volcano Plot of highD against lowD. P-value threshold at -log10(0.05), FC threshold at log2(1.25).

### 3.1.2. Which processes are altered?

In order to get an insight about which biological mechanisms are affected, functional enrichment was realized, using the R-package “topGO” (*topGO*, 2020). DE-genes were subdivided into up-, and down-regulated genes, yielding information about the directionality of the biological process.

#### 3.1.2.1. Functional enrichment of differentially expressed genes

Two tables of GO-terms were obtained (supplementary tables: GOall_density_up, GOall_density_dn).

The GO analysis of the up-regulated gene list revealed GO-terms related to metabolism, energy production, muscle activity and other biological processes.

It is seen that energy production is upregulated, as it can be inferred from the GO-terms “mitochondrial electron transport, NADH to ubiquinone” (Fig. 4. A), “mitochondrial electron transport, ubiquinol to cytochrome” (GO:0006122) and “ATP synthesis coupled proton transport” (GO:0015986) (supplementary table: GOall_density_up).

Furthermore the GO-terms skeletal myofibril assembly (Fig. 4. B), regulation of muscle contraction (GO:0006937) and musculoskeletal movement (GO:0050881) (supplementary table: GOall_density_up), are suggesting that the flies and/or larvae are increasing their muscle activity.

In addition, proteolysis (GO:0006508) is up-regulated. As well as chitin metabolism associated GO-terms, for instance “chitin metabolic process” (GO:0006030), “chitin-based cuticle development” (GO:0040003) is up-regulated.

In contrast to the up-regulated genes, the GO analysis of the down-regulated gene list showed that multicellular reproduction (Fig. 4. C) was reduced.

Moreover, the flies significantly down-regulated response to bacteria (GO:0009617), the response to fungus (Fig. 4. D, GO:0009620) and the humoral immune response (GO:0006959), indicating that in general the immune system is down-regulated in highD.

Furthermore, the fly also showed a down-regulation in some metabolic processes, like the tyrosine metabolic process (GO:0006570), the gluconeogenesis (GO:0006094) or the cellular lipid metabolic process (GO:0044255).

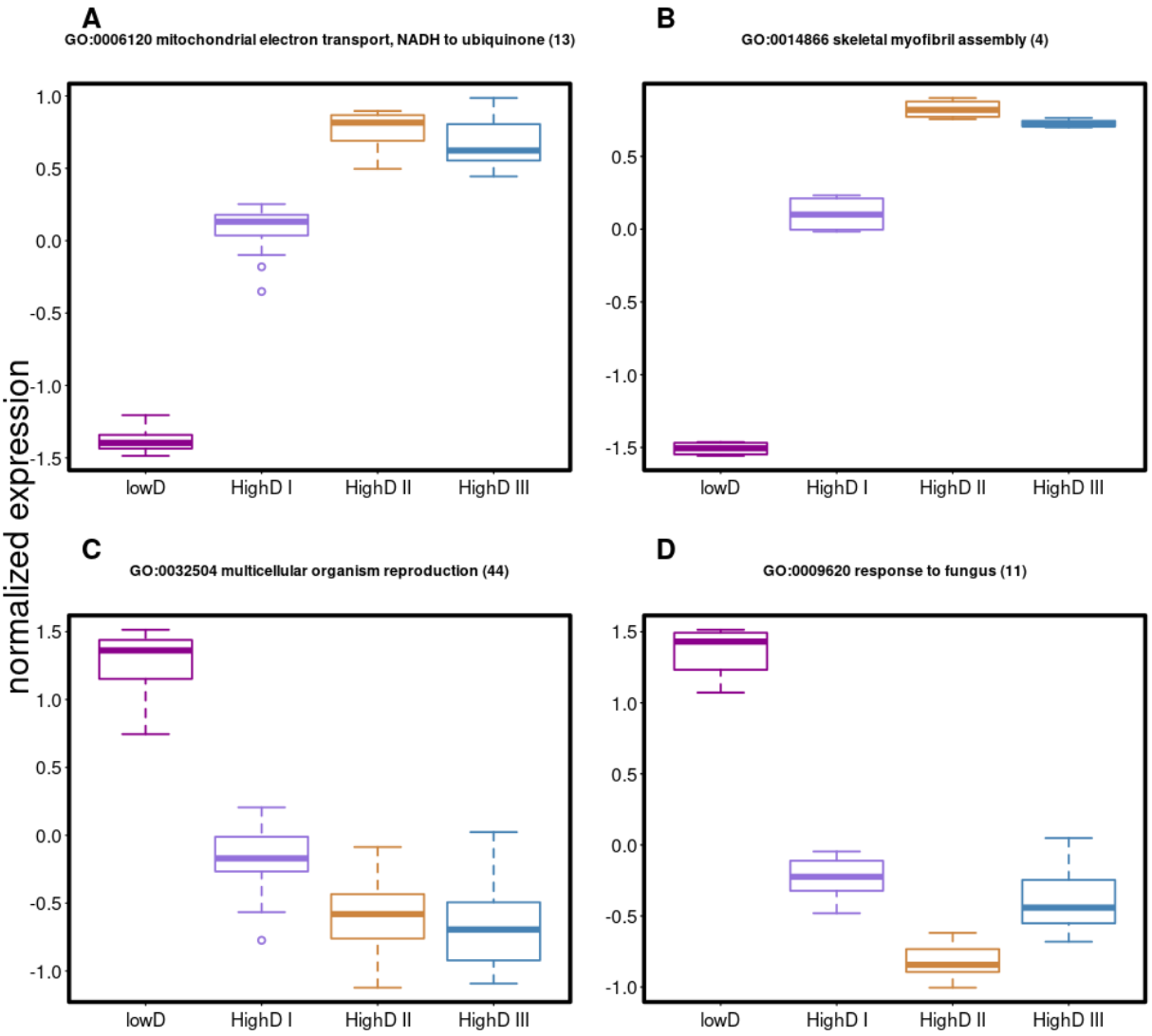


Fig. 4. Normalized expression levels of genes assigned to important GO-terms. Number of significant genes in GO-term visible in round brackets

#### 3.1.2.2. Weighted gene co-expression network analysis

Aiming to obtain a deeper insight in the changing expression of different biological functions, gene expression patterns were clustered, followed by another functional enrichment. Clustering was implemented via the generation of a weighted gene co-expression network (WGCN) with the R-package “WGCNA” (Langfelder and Horvath, 2008). Afterwards a functional enrichment on each cluster or module was realized, utilising the R-package “topGO” (*topGO*, 2020).

Modules have a unique expression pattern (Fig. 5.). The assigned number of genes in a module is highest in Module 0 and decreases gradually with successive module number. Module 1 and Module 2 contain genes, which are up-, and down-regulated significantly in lowD and highD, respectively (Fig. 5.). The overlap of Module 1 and 2 to the significant up-, or down-regulated DE-genes was tested via Fisher's exact test. In both cases, it was highly significant (for both p-value < 2.2e-16), suggesting that many genes are contained by both groups. However, in the contingency table used for the fisher’s tests numerous additional genes in the WGCNA Modules can be found.

A lot of genes were also clustered in modules displaying a decreasing (Module 4) or an increasing (Module 5) gene expression pattern. Indicating general trends of up-, or down-regulation due to the respective harvesting time in the highD groups.

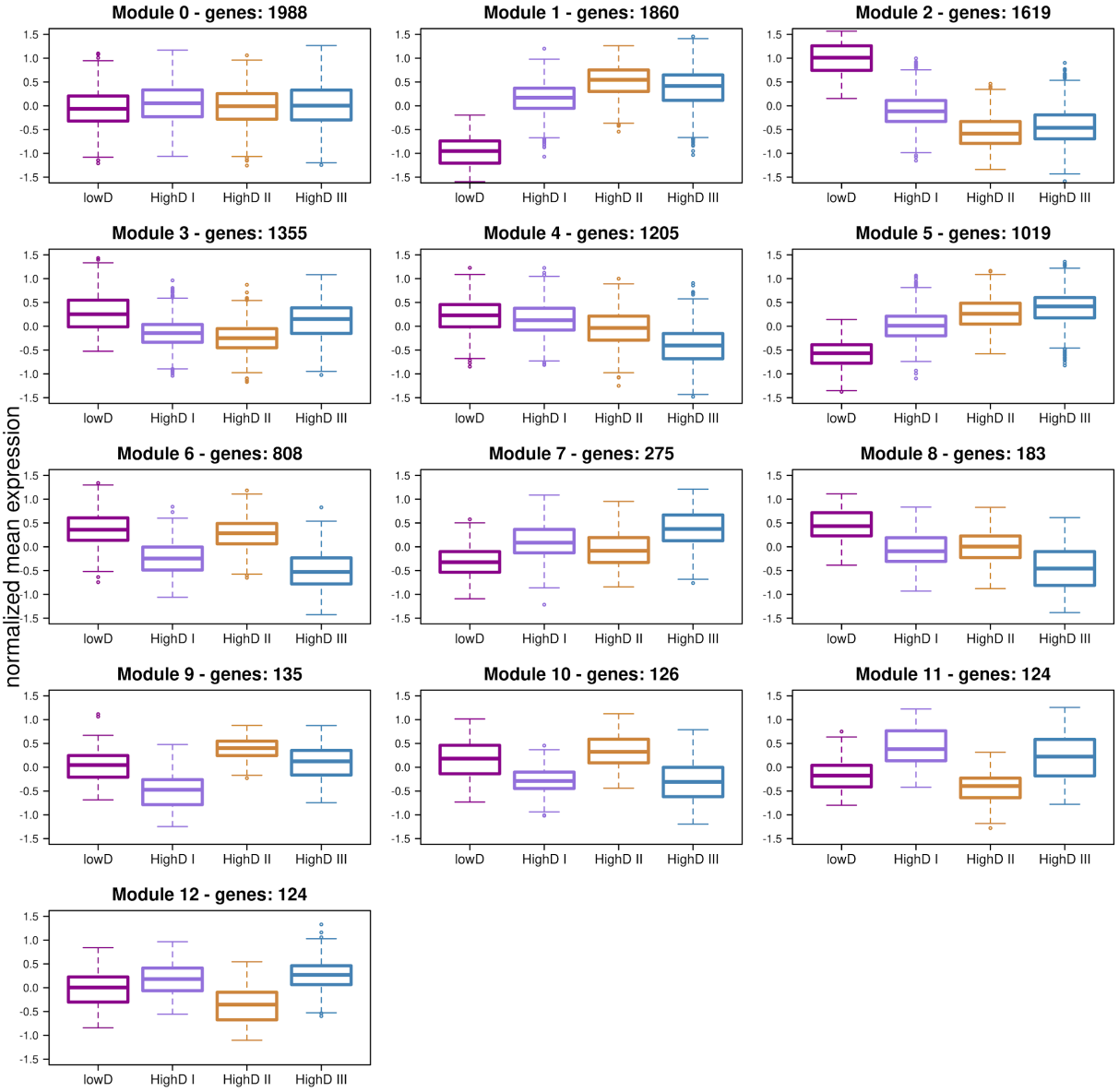


Fig. 5. Gene expression patterns and sizes of each module.

### 3.1.2.3. Functional enrichment of different modules

Functional enrichment for each of the 13 Modules was realized. The obtained GO-terms of each module can be interpreted with their respective gene expression pattern (Fig. 5.). The WGCNA also includes genes which were not significantly differentially expressed. As a consequence, we gain more and broader results from the GO analysis.

In Module 0 there is minimal gene expression change. The top GO-term of Module 0 (Tab. 3.) and most of the other obtained GO-terms in this module indicate that the corresponding genes are crucial for survival and housekeeping.

Module 1 seems to comprise genes which are up-regulated in highD (supplementary table: Module1_GO_res). The results gained from the DE analysis are indicating that energy production in highD is enhanced and the obtained GO-terms in this module likewise. This includes the up-regulation of mitochondrial translation (GO:0032543), diverse processes in the mitochondrial electron transport (GO:0006120, GO:0006122, GO:0034551) and ATP biosynthetic processes (GO:0006754). Besides, GO-terms like musculoskeletal movement (GO:0050881) and skeletal myofibril assembly (GO:0014866), potentially associated with muscle activity were acquired.

Module 2 shows an opposite gene expression pattern to Module 1. Among others, it comprises genes which were significantly down-regulated in the DE analysis (supplementary table: Module2_GO_res). Similar to the down-regulated genes in the DE analysis, this module includes the down-regulation of reproduction associated genes, like multicellular organism reproduction (GO:0032504) and sperm storage (GO:0046693). It is also seen that functions of the immunsystems, like the response to fungus (GO:0009620) and the innate immune response (GO:0045087) are down-regulated. Furthermore, it can be asserted that metabolic processes, such as oxidation-reduction processes (Tab. 3. ; GO:0055114), carbohydrate metabolic processes (GO:0005975) and alpha-amino acid catabolic processes (GO:1901606) are down-regulated.

The GO analysis of the fourth module (supplementary table: Module3_GO_res) revealed that some genes are regulating functions and characteristics of the nervous system. Among other GO-terms axon guidance (Tab. 3. ; GO:0007411), nervous system development (GO:0007399) and long-term memory (GO:0007616) were asserted. Moreover, axon guidance (GO:0007411), long and short-term memory (GO:0007616; GO:0007614) and synaptic growth at neuromuscular junctions (GO:0051124) are altered.
The results of Module 4 and 5 are introduced in 3.2. Differences between highD groups since they show essentially differences between the highD groups.

Tab. 3. GO terms of the modules of WGCNA

| Module ID | # of genes | Top GO-term |
|---|---|---|
| Module 0 | 1988 | regulation of transcription, DNA-templated |
| Module 1 | 1860 | cytoplasmic translation |
| Module 2 | 1619 | oxidation-reduction process |
| Module 3 | 1355 | axon guidance |
| Module 4 | 1205 | proteasome-mediated ubiquitin-dependent protein catabolic process |
| Module 5 | 1019 | axon guidance |
| Module 6 | 808 | cilium-dependent cell motility |
| Module 7 | 275 | rRNA processing |
| Module 8 | 183 | mitotic DNA replication checkpoint |
| Module 9 | 135 | sensory perception of smell |
| Module 10 | 126 | appendage morphogenesis |
| Module 11 | 124 | regulation of histone acetylation |
| Module 12 | 124 | ribosome disassembly |

## 3.2. Differences between highD groups

### 3.2.1. Module 4 and 5 of WGCNA

Two modules, namely 4 and 5 from the WGCNA also showed primarily changes between the highD groups (Fig. 5.).

By taking a closer look at Module 4 (supplementary table: Module4_GO_res), it can be asserted that this module comprises genes related to metabolism, the proteasome and transcription. In this module the gene expression pattern is gradually decreasing, with the highest expression level in lowD and the lowest expression in highD III. Among other GO-terms, functional enrichment yielded oxidative phosphorylation (GO:0006119), proteasome assembly (GO:0043248), malate metabolic process (GO:0006108) and acyl-CoA metabolic process (GO:0006637). Interestingly, Module 4 also comprises a response to cold (GO:0009409).

Lastly Module 5 seems to mirror Module 4, by having the lowest expression level in lowD and the highest in highD. Several GO-terms associated with the regulation of neural systems and behaviour were obtained (supplementary table: Module5_GO_res). On the contrary to module 4, it can be asserted that module 5 comprises many GO-terms associated with behaviour. The flies show change their larval locomotory behaviour (GO:0008345), male courtship behaviour (GO:0008049), social behaviour (GO:0035176), chemosensory behavior (GO:0007635) and also a changed behavioral response to nutrients (GO:0051780). GO-analysis of Module 5 also revealed a response to light stimuli (GO:0071482) and phototaxis (GO:0042331).

Furthermore some responses to diverse conditions have been found in Module 5. The flies showed a response to salt stress (GO:0009651), starvation (GO:0042594) and anesthetics (GO:0072347). Besides, a cellular response to drugs (GO:0035690) and decreased oxygen levels (GO:0036294) were asserted. Intriguingly also a response to heat (GO:0009408) is seen.

### 3.2.2. DE-analysis of highD groups

To assess the differences between highD I, high D II and highD III three DE analyses were conducted, contrasting the respective highD group with the reference lowD. The result was plotted in a Venn diagram (Fig. 6.), showing the intersecting DE-genes between every contrast.

Overall, 574 DE-genes are shared, indicating the abundance of gene expression difference between lowD and highD. A relatively large amount of highD II-specific (262) and highD III-specific (194) DE-genes were obtained. Although 235 DE-genes are shared, this suggests large differences between highD II and highD III. Furthermore there are relatively few highD I-specific genes (69), which indicates that in addition to the high population density highD II and highD III have to acclimate to other factors as well.

As it is already pointed out in the Module 4 and Module 5 in the WGCNA, several genes change their expression depending on the time of eclosion. However the extent of the difference between the highD groups cannot be seen in the significantly differentially expressed genes.

Moreover, it is notable that the result of the PCA located the highD III data points between highD I and highD II data points. This pattern is also visible in more specific groups of genes (Fig. 4. A, B, D; Fig. 5. Module 1, 2).

Since the gene expression is a direct consequence to conditions in the environment, it could be suggested that diverse conditions are changing over the picking time period. This could include metabolic waste products, scarcity of space and abundance of food.

A multiple-site similarity index (Diserud and Ødegaard, 2007) of 0.93 shows the high similarity of every comparison between lowD and the respective highD group. The multiple-site similarity index can be any number between zero and one, whereas one means 100 % similarity and vice versa. This shows the high influence of high rearing density, even so the harvesting time also proves to have an impact on the transcriptome.

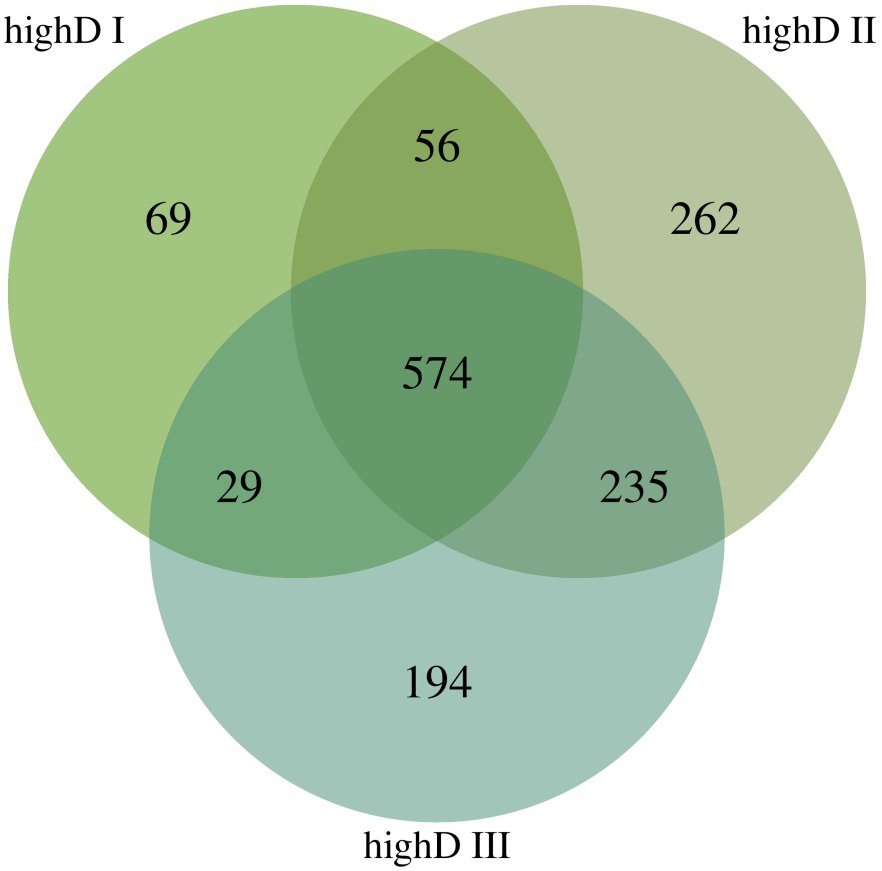


Fig. 6. Three-way Venn diagram of differentially expressed genes of lowD and respective highD group

The GO-analysis of the respective comparison of the highD group to lowD did not yield more information than the GO analysis in chapter 3.1.2.1. Functional enrichment of differentially expressed genes.

Furthermore not many significant differentially expressed genes were obtained in the DE-analysis between each highD group. Thus, another GO analysis of these DE-genes was not indicated.

## 3.3. Comparison of acclimation and adaptation to high rearing density

To be able to compare the acclimation to the adaptation, a DE analysis successive to an evolution experiment had to be done. The evolution experiment includes populations with the same ancestry as the studied populations adapting to laboratory environments. Evolved populations were compared to their ancestors, identifying the genes that are putatively responsible for the adaptation to laboratory conditions. Sequentially, a comparison of the plasticity, seen in the contrast of lowD to highD, and the adaptation to laboratory was realized. First, we tested whether the DE-genes were coinciding. The directionality of expression changes during acclimation and adaptation revealed the fitness effect of the plasticity. The following GO enrichment further provided insights into the biological processes involved in adaptive/mal-adaptive plasticity.

### 3.3.1. DE-genes in acclimation and adaptation

A scatterplot was generated, where the x-axis shows the adaptation to laboratory and stress conditions and the y-axis shows the acclimation to rearing density (Fig. 7.). Interestingly, there are more acclimation-specific DE-genes than evolution-specific DE-genes.

As it can be seen there are 26 consistently regulated genes, which are genes that are up-regulated or down-regulated in both contrasts. More common are the 46 inconsistent genes, which are up-regulated in one contrast and down-regulated in the other contrast.

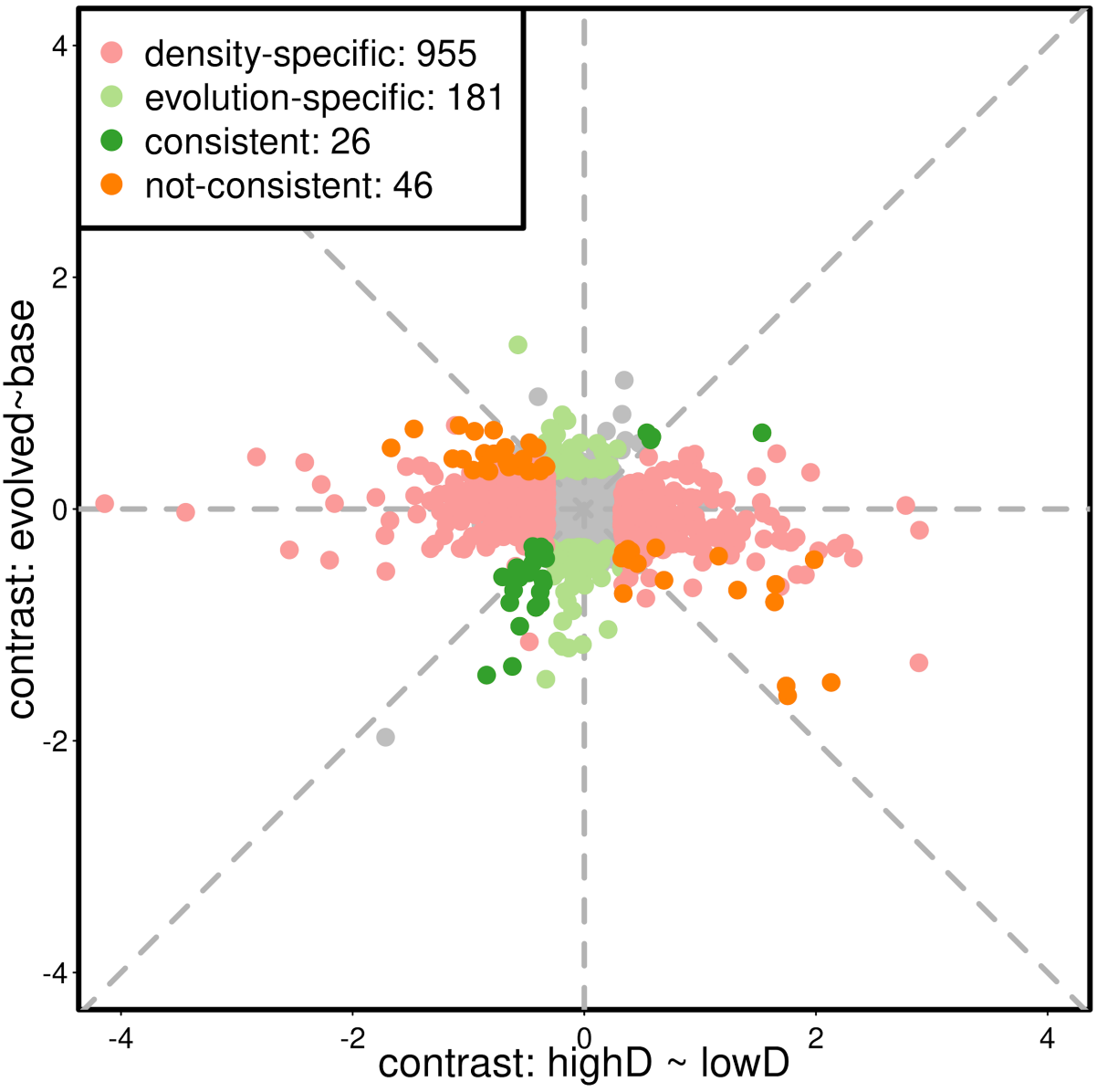


Fig. 7. Scatterplot of expression changes [log2FC]. Y-axis: adaption; x-axis: acclimation. Positive FC values stand for an up-regulation, whereas lowD and the base group is the reference.

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In order to test if the DE-genes of density are significantly overlapping with the DE-genes of evolution the Fisher's Exact Test was used.

Significant overlap was revealed (Tab. 3.), indicating that density induced changes coincide with adaptive responses to a laboratory condition which includes high rearing density.

Interestingly, the laboratory adaptive populations evolved for a compensatory up-regulation that counteracts a substantial portion of the plastic down-regulation changes (Tab. 4. B, C). This suggests that parts of the density-induced phenotypic plasticity are maladaptive.

Nevertheless the down-regulation of some genes in the acclimation and the adaption is evidence for the fitness benefit of some plastic responses (Tab. 4. D). By contrast, the up-regulation of most genes in the density acclimation is not beneficial (Tab. 4. A), as it can be seen in the not significant overlap and on the exaggerated genes in the first quadrant of the scatterplot (Fig. 7.).

Tab. 4. Fisher's Exact Test results. Asterisk marks significant test result

| | Comparison | Odds ratio | *p* value |
|---|---|---|---|
| A | density up ~ evolution up | 1.23 | 0.4174 |
| B | density dn ~ evolution up | 11.15 | < 2.2e-16 * |
| C | density up ~ evolution dn | 5.62 | 9.665e-09 * |
| D | density dn ~ evolution dn | 5.8 | 6.327e-10 * |

### 3.3.2. Functional enrichment of the compared DE-genes

After testing whether the DE-genes of density and evolution overlap, 4 lists of intersecting genes were created. Sequentially a GO analysis was implemented on each of the intersecting gene lists.

As it is also depicted in Figure 8, the measurement of gene expression level changes induced by stress and laboratory conditions occurred in low rearing density.

Since the first overlap (Tab. 4. A) was not significant, the odds-ratio and the results of the GO-analysis do not contain much informative value.

Obverse to the first comparison, the down-regulation of genes by acclimation and adaptation coincided significantly (Tab. 4. D). Hence the results of the GO analysis can be asserted. The plastic and advantageous responses of the flies, includes the down-regulation of the response to DDT (GO:0046680) and digestive system processes (GO:0022600). Intriguingly sphingomyelin metabolic processes (GO:0006684) and phospholipid catabolic processes (GO:0009395) are significantly down-regulated in the acclimation and adaptation.

The second and third comparison (Tab. 4. B, C), show opposite gene expression changes in acclimation and adaptation (Fig. 8. B,C).
Although *Drosophila* is up-regulating the metabolism of sugars for acclimation, it is disadvantageous. By taking a closer look at the detrimental up-regulation in acclimation (Fig. 8. B), it can be asserted that many GO-terms like regulation of glycogen metabolic process (GO:0070873), regulation of carbohydrate catabolic process (GO:0043470) and chitin metabolic process (GO:0006030) are associated with the sugar metabolism.

In the obverse comparison (Fig. 8. C) GO-terms like the lipid catabolic process (GO:0016042), aromatic amino acid family catabolic process (GO:0009074) were found. Therefore, it can be inferred that the up-regulation of the lipid and amino acid metabolism and the down-regulation of the sugar metabolism is beneficial. It can also be asserted that water homeostasis (GO:0030104) is more expressed after selection.

Although, most of the plastic response in metabolism is detrimental, it is seen that the down-regulation of the metabolism related with membrane lipids is advantageous.

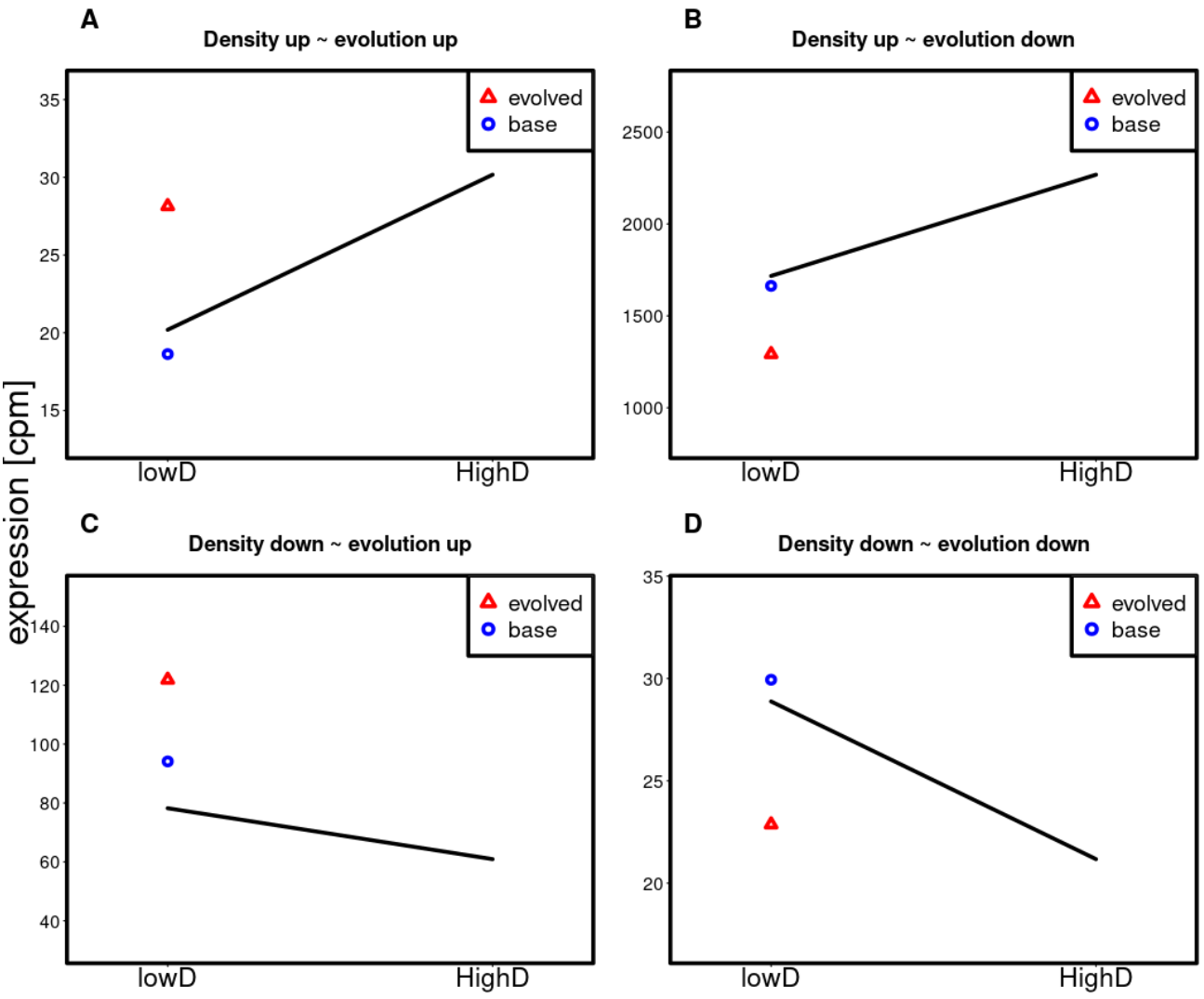


Fig. 8. Gene-expression of evolved and acclimated data. Depicting one gene representative for each comparison

# 4. Discussion

## 4.1. Effects of rearing density

Overall, it was asserted that high larval density affects the expression of 22.5 % of all expressed genes. It can be seen that important biological processes are altered. This includes the metabolism, cellular energy production, the immune system, reproduction, behaviour and other things. These findings are concordant with some observations and measurements of other studies.

Importantly, the functional enrichment of DE genes and the modules of WGCNA show that the fly is reacting to several kinds of conditions.

### 4.1.1. Altered responses to abiotic stress factors

In highD flies showed a response to chemicals (GO:0042221), to salt stress (GO:0009651), to starvation (GO:0042594), to heat (GO:0009408), to cold (GO:0009409), to anesthetic (GO:0072347) and a cellular response to drugs (GO:0035690). This is putatively indicating that the cause for several effects observed under high rearing density is caused by a combination of different responses of the flies. The tolerance to starvation (Zwaan, Bijlsma and Hoekstra, 1991) and the tolerance to heat and cold (Henry, Renault and Colinet, 2018) might be the consequence of the respective previously mentioned responses. Although temperature is constant, the flies react to heat and to cold. This suggests that some stress mechanisms associated with heat and cold are also turned on in highD.

The induced response to heat and cold in highD might be the reason for the observed (Henry, Renault and Colinet, 2018) higher heat tolerance. This is also supported by the fact that pretreatment with heat causes higher heat tolerance in lowD and also highD (Arias *et al.*, 2012).

Given that high amounts of urea and uric acid emerge at high larval rearing densities (Botella et al., 1985), the stress response to salt could be reflecting among other salts a higher concentration of urea (Henry, Tarapacki and Colinet, 2020).

It is well known that high larval crowding increases competition for limited food resources reflecting the response to starvation, which might be responsible for the increased starvation resistance seen in highD (Zwaan, Bijlsma and Hoekstra, 1991).

Many GO-terms in the DE analysis indicate an increase of energy production, which might be the result of a physiological stress response of the fly. The sugar metabolism is up-regulated in contrast to the amino acid and lipid metabolism. Another indicator of stress might be that the immune system of the fly is down-regulated.

### 4.1.2. Changed behaviour and other altered traits

Given that several GO-terms found in the up-regulated DE genes as well as in Module 2 were associated with increased muscle activity, it is indicated that the larvae show increased locomotor activity. Based on that the question arises, why the larvae are moving more in highD. This might be answered by some behavioral alterations seen in Module 5 of WGCNA. It is seen that the larvae exhibit a response to light stimuli (GO:0071482) and show phototaxis (GO:0042331). Moreover, the flies show changed expression in behavioral response to nutrients (GO:0051780) and chemosensory behavior (GO:0007635). These GO-terms suggest that the flies might be moving in response to food, toxic waste gradients and light. It further might show the behavioral stress avoidance in the fly. Besides, the altered behaviour might have been the basis for the evolution of phenotypic plasticity in high larval crowding (Price, Qvarnström and Irwin, 2003).

Intriguingly, it has been found that in high densities of adult flies movement does not decrease, although collision between the flies or interactions are increasing (Rooke *et al.*, 2020).

Taking into account that these flies are up-regulating genes related to muscle activity, the increased tracheal morphogenesis (GO:0060439) and aerobic respiration (GO:0009060), seen in the WGCNA Module 1, might be explained as a compensatory mechanism as the fly acclimates to higher oxygen consumption (Harrison *et al.*, 2018). The up-regulation of tracheal morphogenesis is detrimental, as the comparison to adaptation revealed.

Furthermore, it can be seen that the sensory perception (GO:0007608) and response to pheromones (GO:0019236) is down-regulated. Indicating that the flies are acclimating to the excessive supply of pheromones and stimuli from other flies.

Concordant with several observations (Pearl and Parker, 1922; Lints and Lints, 1971) reproduction is impacted, as multicellular organism reproduction (GO:0032504) and sperm storage (GO:0046693) is down-regulated in highD.

## 4.2. Differences in gene expression considering development time

Flies develop more delayed and more distributed in time due to high larval density (Lints and Lints, 1971). The question why this is the case has already been addressed in several studies. These studies investigated whether the main reason for this is the limited food availability due to scramble competition (Scheiring *et al.*, 1984; Klepsatel, Procházka and Gáliková, 2018), increased concentrations of waste products (Botella *et al.*, 1985), scarcity of space (Scheiring *et al.*, 1984) or a combination of all these factors (Henry, Renault and Colinet, 2018).

The viability of the flies is negatively correlating with the eclosion time (highD I - III) (Horváth and Kalinka, 2016). The reason for this might be the increasing malnutrition, experienced by the larvae. We have already seen that by decreasing yeast in the nutrition similar effects as highD arise in Drosophila (Henry, Renault and Colinet, 2018). Since it is also proven that high population density results in a lack of yeast (Birch, 1955), it can be inferred that this would change the metabolism of the larvae and therefore flies fundamentally. The GO analysis of several DE genes and WGCNA modules comprise many genes regarding metabolism, potentially explaining the huge impact of nutrition.

Another major change between highD groups is the behaviour and neural responses to different stimuli, as it can be asserted from the fourth module in the WGCNA.

Furthermore, it can be seen that the most gene expression change is usually asserted in highD II. highD I had a time advantage (Horváth and Kalinka, 2016), possibly reflecting more untouched food and less toxic waste products. In the case of highD III, finding a reasonable explanation is more difficult. Since these larvae possibly had to deal with the highest amount of toxic waste and least amount of nutrients, the reason for this gene pattern has to depend on a third factor. Based on the experiment setup, highD III experienced the smallest population density on the last day before going into pupal state. This reduction of stress response could be the consequence of less collision between the larvae or less scramble competition.

## 4.3. Fitness effect of plasticity

The effects of evolution under high larval density has been thoroughly studied. It is known that due to the high selection pressure, allele frequencies can change fast in high larval crowding (Birch, 1955).

Though suggestive and interesting, this discussion on the fitness effect of density-related plasticity has a fundamental caveat: the contrast between evolved and ancestral populations was made in a low-density controlled experiment only. Regardless, the obtained results reveal the direction of evolution. Which is indicating if the observed gene expression in acclimation is advantageous or detrimental for the flies. But we do not have sufficient evidence to infer whether the plasticity evolved or not. Two equally possible scenarios would need careful consideration on top of our inference. In order to visualize the following cases a figure was made (Fig. 9.), whereas the plasticity is depicted as the slope of the lines.

First, it is possible that the evolution of gene expression is realized by constitutive up-, or down-regulation. And the plasticity, which is the expression difference between high and low density, is not affected (Fig. 9. A). In this case, the gene expression change between base and evolved populations in highD could be similar to the change between the groups of lowD flies. Assuming this scenario, the expression difference detected at low density would implicate the fitness consequence of the regulation induced by high density.

Second, there is the potential of a change in plasticity. In this case, the implication of the expression difference at low density after the adaptation would be more complicated. For the genes whose expression levels in evolution and acclimation change into opposite directions, it is likely that plasticity becomes exaggerated (Fig. 9. B). Assuming beneficial plasticity, it will become more extreme during evolution. This implies that expression levels in evolved flies will be more extreme at high and low densities. Our basic assumption of a detrimental plasticity would be contradicted. Nevertheless, it is equally possible that plasticity stays detrimental and becomes diminished during evolution (Fig. 9. C). The same applies for the case in which the gene expression in evolution and acclimation changed into the same direction. Hence, the current dataset cannot capture all possible scenarios of evolution.

Another possible case which could be potentially missed by the current approach is that the genes lose or exaggerate their plasticity but exhibit no difference in expression at low density

(Fig. 9. D). Future studies would particularly benefit from a common garden experiment in high density.

In the comparison between the acclimation and the adaptation, detrimental effects of metabolism associated gene regulations were asserted. In more detail, the down-regulation of the amino acid and lipid metabolism was disadvantageous similar to the up-regulation of the carbohydrate metabolism, putatively suggesting that the metabolism of the fly is not optimally acclimated to the available nutrition. In more detail, it might reflect the lack of important nutrients for the fly in a high density environment, for example yeast (Klepsatel, Procházka and Gáliková, 2018).

Furthermore, the detrimental changes in metabolism could be connected to the stress response of the fly. By metabolising carbohydrates for a rapid energy production in stress situations, the fly might benefit but for a long duration it is disadvantageous.

In Figure 7 it can be seen that 955 genes are affected by the sudden response to highD, whereas only 181 were affected by the evolution. Since many more genes show a gene expression change in acclimation, it can be inferred that plasticity is important in highD and advantageous for the flies. Otherwise the flies would not be able to evolve.

It has been shown that the phospholipid fatty acid composition is linked to longevity of the fly (Moghadam *et al.*, 2015) and therefore could influence selection. Possibly linked transcriptional processes were found in evolution.

In sum, it is not clear whether only one of the above mentioned two scenarios is the case. It might as well be a mixture of a change in plasticity and base expression (Fig. 9. C). Hence, to determine which theory depicts the truth better, more experiments have to be done.

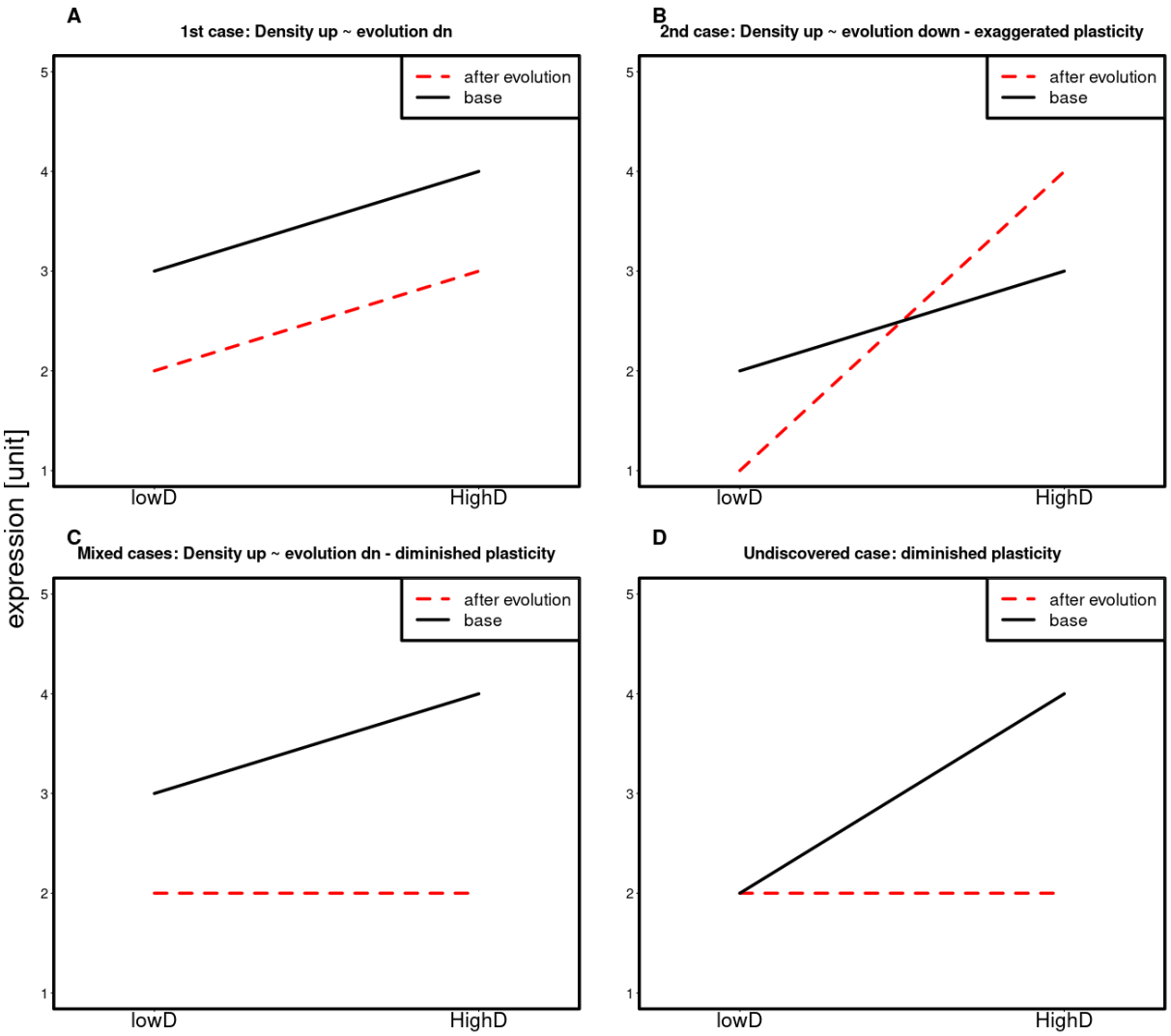


Fig. 9. Explanatory graph depicting possible cases for missing data points.

# 5. Summary

In natural populations of *Drosophila simulans*, high larval density often leads to strong competition for essential resources. It is proven that high rearing density is a stressful environment for *Drosophila* owing to limitation of resources, habitat degradation and spread of disease. Under high larval density, many altered phenotypes have been observed. For instance the fly becomes smaller and its development is delayed and much more. Nevertheless, the field is missing the molecular explanation behind the observed plastic responses. This study aims to fill the gap with a high-throughput transcriptomic resequencing approach and also provide a more thorough scan of phenotypic plasticity in response to high density.

In this study, fly populations were reared in a Common Garden experiment at a relatively sparse (400 eggs per bottle) and at a four-fold denser environment (1600 eggs per bottle). As a next step in the workflow (Fig. 10.), the RNA of the adult fly was extracted and sequenced. Sequential analyses comprise a differential expression analysis, a weighted gene co-expression network analysis and a comparison to stress and laboratory evolved differential regulation followed by a functional enrichment for biological implications.

Overall, the gene expression patterns found in this study can be associated with the previously observed phenotypic responses in metabolism, behaviors and stress resistance. Several transcriptomic responses have been asserted, possibly explaining intriguing phenomenons seen in *Drosophila*. Interestingly, we found that the flies in highD displayed a response to heat and cold. Additionally, a change in behaviour can be inferred from the transcriptomic responses. Moreover, major changes in the carbohydrate, lipid and amino acid metabolism were found, which were inferred to be partially detrimental for the fly.

Concludingly, this bachelor thesis comprises the first steps in revealing important molecular mechanisms in response to density stress, potentially being helpful in further studies with *Drosophila*.

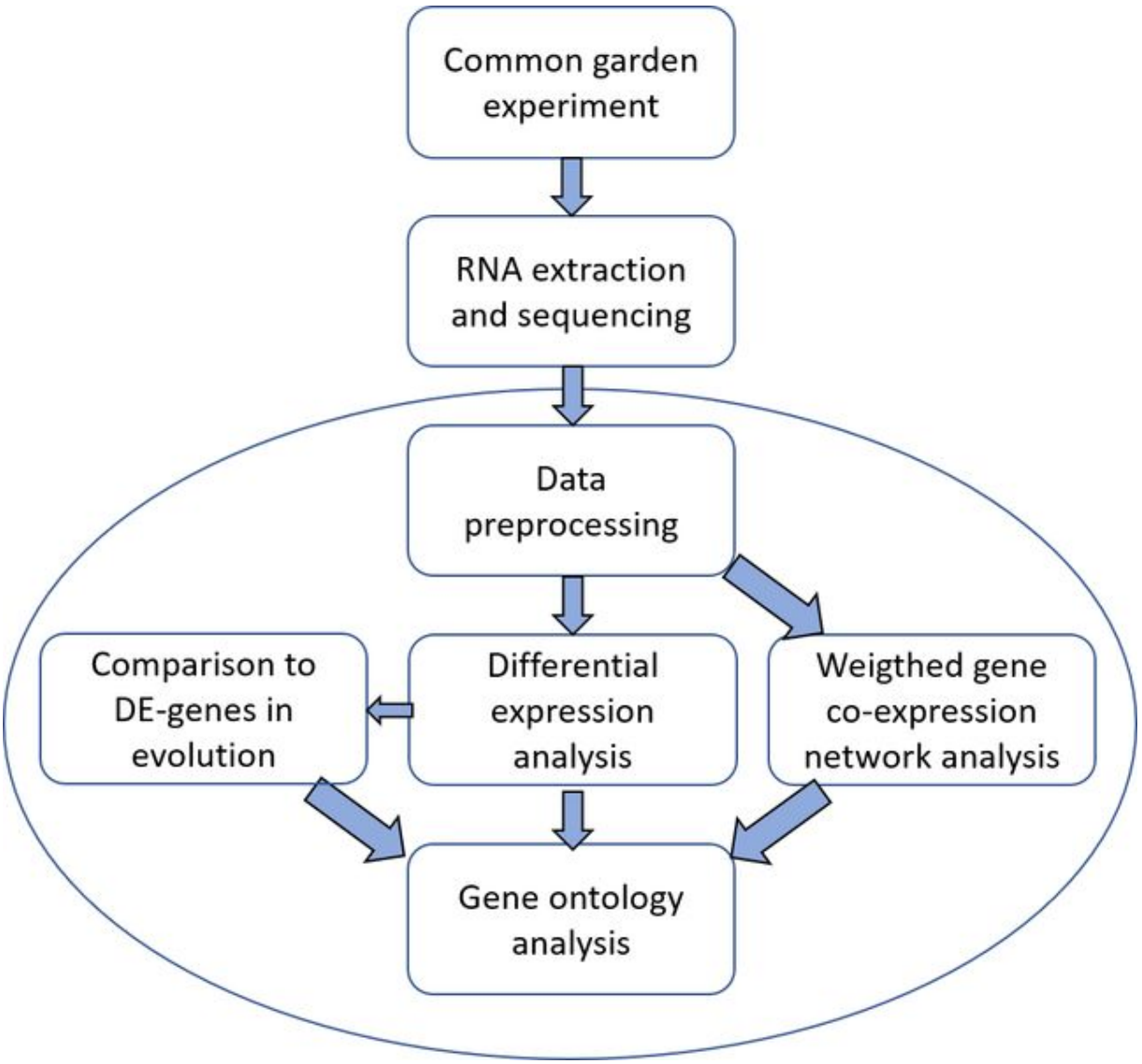


Fig. 10. Workflow of this bachelor thesis

# 6. Zusammenfassung

Unter natürlichen Konditionen, führt hohe larvale Populationsdichte bei *Drosophila simulans* zu einer starken Kompetition von überlebenswichtige Ressourcen. Es ist bewiesen, dass hohe Dichte bei der Haltung durch eine Limitation von Ressourcen, Verfall des Habitates und Aufkommen von Krankheiten zum Stress der Fliegen führt. Unter einer hohen larvalen Dichte wurden schon viele veränderte Phänotypen entdeckt. Zum Beispiel werden die Fliegen kleiner, ihre Entwicklung ist verzögert und auch noch mehr. Trotzdem fehlen die Erklärungen über die molekularen Abläufe hinter diesen beobachteten plastischen Reaktionen. Diese Studie soll diese Wissenslücke, anhand eines “high-throughput” transkriptomischen resequenzierungs Ansatzes füllen. Zusätzlich dazu könnten neue plastische Reaktionen der Fliegen entdeckt werden.

In dieser Studie wurden Fliegen im Labor unter einer wenig dichten (400 Eier pro Flasche) und einer viermal dichteren Bedingung (1600 Eier pro Flasche) gehalten. Dem Arbeitsablauf folgend (Fig. 10.) wurde danach die RNA von den erwachsenen Fliegen extrahiert und sequenziert. Die darauffolgende Analyse beinhaltete eine differenzielle Expressionsanalyse, eine gewichtete Co-Expressions-Netzwerkanalyse und ein Vergleich zu unter Laborbedingung und Stress evolvierten Fliegen. Danach wurden die Expressionsprofile noch auf die veränderte biologische Funktion untersucht.

Im Allgemeinen konnten die in dieser Studie gefunden Expressionsmuster mit den vorher beobachteten phänotypischen Veränderungen im Stoffwechsel, im Verhalten und in der Stressresistenz assoziiert werden. Es wurden einige Veränderungen im Transkriptom gefunden die möglicherweise faszinierende Phänomene in diesem Kontext erklären könnten. Interessanterweise, konnten wir feststellen, dass Fliegen in hoher Populationsdichte eine Reaktion auf Hitze und Kälte zeigen. Zusätzlich dazu konnte eine Änderung des Verhaltens durch die transkriptomischen Reaktionen erklärt werden. Außerdem wurden bedeutende Veränderungen im Zucker- , Fett- und Aminosäurestoffwechsel gefunden, welche sich als schädlich für die Fliegen entpuppten.

Zusammenfassend enthält diese Bachelorarbeit die ersten Schritte um wichtige molekulare Mechanismen, involviert als Reaktion auf Dichtestress, zu enthüllen. Sie ist potentiell ein wichtiger Anhaltspunkt für weitere Studien mit *Drosophila* als Modellorganismus.

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# 8. List of figures and tables

List Of Tables



List Of Images