Intraspecific phenotypic variation and its genetic basis in *Daphnia*

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Thesis abstract

Organisms live in a dynamic and often challenging world. Coping with stress due to environmental changes is a vital skill for organisms to ensure their survival as well as a valuable capability to pass on to their progeny. Organisms evolved a variety of mechanisms such as changes in morphology, life history traits or behavior to cope with environmental changes. These phenotypic plastic responses allow organisms to rapidly adjust their lifestyle to a new environmental situation. Phenotypic plastic responses to vertebrate and invertebrate predators are reported for the ecological and genomic model organism *Daphnia*, a grazing freshwater zooplankter occupying a key position within aquatic food webs. However, the inter- and intra-population variation in *Daphnia* is rarely addressed explicitly. Furthermore, the genetic basis of these predator-induced responses is not well understood.

The present thesis aims to assess the intraspecific phenotypic variation and its genetic basis in European *Daphnia galeata*. Life history traits were recorded in the presence and absence of fish kairomones for a total of 24 clonal lines consisting of four populations with six clonal lines each. High intraspecific phenotypic variation was revealed within and between all four *D. galeata* populations. In addition, the potential to locally adapt to a vertebrate predator regime as well as an effect of the fish kairomones on morphology of *D. galeata* was investigated. To bring light into the genetic level of predator-induced response, the transcriptional profile of two *D. galeata* clonal lines exposed to fish kairomones were established identifying candidate transcripts being involved in predator-induced shifts of life history traits. The differential gene expression analysis revealed a surprisingly high variance between clonal lines reflecting their opposing life history strategies. A total of 125 differentially expressed transcripts (DETs) were identified to be related to fish kairomone exposure. The additional gene co-expression network analysis identified clusters of tightly linked transcripts. Genetic pathways of predator-induced responses were thereby revealed including transcripts being involved in remodeling of the cuticle, growth and digestion. By applying a genome-wide association approach to genotypes and phenotypes of all 24 clonal lines, two life history traits were discovered to have a genetic basis at sequence level in the presence and absence of fish kairomones.

Furthermore, a gene co-expression network analysis of all 24 clonal lines in the absence of fish kairomones identified 44 gene clusters of which one module correlated to one life history trait, the total number of broods. By integrating a transcriptome-wide association analysis and a gene co-expression analysis a list of 156 candidate transcripts was established. To enhance the understanding of the functional roles of the transcripts, orthologs and paralogs from related species were identified using common ontologies to annotate the candidate transcripts of interest.

Interestingly, the integrative approach emphasized the importance of the identity of a clonal line both at the phenotypic and genetic level in the studied 24 clonal lines of European *D. galeata* in an environment of predation risk. The data of the present thesis provides valuable information for predator-induced responses in *Daphnia*, while contributing substantially to our understanding of the genetic basis of intraspecific phenotypic variation.

Zusammenfassung

Organismen leben in einer dynamischen und häufig herausfordernden Welt. Wechselnde Umweltbedingungen zu bewältigen, ist eine wesentliche Fähigkeit von Organismen um ihr Überleben zu sichern und somit eine wichtige Fähigkeit, diese an ihre Nachkommen weiterzugeben. Organismen entwickelten eine Reihe verschiedener Mechanismen wie z. B. Veränderungen in der Morphologie, ihrem Verhalten oder in Merkmalen ihrer Lebensgeschichte ('life history traits') um Umweltveränderungen zu bewältigen. Diese phänotypisch plastischen Antworten ermöglichen den Lebewesen, sich schnell einen neuen Lebensstil anzueignen, wenn eine neue Umweltsituation eintritt. Phänotypisch plastische Antworten auf Prädatoren wurden für den ökologischen und genomischen Modellorganismus *Daphnia* berichtet. Dieser ist ein Zooplankter, der als Weidegänger im Süsswasser eine Schlüsselposition in aquatischen Nahrungsnetzen einnimmt. Allerdings wurde bisher die Variabilität innerhalb von *Daphnia*-Populationen selten explizit adressiert. Des Weiteren ist die genetische Basis dieser Räuber-induzierten Antworten bisher nicht gut verstanden.

Die vorgelegte Arbeit hat zum Ziel, die intraspezifische phänotypische Variation sowie ihre genetische Basis in der europäischen Art *Daphnia galeata* zu untersuchen. 'life history traits' von insgesamt 24 klonalen Linien wurden in An- und Abwesenheit von Fischkairomonen dokumentiert und zeigten hohe intraspezifische phänotypische Variation innerhalb und zwischen den vier untersuchten *D. galeata* Populationen. Die Ergebnisse zeigten weiter, dass das Potential zur lokalen Anpassung an die Anwesenheit von Prädatoren gegeben ist und dass Fischkairomone einen Einfluss auf die Morphologie von *D. galeata* haben. Um Licht ins Dunkel der genetischen Basis von Räuber-induzierten Antworten zu bringen, wurden Transkriptionsprofile von zwei klonalen Linien, die Fischkairomonen ausgesetzt waren, erstellt und Kandidaten-Transkripte identifiziert, die in Räuber-induzierte Veränderungen von 'life history traits' involviert waren. Die differenzierende Genexpressionsanalyse zeigte eine hohe Varianz zwischen den klonalen Linien, die die konträre Strategie der 'life history traits' reflektiert. Insgesamt wurden 125 unterschiedlich exprimierte Transkripte in der Anwesenheit von Fischkairomonen identifiziert. Die zusätzliche Gen-Co-Expressionsanalyse identifizierte Gruppen von eng verbunden Transkripten (Module), die genetische Pfade in Räuber-induzierten Antworten aufzeigen. Sie beinhalten Transkripte, die in der Remodellierung der Kutikula, in Wachstum

und Verdauung involviert sind. Bei der Anwendung einer genom-weiten Assoziationsanalyse auf die Genotypen und Phänotypen der 24 untersuchten klonalen Linien wurden zwei 'life history traits' entdeckt, die eine genetische Basis auf der Sequence Ebene in der An- und Abwesenheit von Räubern hat. Des Weiteren identifizierte die Gen-Co-Expressionsanalyse 44 Module, von dem eines mit dem 'life history trait' Gesamtanzahl von Bruten korrelierte. Durch Integration einer transkriptom-weiten Assoziationsanalyse und einer Gen-Co-Expressionsanalyse konnte eine Liste mit 156 Kandidaten-Transkripten erstellt werden. Um das Verständnis der funktionalen Rolle der Transkripte zu verbessern, wurden orthologe und paraloge Transkripte von verwandten Arten hinzugezogen und gemeinsame Gruppen orthologer Transkripte verwendet, um interessante Kandidaten-Transkripte zu annotieren.

Dieser integrative Ansatz von verschiedenen Methoden bestärkte, dass die Identität einer klonale Linie an sich wichtig ist, sowohl auf phänotypischer als auch genetischer Ebene. Dies wurde in den 24 untersuchten klonalen Linien der europäischen *Daphnia galeata* gezeigt, die dem Risiko einem Räuber zu begegnen ausgesetzt waren. Die Daten dieser Doktorarbeit stellen wertvolle Information über Räuber-induzierte Antworten in *Daphnia* zur Verfügung, während sie gleichzeitig wesentlich zum Verständnis der Bedeutung der genetischen Basis zur intraspezifischen phänotypischen Variation beiträgt.

Acknowledgements

I was happy to meet a large number of faithful and inspiring people during my journey called life helping me to become the person I am now. I am grateful for all of them although I won't provide a complete list here.

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I always consider myself very lucky, when I am asked about my supervisor and my work group. I couldn't feed the negative expectation of those people about being a doctoral candidate. We've been a hard-working team AND we never missed an opportunity to laugh together and have some fun. This was so important for me. I always felt welcome. I always enjoyed coming to work because of you people. Thank you. Having colleagues like you is priceless. I would have been lost so many times without you. Suda Parimala Ravindran, you have been such a great companion throughout our PhDs! I am so grateful that I shared this intense time with such a beautiful soul. Many, many thanks for answering all my 'funny' questions concerning statistics and bioinformatics. Jennifer Lüneburg, Laura Seddar, Jana Nickel and Anne Ehring, thank you for spending over 8 months of every day experiment in the lab with our *Daphnia* mothers and their 'kindergarten'. It was so much fun to get through the days with you, good music and homemade cookies! Many thanks to Michael Engelmohn, Tatjana Usinger and Jonny Schulze our valuable supporters and caretakers for keeping the fish and the *Daphnia* happy! Without you people I would be probably still running experiments....

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Dedication

Had my father told me *again* 16 years ago, that I would be as close as putting a “Dr.” in front of my name as I am now, I would have shaken my head with a smile on my face and the ocean in mind.

Had anyone told me 8 years ago when I handed in my diploma thesis, that I would end up working in the lab instead of working in the field under water, I simply would have called that person crazy and would have made a secret promise to myself that the ocean still has to be involved in my future life.

Had anyone told me 4 years ago, that I am going to do my doctoral thesis in a lab in the big Northern city of Hamburg, I simply would not have believed it.

Yet, here I am an ocean lover and coastward bound scientist in the middle of a concrete jungle, being satisfied with the research questions I worked on and with life, which took yet another unexpected turn. Lessons learned. “Things always turn out differently than expected or hoped for. Things are not as bad as you think. Enjoy the ride, wherever it may take you. Diversity is an essential element for a healthy group of organisms. Like salt and fish are essential elements in a healthy ocean.”

I would not be in this place of my life had it not been for my beloved father and soulmate Hans-Jörg Tams. You gave me all the faith and freedom to explore this planet way before I started the journey of science. Your absences during your life time helped me now, after your death, to live my life and to fulfill my dreams. Reflecting on life by your death helped me to appreciate my intuition as a compass and to navigate by stars with confidence. I am still missing you. Your free spirit and wisdom is still omnipresent and lives on.

This doctoral thesis is dedicated to him.

To life, in general. To life on this blue planet, in particular.

And to love.

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AFR	Age at first reproduction
ANOVA	Analysis of variance
BP	Biological Process
brood1	Number of offspring 1st brood
brood2	Number of offspring 2nd brood
brood3	Number of offspring 3rd brood
brood4	Number of offspring 4th brood
broods	Total number of broods per female
CC	Cellular compound
CEM	Gene co-expression module
DETs	Differentially expressed transcripts
DNA	Desoxyribonucleic acid
dSGR	Differences of somatic growth rate
DVM	Diel vertical migration
FK	Fish kairomone
GLMM	Generalized linear mixed models
GO	Gene Ontology
GPA	General Procrustes Analysis
GSEA	Gene set enrichment analysis
GWA	Genome-wide association
GWAS	Genome-wide association study
GxE	Genotype-environment interaction
LHT	Life history traits
MAF	Minor allele frequency
ME	Module eigengene
MF	Molecular function
offspring	Total numbers of neonates per female
PCA	Principal component analysis
popG	Population Greifensee
popJ	Population Jordan reservoir
popLC	Population Lake Constance
popM	Population Müggelsee
QTL	Quantitative Trait Loci
relclone	Relative fitness of clonal lines among populations
relnest	Relative fitness of clonal lines within a population
RNA	Ribonucleic acid
SGR	Somatic growth rate
size	Body length
SNP	Single Nucleotide Polymorphism
TOM	Topological Overlap Matrices
TPS	Thin plate spline
WGCNA	Weighted gene co-expression network analysis

General introduction

Planet Earth exhibits a fascinating diversity of life forms. Organisms found countless ways to survive and thrive under a variety of circumstances. Over time, organisms developed varying ways to exploit different energy resources, from oxygen consuming mammals, to sunlight transforming plants, to deep sea ciliates hosting a sulphur-transforming bacteria. All organisms together form a community, a mosaic of diverse life forms sharing a habitat, collaborating or competing for resources. All have one goal: to pass on their genes to the next generation.

Environments change constantly over the course of time: from the scale of geological eras (from millions to thousands of years), to lifespans of individuals (from days to years up to decades). Local environmental conditions alter due to climate change which influences a number of abiotic factors. In turn, local environmental conditions affect the biotic factors such as the abundance of individuals, populations and species and therefore the composition of whole communities or ecosystems (reviewed by Beaugrand & Kirby 2018). In general, there are four ways for organisms to deal with environmental change: move, adapt, cope or die (Gienapp *et al.* 2008). This simplistic point of view describes a rather complex relationship of individuals within their environment. Changing one factor in this relationship consequently affects another. For example the change of one abiotic factor such as the increase in sea surface temperature affects the biotic level, for one the marine plant *Zostera marina*, a habitat foundation species (Franssen *et al.* 2011). Northern populations of *Zostera* fail to recover from a simulated heat wave compared to Southern populations. In consequence, if a seagrass population does not recover from a heatwave, the whole community living in seagrass meadows is going to change. Seagrass meadows form a unique habitat for other invertebrate as well as vertebrate species, whose abundance changes depending on the seagrass distribution (Boström & Bonsdorff 1997; Frost *et al.* 1999; Mattila *et al.* 1999; Pihl *et al.* 2006).

The omnipresence of variation in organisms can be explained from an ecological point of view. Ecologists investigate the relationships of organisms (phenotypes) and their environment to understand their interplay and the successful survival of organisms. One of the key concepts explaining phenotypic variation is phenotypic plasticity which describes

how one genotype can produce different phenotypes in different environments (e.g., Agrawal 2001; Stearns 1989). The benefit of being different compared to a conspecific might result in an advantage of survival and potential reproductive success after the environment has changed thus leading to an increased contribution of genes to the gene pool of its population. Here, it becomes evident how tightly linked ecology and evolution are because evolution describes a process in populations over successive generations by using the change of heritable characteristics as a measure. The unit of evolution is an allele which is a variant of a gene. If one allele is involved in the successful survival and reproduction of an organism, it should be passed on to the next generation. Therefore, allele frequencies can be tracked within populations over time explaining different phenomena such as bottlenecks and migration events that are of interest for population geneticists. Since both phenotype and genotype are tightly linked because a genotype, the environment and their interaction define a phenotype (Agrawal 2001; Stearns 1989), I propose that combining an ecological with an evolutionary perspective is a constructive approach to understand the intraspecific phenotypic variation and its genetic basis.

Association of phenotypes and genotypes

Approaches and challenges

The interdisciplinary field of ecological genomics aims to understand the genetic basis of phenotypic variation of ecologically relevant traits (Ungerer *et al.* 2008). By using different approaches such as candidate genes, proteomics and Quantitive Trait Loci (QTL) mapping in an ecological context investigators aim to shed light on whole genome function and its evolution (Ungerer *et al.* 2008). There are other possibilities to link the genetic and phenotypic level. Genotype-phenotype associations can be done at two genetic levels: sequence-based or regulatory-based. Genome-wide association studies (GWAS) are mainly applied in medical sciences since traditionally genome-wide association (GWA) tools were designed to detect associations of single nucleotide polymorphisms (SNPs), here a molecular marker representing the genotype and common human diseases such as heart diseases or diabetes which represents the phenotype (Visscher *et al.* 2012).

Merely the association of phenotypes and genotypes is not enough to understand the genetic basis of phenotypic variation. One could say that a phenotype is the expressed

genotype in a certain environment. Numerous investigations link genotypes and gene regulation (i.e., the molecular phenotype), providing insights into the molecular response at the transcript level, e.g. in plants (Franssen *et al.* 2011), rabbits (Lavergne *et al.* 2014), fish (Windisch *et al.* 2014), corals (Barshis *et al.* 2013), mussels (Place *et al.* 2008) and crustaceans such as *Daphnia* (Chowdhury *et al.* 2015; Connon *et al.* 2008; Orsini *et al.* 2016; Schwarzenberger & Fink 2018; Windisch & Fink 2018).

A subsequent gene co-expression network analysis links clusters (modules) of co-expressed genes to phenotypes, e.g. life history traits, giving insights of potential genotype-phenotype correlations (e.g., Langfelder & Horvath 2008). Since co-expressed genes often share similar biological functions (Subramanian *et al.* 2005), the application of this approach helps to identify candidate transcripts being involved in a genotype-phenotype relationship. Gene co-expression analyses have been applied to different organisms, such as plants (Schaefer *et al.* 2018), fish (Sutherland *et al.* 2018) and mussels (Zhao *et al.* 2016).

To gain a holistic view on the genetic basis of phenotypic variation one still faces challenges. First, a fully annotated genome does not exist for all organisms to apply e.g. a QTL mapping approach. Second, the existing tools for genome-wide association are not appropriate for all organisms due to different reproductive modes such as sexual or asexual. Third, although sequencing costs dropped over the past years, conducting an extensive gene expression study is still cost-intensive and not always affordable. However, investigating genotype-phenotype associations will contribute to our understanding of the genotype-phenotype relationship and its overall importance for population and species persistence.

An ecological perspective

Linking genotypes and phenotypes at the sequence level has rarely been addressed by the scientific community in an ecological or environment-dependent context, at least for animals. In plant sciences several genome- or transcriptome-wide studies were conducted, e.g. for oak (Gugger *et al.* 2016), conifers (Housset *et al.* 2018) and maize (Wang *et al.* 2012). Applying the traditional GWA methods on a non-model organism brings its

difficulties and so far only a few tools have been developed to overcome certain constraints such as a repeated measurements or clonal reproduction. By using a previously adjusted GWA method for repeated measurements implemented in the R package '*RepeatABLE*' (Ronnegard *et al.* 2016) the association of avian breeding time, a highly variable phenotypic trait, to numerous genetic loci (SNPs) revealed no significant SNP association in great tits (Gienapp *et al.* 2008). In addition, a novel phylogenetic approach was developed to overcome clonal population structure in microbes which was implemented in the R package '*treeWAS*' (Collins & Didelot 2018). Another example made use of several previous studies including a GWAS to successfully synthesize the phenotypic, genetic and environmental data in a landscape genomics and association mapping approach giving rise to six candidate genes being under selection for cold-hardiness adaptation in coastal douglas fir (Vangestel *et al.* 2018).

Although methodical challenges exist to identify promising candidate genes or transcripts linked to genotype-phenotype-environment relationships, the results of such approaches help to gain ecological annotations of genes (i.e., ecological genomics). Examples are provided in the well-written synthesis by Aubin-Horth (2016) in which the behavioral phenotypic variation in several fish species was linked to their molecular, cellular and physiological traits.

Variation matters in the light of evolution

Natural variation of traits (phenotype) and their underlying genetic basis (genotype) are the material on which natural selection acts on, it favors phenotypes with a higher fitness (survival and reproductive success) and genes of the latter are passed on to the next generation (Stearns 1989). Variation exists at different interconnected biological levels (Beaugrand & Kirby 2018) (Figure I-1). First, variation at community level describes the interspecific variation, species diversity within one habitat, also known as biodiversity. The importance of variation becomes evident when biodiversity is at stake in highly diverse habitats such as coral reef ecosystems (McWilliam *et al.* 2018). A loss of species in coral reef ecosystems enhances the possibility of functional collapse, since the functional redunancy, defined as multiple species sharing similar functions, decreases (McWilliam *et al.* 2018). In turn, a meta-analysis revealed that the successful establishment of plants and

animals increases with an increased phenotypic and genetic diversity of founder groups (Forsman 2014). Second, variation at population level usually refers to intraspecific variation assessing differences of populations within one species. Third, variation at the individual level can be described in three ways depending on the perspective: (i) among genotypes (phenotypic variation), (ii) among isogenic phenotypes in a given environment (phenotypic variability) and (iii) among environmental conditions (phenotypic plasticity) (Ziv *et al.* 2017).

A phenotypic plastic response describes the ability of a genotype to produce varying phenotypes depending on its current environmental condition to secure its survival and reproductive success (Agrawal 2001; Stearns 1989). Phenotypic plasticity exists in a variety of organisms responding to abiotic and biotic factors of their environment and changing their behavior, physiology, morphology, growth and life history (e.g., reviewed by Harvell 1990). Phenotypic plasticity can influence population and community structure by altering the interactions of individuals and their environments emphasizing its ecological importance (reviewed by Bolnick *et al.* 2011; Miner *et al.* 2005). Phenotypic plastic responses have a reversible (Stearns 1989) as well as an adaptive potential (Agrawal 2001). Although phenotypic plasticity has advantages for organisms, it does have costs and limitations as well (DeWitt *et al.* 1998; Scheiner & Holt 2012). Costs include maintenance, production, information acquisition, development and the genetic level, while limitations include information reliability, lag-time, developmental range and the epiphenotype problem (DeWitt *et al.* 1998). The persistence of a population/species depends on its phenotypic and its genetic variation (Bolnick *et al.* 2011; Forsman 2014; Scheiner & Holt 2012). Sources of phenotypic variation can result from environmental change and genetic variation (Bolnick *et al.* 2011). Genetic variation originates from mutation, recombination and gene flow (Griffiths *et al.* 2000). Another, often forgotten, source of genetic and hence phenotypic variation are seed pools of plants (e.g., Honnay *et al.* 2008) or egg banks of diapausing organisms such as *Daphnia* (Brednock & De Meester 2003; Hairston 1996). Genetic variation can decrease over time e.g. due to genetic drift (the random loss of genes) (Bolnick *et al.* 2011; Vanoverbeke & De Meester 2010), inbreeding depression (Lynch 1991; Swillen *et al.* 2015) or local adaptation (Kawecki & Ebert 2004). The strongest driver for loss of genetic variation, however is positive selection (e.g., Biswas & Akey 2006).

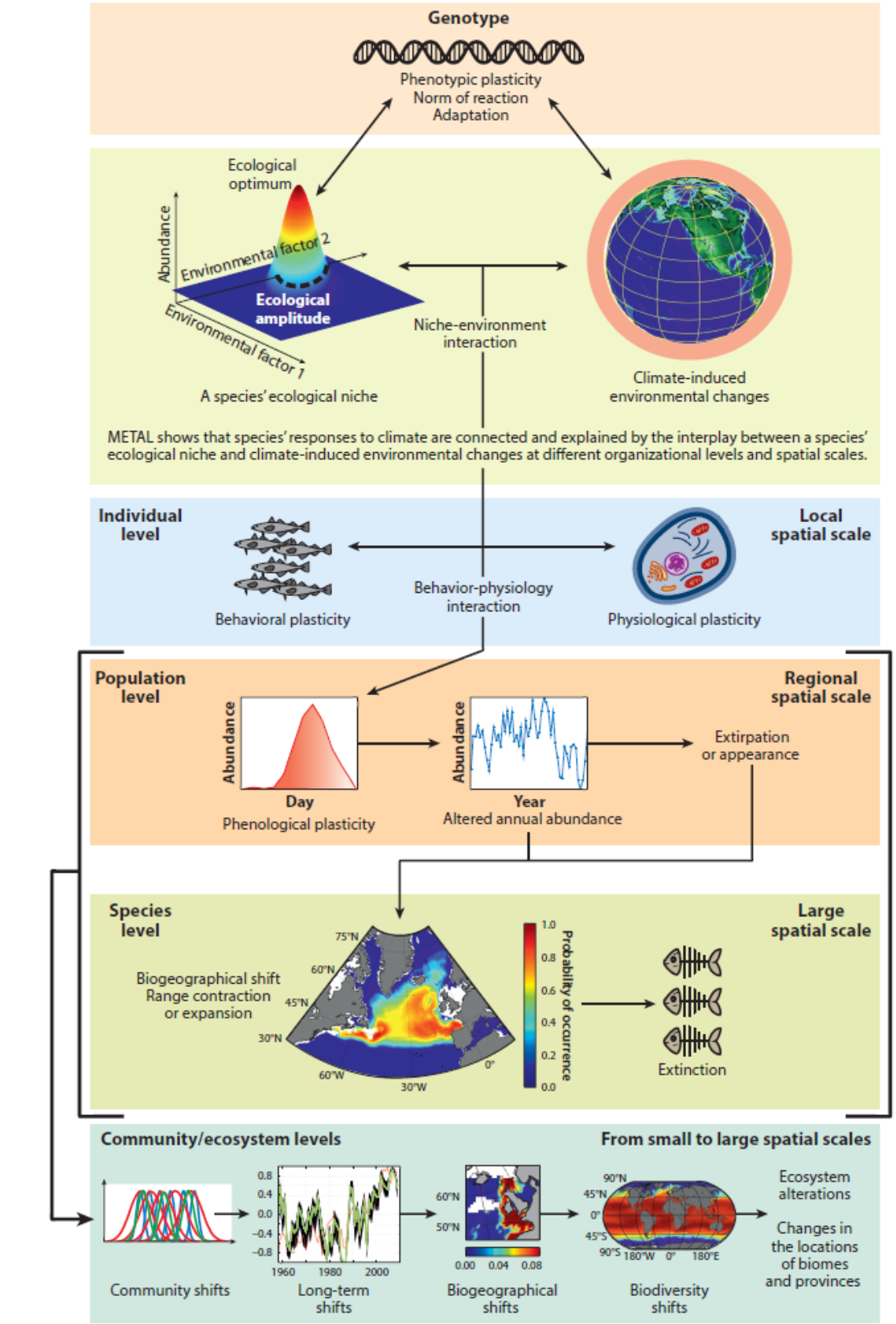

Figure I-1: The interconnectivity of organismal levels. The theoretical diagram was published by Beaugrand & Kirby 2018. The connectivity of different levels is shown from the genetic level up to the community level (top to bottom).

Daphnia, a model system for ecology and evolution

Individuals of the genus *Daphnia*, commonly called water fleas, are microcrustaceans belonging to the Cladocera order. They play a key role in aquatic pelagic food webs of freshwater ecosystems (reviewed by Miner *et al.* 2012). They shape microbial communities (Degans *et al.* 2002) and filter feed upon phytoplankton at the first consumer level (Sommer *et al.* 2003). At the second consumer level of the food web, they become a food source for planktivorous fish themselves (e.g., Ebert 2005). Within the past decades their ecology has been investigated intensely, e.g. their behavior (e.g., Cousyn *et al.* 2001; O'Keefe *et al.* 1998; Stich & Lampert 1981), predator response (e.g., Boersma *et al.* 1998; Weider & Pijanowska 1993), digestion (e.g., Agrawal *et al.* 2005; Schwarzenberger *et al.* 2012) as well as life history (e.g., Lüning 1995; Machacek 1995).

Daphnids are an ideal model organism due to their short reproduction time (~10 days) with respect to an individual's lifespan. Due to their small body size as well as their easy rearing in the laboratory, large numbers of individuals from different populations can be maintained under laboratory conditions. Moreover, their cyclic parthenogenic life cycle (Figure I-2) makes them ideal for experimentation. Parthenogenesis is a type of asexual reproduction that results in offspring genetically identical to their mothers. Each group of offspring from one maternal line is referred to as clonal line, clone or genotype. Therefore, parthenogenetic daughters are ideal to conduct experiments on phenotypic variation as they all share the same genotype. On the other hand, daughters hatched from ephippia, the protective shells containing sexual resting eggs of *Daphnia*, are ideal for evolutionary studies to understand the consequences of genetic changes due to recombination. Sex determination in *Daphnia* is not chromosomal (Huylmans *et al.* 2016) but epigenetic (long noncoding RNAs) (Kato *et al.* 2018) and depends strongly on environmental factors (Huylmans *et al.* 2016; Kato *et al.* 2018). Sexual reproduction in a *Daphnia* life is triggered by a combination of unfavorable environmental conditions such as lowered temperature and shorter day light length (Ebert 2005). Resting eggs can endure the unfavorable environmental conditions in sediments and can still be viable after decades (e.g., Cousyn *et al.* 2001; Goitom *et al.* 2018; Kerfoot & Weider 2004). Egg banks from a diapausing organism like *Daphnia* are a valuable source for genetic variation of a species when environmental factors change (Brednock & De Meester 2003; Honnay *et al.* 2008; Weider *et al.* 1997).

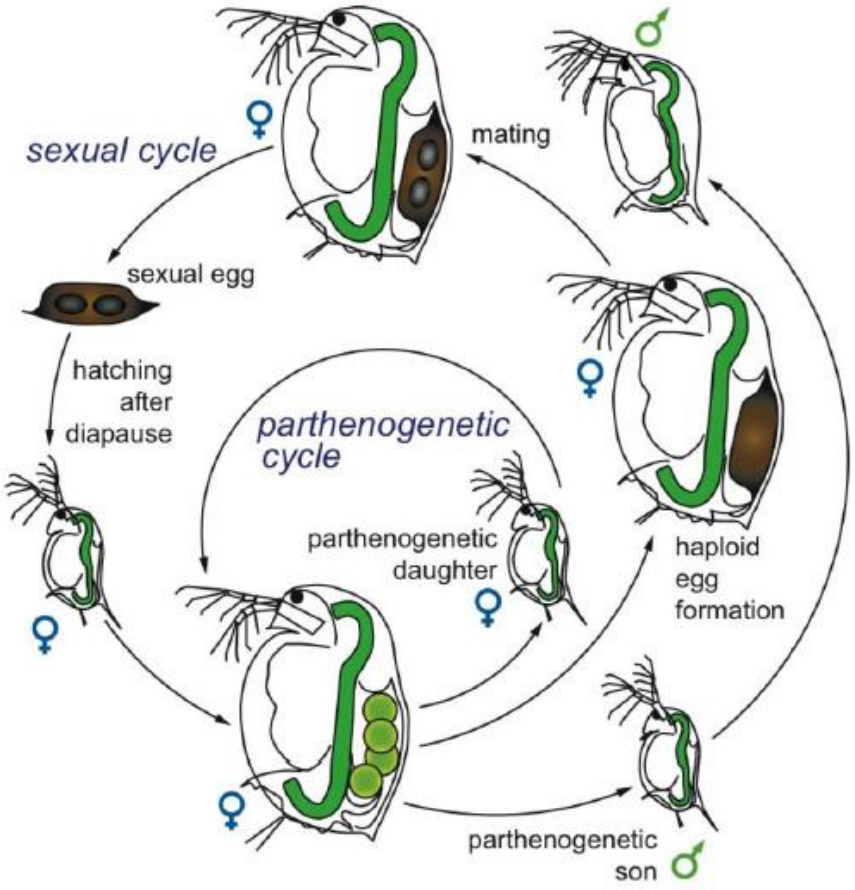

Figure I-2: The life cycle of parthenogenetic *Daphnia*. The figure was published in Ebert 2005. The sexual and asexual (parthenogenetic) life cycle is shown. In the parthenogenetic life cycle females produce diploid eggs which develop directly into isogenic daugthers. The same female may produce diploid asexual eggs that develop into sons. Male production is controlled by environmental factors. Furthermore, the same female may produce haploid eggs that require fertilization by males. These eggs are then enclosed in a protective shell (ephippium) and need to undergo diapause before female offspring will hatch from them.

Daphnids inhabit small, temporary ponds to large, permanent lakes. *Daphnia magna* is the largest daphnid usually found in small, temporary ponds across Europe and North America (Ebert 2005). Due to their key role in aquatic food webs and the deep understanding of their ecology *D. magna* became indicator species in ecotoxicology. Individuals have been exposed to anthropogenic residues such as ibuprofen (Heckmann *et al.* 2006; Heckmann *et al.* 2008), silver (Ashgari *et al.* 2012) and microplastics (Rosenkranz *et al.* 2009) among many more toxic compounds. The *D. pulex* - species complex contains several species such as *D. ambigua*, *D. parvula*, *D. obtusa*, *D. pulicaria* and *D. pulex* which are found in freshwater bodies across North America (e.g., Colbourne *et al.* 1998) and Europe (e.g.,

Dufresne *et al.* 2011) among other places. The closely related *D. longispina* – species complex is mainly composed of *D. cucullata*, *D. longispina* and *D. galeata* that are known to hybridize and occurs across Europe in habitats of varying sizes. This species complex includes the previously described species *D. hyalina* and *D. rosea* (Petrusek *et al.* 2008). The correct species identification in daphnids is difficult and hence the combined application of morphological and genetic markers is recommended to gain the best information (e.g., Petrusek *et al.* 2008). With the rise of transcriptomics and genomics as a result of sinking costs of sequence technology, whole genomes of *Daphnia* were made available in the past decade. In 2011 the first complete genome of *D. pulex* was published and its eco-responsiveness described (Colbourne *et al.* 2011). Six years later a more complete and less fragmented assembly of another *D. pulex* genotype was released (Ye *et al.* 2017). A draft version of a complete genome of *D. magna* was made public in 2010 (http://server7.wfleabase.org/genome/Daphnia_magna/) and was updated in 2016 (NCBI BioProject PRJNA298946). Other valuable genetic resources have been recently published, such as reference transcriptomes of *D. magna* (Orsini *et al.* 2016) and *D. galeata* (Huylmans *et al.* 2016). These ressources were and are used in numerous analyses aiming at linking ecological traits to the underlying genetic pathways. Yet, daphnids are still developing as an important model organism in adjacent fields such as ecological or functional genomics (Miner *et al.* 2012; Stollewerk 2010) as well as in epigenetics (Vandegehuchte & Janssen 2011, 2014; Wojewodzic & Beaton 2017).

Phenotypic variation in Daphnia *and the influences of predation risk*

Daphnids are a group of crustaceans with well-documented, predator-induced phenotypic variation in behavior, morphology and life history of several species. For example, daphnids may alter the diel vertical migration (DVM) behavior due to predators differently: *D. hyalina* migrates into deeper water layers while *D. galeata* stays close to the water surface (Stich & Lampert 1981). Behavioral strategies in *Daphnia* to avoid predation include DVM (Dodson *et al.* 1997), increased alertness (Boersma *et al.* 1998), swarming (Pijanowska & Kowalczewski 1997) and altered swimming behavior (O'Keefe *et al.* 1998).

Most popular examples for inter-specific phenotypic variation in *Daphnia* were documented for morphological changes in the presence of invertebrate and vertebrate

predator cues (kairomones) which are released to the surrounding water. These predator-induced responses are predator-specific. For example, in the presence of kairomones of the invertebrate predator *Chaoborus, D. pulex* increases its body size (Spitze 1991) and develop neck teeth (Lüning 1995). On the other hand, *D. cucullata* (Laforsch & Tollrian 2004b) as well as *D. lumholtzi* (Tollrian 1995) generate longer helmets and tailspines to reduce their vulnerability. A change of body symmetry to an S-shape in *D. barbata* in the presence of kairomones of the invertebrate predator, *Triops*, supposedly impedes the ingestion of the prey by its predator (Herzog *et al.* 2016). The morphological changes and the increased growth rate in the presence of invertebrate predator kairomones is best explained by the gap-limitation of these predators, they are not able to ingest large *Daphnia* selecting (Lüning 1995; Spitze 1991). *Daphnia* exposed to invertebrate kairomones use their energy resources to grow and/or invest in morphological changes to become bigger, so that they outgrow the capacity of an invertebrate to feed on them. An opposing life history strategy sets in when positive size-selecting vertebrate predators are present than *Daphnia* exposed to fish kairomones mature earlier and stay smaller (Boersma *et al.* 1998; Castro *et al.* 2007; Machacek 1995; Weber 2003). Thus, by becoming smaller, *Daphnia* reduce their chances to be detected by the visually-hunting fish that can easily detect large prey (Weber & Van Noordwijk 2002). Studying the life history strategies in *Daphnia* revealed predator-induced shifts in life history strategies as mentioned above. Life history traits are closely related to the fitness of a phenotype which can be estimated by its survival and reproductive success (reviewed by Brommer 2000). A fit phenotype passes on its genes to the next generation, thereby contributing to the persistence of a population. Thus a large variety of phenotypes within one population adds to its long-term persistence (Bolnick *et al.* 2011; Forsman 2014).

Intraspecific phenotypic variation has important consequences for population dynamics as well as ecological consequences at the community level (Bolnick *et al.* 2011; Hairston *et al.* 2005; Post *et al.* 2008). A change species composition and its effect on a whole lake community has been shown for *D. dentifera* (Duffy 2010). Yet, little is known of intraspecific phenotypic variation in *Daphnia* at the population level, although many studies have been investigated predator-induced responses in different *Daphnia* species. Generally, single clonal lines are used in experiments drawing conclusions for an entire

species, except for two investigations looking at local adaption to predation risk in *Daphnia* using several clonal lines per population (Reger *et al.* 2018; Cousyn *et al.* 2001).

The adaptive potential of Daphnia *– coping with rapid environmental change*

The above described predator-induced responses in *Daphnia* are textbook examples for phenotypic plasticity. Phenotypic plasticity implies an adaptive potential to locally adapt to a changed environment (Reger *et al.* 2018; Stearns 1989). If the phenotypically plastic organism produces a modified and successful phenotype whose fitness (measured as higher reproductive success) is higher than an unmodified phenotype, then the underlying genotype contributes more to the genetic set-up of the whole population. In other words, the environment influences phenotypic plasticity while phenotypic plasticity promotes diversification among populations within one species (reviewed by Pfennig *et al.* 2010). The adaptive potential of phenotypic plasticity in *Daphnia* to locally adapt has been shown in earlier studies (Altshuler *et al.* 2011; Hesse *et al.* 2012; Reger *et al.* 2018; Yin *et al.* 2011). For example, Jansen *et al* (2011) revealed the adaptive potential of *D. magna* to the pesticide carbaryl and Reger *et al* (2018) revealed local adaptation of phenotypic plasticity to predation in *D. pulex*. Given that, it is known that *Daphnia* respond phenotypically plastic to environmental changes and they are able to adapt rapidly to local environmental stressors. However, the gap of knowledge for the genetic basis of predator-induced phenotypic variation in *D. galeata* is yet unexplored.

Thesis outline

The aim of my thesis was to assess intraspecific phenotypic variation in European *Daphnia galeata* populations and to understand their underlying genetic basis of intraspecific phenotypic variation.

In the **first chapter**, I assessed the intraspecific phenotypic variation of life history traits in *D. galeata* in the presence and absence of fish kairomones to simulate predation risk. A common garden experiment with a total of 24 clonal lines with 6 clonal lines per population revealed high intraspecific phenotypic variation of life history traits within and among four European *Daphnia galeata* populations (Figure I-3). The research question

was: Which factor (genotype, environment, population or their interaction) drives the intraspecific phenotypic variation in *D. galeata* in the presence of fish kairomones at the population level? The analysis revealed that there is not one driving force influencing the intraspecific phenotypic variation. Instead, the study confirms the complexity of the interacting elements population, genotype and environment.

In the **second chapter**, I took the investigation to another level to examine differential gene expression and co-expression networks in the context of fish kairomone exposure. The previous experiment on fish predation risk allowed me to identify clonal lines with opposing life history strategies. To understand the genetic basis of this phenotypic variation, I conducted a smaller experiment with two clonal lines from one population. The research question was: Does the presence of fish kairomones affect gene expression in *D. galeata*? Using an RNA-seq approach, I identified differentially expressed transcripts and constructed a gene co-expression network to reveal underlying pathways (Figure I-3). The expression analysis revealed surprisingly high variances between clonal lines and identified 125 differentially expressed transcripts related to fish kairomone exposure. Taking advantage of available transcriptomic data on other *Daphnia* species, I assessed similarities of stress responses and reproduction in *Daphnia*. A total of 76 orthogroups contained transcripts of *D. galeata* and *D. magna* or *D. pulex* and related to a predator-induced response or reproduction.

Finally, in the **third chapter**, I associated the existing phenotype and genotype information of the 24 clonal lines by using a combined approach of genome-wide association and gene co-expression network analysis (Figure I-3). The research question was: Do genotypes and phenotypes of *D. galeata* have an association at the sequence level? The univariate transcriptome-wide association analysis showed a genetic basis for two life history traits in both environments with or without fish kairomones, while the multivariate analysis revealed more associations of a SNP to all life history traits only for the control environment. The gene co-expression analysis identified 44 gene co-expression modules of which one correlates to one life history trait, the total number of broods. Overall, biologically significant candidate transcripts being involved in predator-induced responses were identified laying a valuable cornerstone for further investigations of environment-dependent genotype-phenotype relationships.

By combining an integrative approach of transcriptome-wide association (**Chapter 3**), gene expression, and gene co-expression analyses (**Chapter 2**), I laid a cornerstone for the understanding of the intraspecific phenotypic variation of life history traits in European *D. galeata* in the presence of fish kairomones (**Chapter 1**) (Figure I-3).

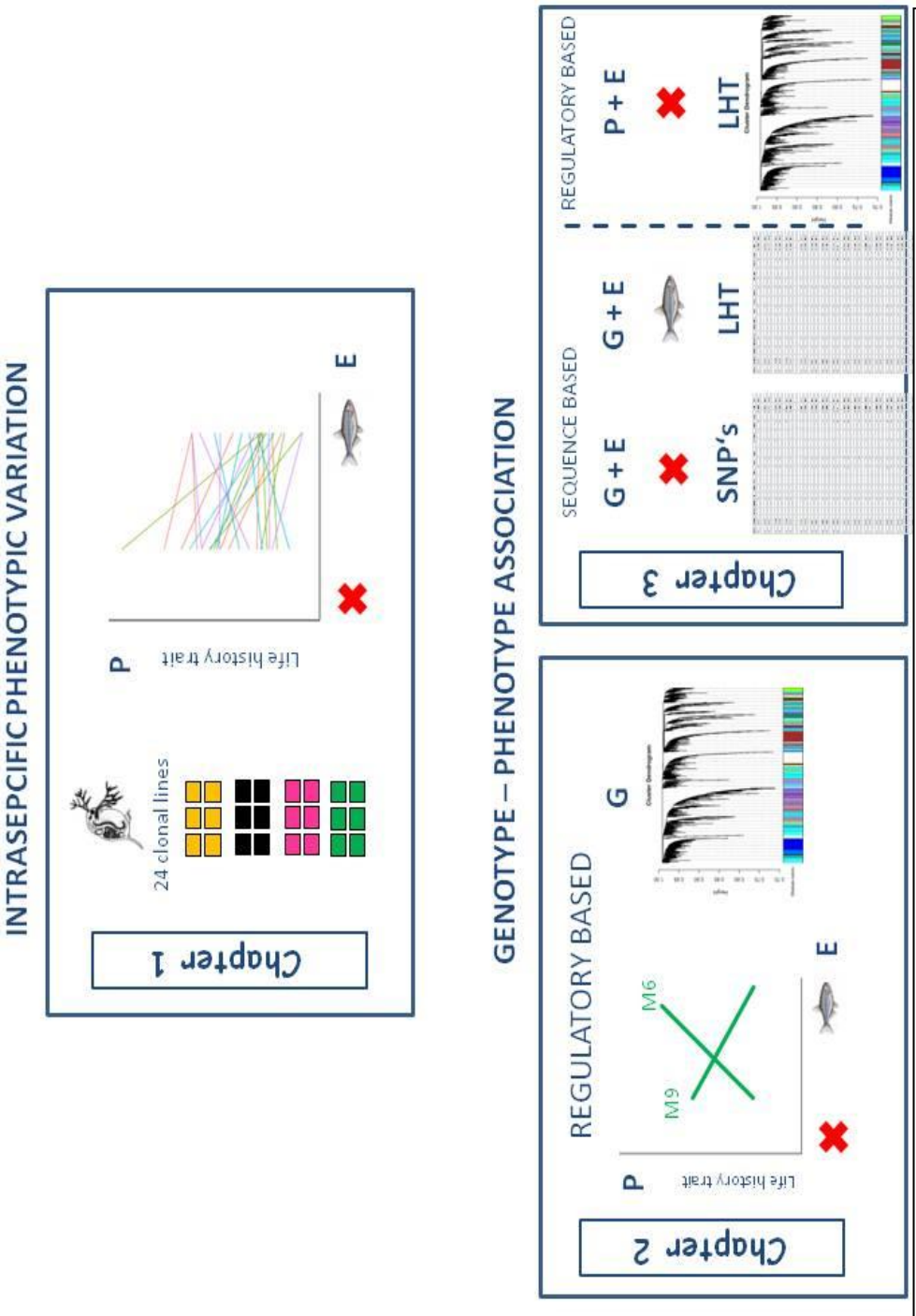

Figure I-3: Graphical thesis overview. In Chapter 1 the intraspecific phenotypic variation was assessed for 24 clonal lines of European *D. galeata* in the presence and absence of fish kairomones. In Chapter 2 a regulatory-based genotype - phenotype association was established via gene expression and co-expression network analysis. In Chapter 3 a sequenced-based genotype-phenotype association was established via a transcriptome-wide association. In addition a gene co-expression analysis for all 24 clonal lines in the absence of fish kairomone was applied. 'P' = phenotype. 'G' = genotype. 'E' = environment. 'red cross' = control environment. 'fish' = fish environment. 'LHT' = life history traits.' SNPs' = single nucleotitide polimorphism. 'M6' = clonal line M6. 'M9' = clonal line M9.

Chapter 1

Intraspecific phenotypic variation in life history traits of *Daphnia galeata* populations in response to fish kairomones

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Abstract

Phenotypic plasticity is the ability of a genotype to produce different phenotypes depending on the environment. It has an influence on the adaptive potential to environmental change and the capability to adapt locally. Adaptation to environmental change happens at the population level, thereby contributing to genotypic and phenotypic variation within a species. Predation is an important ecological factor structuring communities and maintaining species diversity. Prey developed different strategies to reduce their vulnerability to predators by changing their behavior, their morphology or their life history. Predator-induced life history responses in *Daphnia* have been investigated for decades, but intra-population variability was rarely addressed explicitly. We addressed this issue by conducting a common garden experiment with four European *Daphnia galeata* populations, each represented by six genotypes. We recorded life history traits in the absence and presence of fish kairomones. Additionally, we looked at the shape of experimental individuals by conducting a geometric morphometric analysis, thus assessing predator-induced morphometric changes. Our data revealed high intraspecific phenotypic variation within and between four *D. galeata* populations, the potential to locally adapt to a vertebrate predator regime as well as an effect of the fish kairomones on morphology of *D. galeata*.

Introduction

Intraspecific phenotypic variation is crucial for the persistence of a population, since low intra-population variation increases the risk of extinction (Bolnick *et al.* 2011; Forsman 2014; Scheiner & Holt 2012). Loss of phenotypic variation can be caused by the reduction of genetic variation e.g. due to genetic drift (random loss of alleles) (e.g., Bolnick *et al.* 2011; Vanoverbeke & De Meester 2010), inbreeding depression (e.g., Lynch 1991; Swillen *et al.* 2015) or positive selection (e.g., Biswas & Akey 2006). On the contrary, phenotypic variation can increase as a consequence of environmental change (biotic and/or abiotic) as well as through an increase in genetic variation, which in turn occurs through gene flow (migration), mutation and recombination (Griffiths *et al.* 2000). Phenotypic variation 'is the fuel that feeds evolutionary change' because natural selection acts on it (Stearns 1989). Phenotypic plasticity describes the ability of genotypes to produce different phenotypes depending on the environment, helping organisms to survive and reproduce in heterogeneous environment (Agrawal 2001; Stearns 1989). Phenotypic plasticity implies an adaptive potential to locally adapt to a changed environment (Reger *et al.* 2018, Stearns 1989). If the phenotypically plastic organism produces a modified and successful phenotype whose fitness (higher reproductive success) is higher than an unmodified phenotype, the underlying genotype contributes more to the genetic make-up of the whole population.

Predation structures whole communities (Aldana *et al.* 2016; Beschta & Ripple 2009; Boaden & Kingsford 2015; Werner & Peacor 2003), drives natural selection within populations (Kuchta & Svensson 2014; Morgans & Ord 2013) and maintains species diversity (Estes *et al.* 2011; Fine 2015). Aquatic predators release chemical substances, so called kairomones, into the surrounding waters which can be detected by their prey. Both vertebrates (e.g., Schoeppner & Relyea 2009; Stibor 1992) and invertebrates (e.g., Machacek 1991; Stibor & Lüning 1994) release kairomones, triggering specific phenotypic plastic responses such as morphological or behavioral changes (e.g., Dodson 1989; Schoeppner & Relyea 2009). The predator-induced defenses can be highly variable within a species, depending on factors such as the predator and colonization histories (e.g., Edgell & Neufeld 2008; Ekloev & Svanbaeck 2006; Kishida *et al.* 2007).

Invertebrate as well as vertebrate predator kairomones have been shown to cause phenotypic plastic responses in *Daphnia*. These induced responses are predator specific and vary across *Daphnia* species. Behavioral changes such as diel vertical migration (DVM) (Effertz & von Elert 2015) and the associated metabolic costs (Dawidowicz & Loose 1992), depth selection (Cousyn *et al.* 2001), increased alertness (Boersma *et al.* 1998) and diapause (production of resting eggs = ephippia) (Pijanowska & Stolpe 1996) were reported for different *Daphnia* species exposed to vertebrate predator kairomones (fish). Diverse morphological changes have been shown to occur in the presence of kairomones of the invertebrate predator *Chaoborus*, such as the production of neck teeth in *D. pulex* (Lüning 1995; Tollrian 1995) or the famous helmets of *D. longispina* (Brett 1992) and *D. cucullata* (Agrawal *et al.* 1999). Recently Herzog *et al.* (2016) observed a remarkable morphological change of *D. barbata* exposed to *Triops* kairomones. *D. barbata* changes its whole body symmetry to an S-shape, presumably to impede ingestion by their invertebrate predator. Apart from morphology, physiology and behavior, predator kairomones were also shown to influence life history traits in different *Daphnia* species. Among others, size and fecundity, two important traits for population survival, were affected, resulting in earlier maturation (Castro *et al.* 2007; De Meester & Weider 1999; Riessen 1999; Weber 2003) and smaller size (Castro *et al.* 2007; Stibor & Lüning 1994). Size is a very important factor for survival in the face of fish predation, since small individuals are more likely to go undetected. These predator-induced responses are the result of phenotypic plasticity and their magnitude might play a role in adaptation.

Although clonal variation of *Daphnia* within one population has been regularly reported (Beckerman *et al.* 2010; Castro *et al.* 2007; Cousyn *et al.* 2001; De Meester 1996; Machacek 1991), and many experimental studies compare several populations of *Daphnia* (Boeing *et al.* 2006; Boersma *et al.* 1998; Declerk & Weber 2003; Gliwicz & Boavida 1996; Hamrova *et al.* 2012; Lind *et al.* 2015), we are aware of only two studies which addressed the intra-population level. Boersma *et al.* (1998) used four clonal lines for each of the four populations shoowing that the strength and combination of responsive traits can differ across genotypes (clonal lines). Recently, Reger *et al.* (2018) revealed that predation drives local adaptation in phenotypic plasticity in 70 clonal lines of *D. pulex*. Others rarely used more than one or two genotypes per population, drawing conclusions based on single genotypes. Although intra-population variation or lack thereof is relevant to population

maintenace in the face of predation pressure, the relative importance of the intra- and inter-population variation was rarely measured. The ability of *Daphnia* to locally adapt to different stressors has been demonstrated e.g. for fish as a vertebrate predator (Boersma *et al.* 1998; Cousyn *et al.* 2001; Declerk & Weber 2003) and pesticides (Jansen *et al.* 2011). We therefore expect the populations to be locally adapted, which translates into a population specific response.

In the present study, we assess the intraspecific phenotypic variation among four European *Daphnia galeata* populations in the presence of fish kairomones, measuring shifts in life history traits as well as morphological changes. We expect that (i) there is intraspecific phenotypic variation within each population. Our experimental setup allows us to (ii) assess the relative importance of the factors (environment, genotype, population or their interaction) driving phenotypic variation in the different populations. We hypothesize that (iii) the potential for local adaptation is reflected in phenotypic predator-induced life history responses. Finally, we expect that (iv) the exposure to fish kairomone affects the morphology. We hypothesize that a correlation between life history change and morphology exist. Sepcifically, we hypothesize that females which increased their total number of offspring in the presence of fish kairomones, change their morphology towards a bulkier shape to accommodate more eggs.

Materials and methods

Experimental organisms and lakes of origin

This study integrated 24 *D. galeata* clonal lines from four different locations: Lake Constance (popLC), Germany; Greifensee (popG), Switzerland; Müggelsee (popM), Germany and Jordan Reservoir (popJ), Czech Republic. These are all permanent lakes with a large water body and varying fish densities (Table C1-S1). Clonal lines were established from dormant eggs from sediment cores and have been used in previous studies (Henning-Lucass *et al.* 2016; Herrmann *et al.* 2017). The clonal lines were maintained in lab cultures (18°C, 16h light / 8h dark cycle, food: *Acutodesmus obliquus*, medium: Aachener Daphnien Medium (ADaM) (Klüttgen *et al.* 1994) for up to 5 years and no less than 3 years prior to the present experiment.

Media preparation

The basic medium was ADaM for fish and *Daphnia* cultures. Two types of media were used for breeding and experimental conditions: fish kairomone and control medium. In total forty ide (*Leuciscus idus*) were maintained in an aerated, separate 200L aquarium, in which they were fed with frozen *Daphnia* cubes and dry food. The ide or closely related species are present in all the studied lakes (Table C1-S1). Previous studies showed that ide elicit plastic responses in *D. galeata* clonal lines from Lake Constance (Sakwinska 2002) and Greifensee (Wolinska *et al.* 2007). Fish medium was obtained by keeping 5 randomly chosen ide in an aerated 20L aquarium for 24h to produce fish kairomone medium. The fish were not fed in the fish medium production tank to avoid *Daphnia* alarm cues to be mixed with the fish kairomones. The fish kairomone media imitates a scenario of high fish density (Cousyn *et al.* 2001; Swillen *et al.* 2015). Control medium was produced in an aerated, separated aquarium and handled first, before handling of fish and fish medium. All media was filtered before use to remove feces from predators and bacteria larger than 1.2µm (Whatman, membrane filters, ME28, Mixed cellulose-ester, 1.2µm). All media were supplemented with 1.0 mg C L-1, P rich *Acutodesmus obliquus* before use and exchanged daily (1:2) to guarantee a nutrient rich environment and a constant fish kairomone concentration. The algae concentration was calculated from photometric measurement of the absorbance rate at 800 nm.

Because fish was used to produce fish kairomone media, this experiment was subject to approval through the "Behörde für Gesundheit und Verbraucherschutz" of the City of Hamburg (#75/15).

Experimental design and procedures: life table experiment

Prior to the experiment, each clonal line was bred in kairomone-free water (control environment) and in kairomone water (fish environment) for two subsequent generations to minimize inter-individual variances. To this end, 10-15 egg-bearing females per clonal line were randomly selected from mass cultures. From these females of unknown age, neonates were collected and raised under experimental conditions and served as grandmothers (F0) for the experimental animals (F2). Neonates of the 3rd to 5th brood carried by the F0 animals were used as breeding (F1) animals. Neonates of the 3rd to 5th

brood carried by the F1 animals were used in turn as experimental individuals (F2). A pair of neonates was introduced in the experimental vessels (50 mL glass tube) at the start of the experiment to compensate for eventual mortality. One of the individuals was randomly discarded when necessary at day 4 (t4), so that one individual remained in each vessel. This procedure was applied to F1 and F2 individuals. Fifteen replicates were used per environment and per genotype (clonal line). Sister neonates of F2 (n=15) were collected in 70% ethanol for size measurements at day 0 (t0). Life history parameters were recorded daily during the experiment. Before media renewal, females were checked for maturation and neonates were counted, removed and preserved in ethanol every day. Adults were preserved in ethanol as well at the end of the experiment. The experiment lasted for 14 days (t14) for each experimental individual to monitor the performance of each clonal line within a fixed period of time.

Cetyl alcohol was used to break the surface tension of the media 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).

The experiment was conducted in three experimental rounds due to logistic reasons. In each round clonal lines from all four populations were present (Table C1-S2). Previous pilot studies showed that ensuring synchronicity of so many clonal lines at once is extremely difficult.

Data collection and analysis

Life history traits

Life history parameters such as age at first reproduction ('AFR') [d], number of neonates per brood per female, total number of broods per female ('broods'), total numbers of neonates per female ('offspring'), size of first clutch ('brood1') [number of neonates per female], 'survival' [%] and somatic growth rate ('SGR') [µm d^{-1}] were recorded. Age at first reproduction was the day of releasing the first brood from the brood pouch, with neonates swimming in the vessel. For further analysis the average value of the 15 individuals per clonal line ('Genotype') per environment ('Treatment') was calculated for

each life history trait to estimate the clonal response to a kairomone (fish) vs. kairomone-free (control) environment. Survival rate was defined as the proportion of females surviving from the day of separation (t4) until the end of the experiment (t14). Reproductive rate was calculated by dividing the total number of offspring per female by the total number of broods per female. Relative fitness (w) was calculated by multiplying survival and reproductive rate of a genotype before dividing by the maximum survival and reproductive rate of the other genotypes within population and among all populations. Some genotypes produced male offspring during breeding and the experiment. Males occurred at very low frequencies and were excluded from the data analysis. We aimed to test a total of 720 individuals in this experiment (24 clonal lines x 2 treatments x 15 replicates). In total we measured life history traits for 684 experimental individuals (Table C1-S2).

Digitizing of experimental animals for 'size' and 'shape' analysis

Digital photographs of *Daphnia* preserved in ethanol were taken with a stereomicroscope (Nikon SMZ800N) at a magnification of 60x for neonates (t0) and 40x for adults (t14) with NIS-elements 4.3 software. All experimental individuals were photographed in lateral view (left body side up).

Measurement of body length ('size')

Body length ('size') was measured from the top of the head through the middle of the eye to the ventral basis of the spine, excluding the spine itself. Somatic growth rate ('SGR', µm/day) was calculated by subtracting the average 'size' of neonates at the beginning of the experiment (t0; n=15) from the 'size' of each adult individual at the end of the experiment (t14), divided by the complete experimental time in days. The measurement error of digitizing and measuring the body length 10 times of the same individual was +/- 3.24 µm (SD). The measurement error of measuring 10 times the body length of an individual using the exact same picture was +/-1.67 µm (SD).

Geometric morphometric analysis of the 'shape' of the body

Since the morphology of *Daphnia* does not allow the assignment of many landmarks, we decided to integrate the semilandmark approach. Semilandmarks are a set of individual landmarks which are interpolated to represent the curve of a structure (Zelditch *et al.* 2004). Landmarks and semilandmarks were assigned on a subset of digital images of adult experimental individuals (max. n=10 per clonal line and environment, with a total of 459 individuals) according to Zelditch et al. 2004. In total three landmarks and 115 semilandmarks were assigned on each individual photograph. The first landmark was appointed to the tip of the rostrum, the second in the middle of the eye and the third at the ventral basis of the spine. In our study the first curve consisted of 70 interpolated landmarks (=semilandmarks) along the dorsal body outline, starting at the first landmark and ending on the dorsal basis of the spine. The second set of semilandmarks consisted of 45 semilandmarks along the ventral body outline, starting at landmark three and ending opposite of the dorsal basis of antenna. After the assignment of landmarks and semilandmarks, X and Y coordinates were recorded using 'TpsDig2' (Rholf 2015). A General Procrustes Analysis (GPA) was performed using the package '*geomorph*' in R (Adams *et al.* 2013). The measurement variance for assigning landmarks and semilandmarks of an individual using the exact same picture was <0.0001. Investigators of 'shape' measurements worked with a blind data set, not knowing which individual belongs to which group (environment, genotype and population).

Statistical analysis

All statistical analyses for life history traits were performed and all figures were created using R version 3.3.1 (R CoreTeam 2018). For the generalized linear mixed models (GLMM) the package *'lme4'* was used (Bates *et al.* 2015). Subsequent post-hoc tests were performed with the package '*lsmeans'* (Lenth 2016). To account for multiple testing, strict Bonferroni correction was applied. Visualization of life history traits were performed by using the package '*ggplot2'* (Wickham 2010). For the geometric morphometric analysis the package '*geomorph'* was used (Adams *et al.* 2013). The visualization of 'shape' differences was performed with the R package '*shapes*' (Dryden 2017). R scripts are provided in supplementary materials.

To compare life history traits between the different populations in the presence and absence of fish kairomones, we applied generalized linear mixed effect models for each trait, except 'shape'. Visual inspection of residual plots as well as the Shapiro-Wilk-Test revealed deviations from homoscedasticity for each trait, supporting the decision to use nonparametric models for statistical analysis. Hence, error distributions were assigned individually per trait. We used 'Treatment' and the interaction of 'Treatment x Population' as fixed categorical factors in our models. To account for genotype differences among populations, we included 'Clone' ('Genotype') nested within 'Population' as a random factor. We checked for the necessity of random slopes and intercepts, finally resulting in a general random intercept model for 'Treatment' (response ~ T + (1|pop/clone)) and 'Treatment x Population' (response ~ T*P + (1|pop/clone)). Statistical significances for life history traits were obtained by likelihood ratio tests of the full model with the effect in question against the model without the effect in question using the function (Anova(model,type=2)) which performs a Wald Chi-Square test.

To assess shape variation we used the principal component analysis (PCA) after the General Procrustes Analysis (GPA) in the R package '*geomorph*'. Subsequently the statistical analysis was done with Procrustes ANOVA and pairwise tests to reveal statistically relevant 'shape' differences between environments.

Results

Effects of fish kairomones on life history traits: 'Treatment' effect

Fish kairomones significantly affected age at first reproduction ('AFR'), total number of broods ('broods'), somatic growth rate ('SGR') and body length ('size') (Table C1-1, Figure C1-1, Figure C1-2). *D. galeata* exposed to fish kairomones matured 1.7 hours earlier compared to a mean of 9 days, grew 2.53µm less per day (+/- 0.63 SE) and were smaller by 59.82µm (+/- 9.71 SE) at the end of the experiment (day 14). The probability of having more than two broods decreased from 0.55 in the control environment to 0.43 in the fish environment (Figure C1-3).

The 'Population' effect was small for 'broods' (4.11% of total random effect variation) and estimated to be zero for 'AFR', 'SGR' and 'size'. The 'Clone' effect was small for 'AFR' (0.12%

of the total random effect variation) and estimated to be zero for 'size', while it was high 'broods' (47.46% of total random effect variation) and 'SGR' (65.09% of total random effect variation).

The presence of fish kairomones did not affect the relative fitness of females within each population ('relnest') as well as the relative fitness among all populations ('relclone') (Table C1-1). There was no random 'Population' effect for the relative fitness of females within one population, since we did not compare across several populations, while there was a 'Population' effect of 19% of total random effect variation for the relative fitness of females among all populations. The 'Clone' effect was substantial for the relative fitness of females within one population (109.4% of total random effect variation) and among populations (159.89% of total random effect variation). Further details of relative fitness for each clonal line within their population can be found in Table C1-2A and C1-2B. The fittest population in control environment was popJ (w=1), followed by popM (w=0.83), popLC (w=0.78) and popG (w=0.67). In fish environment a small change of positions occurred for popLC and popM. Here the decreasing order was popJ (w=1), followed by popLC (w=0.80), popM (w=0.77) and popG (w=0.63) among all populations.

Table C1-1: General linear mixed effect model (GLMM) testing for the effect of presence/absence of fish kairomones ('Treatment') and individual origin ('Population') on various life history traits. For the trait 'shape' Procrustes ANOVA/regression was used as a model to test for effects. Significant values (p<0.05, p<0.01, p<0.001) are highlighted in bold. Values are rounded.

	'Treatment'			'Treatment x Population'		
Model	**Response ~ T + (1|pop:clone)**			**Response ~ T * P + (1|pop:clone)**		
Life history trait	**Chisq**	**Df**	**Pr(>Chisq)**	**Chisq**	**Df**	**Pr(>Chisq)**
Age at first reproduction ('AFR')	34	1	**<0.001**	20	3	**<0.01**
Total number of broods ('broods')	11	1	**<0.001**	4	3	0.26
Total number of offspring ('offspring')	3	1	0.65	9	3	**0.05**
Total number of offspring first brood ('brood1')	0.04	1	0.84	4	3	0.24
'Survival' (surv)	3	1	0.07	0.06	3	0.70
Somatic growth rate ('SGR')	16	1	**<0.001**	22	3	**<0.001**
Relative fitness within populations ('relnest')	0.59	1	0.443	2	3	0.59
Relative fitness among populations ('relclone')	0.09	1	0.76	2	3	0.64
Body length ('size')	38	1	**<0.001**	35	3	**<0.001**
Morphological trait	**F**	**Df**	**Pr(>F)**	**F**	**Df**	**Pr(>F)**
Body shape ('shape')	4	454	**<0.001**	3	451	**0.004**

Table C1-2: Relative fitness (w) within and among populations. A. Relative fitness within and among populations for genotype means. B. Range of relative fitness among populations for genotype means. Fittest genotype or population (w=1.0) is highlighted in bold.

(A)

		w within population ('relnest')		w among populations ('relclone')	
population	clone	control	fish	control	fish
G	G1.11	0.53	0.84	0.36	0.50
	G1.12	0.35	0.66	0.24	0.40
	G1.6	0.46	0.31	0.32	0.19
	G1.7	0.95	0.86	0.65	0.51
	G2.1	0.81	0.86	0.56	0.52
	G3.1	**1.00**	**1.00**	0.69	0.60
J	J1	0.73	0.75	0.73	0.75
	J2	0.64	0.68	0.64	0.68
	J2.1	0.50	0.69	0.50	0.69
	J2.4	**1.00**	**1.00**	**1.00**	**1.00**
	J3	0.67	0.70	0.67	0.70
	J4	0.63	0.55	0.63	0.55
LC	LC3.1	0.73	0.59	0.55	0.45
	LC3.3	0.56	0.63	0.42	0.47
	LC3.5	0.78	0.96	0.59	0.72
	LC3.6	**1.00**	**1.00**	0.75	0.75
	LC3.7	0.46	0.54	0.35	0.41
	LC3.9	0.78	0.95	0.59	0.71
M	M10	0.72	0.97	0.43	0.66
	M12	0.87	0.71	0.52	0.48
	M2	0.98	0.86	0.59	0.59
	M5	0.95	**1.00**	0.57	0.69
	M6	0.82	0.88	0.50	0.60
	M9	**1.00**	0.78	0.60	0.54

(B)

population	w control	w fish
G	0.24-0.69	0.19-0.60
J	**0.50-1.00**	**0.55-1.00**
LC	0.35-0.75	0.41-0.75
M	0.43-0.60	0.54-0.69

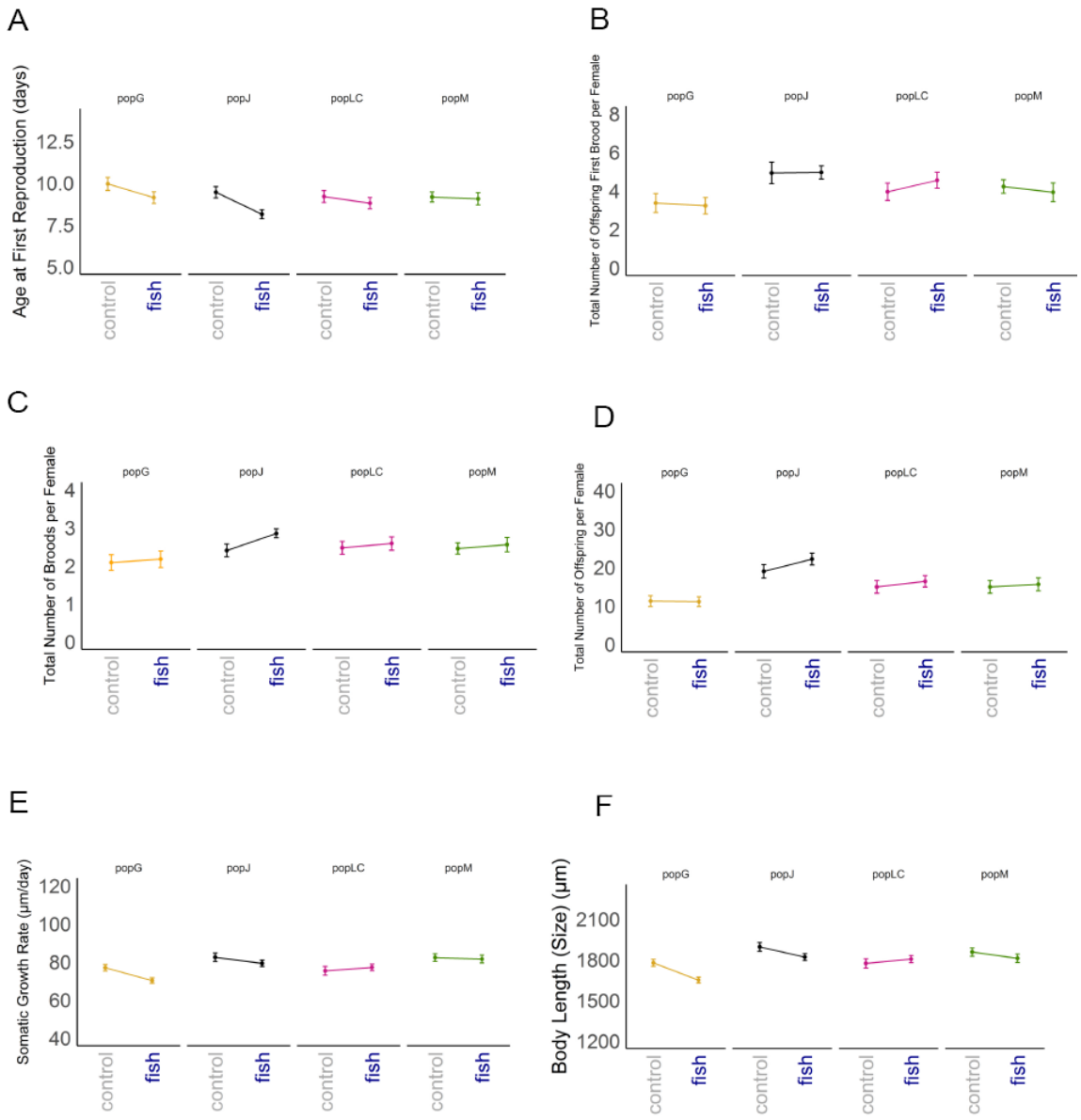

Figure C1-1: Reaction norms for selected life history traits showing population differences (mean +/- SE). Population Greifensee (popG, yellow), population Jordan reservoir (popJ, black), population Lake Constance (popLC, magenta) and population Müggelsee (popM, green). A. Age at first reproduction ('AFR'). B. Total number of offspring first brood ('brood1'). C. Total number of broods ('broods'). D. Total number of offspring ('offspring'). E. Somatic growth rate ('SGR'). F. Body length ('size').

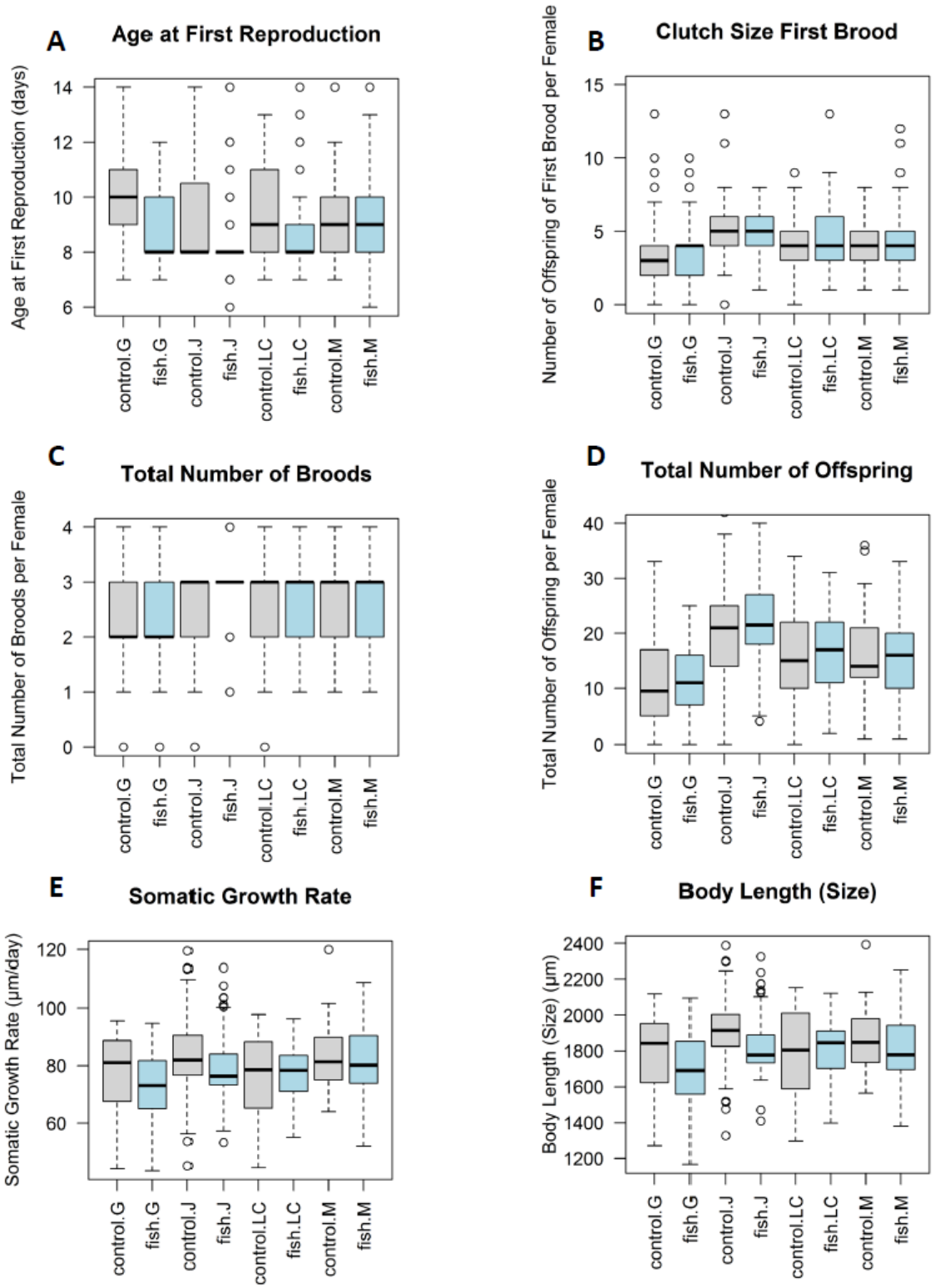

Figure C1-2: Boxplots for selected life history traits showing population differences (median +/- SD). A. Age at first reproduction ('AFR'). B. Total number of offspring first brood ('brood1'). C. Total number of broods ('broods') D. Total number of offspring ('offspring'). E. Somatic growth rate ('SGR'). F. Body length ('size'). 'grey' = control environment. 'lightblue' = fish environment.

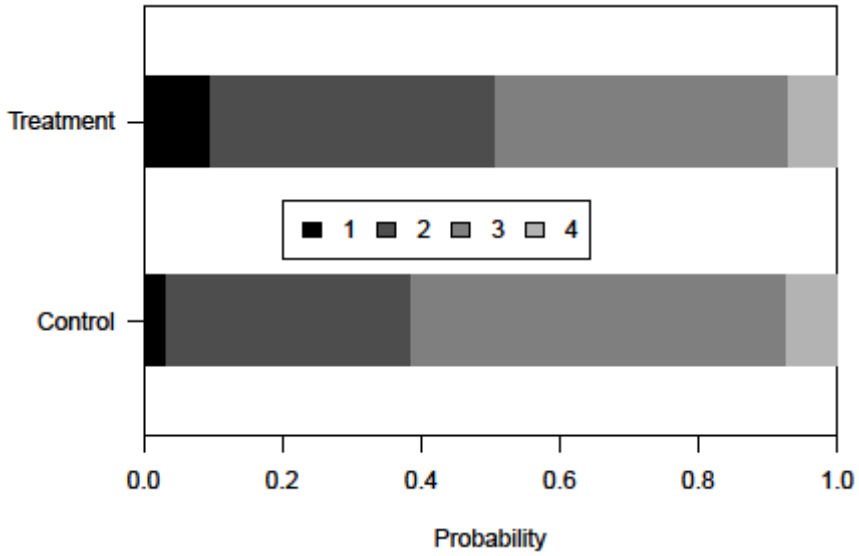

Figure C1-3: Probability plot showing the probability of having two broods within each environment. 'Control' = environment without fish kairomones. 'Treatment' = environment with fish kairomones.

Effect sizes of the factors 'Treatment', 'Genotype' and 'Population'

We summarized the effect sizes of the fixed factor 'Treatment' (environment) and the random factors 'Genotype' and 'Population' by plotting their effect sizes (Figure C1-4). Effect sizes were standardized by dividing the standard error (SE) of one trait by its residual, turning the effect size of residuals into 1 and thus allowing comparisons across the different data types.

Three traits ('brood1', 'relnest' and 'relclone') were not influenced by any of the three factors. 'Treatment' seemed to be the main driver for the two traits 'AFR' and 'size'. The two traits 'offspring' and 'SGR' seemed to be mainly influenced by 'Genotype', while the trait 'broods' was influenced by 'Treatment' and 'Genotype'. The random factor 'Population' had overall no to little effect on the predator-induced response.

Effects of genotype origin on predator-induced responses in life history traits: 'Treatment x Population' effect

A significant interaction effect of 'Treatment x Population' was revealed mainly for within-population differences in the population from Greifensee (popG) and Jordan Reservoir (popJ) as well as among those two populations.

In fish environment, the age at first reproduction ('AFR') differed significantly within popJ (p=<.0001) and within popG (p=0.0023). Additionally, 'AFR' differed significantly between popG and popJ (p=0.0347) in control environment, meaning that clonal lines of popG reproduce later compared to clonal lines of popJ regardless of the environment.

The total number of 'offspring' differed significantly between popG and popJ in control environment (p=0.0198) and fish environment (p=0.0023), as well as between the two environments (popG-fish vs. popJ-control (p=0.0311)). Additionally, the total number of 'offspring' differed significantly between environments within popJ (p=0.0243) resulting in an increase of 'offspring' for popJ exposed to fish.

In fish environment, the somatic growth rate ('SGR') differed significantly within popG (p=<.0001) and popJ (p=0.0135) (Figure C1-1E, Figure C1-2E). The visualization of growth differences between environments and populations (dSGR, Figure C1-5) showed that all clonal lines from popG had a negative somatic growth rate in fish environment, resulting in a smaller body size. Four out of six clonal lines from popJ had a negative somatic growth rate, while clonal lines from popLC and popM vary in somatic growth rate across environments.

In fish environment, body length ('size') differed significantly within popG (p=<0.001), popJ (p=0.0002) and popM (p=0.0042) (Figure C1-1F, Figure C1-2F).

Genotype origin (‘Population’) had a significant effect on total number of offspring in first brood ('brood1') (Chisq=11.6722, Df=3, Pr(>Chisq)=0.008595). The trait 'brood1' differed significantly between popG and popJ in control (p=0.0073) and fish (p=0.0301) environment, meaning that the total number of offspring in the first brood for popG was overall smaller compared to popJ regardless of the environment (Figure C1-1B, Figure C1-2B).

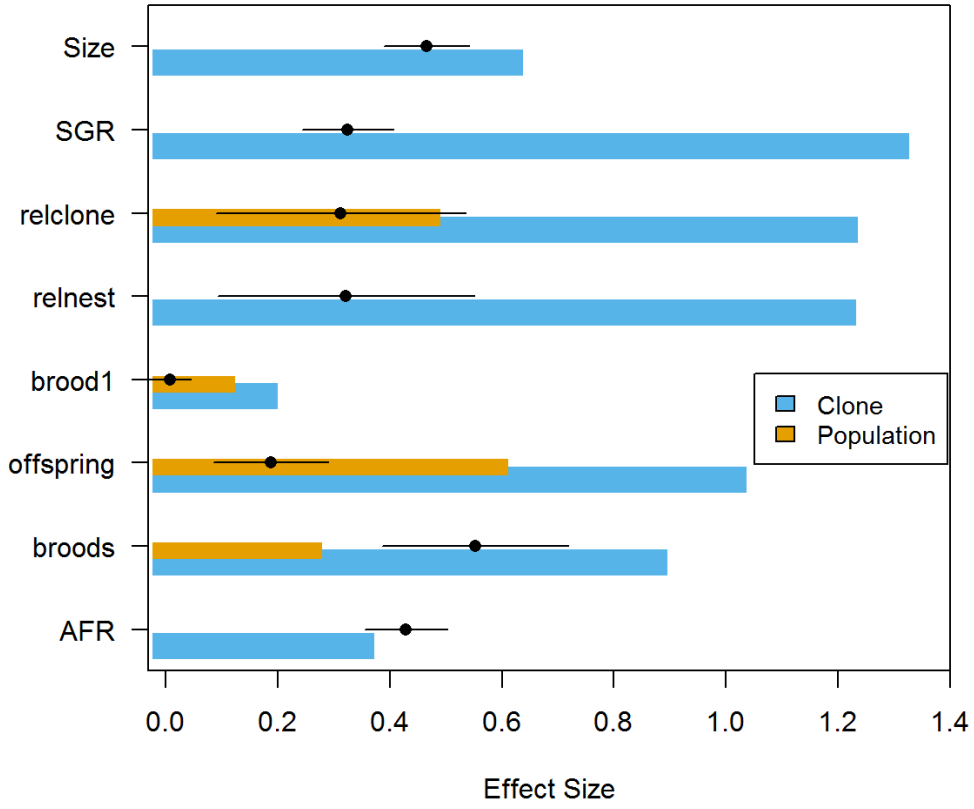

Figure C1-4: Visualization of standardized effect sizes. Absolute values of the fixed effect 'Treatment' are plotted with black dots (+/- 1 SE). The effect of random factors are displayed in orange bars for 'Population' and blue bars for 'Clone' (clonal line) nested in 'Population'. The life history traits are 'Size' = body length, 'SGR' = somatic growth rate, 'relclone' = relative fitness among population, 'relnest' = relative fitness within population, 'brood1' = total number of offspring first brood, 'offspring' = total number of offspring, 'broods' = total number of broods, 'AFR' = age at first reproduction.

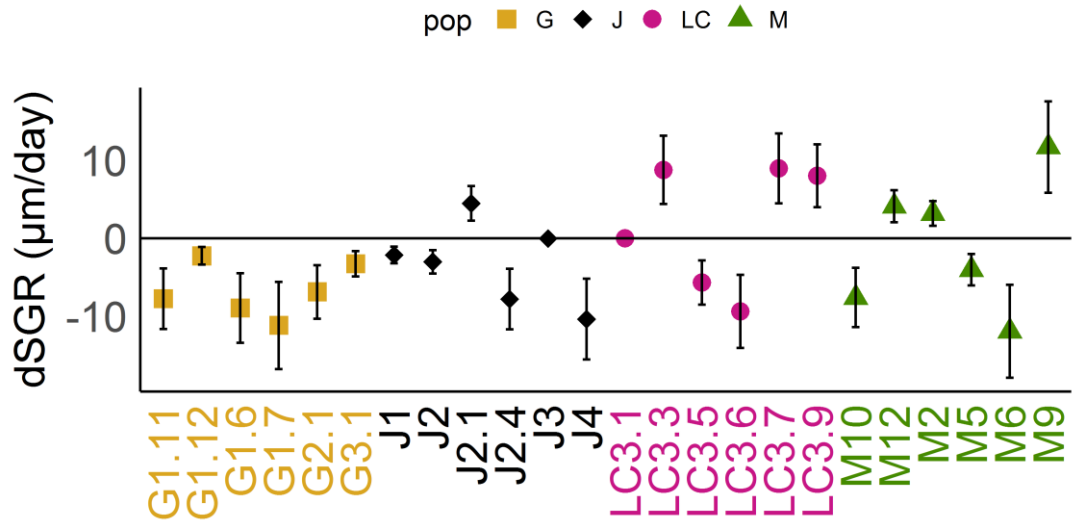

Figure C1-5: Differences of somatic growth rate (dSGR) as µm per day (mean +/-SD). dSGR was calculated as the mean of 'SGR' (fish) minus mean 'SGR' (control) equals dSGR per genotype. Values are sorted by populations.

Effect of fishkairomones on the morphological trait 'shape'

A total of 83% of 'shape' variation was explained by the first four principal components (PC1= 42%, PC2=24%, PC3=11% and PC4= 6%) (Figure C1-S1). The geometric morphometric analysis showed that 'Treatment' was a meaningful factor for 'shape' variation (Df=454, F=3.4177, Z=3.1515, Pr(>F)=0.001). Visualization revealed an overall 'shape' change towards a smaller body. In detail, the head area changed to a ventral position, while the tail area changed to a dorsal position (Figure C1-6A).

A significant 'Treatment' effect on 'shape' existed within populations, except for popG (Table C1-3A) as well as among populations (Table C1-3B). The 'shape' of individuals of popM differed compared to all the other three populations (p=0.001) and the 'shape' of individuals differed between popG and popJ (p=0.011) (Table C1-3B).The visualizations showed a homogenous change from all directions to a smaller body form for popG (Figure C1-6B). Within popJ the overall 'shape' change towards a smaller body was shown with the strongest change in the head area (bending of the thin plate spline) and an anterior-posterior direction (Figure C1-6C). Within popLC the head position changed from dorsal to ventral direction, while a small change of the tail area from a ventral to dorsal direction (Figure C1-6D) occurred. Within popM the overall shape change towards a smaller body size was shown in the head area from a dorsal to ventral direction and in the tail area from a ventral to dorsal direction (Figure C1-6E).

There was a significant interaction effect of 'Treatment x Population' on 'shape' (Df=451, F=2.5725, Z=2.3747, Pr(>F)=0.004). The *p*-value matrix revealed that there was a statistical significance difference within popLC between environments (p=0.043; Table C1-3C). Further analysis revealed significant 'shape' differences among populations within each environment (control: Df=3, F=2.1558, Z=1.9388, P=0.002 Pr(>F)=0.002; fish: Df=3, F=5.2562, Z=4.6072, Pr(>F)=0.001).

The 'shape' of females with lots of offspring (n>22 = upper quartile of total number of offspring) differed significantly among populations in the control environment (Df=1, F=2.3358, Z=1.8997, Pr(>F)= 0.049), but not in the fish environment (Df=1, F=0.93, Z=0.72905, Pr(>F)=0.431). There is no association of 'shape' and a high number of 'offspring' in the fish environment. Further analysis revealed that the 'shape' of females

with lots of 'offspring' did not differ significantly between environments within each population.

Statistical analysis revealed no block effect for all traits in our experiment, except for 'brood1' (GLMM: Pr(>Chisq)= 0.001867), 'SGR' (GLMM: Pr(>Chisq) <0.001) and 'shape' (Procrustes ANOVA: Pr(>F)=0.001).

Detailed experimental information for each clonal line can be found in the supplementary material (Figure C1-S2 to Figure C1-S7).

Table C1-3: Results of geometric morphometric analysis. A. P-values of 'Treatment' effect on 'shape' differences within populations. B. P-value matrix of 'Treatment' effect on 'shape' among populations. C. P-value matrix of the interaction of 'Treatment x Population' on 'shape'. Statistical significant F-values (Pr(>F)<0.05) are displayed in bold.

(A)

Population	Df	F	Pr(>F)
G	1	0.32	0.897
J	1	4.54	**0.001**
LC	1	3.43	**0.011**
M	1	2.49	**0.014**

(B)

-	G	J	LC	M
G	-	**0.011**	0.180	**0.001**
J	**0.011**	-	0.354	**0.001**
LC	0.180	0.354	-	**0.003**
M	**0.001**	**0.001**	**0.003**	-

(C)

-	G:control	G:fish	J:control	J:fish	LC:control	LC:fish	M:control	M:fish
G:control	-	0.512	0.569	0.398	0.077	0.614	0.666	0.972
G:fish	0.512	-	0.960	0.168	0.695	0.175	0.098	0.158
J:control	0.569	0.960	-	0.192	0.867	0.225	0.508	0.417
J:fish	0.398	0.168	0.192	-	0.083	0.463	0.964	0.313
LC:control	0.077	0.695	0.867	0.083	-	**0.043**	0.165	0.229
LC:fish	0.614	0.175	0.225	0.463	**0.043**	-	0.959	0.772
M:control	0.666	0.098	0.508	0.964	0.165	0.959	-	0.403
M:fish	0.972	0.158	0.417	0.313	0.229	0.772	0.403	-

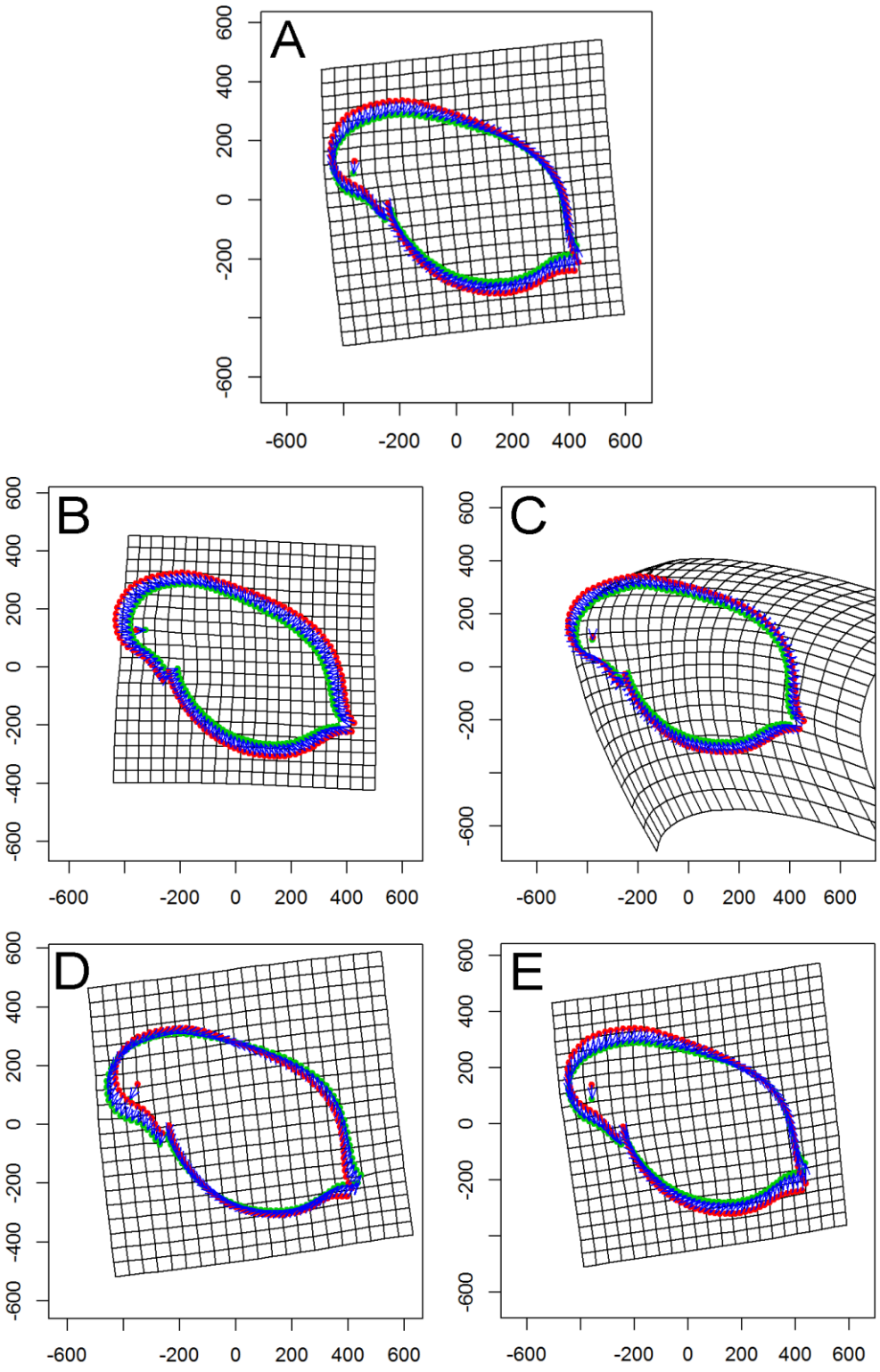

Figure C1-6: Thin plate spline (TPS) grids of consensus shapes of superimposed Procrustes coordinates. 'red' = control environment. 'green' = fish environment. A. All specimens. B. Population Greifensee (popG). C. Population Jordan Reservoir (popJ). D. Population Lake Constance (popLC). E. Population Müggelsee (popM).

Discussion

Intraspecific phenotypic variation in life history traits within and among populations

Concordant to previous studies by Boersma *et al.* (1998) as well as Stibor and Lüning (1994), our results showed a decrease of age at first reproduction, a decrease of somatic growth rate and a decrease of body length in the presence of fish kairomones in *Daphnia galeata.* Our experimental design further allowed us to assess the distribution of variance at different levels, clonal and population level. We thus detected phenotypic variation within each as well as among several populations independent of the environment. We identified two different strategies of phenotypic plastic responses of *Daphnia galeata* by comparing the 'Treatment' effect within as well as among the populations. In popJ, the variation of a trait itself, not the change in the trait median value as a response was extremely reduced for two life history traits, 'AFR' and total number of 'broods' (Figure C1-2C). Almost all individuals of popJ started to reproduce at the same age and produce the same amount of broods in the fish environment, showing a striking homogeneity under stress. On the contrary, in popM the variation for 'AFR' increased, resulting in a broader range of ages at first reproduction in fish environment. Overall our study with a total of 24 clonal lines revealed a broad spectrum of phenotypic variation in European *Daphnia galeata.*

Driving forces of phenotypic variation ('Effect Sizes')

Our analyses brought to light that the effect size of the fixed factor 'Treatment' was largest for 'AFR' and 'size' implying that the environment, here predation risk, influences the life history of its prey. In our study 13 out of 24 genotypes matured early (Figure C1-S2) and 17 of 24 genotypes reduced their body length (Figure C1-S7) in the presence of fish kairomones, which thus concur with previous findings. Indeed, early maturation and a reduced size of *Daphnia* in the presence of vertebrate predators have been reported before (Gliwicz & Boavida 1996; Lampert 1993; Machacek 1991; Weider & Pijanowska 1993). The ecological benefit lies in a successful reproduction before reaching a body size making the individual vulnerable to fish predation (Lampert 1993; Lynch 1980).

We observed that the random factor 'Genotype' was the main driver for the observed phenotypic variation of the two traits total number of 'offspring' and somatic growth rate

('SGR'). The phenotypic variation between clonal lines was best visualized by plotting the differences of somatic growth rate (dSGR) between the environments (Figure C1-5), unifying the environmental and clonal effect. All six clonal lines of popG and four out of six clonal lines of popJ decreased their somatic growth in fish environment, while the direction of response varies for popLC and popM. The main effect of 'Genotype' on the traits 'offspring' and 'SGR' implies that the presence or absence of certain clonal lines within one population might have an effect on overall population survival, depending on environmental factors such as predation risk. Hence, if the phenotypic diversity within one population is reduced and the majority produces relatively less offspring in a fish environment, the result could be an overall low number of offspring in the following cohorts, which would threaten the persistence of the whole population. Notably, individuals of popG produced less offspring and less broods compared to the other three populations regardless of the environment and their relative fitness was comparatively low. Potential explanations for this relative low performance of popG could be genetic drift and inbreeding depression which have a negative effect on genetic diversity (Vanoverbeke & De Meester 2010). However, low genetic variation for *D. galeata* in Greifensee was not identified (Herrmann *et al.* 2017), making these two explanations unlikely at first glance. Yet, Herrmann *et al.* (2017) showed that most clonal lines in Greifensee (four out of six) had a lower heterozygosity than expected, perhaps as result of inbreeding in this population. Therefore, inbreeding depression could explain lower fitness in popG and should be further investigated in a future study.

For three life history traits we found a statistically significant block effect. The difference between experimental rounds for somatic growth rate, total number of offspring in first brood ('brood1') and 'shape' could be attributed to the high clonal variation we observed in all life history traits. Since we did not find a significant 'Treatment' effect for 'brood1', we rule out that the block effect was connected to the presence of fish kairomones or differences of effectiveness of fish kairomones between rounds which we accounted for by providing same conditions (number and size of fish per l) in experimental rounds. For these reasons, we neglect the block effect although we are aware that we cannot completely rule out this constraint in our experimental design. It would be beneficial to change the strategy for follow up studies and prepare a single stock of kairomones solution to be used throughout experiments (see Von Elert & Stibor 2006 for details). Block

effects due to variation in kairomone concentration could be thus avoided. However, synchronizing many different clonal lines from various populations was the main limitation in our case, and is difficult to avoid.

To our surprise the effect size of the random factor 'Population' was overall non-existent to small on the predator-induced response, although we observed population differences, especially between the two extremes popG and popJ. The effect size of population was large for two traits only: total number of offspring ('offspring') and relative fitness among populations ('relclone'), while the latter was calculated based on the total number of offspring (Figure C1-4). The best explanation for the observed population difference could be the extreme difference of total number of offspring between popG and popJ. In general, clonal lines in popJ produced the highest number of offspring among all populations. In contrast, the total number of offspring of clonal lines in popG was overall lower compared to the other three populations, regardless of the environment. This implies that even the increased number of offspring for clonal lines of popG in fish environment is less than the numbers of offspring for clonal lines of popJ in control envrionment. Hence, the genotype origin ('Population') itself had little to no main effect on life history traits in *Daphnia* implying that the identity of a clonal line within population seems to be more important than the origin of the clonal line *per se*.

In the end, we were not able to identify one main driving force influencing the phenotypic variation in life history traits. Instead, our study displays the complexity of the interacting factors environment and genotype to produce a variety of phenotypes within one species, thereby contributing to the understanding of intraspecific phenotypic variation.

Potential for local adaptation to fish kairomones

Our findings allow the conclusion that there is potential for local adaptation to predation risk in the investigated European populations of *D. galeata*. This conclusion was based on three outcomes of our study. Firstly, an effect of the interaction of exposure to fish kairomones ('Treatment') and genotype origin ('Population') was found for many of the measured traits: age at first reproduction, total number of offspring, total number of offspring first brood, somatic growth rate, body length, and body shape. Furthermore, we

observed an extreme predator-induced life history response for popJ. The variation of the phenotypic response was reduced to a minimum in popJ, so that almost all individuals of the six genotypes and 15 replicates reproduce at the very same age when exposed to fish (Figure C1-2A). On top of that, we observed a similar reduction of variation for the life history trait total number of broods (Figure C1-2C). These strong responses could be explained by local adaptation to the presence of fish. The Jordan Reservoir is an artificial inner city water reservoir, used for recreational purposes such as fishing since 1900 (Kubecka & Bohm 1991) and had been regularly stocked with fish (Seda *et al.* 2000). Therefore, *D. galeata* of Jordan reservoir had the possibility to adapt to an environment with a higher predation risk for more than a century. Such microevolutionary changes for *Daphnia* species have been described in other contexts before. For instance, Jansen *et al*. (2011) showed that *D. magna* was able to evolve resistance to a pesticide (carbaryl) within experimental time. Further, Declerck *et al.* (2001) showed that populations of *D. galeata* were able to locally adapt to fish kairomones as well as Reger et al (2018) for *D. pulex*. Alternatively, since the reservoir, unlike the other lakes in this study, has been created specifically with fishing in mind, differential colonization might also be the source of the observed pattern. This habitat might have been colonized only by *Daphnia* pre-adapted to fish, with very specific life histories, leading to the present-day striking pattern. Finally, the relative fitness within and among populations of individuals of popJ suggests that females exposed to fish kairomones are fitter, concurring with results obtained by Castro *et al*. (2007) and Jansen et al. (2011). Since local adaptation to a certain stressor implies a better performance in the 'stress' environment than without this stressor (Joshi *et al.* 2001; Lenormand *et al.* 1999) we suggest that the local adaptive potential exists for at least three populations because the relative fitness in the presence of fish kairomones increased overall for 13 out of 24 clonal lines (popG=2, popJ=4, popLC=4, popM=3) (Table C1-2A and C1-2B). Our results are in line with earlier studies showing the adaptive potential of phenotypic plasticity in *Daphnia* exposed to different stressors (e.g., Altshuler *et al.* 2011; Hesse *et al.* 2012; Reger *et al.* 2018; Yin *et al.* 2011).

Predation risk and morphological changes

In general, we did not observe any predator-induced extreme morphological changes such as the formation of helmets for fish kairomone exposed *Daphnia* as those reported for *D. lumholtzi* (Laforsch & Tollrian 2004a). We presented here the first study using the geometric morphometric analysis, hence complementing the traditional approaches (life history traits and behavior) by measuring morphometric changes to an environmental factor in an intraspecific context in *D. galeata.* Our morphometric analysis revealed that the presence of fish kairomones had an effect on the body shape of *Daphnia*. However, no overall pattern was recognizable among the populations and no effect was observed at all for popG. Instead we observed different changes of 'shape' in each population. We suggest that the morphological trait 'shape' is phenotypically plastic due to high clonal variation, which is consistent with the results reported by Dlouhá *et al.* (2010) and Zuykova *et al* (2012).

We hypothesized that life history change and morphological change are correlated, meaning that females with a higher number of offspring (n>22, upper quartile of observed total number of offspring) would change their 'shape' towards a bulkier body form to accommodate a greater number of offspring within their brood pouch. This correlation was found only for individuals in control environment and not for individuals in fish environment. Changing the 'shape' of the body might come along with some drawbacks: the bulkier the 'shape', the higher the detection risk by the predator and the slower the swimming ability due to drag. In fact, fish prey size-selectively on *Daphnia* meaning that larger *Daphnia* are preyed upon more often than smaller *Daphnia* (e.g., Beckerman *et al.* 2010; Weber & Van Noordwijk 2002). Since fish prey on faster swimming individuals of *Daphnia* (O'Keefe *et al.* 1998), being a slow swimming *Daphnia* would be beneficial. Alternatively, accommodating more offspring without changing the 'shape' of the body might be achieved through the production of smaller offspring (Castro *et al.* 2007; Lampert 1993). In line with previous studies showing a predator-induced reduction in neonate size, we can speculate that this is also the case in our experiment and plan to further explore this dimension.

Conclusion

The study presented here focused on the intraspecific phenotypic variation among and within populations. By comparing the range of phenotypic response of four populations with six clonal lines per population, we contribute to the understanding of the effect of environmental change on intraspecific phenotypic variation at the population level. We observed high clonal variation in all studied life history traits and identified high inter-clonal variation, leading to the suggestion that single genotype studies on *Daphnia* might deliver biased conclusions.

Acknowledgments

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Chapter 2

Gene co-expression in *Daphnia galeata* exposed to fish kairomones

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Abstract

Organisms live in a dynamic and often challenging world. Phenotypic plastic responses allow organisms to rapidly adjust to new environmental conditions. Although phenotypic plastic responses to predation risk are 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* exposed to fish kairomones. First, we investigated the differential gene expression identifying candidate transcripts involved in shifts of life history traits in fish kairomone exposed *D. galeata* identifying a total of 125 differentially expressed transcripts (40 up- and 85 downregulated). Gene expression analysis revealed a surprisingly high variance between clonal lines reflecting their different life history strategies in response to fish kairomones. Second, we applied a gene co-expression network analysis to find clusters of tightly-linked transcripts and characterize their function to 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 to previous studies on other *Daphnia* species to assess similarities in the stress responses and *Daphnia* reproduction. Orthologs of *D. pulex* related to reproduction were found in D. *galeata.* We also found *D. galeata* orthologs related to predator-induced responses in *D. magna*. The unique combination of methods and comparative approach allowed the identification transcript sets of interest involved in predator-induced responses and reproduction in *Daphnia*.

Introduction

Organisms are challenged throughout their lives by a range of environmental stressors 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 might be advantageous in one environment but might become disadvantageous in another. In general, organisms have two possibilities to cope with stress: 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 phenotypic level describing phenotypic plastic responses (reversible), while the latter applies to the genotype level when a successful phenotype passes on its' abilities coded in their alleles to the next generation (irreversible).

Predation is an important biotic factor structuring whole communities of organisms (e.g., Aldana *et al.* 2016; Boaden & Kingsford 2015), maintaining species diversity (e.g., Estes *et al.* 2011; Fine 2015) and driving natural selection in populations (e.g., Kuchta & Svensson 2014; Morgans & Ord 2013). Aquatic predators, vertebrate as well as invertebrate, release kairomones into the surrounding water (Machacek 1991; Schoeppner & Relyea 2009; Stibor 1992; Stibor & 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 often phenotypic plasticly and are reported in detail for different *Daphnia* species (Boeing *et al.* 2006; Boersma *et al.* 1998; Brett 1992; Duffy 2010; Effertz & von Elert 2015; Herzog *et al.* 2016; Jansen *et al.* 2011; Laforsch & Tollrian 2004b; Lampert 1993; Lüning 1992; Machacek 1991; Rabus *et al.* 2013; Reede & Ringelberg 1998; Sakwinska 2002; Stibor & Lüning 1994; Tollrian 1995; Weber 2003; Weider & Pijanowska 1993; Yin *et al.* 2011).

Daphnids, are small branchiopod crustaceans that 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). Shifts of behavior, morphology or life history were observed in response to predation and predation risk at different components of phenotypes. Induced responses 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) as well as the formation of neck teeth in *D. pulex* (e.g., Tollrian 1995). Vertebrate predators induced behavioral changes linked to the diel vertical migration (DVM), an avoidance strategy (Cousyn *et al.* 2001; Effertz & von Elert 2015) as well as changes in life history traits (Boersma *et al.* 1998; Effertz & von Elert 2015) in *D. magna.* The specificity of predator-induced responses by vertebrate and invertebrate kairomones had been shown e.g. for a *Daphnia* species complex (*D. galeata*/*hyalina*/*cucullata*) from the Swiss lake Greifensee (Wolinska *et al.* 2007). The documented changes of life history traits included a decrease of 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 kariomones do not show a diel vertical migration behavior (Spaak & Boersma 2001; Stich & Lampert 1981), nor do they produce morphological changes like helmets or neck teeth (**Chapter 1**). The effect of long-term (14 days) exposure to fish kairomones in *D. galeata* life history traits revealed substantial variation within and among populations, as well as trends congruent to previous studies such as a decrease in both age at first reproduction ('AFR') and somatic growth rate ('SGR') in the presence of fish kairomones (**Chapter 1**) (e.g., Boersma *et al.* 1998; Stibor & Lüning 1994).

Stress responses have been investigated in different contexts using gene expression approaches. Combined approaches are necessary to understand the complexity of stress responses such as predator-induced responses. Today gene expression profiling as well as the gene co-expression analysis is 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). The benefit of the co-expression analysis lies in the modular structure of the co-expressed genes and their functional relationships (Bergmann *et al.* 2004). A gene co-expression network consists of several modules, in which co-expressed genes are clustered (Langfelder & Horvath 2008). 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 attempt to link the predator-induced response to the underlying gene expression pattern has rarely been addressed. Short-term exposure to fish kairomones (several hours) in *D. magna* revealed no gene expression response (Orsini *et al.* 2017). Other transcriptomic approaches linked stress responses in daphnids to environmental stressors, such as food quality and anthropogenic stressors in *D. pulex* revealing 258 transcripts to be involved in *Daphnia* reproduction (Asselman *et al.* 2017). In response to invertebrate predation risk, 230 differentially expressed genes were identified in *D. pulex* of which the most prominent classes of upregulated genes included cuticle genes, zink-metalloproteinases and vitellogenin genes (Rozenberg *et al.* 2015). In response to vertebrate predation risk, ~50 responsive genes involved in reproduction, digestion and exoskeleton structure were revealed in *D. ambigua* as a transgenerational effect (Hales *et al.* 2017).

Predator-induced responses vary in *Daphnia* phenotypes across species, yet it is unknown if the underlying gene expression to these responses is conserved across species. A comparative transcriptomic approach could reveal common transcripts involved in stress responses across *Daphnia* species. Therefore, we compare our results of a long-term exposure to fish kairomones in *D. galeata* to the results the short-term exposure to fish kairomones in *D. magna* (Orsini *et al.* 2017) and to the predicted reproduction-related transcripts after the long-term exposure to cyanobacteria, insecticides and their combination in *D. pulex* (Asselman *et al.* 2017)

Our study goal is to unravel the underlying genetic basis of a predator-induced response in the freshwater grazer *Daphnia 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 fish kairomone exposure and life history traits in *D. galeata*?, (iii) which GO terms are enriched in transcript sets of interest? Since most of the predator-induced responses described earlier related to *Daphnia* reproduction and growth including morphological changes, we expect to identify transcripts with Gene Ontology (GO) annotations linked to either reproduction, growth and/or kairomone perception. Here, we lay a valuable cornerstone for 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 lines originally sampled in the Müggelsee (northeast Germany) which differ in their life history responses in the presence of fish kairomones (**Chapter 1**). A large phenotypic experiment involving 24 clonal lines from four different lakes revealed that within the Müggelsee population the variation for some life history traits increased when these clonal lines where exposed to fish kairomones. An increase in life history variation means that a broader range of phenotypes are displayed for that life history trait. We chose to use the clonal line 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 C2-S1).

Media preparation

The basic medium was ADaM (Klüttgen *et al.* 1994) for fish and *Daphnia* cultures. The two types of media, fish kairomone (FK) and control, were used for breeding and experimental conditions and their preparation is detailed in **Chapter 1**. Fish kairomone medium was obtained by maintaining 5 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 supplemented with 1.0 mg C L-1, P rich *Acutodesmus obliquus*. Media were 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, Typ 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 (**Chapter 1**), 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 eventual mortality. Before the release of the first brood, at day 6, one of the individuals was randomly discarded if necessary so that 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 (+ 1 day) since 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 treatment and per clonal line resulting in a total of 400 individuals (two clonal lines x two treatments x 20 individuals x 5 biological replicates). The experiment lasted for 14 days for each experimental individual to measure the long-term effect of fish kairomones on gene expression level in *D. galeata*.

Data collection and analysis

RNA isolation and preparation

Appropriate amounts of RNA were not available from single individuals 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.* 2017;

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, an acid-guanidinium-phenol reagent, (Bio Budget technologies, Krefeld, Germany) 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 cleanly. The upper aqueous phase was transferred into a clean microcentrifuge and the RNA precipitated with absolute ethanol. RNA purification and DNAse treatment were done 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 treatments 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 platform was used for paired-end library sequencing with 101bp 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). This workflow was also applied to the 12 *D. magna* datasets published in Orsini *et al*. (2016), which exposed two *Daphnia magna* clonal lines to a variety of environmental stressors including fish kairomones. RNA-seq data and reference *D. magn*a transcriptome were available from the International Nucleotide Sequence Database Collaboration BioProject PRJNA284518 (http://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA284518).

Differential gene expression analysis

Differential gene expression analysis was performed in the R environment v.3.4.2 (R Core Team 2018) 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 treatment compared to the control group 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 & 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 treatments, between clonal lines and between treatments 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 2010). The web tool jvenn (Bardou *et al.* 2014) was used to visualize numbers of shared transcripts between groups. The same workflow was applied to the *D. magna* dataset (Orsini *et al.* 2016).

Gene co-expression network analysis

The terminology of weighted gene co-expression network analysis has been described previously (Langfelder & Horvath 2008). Variance-stabilized read counts obtained from the previous DESeq2 analysis were used in this procedure. Subsequent analysis was performed in the R environment v.3.4.2 (R Core Team 2018). First, an automatic, signed weighted, single gene co-expression network construction was performed on a workstation with the R environment v.3.2.3 with the R package '*WGCNA*' v.1.61 (Langfelder & Horvath 2008). Second, gene co-expression modules were identified using the Topological Overlap Matrices (TOM) with a soft cut-off threshold 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 a resampling procedure outlined below. Finally, the most interconnected genes per module, so-called 'hub-genes' were identified. We applied a gene co-expression network analysis on the *D. magna* dataset as well (Orsini *et al.* 2016).

Module eigengene – trait correlation

Modules were related to external trait information that originated from a previous life history study of *D. galeata* (**Chapter 1**) in which several 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. 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 artefact, 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. For example, we had trait values for 15 individuals for the trait 'broods' in the clonal line M6 exposed to fish kairomones (Table C2-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 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 correlations. 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 occurring in more than 95% of the iterations. Further details can be found in the supplementary material (R script: Tams-et-al_Resampling_DaphniaFK.Rmd). No life history trait data existed for *D. magna*, thus no ME-trait correlation was performed.

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 & Rahnenführer 2016). The default algorithm 'weight01' was used taking the hierarchy of GO terms into account which results in fewer false positive results (Alexa & 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 GO domains, 'Molecular Function' (MF), 'Biological Process' (BP) and 'Cellular Compounds' (CC) with a p-value < 0.05 were considered significant. The same workflow was applied to the *D. magna* dataset (Orsini *et al.* 2016).

A priori, a list of expected GO terms was created by using the AMIGO database (Carbon *et al.* 2009). We searched for 'annotations' and used following terms to extract GO classes with direct annotations, 'eukaryota', 'metazoa' and '*Drosophila melanogaster*'. We expected changes of genes related to growth, reproduction and kairomone perception. Search terms were cell death, cell growth, chitin and molting; hatching, metabolism, reproduction, vitellogenesis, vitellogenin and yolk as well as external stimulus and sensory perception. Since our data mining approach does not focus on the direction of gene expression changes we excluded GO classes containing positive and negative regulation of terms 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 C2-S2).

Comparative transcriptomics

Orthologous clusters were obtained from Huylmans *et al.* (2016) who applied OrthoMCL to cluster amino acid sequences of *D. galeata*, *D. pulex*, *D. magna*, *Drosophila melanogaster* and *Nasonia vitripennis.* With these orthologous clusters we were able to make an interspecies comparison of transcripts of *D. magna* (Orsini *et al.* 2016), *D. pulex* (Asselman *et al.* 2017) and our *D. galeata*. A custom python script was used to annotate orthologous cluster to the lists of transcripts before extracting orthogroups (supplementary script: OMCLFinal.py). To compare the interspecies response to short-term vs. long-term predation risk we used the orthogroups to identify overlaps between the gene co-expression modules with the highest negative and positive correlation to fish kairomones for *D. galeata* and *D. magna* as well as between the 'hub-genes' for each module. Despite differing exposure durations between the experiments, we expected to find transcripts involved in the response to fish kairomones in both species. To identify common reproduction-related transcripts in *Daphnia* species exposed to environmental stressors we compared reproduction-related transcripts from our gene co-expression network analysis with the gene list of reproduction-related transcripts of *D. pulex* (Asselman *et al.* 2017). Although stressors and exposure durations varied between the experiments, we expect to find transcripts involved in reproduction in both species.

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 101bp 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 dataset.

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 clonal line M6 and 21,813 for clonal line M9.

A principal component analysis (PCA) was performed visualizing the grouping of read counts to 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 treatment (Figure C2-1). PC 2 explained just 10% of the variance, which seems related to variance between replicates. To improve the visualization of replicate and treatment differences, separate plots per clonal line were produced (not shown) resulting in no visible treatment effect.

The differential expression analysis revealed that there were no differentially expressed transcripts (DETs) between treatment groups, but a total of 5283 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 C2-1A). Within clonal line M6 there were 30 DETs between treatments of which 27 were down-regulated (90%) and 3 were up-regulated (10%). For clonal line M9 57 DETs were found between treatments 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 interaction (GxE) a two-factor analysis was applied (Table C2-1B). Between treatments, clonal line M6 had four DETs (up: 1 (25%); down: 3 (75%)), while clonal line M9 had 68 DETs (up: 29 (43%); down: 39 (57%)). The GxE resulted in 22 DETs (up: 7 (32%); down: 15 (68%)).

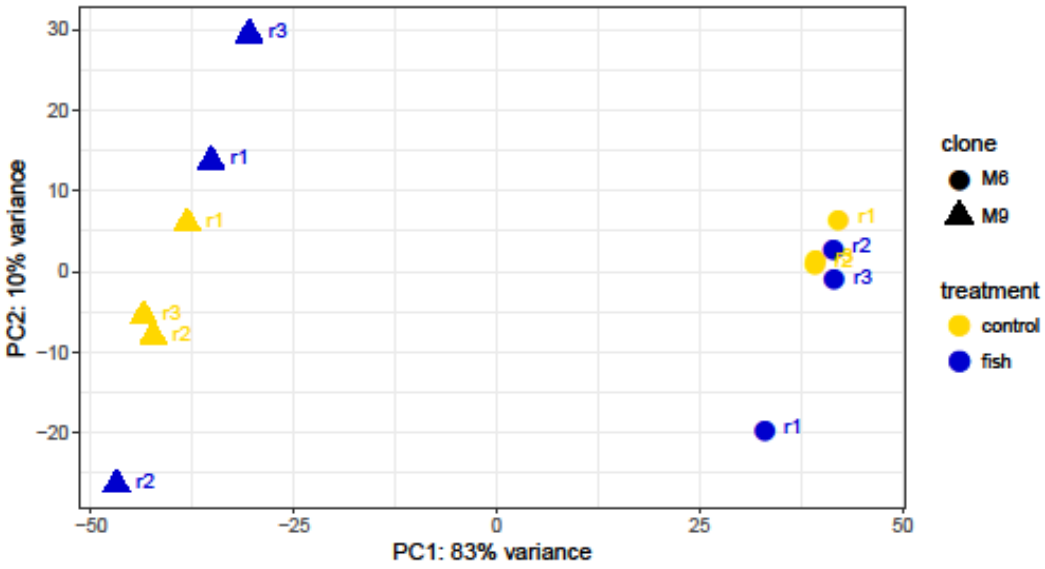

Figure C2-1: Principal component (PC) plot of the biological RNA-seq samples in *D. galeata*. Yellow: control environment. Blue: fish environment. Triangles: clonal line M9. Circles: clonal line M6.

There were no shared DETs between the two clonal lines (genotype) in regard to fish kairomone exposure (environment) and only a small number of DETs were shared within one clonal line for the one and two factor analysis (Figure C2-2). In total 125 DETs related to fish environment (hereafter, 'FK') of which 40 were up- and 85 were downregulated (Figure C2-2, Table C2-S3). The expression of most of the FK-related DETs (~50%) was strong (fold change >2) (Table C2-1).

No differentially expressed transcripts were found for the *D. magna* dataset. Further corresponding results for *D. magna* can be found in the supplementary material section (Table C2-S4, Figure C2-S1).

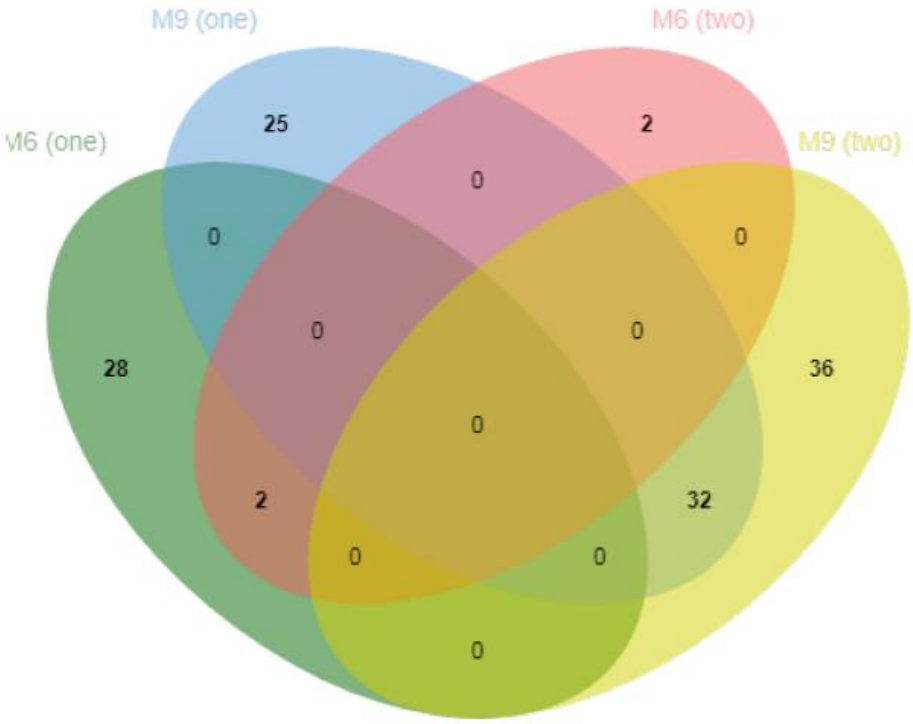

Figure C2-2: Venn diagram of the 125 differentially expressed transcripts (DETs) related to fish kairomone exposure (FK) in *D. galeata*. The set of FK-related DETs originates from the one and two factor analysis. 'M6 (one)' = DETs from the one factor analysis for the clonal line M6. 'M9 (one)'= DETs 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.

Table C2-1: 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 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

Gene co-expression network analysis

The single network analysis revealed that the expressed transcripts clustered into 16 co-expression modules (CEM) (Figure C2-3, Table C2-2). 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=1525) of all transcripts. For each module the hub-gene, or the most highly interconnected gene within a gene co-expression module, was identified. An overview of the modules, transcript numbers and hub-genes is provided in Table C2-2.

A total of five modules were significantly ($p \leq 0.05$) associated 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 (Table C2-2). 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 in each clonal line. 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 were identified for the FK-related DETs (Table C2-2), namely 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 *D. magna* dataset including one module 'royalblue' being positively correlated to fish kairomone exposure. Details of results can be found in the supplementary material section (Table C2-S5, Figure C2-S2).

Table C2-2: 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 clone (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 transcript	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		

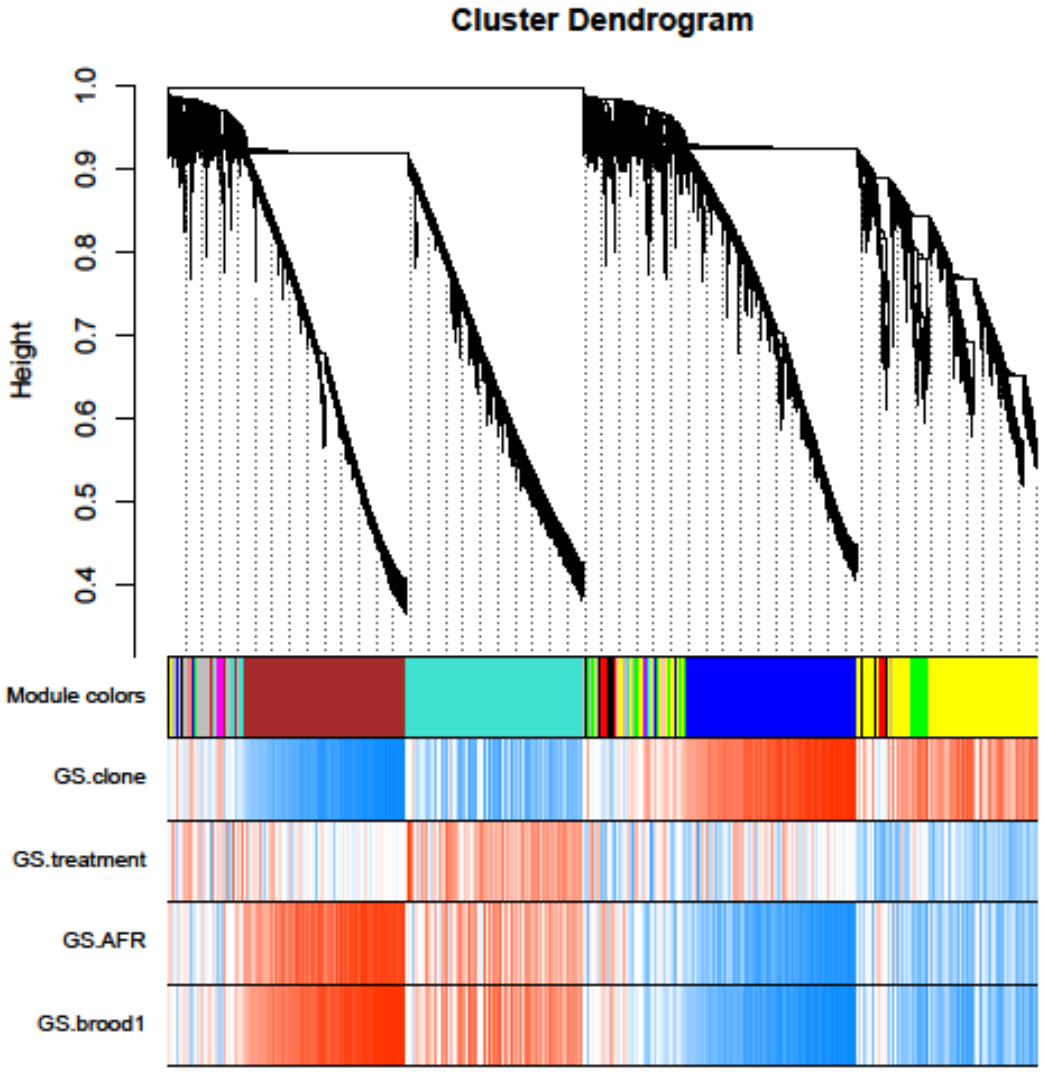

Figure C2-3: Cluster dendrogram of transcripts in *D. galeata*, with dissimilarity based on the topological overlap matrices (TOM). Additional assignments are module colors, the gene significances (GS) for the trait clone (clonal line), treatment (fish kairomone exposure), age at first reproduction ('AFR') 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.

Annotation and gene set enrichment analysis (GSEA)

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 our analysis had a GO annotation and thus constituted our 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 C2-4).

28% of transcripts deriving from the co-expression modules of interest were annotated ('blue-brown' n= 2,681; '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 C2-2).

Although not all modules were correlated to either life history traits or fish environment (FK), it is of interest that significantly enriched GO terms were detected for three hub-genes. The hub-gene of the 'green' module was involved in 'structural constituent of cuticle' and related to fish kairomone exposure. The hub-gene of the 'black' module was involved in 'protein binding' and related to reproduction. The hub-gene of the 'greenyellow' module had a GO term related to fish environment and reproduction, 'calcium ion binding'.

In total we found 29 unique GO terms to be significantly enriched for all three categories in the FK transcript set (hereafter, “uniqueGO_FK”, Table C2-3A) and 47 in the reproduction transcript set (hereafter, “uniqueGO_re”, Table C2-3B). A total of 2,465 transcripts (~30%) had at least one observed FK-related GO term, while 3,263 transcripts (~40%) had at least one observed reproduction-related GO term. A total of 12 significantly enriched GO terms related to fish kairomone exposure were found for FK-related DETs as well as 15 significantly enriched GO terms related to reproduction.

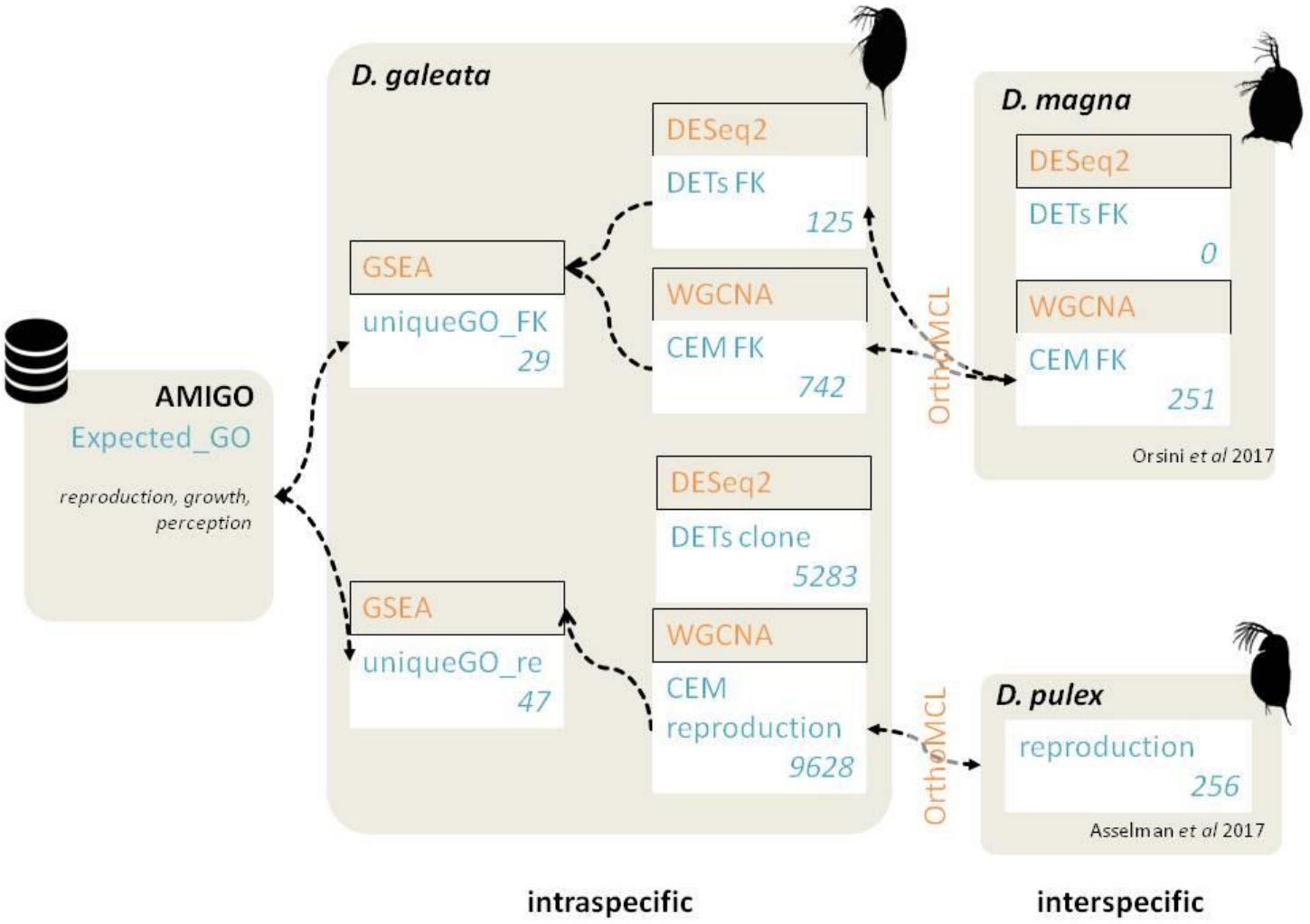

Figure C2-4: Overview of datasets created by gene expression and gene co-expression analysis and used for comparative transcriptomics. 'DESeq2' = gene expression analysis. 'W'GCNA' = gene co-expression analysis. 'GSEA' = gene set enrichment analysis. 'OrthoMCL' = identification of orthologous clusters. 'DETs' = differentially expressed transcripts. 'CEM' = co-expression module. 'expected_GO' = expected GO terms (Table C2-S2). 'uniqueGO_FK' = significantly enriched GO terms of FK-related transcripts (Table C2-3A). 'uniqueGO_re = significantly enriched GO terms of reproduction-related transcripts (Table C2-3B).

We expected to find Gene Ontology (GO) terms related to reproduction, growth and kairomone perception to be overrepresented in the gene set enrichment analysis. There was only a small overlap between “expected_GO” terms (Table C2-S2), “uniqueGO_FK” (Table C2-3A) as well as “uniqueGO_re” (Table C2-3B). A total of five expected GO terms were identified (Figure C2-5): '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'.

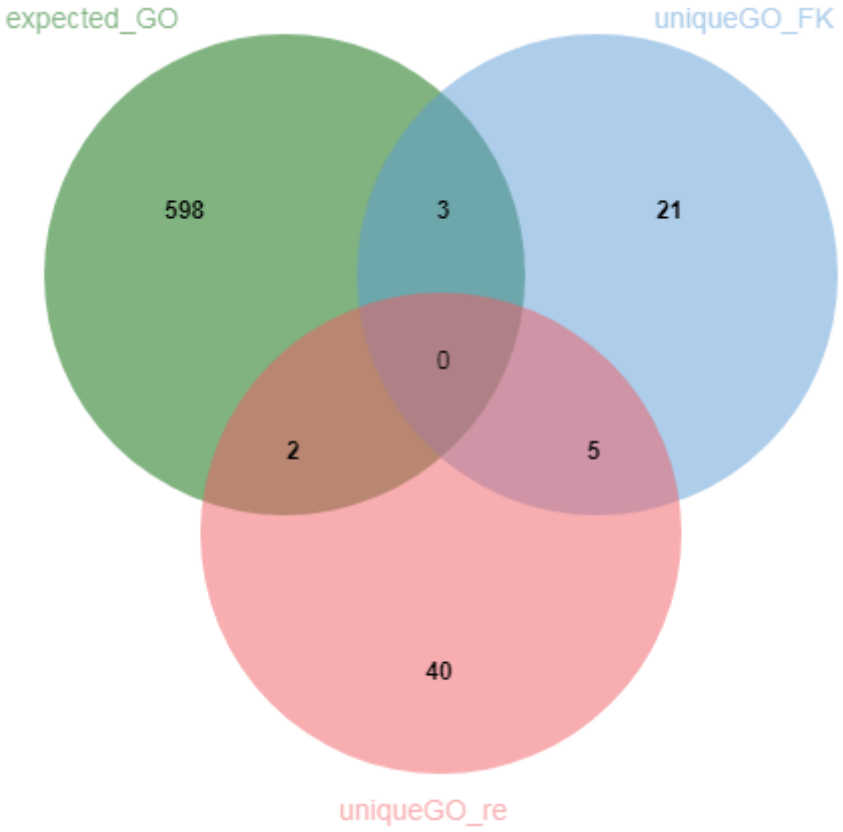

Figure C2-5: Venn diagram of Gene Ontology (GO) classes of *D. galeata* datasets. 'expected_GO' = classes derived from AMIGO database (Table C2-S2). 'uniqueGO_FK' = significantly enriched GO class related to fish environment (Table C2-3A). 'uniqueGO_re' = significantly enriched GO class related to reproduction (Table C2-3B).

Table C2-3: List of 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	Transcript set	category
GO:0003824	catalytic activity	4470	71	60.11	0.025	red	MF
GO:0003951	NAD+ kinase activity	2	1	0.03	0.0267	red	MF
GO:0004181	metallocarboxypeptidase activity	47	1	0.04	0.035	M6	MF
GO:0004252	serine-type endopeptidase activity	518	9	1.78	0.000043	salmon	MF
GO:0004252	serine-type endopeptidase activity	518	7	1.67	0.0011	M9	MF
GO:0004867	serine-type endopeptidase inhibitor acti...	21	2	0.28	0.0319	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:0005201	extracellular matrix structural constitu...	100	3	0.32	0.004	M9	MF
GO:0005509	calcium ion binding	127	5	1.71	0.0281	red	MF
GO:0005581	collagen trimer	100	3	0.13	0.00015	M9	CC
GO:0005622	intracellular	1118	12	16.21	0.016	red	CC
GO:0006508	proteolysis	929	9	3.76	0.008	salmon	BP
GO:0006508	proteolysis	929	9	3.16	0.002	M9	BP
GO:0006741	NADP biosynthetic process	2	1	0.03	0.027	red	BP
GO:0006801	superoxide metabolic process	13	1	0.04	0.035	tan	BP
GO:0006850	mitochondrial pyruvate transmembrane tra...	2	1	0.03	0.027	red	BP
GO:0007156	homophilic cell adhesion via plasma memb...	22	3	0.3	0.0031	red	BP
GO:0007186	G-protein coupled receptor signaling pat...	250	4	0.69	0.024	tan	BP
GO:0007218	neuropeptide signaling pathway	4	1	0.01	0.011	tan	BP
GO:0008083	growth factor activity	17	2	0.23	0.0214	red	MF
GO:0008083	growth factor activity	17	1	0.04	0.04	tan	MF
GO:0008234	cysteine-type peptidase activity	147	2	0.35	0.047	tan	MF
GO:0008762	UDP-N-acetylmuramate dehydrogenase activ...	5	1	0.02	0.017	salmon	MF
GO:0016614	oxidoreductase activity, acting on CH-OH...	68	4	0.91	0.0131	red	MF
GO:0016788	hydrolase activity, acting on ester bond...	219	3	0.75	0.039	salmon	MF
GO:0016887	ATPase activity	129	3	0.44	0.03	salmon	MF
GO:0016972	thiol oxidase activity	2	1	0.03	0.0267	red	MF
GO:0030131	clathrin adaptor complex	10	1	0.02	0.02	salmon	CC

GO:0042302	structural constituent of cuticle	209	4	0.16	0.0000082	M6	MF
GO:0042302	structural constituent of cuticle	209	6	0.67	0.000045	M9	MF
GO:0050660	flavin adenine dinucleotide binding	58	4	0.78	0.0076	red	MF
GO:0055114	oxidation-reduction process	427	11	5.81	0.0289	red	BP
GO:0080019	fatty-acyl-CoA reductase (alcohol-formin...	13	2	0.17	0.0127	red	MF

B							
GO.ID	**Term**	**Annotated**	**Significant**	**Expected**	**classicFisher**	**Transcript set**	**category**
GO:0000062	fatty-acyl-CoA binding	10	4	1.35	0.03531	blue	MF
GO:0001522	pseudouridine synthesis	39	9	4.84	0.04564	brown	BP
GO:0004013	adenosylhomocysteinase activity	3	2	0.4	0.04966	blue	MF
GO:0004252	serine-type endopeptidase activity	518	93	69.88	0.0019	blue	MF
GO:0004252	serine-type endopeptidase activity	518	101	64.59	0.0000018	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:0004517	nitric-oxide synthase activity	3	2	0.37	0.04274	brown	MF
GO:0004553	hydrolase activity, hydrolyzing O-glycos...	317	73	42.77	0.0000036	blue	MF
GO:0004672	protein kinase activity	763	123	102.94	0.00681	blue	MF
GO:0004672	protein kinase activity	763	121	95.14	0.00266	brown	MF
GO:0004677	DNA-dependent protein kinase activity	2	2	0.25	0.01554	brown	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:0005201	extracellular matrix structural constitu...	100	23	13.49	0.00639	blue	MF
GO:0005328	neurotransmitter:sodium symporter activi...	19	6	2.37	0.02393	brown	MF
GO:0005506	iron ion binding	164	40	22.13	0.0006	blue	MF
GO:0005506	iron ion binding	164	28	20.45	0.04395	brown	MF
GO:0005515	protein binding	2135	292	266.21	0.00539	brown	MF
GO:0005524	ATP binding	1009	147	125.81	0.01983	brown	MF
GO:0005581	collagen trimer	100	23	12.07	0.0014	blue	CC
GO:0005975	carbohydrate metabolic process	385	77	52.42	0.000023	blue	BP
GO:0006303	double-strand break repair via nonhomolo...	5	3	0.62	0.01571	brown	BP
GO:0006468	protein phosphorylation	759	121	103.34	0.02786	blue	BP
GO:0006468	protein phosphorylation	759	120	94.25	0.00239	brown	BP

GO:00064 86	protein glycosylation	192	36	26.14	0.02624	blue	BP
GO.ID	**Term**	**Annotated**	**Significant**	**Expected**	**classicFisher**	**Transcript set**	**category**
GO:00065 08	proteolysis	929	145	126.48	0.00882	blue	BP
GO:00065 08	proteolysis	929	148	115.35	0.00012	brown	BP
GO:00068 09	nitric oxide biosynthetic process	3	2	0.37	0.04239	brown	BP
GO:00068 12	cation transport	121	18	16.47	0.02918	blue	BP
GO:00068 36	neurotransmitter transport	19	6	2.36	0.02342	brown	BP
GO:00080 13	beta-catenin binding	2	2	0.27	0.01819	blue	MF
GO:00081 99	ferric iron binding	9	4	1.21	0.02367	blue	MF
GO:00082 72	sulfate transport	11	5	1.5	0.0105	blue	BP
GO:00084 17	fucosyltransferase activity	125	28	16.86	0.00418	blue	MF
GO:00099 82	pseudouridine synthase activity	37	9	4.61	0.03447	brown	MF
GO:00150 74	DNA integration	56	18	7.62	0.00028	blue	BP
GO:00150 74	DNA integration	56	15	6.95	0.00267	brown	BP
GO:00151 16	sulfate transmembrane transporter activi...	11	5	1.48	0.01012	blue	MF
GO:00152 99	solute:proton antiporter activity	10	5	1.35	0.0062	blue	MF
GO:00159 30	glutamate synthase activity	3	2	0.4	0.04966	blue	MF
GO:00160 20	membrane	1595	237	192.58	0.0000009 3	blue	CC
GO:00160 21	integral component of membrane	748	111	90.32	0.0061	blue	CC
GO:00161 92	vesicle-mediated transport	67	16	8.32	0.03683	brown	BP
GO:00165 67	protein ubiquitination	19	8	2.36	0.00117	brown	BP
GO:00167 05	oxidoreductase activity, acting on paire...	143	34	19.29	0.00016	blue	MF
GO:00168 05	dipeptidase activity	8	4	1.08	0.0147	blue	MF
GO:00200 37	heme binding	149	33	20.1	0.0024	blue	MF
GO:00200 37	heme binding	149	27	18.58	0.02823	brown	MF
GO:00301 26	COPI vesicle coat	3	2	0.31	0.03	brown	CC
GO:00301 51	molybdenum ion binding	3	2	0.37	0.04274	brown	MF
GO:00332 27	dsRNA transport	4	3	0.54	0.00904	blue	BP
GO:00510 33	RNA transmembrane transporter activity	4	3	0.54	0.00881	blue	MF
GO:00551 14	oxidation-reduction process	427	73	58.14	0.01863	blue	BP

Comparative transcriptomics

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

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 clusteres comprised at least one transcript for each of the three species (Huylmans *et al.* 2016). No orthogroups were found between the 'salmon' *D. galeata* module and the 'royalblue' *D. magna* module. However, 34 orthogroups were identified between the 'royalblue' *D. magna* module and the negatively correlated 'red' *D. galeata* module.

Interspecies comparison of reproduction-related stress response in Daphnia

The **intra**specific comparison of orthogroups within *D. galeata* revealed 445 orthogroups that contain transcripts of the co-expression modules 'blue' and 'brown' related to reproduction. The **inter**specific comparison of orthogroups between *D. pulex* and *D. galeata* resulted in 42 orthogroups related to reproduction (Figure C2-6). Within these 42 orthogroups, 221 *D. galeata* transcripts exist of which 140 were annotated and 300 *D. pulex* transcripts of which 50 belonged to the 258 predicted reproduction-related transcripts of (Asselman *et al.* 2017). In general, annotated *D. galeata* transcripts were identified in 28 orthogroups and their GO terms (n=50) were extracted (Table C2-S6).

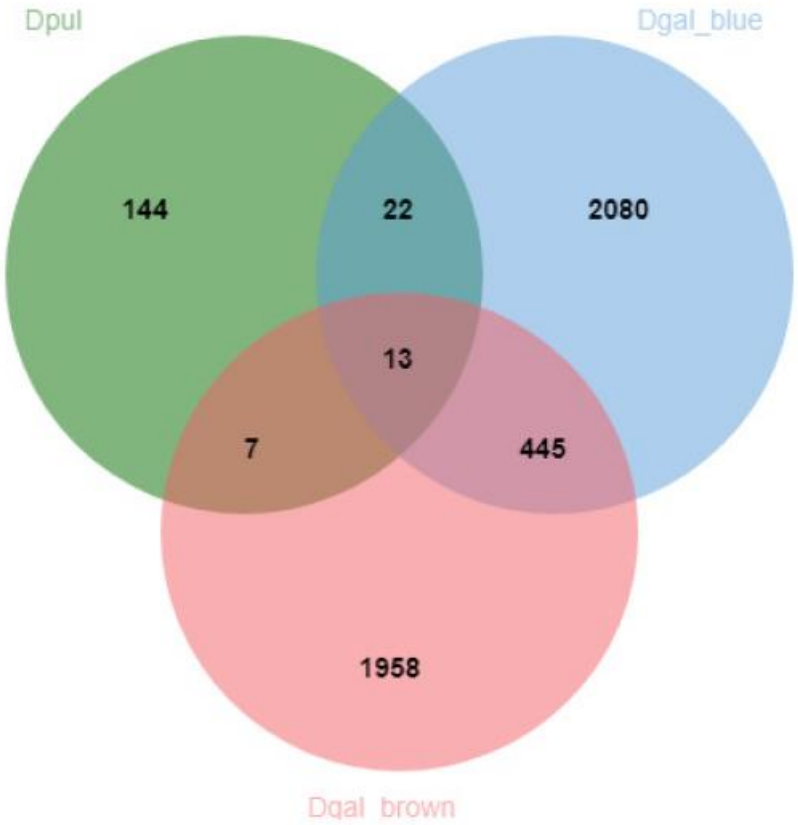

Figure C2-6: Venn diagram of orthologous clusters comprising reproduction-related transcripts. 'Dpul' = orthologous clusters for *D. pulex* (Asselman et al 2017). 'Dgal_blue'& 'Dgal_brown' = orthologous clusters for *D. galeata* transcripts of the co-expression modules blue and brown.

Discussion

Predator-induced responses in *Daphnia* have long been studied but, few studies so far have addressed the link between the ecological traits and the underlying genetic pathways. To gain insight into the genetic basis of predator-induced responses, gene expression profiling was performed 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 their potential functions of previously unknown biological pathways. The interspecies comparison revealed common transcripts involved in reproduction and the stress response of *Daphnia*.

Interclonal variance

Regardless of the experimental setup, it is important to account for variance between clonal lines. Predator-induced responses vary, e.g. *D. pulex* clonal lines display different

numbers of neck-teeth to *Chaoborus* kairomones or even none at all (Lüning 1995) and *D. galeata* clonal lines exhibit different life history strategies in response to fish kairomones even in the same population (**Chapter 1**). Stress responses at the organism level vary between genotypes, often known as phenotypic plasticity (West-Eberhard 1989). This phenotypic plasticity is associated, among others, to variation in gene expression as a product of the genotype-environment interaction (GxE) (Hodgins-Davis & Townsend 2009). 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 one population.

Since clonal lines were chosen for their antithetical life history response to fish kairomones, some interclonal variation was expected to occur. A previous study identified 5,492 transcripts differentially expressed between the *D. galeata* clonal lines M10 (from the same location as clonal lines M6 and M9) and clonal line J2 from Jordán Reservoir in the Czech Republic (Huylmans *et al.* 2016). This rather large difference was attributed to the geographic distance between the two locations. Surprisingly, the clonal lines of this study, which originated from the same population, had a similar amount of DETs (5,283 DETs). 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. The within-population diversity of *Daphnia* can vary across the species distributional range (Walser & Haag 2012), which makes it challenging to compare differential expression between genotypes (clonal lines) across species and populations.

The observed clonal variance does not seem to be directly correlated with distance or differences between habitats. The variance between clonal lines from the Müggelsee in our study was approximately the same as from clonal lines from Müggelsee and Jordán Reservoir (~400 km apart) (Huylmans *et al.* 2016). For the two *D. magna* clonal lines, Xinb3 and Inb1, 2,929 transcripts were differentially expressed. The clonal lines were collected from two habitats in Southern Germany and Southwest Finland, representing the species distributional range (Orsini *et al.* 2016). Even though they derived from a temporary rock

pool system connected to the Baltic Sea and a fish-rearing pond more than 2000 km apart, the *D. magna* clonal lines showed the least variance. Alltogether, we hypothesize that geographical distance does not necessarily play a role for intraspecific clonal variation in life history traits as well as gene expression.

The variance between clonal lines in *D. galeata* was also reflected in the gene co-expression network analysis. A 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 different reproduction strategies of each clonal line (**Chapter 1**). 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 being exposed to fish kairomones. This is not surprising since the differential expression analysis of *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 if the *D. magna* gene co-expression network correlates to life history traits or reproduction strategies.

Effect of fish kairomones on gene expression

In contrast to the large differences between clonal lines M6 and M9, 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 some overlap of 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 treatments 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 the two *D. galeata* clonal lineages. In comparison, Hales *et al.* (2017) observed 48 significantly differentially expressed genes after one generation of fish kairomone exposure but found 223 and 170 genes differentially expressed in the second and third generation, respectively, without any predator kairomones. The clonal line used in this study showed strong transgenerational plasticity in which phenotypic defenses persist for multiple generations. It is unknown whether *D. galeata* clonal lines display this effect and pass on epigenetic modifications after the 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 with decreasing body size, reducing 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 with a bidirectional change. *Daphnia* have the ability to rapidly adapt to local predator regimes (e.g., Declerk & Weber 2003). The different life history strategies of the clonal lines could be a result of clonal liones 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 predator-induced response

The 'salmon' module 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* the exposure to predator kairomones for one generation also leads 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). Therefore, we hypothesized 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' were 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 found 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 treatments showed enrichment of genes related to body remodeling and activation of cuticle proteins (Orsini *et al.* 2017). No pronounced morphological defenses are described for the *D. galeata* clonal lines but they displayed changes in body size and symmetry especially with regard to head shape (**Chapter 1**). Furthermore, for *D. magna*, *D. pulex* and *D. cucullata*, not only visible morphology changes have been recorded, but also fortification of the carapace in the presence of predator kairomones (Laforsch & Tollrian 2004b; 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 predator presence. 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 were 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 our analysis which could be explained by the small amount 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.

Interspecific comparisons of gene expression

We discuss our findings in the context of two recent transcriptomic studies on *Daphnia*. First, we compare our results with the results of Orsini *et al*. (2017) who investigated the short-term exposure to fish kairomones in *D. magna* to find common transcripts involved in predator-induced responses. Second, we compare our results with transcripts from Asselman *et al.* (2017) who predicted reproduction-related transcripts after the long-term exposure to cyanobacteria, insecticides and their combination in *D. pulex* to find common transcripts involved in reproduction after the exposure to stressors.

No biologically relevant transcripts were identified for *D. magna*. The experimental design for *D. magna* was different than in *D. galeata* with juvenile *D. magna* exposed to fish kairomones for only 4 hours that could explain the weak response. Orsini *et al.* (2017) focused on characterizing the early transcriptional stress response to abiotic and biotic stressors, while the present study examined the long-term life history response to fish kairomones across generations. The biotic stressors Orsini *et al.* (2017) tested had little impact on the differential expression, while the abiotic stressors caused stronger responses to *Daphnia* after a 24 h exposure. This difference in design made detecting similarities between the predator-induced responses in different *Daphnia* species difficult. However, orthogroups of *D. magna* and *D. galeata* were discovered suggesting that similar transcripts could be involved in the predator-induced responses in both species. It remains unclear whether the predator-induced response affects species-specific transcripts and how the early stress response deviates from long-time exposure. We found some similarities to *D. ambigua*, which is a species from the *D. pulex*-complex and more closely related to *D. galeata* than *D. magna* is (Petrusek *et al.* 2005).

To shed light into reproduction strategies after exposure to stressors, we compared *D. galeata* transcripts to *D. pulex* transcripts that were predicted to be involved in reproduction (Asselman *et al.* 2017). We identified 28 reproduction-related orthogroups containing at least one annotated *D. galeata* (Huylmans *et al.* 2016) and one predicted *D. pulex* transcript yielding 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 S6): 'DNA binding', 'carbohydrate metabolic

process', 'signal transduction', 'zinc ion binding' and 'integral component of membrane'. Little to no information was found 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 molecular mechanisms of reproduction in *Daphnia*.

In summary, the aim of this study was to characterize the genetic basis of predator-induced responses in the freshwater grazer *Daphnia galeata*. Our transcriptional profiling revealed differentially expressed transcripts and gene co-expression modules in connection to the presence of fish kairomones. The discovered functional pathways represent a valuable starting point for future investigations addressing the functionality of certain transcripts per se or in respect to a stress response.

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Chapter 3

An environment-dependent genotype-phenotype association in European *Daphnia galeata*

Verena Tams, Suda Parimala Ravindran and Mathilde Cordellier

Abstract

Environment-dependent phenotypic plasticity is exhibited to some extent by all organisms. To cope with environmental change, organisms adapt through a variety of mechanisms such as changes in morphology, physiology, life history traits or behavior that do not require genotypic changes known as phenotypic plasticity. Understanding the genetic basis of varying phenotypic responses is essential and thus the identification of candidate genes that mediate the phenotypic variation is important. To this aim, we used *Daphnia* as a model organism to understand the genetic basis of phenotypic variation in a predation risk environment using a genome-wide association approach. Furthermore, we used a gene co-expression network analysis to identify gene clusters correlated to life history traits. To enhance our understanding of the functional roles of the transcripts, we identified orthologs and paralogs from related species and used ontologies to annotate the candidates of interest. Our association analysis revealed two life history traits to have a genetic basis in the presence and absence of fish kairomones, while our gene co-expression analysis identified 44 modules, of which one module correlated to another life history trait, the total number of broods. Our combined use of gene co-expression network and transcriptome-wide association analysis provided a systems-level approach to understand the genetic basis of phenotypic variation in *Daphnia*.

Introduction

Global change and its impact on biodiversity is currently a major focus of scientific inquiry (reviewed in Beaugrand & Kirby 2018). Natural populations are subject to novel environmental conditions due to climate change, habitat degradation and/or shifts in population ranges thereby expressing new phenotypic characteristics (Grether 2005). This phenomenon may facilitate adaptive evolution (Grether 2005; Price *et al.* 2003). When environments impose strong constraints and when adaptive potential exists in populations, selection favors trait values that increase the fitness of individuals in their local habitat. Hence, individuals have better fitness compared to other populations in their local environment and this is known as local adaptation (e.g., de Villemereuil *et al.* 2018; Kawecki & Ebert 2004).

Phenotypes and genotypes are tightly linked, since a genotype and its environment define the phenotype (e.g., Agrawal 2001; Stearns 1989). Genetic as well as phenotypic variation is crucial for an organism to survive environmental changes and to successfully reproduce and pass on their alleles to the next generation. Identifying the genetic basis of local adaptation is critical in addressing the central questions in evolutionary biology (Rausher & Delph 2015). Resolving whether natural selection acts on standing genetic variation or on novel mutations, and identifying the loci contributing to regulatory, coding and structural variation helps to understand the adaptation and speciation processes (Hoban *et al.* 2016).

Phenotypic plasticity is an important mechanism that helps coping with environmental perturbations (Alberto *et al.* 2013; Charmantier *et al.* 2008). Phenotypic plasticity is the ability of an organism to produce multiple phenotypes from a single genotype depending on the environment (Miner *et al.* 2005). Although phenotypic plasticity is advantageous in heterogenous and/or fast changing habitats, its maintenance is associated with costs (DeWitt *et al.* 1998; Van Buskirk & Steiner 2009) and sometimes becomes maladaptive (Ghalambor *et al.* 2007; Langerhans & DeWitt 2002). A wide diversity of organisms exhibit phenotypic plasticity in response to biotic and abiotic factors in their environments (reviewed in DeWitt & Scheiner 2004; Harvell 1990; Karban & Baldwin 1997; Karban & Myers 1989; Sultan 2000) leading to changes in behavior, morphology, physiology and life history traits. These plastic responses can be expressed either within the lifespan of a single individual (Young *et al.* 2003) or across generations (Miner *et al.* 2005).

Individuals in a population show differences in their phenotypic traits, which is influenced by both genetic and environmental sources. Understanding the mechanisms of variation is the key to assess the adaptive potential of a population to changing environments (Fuhrman *et al.* 2018). Three factors influence the phenotypic trait value of any individual: (i) genetic factors that define heritable differences within an environment (ii) environmental factors that influence the genotypes and (iii) the intrinsic capability of the phenotypic trait, given that genetic and environmental factors are identical (Ziv *et al.* 2017). Understanding how each of these factors contributes to variation in quantitative traits remains a challenge.

Examining the genetic architecture of phenotypic traits not only identifies causal mutations but also helps in understanding past and predicting future evolutionary processes of adaptation (Ronnegard *et al.* 2016). Genetic variation can be studied at two levels of organization: patterns of gene expression and the DNA/RNA sequence level. Associating the regulatory level of genetic variation to phenotypic traits can be accomplished by constructing gene co-expression networks, which identify clusters of co-expressed genes. Often co-expressed genes within one module (cluster) share conserved biological functions revealing their potential genetic pathways (Subramanian *et al.* 2005). The benefit of gene co-expression network analysis lies in the opportunity to correlate the gene co-expression information to biological information, by gathering insights of the biological association of genes and traits hence candidate genes can be identified. For example, a study on lake whitefish (Filteau *et al.* 2013) used a weighted gene co-expression network analysis to identify gene clusters correlating to three phenotypic traits such as trophic behavior, trophic morphology (gill rakers), and reproduction. On the other hand, associating the sequence level of genetic variation to phenotypic traits is accomplished in genome-wide association studies (GWAS) which make use of single nucleotice polymorphisms (SNPs) (Visscher *et al.* 2012). GWAS have been applied extensively in humans (for e.g., Busch *et al.* 2016; Eising *et al.* 2016; Kao *et al.* 2017), animals (for e.g., pigs (Duijvesteijn *et al.* 2010), cows (Hayes *et al.* 2009), dogs (Wood *et al.* 2009)) and plants (for e.g., rice (Zhao *et al.* 2016), *Arabidopsis* (Atanasov *et al.* 2016), sunflower (Kim & Rieseberg 1999)).

The genotype-environment interaction (GxE) is a common phenomenon describing how a genetic variant has different phenotypic effects in different environments (Smith &

Kruglyak 2008). For example, human individuals with sickle cell anemia have a survival advantage in endemic areas of malaria but are at a disadvantage in areas without malaria (Ferreira *et al.* 2011). Recently, biologists have applied genomic data and traditional pedigree information to explain phenotypic differences in life history traits such as horn shape in soay sheep (Johnston *et al.* 2013), clutch size in collared flycatchers (Husby *et al.* 2015; Ronnegard *et al.* 2016) and Glanville fritillary butterfly (Duplouy *et al.* 2017) where life history trade-offs may be involved in promoting genetic variation at one or several loci in the species.

Daphnia is a well-established organism for population genetic studies and plays a vital role in the trophic cascade of freshwater ecosystems (e.g., Carpenter *et al.* 2001; Lampert 2011; Sommer *et al.* 2003). This small, filter feeding crustacean has become a widely used isogenic model organism in ecology, ecological toxicology and ecological evolution because of its ability to reproduce parthenogeneticly. Due to their short generation times and easy handling, *Daphnia* researchers use several individuals of every clonal line in their analyses for measuring both genetic and phenotypic traits. A few studies in *Daphnia* research exist that have associated the phenotypic traits to their genotype by using information of single nucleotide polymorphisms (SNPs). For example, two previous studies (Henning-Lucass *et al.* 2016; Herrmann *et al.* 2017), showed the effects of temperature on fitness in *D. galeata*. A study (Schwerin *et al.* 2009) in *D. magna* revealed the effects of temperature on gene expression patterns of several genes. Another study investigated the association of parasite resistant traits to genotypes in *D. magna* (Bento *et al.* 2017). All the above-mentioned studies use an average trait value for every clonal line and associated them to the genotype.

In the present study, we associate genotypic and phenotypic data of 24 clonal lines of European *D. galeata* by integrating two approaches, a genome-wide association (GWA) analysis and a gene co-expression network analysis. We applied a GWA analysis to the phenotypic dataset (**Chapter 1**) and the corresponding genetic dataset (Herrmann *et al.* 2017, Ravindran et al submitted). We took advantage of the well designed experiment with 15 individuals as replicates for each clonal line in the control and fish environment (**Chapter 1**) and applied a GWA to the complete phenotypic dataset. We then compared these results to the results from GWA analysis obtained with the mean phenotypic trait value. Further, we applied a weighted gene co-expression network analysis to understand

the genotype-phenotype associations at the gene co-expression network level. Based on these analyses, we addressed following research questions: (1) Which SNPs (genotype) associate to different phenotypic life history traits in the two different environments? and (2) Which gene co-expression modules are correlated to life history traits in control environment? We were able to synthesize the two levels of genotype-phenotype associations: genotype (SNPs) – phenotype (life history traits in control and fish environment) and gene co-expression modules – phenotype (life history traits in control environment). By answering these questions, we contribute to the understanding of the genetic basis of phenotypic variation in the absence and presence of fish kairomones in *Daphnia galeata*.

Material and methods

The genotypic (SNPs) and phenotypic (life history trait) datasets used in the present study have been described in Herrmann *et al.* (2017) and **Chapter 1**, respectively. Variance-stabilized normalized read counts for the gene co-expression network analysis were obtained from Ravindran *et al.* (*submitted*). Functional annotation of the *D. galeata* transcriptome has been described in Huylmans *et al.* (2016) and Ravindran *et al*. (*submitted*). We present here a brief overview of methods used for the creation of input datasets for the GWA analysis as well as a detailed description of the GWA and gene co-expression network analysis.

Study organism

The cladoceran *D. galeata* is a widely distributed keystone species in freshwater ecosystems. Their parthenogenic life cycle allows rearing of many genetically identical individuals from one genotype. Despite their identical genetic makeup, one clonal line can result in different phenotypes. For all datasets (i.e., genotype (SNP), phenotype, and gene expression) summarized below, we used 24 clonal lines of *D. galeata* from four European lake populations (six clonal lines per population): Greifensee (Switzerland), Jordan reservoir (Czech Republic), Lake Constance (South Germany) and Müggelsee (North Germany). Clonal lines were established from dormant eggs extracted from sediment cores, which have been used and described in previous studies (Henning-Lucass *et al.*

2016; Herrmann *et al.* 2017). They were maintained in lab cultures (18°C, 16h light / 8h dark cycle, food: *Acutodesmus obliquus*, medium: Aachener Daphnien Medium (ADaM) (Klüttgen *et al.* 1994).

Phenotype dataset and design of life history experiment

Phenotypic data originates from the life history experiment investigating the effect of fish kairomones on *D. galeata* (described in **Chapter 1**) for a total of 684 experimental individuals (aim: 24 clonal lines x 2 treatments x 15 replicates=720 individuals). Prior to the experiment, each clonal line was bred in kairomone-free water (control) and in kairomone water (fish) for two subsequent generations to minimize inter-individual variances (Figure C3-S1). 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, Typ 3201D). Experimental individuals (F2) were female neonates of the 3rd to 5th brood. Ten life history traits were recorded: age at first reproduction ('AFR') [day of releasing offspring from brood pouch], numbers of broods per female including numbers of neonates per brood per female ('brood1', 'brood2', 'brood3', 'brood4'), total numbers of neonates per female ('offspring'), total number of broods ('broods'), 'survival' [in days], body length ('size') [in µm] and somatic growth rate ('SGR') [in µm d^{-1}] (Table C3-S1). The experiment lasted for 14 days for each experimental individual. The experiment revealed a change of life history trait values when exposed to fish kairomones concordant to previous studies, e.g. early maturation in the presence of fish kairomones. Nevertheless, we found high intraspecific phenotypic variation of life history traits within each population as well as among the four populations. Further details can be found in **Chapter 1**.

In this study we used two phenotypic datasets originating from the above described experiment. First, we used the complete raw dataset with up to 15 individuals as replicates per clonal line (Table C3-S1). Second, we created a dataset containing the means of each clonal line for each of the life history traits (hereafter, “mean dataset”) (Table C3-S2). To avoid confusion with terminology, we use the term 'clonal line' for the 24 genotypes and the term 'genotype' for SNPs throughout the manuscript.

Genotype dataset and SNP calling

The SNP calling procedure has already been described in Herrmann et al (2017). Briefly, aligned reads from RNAseq experiment were merged with samtools (Li *et al.* 2009) and realignment around indels was performed using GATK's (DePristo *et al.* 2011) IndelRealigner tool. The initial variant calls were made using GATK's HaplotypeCaller. Using GenotypeGVCF tool in GATK, samples were jointly genotyped and a single vcf file was obtained. Variants were further filtered using VariantFiltration tool implemented in GATK with the following criteria: (i) clusterWindowSize = 35; (ii) Quality by depth (QD) < 2.0; (iii) Fisher Strand (FS) > 30.0.

To use the SNP data for GWA analysis, we further filtered variants with a minor allele frequency (MAF) of 0.1 to exclude rare variants. Only biallelic sites in the MAF filtered SNP data were considered for further analysis. A total of 155,638 SNPs were used for the association analysis. We used this as input for GWA analysis and the mean values of the life history traits ("mean dataset") for both univariate and multivariate analysis. However, for the GWA analysis using the values per individual for the life history traits, we artificially inflated the SNP data (hereafter, "inflated dataset") as follows (Figure C3-S2): For every individual from every clonal line, we assumed they contain the same haplotype as *Daphnia* have the ability to reproduce clonally and hence are genetically identical. Therefore, we replicated the haplotypes for every individual in every clonal line in every treatment (24 clonal lines x 15 individuals x 2 treatments = 720) and created two vcf files with SNP information for both the control and fish environments, separately. For the univariate and multivariate GxE analysis, we combined the SNP information from the two files into a single vcf file.

Genotype-phenotype association analysis

The genome-wide association (GWA) approach was applied by using the program PLINK v.1.07 (Purcell *et al.* 2007) to test for association between transcriptome-based SNPs (genotype) and life history traits (phenotype) in the presence (hereafter, fish environment) and absence (hereafter, control environment) of fish kairomones. PLINK was used to perform (a) a univariate approach where each SNP was tested for association to each of the ten life history traits individually, and (b) a multivariate approach where each SNP was tested for association to the combination of all ten life history traits. Both univariate and

multivariate analyses were performed on the “mean dataset", while only a univariate analysis was performed on the “inflated dataset".

The univariate association was performed using the “assoc” command on each phenotypic trait (“--all-pheno”). The multivariate association was performed using MV-PLINK tool using the "--mult-pheno –mqfam" option. PLINK was further used to test for differences in genotype–phenotype associations between the two environments. Therefore the "gxe" command was used to reveal genotype-environment interactions (GxE).

Both univariate and multivariate analyses were performed for the control and fish environment and the GxE interaction separately. Settings were applied to correct for population stratification in the data set by permuting for 1,000 iterations within populations. All *p*-values were corrected for multiple testing using the Bonferroni correction method in R (R Core Team 2018). The $-\log_{10}$ *p*-values were calculated on adjusted *p*-values and visualized using Manhattan plots in R. A SNP was said to be associated to a phenotypic trait if it had a $-\log_{10}$ *p*-value of 1.5.

Gene co-expression network analysis – Linking gene co-expression and life history traits

The gene co-expression network analysis was based on variance stabilized read counts obtained from HTSeq data used in the R package ‘*DESeq2*’ (Love *et al.* 2014) by Ravindran et al (*submitted*) to investigate differential gene expression at population level between the four European *D. galeata* populations in control environment. We applied a weighted gene co-expression network analysis (WGCNA) by using the R package ‘*WGCNA’* v. 1.6.1 to find putative pathways from the highly correlated genes clustered in modules (Langfelder & Horvath 2008).

A single, signed, weighted gene co-expression network was constructed on a workstation with the R environment v.3.2.3 while subsequent analysis was performed in the R environment v.3.4.2 (R Core Team 2018). Gene modules containing co-expressed genes were identified using the topological overlap matrices (TOM) with a soft cut-off threshold of 8 in ‘*WGCNA’*. Module eigengenes (ME) were calculated as the most representative gene within a module and were clustered in an eigengene dendrogram to reveal their relationships. Gene co-expression modules were correlated to phenotypic life history traits following methodology in **Chapter 2**. Further details can be found in the supplementary

material (R script: Tams-et-al_ResamplingAll_Daphnia.Rmd). Significant ME-trait correlations resulted in transcript sets of interest which were extracted to investigate their biological importance. In addition, hub-genes, which represent the most interconnected gene per module, were identified and their biological relevance explored.

Functional annotation

For every SNP/transcript associated to a life history trait in both GWA and gene co-expression network analysis, we assigned Gene Ontology (GO) terms using annotations from Huylmans *et al.* (2016) and performed an enrichment analysis with '*topGO*' (Alexa & Rahnenführer 2016) to investigate their biological relevance. Additionally, we identified orthologs and paralogs for the transcripts associated to a phenotypic trait using orthoMCL data from Huylmans *et al*. (2016). To enhance our understanding of the ecological role of transcripts associated to life history traits, we performed a BLAST analysis on the *Daphnia* stressor database (Ravindran et al. *in preparation*). This enabled us to identify stressors for the candidate transcripts of interest from our GWA and gene co-expression network analysis.

Results and discussion

Our integrative approach revealed genotype-phenotype associations at sequence and regulatory level. An univariate analysis identified a total of eight SNPs associated with three life history traits (4 SNPs in fish environment, 2 SNPs in control environment, and 2 SNPs by GxE). The multivariate analysis revealed 38 SNPs in the control environment, no SNPs in the fish environment, and 51 SNPs by the GxE interaction. A correlation analysis of module eigengenes and life history traits revealed only one association of a life history trait and a gene co-expression module, 'darkorange' (85 transcripts). We provide a list of overall 156 candidate transcripts being involved in the intraspecific phenotypic variation in *D. galeata*.

Genotype-phenotype association analysis

"Inflated dataset": univariate analysis

We wanted to take advantage of our well replicated life history measurements and performed a GWA analysis considering every individual, rather than phenotypic mean values, and inflated the genotype data. Our univariate analysis revealed associations between the genotype and phenotype for all traits except 'survival' in both control and fish environments (Table C3-S3). In the GxE analysis, we found seven traits ('brood2', 'brood3',' AFR', 'broods', 'survival', 'size' and 'SGR') to be associated with a genotype (SNP). Although we found a large number of SNPs to be associated to life history traits in the "inflated dataset", we could not differentiate between the true positive and false positive associations.

One reason is the lack of available literature for comparisons that use such experimental setups and inflated genotype calls. GWAS in an ecological context are rare, where several clonal lines and their replicates were used for experiments. Traditional GWAS tools such as PLINK were designed for identifying and analyzing disease-causing SNPs in humans and correlating them to disease phenotypes (Visscher *et al*. 2012), where replication of individuals is not feasible. To overcome some shortcomings of a traditional GWAS approach, tools like RepeatABEL (Ronnegard *et al.* 2016) or treeWAS (Collins & Didelot 2018) were designed. RepeatABLE is a tool used for repeated measurements of the same individual and it has been applied previously in the collared flycatchers (Ronnegard *et al.* 2016), while treeWAS was designed to account for clonal population structure in microbes via a phylogenetic approach (Collins & Didelot 2018). Unfortunately, the existing GWAS tools are not capable of handling replicated data as such for organisms like *Daphnia*.

Since our GWA analysis of the "inflated dataset" leads to excessive background noise, we could not infer true associations. Therefore we used the “mean dataset" for further analysis.

“Mean dataset”: univariate analysis

While taking the mean values of replicates for each clonal line, we observed associations between genotypes and two life history traits, namely 'brood3' and 'brood4' in the univariate analysis (Table C3-1). In the fish environment, the phenotypic trait 'brood3' was associated with three SNPs (in three transcripts). 'brood4' had one SNP in the fish

environment and two SNPs (in two transcripts) associated in the control environment. Significant GxE interactions across the two environments were found for one phenotypic trait, namely 'offspring', associated with two SNPs (in one transcript). No associations were found for the other life history.

We found no direct evidence of genotype-phenotype associations for most of the life history traits except for 'brood3' and 'brood4'. The genotype-phenotype associations for 'brood3' and 'brood4' are promising candidates for true associations. To verify these associations, a subsequent investigation should test the candidate SNPs for their biological relevance for the trait. The observed significant GxE interaction effect for the trait 'offspring' could be best explained by the significant associations of SNPs to 'brood3' and 'brood4' since these life history traits are not independent. The trait 'offspring' describes the total number of offspring per female and thus includes the total number of offspring for the first, second, third and fourth brood ('brood1' to 'brood4'). This correlation of life history traits might have biased the statistical genotype-phenotype association and led to a false positive association in the GxE analysis.

"Mean dataset": multivariate analysis

As our univariate approach showed weak association signals between SNPs and life history traits, we performed a multivariate testing. Multivariate testing has been shown to be more powerful compared to univariate analysis (Galesloot *et al.* 2014). Therefore, we assessed the association of one SNP on all phenotypic traits combined, thus taking the interdependence of the life history traits into account. We identified 38 SNPs (in 24 transcripts) to be significantly associated with all life history traits in the control environment (Table C3-2). However, no SNPs were significantly associated to all phenotypic traits in the fish environment. Our multivariate GxE analysis showed 51 SNPs (in 40 transcripts) to be associated to all life history traits.

In general, the association of SNPs to complex traits has been reported to be successful (Galesloot *et al.* 2014). Since PLINK is known to perform better compared to other tools available for multivariate genome-wide association studies (Galesloot *et al.* 2014), we do not expect a statistical/analytical bias *per se*. The number of identified genotype-phenotype associations in the control environment as well as for the genotype-environment interaction (GxE) is concordant to a previous study in wing shape of *D. melanogaster* that identified 139 genotype-phenotype associations (Pitchers *et al.* 2017).

The number of SNPs associated to life history traits in the multivariate GxE might exist because the trait values in the control environment drive the statistical associations even though there is a lack of association in the fish environment. Further analysis is required to test this interpretation such as knock-down studies on the specific transcripts that are inferred to be associated to the life history traits.

Table C3-1: Number of significant SNPs and corresponding transcript associations of univariate analysis for the "mean dataset". Association analysis was applied to data from control and fish environment as well as the GxE interaction. Numbers of genotype-phenotype associations are highlighted in bold.

	Control -log10P > 1.5	**Fish -log10P > 1.5**	**GxE -log10P > 1.5**
brood1_snps	0	0	0
brood1_transcripts	0	0	0
brood2_snps	0	0	0
brood2_transcripts	0	0	0
brood3_snps	0	**3**	0
brood3_transcripts	0	**3**	0
brood4_snps	**2**	**1**	0
brood4_transcripts	**2**	**1**	0
afr_snps	0	0	0
afr_transcripts	0	0	0
broods_snps	0	0	0
broods_transcripts	0	0	0
offspring_snps	0	0	**2**
offspring_transcripts	0	0	**1**
survival_snps	0	0	0
survival_transcripts	0	0	0
length_snps	0	0	0
length_transcripts	0	0	0
sgr_snps	0	0	0
sgr_transcripts	0	0	0

Table C3-2: Number of significant SNPs and corresponding transcript associations of multivariate analysis for the "mean dataset". Association analysis was applied to data from control and fish environment as well as the GxE interaction. Numbers of genotype-phenotype associations are highlighted in bold.

	Control -log10P > 1.5	**Fish -log10P > 1.5**	**GxE -log10P > 1.5**
All_SNPs	**38**	0	**51**
All_Transcripts	**24**	0	**40**

Although significant phenotypic differences were observed in the life history traits of all 24 clonal lines exposed to fish kairomones (**Chapter 1**), our present GWA results did not allow pinpointing associated sequence polymorphisms of coding regions to life history traits. Several possible explanations exists for the lack of genotype-phenotype associations at the sequence level. First, the traits are phenotypically plastic (Ayrinhac *et al.* 2004) and thus not necessarily under immediate selection (Merila & Hendry 2014). Second, the traits are complex and have a polygenic nature which we were unable to detect with the present multivariate GWA approach. Instead, an additional multivariate approach that test the association of all SNPs with each life history trait may provide further insights into a potential polygenic basis of phenotypic variation in life history traits. Third, we investigated genotype-phenotype associations at the transcript level, thereby introducing the limitation of looking only at genotype-phenotype associations in coding regions. Our study design did not allow to test for the role of non-coding regions since genomic data of *D. galeata* is not available. Associations between phenotypic traits and polymorphisms in non-coding regions have been reported by McKown *et al.* (2014) in *Populus trichocarpa,* where 152 out of 275 identified associated polymorphisms were in non-coding regions. Non-coding regions include essential regions for the complex mechanism of gene expression regulation, such as transcription factors and promotors which are spatially located in the close vicinity of a gene, while gene regulatory elements such as enhancers, locus control regions or insulators can be located several kilobases away from the expressed gene of interest (Babu *et al.* 2008). Future investigations should include non-coding regions to explore the genetic basis of phenotypic variation of life history traits as well as at the epigenetic level (Tak & Farnham 2015) which we did not investigated in the present study. Fourth, low levels of genetic variation in specific ecological relevant traits can also prevent an adaptive response in populations as seen for example in *Drosophila birchii* (Hoffmann *et al.* 2003). Further, the observed levels of heterozygosity patterns could confound the absence of genotype-phenotype associations in this study. Most of the clonal lines (19 out 24) in the sampled populations showed higher observed heterozygosity than the expected levels (Ravindran et al. submitted) which would help to buffer environmental influences on the organism (Pigliucci 2005). On the other hand, five out of 24 clonal lines showed less heterozygous levels (Ravindran et al. submitted) and such patterns are observed not only in individuals with an inbreeding depression (Keller &

Weller 2002). This effect may also be due to lack of variation in the source population, which is caused by a founder effect or a severe bottleneck event during colonization (Luikart *et al.* 1998).

Gene co-expression network analysis – Linking gene co-expression and life history traits

The single network construction resulted in 44 modules of co-expressed transcripts in control environment (Figure C3-S3). Most transcripts were assigned to the module 'turquoise', 'blue', 'brown' and 'yellow'. The 'grey' module is the largest and includes all transcripts which were not assigned to any module (22%; n=7,297). For each module, the hub-gene, or the most highly interconnected gene within a gene co-expression module, was identified. To assess the biological meaning of modules we correlated life history trait information to the module eigengenes (ME). Only one module was significantly correlated to one life history trait, namely the module ‘darkorange’ and the trait ‘broods’ correlated 9,782 out of 10,000 iterations. A detailed overview of modules, number of transcripts and hub-genes are listed in Table C3-S4.

Functional annotation

Gene Ontology analysis

Gene Ontology (GO) terms were assigned to transcripts identified in the univariate and multivariate GWA, in addition to hub-genes and transcripts of the 'darkorange' module which correlated to the trait 'broods'. In total, GO terms were assigned to 68 transcripts (44 transcripts in GWA and 24 transcripts in WGCNA) (Table C3-S5) and to 15 out of the 44 hub-genes (Table C3-S4). GO terms identified in the GWA were enriched for ‘spermatogenesis’ and other metabolic processes; and those identified in the WGCNA were enriched for metabolic processes (Table C3-S6). There were 18 GO terms assigned to the hub-genes and included functions for enzyme activities, binding and transport activities which are important for general metabolic processes.

Surprisingly, we found GO terms of the GWA analysis to be enriched for 'spermatogenesis'. There is no sound explanation to this observation. Only parthenogenetically reproduced females were used in the experiment. We suggest that the result for 'spermatogenesis' is a

false positive because most of the transcripts are not annotated. Only 34% of the *D. galatea* reference transcriptome has GO annotations. Thus, we cannot exclude that a potential bias in the gene set enrichment analysis exists due to the lack of additional GO terms. In general, the gene set enrichment analysis emphasizes the need of further functional annotations for the existing *Daphnia* genomes to improve biological valid conclusions.

The 17 enriched GO terms (Table C3-S6) assigned to the transcripts of the 'darkorange' module from the WGCNA were for enzymatic activities and metabolic processes. Since these functions are generally important for the survival of an organism, no specific conclusions can be drawn in the context of our study.

We highlight hub-genes of modules with biological functions we identified earlier to be involved in predator-induced responses (**Chapter 2**). Growth-related GO terms, such as 'chitin binding' and 'chitin metabolic process' were identified for the 'turquoise' hub-gene (3017 transcripts), while the GO term 'structural constituent of cuticle' was identified for the hub-genes 'paleturquoise' (222 transcripts) and 'darkgreen' (371 transcripts). Growth-related functions are interesting since previous studies showed that predator-induced responses in *Daphnia* are phenotypic plastic and include changes of body size as well as morphological modifications (e.g., Laforsch 2004; Laforsch & Tollrian 2004a; Laforsch & Tollrian 2004b; Tollrian 1995). For example, a smaller body size in the presence of fish kairomones was observed in *D. galeata* from Greifensee, in contrast to a larger body size observed in the presence of *Chaoborus* kairomones, an invertebrate predator (Wolinska *et al.* 2007).

Digestion-related GO terms for 'cystein-type peptidase activity' were found for the hub-genes of the 'royalblue' (405 transcripts) and 'lightcyan1' (70 transcripts) module. Peptidases are major digestive proteases in the gut of *Daphnia* (von Elert *et al.* 2004). Juvenile growth rate in four clonal lines of *D. magna* declined in the presence of a cyanobacterial strain containing effective peptidase inhibitors (Schwarzenberger *et al.* 2012) illustrating the importance of peptidase activity in energy allocation for *Daphnia* growth. Although we do not have gene expression data for all 24 clonal lines exposed to fish kairomones we would still like to highlight, that the identified gene co-expression modules with hub-genes annotated to relevant GO terms to predator-induced responses are likely important for the observed phenotypic variation of predator-induced responses

in *D. galeata* (**Chapter 2**). However, this needs to be verified e.g. by differential gene expression and gene co-expression analysis of all 24 clonal lines exposed to fish kairomones.

Comparative genomics

To gain further insights into the biological relevance of our candidate transcripts identified in the GWA analysis and the WGCNA, we performed an orthoMCL analysis. All candidate transcripts of the GWA analysis and 58 transcripts from WGCNA were assigned to an orthogroup (Table C3-S5). In total transcripts of the GWA analysis were assigned to 67 orthogroups, while transcripts of the WGCNA were assigned to 53 orthogroups. There was only one overlap between the orthogroups identified in the GWA analysis and the WGCNA, 'ORTHO_ALL24' containing transcripts with the GO term 'protein binding'.

The aim of our orthoMCL approach was to reveal further biological relevance of identified candidate transcripts by making use of annotated genomes of other *Daphnia* species. The identification of orthogroups via e.g. orthoMCL, facilitates functional and evolutionary analyses of genomes and is useful in comparative genomics and genome annotation (Li *et al.* 2003). An orthogroup contains a set of protein-coding genes which help to characterize their functions by inferring protein functions from other genomes (Li *et al*. 2003). The orthoMCl tool clusters highly similar protein-coding sequences into one orthogroup by identifying orthologs between species deriving from a speciation event or "recent" paralogs within species deriving from a recent gene duplication event (Li *et al.* 2003). In the end, our orthoMCL analysis allowed two obvious conclusions. First, an integrative approach is beneficial to identify putative candidate transcripts/genes. Our integrative approach resulted in a candidate transcript list of overall 156 transcripts (71 from GWA and 85 from WGCNA) being involved in phenotypic variation of life histroy traits in *D. galeata*. Second, genomes still lack functional annotations hindering the interpretation of biological relevant transcripts. Only about one third of the our candidate transcripts had a GO annotation limiting our conclusions.

Identifying transcript-specific stressors is another way of looking into the functional aspects of a transcript. In the present study, we identified stressors for a total of 22 transcripts (10 from GWA and 12 from WGCNA) (Table C3-S5). The hits of stressors identified for the candidate transcripts are mostly abiotic factors such as phosphorus (5),

salinity (4), temperature (3) and light dark cycle (2) among others, while only three biotic factors were associated to a transcript including *Chlamydomonas* (1), microcystin, a toxin produced by a cyanobacteria (1) and fish kairomones (1). The transcript with the stressor-based hit for fish kairomones is grouped to the orthogroup 'ORTHO_ALL324' which contains protein coding transcripts of *D. galeata* (10), *D. pulex* (3), *D. magna* (2) and *Nasonia* (1) (Table C3-S5). Unfortunately, no GO annotation exists for one of the transcripts clustered in this orthogroup. Depending on the genes or transcripts of interest, further research can be conducted on these candidates that have an identified stressor in literature (Figure C3-1). Furthermore, expansion of the *Daphnia* stressor database (Ravindran *et al.* in preparation) may help researchers to identify biological relevant transcripts and to to infer stress responses in other *Daphnia* and related species.

Limitations and conclusions

In this study, we explored the association of phenotype, genotype and environment in European *Daphnia galeata* emphasizing the complexity of their interactions. The present study helped us identify a few candidate transcripts for understanding the genetic basis of phenotypic variation and also brought to light some shortcomings. First, an appropriate GWA approach is missing to account for the clonal nature of *Daphnia*. We would have gained more information and statistical power by using the complete phenotypic dataset of individuals (n= ~700) rather than phenotypic means per clonal line (n= 24). Second, although we found very little evidence of genotype-phenotype associations at the transcriptome level, we cannot exclude the role of non-coding regions in shaping phenotypic variation. Once genomic information is available, investigating the role of non-coding regions can help to understand the interplay of genotype, phenotype and environment better. Hence, genotype-phenotype associations can be explored at the epigenetic level in non-coding regions and/or coding regions. Third, easy access to annotation information for *Daphnia* would help to identify biologically meaningful transcripts. Finally, to better understand the influence of predation risk here simulated by the presence of fish kairomones on *Daphnia* life history traits, gene expression profiles are needed for all 24 clonal lines. These gene expression profiles would allow the application of a differential gene co-expression network analysis between the two gene co-expression networks (control vs. fish environment), further revealing biologically significant pathways

and hence candidate transcripts. Overall, the identification of biologically significant transcripts being involved in predator-induced responses in *Daphnia* provide a valuable source to design future investigations of the environment-dependent genotype-phenotype relationships in *Daphnia*.

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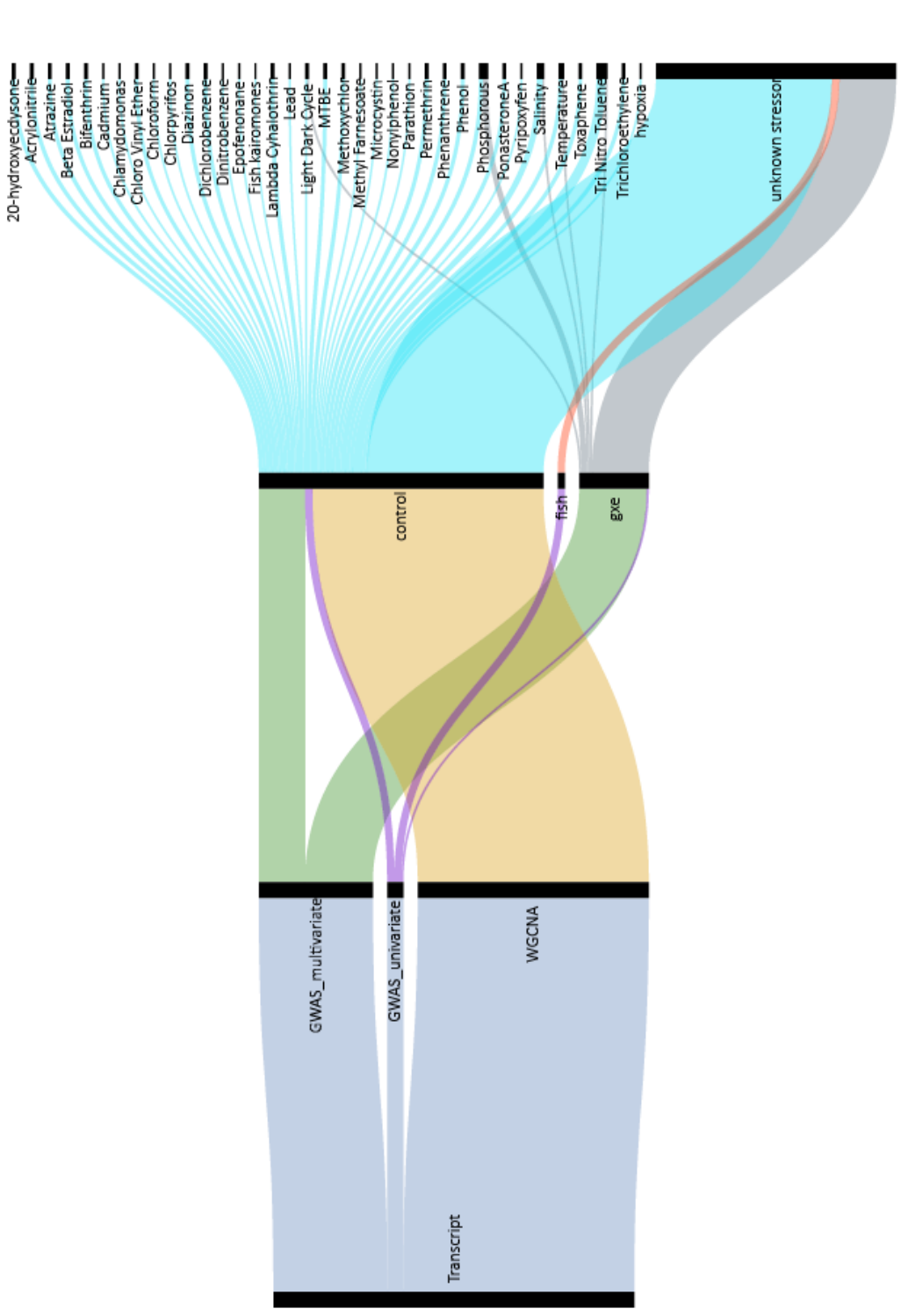

Figure C3-1: Flow diagram representing the proportion of candidate transcripts as identified in GWA and WGCNA and their associated stressors. Each rectangle bar is called a 'node' and each vertical group of nodes is called a 'step'. The colored areas linking the nodes are called 'flows'. The step 'Transcript' contains the candidate transcripts as identified in GWA and WGCNA. The step 'Analysis' contains three nodes: 'GWAS_multivariate', 'GWAS_univariate' and 'WGCNA' representing the analysis from which the candidate transcripts were obtained. The step 'Interaction' contains three nodes: 'control', 'fish' and 'gxe' representing the control and fish environments and gxe interactions. The step 'Stressors' represents the identified stressor for each transcript based on sequence similarity from *Daphnia* Stressor database.

General discussion and conclusion

In my thesis, I focused on intraspecific phenotypic variation and its genetic basis in *Daphnia* populations. To this aim, I analyzed the intraspecific phenotypic variation of life history traits in 24 clonal lines in four European *Daphnia galeata* populations exposed to fish kairomones simulating predation risk (**Chapter 1**). I observed high intraspecific phenotypic variation within and among the populations and identified the underlying driving forces, environment, genotype or population. The study displays the complexity of the interacting factors 'genotype' (clonal line) and 'environment' to produce a variety of phenotypes within one species. Surprisingly, 'population' was not one of the important driving factors for the observed phenotypic variation. To further elucidate these findings, I applied an RNAseq approach on two clonal lines from one population with opposing life history strategies in the presence of fish kairomones (**Chapter 2**). Differential gene expression showed strong differences between the clonal lines so that only a clone-wise analysis revealed differentially expressed transcripts related to fish kairomones. An additional gene co-expression analysis expanded the list of transcripts being in a predator-induced response to a total of 125 candidate transcripts. Lastly, I utilized an existing genotype (SNP) dataset (Ravindran et al. *submitted*) to find genotype-phenotype associations in the phenotypic life history traits of 24 clonal lines of *D. galeata* (**Chapter 3**). The multivariate association analysis yielded 38 SNPs in the control environment, no SNP in the fish environment and further 51 SNPs by the GxE interaction. By integrating a transcriptome-wide association analysis and a gene co-expression analysis 151 candidate transcripts were identified. These results carry important implications for my initial research questions which adressed the driving force for the intraspecific phenotypic variation in *D. galeata* in the presence of fish kairomones at population level (**Chapter 1**), the effect of fish kairomones on gene expression in *D. galeata* (**Chapter 2**) and the genotype-phenotype association of *D. galeata* at sequence level (**Chapter 3**).

Driving forces of intraspecific phenotypic variation

The origins of phenotypic variation are environmental as well as genetic (Griffiths *et al.* 2000; Stearns 1989; Stearns *et al.* 1991; West-Eberhard 1989). A single genotype has the ability to produce a variety of phenotypes in different environments. This phenomenon is

named phenotypic plasticity, and also contributes to the observed phenotypic variation within species (Pfennig *et al.* 2010). Prey develops different strategies to reduce its vulnerability to predators by changing its behavior, its morphology or its life history (e.g., Bourdeau *et al.* 2015; Lass & Spaak 2003). Phenotypic plastic responses in *Daphnia* exposed to fish kairomones have been reported in numerous studies (e.g., Gliwicz & Boavida 1996; Lampert 1993; Machacek 1991; Weider & Pijanowska 1993). The results I presented in **Chapter 1** are concordant with previous studies reporting shifts of life history to early maturation and smaller body size in several clonal lines exposed to fish kairomones. Interestingly, the results show that phenotypic traits were affected by different factors, namely 'Environment' (or 'Treatment'), 'Genotype' (here, clonal line) or 'Population'. The 'Environment' affected how quickly an individual reproduces and how tall they become, while the 'Genotype' had the strongest effect on how many offspring were produced and much they grow. Surprisingly, 'Population' had little to no effect on the phenotypic traits. These results suggest that, the genotypic variation within a population seems to be more important than the origin of the 'Genotype' *per se*.

Local adaptation describes a pattern or process in which genotypes of a population have a higher relative fitness in their local habitat than genotypes originating from other habitats (Joshi *et al.* 2001; Kawecki & Ebert 2004; Lenormand *et al.* 1999). Local adaptation is the consequence of divergent selection on genotypes which produce phenotypes with a higher relative fitness in a local habitat and which are selected (Kawecki & Ebert 2004). On the other hand, adaptive phenotypic plasticity results in optimized phenotypes in a local population without any genetic changes (Schlichting & Pigliucci 1998) promoting diversification among populations and hence speciation (Pfennig *et al.* 2010). Thus, phenotypic plasticity can be seen as the potential to locally adapt to a changed environment (Stearns 1989). Adaptive changes have been described in *Daphnia* species before, emphasizing their adaptive potential of phenotypic plastic responses to environmental changes including predation risk (e.g., Altshuler *et al.* 2011; Declerk *et al.* 2001; Declerk & Weber 2003; Dlouhá *et al.* 2010; Hesse *et al.* 2012; Jansen *et al.* 2011; Yin *et al.* 2011; Zuykova *et al.* 2012). Although my results in **Chapter 1** support the hypothesis that the potential to locally adapt to predation risk exists at least for one population (popJ), it is unclear if this phenotypic divergence is due to adaptive phenotypic plasticity or local adaptation. In the end, I was not able to identify one main driving force influencing

the phenotypic variation in life history traits. Instead, my results emphasize the complexity of the genotype-environment interaction to produce a variety of phenotypes within one species. Accordingly, I looked at the genetic basis of phenotypic variation integrating three different approaches: differential gene expression, gene co-expression and transcriptome-wide association analysis.

The genetic basis of phenotypic variation

... at the regulatory level: the effect of fish kairomones on gene expression

In the previous section I discussed phenotypic plasticity with respect to phenotypic variation in life history traits. Here, phenotypic variation refers to variation in gene expression. As an aquatic key stone species in freshwater food webs *Daphnia* are exposed to predations risks varying in intensity and mode (Lampert 2011). Predator-induced responses are well studied in *Daphnia*, yet few studies have addressed the link between the ecological traits and the underlying genetic pathways. As described in **Chapter 2**, I identified a total of 125 candidate transcripts being correlated to life history trait changes in the presence of fish kairomones as well as their potential biological function. In addition, the interspecies comparison via orthogroups revealed common transcripts to be involved to reproduction in the stress response of *Daphnia*. To account for the omnipresent inter-clonal variation (e.g., Lüning 1995, Chapter 1) two clonal lines from the same population were chosen. The effect of fish kairomones on the gene expression was obscured by the huge phenotypic differences of the two clonal lines probably reflecting their opposing life history strategies in the presence of fish kairomones (**Chapter 1**). By applying the differential gene expression analysis on each clonal line, the effect of fish kairomones at gene expression was revealed but no shared DETs between environments were identified. Hence, a completely different set of transcripts seems to be linked to fish kairomone response within each clonal line. The gene co-expression network analysis confirmed the substantial clonal differences at the gene expression level because the majority of the transcripts correlated to clonal line and were assigned to either the 'brown' or 'blue' module.

Causes of variation in gene expression are the genotype-environment interaction (GxE) (Hodgins-Davis & Townsend 2009) and epigenetic modifications such as regulation by non-

coding RNAs (e.g., Klimenko 2017), or additional posttranslational process (e.g., Schwarzenberger *et al.* 2009). The field of epigenetics is a controversial topic in the scientific community. One can define epigenetics as heritable changes without the alteration of the DNA sequence itself, including DNA methylation, histone modification and RNA interferences (e.g., Bossdorf *et al.* 2008; Richards 2006). Environmental factors induce epigenetic responses in animals and plants (Richards 2006) including *Daphnia* (Vandegehuchte & Janssen 2014; Wojewodzic & Beaton 2017). Epigenetic mechanisms mediate phenotypic plasticity/variation and play a role in species adaptation to environmental change in freshwater ecosystems (reviewed in Jeremias *et al.* 2018). It is likely that epigenetic modifications play a role in the transmission of phenotypic plastic traits such as predator-induced responses. DNA methylation shifted in *D. ambigua* when exposed to fish kairomones over two generations (Schield *et al.* 2016). Yet, it is unknown whether *D. galeata* clonal lines display epigenetic modifications after exposure to fish kairomones. Further investigations are required to explore the epigenetic transmission of predator-induced phenotypicly plastic responses in *D. galeata*.

... at the sequence level: genotype-phenotype associations

The transcriptome-wide association analysis (**Chapter 3**) resulted only in a few associations of life history traits (phenotype) and SNPs (genotype). The univariate analysis, which tested the association of each SNP and each trait revealed only a total of four SNPs associated to two life history traits. The multivariate analysis, tested the association of each SNP to all traits at once, revealed a few SNPs to be associated to all traits (38 SNPs in control, no SNP in fish, 51 by GxE). In summary, only a few genotype-phenotype associations were identified at sequence level, so that I was unable to pinpoint genotype-phenotype association to the observed significant, phenotypic variation in the life history traits of 24 clonal lines in *D. galeata* exposed to fish kairomones (**Chapter 1**).

A few possible explanations exist why no sequence-based genetic differences for the intraspecific phenotypic variation life history traits in *D. galeata* was found. First, life history traits are phenotypically plastic (Ayrinhac *et al.* 2014) and are not under divergent selection (Merila & Hendry 2014). Second, the traits are complex and have a polygenic basis. Third, the genetic basis of phenotypic variation is not on the coding sequence, but

rather on the non-coding regions of the genome and can include epigenetic modifications (e.g., Jeremias *et al.* 2018; McKown *et al.* 2014). I think chances are high to find a genetic basis of phenotypic traits in the non-coding regions because switching of epigenetic phenotypes can help to cope with environmental stress (e.g., Burggren 2016). Fourth, low levels of genetic variation of ecological relevant traits may result in non-adaptive resonses (e.g., Hoffmann *et al.* 2003). Further, a higher proportion of observed than of expected heterozygosity buffers environmental influences on the phenotype (Pigliucci 2005).
Finally, I identified a few candidate transcripts with SNPs being associated to one trait or all traits. Thereby I laid a foundation to understand the genetic basis of intraspecific phenotypic variation in the presence or absence of fish kairomones. My data provide a valuable source for further investigations into the environment-dependent genotype-phenotype relationships in *Daphnia*.

... at the functional level: the biological importance of identified transcripts

The genome-wide association analysis is a statistical approach to test for associations between genotypes and phenotypes (e.g., McClellan & King 2010). Whether these associations are biologically important or not, could be inferred by identifying the functions of transcripts. For example, gene ontologies (GO) and a subsequent a gene set enrichment analysis helps to reveal this importance. Most fundamental is the experimental validation of transcripts being involved in the potential biological process

To understand the biological relevance of the candidate transcripts, a GO enrichment analysis identified underlying genetic pathways, here linked to predator-induced responses. Overall GO terms relating to enzymatic activities and 'structural constituent of cuticle' were assigned to the candidate transcripts identified in **Chapter 2** and **Chapter 3**. A number of pathways that were hypothesized to be involved in predator-induced response were confirmed for transcripts related to body remodeling and growth. Several studies on *Daphnia* species linked the assigned GO terms to a biological important function. For example, digestion related enzymes like serine-type endopeptidase (Agrawal *et al.* 2005; Hales *et al.* 2017; Schwarzenberger *et al.* 2010; von Elert *et al.* 2004) were involved in resource allocation important for growth and reproduction (Schwarzenberger *et al.* 2012). Growth or morphology-related GO terms such as the 'structural constituent of cuticle'

were involved in the remodeling of the cuticle (Otte *et al.* 2015; Rozenberg *et al.* 2015; Tarrant *et al.* 2014) as well as the formation of ultrastructural defenses (fortification) of the cuticle (Rabus *et al.* 2013). Altogether, cuticle-associated and digestion-associated proteins seem to play an essential role in the response to predation risk. The transcriptome-wide association analysis revealed similar assigned GO terms (**Chapter 3**). The annotated hub-genes of the gene co-expression network of the 24 clonal lines (**Chapter 3**) included functions such as enzymatic activities, binding and transport activities. Growth-related GO terms, like 'chitin binding' and 'chitin metabolic process', were indeed associated to three hub-genes and digestion-related GO terms such as 'cysteine peptidase activity' were assigned to two hub-genes. By comparing the lists of candidate transcripts resulting from two clonal lines (**Chapter 2**) and 24 clonal lines (**Chapter 3**) I could identify one overlapping transcript 'soapsoap392443'. This transcript was involved in the predator-induced response in the clonal line M9 (**Chapter 2**) and contains a significant association of one SNP and all traits revealed by the multivariate GxE analysis (**Chapter 3**). Unfortunately, there was no functional annotation for this transcript. Overall, functional annotation and enrichment results from **Chapter 2** and **Chapter 3** emphasize the need of further functional annotations in existing *Daphnia* genomes and transcriptomes to improve biologically valid conclusions. By now, most of the transcripts are not annotated, which could also lead to a bias of false positives if one applies gene set enrichment analysis to reveal biological information.

To shed light into a common reproduction strategy after exposure to stressors between *Daphnia* species, I identified 42 unique interspecies reproduction-related orthogroups with at least one *D. galeata* and one *D. pulex* transcript (**Chapter 2**). Their functions can be summarized into enzymatic activities, metabolic processes, transport and binding, but no further information was found for their relevance in *Daphnia* reproduction, physiology or stress responses.

To further elucidate the biological importance of candidate transcripts I used available orthogroup information. Orthology and paralogy are key concepts in evolutionary genomics. Orthologs are genes originating from a single ancestral gene in the last common ancestor of the compared genomes, while paralogs are genes which are related via gene duplication (Koonin 2005). The benefit of using orthogroup information is that annotations of genes/transcripts in other species can be used (carefully) to infer biological functions of

un-annotated genes/transcripts of interest binned in the same orthogroup. Protein-coding genes/transcripts within one orthogroup have a similar sequence and hence are likely to have the same biological function (Emms & Kelly 2015; Koonin 2005). I was able to identify 42 orthogroups related to reproduction containing minimum one transcript each of *D. galeata* and *D. pulex* (**Chapter 2**), 34 orthogroups related to predation risk with *D. galeata* and *D. magna* transcripts (**Chapter 2**) as well as 67 orthogroups from GWA analysis and 53 orthogroups from WGCNA related to predation risk in *D. galeata* (**Chapter 3**). One orthogroup overlapped the set of candidate transcripts identified in GWA analysis and WGCNA (**Chapter 3**) that were related to predation risk. This group was 'ORTHO_ALL24' containing transcripts with a GO term for 'protein binding'. Another orthogroup overlapped the set of candidate transcripts related to reproduction (**Chapter 2**) and fish kairomone exposure (**Chapter 3**). This group was 'ORTHO_ALL63' containing transcripts with several GO terms, namely 'hydrolase activity', 'carbohydrate metabolic process', 'protein phosphorylation' and 'protein kinase activity'. In general, the information of orthologs and paralogs I provide in this study can help to infer stress responses in other *Daphnia* and related species.

The genotype-phenotype-environment relationship triangle

The results I presented in **Chapter 1**, **Chapter 2** and **Chapter 3** underline the complex relationship between genotypes, phenotypes and their environment. I looked at the variation of phenotypes as organisms revealing high intraspecific phenotypic variation of life history traits and morphology under predation risk (**Chapter 1**). By integrating gene expression profiling, I investigated the biochemical phenotypes under predation risk using two clonal lines with opposing life history strategies (**Chapter 2**) displaying differential gene expression congruent to reproduction strategies. Applying a genotype-phenotype association analysis revealed only a few sequenced-based associations of SNPs (genotype) and life history traits (phenotype) (**Chapter 3**).

The observed phenotypic variation fits in the concepts of phenotypic plasticity and genotype-environment interactions (e.g., Agrawal 2001; Stearns 1989) as well as the numerous studies describing the influence of genotype (clonal line) and environment on *Daphnia*'s response. Yet, it is puzzling to find little associations to the underlying genetic

level. Only a few associations were found at the sequence level in protein-coding regions. Instead, I hypothesize that changes at the regulatory level and/or non-coding regions have a major effect on the observed phenotypic variation.

Conclusions and future perspectives

I explored the relationship of phenotypes, genotypes and environment in the context of intraspecific phenotypic variation in European *D. galeata* emphasizing their complex interplay and contributing to the understanding of the genetic basis of intraspecific phenotypic variation.

After dedicating four years of research to understand the underlying mechanisms of intraspecific phenotypic variation, I realize how complex the relationship between the three components phenotype, genotype and environment is. Each component contributes countless possibilities to the variation. It is fascinating, that the intraspecific variation of these tiny crustaceans is so diverse at both levels phenotype and genotype.

As a result of these studies, I am interested in further investigations to understand the genetic basis of intraspecific phenotypic variation. Specifically, I am interested in investigating gene expression patterns of all 24 clonal lines to identify further transcripts of interests being associated to fish kairomone exposure. This RNAseq experiment could either confirm that gene expression profiles are genotypically dependent or reveal environment dependency based on fish kairomone exposure. In addition, a differential gene co-expression analysis could be applied between the two gene co-expression networks (control vs. fish environment) revealing further candidate transcripts being associated to predation risk/fish kairomone exposure.

One of the main challenges was the lack of an existing whole genome assembly of our model organism *D. galeata*. A well-annotated genome would open new opportunities to find answers for my research questions. With a whole genome assembly, I could test the role of non-coding regions and their associations to life history traits, thereby exploring the epigenetic basis for phenotypic plastic responses such as predator-induced changes in *D. galeata*.

The scientific community still has lots of research to do: experiments are needed testing the functions of candidate transcripts and providing further functional annotations for the existing *Daphnia* genomes and transcriptomes. An easy access of annotations is needed to share this biological information. Last but not least, the development of an appropriate GWA approach accounting for the clonal nature of *Daphnia* reproduction would improve the statistical power of the analysis and would allow me to re-analize the data using the complete phenotypic dataset of ~700 individuals instead of reducing the phenotypic data to mean values.

I am convinced that these future investigations in *D. galeata* will help to understand general mechanisms of how intraspecific phenotypic variation is passed on to the next generations.

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Supplementary material

Supplementary tables

Table C1-S1: Background information of ecological aspects of the four European lakes of which experimental clonal lines originate from. Number of clonal line (N). Altitude (Alt.). Volume (Vol.). Maximum depth (Dep.). Average depth (Av. Dep.).

Lake	Greifensee	Jordán Reservoir	Lake Constance	Müggelsee
Abbreviation	popG	popJ	popLC	popM
Location	Switzerland	Czech Republic	Austria, Germany, Switzerland	Germany
GPS coordinates	47° 21‘20‘‘ N, 8° 40‘10‘‘ E	49° 24‘55‘‘ N, 14° 39‘49‘‘ E	47° 37‘21‘‘ N, 9° 26‘24‘‘ E	52° 26‘6‘‘ N, 13° 38‘6‘‘ E
N	6	6	6	6
Alt. [m]	435	437	395	32
Vol. [km3]	0.1485	0.0027	48	0.0366
Dep. [m]	34	14	254	8
Av. Dep. [m]	18	4.5	90	4.9
Stratification	dimictic	dimictic	monomictic	polymictic
Fish biomass [kg/ha]	19	607.5	54	70-100
Presence of *Leuciscus* sp.	yes	yes	yes	yes

Table C1-S2: Overview of all *D. galeata* clonal lines used in experimental rounds.

round	start breeding	end experiment	total number of days	pop	clone	Number of replicates for life history trait analysis (t4-t14)		Number of replicates for morphometric analysis	
						control	fish	control	fish
1	27.07.2015	16.09.2015	51	LC	LC3.1	15	13	9	10
					LC3.6	15	15	10	10
				J	J2	15	15	10	10
					J1	15	14	10	10
					J4	9	13	8	10
				G	G3.1	14	15	10	10
					G1.11	15	15	10	10
				M	M5	15	15	10	10
					M12	12	13	7	10
					M6	14	14	10	10
2	21.10.2015	17.12.2015	57	LC	LC3.5	15	15	10	8
					LC3.7	10	15	4	10
					LC3.9	13	15	10	10
				J	J3	13	13	10	9
					J2.1	15	15	10	10
				G	G1.12	15	15	8	10
					G1.6	15	12	10	6
				M	M2	15	15	10	10
3	17.05.2016	10.07.2016	54	LC	LC3.3	14	15	10	10
				J	J2.4	15	15	10	10
				G	G1.7	15	15	10	10
					G2.1	15	15	10	10
				M	M9	15	13	10	10
					M10	15	15	10	10

Table C2-S1: Phenotypic data of life history traits for *D. galeata* clonal lines M6 and M9 (Chapter 1). The life history traits are 'clone' (6=M6; 9=M9), 'treatment' (0= control; 1= fish), total number of offspring per brood (1st brood= 'brood1', etc up to 'brood4'), age at first reproduction ('AFR', day of releasing neonates from brood pouch), total number of broods ('broods'), total number of offspring ('offspring'), body length ('size', µm) and somatic growth rate ('SGR', µm/day).

available on supplementary CD

Table C2-S2: Expected GO terms (direct) in response to vertebrate predation. (A) search class 'growth'. (B) search class 'perception.' (C) search class 'reproduction'.

	search term	Expected GO class (direct)
A	cell death	activation of cysteine-type endopeptidase activity involved in apoptotic process
		activation of cysteine-type endopeptidase activity involved in apoptotic process by cytochrome c
		activation of MAPK activity
		apoptosome
		apoptotic DNA fragmentation
		apoptotic mitochondrial changes
		apoptotic process
		apoptotic process involved in morphogenesis
		apoptotic signaling pathway
		autophagic cell death
		border follicle cell migration
		cell death
		cell proliferation
		compound eye retinal cell programmed cell death
		cysteine-type endopeptidase activator activity involved in apoptotic process
		cysteine-type endopeptidase activity involved in apoptotic process
		cysteine-type endopeptidase activity involved in apoptotic signaling pathway
		cysteine-type endopeptidase activity involved in execution phase of apoptosis
		cysteine-type endopeptidase inhibitor activity involved in apoptotic process
		cytokinesis
		cytoplasm
		cytoplasmic side of endoplasmic reticulum membrane
		cytoplasmic transport, nurse cell to oocyte
		cytosol
		dendrite morphogenesis
		DNA binding
		dolichyl-diphosphooligosaccharide-protein glycotransferase activity
		ecdysone-mediated induction of salivary gland cell autophagic cell death
		ectopic germ cell programmed cell death
		embryonic hemopoiesis
		endomembrane system
		execution phase of apoptosis
		extrinsic apoptotic signaling pathway
		extrinsic apoptotic signaling pathway in absence of ligand
		germ cell migration
		germarium-derived female germ-line cyst formation
		glial cell apoptotic process
		hemocyte development
		inhibition of cysteine-type endopeptidase activity involved in apoptotic process
		integral component of membrane
		intracellular

	search term	Expected GO class (direct)
		intracellular signal transduction
		intrinsic apoptotic signaling pathway
		intrinsic apoptotic signaling pathway by p53 class mediator
		intrinsic apoptotic signaling pathway in response to DNA damage
		intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator
		intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress
		larval midgut cell programmed cell death
		maturation of 5.8S rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)
		maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)
		mitochondrial envelope
		mitochondrial fragmentation involved in apoptotic process
		mitochondrial outer membrane
		mitochondrial outer membrane permeabilization
		mitochondrion
		mitotic spindle assembly
		molecular_function
		mRNA processing
		neuron apoptotic process
		neuron cellular homeostasis
		neuron remodeling
		neuronal cell body
		neurotransmitter secretion
		nuclease activity
		nucleolus
		nucleus
		nurse cell apoptotic process
		oligosaccharyl transferase activity
		oligosaccharyltransferase complex
		oogenesis
		ovarian nurse cell to oocyte transport
		peptidoglycan recognition protein signaling pathway
		perinuclear region of cytoplasm
		peripheral nervous system neuron development
		postsynaptic membrane
		programmed cell death
		programmed cell death involved in cell development
		protein N-linked glycosylation
		regulation of alternative mRNA splicing, via spliceosome
		regulation of apoptosis involved in tissue homeostasis
		regulation of apoptotic process
		regulation of apoptotic signaling pathway
		regulation of cell cycle

	search term	Expected GO class (direct)
		regulation of cell death
		regulation of compound eye retinal cell programmed cell death
		regulation of cysteine-type endopeptidase activity involved in apoptotic process
		regulation of cytoplasmic translation
		regulation of execution phase of apoptosis
		regulation of extrinsic apoptotic signaling pathway via death domain receptors
		regulation of mitochondrial membrane permeability
		regulation of mitochondrial membrane permeability involved in programmed necrotic cell death
		regulation of neuron apoptotic process
		regulation of neuron death
		regulation of nurse cell apoptotic process
		regulation of oxidative stress-induced intrinsic apoptotic signaling pathway
		regulation of programmed cell death
		regulation of Rab protein signal transduction
		regulation of retinal cell programmed cell death
		regulation of signal transduction
		retinal cell programmed cell death
		ribosomal small subunit binding
		ribosomal small subunit biogenesis
		RNA binding
		salivary gland cell autophagic cell death
		sensory organ precursor cell division
		small-subunit processome
		somatic stem cell division
		sterol regulatory element binding protein cleavage
		synaptic membrane
		synaptic vesicle
		terminal bouton
		tumor necrosis factor-activated receptor activity
		zinc ion binding
	cell growth	activation of MAPKKK activity
		activin receptor activity, type I
		activin receptor complex
		activin receptor signaling pathway
		activin-activated receptor activity
		apoptotic cell clearance
		axon
		axon extension
		axon extension involved in axon guidance
		axon guidance
		axonal growth cone
		basal plasma membrane

	search term	Expected GO class (direct)
		BMP signaling pathway
		BMP signaling pathway involved in Malpighian tubule cell chemotaxis
		BMP signaling pathway involved in spinal cord dorsal/ventral patterning
		branched duct epithelial cell fate determination, open tracheal system
		cell adhesion
		cell competition in a multicellular organism
		cell differentiation
		cell fate commitment
		cell fate determination
		cell growth
		cell migration
		cell projection assembly
		cell-cell adhesion
		cell-cell signaling
		chorion-containing eggshell formation
		chorion-containing eggshell pattern formation
		collateral sprouting of injured axon
		compound eye cone cell fate commitment
		compound eye photoreceptor cell differentiation
		compound eye photoreceptor fate commitment
		cytokine activity
		cytoneme
		cytoneme assembly
		decapentaplegic signaling pathway
		dendrite extension
		dense core granule
		determination of genital disc primordium
		determination of muscle attachment site
		developmental growth
		dorsal appendage formation
		dorsal closure, elongation of leading edge cells
		dorsal closure, spreading of leading edge cells
		early endosome
		early endosome to late endosome transport
		endocytic recycling
		endocytosis
		endosomal transport
		endosome
		endosome transport via multivesicular body sorting pathway
		engulfment of apoptotic cell
		epidermal growth factor receptor ligand maturation
		epidermal growth factor receptor signaling pathway

	search term	Expected GO class (direct)
		epidermal growth factor-activated receptor activity
		epithelial cell migration, open tracheal system
		epithelial cell proliferation involved in Malpighian tubule morphogenesis
		ESCRT-0 complex
		establishment or maintenance of apical/basal cell polarity
		exocytosis
		exon-exon junction complex
		exosomal secretion
		fibroblast growth factor receptor binding
		fibroblast growth factor receptor signaling pathway
		fibroblast growth factor-activated receptor activity
		filopodium assembly
		filopodium tip
		flagellated sperm motility
		G2/M transition of mitotic cell cycle
		gap junction
		germ cell development
		germarium-derived oocyte differentiation
		germ-line stem cell division
		germ-line stem cell population maintenance
		glial cell growth
		glial cell migration
		Golgi apparatus
		Golgi membrane
		growth cone
		growth cone lamellipodium
		growth cone membrane
		growth factor activity
		growth of a germarium-derived egg chamber
		gurken signaling pathway
		hemocyte differentiation
		hemocyte migration
		histone acetyltransferase complex
		hormone activity
		insulin receptor signaling pathway
		insulin-like growth factor binding
		insulin-like growth factor receptor signaling pathway
		integral component of plasma membrane
		integrin binding
		JNK cascade
		long-term strengthening of neuromuscular junction
		MAP kinase kinase kinase activity

	search term	Expected GO class (direct)
		maternal determination of dorsal/ventral axis, ovarian follicular epithelium, soma encoded
		microtubule
		motile cilium
		MOZ/MORF histone acetyltransferase complex
		multivesicular body
		muscle cell fate specification
		neuroblast proliferation
		neurogenesis
		neuromuscular synaptic transmission
		neuron development
		neurotrophin receptor activity
		neurotrophin TRK receptor signaling pathway
		notum cell fate specification
		NuA4 histone acetyltransferase complex
		oenocyte differentiation
		ommatidial rotation
		oocyte anterior/posterior axis specification
		oocyte axis specification
		oocyte dorsal/ventral axis specification
		oocyte growth
		oocyte growth in germarium-derived egg chamber
		ovarian follicle cell development
		peptide bond cleavage involved in epidermal growth factor receptor ligand maturation
		perineurial glial growth
		phagocytosis
		phagocytosis, engulfment
		phototransduction
		plasma membrane
		postsynapse
		presynapse
		R7 cell differentiation
		receptor activator activity
		receptor-mediated endocytosis
		recycling endosome
		regulation of axon extension
		regulation of axon extension involved in axon guidance
		regulation of BMP secretion
		regulation of BMP signaling pathway
		regulation of cell differentiation
		regulation of cell growth
		regulation of cell size
		regulation of cell-cell adhesion

	search term	Expected GO class (direct)
		regulation of epidermal growth factor receptor signaling pathway
		regulation of epidermal growth factor-activated receptor activity
		regulation of epithelial cell migration, open tracheal system
		regulation of hemocyte differentiation
		regulation of insulin-like growth factor receptor signaling pathway
		regulation of mitotic cell cycle
		regulation of Notch signaling pathway
		regulation of planar cell polarity pathway involved in axis elongation
		regulation of R8 cell spacing in compound eye
		regulation of Rho protein signal transduction
		regulation of synaptic growth at neuromuscular junction
		regulation of transforming growth factor beta receptor signaling pathway
		retrograde transport, endosome to Golgi
		Rho protein signal transduction
		RIC1-RGP1 guanyl-nucleotide exchange factor complex
		second mitotic wave involved in compound eye morphogenesis
		signal transducer activity
		signal transducer, downstream of receptor, with protein tyrosine kinase activity
		signal transducer, downstream of receptor, with serine/threonine kinase activity
		signal transduction
		Sin3-type complex
		site of polarized growth
		sprouting of injured axon
		stem cell fate commitment
		synaptic vesicle cycle
		synaptic vesicle endocytosis
		synaptic vesicle exocytosis
		synaptic vesicle priming
		torso signaling pathway
		transforming growth factor beta receptor activity, type I
		transforming growth factor beta receptor activity, type II
		transforming growth factor beta receptor complex assembly
		transforming growth factor beta receptor signaling pathway
		transforming growth factor beta receptor signaling pathway involved in endodermal cell fate specification
		transforming growth factor beta receptor, common-partner cytoplasmic mediator activity
		transforming growth factor beta receptor, inhibitory cytoplasmic mediator activity
		transforming growth factor beta receptor, pathway-specific cytoplasmic mediator activity
		transforming growth factor beta-activated receptor activity
		transmembrane receptor protein serine/threonine kinase activity
		transmembrane receptor protein serine/threonine kinase signaling pathway
		transmembrane receptor protein tyrosine kinase activity

	search term	Expected GO class (direct)
		transmembrane receptor protein tyrosine kinase signaling pathway
		transmembrane signaling receptor activity
		type III terminal bouton
		vascular endothelial growth factor receptor signaling pathway
		vascular endothelial growth factor-activated receptor activity
	chitin	adult chitin-based cuticle development
		adult chitin-based cuticle pattern formation
		adult chitin-containing cuticle pigmentation
		apical part of cell
		carbohydrate binding
		carbohydrate metabolic process
		cell periphery
		cell septum
		cell wall chitin biosynthetic process
		chitin binding
		chitin biosynthetic process
		chitin catabolic process
		chitin deacetylase activity
		chitin metabolic process
		chitin synthase activity
		chitinase activity
		chitin-based cuticle attachment to epithelium
		chitin-based cuticle development
		chitin-based cuticle sclerotization
		chitin-based embryonic cuticle biosynthetic process
		chitin-based larval cuticle pattern formation
		cuticle chitin biosynthetic process
		cuticle chitin catabolic process
		ecdysis, chitin-based cuticle
		embryonic epithelial tube formation
		extracellular region
		extracellular space
		galactose binding
		hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds
		larval chitin-based cuticle development
		molting cycle, chitin-based cuticle
		multicellular organism reproduction
		perivitelline space
		puparial adhesion
		regulation of adult chitin-containing cuticle pigmentation
		regulation of chitin-based cuticle tanning
		regulation of multicellular organism growth

	search term	Expected GO class (direct)
		structural constituent of chitin-based cuticle
		structural constituent of chitin-based larval cuticle
	molting	determination of adult lifespan
		ecdysone biosynthetic process
		long-term memory
		nucleic acid binding

	search term	Expected GO class (direct)
B	external stimulus	adaptation of rhodopsin mediated signaling
		axon choice point recognition
		axon guidance receptor activity
		axon midline choice point recognition
		branchiomotor neuron axon guidance
		cell chemotaxis
		cellular response to lipopolysaccharide
		chemoattractant activity
		chemorepulsion of axon
		chemotaxis
		deactivation of rhodopsin mediated signaling
		defense response to other organism
		dendrite guidance
		DNA protection
		dorsal/ventral axon guidance
		germ cell attraction
		germ cell repulsion
		G-protein coupled photoreceptor activity
		induction of negative chemotaxis
		lipopolysaccharide receptor activity
		lipopolysaccharide-mediated signaling pathway
		mechanosensory behavior
		metarhodopsin inactivation
		netrin receptor activity involved in chemorepulsion
		olfactory bulb axon guidance
		phospholipase C-activating rhodopsin mediated signaling pathway
		photoreceptor cell axon guidance
		positive chemotaxis
		proboscis extension reflex
		regulation of axon guidance
		regulation of macrophage chemotaxis
		regulation of photoreceptor cell axon guidance
		regulation of response to food

	search term	Expected GO class (direct)
		regulation of rhodopsin mediated signaling pathway
		response to lipopolysaccharide
		response to peptidoglycan
		retinal ganglion cell axon guidance
		rhodopsin mediated signaling pathway
		Roundabout signaling pathway involved in muscle cell chemotaxis toward tendon cell
		semaphorin-plexin signaling pathway involved in axon guidance
		semaphorin-plexin signaling pathway involved in regulation of photoreceptor cell axon guidance
		sensory neuron axon guidance
		startle response
		synaptic target attraction
		synaptic target inhibition
		taxis
	sensory perception	detection of chemical stimulus involved in sensory perception
		detection of chemical stimulus involved in sensory perception of smell
		detection of mechanical stimulus involved in sensory perception
		detection of mechanical stimulus involved in sensory perception of touch
		detection of stimulus involved in sensory perception
		olfactory receptor activity
		sensory perception
		sensory perception of chemical stimulus
		sensory perception of light stimulus
		sensory perception of mechanical stimulus
		sensory perception of smell
		sensory perception of touch
		visual perception

	search term	Expected GO class (direct)
C	hatching	amnioserosa maintenance
		dorsal closure
		dorsal closure, amnioserosa morphology change
		dorsal closure, leading edge cell differentiation
		dorsal closure, leading edge cell fate determination
		embryo development ending in birth or egg hatching
		embryonic development via the syncytial blastoderm
		garland nephrocyte differentiation
		germ-band shortening
		hatching
		hatching behavior
		head involution
		nuclear axial expansion

	search term	Expected GO class (direct)
		nuclear cortical migration
		pseudocleavage involved in syncytial blastoderm formation
		suture of dorsal opening
		syncytial nuclear migration
	metabolism	aminoacyl-tRNA editing activity
		D-tyrosyl-tRNA(Tyr) deacylase activity
		Gly-tRNA(Ala) hydrolase activity
		Ser-tRNA(Ala) hydrolase activity
		trehalose metabolism in response to stress
	reproduction	achiasmate meiosis I
		acrosome assembly
		anterior/posterior axis specification, follicular epithelium
		astral spindle assembly involved in male meiosis
		attachment of spindle microtubules to kinetochore involved in homologous chromosome segregation
		attachment of spindle microtubules to kinetochore involved in meiotic chromosome segregation
		bicoid mRNA localization
		border follicle cell delamination
		centripetally migrating follicle cell migration
		chromosome organization involved in meiotic cell cycle
		clathrin-dependent endocytosis involved in vitellogenesis
		cystoblast division
		cytoskeleton-dependent cytoplasmic transport, nurse cell to oocyte
		distributive segregation
		dorsal/ventral axis specification, ovarian follicular epithelium
		double-strand break repair involved in meiotic recombination
		early meiotic recombination nodule assembly
		egg activation
		eggshell chorion assembly
		eggshell chorion gene amplification
		eggshell formation
		establishment of meiotic spindle localization
		establishment of meiotic spindle orientation
		establishment of pole plasm mRNA localization
		external genitalia morphogenesis
		female courtship behavior
		female gamete generation
		female genitalia development
		female germ-line cyst encapsulation
		female germ-line cyst formation
		female germline ring canal formation
		female germline ring canal formation, actin assembly
		female germline ring canal stabilization

	search term	Expected GO class (direct)
		female germ-line sex determination
		female germ-line stem cell asymmetric division
		female gonad development
		female gonad morphogenesis
		female meiosis chromosome segregation
		female meiosis I
		female meiosis II
		female meiosis sister chromatid cohesion
		female meiotic nuclear division
		female pigmentation
		female pronucleus assembly
		female sex differentiation
		female somatic sex determination
		fertilization, exchange of chromosomal proteins
		fusome organization
		G2/MI transition of meiotic cell cycle
		gamete generation
		generative cell mitosis
		genitalia development
		germ cell proliferation
		germarium-derived cystoblast division
		germarium-derived egg chamber formation
		germarium-derived female germ-line cyst encapsulation
		germarium-derived oocyte fate determination
		germline cell cycle switching, mitotic to meiotic cell cycle
		germ-line cyst formation
		germline ring canal formation
		germ-line sex determination
		gonad development
		gonad morphogenesis
		gonadal mesoderm development
		homologous chromosome segregation
		imaginal disc-derived female genitalia development
		imaginal disc-derived female genitalia morphogenesis
		imaginal disc-derived genitalia development
		imaginal disc-derived male genitalia morphogenesis
		insemination
		internal genitalia morphogenesis
		late meiotic recombination nodule assembly
		maintenance of pole plasm mRNA location
		maternal determination of dorsal/ventral axis, ovarian follicular epithelium, germ-line encoded
		maternal specification of dorsal/ventral axis, oocyte, germ-line encoded

	search term	Expected GO class (direct)
		maternal specification of dorsal/ventral axis, oocyte, soma encoded
		mating plug formation
		meiosis I cytokinesis
		meiosis II cytokinesis
		meiotic cell cycle
		meiotic chromosome condensation
		meiotic chromosome segregation
		meiotic chromosome separation
		meiotic cytokinesis
		meiotic DNA double-strand break formation
		meiotic DNA double-strand break formation involved in reciprocal meiotic recombination
		meiotic DNA double-strand break processing
		meiotic DNA double-strand break processing involved in reciprocal meiotic recombination
		meiotic DNA integrity checkpoint
		meiotic DNA recombinase assembly
		meiotic DNA repair synthesis
		meiotic DNA repair synthesis involved in reciprocal meiotic recombination
		meiotic gene conversion
		meiotic metaphase I plate congression
		meiotic metaphase plate congression
		meiotic mismatch repair
		meiotic mismatch repair involved in reciprocal meiotic recombination
		meiotic nuclear envelope disassembly
		meiotic recombination checkpoint
		meiotic sister chromatid cohesion
		meiotic sister chromatid cohesion, centromeric
		meiotic spindle assembly checkpoint
		meiotic spindle checkpoint
		meiotic spindle midzone assembly
		meiotic spindle organization
		micropyle formation
		Nebenkern assembly
		oocyte construction
		oocyte development
		oocyte differentiation
		oocyte fate commitment
		oocyte karyosome formation
		oocyte localization involved in germarium-derived egg chamber formation
		oocyte maturation
		oocyte microtubule cytoskeleton organization
		oocyte microtubule cytoskeleton polarization
		oocyte morphogenesis

	search term	Expected GO class (direct)
		oocyte nucleus localization involved in oocyte dorsal/ventral axis specification
		oocyte nucleus migration involved in oocyte dorsal/ventral axis specification
		ovarian follicle cell migration
		ovarian follicle cell stalk formation
		ovarian follicle cell-cell adhesion
		ovarian follicle development
		ovarian fusome organization
		oviduct morphogenesis
		ovulation
		P granule organization
		pole cell development
		pole cell fate determination
		pole cell formation
		pole cell migration
		pole plasm assembly
		pole plasm mRNA localization
		pole plasm oskar mRNA localization
		pole plasm protein localization
		pole plasm RNA localization
		premeiotic DNA replication
		primary spermatocyte growth
		pronuclear fusion
		pronuclear migration
		reciprocal meiotic recombination
		regulation of bicoid mRNA localization
		regulation of border follicle cell delamination
		regulation of female receptivity, post-mating
		regulation of fertilization
		regulation of meiotic cell cycle
		regulation of meiotic nuclear division
		regulation of oogenesis
		regulation of ovulation
		regulation of pole plasm oskar mRNA localization
		regulation of transcription from RNA polymerase II promoter involved in spermatogenesis
		reproduction
		reproductive process
		reproductive structure development
		resolution of meiotic recombination intermediates
		single fertilization
		spindle assembly involved in female meiosis
		spindle assembly involved in female meiosis I
		spindle assembly involved in female meiosis II

	search term	Expected GO class (direct)
		spindle assembly involved in male meiosis I
		spindle assembly involved in meiosis
		synapsis
		synaptonemal complex assembly
		vitelline membrane formation involved in chorion-containing eggshell formation
	vitellogin	vitellogenesis
		acylglycerol transport
		basal part of cell
		lipid transporter activity
		lipoprotein particle receptor binding
		sterol transport
	yolk	vitellogenin receptor activity
		ACF complex
		calcium ion binding
		carboxylic ester hydrolase activity
		coated vesicle
		cytoplasmic vesicle
		embryo development
		Ku70:Ku80 complex
		P granule
		protein heterodimerization activity
		regulation of embryonic development
		sex differentiation

Table C2-S3: List of all differentially expressed transcripts (DETs) in *D. galeata* in response to fish kairomones including co-expression module and GO annotation. Hub-genes are highlighted in bold.

transcript	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	gene.set	module	GO.ID	GO.class
trinitytrinloc18424c0t1	73.58148842	-7.193287423	1.666046582	-4.317578812	1.5775E-05	0.006908736	M6	midnightblue		
oasesvelvLoc9845t1	511.6437653	-6.786573381	0.688668539	-9.854629617	6.54576E-23	1.22694E-18	M6	midnightblue	GO:0042302	structural constituent of cuticle
soapsoap331593	122.1465034	-6.674898155	1.694974136	-3.938053102	8.21454E-05	0.026200538	M9	yellow	GO:0042302	structural constituent of cuticle
oasesvelvLoc20812d40883t2	56.87223868	-5.522145988	0.864075535	-6.390813953	1.65005E-10	5.61375E-07	M9	yellow		
trinitytrinloc7528c0t1	12.1944208	-5.205718746	1.240685925	-4.195839285	2.71863E-05	0.009991764	M6	cyan		
soapsoap92491	37.72818101	-5.163751913	1.01732319	-5.075822477	3.85823E-07	0.000328159	M9	yellow	GO:0042302	structural constituent of cuticle
trinitytrinloc19841c0t1	814.5734557	-4.915207127	0.89383897	-5.49898504	3.81984E-08	7.1599E-05	M6	midnightblue	GO:0042302	structural constituent of cuticle
trinitytrinloc20954c0t1	43.21888867	-4.881474475	0.631391131	-7.731300359	1.06454E-14	6.65122E-11	M6	midnightblue		
trinitytrinloc38536c0t1	18.58937561	-4.804299369	1.126584383	-4.264482485	2.00366E-05	0.008164481	M6	grey		
soapsoap373159	358.5424908	-4.621462095	1.288151547	-3.587669561	0.000333647	0.045085831	M9.2	brown		
oasesvelvLoc20887t7	53.11697184	-4.605233035	0.874891851	-5.263774063	1.41128E-07	0.00018895	M6	purple		
trinitytrinloc20807c0t7	55.76811804	-4.605194306	1.263312444	-3.645332814	0.000267046	0.038457436	M9.2	brown		
abyssk80_i_452081	366.8438527	-4.520529555	0.600417897	-7.52897203	5.11413E-14	2.39648E-10	M6	midnightblue		
trinitytrinloc21379c0t1	66.12346623	-4.298507228	0.709945972	-6.05469627	1.40683E-09	5.46553E-06	M9.2	yellow		
trinitytrinloc18166c0t1	38.99444305	-4.081686071	0.716108299	-5.699816744	1.19936E-08	3.72762E-05	M9.2	yellow		
soapsoap442201	164.1313255	-4.078846734	0.744967018	-5.475204455	4.37007E-08	9.70155E-05	M9.2	yellow		
trinitytrinloc13810c0t3	40.97885816	-3.892065636	0.906152974	-4.295152967	1.74573E-05	0.007271544	M6	brown		
oasesvelvLoc8694260887t2	9.60245038	-3.814185194	0.957581896	-3.983142549	6.80099E-05	0.018211117	M6	purple	GO:0042302	structural constituent of cuticle
trinitytrinloc45590c0t1	9.777710988	-3.709788257	0.950292385	-3.90383877	9.46789E-05	0.029733534	M9	yellow		
soapsoap324103	110.81238822	-3.563026725	0.596409048	-5.974132579	2.31318E-09	5.24654E-06	M9	yellow		
soapsoap367845	34.40586671	-3.550405233	0.697179577	-5.092526155	3.53324E-07	0.000313583	M9	blue		
soapsoap405689	20.6532323	-3.415956997	0.692238487	-4.934653392	8.02932E-07	0.000591038	M9	yellow		
oasesvelvLoc1179d42471t2	687.4094772	-3.400104703	0.502915115	-6.760792431	1.37239E-11	5.60292E-08	M9	yellow	GO:0042302	structural constituent of cuticle
soapsoap376028	405.631614	-3.274843528	0.858924892	-3.812723975	0.000137444	0.030669553	M6	midnightblue	GO:0003700	DNA binding transcription factor activity
									GO:0043565	sequence-specific DNA binding
									GO:0006355	regulation of transcription, DNA-templated
trinitytrinloc15510c0t3	92.36899026	-3.218085638	0.62408558	-5.156481328	2.51634E-07	0.0002446	M9	yellow	GO:0042302	structural constituent of cuticle
abyss8406	158.4719798	-3.176698745	0.719678548	-4.414052293	1.01453E-05	0.005051144	M9	yellow		
trinitytrinloc20573c0t5	54.69309998	-3.140549734	0.737756114	-4.256894215	2.07286E-05	0.008635381	M9	yellow		
trinitytrinloc24791c0t2	368.7737359	-3.041827522	0.668617609	-4.549427778	5.3792E-06	0.025701814	M6.2	brown		
oasesvelvLoc3277d15830t1	337.3535463	-3.031269308	0.601929138	-5.035923863	4.7555E-07	0.000821116	M9.2	tan		
oasesvelvLoc1751d464858t2	2779.584509	-2.812527426	0.659919453	-4.261925321	2.02673E-05	0.008619099	M9	yellow	GO:0042302	structural constituent of cuticle
oasesvelvLoc100343	188.0366161	-2.687585839	0.642115216	-4.185519625	2.84514E-05	0.009408742	M9.2	yellow	GO:0006629	lipid metabolic process
trinitytrinloc16995c0t1	647.7384431	-2.640164742	0.602647848	-4.380941131	1.18168E-05	0.005743236	M9	yellow		
oasesvelvLoc795t10	75.97151381	-2.615041869	0.389167507	-6.719579158	1.8225E-11	5.69349E-08	M6	purple		
oasesvelvLoc10386t1	150.3431379	-2.585167546	0.524875726	-4.92529454	8.42334E-07	0.000592916	M9	yellow		
oasesvelvLoc1822t7	40.22588989	-2.581038018	0.621396621	-4.153608066	3.27274E-05	0.011153482	M6	turquoise		
trinitytrinloc9725c0t1	14.29183957	-2.465140817	0.669634327	-3.681323846	0.000232026	0.04727279	M6	yellow		
soapsoap179907	30.0833106	-2.444791328	0.511420411	-4.780394515	1.74952E-06	0.001510415	M9.2	tan		
trinitytrinloc18410c0t1	115.5613716	-2.434944494	0.274952858	-8.855861735	8.30398E-19	7.78249E-15	M6	midnightblue		
trinitytrinloc27145c0t1	53.68623896	-2.401111415	0.499458803	-4.807426358	1.52886E-06	0.001456803	M9.2	brown		
soapsoap341796	99.95178454	-2.3941731	0.483118866	-4.955660542	7.2085E-07	0.00056595	M9	yellow	GO:0004867	serine-type endopeptidase inhibitor activity
oasesvelvLoc13563t1	278.384404	-2.381088893	0.534817019	-4.452156176	8.50123E-06	0.004718182	M9.2	brown		
soapsoapd29821301309	27.53117094	-2.270224822	0.571820004	-3.970173841	7.18202E-05	0.023646225	M9	blue		
trinitytrinloc6156c0t1	96.65865041	-2.253072748	0.495869392	-4.543681831	5.52801E-06	0.003721355	M9.2	tan		
trinitytrinloc15029c0t1	150.4054682	-2.243906649	0.49089908	-4.571014169	4.85369E-06	0.003428473	M9.2	tan		
oasesvelvLoc17963	2068.271482	-2.223542229	0.432131361	-5.14552386	2.66775E-07	0.000247531	M9	yellow		
soapsoap375515	34.56866786	-2.213622182	0.496831613	-4.455476994	8.37069E-06	0.004718182	M9.2	blue		
abyss5311	36.12179456	-2.197347378	0.433681448	-5.066731322	4.04705E-07	0.000505719	M6	midnightblue		
trinitytrinloc22348c0t1	232.238728	-2.193579615	0.410523949	-5.343365764	9.12364E-08	0.000142511	M6	midnightblue		
soapsoap392443	142.4371123	-2.123827812	0.482822395	-4.398776515	1.08863E-05	0.005639096	M9.2	yellow		
trinitytrinloc23759c0t1	161.6924017	-2.090692312	0.462839899	-4.517096122	6.26935E-06	0.003456253	M6	grey		
trinitytrinloc10829c0t1	44.6312329	-2.028497275	0.425472208	-4.767637546	1.86399E-06	0.001519069	M6	midnightblue		

transcript	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	gene.set	module	GO.ID	GO.class
trinitytrinloc21562c0t1	55.08639788	-2.0194960944	0.488433653	-4.134637495	3.55515E-05	0.011049417	M9.2	blue		
oasesvelvLoc18913t4	112.185022	-1.96156471	0.469507728	-4.177917836	2.9419E-05	0.010211658	M6	purple	GO:0042302	structural constituent of cuticle
soapsoap259269	398.6047946	-1.8786023833	0.464693065	-4.042673594	5.28452E-05	0.013918878	M9.2	tan		
soapsoap441829	71.61585989	-1.8339563693	0.431776874	-4.247463172	2.16205E-05	0.008509008	M9.2	blue	GO:0005201	extracellular matrix structural constituent
									GO:0005581	collagen trimer
trinitytrinloc17691c0t4	62.68875512	-1.7583809453	0.400607079	-4.389290748	1.13721E-05	0.005700721	M9.2	red	GO:0007155	cell adhesion
									GO:0016020	membrane
oasesvelvLoc7362t2	251.1168428	-1.707166853	0.360484493	-4.735756695	2.18239E-06	0.001704449	M6	brown		
oasesvelvLoc15430t2	162.5946632	-1.6769209122	0.422065885	-3.973125927	7.09355E-05	0.018466881	M6	purple		
abyss4675	24.95859572	-1.6495198362	0.408702293	-4.035993596	5.43717E-05	0.01408227	M9.2	yellow		
soapsoap376847	54.95373228	-1.647888193	0.380300302	-4.333123533	1.47009E-05	0.006720801	M6	midnightblue		
soapsoapd37724381901	42.55329626	-1.6443449382	0.430767138	-3.817247864	0.000134949	0.025574402	M9.2	brown	GO:0004672	protein kinase activity
									GO:0005524	ATP binding
									GO:0006468	protein phosphorylation
oasesvelvLoc2888t2	72.1671046	-1.5789103441	0.401383486	-3.933670416	8.36585E-05	0.019805922	M9.2	brown		
trinitytrinloc21707c0t1	411.4615713	-1.5163986	0.405611566	-3.738548715	0.000185086	0.031263377	M9.2	yellow	GO:0008234	cysteine-type peptidase activity
									GO:0006508	proteolysis
trinitytrinloc18518c0t1	103.6410422	-1.4881678142	0.375021616	-3.968218769	7.24118E-05	0.018592981	M6	tan	GO:0004180	carboxypeptidase activity
									GO:0006508	proteolysis
									GO:0004181	metallocarboxypeptidase activity
									GO:0008270	zinc ion binding
abyssk76_f_63836	66.39204479	-1.4315495262	0.300154318	-4.769378415	1.84795E-06	0.001519069	M6	midnightblue		
oasesvelvLoc22176t1	176.090533	-1.4124793362	0.363788874	-3.882689761	0.000103307	0.022601371	M9.2	yellow	GO:0006629	lipid metabolic process
trinitytrinloc22909c0t1	86.96867463	-1.3987128183	0.344921879	-4.055158289	5.01004E-05	0.018262477	M9	yellow	GO:0042302	structural constituent of cuticle
trinitytrinloc21949c0t1	359.3821724	-1.3929240743	0.32816684	-4.244560701	2.19022E-05	0.008509008	M9.2	red		
oasesvelvLoc12814t2	405.7499312	-1.3631406343	0.348561965	-3.910755539	9.20079E-05	0.020921237	M9.2	tan		
trinitytrinloc22209c0t3	663.2249835	-1.3152481513	0.341305445	-3.85358092	0.000116403	0.023492195	M9.2	yellow	GO:0004252	serine-type endopeptidase activity
									GO:0006508	proteolysis
oasesvelvLoc16871d40318t1	381.9172302	-1.2719246272	0.280438824	-4.535479828	5.74727E-06	0.003721355	M9.2	tan		
oasesvelvLoc2240d36779t1	118.6228052	-1.2665489752	0.2472894	-5.121727727	3.02749E-07	0.00058809	M9.2	yellow		
oasesvelvLoc1810t4	128.7074402	-1.237801	0.335639113	-3.68789439	0.000226117	0.035138653	M9.2	yellow		
oasesvelvLoc7976d18591t2	168.9694477	-1.2030336153	0.319241624	-3.768410898	0.00016429	0.028729694	M9.2	yellow		
soapsoap245579	379.0147278	-1.195851913	0.314550701	-3.801777923	0.000143661	0.031311518	M6	red		
abyss10349	62.89296022	-1.1806888113	0.286620476	-4.119345646	3.7995E-05	0.011354655	M9.2	blue		
oasesvelvLoc15968d221251t1	54.62958817	-1.172934232	0.301798885	-3.886476249	0.00010171	0.022579592	M9.2	blue	GO:0005201	extracellular matrix structural constituent
									GO:0005581	collagen trimer
oasesvelvLoc11597t2	283.2654188	-1.1698372433	0.224355905	-5.214203052	1.84609E-07	0.000198338	M9	brown	GO:0004222	metalloendopeptidase activity
									GO:0006508	proteolysis
trinitytrinloc1250c0t1	44.00359523	-1.1162522973	0.272863294	-4.090884785	4.29731E-05	0.012275493	M9.2	yellow		
trinitytrinloc23306c0t2	377.9805617	-1.1138354533	0.258311683	-4.311982492	1.61797E-05	0.006908736	M6	turquoise	GO:0008061	chitin binding
									GO:0006030	chitin metabolic process
									GO:0005576	extracellular region
abyssk84_f_251810	124.0320807	-1.1033162223	0.302889291	-3.642638598	0.000269858	0.038457436	M9.2	brown	GO:0005319	lipid transporter activity
									GO:0006869	lipid transport
abyssk28_b_682971	109.1724854	-1.0599838343	0.297748206	-3.560000742	0.000370854	0.049681614	M9.2	yellow		
abyssk60_f_422112	887.3547713	-1.049908473	0.190428662	-5.513395192	3.51977E-08	9.1162E-05	M9.2	yellow		
oasesvelvLoc3120d27765t1	97.54672307	-1.0214663443	0.230765794	-4.426420086	9.58099E-06	0.005134087	M9.2	tan	GO:0005201	extracellular matrix structural constituent
									GO:0005581	collagen trimer
trinitytrinloc25201c0t3	290.7885847	-1.0155249713	0.267538408	-3.795810032	0.000147162	0.026286206	M9.2	yellow	GO:0016614	oxidoreductase activity, acting on CH-OH group of donors
									GO:0050660	flavin adenine dinucleotide binding
									GO:0055114	oxidation-reduction process
oasesvelvLoc364d3560t4	1094.400697	1.0017567344	0.145844181	6.868678126	6.47995E-12	3.30688E-08	M9	salmon		
abyssk84_f_262622	732.8258622	1.0113472922	0.260697197	3.879394572	0.000104717	0.022601371	M9.2	salmon		

transcript	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	gene.set	module	GO.ID	GO.class
soapsoap246465	11252.36708	1.01201852	0.239988174	4.216951616	2.47627E-05	0.009109483	M9.2	salmon		
oasesvelvLoc6472d10905t1	273.0639044	1.02942143	0.22163876	4.644591182	3.40751E-06	0.002647632	M9.2	turquoise		
oasesvelvLoc6902t2	826.6113556	1.043329845	0.243230396	4.289471479	1.79099E-05	0.007731099	M9.2	salmon		
oasesvelvLoc671813	3719.817475	1.045570596	0.248951232	4.199901266	2.67031E-05	0.009408742	M9.2	salmon		
oasesvelvLoc6t3404	869.9013152	1.094843216	0.298272723	3.6706168	0.000241966	0.048767831	M6	magenta		
oasesvelvLoc163066t2	171.7785778	1.133866577	0.29775622	3.808036576	0.000140075	0.025608925	M9.2	salmon		
oasesvelvLoc3503d3931t1	2375.671862	1.162991042	0.24127718	4.820145212	1.43454E-06	0.001344449	M6	turquoise		
trinitytrinloc18280c0t2	260.1778236	1.176651143	0.318807844	3.690784793	0.000223563	0.035092652	M9.2	turquoise	GO:0005506	iron ion binding
									GO:0016705	oxidoreductase activity, acting on paired donors, with ....
									GO:0020037	heme binding
									GO:0055114	oxidation-reduction process
oasesvelvLoc8366t1	318.3111463	1.206818546	0.266910431	4.521436437	6.14214E-06	0.003817954	M9.2	turquoise		
trinitytrinloc22525c0t5	152.0458847	1.25742822	0.332718408	3.779256543	0.000157297	0.04587014	M9	turquoise	GO:0016747	transferase activity, transferring acyl groups other than amino-acyl groups
soapsoap271880	28.20444562	1.258094017	0.349393445	3.600794561	0.000317246	0.043628369	M9.2	turquoise		
soapsoap410607	57.1032533	1.35807677	0.331159163	4.100978995	4.11406E-05	0.016079554	M9	turquoise		
oasesvelvLoc18904d46506t1	1944.949078	1.369881182	0.285444456	4.79911644	1.59367E-06	0.001456803	M9.2	brown	GO:0004252	serine-type endopeptidase activity
									GO:0006508	proteolysis
trinitytrinloc23987c1t2	207.1757706	1.380084666	0.374794451	3.682244131	0.00023119	0.035571192	M9.2	salmon	GO:0004252	serine-type endopeptidase activity
									GO:0006508	proteolysis
trinitytrinloc18575c0t1	146.2981904	1.49139131	0.386118455	3.862522731	0.000112222	0.02596903	M6	turquoise		
abyss10288	291.42496	1.499763074	0.366630736	4.090663788	4.3014E-05	0.0162601	M9	salmon	GO:0004252	serine-type endopeptidase activity
									GO:0006508	proteolysis
oasesvelvLoc212t1	156.6073497	1.51270863	0.407307364	3.713924086	0.00020407	0.033033837	M9.2	turquoise		
oasesvelvLoc23086t7	1396.25577	1.534879252	0.311624725	4.925409081	8.4184E-07	0.001090183	M9.2	brown	GO:0004252	serine-type endopeptidase activity
									GO:0006508	proteolysis
soapsoap442711	165.1179918	1.578227825	0.364227733	4.333079775	1.47038E-05	0.006821551	M9	turquoise	GO:0016758	transferase activity, transferring hexosyl groups
									GO:0008152	metabolic process
trinitytrinloc21451c0t1	1843.937008	1.65801309	0.453439461	3.656525801	0.000255657	0.03783719	M9.2	turquoise	GO:0004672	protein kinase activity
									GO:0005524	ATP binding
									GO:0006468	protein phosphorylation
soapsoap416435	226.4697911	1.731737276	0.359649708	4.815066537	1.47151E-06	0.001456803	M9.2	turquoise	GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds
									GO:0005975	carbohydrate metabolic process
oasesvelvLoc4t3567	3353.796259	1.765568408	0.441802825	3.996281393	6.43452E-05	0.015871827	M9.2	turquoise		
trinitytrinloc20637c0t2	882.2875216	1.775844959	0.421264375	4.215511838	2.49212E-05	0.009109483	M9.2	salmon		
soapsoap343869	57.67717388	1.840471787	0.453283387	4.060311583	4.90073E-05	0.013130569	M9.2	brown		
soapsoap184741	517.3847311	1.851368798	0.451865349	4.097169218	4.18233E-05	0.012262912	M9.2	turquoise		
oasesvelvLoc5985d8251t1	90.27436258	1.85241143	0.494571942	3.745448427	0.000180046	0.030746339	M9.2	turquoise		
oasesvelvLoc7961t1	293.41572007	1.868169954	0.475076661	3.932354729	8.41178E-05	0.019805922	M9.2	turquoise	GO:0004252	serine-type endopeptidase activity
									GO:0006508	proteolysis
soapsoapd36237363089	116.626636	1.99767094	0.325340555	6.140245689	8.23939E-10	4.26801E-06	M9.2	salmon	GO:0004252	serine-type endopeptidase activity
									GO:0006508	proteolysis
abyssk84_f_61659	105.9549528	2.065551922	0.386188739	5.34855555	8.8659E-08	0.000120653	M9	salmon	GO:0016491	oxidoreductase activity
									GO:0008152	metabolic process
soapsoap436175	160.0582483	2.118125079	0.398407816	5.316474718	1.05797E-07	0.000127037	M9	turquoise		
oasesvelvLoc14505d32126t1	1769.071083	2.599216753	0.366983111	7.082660419	1.41413E-12	2.19756E-08	M9.2	salmon	GO:0016788	hydrolase activity, acting on ester bond
abyss11447	459.2093434	2.616922812	0.380271476	6.881722602	5.91331E-12	4.59464E-08	M9.2	salmon	GO:0016788	hydrolase activity, acting on ester bond
trinitytrinloc59114c0t1	908.1201337	2.661240186	0.68089553	3.908441265	9.28935E-05	0.020921237	M9.2	brown		
oasesvelvLoc38047t1	100.4425369	2.783499935	0.711637963	3.911398885	9.17631E-05	0.020921237	M9.2	turquoise	GO:0015074	DNA integration
oasesvelvLoc31634t1	32.00241297	3.157951347	0.721742419	4.375454821	1.2118E-05	0.005884781	M9.2	brown		
oasesvelvLoc10896d11543t1	52.96604392	4.981164339	1.25852682	3.957932609	7.56013E-05	0.018356936	M9.2	brown		
trinitytrinloc19074c0t1	11843.14238	6.156895605	1.639857171	3.754531624	0.000173666	0.029986315	M9.2	turquoise		
oasesvelvLoc43t10	66.62092794	19.21249641	3.593880032	5.345892527	8.99726E-08	0.002149445	M6.2	green		

Table C2-S4: Number of differentially expressed transcripts (DETs) in *D. magna* (p.adj=0.05). After excluding low-count reads 20,696 transcripts remained for differential expression analysis. No DETs were found between *D. magna* exposed to fish kairomone (FK) and in control condition. (A) Results of one-factor analysis. 'Clone' = DETs between clonal lines (Inb1 over Xinb3). (B) Results of two-factor analysis. 'Inb1 vs Xinb3' = differences between the two clonal lines in control condition (Inb1 over Xinb3). 'Inb1 vs Xinb3 FK' = differences between clonal lines exposed to FK (Inb1 over Xinb3).

A

	All	<2-fold	2- to 4-fold	4- to 6-fold	< 6-fold
Clone	2929	1863	901	146	19
up	1597	996	420	45	19
down	1332	867	481	101	0

B

	All	<2-fold	2- to 4-fold	4- to 6-fold	< 6-fold
Inb1 vs Xinb3	2838	1749	914	157	18
up	1544	936	488	102	18
down	1294	813	426	55	0
Inb1 vs Xinb3 FK	2475	1496	821	138	20
up	1153	706	408	39	0
down	1322	790	413	99	20

Table C2-S5: Overview of gene co-expression modules in *D. magna*. The table summarizes module color, total number of transcripts per module, the name of the most interconnected gene (hub-gene) and gene significances (GS) and its *p*-value for treatment (fish environment) and clone (clonal line). The module 'grey' contains all co-expressed genes which were not assigned to a co-expression module.

module	Total number of transcript	hub-gene of co-expression module	GS.treat-ment	p.GStreat-ment	GS.clone	p.Gsclone
turquoise	3911	Dapma7bEVm017592t1	−0.0027	1.00	−1	1e−11
blue	3774	Dapma7bEVm012433t1	0.04	0.90	0.99	**6e−10**
brown	1491	Dapma7bEVm027596t1	−0.26	0.40	0.47	**0.10**
yellow	1146	Dapma7bEVm000539t1	−0.13	0.70	0.1	0.7
green	1127	Dapma7bEVm001258t1	0.12	0.70	−0.061	0.8
red	1060	Dapma7bEVm003400t1	−0.11	0.70	−0.6	0.04
black	914	Dapma7bEVm002170t1	0.12	0.70	0.61	0.04
pink	686	Dapma7bEVm005025t1	−0.25	0.40	−0.52	0.09
magenta	677	Dapma7bEVm029411t1	0.22	0.50	0.49	0.1
purple	535	Dapma7bEVm001058t1	0.20	0.50	0.24	0.5
greenyellow	496	Dapma7bEVm007702t1	0.02	0.90	−0.49	0.1
tan	495	Dapma7bEVm011147t1	−0.16	0.60	0.25	0.4
salmon	431	Dapma7bEVm002963t1	−0.2	0.50	−0.31	0.3
cyan	430	Dapma7bEVm029689t1	0.22	0.50	0.28	**0.4**
midnightblue	331	Dapma7bEVm029214t1	0.11	0.70	−0.55	0.06
lightcyan	316	Dapma7bEVm003072t1	−0.032	0.90	−0.65	0.02
grey60	264	Dapma7bEVm011904t1	−0.25	0.40	−0.54	0.07
lightgreen	262	Dapma7bEVm011198t1	0.09	**0.80**	0.3	0.3
lightyellow	254	Dapma7bEVm017130t1	0.13	0.70	0.57	0.05
royalblue	251	Dapma7bEVm002299t1	0.40	0.20	−0.54	0.07
darkred	250	Dapma7bEVm007405t1	0.00	1.00	0.18	0.6
darkgreen	244	Dapma7bEVm029060t1	0.20	0.50	−0.55	0.06
darkturquoise	231	Dapma7bEVm011418t1	0.06	0.90	0.76	0.004
darkgrey	158	Dapma7bEVm019807t1	−0.025	0.90	0.49	0.1
orange	145	Dapma7bEVm006913t1	−0.2	0.50	−0.095	0.8
darkorange	136	Dapma7bEVm019167t1	−0.13	0.70	−0.48	0.1
white	134	Dapma7bEVm025508t1	0.35	0.30	0.3	**0.3**
skyblue	128	Dapma7bEVm010777t1	−0.038	0.90	−0.64	0.02
saddlebrown	110	Dapma7bEVm009300t1	0.13	0.70	−0.52	0.08
steelblue	95	Dapma7bEVm002018t1	−0.23	0.50	0.51	0.09
paleturquoise	89	Dapma7bEVm019164t1	−0.044	**0.90**	0.25	0.4
violet	52	Dapma7bEVm005794t1	0.26	0.40	−0.2	0.5
grey	73	Genes not assigned to a module	x	x	x	x

Table C2-S6: List of unique, enriched GO terms with orthogroups containing reproduction-related transcripts of *D. galeata* and *D. pulex*.

GO.ID	GO.class	orthogroup 1	orthogroup 2	orthogroup 3	orthogroup 4	orthogroup 5	orthogroup 6
GO:0000166	nucleotide binding	ORTHO_ALL503					
GO:0003677	DNA binding	ORTHO_ALL26					
GO:0004180	carboxypeptidase activity	ORTHO_ALL4431					
GO:0004181	metallocarboxypeptidase activity	ORTHO_ALL4431					
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	ORTHO_ALL63					
GO:0004672	protein kinase activity	ORTHO_ALL368	ORTHO_ALL63				
GO:0004930	G-protein coupled receptor activity	ORTHO_ALL11467	ORTHO_ALL20474	ORTHO_ALL6318			
GO:0005230	extracellular ligand-gated ion channel activity	ORTHO_ALL1555					
GO:0005328	neurotransmitter:sodium symporter activity	ORTHO_ALL2244					
GO:0005506	iron ion binding	ORTHO_ALL37					
GO:0005515	protein binding	ORTHO_ALL2670	ORTHO_ALL548				
GO:0005524	ATP binding	ORTHO_ALL104	ORTHO_ALL21	ORTHO_ALL73			
GO:0005525	GTP binding	ORTHO_ALL445	ORTHO_ALL9194				
GO:0005975	carbohydrate metabolic process	ORTHO_ALL63					
GO:0006310	DNA recombination	ORTHO_ALL26					
GO:0006468	protein phosphorylation	ORTHO_ALL368	ORTHO_ALL63				
GO:0006508	proteolysis	ORTHO_ALL4431					
GO:0006629	lipid metabolic process	ORTHO_ALL883					
GO:0006810	transport	ORTHO_ALL1555	ORTHO_ALL73				
GO:0006811	ion transport	ORTHO_ALL1555					
GO:0006812	cation transport	ORTHO_ALL503					
GO:0006836	neurotransmitter transport	ORTHO_ALL2244					
GO:0006904	vesicle docking involved in exocytosis	ORTHO_ALL2429					
GO:0007165	signal transduction	ORTHO_ALL548					
GO:0007186	G-protein coupled receptor signaling pathway	ORTHO_ALL11467	ORTHO_ALL20474	ORTHO_ALL6318			
GO:0007264	small GTPase mediated signal transduction	ORTHO_ALL9194					
GO:0008146	sulfotransferase activity	ORTHO_ALL495					
GO:0008152	metabolic process	ORTHO_ALL82	ORTHO_ALL968				
GO:0008270	zinc ion binding	ORTHO_ALL4431	ORTHO_ALL5324				
GO:0009190	cyclic nucleotide biosynthetic process	ORTHO_ALL368					
GO:0015074	DNA integration	ORTHO_ALL26					
GO:0016020	membrane	ORTHO_ALL104	ORTHO_ALL1555	ORTHO_ALL21			
GO:0016021	integral component of membrane	ORTHO_ALL11467	ORTHO_ALL21	ORTHO_ALL20474	ORTHO_ALL2244	ORTHO_ALL503	ORTHO_ALL6318
GO:0016192	vesicle-mediated transport	ORTHO_ALL2429					
GO:0016491	oxidoreductase activity	ORTHO_ALL10872	ORTHO_ALL5324				
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	ORTHO_ALL37					
GO:0016758	transferase activity, transferring hexosyl groups	ORTHO_ALL82	ORTHO_ALL968				
GO:0016831	carboxy-lyase activity	ORTHO_ALL210					
GO:0016849	phosphorus-oxygen lyase activity	ORTHO_ALL368					
GO:0016887	ATPase activity	ORTHO_ALL104	ORTHO_ALL21	ORTHO_ALL503			
GO:0019752	carboxylic acid metabolic process	ORTHO_ALL210					
GO:0020037	heme binding	ORTHO_ALL37					
GO:0030170	pyridoxal phosphate binding	ORTHO_ALL210					
GO:0042626	ATPase activity, coupled to transmembrane movement of substances	ORTHO_ALL73					
GO:0055085	transmembrane transport	ORTHO_ALL73					
GO:0055114	oxidation-reduction process	ORTHO_ALL37	ORTHO_ALL5324				
GO:0080019	fatty-acyl-CoA reductase (alcohol-forming) activity	ORTHO_ALL79					
GO:0098599	palmitoyl hydrolase activity	ORTHO_ALL7855					

Table C3-S1: Raw life history trait data used as input for GWA analysis in the control and fish environments. The life history traits are clonal line ('clone'), total number of offspring per brood (1st brood= 'brood1', etc. up to 'brood4'), age at first reproduction ('AFR', day of releasing neonates from brood pouch), total number of broods ('broods'), total number of offspring ('offspring'), body length ('size', µm) and somatic growth rate ('SGR', µm/day).

available on supplementary CD

Table C3-S2: Mean values of the life history trait data used as input for GWA analysis in the control and fish environments. The life history traits are clonal line ('clone'), total number of offspring per brood (1st brood= 'brood1', etc. up to 'brood4'), age at first reproduction ('AFR', day of releasing neonates from brood pouch), total number of broods ('broods'), total number of offspring ('offspring'), body length ('size', µm) and somatic growth rate ('SGR', µm/day).

available on supplementary CD

Table C3-S3: GWA results of the "inflated dataset" in control and fish environment as well as the GxE interaction.

	Control: -log10P > 1.5	**Fish: -log10P > 1.5**	**GxE: -log10P > 1.5**
brood1_snps	5258	7309	0
brood1_transcripts	2457	3049	0
brood2_snps	13018	14420	8
brood2_transcripts	4686	5008	5
brood3_snps	11231	18383	49
brood3_transcripts	4206	5716	40
brood4_snps	3	7	0
brood4_transcripts	3	7	0
afr_snps	165	8625	24
afr_transcripts	115	3071	20
broods_snps	2656	3306	74
broods_transcripts	1414	1652	50
offspring_snps	23284	32126	0
offspring_transcripts	6374	7486	0
survival_snps	0	0	6
survival_transcripts	0	0	3
length_snps	22085	19998	23
length_transcripts	6335	6026	21
sgr_snps	18763	18838	10
sgr_transcripts	5774	5837	10

Table C3-S4: Overview of gene co-expression modules in *D. galeata* in control environment from WGCNA. The table summarizes module color, total number of transcripts per module, the name of the most interconnected gene (hub-gene), as well as Gene Ontology (GO) IDs and classes. The module 'grey' contains all co-expressed genes which were not assigned to a co-expression module. .

moduleColor	total number of transcripts	hub-gene of co-expression modules	GO.ID	GO.class
grey	7297			
turquoise	3017	trinitytrinloc25363c0t1	GO:0008061	chitin binding
			GO:0006030	chitin metabolic process
blue	2570	oasesvelvLoc107d35313t1		
brown	2213	oasesvelvLoc12896t2	GO:0015031	protein transport
			GO:0016021	integral component of membrane
yellow	2196	abyssk26_j_731017	GO:0016020	membrane
green	1036	oasesvelvLoc27382t4	GO:0004672	protein kinase activitiy
			GO:0005524	ATP binding
red	996	soapsoapd12459370536		
black	958	oasesvelvLoc5318t1		
pink	828	oasesvelvLoc18341d44940t1		
magenta	797	soapsoapd37772382671	GO:0003700	DNA binding transcription factor activity
			GO:0008270	zinc ion binding
purple	780	oasesvelvLoc889t6		
greenyellow	648	soapsoap351951		
tan	622	soapsoap449937		
salmon	621	abyssk32_j_646314	GO:0005634	nucleus
cyan	577	oasesvelvLoc10279t3	x	x
midnightblue	543	soapsoapd376202061	x	x
lightcyan	535	abyssk34_f_723421	x	x
grey60	526	abyssk84_f_37405	x	x
lightgreen	524	abyss840	GO:0005515	protein binding
lightyellow	458	trinitytrinloc24022c0t2		
royalblue	405	trinitytrinloc32092c0t1	GO:0008234	cysteine-type peptidase activity
			GO:0006508	proteolysis
darkred	394	oasesvelvLoc7394d43926t2		
darkgreen	371	soapsoap356503	GO:0042302	structural constituent of cuticle
darkturquoise	309	soapsoap174291		
darkgrey	308	trinitytrinloc23766c0t3	GO:0005515	protein binding
			GO:0016020	membrane
orange	295	abyssk28_f_692990	GO:0015078	proton transmembrane transporter activity
			GO:0015986	ATP synthesis coupled proton

				transport
moduleColor	**total number of transcripts**	**hub-gene of co-expression modules**	**GO.ID**	**GO.class**
darkorange	270	soapsoap384802		
white	242	oasesvelvLoc7501d8444t1		
skyblue	238	oasesvelvLoc20412d23507t1		
saddlebrown	236	abyssk30_f_3437		
steelblue	231	oasesvelvLoc3461t3		
paleturquoise	222	abyssk72_f_479667	GO:0042302	structural constituent of cuticle
violet	171	soapsoapd11549355087		
darkolivegreen	166			
darkmagenta	154	trinitytrinloc25721c1t2	GO:0003677	DNA binding
			GO:0005524	ATP binding
sienna3	131	oasesvelvLoc917d9903t2		
yellowgreen	108	oasesvelvLoc1851t4		
skyblue3	94	trinitytrinloc15529c0t1		
plum1	86	oasesvelvLoc10900t4		
orangered4	85	abyssk24_b_768638		
mediumpurple3	84	abyssk34_f_188870	GO:0003678	DNA helicase activity
			GO:0005524	ATP binding
lightsteelblue1	73	oasesvelvLoc4832t3		
ivory	70	oasesvelvLoc401t4		
lightcyan1	70	oasesvelvLoc312d7487t3	GO:0008234	cysteine-type peptidase activity
			GO:0006508	proteolysis

Table C3-S5: Functional annotation of candidate transcripts of interest as identified in the univariate and multivariate GWA analysis and WGCNA. A total of 156 candidate transcripts are listed. 'orthogroup' = orthoMCL cluster with the assigned transcripts. 'dgal' = number of *D. galeata* transcripts present in the assigned orthoMCL cluster. 'dpul' = number of *D. pulex* transcripts present in the assigned orthoMCL cluster. 'dmag' = number of *D. magna* transcripts present in the assigned orthoMCL cluster. 'dme' = number of *Drosophila melanogaster* transcripts present in the assigned orthoMCL cluster. 'nvi' = number of *Nasonia vitripennis* transcripts present in the assigned orthoMCL cluster. 'tBLASTx' = search in translated nucleotide database. 'BLASTx' = search in protein database. 'hit' = transcript that is significantly similar in *Daphnia* stressor database to the candidate transcript of interest. 'identiy percent' = BLAST identity percentage corresponding to the *Daphnia* stressor database hit.

											tBLASTx			BLASTx			
Transcripts	Analysis	Environment	Trait	orthogroup	dgal	dpul	dmag	dme	nvi	GO_annotation	tBLASTx_hit	tBLASTx_iden	Stressor_tBLASTx	BLASTx_hit	BLASTx_iden	Stressor_BLASTx
trinitytrinloc25908c0t3	GWAS_univariate	fish	brood4	ORTHO_ALL527	2	1	1	0	1							
soapsoap324748	GWAS_univariate	control	brood4	ORTHO_ALL18855	1	0	0	0	0							
abyssk32_f_282383	GWAS_univariate	control	brood4	ORTHO_ALL12465	1	1	0	0	0	GO:0004222, GO:0006508,	DappuDraft_302051	59,2106	Salinity			
abyssk22_f_855011	GWAS_univariate	fish	brood3	ORTHO_ALL268	6	4	4	4	5	GO:0005524, GO:0016887,						
abyssk40_f_658599	GWAS_univariate	fish	brood3	ORTHO_ALL5300	1	1	1	1	1							
trinitytrinloc25796c0t2	GWAS_univariate	fish	brood3	ORTHO_ALL6438	1	1	1	1	1	GO:0005515,						
trinitytrinloc15632c0t6	GWAS_univariate	gxe	offspring	ORTHO_ALL5309	1	1	2	0	0	GO:0007283,						
oasesevlvloc2202d2711344	GWAS_multivariate	control	all	ORTHO_ALL7667	1	1	1	0	0							
soapsoap398707	GWAS_multivariate	control	all	ORTHO_ALL344	18	0	0	0	0							
trinitytrinloc24633c0t1	GWAS_multivariate	control	all	ORTHO_ALL588	2	3	3	0	0	GO:0004553, GO:0005975,	DappuDraft_326098	72,1866	Light Dark Cycle			
oasesevlvloc6273d61385	GWAS_multivariate	control	all	ORTHO_ALL590	2	2	2	2	1	GO:0016491, GO:0055114,	DappuDraft_188248	70,9645	Phosphorous			
oasesevlvloc1345t5	GWAS_multivariate	control	all	ORTHO_ALL301	2	4	4	3	4	GO:0003774, GO:0005524, GO:0016459, GO:0005515, GO:0005856,						
soapsoap378541	GWAS_multivariate	control	all	ORTHO_ALL10824	1	1	1	0	0	GO:0003777, GO:0005524, GO:0008017, GO:0007018,						
abyssk84_f_13467	GWAS_multivariate	control	all	ORTHO_ALL8014	1	2	2	0	0							
soapsoap449363	GWAS_multivariate	control	all	ORTHO_ALL542	3	3	5	1	1	GO:0003824, GO:0008152,	Dapma7bEVm004083t1	67,4772	Tri Nitro Toluene			
oasesevlvloc1384d265212	GWAS_multivariate	control	all	ORTHO_ALL5612	1	1	1	1	1	GO:0005515, GO:0005737,						
trinitytrinloc25844c0t12	GWAS_multivariate	control	all	ORTHO_ALL333	4	2	3	3	2	GO:0005509, GO:0007156, GO:0016020, GO:0005515,						
oasesevlvloc546662	GWAS_multivariate	control	all	ORTHO_ALL3010	1	2	1	1	1							
trinitytrinloc20144c2t5	GWAS_multivariate	control	all	ORTHO_ALL4658	1	2	1	1	1	GO:0005515,						
trinitytrinloc25497c0t6	GWAS_multivariate	control	all	ORTHO_ALL390	1	1	1	1	0							
oasesevlvloc4766d106241t1	GWAS_multivariate	control	all	ORTHO_ALL7635	1	1	0	0	0							
oasesevlvloc593t7	GWAS_multivariate	control	all	ORTHO_ALL342	5	7	7	3	2	GO:0005328, GO:0006836, GO:0016021,						
trinitytrinloc22913c0t7	GWAS_multivariate	control	all	ORTHO_ALL6660	1	1	1	2	1	GO:0004332, GO:0006096,						
trinitytrinloc20791c0t3	GWAS_multivariate	control	all	ORTHO_ALL1143	2	2	1	1	2	GO:0004672, GO:0005524, GO:0006468,						
trinitytrinloc24368c0t5	GWAS_multivariate	control	all	ORTHO_ALL152	7	1	2	0	0	GO:0003676,						
oasesevlvloc9097[illegible]5772	GWAS_multivariate	control	all	ORTHO_ALL838	1	4	2	0	0	GO:0005515, GO:0000228,						
trinitytrinloc24095c0t1	GWAS_multivariate	control	all	ORTHO_ALL3185	2	1	1	0	0							
oasesevlvloc116206946t2	GWAS_multivariate	control	all	ORTHO_ALL5376	1	1	1	1	1	GO:0005515,						
trinitytrinloc24918c0t1	GWAS_multivariate	control	all	ORTHO_ALL367	4	5	3	0	1	GO:0055085, GO:0016021,						
trinitytrinloc25138c1t20	GWAS_multivariate	control	all	ORTHO_ALL4665	1	1	1	0	0							
trinitytrinloc21653c0t1	GWAS_multivariate	control	all	ORTHO_ALL6333	1	1	1	1	1							
abyssk34_f_729597	GWAS_multivariate	gxe	all	ORTHO_ALL2541	1	1	1	1	2	GO:0003723, GO:0006396, GO:0005515,						
trinitytrinloc20056c0t3	GWAS_multivariate	gxe	all	ORTHO_ALL7934	1	1	0	1	1	GO:0004222, GO:0006508,	DappuDraft_254737	74,11	Salinity; Light Dark Cycle; Phosphorous			
abyssk34_f_231353	GWAS_multivariate	gxe	all	ORTHO_ALL10125	1	1	1	0	0							
soapsoap324748	GWAS_multivariate	gxe	all	ORTHO_ALL18855	1	0	0	0	0							
abyssk60_f_293849	GWAS_multivariate	gxe	all	ORTHO_ALL15855	1	0	0	0	0	GO:0004672, GO:0006468,						
trinitytrinloc22396c0t2	GWAS_multivariate	gxe	all	ORTHO_ALL3492	1	2	3	0	0							
soapsoap390254	GWAS_multivariate	gxe	all	ORTHO_ALL14997	1	1	1	0	0							
trinitytrinloc23826c0t5	GWAS_multivariate	gxe	all	ORTHO_ALL10752	1	1	1	0	0							
trinitytrinloc23664c0t1	GWAS_multivariate	gxe	all	ORTHO_ALL7919	1	1	1	0	0							
oasesevlvloc764215	GWAS_multivariate	gxe	all	ORTHO_ALL7034	3	0	0	0	0							
abyssk32_f_282383	GWAS_multivariate	gxe	all	ORTHO_ALL12465	1	0	0	0	0	GO:0004222, GO:0006508,	DappuDraft_302051	59,2106	Salinity			
trinitytrinloc14146c0t1	GWAS_multivariate	gxe	all	ORTHO_ALL11216	1	0	3	0	0	GO:0008417, GO:0006486, GO:0016020,						
soapsoap858198123	GWAS_multivariate	gxe	all	ORTHO_ALL2635	2	1	1	1	1	GO:0005515,						
abyssk26_f_347901	GWAS_multivariate	gxe	all	ORTHO_ALL581	5	6	6	1	1	GO:0005506, GO:0016705, GO:0020037, GO:0055114,						
trinitytrinloc19638c1t2	GWAS_multivariate	gxe	all	ORTHO_ALL3311	1	1	1	1	1	GO:0004114, GO:0007165,	DappuDraft_321218	63,6142	Phosphorous			
trinitytrinloc19154c0t4	GWAS_multivariate	gxe	all	ORTHO_ALL3403	1	1	1	1	1	GO:0002161,						
trinitytrinloc18105c1t3	GWAS_multivariate	gxe	all	ORTHO_ALL3317	1	2	2	1	1	GO:0008270,						
oasesevlvloc564d380441t1	GWAS_multivariate	gxe	all	ORTHO_ALL10025	1	1	1	0	0							
trinitytrinloc25497c0t6	GWAS_multivariate	gxe	all	ORTHO_ALL390	1	1	1	1	0							
abyss9699	GWAS_multivariate	gxe	all	ORTHO_ALL5025	1	1	1	1	1	GO:0005525, GO:0005634, GO:0005737,						
oasesevlvloc1699804345t1	GWAS_multivariate	gxe	all	ORTHO_ALL4372	1	0	0	0	0							
oasesevlvloc1135212	GWAS_multivariate	gxe	all	ORTHO_ALL14353	1	1	1	0	0	GO:0004797, GO:0005524,						

												tBLASTx			BLASTx		
Transcripts	Analysis	Environment	Trait	orthogroup	dgal	dpul	dmag	dme	nvi	GO_annotation	tBLASTx_hit	tBLASTx_iden	Stressor_tBLASTx	BLASTx_hit	BLASTx_iden	Stressor_BLASTx
trinitytrinloc11417c0t1	GWAS_multivariate	gxe	all	ORTHO_AUL15047	1	1	1	0	0							
oasesvelvloc7501d2993t2	GWAS_multivariate	gxe	all	ORTHO_AUL510	1	1	0	0	0	GO:0004222, GO:0008270, GO:0006508, GO:0031012,						
abyss3928	GWAS_multivariate	gxe	all	ORTHO_AUL7076	1	1	1	0	0	GO:0005515,	DappuDraft_319446	56,1507	Temperature			
abyssk24_f_775461	GWAS_multivariate	gxe	all	ORTHO_AUL5191	1	1	1	1	1							
soapsoap392443	GWAS_multivariate	gxe	all	ORTHO_AUL12802	1	1	0	0	0							
trinitytrinloc24609c0t3	GWAS_multivariate	gxe	all	ORTHO_AUL2402	1	1	1	1	2		Dapma7bEVm005424t1	64,9378	Tri Nitro Toluene			
oasesvelvloc557911	GWAS_multivariate	gxe	all	ORTHO_AUL918	4	3	2	1	1	GO:0005515, GO:0004435, GO:0006629, GO:0007165, GO:0035556,						
soapsoap389937	GWAS_multivariate	gxe	all	ORTHO_AUL13425	1	1	1	1	1	GO:0016020,	DappuDraft_191157	52,7836	Phosphorous			
trinitytrinloc12328c0t2	GWAS_multivariate	gxe	all	ORTHO_AUL10265	1	1	1	0	0	GO:0003700, GO:0043565, GO:0006355,						
oasesvelvloc5887d17451t1	GWAS_multivariate	gxe	all	ORTHO_AUL1702	1	2	1	1	1	GO:0005515, GO:0004672, GO:0006468,						
oasesvelvloc13048d2097571	GWAS_multivariate	gxe	all	ORTHO_AUL8804	1	1	1	1	1							
oasesvelvloc5843d43690t1	GWAS_multivariate	gxe	all	ORTHO_AUL253	3	4	6	4	3	GO:0035556, GO:0005515, GO:0004672, GO:0005524, GO:0006468,						
trinitytrinloc21598c0t2	GWAS_multivariate	gxe	all	ORTHO_AUL24	20	6	22	0	0	GO:0005515,						
oasesvelvloc1345t5	GWAS_multivariate	gxe	all	ORTHO_AUL301	2	4	4	3	4	GO:0003774, GO:0005524, GO:0016459, GO:0005515, GO:0005856,						
trinitytrinloc18365c0t1	GWAS_multivariate	gxe	all	ORTHO_AUL6050	1	2	1	0	1	GO:0004866, GO:0005615, GO:0005576,						
trinitytrinloc25696c1t2	GWAS_multivariate	gxe	all	ORTHO_AUL2696	1	1	3	1	1	GO:0003677, GO:0006355,						
trinitytrinloc24931c0t1	GWAS_multivariate	gxe	all	ORTHO_AUL1336	1	1	1	1	1	GO:0045454,						
trinitytrinloc25838c2t1	GWAS_multivariate	gxe	all	ORTHO_AUL2522	1	1	4	1	1	GO:0005509, GO:0005515,						
soapsoapd44175363445	WGCNA	control	broods	ORTHO_AUL156	15	0	0	0	0	GO:0004252, GO:0006508,						
trinitytrinloc17566c0t1	WGCNA	control	broods	ORTHO_AUL10786	1	1	1	0	0							
soapsoap312581	WGCNA	control	broods	ORTHO_AUL280	11	0	0	0	0							
oasesvelvloc2111t0d237201t1	WGCNA	control	broods							GO:0008277,						
soapsoap417471	WGCNA	control	broods	ORTHO_AUL280	11	0	0	0	0							
abyssk84_f_236803	WGCNA	control	broods	ORTHO_AUL187	20	0	0	0	0							
trinitytrinloc21568c0t2	WGCNA	control	broods													
trinitytrinloc42914c0t1	WGCNA	control	broods													
abyssk36_b_702278	WGCNA	control	broods	ORTHO_AUL404	7	0	0	0	0	GO:0004672, GO:0005524, GO:0006468,						
soapsoap391499	WGCNA	control	broods	ORTHO_AUL15593	1	0	0	0	0		Dapma7bEVm000945t1	62,1952	Chlamydomonas; Microcystin; Lead; Tri Nitro Toluene			
soapsoapd309283087 39	WGCNA	control	broods	ORTHO_AUL166	21	1	0	0	0	GO:0004672, GO:0005524, GO:0006468,						
trinitytrinloc22220c0t1	WGCNA	control	broods	ORTHO_AUL125	15	3	2	0	0							
abyssk60_j_540606	WGCNA	control	broods	ORTHO_AUL2147	1	1	2	1	1		Dapma7bEVm015471t1	50,5882	Acrylonitrile; Chloro Vinyl Ether; 20-hydroxyecdysone; Phenol; MTBE; PonasteroneA; Trichloroethylene; Atrazine; Dichlorobenzene; Beta Estradiol; Parathion; Diazinon; Phenanthrene; Pyripoxyfen; Methoxychlor; Toxaphene; Bifenthrin; Lambda Cyhalothrin; Permethrin; Tri Nitro Toluene;			
trinitytrinloc21412c0t10	WGCNA	control	broods	ORTHO_AUL3050	3	1	2	5	0	GO:0004222, GO:0006508,	DappuDraft_93694	62,7273	Salinity	DAPPUDRAFT_347623	63,7501	hypoxia
trinitytrinloc17214c0t3	WGCNA	control	broods	ORTHO_AUL940	2	1	0	0	0							
oasesvelvloc188161	WGCNA	control	broods	ORTHO_AUL19804	1	0	1	0	0	GO:0004550, GO:0005524, GO:0006165, GO:0006183, GO:0006228, GO:0006241,	NM_008704.2	62,0226	Dinitrobenzene			
abyssk22_f_12916	WGCNA	control	broods													
trinitytrinloc30564c0t1	WGCNA	control	broods	ORTHO_AUL8054	1	0	4	0	0							
trinitytrinloc37310c0t1	WGCNA	control	broods													
abyss5310	WGCNA	control	broods	ORTHO_AUL737	9	1	0	0	0	GO:0005515,						
oasesvelvloc85t12	WGCNA	control	broods	ORTHO_AUL9371	1	1	1	1	1	GO:0005524, GO:0042626, GO:0006810, GO:0055085, GO:0016021, GO:0016887,	Dapma7bEVm001092t1	58,7793	Tri Nitro Toluene			
abyss7590	WGCNA	control	broods	ORTHO_AUL12363	2	0	0	0	0							
soapsoap359419	WGCNA	control	broods	ORTHO_AUL4987	2	0	0	0	0	GO:0004672, GO:0006468,						
trinitytrinloc20470c0t1	WGCNA	control	broods	ORTHO_AUL13117	2	0	0	0	0							
soapsoapd396832678 49	WGCNA	control	broods													
trinitytrinloc49177c0t1	WGCNA	control	broods													
trinitytrinloc34171c0t1	WGCNA	control	broods													
abyssk40_f_333407	WGCNA	control	broods	ORTHO_AUL779	8	1	2	0	0							
trinitytrinloc36041c0t1	WGCNA	control	broods	ORTHO_AUL9848	1	1	1	1	1	GO:0003700, GO:0043565, GO:0006355,						
oasesvelvloc237913	WGCNA	control	broods	ORTHO_AUL3762	1	1	1	1	2	GO:0015116, GO:0008272, GO:0016021,						
soapsoap395775	WGCNA	control	broods													
soapsoap384802	WGCNA	control	broods	ORTHO_AUL6261	1	1	1	0	1							
oasesvelvloc2330t1	WGCNA	control	broods	ORTHO_AUL2424	1	1	1	1	1	GO:0005524, GO:0004222, GO:0006508,						
oasesvelvloc97678892t2	WGCNA	control	broods													
trinitytrinloc23036c0t1	WGCNA	control	broods	ORTHO_AUL4199	1	1	1	1	1							

Transcripts	Analysis	Environment	Trait	orthogroup	dgal	dpul	dmag	dme	nvi	GO_annotation	tBLASTx			BLASTx		
											tBLASTx_hit	tBLASTx_iden	Stressor_tBLASTx	BLASTx_hit	BLASTx_iden	Stressor_BLASTx
abyss5709	WGCNA	control	broods	ORTHO_ALL4983	2	0	0	0	0	GO:0003676, GO:0005524,						
abyss5632	WGCNA	control	broods													
soapsoap233253	WGCNA	control	broods											ABD19215	73,9335	Cadmium
abyssk84_f_221570	WGCNA	control	broods	ORTHO_ALL7	124	0	0	0	0		DappuDraft_257184	52,941	Temperature			
abyssk76_f_462372	WGCNA	control	broods	ORTHO_ALL5561	1	1	1	1	1							
oasesvelvLoc395t1	WGCNA	control	broods							GO:0008483, GO:0030170,	Dapma7bEVm010837t1	60,3632	Acrylonitrile; Chloro Vinyl Ether; 20-hydroxyecdysone; Phenol; MTBE; Chloroform; PonasteroneA; Trichloroethylene; Atrazine; Dichlorobenzene; Beta Estradiol; Diazinon; Phenanthrene; Methoxychlor; Chlorpyrifos; Toxaphene; Methyl Farnesoate; Bifenthrin; Lambda Cyhalothrin; Nonylphenol; Permethrin; Tri Nitro Toluene; Epofenonane;			
trinitytrinloc24577c0t1	WGCNA	control	broods	ORTHO_ALL2100	1	2	0	0	0							
trinitytrinloc20212c0t4	WGCNA	control	broods	ORTHO_ALL6418	5	0	0	0	0							
trinitytrinloc33183c0t1	WGCNA	control	broods													
oasesvelvLoc6289d17688t1	WGCNA	control	broods	ORTHO_ALL19907	1	1	0	0	0	GO:0005515,						
soapsoapd45262380885	WGCNA	control	broods													
soapsoapd23587267991	WGCNA	control	broods													
soapsoap371088	WGCNA	control	broods	ORTHO_ALL1350	3	1	2	0	0	GO:0004930, GO:0007186, GO:0016021,						
trinitytrinloc34628c0t1	WGCNA	control	broods													
soapsoapd557203853	WGCNA	control	broods	ORTHO_ALL7014	1	2	1	1	1	GO:0003677,						
soapsoapd14317394433	WGCNA	control	broods													
oasesvelvLoc2927d7883t2	WGCNA	control	broods	ORTHO_ALL48	27	0	0	0	0							
soapsoap375229	WGCNA	control	broods	ORTHO_ALL398	9	0	0	0	0							
oasesvelvLoc3698t3	WGCNA	control	broods	ORTHO_ALL324	10	3	2	0	1		hxAUG25s183g211t1	67,5888	Fish kairomones			
soapsoap378521	WGCNA	control	broods	ORTHO_ALL5	87	5	5	0	0							
oasesvelvLoc17558t2	WGCNA	control	broods	ORTHO_ALL8912	1	1	1	1	1							
oasesvelvLoc3505t1	WGCNA	control	broods	ORTHO_ALL38	38	0	0	0	0							
oasesvelvLoc1704d1887t1	WGCNA	control	broods	ORTHO_ALL3008	1	1	2	1	1	GO:0019901, GO:0000079,						
oasesvelvLoc2936t1	WGCNA	control	broods	ORTHO_ALL5	87	5	5	0	0							
oasesvelvLoc13262t1	WGCNA	control	broods	ORTHO_ALL3055	1	1	2	0	0	GO:0008483, GO:0030170,	DappuDraft_209533	67,608	Phosphorous			
soapsoap315181	WGCNA	control	broods	ORTHO_ALL3	138	3	1	1	0	GO:0004672, GO:0006468,						
soapsoapd11156349605	WGCNA	control	broods	ORTHO_ALL166	21	1	0	0	0	GO:0004672, GO:0005524, GO:0006468,						
abyssk72_f_138736	WGCNA	control	broods	ORTHO_ALL3787	2	1	0	0	0							
trinitytrinloc25809c0t15	WGCNA	control	broods	ORTHO_ALL13807	2	0	0	0	0							
abyss7623	WGCNA	control	broods	ORTHO_ALL166	21	1	0	0	0	GO:0004672, GO:0005524, GO:0006468,						
trinitytrinloc32557c0t1	WGCNA	control	broods													
soapsoap309681	WGCNA	control	broods													
soapsoap422203	WGCNA	control	broods	ORTHO_ALL24	20	6	22	0	0	GO:0005515,						
soapsoapd32516319717	WGCNA	control	broods	ORTHO_ALL9	103	2	0	0	0							
soapsoap445117	WGCNA	control	broods	ORTHO_ALL459	12	0	0	0	0							
oasesvelvLoc11854t1	WGCNA	control	broods	ORTHO_ALL63	17	18	19	0	1	GO:0004672, GO:0006468,						
soapsoap297019	WGCNA	control	broods	ORTHO_ALL133	20	0	0	0	0							
trinitytrinloc30618c0t1	WGCNA	control	broods													
abyssk22_f_206059	WGCNA	control	broods	ORTHO_ALL18226	1	0	0	0	0							
abyssk34_b_719141	WGCNA	control	broods													
soapsoap363545	WGCNA	control	broods	ORTHO_ALL13080	1	0	0	0	0							
soapsoap236301	WGCNA	control	broods													
trinitytrinloc5465c0t1	WGCNA	control	broods													
trinitytrinloc37736c0t1	WGCNA	control	broods													
soapsoap337106	WGCNA	control	broods													
soapsoap356485	WGCNA	control	broods													
trinitytrinloc25768c0t5	WGCNA	control	broods	ORTHO_ALL63	17	18	19	0	1							
soapsoap340859	WGCNA	control	broods	ORTHO_ALL15628	1	0	0	0	0							
trinitytrinloc47565c0t1	WGCNA	control	broods	ORTHO_ALL2816	1	7	1	0	0		DappuDraft_328985	76,5217	Temperature			
soapsoap323057	WGCNA	control	broods	ORTHO_ALL207	16	4	0	0	0							

Table C3-S6: List of GO enrichment for candidate transcripts of interest as identified in the GWA analysis and the WGCNA. The three ontologies are molecular function (MF), cellular component (CC) and biological processes (BP).

GO.ID	Term	Annotated	Significant	Expected	Rank in classicFisher	classicFisher	classicKS	elimKS	category	dataset
GO:0004797	thymidine kinase activity	1	1	0	1	0.0041	1	1	MF	GWAS
GO:0004332	fructose-bisphosphate aldolase activity	1	1	0	2	0.0041	1	1	MF	GWAS
GO:0002161	aminoacyl-tRNA editing activity	2	1	0.01	3	0.0081	0.9995	0.9995	MF	GWAS
GO:0005515	protein binding	2098	16	8.57	4	0.0083	0.0882	0.0947	MF	GWAS
GO:0004222	metalloendopeptidase activity	121	3	0.49	5	0.013	0.9189	0.9189	MF	GWAS
GO:0004114	3',5'-cyclic-nucleotide phosphodiesteras...	10	1	0.04	6	0.0401	0.8702	0.8702	MF	GWAS
GO:0004435	phosphatidylinositol phospholipase C act...	11	1	0.04	7	0.044	0.8122	0.8122	MF	GWAS
GO:0000228	nuclear chromosome	8	1	0.03	1	0.029	0.9473	0.3354	CC	GWAS
GO:0005615	extracellular space	10	1	0.04	2	0.037	0.1871	0.1871	CC	GWAS
GO:0007283	spermatogenesis	1	1	0	1	0.0039	1	1	BP	GWAS
GO:0006096	glycolytic process	10	1	0.04	2	0.0382	1	0.9999	BP	GWAS
GO:0008483	transaminase activity	9	2	0.02	1	0.00021	0.8373	0.8373	MF	WGCNA
GO:0004672	protein kinase activity	763	7	1.89	2	0.00185	0.4412	0.7175	MF	WGCNA
GO:0005524	ATP binding	1009	8	2.49	3	0.00205	0.5993	0.5993	MF	WGCNA
GO:0030170	pyridoxal phosphate binding	40	2	0.1	4	0.0043	0.9046	0.9046	MF	WGCNA
GO:0019901	protein kinase binding	2	1	0	5	0.00494	0.7386	0.7386	MF	WGCNA
GO:0004550	nucleoside diphosphate kinase activity	6	1	0.01	6	0.01474	0.3325	0.3325	MF	WGCNA
GO:0015116	sulfate transmembrane transporter activi...	11	1	0.03	7	0.02686	0.6844	0.6844	MF	WGCNA
GO:0004222	metalloendopeptidase activity	121	2	0.3	8	0.0355	0.9251	0.9251	MF	WGCNA
GO:0016021	integral component of membrane	748	3	0.76	1	0.016	0.8169	0.7531	CC	WGCNA
GO:0006468	protein phosphorylation	759	8	2.09	1	0.0017	0.834	0.8451	BP	WGCNA
GO:0000079	regulation of cyclin-dependent protein s...	1	1	0	2	0.0028	1	1	BP	WGCNA
GO:0006241	CTP biosynthetic process	6	1	0.02	3	0.0164	0.344	0.3435	BP	WGCNA
GO:0006183	GTP biosynthetic process	6	1	0.02	4	0.0164	0.344	0.3435	BP	WGCNA
GO:0006228	UTP biosynthetic process	6	1	0.02	5	0.0164	0.344	0.3435	BP	WGCNA
GO:0008272	sulfate transport	11	1	0.03	6	0.0299	0.688	0.6882	BP	WGCNA
GO:0006165	nucleoside diphosphate phosphorylation	16	1	0.04	7	0.0432	0.344	0.8521	BP	WGCNA
GO:0008277	regulation of G-protein coupled receptor...	16	1	0.04	8	0.0432	0.292	0.2924	BP	WGCNA

Supplementary figures

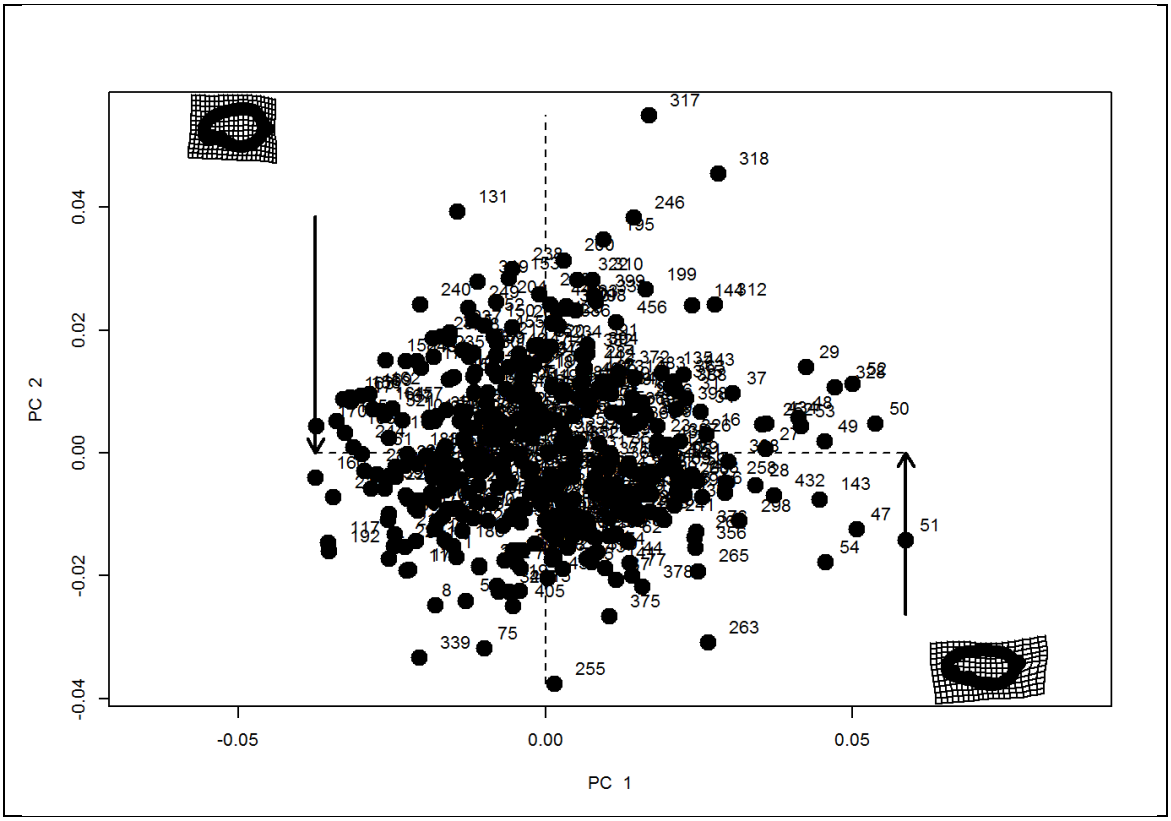

Figure C1-S1: Principal Component (PC) plot of 'shape' variation. PC1 explains 42% of variation whereas PC2 explains 24%.

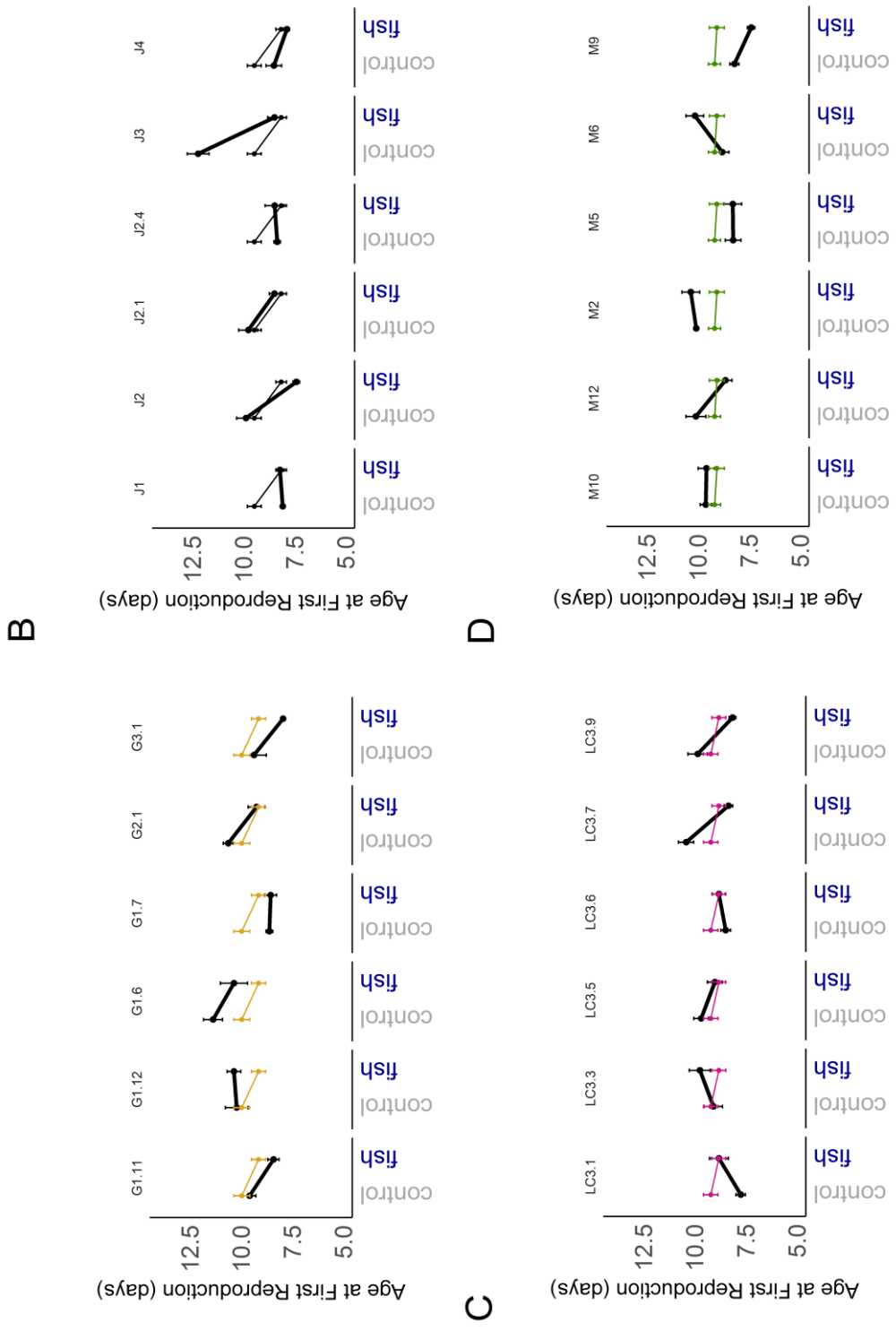

Figure C1-S2: Reaction norms for the life history trait age at first reproduction ('AFR'). Genotype mean (+/-SE) within one population are displayed. The overall within population mean (+/-SE) is displayed in a population specific color. A. Population Greifensee= popG= 'yellow'. B. Population Jordan Reservoir= popJ= 'black'. C. Population Lake Constance= popLC= 'magenta'. D. Population Müggelsee= popM= 'green'

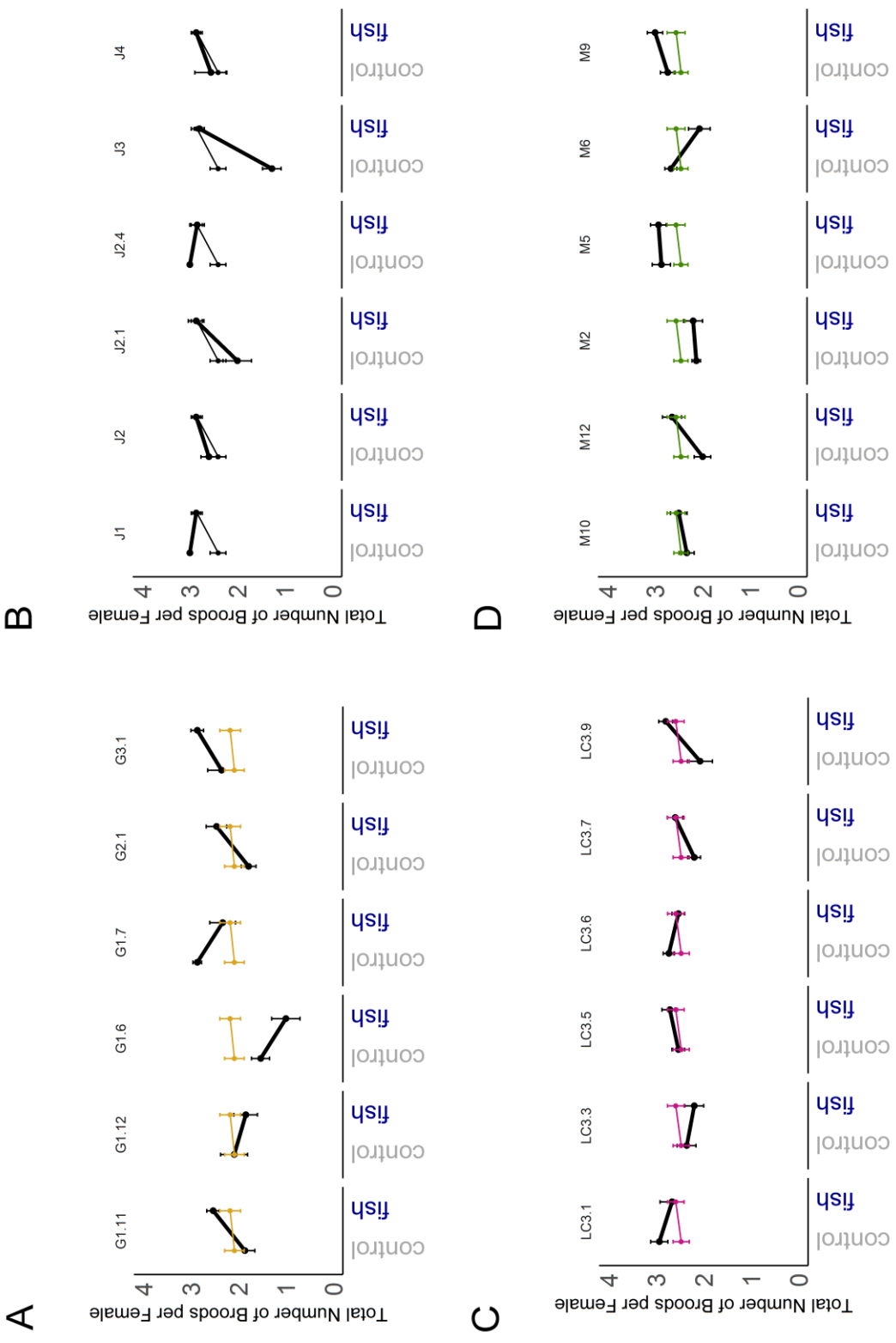

Figure C1-S3: Reaction norms for the life history trait total number of broods ('broods'). Genotype mean (+/-SE) within one population are displayed. The overall within population mean (+/-SE) is displayed in a population specific color. A. Population Greifensee= popG= 'yellow'. B. Population Jordan Reservoir= popJ= 'black'. C. Population Lake Constance= popLC= 'magenta'. D. Population Müggelsee= popM= 'green'.

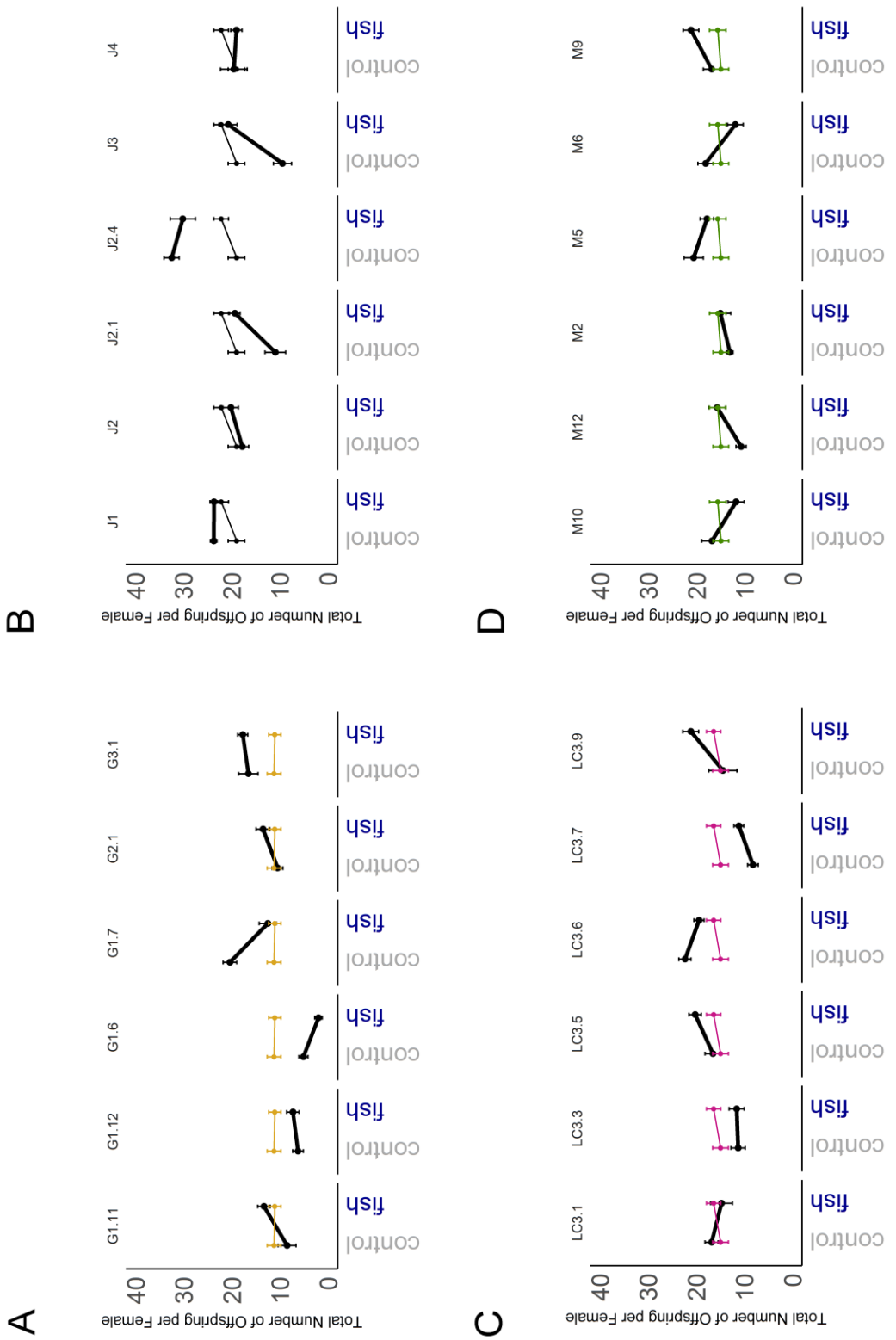

Figure C1-S4: Reaction norms for the life history trait total number of offspring ('offspring'). Genotype mean (+/-SE) within one population are displayed. The overall within population mean (+/-SE) is displayed in a population specific color. A. Population Greifensee= popG= 'yellow'. B. Population Jordan Reservoir= popJ= 'black'. C. Population Lake Constance= popLC= 'magenta'. D. Population Müggelsee= popM= 'green'.

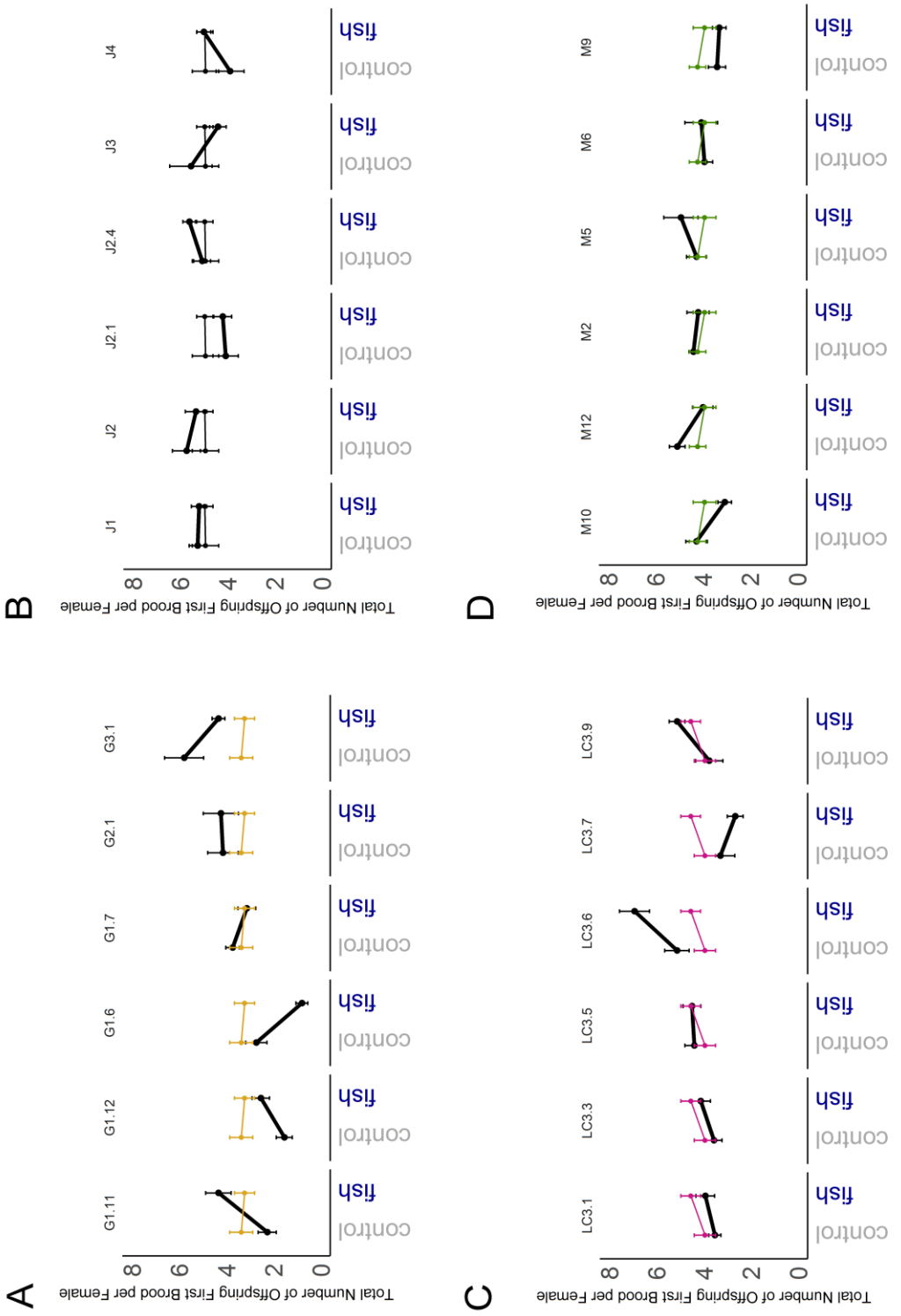

Figure C1-S5: Reaction norms for the life history trait total number of offspring first brood ('brood1'). Genotype mean (+/-SE) within one population are displayed. The overall within population mean (+/-SE) is displayed in a population specific color. A. Population Greifensee= popG= 'yellow'. B. Population Jordan Reservoir= popJ= 'black'. C. Population Lake Constance= popLC= 'magenta'. D. Population Müggelsee= popM= 'green'.

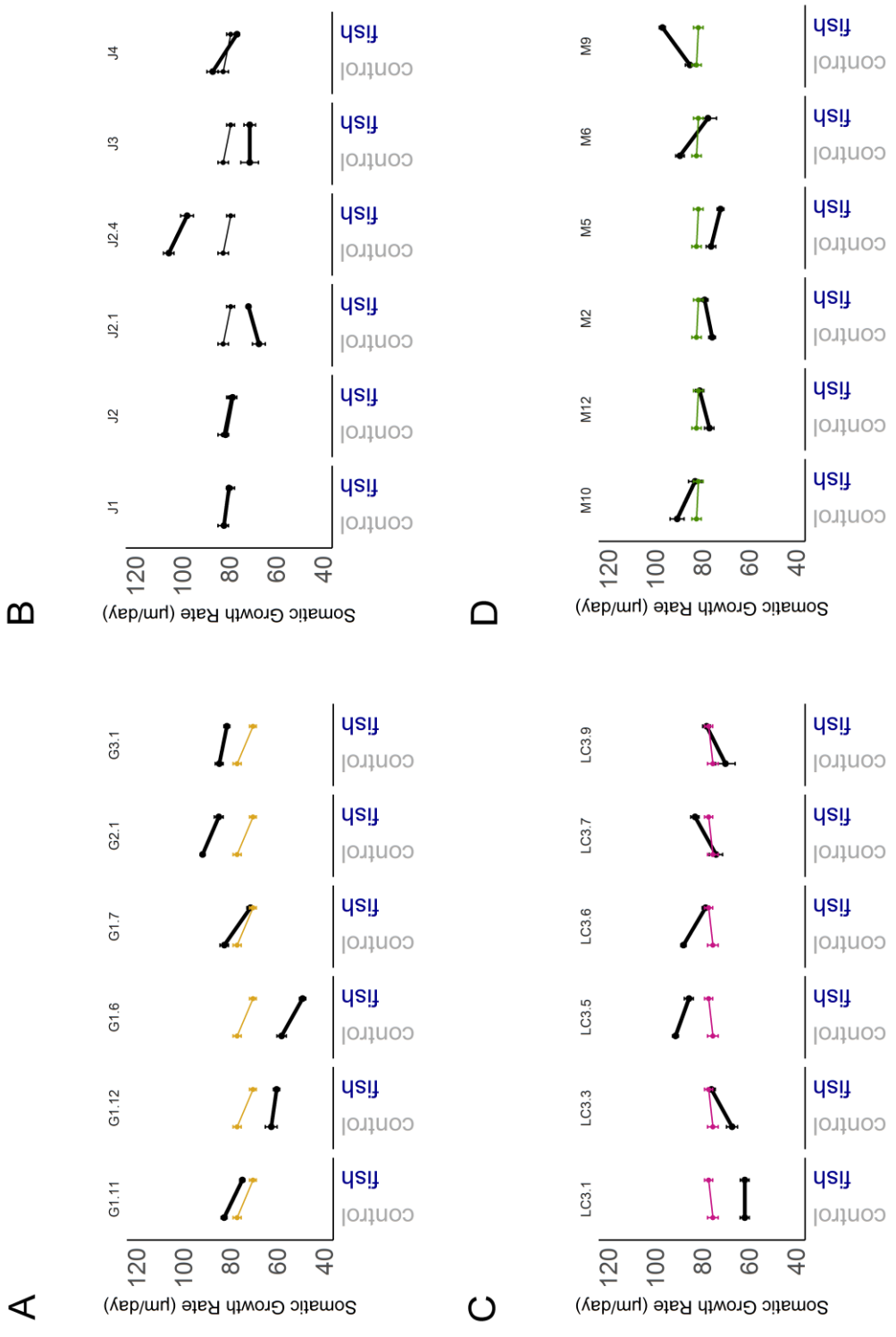

Figure C1-S6: Reaction norms for the life history trait somatic growth rate ('SGR'). Genotype mean (+/-SE) within one population are displayed. The overall within population mean (+/-SE) is displayed in a population specific color. A. Population Greifensee= popG= 'yellow'. B. Population Jordan Reservoir= popJ= 'black'. C. Population Lake Constance= popLC= 'magenta'. D. Population Müggelsee= popM= 'green'.

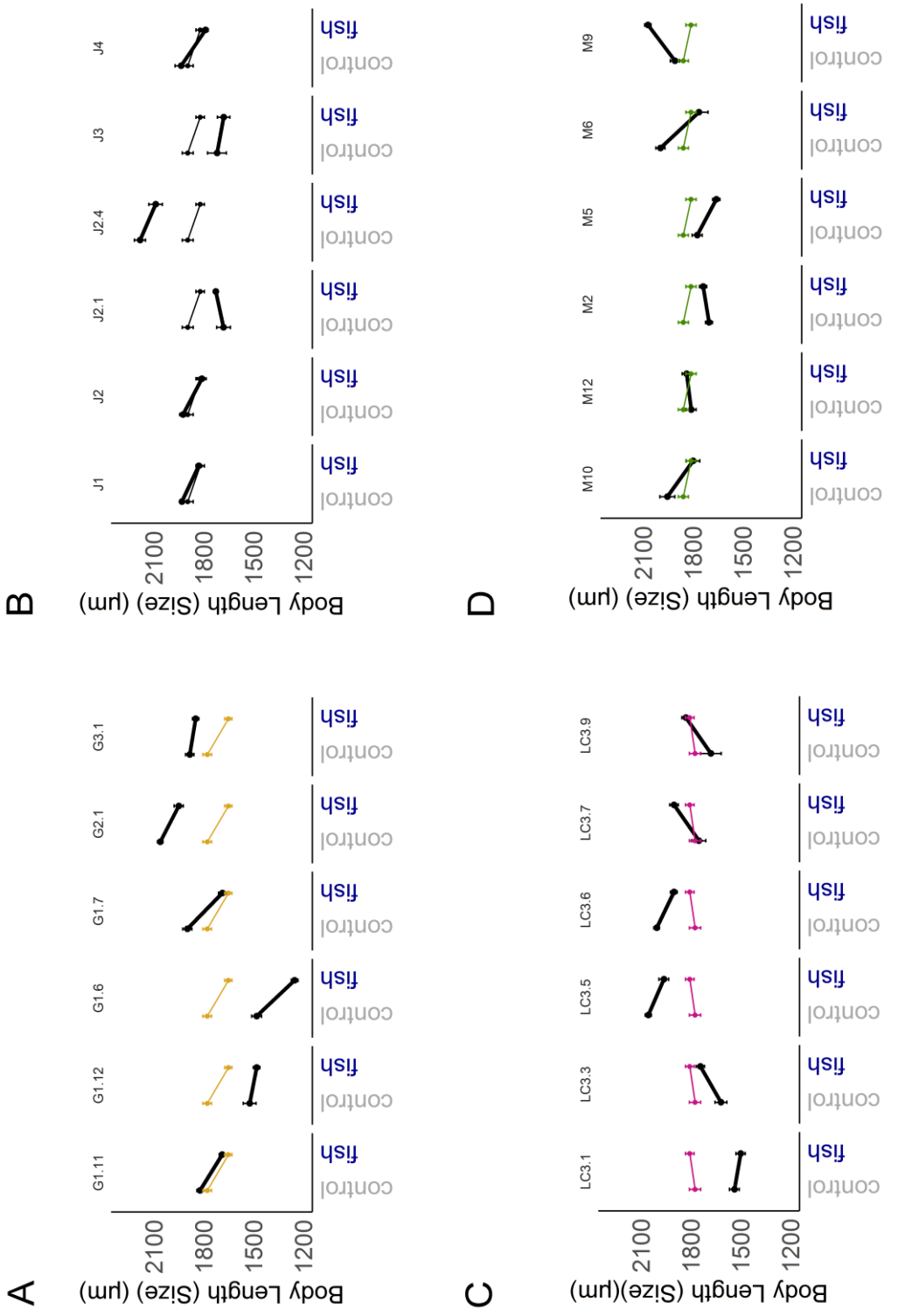

Figure C1-S7: Reaction norms for the life history trait body length ('size'). Genotype mean (+/-SE) within one population are displayed. The overall within population mean (+/-SE) is displayed in a population specific color. A. Population Greifensee= popG= 'yellow'. B. Population Jordan Reservoir= popJ= 'black'. C. Population Lake Constance= popLC= 'magenta'. D. Population Müggelsee= popM= 'green'.

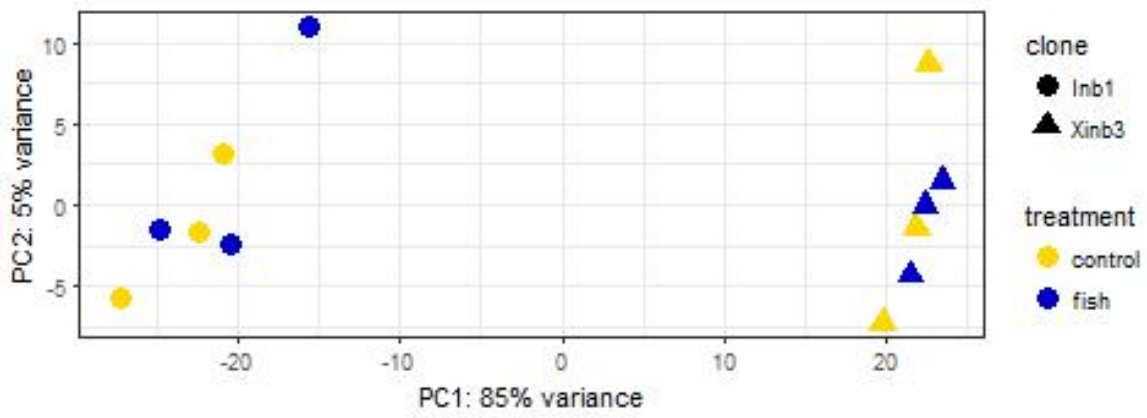

Figure C2-S1: Principal component (PC) plot of the biological *D. magna* RNA-seq samples. 'yellow' = control environment. 'blue' = fish environment. 'triangles' = clonal line Xinb3. 'circles' = clonal line Inb1.

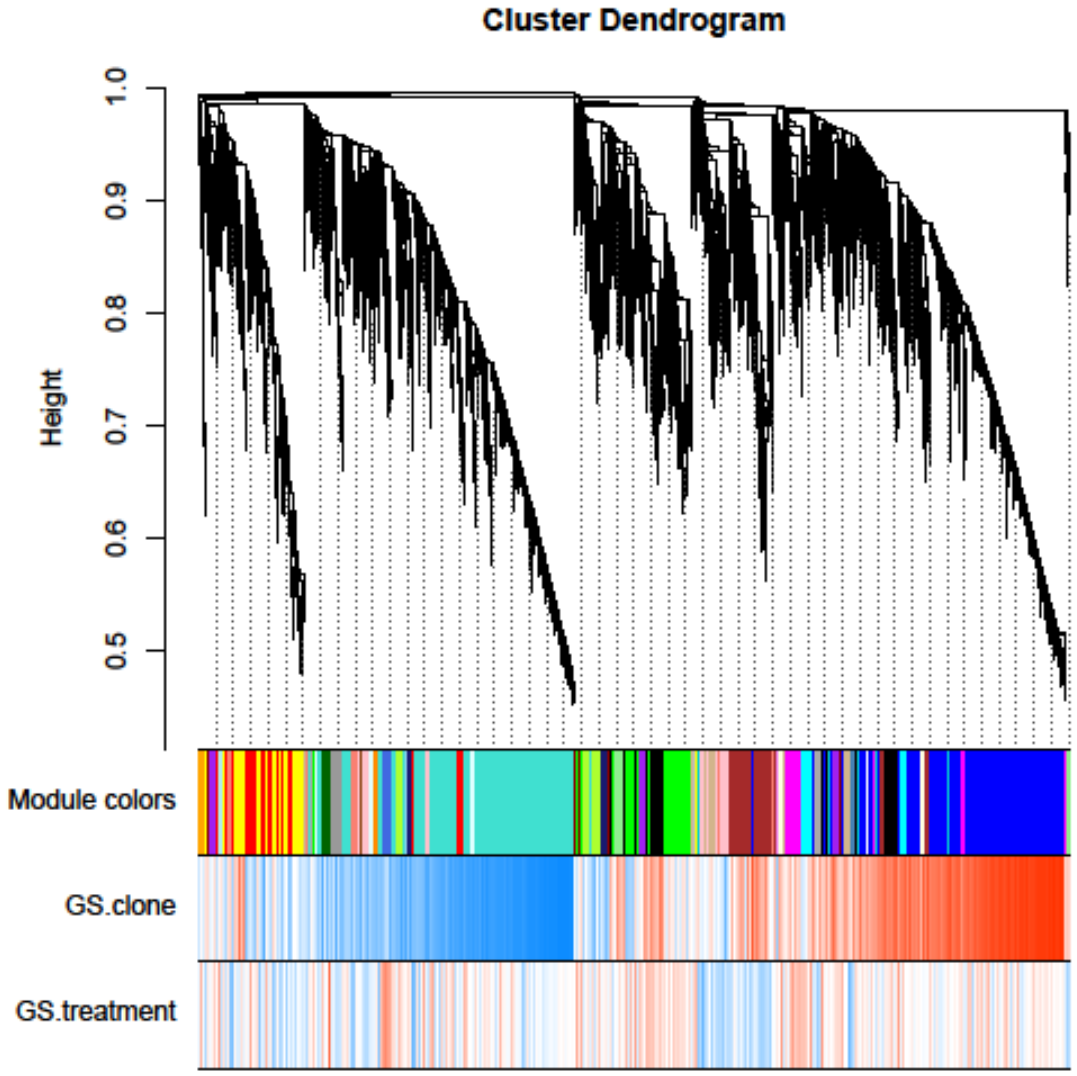

Figure C2-S2: Cluster dendrogram of transcripts in *D. magna*, with dissimilarity based on the topological overlap matrices (TOM). Additional assignments are module colors, the gene significances (GS) for the trait' clone' and 'treatment' (fish environment). Red and blue indicate a positive and negative correlation of the module with the respective trait. Darker hues indicate higher correlation values.

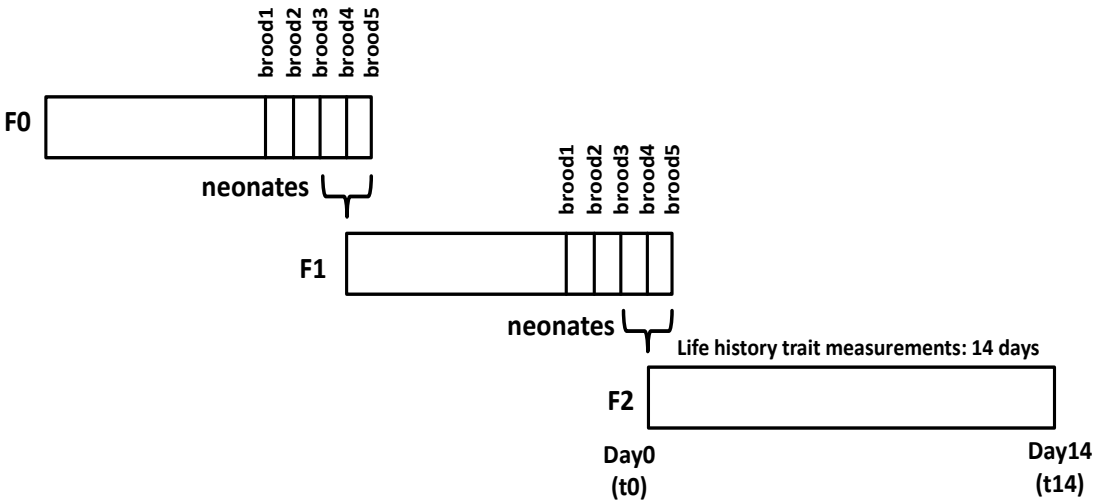

Figure C3-S1: Breeding design of life history experiment in the absence or presence of fish kairomones (Chapter 1). Each clonal line was bred in kairomone-free water (control envionment) and in kairomone water (fish envionment) for two subsequent generations (F0 & F1). Neonates from 3rd to 5th brood were used to start a new generation. Life history traits of experimental individuals (F2) were measured for 14 days. Neonates were preserved in ethanol at the beginning of the experiment (t0) and experimental individuals at the end of the experiment (t14) to measure the trait 'size'.

			PopG						PopJ						PopLC						PopM					
SNP	Ref	Alt	G1.11	G1.12	G1.6	G1.7	G2.1	G3.1	J1	J2	J2.1	J2.4	J3	J4	LC3.1	LC3.3	LC3.5	LC3.6	LC3.7	LC3.9	M10	M12	M2	M5	M6	M9
abyssk22_f_5797:149	G	C	0/1	0/1	1/1	1/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0	1/1	0/0	0/0	0/0	0/0	0/0	0/0
trinitytrinloc24474c0t10:96	A	G	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/1
basesvelvLoc4568d28579t2:4039	T	G	0/0	0/0	0/1	0/1	0/0	0/2	0/2	0/0	0/0	0/0	0/2	0/0	0/0	0/2	0/0	0/0	0/2	0/0	0/2	0/2	0/2	0/0	0/2	0/2
soapsoap366791:179	A	G	0/0	0/0	0/1	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0

Control condition

	G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11	
	Individual 1		Individual 2		Individual 3		Individual 4		Individual 5		Individual 6		Individual 7		Individual 8		Individual 9		Individual 10		Individual 11		Individual 12		Individual 13		Individual 14		Individual 15	
	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt
abyssk22_f_5797:149	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C

Fish condition

	G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11		G1.11	
	Individual 1		Individual 2		Individual 3		Individual 4		Individual 5		Individual 6		Individual 7		Individual 8		Individual 9		Individual 10		Individual 11		Individual 12		Individual 13		Individual 14		Individual 15	
	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt	Ref	Alt
abyssk22_f_5797:149	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C

Figure C3-S2: A visual representation of how the "inflated dataset" of SNPs was created for GWA analysis.

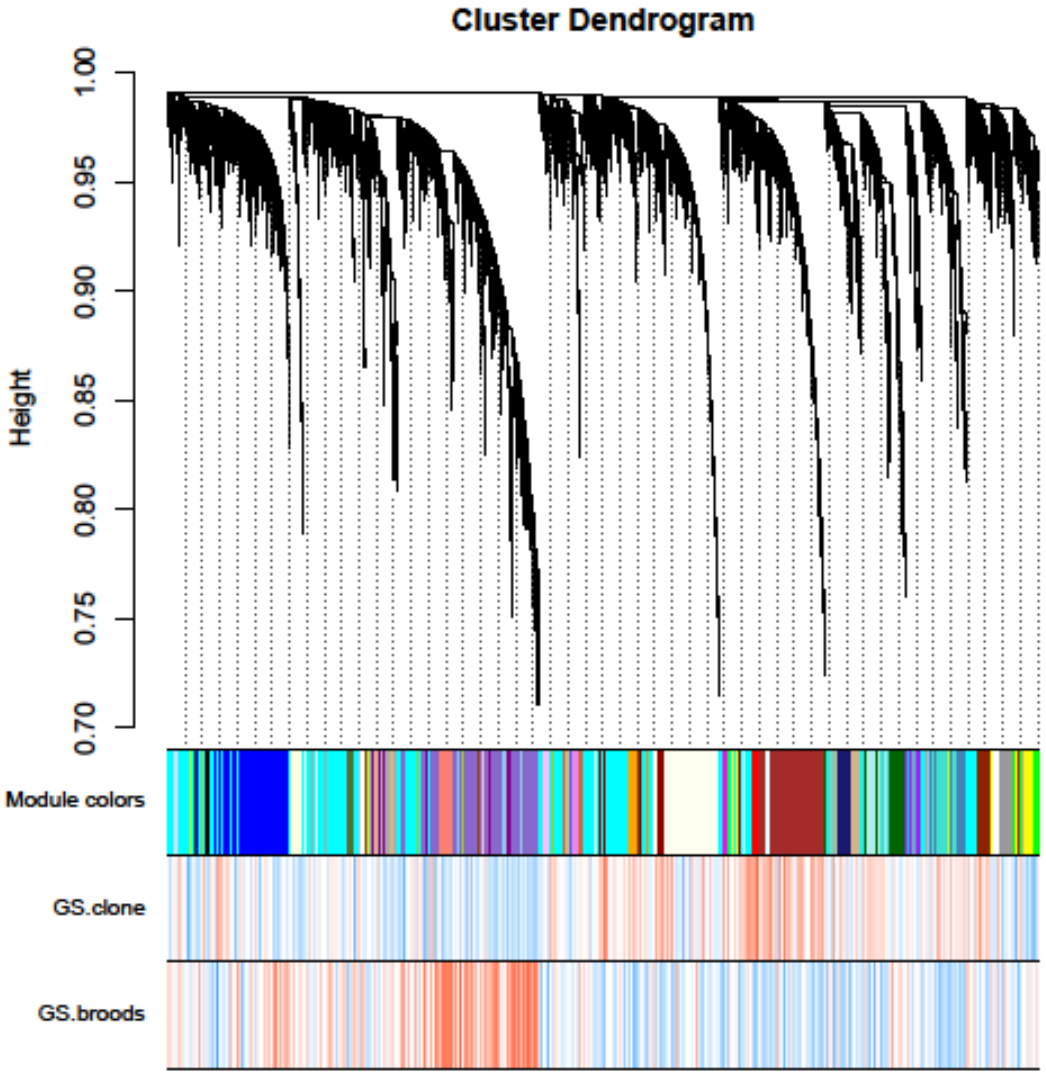

Figure C3-S3: Cluster dendrogram of *D. galeata* transcripts obtained from WGCNA. Dissimilarity based on topological overlap matrices (TOM). Additional assignments are the module colors, the gene significances (GS) for the trait 'clone' (clonal line) and 'broods' (total number of broods). Red and blue indicate a positive and negative correlation of the module with the respective trait. Darker hues indicate higher correlation values.

Data Accessibility

Chapter 2

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).

Chapter 3

SNP data used as input for GWAS analysis has been archived in European Variation Archive (EVA) and can be accessed using (*to be announced*).

Supplementary scripts

Raw data and R scripts are provided on a supplementary CD-ROM. Here, an overview of files and folders is provided.

Chapter 1

Life history analysis	
R script files:	1_FK_LHT1_PeerJ.Rmd
	2_FK_LHT2_PeerJ.Rmd
	3_FK_LHTgraphs_PeerJ_ed.Rmd
Raw data files for R:	dSGR_pop.txt
	FKmaster.txt
	surv_repro_relfit.txt
Morphometric analysis	
R script files:	FK_shape_PeerJ.Rmd
Raw data files for R:	FK_classifier.txt
	all.TPS, all_c.TPS, all_f.TPS, all_G.TPS, all_Gc.TPS, all_Gf.TPS, all_Gf.TPS, all_J.TPS, all_Jc.TPS, all_Jf.TPS, all_LC.TPS, all_LCc.TPS, all_LCf.TPS, all_M.TPS, all_Mc.TPS, all_Mf.TPS

Chapter 2

Differential Gene Expression (DEG)	
R script files:	Tams-et-al_DEG_DaphniaFK.Rmd
Raw data files for R:	Folder 'read counts':1_M9_f_count.tab, 2_M9_f_count.tab, 4_M9_c_count.tab, 5_M9_c_count.tab, 6_M9_c_count.tab, 8_M6_c_count.tab, 9_M6_c_count.tab, 10_M6_c_count.tab, 13_M6_f_count.tab, 14_M6_f_count.tab, 15_M6_f_count.tab, 20_M9_f_count.tab, SampleSheet.csv, SampleSheetM6.csv, SampleSheetM9.csv
Gene co-expression network analysis	
R script files:	Tams-et-al_Network_DaphniaFK.Rmd
	Tams-et-al_Resampling_DaphniaFK.Rmd

Raw data files for R:	folder 'Input':LHT2.csv; Sample_counts_vst.csv Galeata-networkConstruction-auto.RData
	folder 'Resampling': folder 'Input': datExpr.csv; LHT2.csv; MEs.csv
Annotation and GSEA	
R script files:	Tams-et-al_AnnotationGSEA_DaphniaFK.Rmd
Raw data files for R:	Folder 'Input': allBlue.txt, allBrown.txt, allRed.txt, allSalmon.txt, allTan.txt, DEGs_M6.txt, DEGs_M9.txt, Dgal_GOs2.txt, uniqueDETs_M6.txt; uniqueDETs_M9.txt
Orthogroup analysis	
OrthoMCL_MC	
Python script	OMCLFinal.py
Raw data files for python:	Folder 'Input_python': blue-gene.txt, brown-gene.txt, Genelist.General_JA.txt, orthomcl_daphnia_orthology_okayset.txt
Orthogroups_VT	
R script files:	Tams-et-al_Orthogroups_DaphniaFK.Rmd
Raw data files for R:	folder 'Output_python': subsetOMCL.txt, JA_Annotated.txt,
	Dgal_GOs2.txt, expected GOs.csv

Chapter 3

Gene co-expression network analysis	
R script files:	Tams-et-al_NetworkAll_Daphnia.Rmd
	Tams-et-al_ResamplingAll_Daphnia.Rmd
Raw data files for R:	folder 'Input': vst_norm_reads.tab, LHT_control2a.csv, Galeata-networkConstruction-auto_all.RData
	folder 'ResamplingAll': folder 'Input': datExpr.csv, LHT_control2a.csv, MEs.csv

Author contribution

The work presented in **Chapter 1** was published as preprint in PeerJ as “Intraspecific phenotypic variation in life history traits of *Daphnia galeata* populations in response to fish kairomones” by Verena Tams, Jennifer Lüneburg, Laura Seddar, Jan-Philip Detampel and Mathilde Cordellier. V. Tams designed the study, carried out laboratory work, performed the life history trait and geometric morphometric analysis and wrote the mansucript. J. Lüneburg and L. Seddar carried out laboratory work. J. P. Detampel established the geometric morphometric analysis. M. Cordellier designed the study and wrote the manuscript.

The work presented in **Chapter 2** is in preparation for publication in Molecular Ecology as “Gene co-expression in *Daphnia galeata* exposed to fish kairomones” by Verena Tams, Jana Helene Nickel, Anne Ehring, Mathilde Cordellier. The authorship is shared by V. Tams and J. H. Nickel. V. Tams designed the study, carried out laboratory work, performed the gene expression and gene co-expression analysis and wrote the mansucript. J. H. Nickel designed the study, carried out laboratory work, performed the gene expression and gene co-expression analysis and wrote the mansucript. A. Ehring carried out laboratory work. M. Cordellier designed the study and wrote the manuscript.

The work presented in **Chapter 3** is in preparation for publication in G3: Genes|Genomes|Genetics as “An environment-dependent genotype-phenotype association in European *Daphnia galeata*” by Verena Tams, Suda Parimala Ravindran and Mathilde Cordllier. The authorship is shared by V. Tams and S. P. Ravindran. V. Tams designed the study, conducted the experiment (**Chapter 1**), performed the gene co-expression analysis and wrote the manuscript. S. P. Ravindran designed the study, performed the genome-wide association and functional analysis and wrote the manuscript. M. Cordellier designed the study and wrote the manuscript.

Declaration

Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hamburg, 24.07.2018

Correctness of language

Herewith, I confirm the correctness of language of the dissertation “Intraspecific phenotypic variation and its genetic basis in *Daphnia*” written by Verena Tams.

Helsinki 10/7/18
place and date


signature

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