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Phenotypic plasticity in the *Daphnia* model organism: gene expression and maternal control in the adaptation to environmental perturbations

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Abstract

Phenotypic plasticity is the ability of a genotype to express different phenotypes as a function of the environment. The main aim of this thesis was to evaluate the molecular fundamentals and the implications of epigenetic control, gene expression and hormonal regulation underlying phenotypic plasticity in model organisms.

The microcrustacean *Daphnia* is a model species in ecological, toxicological and evolutionary fields due to its eco-responsive genome, that makes it possible to use this organism in epigenetic, transcriptome and microbiome research as well.

In this thesis, laboratory procedures (RT-qPCR, Mi-seq, GWAS, RNA-seq) were applied to understand the molecular pathways involved in phenotypic responses in different clones of two *Daphnia* species: *D. pulex* and *D. magna*. Preliminary experiments, in which differences in plastic life history traits (e.g. fecundity, the production of males and ephippia, morphological defences) were tested, allowed to discriminate among different clones of *D. pulex* (Chapter 2) and *D. magna* (Chapter 3).

Transgenerational plasticity occurs when the maternal response to the environment causes a change in the phenotype of the offspring without a corresponding change in the genome: this factor was evaluated in different *Daphnia* clones (Chapters 2.2 and 3.1).

After exposure to the juvenile hormone Methyl Farnesoate (MF), RT-qPCR was used to detect changes in the expression of genes involved in hormonal regulation and epigenetic control in *D. pulex* clones (Chapter 2.2). After exposure to glyphosate and Roundup, RNA-seq was applied to assess the response of the whole transcriptome and to detect specific co-expression patterns among *D. magna* clones (Chapter 3.2). RNA-seq was also helpful to evaluate the role of the microbiome considering the reaction of the organisms. In the end, an approach based on RT-qPCR was applied for the screening of the hormonal activities of chemicals and endocrine disruptors in the surficial waters (Chapter 4).

Both genetic differences among clones and epigenetic factors (maternal effect via hormonal signals, DNA methylation and microbiome) are involved in the modulation of phenotypic plasticity and transgenerational plasticity.

Different clones used distinct molecular pathways to cope with environmental changes: in *D. magna* clones, divergences were observed in transcriptomics pathways when exposed to glyphosate and Roundup (Chapter 3.2). In addition, other differences were reported in gene expression among the clones of the species *D. pulex* exposed to MF (Chapter 2.2), while *D. magna* clones exhibited diverse resistances to glyphosate and Roundup (Chapter 3.2).

Abbreviations

HH COMBO	High-Hardness COMBO
PCR	Polymerase Chain Reaction
TEA Buffer	Tris-Acetate-EDTA Buffer
w/v	weight/volume
DMSO	Dimethyl Sulfoxide
DMF	Dimethylformamide
20-HE	20-hydroxyecdysone
MF	Methyl Farnesoate
GA	Glufosinate Ammonium
phbr2	prohibitin 2
vtg1	vitellogenin 1
cut12	cuticle 12
cpa1	precursor of carboxypeptidase A1
dhb1	di-domain haemoglobin 1
dsx1	doublesex 1
RXR	retinoid X receptor
JHAMT	Juvenile Acid-O-Methyltransferase
Met	methoprene-tolerant
DNMT3A	DNA methyltransferase 3A
GAPDH	Glyceralddehyde 3 phosphate dehydrogenase
AKR1	Aldo Keto Reductase
NMDA	N-methyl-D aspartate
JHE	Juvenile Hormone Esterase
LdhA	lactate dehydrogenase A)
Rab4	subunit gene of the GTPase family

ND5	NADH dehydrogenase subunit 5
SAM	S-Adenosil-Metionine
EcR	Ecdisteroid Receptor
JH	Juvenile Hormone
RT-qPCR	Real-Time Polymerase Chain Reaction
RNA-seq	RNA sequencing
GWAS	Genome Wide Association Study
MODA	MOdule Differential Analysis
FC	Fold Change
Ct	Threshold cycle
NGS	Next Generation Sequencing
QTL	Quantitative Traits Loci

Chapter 1: Introduction

Phenotypic plasticity

Phenotypic plasticity or polyphenism is the ability of a genotype to express different phenotypes as a function of its environment (Pigliucci, 2001). Phenotypic plasticity is due to "environment-dependent phenotypic expression" (DeWitt and Scheiner, 2004) and it constitutes an important ecological process, because it is a model to understand life on earth, ecological communities and the evolution of organisms (Whitman and Agrawal, 2009). Environmental factors such as food availability, light, temperature, the presence of predators, parasites and competitors can induce phenotypic plasticity (Matos et al., 2009; Siebenkäs et al., 2015; Matsunami et al., 2015; Fischman et al., 2017; Noble et al., 2018). Examples of phenotypic plasticity are mimicry of fish, caste determination in social insects, morphological defences in cladocerans and switches in sex determination in reptiles (Crews, 2003; Laforsch and Tollrian, 2004a; Mori et al., 2005; Janzen and Phillips, 2006; Sumner et al., 2006; Urban et al., 2014; Cortesi et al., 2015). Reaction norms, that describe the pattern of phenotypic expression of a single genotype across a range of environments, can be used as a graphical representation of the values of a trait in each environment (Fig. 1.1) (Aubin-Horth and Renn, 2009).

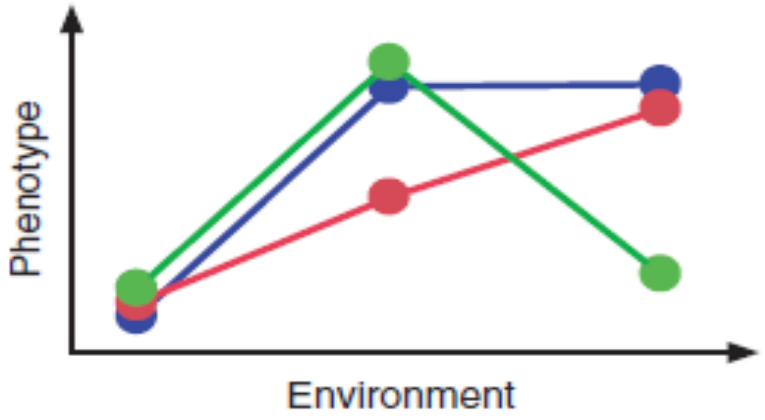


Figure 1.1: Reaction norms are the representation of trait values in relation to the environment. The y-axis represents three steps of phenotypic plasticity. "The red reaction norm shows a progression in phenotype value, the green reaction norm a phenotypic difference only in the intermediate environment and the blue reaction norm a phenotype that changes during the transition, which kept the same value in the endpoint step of development" (Aubin-Horth and Renn, 2009).

The study of phenotypic plasticity is interdisciplinary and encompasses aspects of development, ecology, evolution, genetics, genomics and physiological systems (Price et al., 2003; Kelly et al., 2012). Organisms may express plasticity both at phenotypic level (which tends to be irreversible) and at biochemical and physiological levels (which tend to be reversible and occur in a short time scale) (Pigliucci et al., 2006). However, not many studies integrate phenotypic observation and molecular analysis (see also Zhou et al., 2012; Wellband and Heath, 2017 for phenotypic/molecular approaches). Molecular analysis is indeed regarded as a tool able to clarify the mechanisms at the basis of this phenomenon. Recently, studies of "molecular phenotype", in which the gene expression is linked with phenotypic responses of complex biological systems, have emerged (Zhang et al., 2015). Literature in the field of phenotypic plasticity is numerous. Using the keyword "phenotypic plasticity", PubMed research results show 5949 articles, more than half (53,87%) of which published in the last five years and 9,61% published in 2018. This demonstrates that the efforts to understand the phenomenon of phenotypic plasticity and the interest in the field are increasing. The investigation of molecular reprogramming that occurs in phenotypic plasticity is a premise to characterize the evolutionary process driving the variation of organisms in different environmental conditions.

Phenotypic plasticity can be divided into developmental plasticity that occurs within a single generation and transgenerational plasticity that occurs in subsequent generations (Angilletta, 2009; Munday et al., 2013).

Maternal effect and transgenerational plasticity

Maternal effect is defined as "the causal influence of the maternal genotype or phenotype on the offspring phenotype" (Wolf and Wade, 2009). Maternal effects are ubiquitous in nature and they influence the development and life history, ecology and evolution of plants and animals (Roach and Wulff, 1987; Bernardo, 1996; Donohue, 2009) but their role in the evolutionary dynamics of populations is still poorly established (Räsänen and Kruuk, 2007). Maternal genetic effect is caused by genes transmitted by mothers that are able to influence the phenotype of their offspring (Nordskog and Hassan, 1971; Shaw and Byers, 1998). An organism's phenotype is the result of the expression of its genotype in the environment experienced during development, but it can also be influenced by maternal epigenetic effects that refer to modifications of the genome expression and phenotype without changes in the DNA sequence (Mousseau and Fox, 1998; Agrawal et al., 1999; LaMontagne and McCauley, 2001; Räsänen and Kruuk, 2007; Rohde and Junttila, 2008; Bonduriansky and Day, 2009; Mousseau et al., 2009). The main molecular mechanism of

the maternal epigenetic effect is based on transferring maternal messenger RNAs or proteins to the oocyte (Wolf and Wade, 2009). This process is important in many organisms in the early stages of the embryo, in which transcription is initially inactive (Schier, 2007) and is linked with the inheritance of gene expression in subsequent generations (transgenerational plasticity). The exposure of mothers to environmental stimuli can have indirect effects in non-exposed offspring, by changing their epigenetic profile, gene expression, hormonal regulation and phenotypic traits (Skinner and Guerrero-Bosagna, 2009; Burton and Metcalfe, 2014; Meise et al., 2016; Zimmer et al., 2017). The ability of environmental factors to reprogram the germ line and to promote transgenerational plasticity has significant implications on evolutionary biology, ecology and conservation biology, disease etiology and public health (Anway et al., 2005; Drake and Liu, 2010).

In organisms with sexual reproduction, there are two specific life stages in which the mother can influence the next generations: during early development, but also before and throughout reproduction (Burton and Metcalfe, 2014; Fawcett and Frankenhuis, 2015). In such species, the limitation in transgenerational plasticity studies is due to recombination during reproduction, thus parents and offspring do not have the same genotype (Miller et al., 2012; Seebacher et al., 2014). To avoid this confounding factor and increase the understanding of the ecological and evolutionary role of transgenerational plasticity, clonal organisms are ideal for experimental research on this topic (Kielland et al., 2017).

Microbiome

There are evidences about other types of maternal influences on offspring. In humans, the microbiota present in the mother's gut, vagina, breast milk, mammary areola present high similarity with their infants until six months after birth (Drell et al., 2017). The microbiota includes the entire complex of micro-organisms (such as bacteria, viruses and archaea) that are present in a micro-ecosystem. The microbiome is referred to the genetic composition of the respective microbiota and represents "the ecological community of commensal, symbiotic, and pathogenic micro-organisms that literally share an organism's body space" (Lederberg and McCray, 2001). Microbiome plays an essential role in the immune system, homeostasis, endocrine system and metabolism of organisms and defects in its formation and composition have negative effects on their host's fitness, phenotype and health (Belkaid and Hand, 2014; Clarke et al., 2014; Cani and Knauf, 2016; Rowland et al., 2018; Zhang et al., 2018). In the last years, projects and studies about human microbiota/microbiome composition have been developed and the publications in this field

are increasing (Turnbaugh et al., 2007; Young, 2017; Barko et al., 2018; Gilbert et al., 2018). In the same way, studies aimed to understand the microbiome characteristics in other mammals, amphibians, reptiles, birds and fishes have been performed (Keenan et al., 2013; Colombo et al., 2015; Bahrndorff et al., 2016; Nishida and Ochman, 2018; Egerton et al., 2018; Videvall et al., 2018).

A microbiome may be passed on by vertical transmission (bacteria can be passed directly from parents to offspring), horizontal transmission (bacteria are acquired from the environment during development) or a by combination of these two processes (Bright and Bulgheresi, 2010). A bacterial community may influence the relation between host and environment and the response of organisms to abiotic and biotic factors. Microbiome has a direct impact on phenotypic plasticity, and there are evidences about the role of bacteria (i.e. Proteobacteria) in the expression of phenotypic plasticity (Carrier and Reitzel, 2018).

Due to the importance of microbiome for the health of organisms, it is considered a "second genome" (Grice and Segre, 2012). Studies on invertebrates are increasing; symbiosis and cross talk between invertebrates and microbes represent great potential for new discoveries (Petersen and Osvatic, 2018). However, in an ecological scenario, not many studies have been devoted to understand the role of microbiota in phenotypic plasticity of offspring.

Molecular basis of phenotypic plasticity

As reported by Schlichting and Smith (2002), the hierarchy of phenotypic plasticity is based on the following pattern:

"Environment $\longrightarrow$ Gene expression $\longrightarrow$ mRNA translation $\longrightarrow$ Phenotypic traits".

Both hormones and epigenetic changes have direct effects on gene expression and eventually on phenotypic traits in response to environmental stimuli (Fig. 1.2).

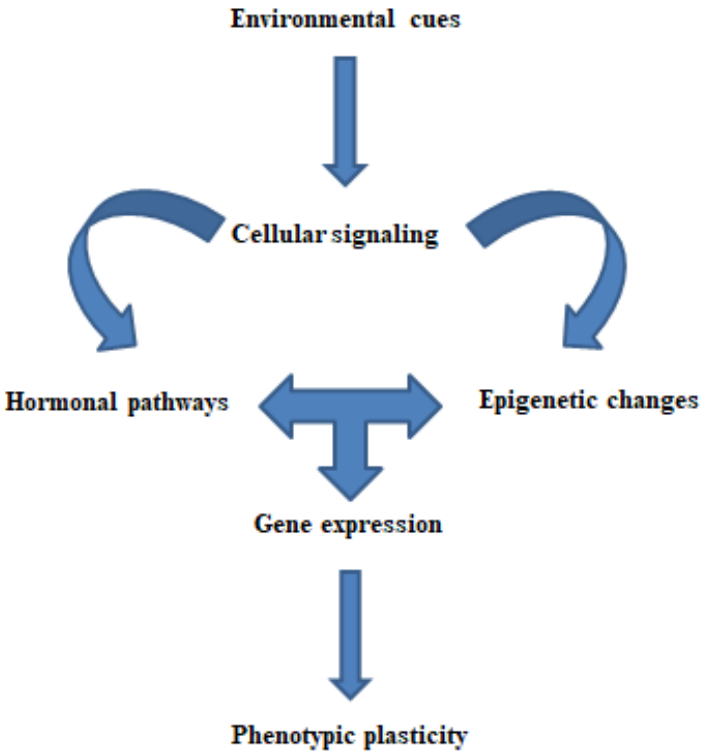


Figure 1.2: The hierarchy of gene expression underlying phenotypic plasticity, considering hormonal signaling and epigenetic control.

The role of hormone and endocrine signaling pathways are fundamental. Hormone secretion can be changed in response to changes in environmental conditions (e.g. photoperiod, temperature) and hormones can recognize specific cellular receptors, change gene expression and determine morphological traits in organisms exposed to specific stimuli (Lema and Kitano, 2013). Hormones are an interacting factor that contribute to the complex orchestration of phenotypic development (Lema, 2014) and specific signaling pathways are involved, such as insulin/insulin like growth signaling (IIS), ecdysteroids (EC), vitellogenesis (VTG) and juvenile hormones (JHs) (Corona et al., 2016). In arthropods, ecdysteroids and juvenile hormones are important in an organism's life cycle and development, ecdysis, metamorphosis, sex differentiation and morphological defences (Newitt and Hammock, 1986; Nijhout, 1999a; De Loof, 2008; Fusco and Minelli, 2010; Dennis et al., 2014; Toyota et al., 2015a). There are examples of endocrine control of phenotypic traits such as the seasonal colour variation in butterfly wings caused by ecdysteroids (Rountree and Nijhout, 1995), and the horn size and growth in beetles caused by juvenile hormones (Moczek and Emlen, 1999). Consequently, hormones regulate various aspects of phenotypic plasticity and there is a dose-response curve in which high

hormone concentrations can induce a negative feedback loop (Kohn and Melnick, 2002; Smith and O'Malley, 2004; Li et al. 2007).

Other factors involved in phenotypic plasticity are epigenetic changes, that are referred to modifications of the DNA and chromatin without changes in the DNA sequence. These modifications (DNA methylation, histone modifications, RNA interference) can induce effects in gene expression and consequently in protein translation. DNA methylation is catalysed by the enzymes DNA methyltransferases (DNMT) that add methyl groups to DNA. Considering that DNA hypo- or hyper-methylation can induce increases or decreases in transcription activity, respectively, DNA methyltransferase 3A (DNMT3A) is responsible for *de novo* methylation (to distinguish DNMT1 that is involved in the maintenance of the metilome status) (Okano et al., 1998; Pradhan et al., 1999). DNMT3A is implicated in epigenetic changes at the basis of the transfer from parents to offspring that are essential for processes such as cellular differentiation and embryonic development (Jia et al., 2016). For more information about epigenetic mechanisms and effects on organisms and phenotypic plasticity see Duncan et al. (2014). Epigenetic changes can be induced by environmental cues such as, heat stress, predators, nutrition, biotic stressors and contaminants (Norouzitallab et al., 2018) and represent a link between environmental variation and the modulation of gene expression affecting the phenotype of organisms (Geng et al., 2012). For example, epigenetic changes are associated with caste determination in ants and honeybees (Kucharski et al., 2008; Bonasio et al., 2012; Simola et al., 2013).

Lately, omics (methylome, transcriptome, microbiome, proteome, metabolome) approaches based on the application of molecular methods at different levels of biological organization are increasing (Lederberg and McCray, 2001). The advent of molecular technologies, advances in next-generation sequencing, the increase of number of genomes available and the knowledge about epigenetic and hormonal pathways are good premises for increasing the comprehension of the phenotypic plasticity process (Kelly et al., 2012). Integrative biology, ecological annotation of genes and the use of comparative methods (bioinformatic analysis) can improve the information about phenotypic plasticity (Aubin-Horth and Renn, 2009). Through the analysis of 16S ribosomal gene (16S rRNA) sequences, it is possible to identify microbial species and to define bacteria community composition (Barko et al., 2018).

Transcriptomic approach

The transcriptome is the entirety of RNA produced by a cell, tissue or organism and includes RNA coding for proteins (mRNA) and non-coding RNA (rRNA, tRNA, lncRNA, miRNA and others). As of today, two techniques are used to detect transcriptomic changes: RNA-seq (NGS - Next-Generation Sequencing technologies), which examines whole transcriptome profiling and detects novel transcripts (isoforms) and splice junctions, and RT-qPCR regarded as the most important gene expression analysis because of its sensitivity and precision (Kerr et al., 2008; Derveaux et al., 2010; Rai et al., 2018; Kolodziejczyk and Lönnberg, 2018).Transcriptomics represents an ideal approach to understand how organisms respond to environmental changes (both biotic and abiotic) (Gracey and Cossins, 2003; Cossins et al., 2006; Gracey, 2007; Ozsolak and Milos, 2011; Evans and Hofmann, 2012) and it can be used to select specific genes that make an impact on relevant traits (Gracey, 2007). Changes in gene expression give information about an organism's biology and details on its diseases (Lowe et al., 2017). Gene modulation and phenotypic plasticity may be linked to transcriptome differences and transcriptomics approaches can be effectively implemented to understand the role of genes implicated in phenotypic plasticity. Recently, the exposure of model organisms to several environmental conditions brought the interaction between specific genes and environmental changes to light. In the nematode *Caenorhabditis elegans* the interactions between genes and the environment affecting life history traits were investigated (Gutteling et al., 2007; Viney and Diaz, 2012) and in the fruit fly *Drosophila melanogaster*, genes involved in detoxification, metabolism, proteolysis and in the synthesis of heat shock proteins are described as phenotypically plastic (Zhou et al., 2012).

***Daphnia* as a model organism**

The study of model organisms with specific characteristics can have a key role in improving the knowledge on phenotypic plasticity and the molecular mechanisms of this process. Characteristics of optimal model organisms are the availability of a sequenced genome and annotated genes, to understand the possible role of unknown genes in different ecological contexts, and the possibility to obtain clonal cohorts, to understand the role of the different mechanisms (epigenetic modifications, hormones regulation, gene expression) avoiding the background noise caused by genome variability/mutations.

Organisms of the genus *Daphnia* (Crustacea: Cladocera) (Fig. 1.3) are key microcrustaceans in freshwater environments, being a principal grazer of algae and a

primary source of nutrition for fish. *Daphnia* exhibits a range of context-dependent specialized phenotypes, such as switching between parthenogenetic (asexual) and sexual reproduction in response to environmental conditions (Yampolsky, 1972; Hobaek and Larsson, 1990; Innes and Singleton, 2000) and typical morphological traits for predator avoidance (Schwartz, 1991). *Daphnia* species are used to assess the ecological impact of environmental changes and are sensitive to toxicants. Short generation time, large brood size, and easy laboratory and field manipulation make *Daphnia* important as a model for ecological and evolutionary research, but also for helping environmental protection agencies in testing chemical safety, setting regulatory standards and monitoring water quality (Forbes et al., 2001).

Figure 1.3: *Daphnia pulex* (doi:10.1371/journal.pbio.0030219)

It is also one of 13 model organisms for biomedical research listed by the US National-Institute- of Health (NIH) (https://www.nigms.nih.gov/Research/models/Pages/default.aspx). In 2005, the *Daphnia* Genomic Consortium (DGC) developed a research program (http://wfleabase.org/) to obtain genetic information and to identify genes with a key role in ecological and evolutionary processes (Colbourne et al., 2005). In 2011, the first sequence of the genome of *Daphnia pulex* was published (Colbourne et al., 2011) and, in 2016, sequencing of *D.*

magna genome was submitted (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA298946). According to Colbourne and collaborators, *Daphnia*'s genome is only 200 Mb and contains 30,097 genes, a higher number than in many species, humans included. The authors reported that 36% of the annotated genes do not have homologous in other organisms and probably have species-specific functions in phenotypic plasticity (Colbourne et al., 2011). *Daphnia* species are considered an interesting model to study environmental influences on developmental stages because of their "eco-responsive" genome (Colbourne et al., 2011; Tautz, 2011) and their somewhat unusual life cycle (Lynch, 1980; Harris et al., 2012). Recently, Ye and collaborators (2017) assembled the genome of another *D. pulex* clone by using an approach based on a combination of paired-end, mate-pair libraries and synthetic long reads, with annotation guidance provided by RNA-seq. These authors reported that 7,320 genes sequenced by Colbourne and collaborators probably represent pseudogenes or other false positives: 592 of these genes are not covered because of mutation-accumulation lines (i.e., contamination from exogenous DNA) and 698, indicated as paralogous, can be annotation errors (Ye et al., 2017). It is known that different clones from different habitats have differences in their DNA sequences. The genome sequencing of a large number of *Daphnia* clones has allowed to study the genetic basis of phenotypic traits, obligate parthenogenesis and environmental sex determination and the molecular responses to environmental cues such as heavy metals and pesticides (Kato et al., 2011; Xu et al., 2015; Schumpert et al., 2015b; Kim et al., 2015).

There are evidences about phenotypic plasticity in *Daphnia* after exposure to some environmental cues. To reduce the risk posed by unfavourable conditions and predation, many species of *Daphnia* have indeed evolved the ability to reprogram their life history and produce different morphological structures such as spines, neckteeth and helmets in response to kairomone, a hormone released by vertebrate and invertebrate predators (Grant and Bayly, 1981; Krueger and Dodson, 1981; Tollrian, 1990; Sorensen and Sterner, 1992; Weider and Pijanowska, 1993; Tollrian, 1995; Dzialowski et al., 2003; Gélinas et al., 2007; Petrusek et al., 2009; Walsh et al., 2015).

Molecular basis of *Daphnia*'s phenotypic plasticity

In *Daphnia*, phenotypic plasticity is under hormonal control and the activation of specific genes (Miyakawa et al., 2010; Dennis et al., 2014; Miyakawa et al., 2017). Hormonal regulation in *D. magna* and variation of hormonal levels at different development stages have been deeply studied (Subramoniam, 2000; Chawla et al., 2001; Mu and LeBlanc,

2002; King-Jones and Thummel, 2005; Kretschmer and Baldwin, 2005; Martin-Creuzburg et al., 2007, Hannas and LeBlanc, 2010; Hannas et al., 2011a; Karimullina et al., 2012; Litoff et al., 2014; Li et al., 2014). The genes accountable for phenotypic responses are involved in the juvenile hormone pathway (Juvenile Hormone Acid O-Methyltransferase-JHAMT and Methoprene-tolerant-Met), in sex differentiation (doublesex 1- dsx and Retinoid X Receptor - RXR) and in the activation of morphological defences (Differential Display 1 - DD1) (Wang et al., 2007; Miyakawa et al., 2010; Kato et al., 2011; Toyota et al., 2013; 2015a). There are evidences about the role of methyl farnesoate (a juvenile hormone) in influencing phenotypic traits, such as morphological defences and sex determination (Miyakawa et al., 2010; Toyota et al., 2015a). In the last years, molecular studies on patterns of DNA methylation in Daphnia are increasing (Asselman et al., 2016; Kusari et al., 2017; Strepetkaite et al., 2016; Kvist et al., 2018).

Considering hormonal and epigenetic regulation and changes of morphological characteristics in different ecological contexts, *Daphnia* can be considered a suitable model to study the molecular fundamentals of phenotypic plasticity. The use of *Daphnia* in epigenetic studies can offer the possibility to distinguish between genetic and epigenetic influences in phenotypic response (Harris et al., 2012). For this purpose, the increasing availability of NGS (Next-Generation Sequencing) technologies applied to daphnids makes it possible to analyse gene expression in response to environmental cues and to answer questions about the relations between genotype and phenotype (Miner et al., 2012). In addition, the use of various genotypes can provide with information on the molecular basis of genotype-specific plasticity. According to Orsini et al. (2018), different genotypes can change the transcriptional activity in a clonal-specific manner by modulating different genes to cope with environmental cues.

In *Daphnia*, gene expression analysis was used to define the transcriptional programs involved in its life-cycle, aging, morphological defences, sex determination (Miyakawa et al., 2010; Toyota et al., 2015a; Christjani et al., 2016; Campos et al., 2018; Hu et al., 2018). RNA-Seq can give more information on levels of all transcripts, isoforms and co-expression networks (Wang et al., 2009; Zhao et al., 2014). This technique can be efficiently used to assess the genetic background of phenotypic plasticity as it gives information about all the transcriptional activity at the basis of the response at organism (e.g. morphological defences) (Rozenberg et al., 2015; An et al., 2018), genotype and single specific gene levels (Orsini et al, 2018). In *Daphnia*, knowledge about gene co-expression and the integration of genomic sequences and gene expression analyses are quickly increasing (Spanier et al., 2017).

Transgenerational effect in *Daphnia*

In transgenerational plasticity, the environmental condition experienced by mothers can influence the performance of the subsequent generations and parents can influence the phenotype of their offspring through non-genetic or epigenetic processes (Donelson et al., 2018). In transgenerational studies, the use of genetically different clones allows to distinguish between the role of genetic variations and epigenetic mechanisms. For this reason, *Daphnia* represents a good model organism to evaluate the transgenerational effects since, in these organisms, epigenetic control contributes in maintaining the mother phenotype in non-exposed generations (Hales et al., 2017). In *Daphnia*, there are many genes that suppress recombination processes and genetic recombination has not been observed (Hebert et al., 1972; Schurko et al., 2009). Thus, by using this clonal organism, genetic changes occurring from mothers to offspring are minimized (Kielland et al., 2017).

In *Daphnia* species there are several examples of transgenerational effects. The exposure to predation risk, microplastics, contaminants and elevated temperatures and changes in food quality and photoperiod have been demonstrated to modify phenotypic traits (i.e. fecundity, size) of subsequent non-exposed generations (Frost et al., 2010; Walsh et al., 2015; Giraudo et al., 2017; Lyu et al., 2017; Coakley et al., 2018; Martins and Guilhermino 2018; Toyota et al., under review). Recent experiments (Vandegehuchte et al., 2009; Hales et al., 2017; Trijau et al., 2018; Jeremias et al., 2018) showed that epigenetic modifications (in particular DNA methylation) could probably induce changes in gene expression that in turn have effects on life history traits in non- exposed organisms. Inheritance of the methylation profile in the subsequent non-exposed generations (F_1, F_2 and F_3) was observed in daphnids after exposure of the parental generation (F_0) to high salinity, chronic γ irradiation and predation risk (Schield et al., 2016; Jeremias et al., 2018; Trijau et al., 2018). Since epigenetic changes have direct consequences on gene expression, transgenerational effects have also been analyzed with a transcriptomic approach to study how environmental cues such as predation risks and contaminants cause alterations in gene expression in non-exposed F_1, F_2 and F_3 generations (Hales et al., 2017; Giraudo et al., 2017). The link among DNA methylation, gene transcription and life history traits in transgenerational plasticity was evidenced in daphnids exposed to predation risk (kairomone), which induces changes in methylome, transcriptome and fitness in non-exposed organisms after exposure of the mother (Walsh et al., 2015; Schield et al., 2016; Hales et al., 2017). The integration of ecological and molecular studies on the inheritance of the methylome and transcriptome can allow to understand the role of maternal effects in the phenotypic pathways of the progeny and its ability to cope with environmental changes

and the molecular processes at the basis of transgenerational plasticity (Bell and Stein, 2017).

Clonal model organisms, with a specific genetic background, are regarded as important "tools" also to investigate the role of microbiome in an environmental context. Clonal organisms can be edited to generate "germ-free" (microbiologically sterile) organisms, so that variations in the response to stress between non-free and "germ-free" animals describe the real impact of the microbiome. In this context, *Daphnia* species represent good candidates for microbiome studies, having all the characteristics reported above and allowing to distinguish the influence of the microbiome from genetic plasticity.

Ecotoxicological studies

Transcriptomic studies have been successfully employed also in ecotoxicological studies by the evaluation of the expression of single specific genes (ecotoxicogenomics) (Poynton et al., 2007; Watanabe et al., 2007; 2008; Connon et al., 2008). Ecotoxicogenomics describe "the integration of genomics (transcriptomics, proteomics, metabolomics) into ecotoxicology" (Snape et al., 2004). The ecotoxicogenomic approach is regarded as a useful tool to understand the environmental risk in ecological context through the application of molecular techniques using several organisms relevant to ecotoxicology (Snape et al., 2004; Ankley et al., 2006; Kim et al., 2015). The toxicogenomic approach has three main aims: recognizing molecular pathways of toxicity, understanding the effect of chemicals on organisms and identifying specific molecular biomarkers to detect environmental risk (Waters et al., 2003; Waters and Fostel, 2004). The -omics techniques applied to *Daphnia* have also been proposed to carry out pre-screening tests in order to identify the pseudo-hormonal effect of chemicals, such as emerging pollutants and endocrine disruptors (Ankley et al., 2006). *D. magna* presents many advantages also in ecotoxicogenomic studies as it shows changes in gene expression in response to the exposure to environmental contaminants (for more information see Kim et al., 2015). This relatively recent framework can be used to understand the potential impact of contaminants in aquatic ecosystems (Piña and Barata, 2011). In *D. magna,* this approach has been successfully employed by evaluating the expression of specific genes (including genes involved in metabolism, energy production, transcription/translation and stress responses) after exposures to different chemicals (Ha and Choi, 2009; Poynton et al., 2011; Kim et al., 2015; Toyota et al., 2017), such as heavy metals (copper, cadmium and zinc), chemotherapeutic agents (beta-naphthoflavone) and pesticides (propiconazole, pentachlorophenol, fenarimol) (Soetaert et a., 2006; 2007a; Poynton et al., 2007; Watanabe

et al., 2007; 2008; Connon et al., 2008). Daphnids can change their expression profile in a short exposure time (i.e. hours) (Ha and Choi, 2009; Poynton et al., 2011; Abe et al., 2015). Poynton et al. (2008) suggested that gene expression analysis in *D. magna* can allow to distinguish between toxic and non-toxic concentrations of metals in the environment and that a No Observed Transcriptional Effect Level (NOTEL) can be defined (Piña and Barata, 2011; Kim et al., 2015). The application of whole transcriptome analysis can allow to discriminate the genes involved in ecological responses through co-expression networks. Co-expressed genes can be used as biomarkers in freshwater monitoring. Consequently, the transcriptome analysis can be applied to evaluate the environmental risk of chemicals with pseudo-hormonal activity (endocrine disruptors) and emerging pollutants in surface waters.

Aims of the thesis

To elucidate the role of genetic and epigenetic controls on phenotypic plasticity and in the attempt to bridge the gap between molecular and ecological aspects of population dynamics, the use of clonal organisms with a sequenced genome, a combination of phenotypic and genomic data seem to be good tools. In this context, genetically different clones of two species of the genus *Daphnia*, *D. magna* and *D. pulex,* were studied. Several questions are at the basis of the present thesis. For example, do different clones use different molecular pathways to cope with environmental changes or do they adopt the same strategies? Are there specific genes involved in phenotypic plasticity? Is the lack of genetic variation a limit to phenotypic plasticity? Are there differences in the role of epigenetic control and hormonal regulation in phenotypic plasticity among clones?

In table 1.1 aims and methodological approaches of each chapter were reported. Experiments based on comparisons of clonal molecular responses after exposure to both biotic (crowding, population density) and abiotic (photoperiod, exogenous hormones, pesticides, heavy metals, chemicals with pseudo-hormonal activity) environmental changes might allow to find answers to our questions.

Table 1.1: Tables of *D. pulex* and *D. magna* experiments. Environmental changes, number of clones, methods and aims for *D. pulex* and *D. magna* experiments.

Daphnia pulex experiments

Chapters	Environmental changes	Number of clones	Methods	Aims
2	(Preliminary test) Crowding Population density	Three	Life history traits evaluation	Clones propensity in male and ephippia production
2.1	Methyl farnesoate 0.8 µM Temperature Conditionate water	Three	Life histoty traits evaluation	Morphological changes (neckteeth) among clones
2.2	Methyl farnesoate 0.8 µM	Three	Life history traits evaluation; RT-qPCR	Phenotypic plasticity and gene expression among clones
2.2	Methyl farnesoate 0.15 µM in F_0 and F_1	Two	Life history traits evaluation; RT-qPCR	Transgenerational plasticity and gene expression between clones

Daphnia magna experiments

Chapters	Environmental changes	Number of clones	Methods	Aims
3	(Preliminary test) Photoperiod changes	Thirty-one	Life history traits evaluation	Clones propensity in male production
3.1	Photoperiod changes in F_0 and F_1	Thirty-one (Experiment1) Four (Experiment2)	Life history traits evaluation; Genome Wide Association Study (GWAS)	Transgenerational plasticity among clones
3.2	Glyphosate 1 mg/l, Roundup 1 mg/l, antibiotics 20 mg/l	Four	Life history traits evaluation RNA-Seq Mi-Seq Comet assay	Influence of whole transcriptome and microbiome in chemicals resistence. Evaluation of effects in DNA integrity. Differences in chemicals resistance among clones
4.1	Artificial and natural waters	One	RT-qPCR	Changing in gene expression by artificial and drinking waters
4.2	Glufosinate ammonium 0.6 µg/l, 6 µ/l, 60 µg/l	One	Life history traits evaluation RT-qPCR	Evaluation of linking between phenotypic and transcriptional processes
4.3	Cadmium, 20-hydroxyecdysone and their mixtures	One	RT-qPCR	Transcriptional activity after mixtures exposure

Chapter 2: Morphological defences and sexual reproduction in *Daphnia pulex* clones

Phenotypic plasticity offers a unique opportunity to disentangle genetic and epigenetic influences on the phenotype of clonal lines. Well-known examples of development plasticity are adaptive polyphenisms triggered by chemical cues released by predators or capable of environmental sex determination (Gilbert, 2001; Janzen and Phillips, 2006). The reproductive model of *Daphnia* is characterized by cyclical parthenogenetic reproduction for most clones, but clonal lineages that only reproduce asexually have also been reported (Decaestecker et al., 2009).

The morphological and behavioural characteristics of the water flea *Daphnia* spp. and its unusual life cycle make it possible to study environmental influences on developmental stages (Lynch, 1980; Harris et al., 2012). In response to variations in the environmental conditions, *Daphnia* species exhibit changes in several traits such as morphological defences, switching from clonal to sexual reproduction and rapid changes in haemoglobin levels (Stibor, 1992; Riessen, 1999; Gorr et al., 2004; Toyota et al., 2013). Considering that a field population is composed by different clones, the use of clonal lineages is a good premise for the application of laboratory results in an ecological field (Lampert et al., 2012). After exposure to environmental cues, differences in plastic response and production of morphological defences were observed among clones of *D. pulex* (Bell and Stein, 2017). *Daphnia* clones can exhibit great variability in the production of males in response to changes of environmental conditions such as photoperiod (Lampert et al., 2012), and clones that inhabit ponds with variable predation risks can have a different inclination towards phenotypic plastic responses (Walsh et al., 2015) .

A linkage between morphological differences was observed after exposure to environmental stimuli (such as predation risk, heat or food availability) and typical clone-specific changes in gene expression were reported (Yampolsky et al., 2014; Christjani et al., 2016; Becker et al., 2018). Adjustments in transcriptional pathways can give clones a different ability to overcome environmental changes (Becker et al., 2018). The analysis of the differences in gene expression among clones that show different phenotypic responses to a specific environmental cue can provide the comprehension of the molecular pathways underlying phenotypic plasticity. However, studies that analyse the effects of environmental changes on both plastic responses and transcriptional regulation in different *Daphnia* clones are still few. In the present study, clonal lineages of *D. pulex* were used to

analyse the responses to environmental factors that elicit morphological defences and male production, both at phenotypic and molecular levels. A preliminary experiment was performed to select clones and evaluate their phenotypic response to different conditions.

Preliminary experiment: clone selection and definition of breeding conditions.

Materials and Methods

Adult parthenogenetic females of *D. pulex* were isolated from zooplankton samples collected in the pond Bodrio del pastore III located in northern Italy (45° 00' 05" N 10° 19' 26" E) and two different ponds located in Czech Republic (pond in Kokořín, 50° 25' 47" N 14° 34' 4" E, and pond in Sedlec, 50° 26' 21" N 14° 36' 29 E) (Rossi et al., 2014, Suppa et al., in review). Females were bred singly in the laboratory, in a commercial natural water, at 20±1 °C with photoperiod 14:10 h L:D and fed the unicellular green alga *Pseudokirchneriella subcapitata* and the yeast *Saccharomyces cerevisiae,* according to the feeding method reported by APAT-IRSA CNR (2003) for *D. magna.* The best-growing and healthy clonal population from each location (named clone I, from the Italian pond, clone K and clone S, from the Czech ponds) was acclimated and synchronized for ten generations to the conditions reported above. Each clonal culture was fed with *P. subcapitata* (at a density of $1.5x10^5$ cells/ml) and the yeast *S. cerevisiae* (at a density of $1.5x10^5$ cells/ml), maintaining a population density of 20 females per clone in 600 ml. The culture medium and the food were renewed twice a week.

Each clone was identified and classified by the analysis of two mitochondrial genes for 12S rRNA, a ~ 711 base-pair (bp) long fragment of the gene coding for the NADH dehydrogenase subunit 5 (ND5) and two nuclear protein-coding loci: Rab4 (subunit gene of the GTPase family) and LdhA (lactate dehydrogenase A). Total genomic DNA was extracted from one to 3 females per clone, and stored in 95% ethanol using the QIAGEN DNeasy Tissue Kit (Valencia, CA). Portions of two mitochondrial genes, 12S rRNA and ND5, were amplified and sequenced with published primers in accordance with the PCR conditions described by Taylor et al. (1996) and Dufresne et al. (2011) respectively. The entire LdhA gene and a part of the gene coding for the small GTPase Rab4 were amplified and sequenced according to Marková et al. (2013) and Omilian et al. (2008). All nucleotide sequence data from the present study will be deposited in the Gen-Bank database under accession numbers DPXA -DPXC (12S rRNA), DPXA -DPXC (ND5), DPXA - DPXG (Rab4) and DPXA (LdhA).

Life-table experiments were also performed on the three clones at conditions that were proven to elicit diversified phenotypic responses in genetically different daphnid clones

(Gorbi et al., 2011). Each clone was maintained for 15 days in three different conditions of population density and food availability:

- high food availability (1.5x105 cells/ml of *P. subcapitata and of S. cerevisiae*) and low daphnid density (1 individual/50 ml), which represents the optimal condition (HFLD treatment);
- low food availability (0.3x105 cells/ml of *P. subcapitata* and of *S. cerevisiae*) and low daphnid density (1 individual/50 ml) that constitute a situation of food shortage due to low algae and yeast density (LFLD treatment);
- high food density ($1.5x10^5$ cells/ml of *P. subcapitata* and of *S. cerevisiae)* and high daphnid density (1 individual/10 ml), representing a situation of food shortage due to crowding (HFHD treatment).

It is worthy to underline that in the second and third condition the food quantity available per individual was the same. Three replicates per treatment were performed, each consisting of five daphnids. Culture medium (the same natural water that was used for the cultures) and food were renewed twice a week. The experiment was performed at high temperature and long-day photoperiod (20 °C, 14:10 L:D). Whenever a daphnid died during the experiment, the volume of the medium was appropriately reduced to maintain a constant population density. The number of female and male offspring produced, along with that of the ephippia, was recorded at each renewal of the medium and discarded. Daphnid growth was checked by measuring carapace length at the beginning of the experiment (neonates aged <24 h) and after 5, 8 and 11 days of treatment.

Results and Discussion

The three selected clonal lineages were labeled as European *D. pulex* and were genetically different. clones I, S and K carried three different haplotypes at both mitochondrial genes 12SrRNA and ND5. Clone I from Bodrio del Pastore III carried the ND5 haplotype DPX A alike its original populations. Clones K from Kokořín and clone S from Sedlec carried local specific haplotypes for Kokořín and Sedlec ponds at both mitochondrial genes 12SrRNA and ND5: haplotype DPX B for clones K and haplotype DPX C for clone S (Figg. 2.1 and 2.2). Two different haplotypes were found in the Italian clone I and in the Czech clones S and K at nuclear genes LdhA and Rab4. The Italian clone I carried the DPX A haplotype for the LDHA gene while clones K and S were heterozygote at the LDHA gene with several hetorozygous positions plus an indel. The Italian clone I carried the DPX E haplotype for the Rab4 gene while clones K and S bore the DPX G haplotype (Fig. 2.3).

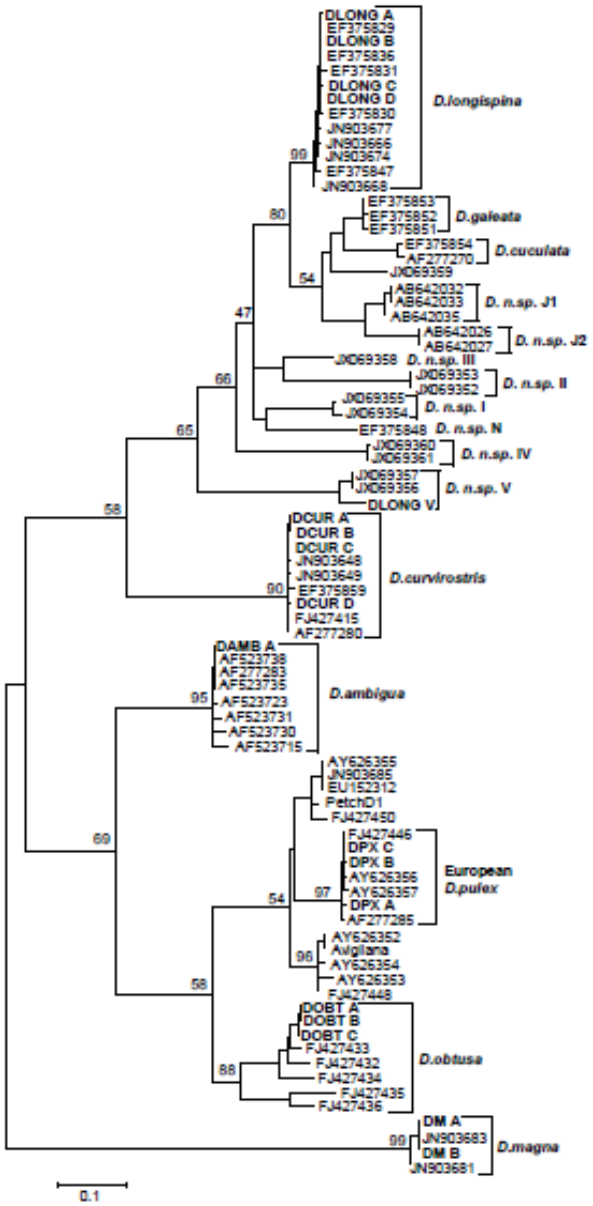


Figure 2.1: Maximum likelihood mitochondrial phylogenetic tree based on 12S rRNA haplotypes of clones I (haplotype DPX A), K (haplotype DPX B) and S (haplotype DPX C).

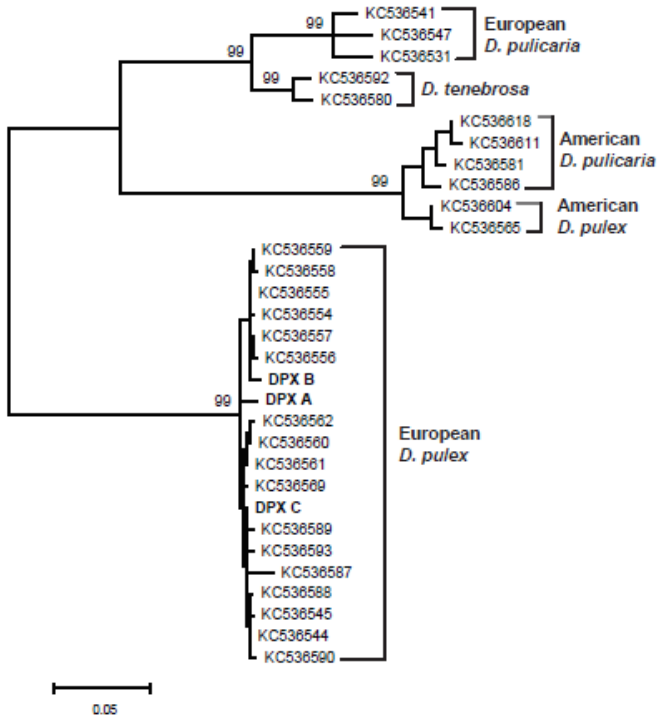


Figure 2.2: Maximum likelihood mitochondrial phylogenetic tree based on NADH dehydrogenase subunit 5 (ND5) haplotypes of clones I (haplotype DPX A), K (haplotype DPX B) and S (haplotype DPX C).

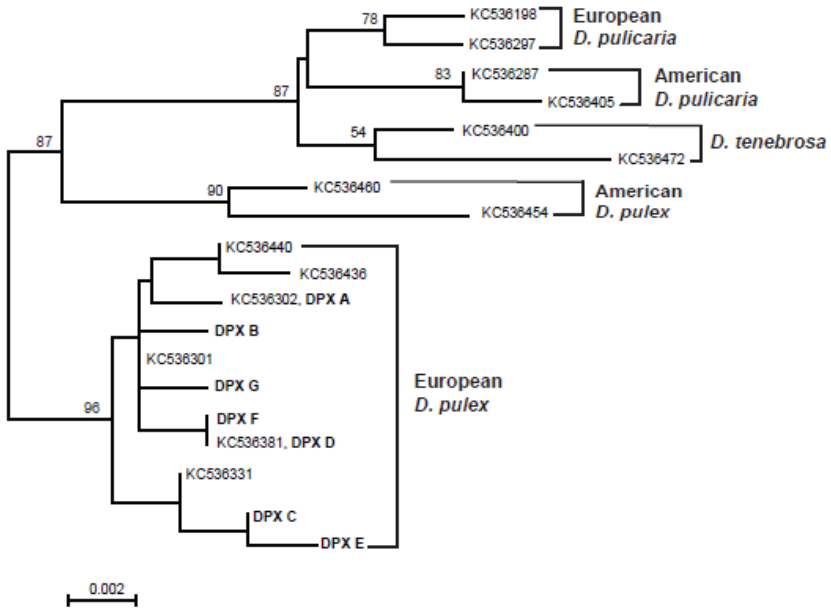


Figure 2.3: Phylogenetic tree of Maximum likelihood nuclear phylogenetic tree based on gene Rab4 (subunit gene of the GTPase family) in clones I (haplotype DPX E), K and S (haplotype DPX G).

The three clones showed the highest fecundity and growth in the HFLD treatment. However significant differences among clones were observed, clone K displaying the lowest fecundity and clone S the highest ($p<0.001$) (Fig. 2.4). Both HFHD and LFLD treatments significantly lowered fecundity, but with diversified responses among clones. In clones I and S the decrease was significantly more pronounced in crowded conditions (HFHD treatment) ($p<0.01$) rather than during situations of food shortage due to low algae and yeast cell density (LFLD treatment); instead, in these two treatments the fecundity of clone K was similar (Fig. 2.4). No males were observed in the progeny of clone I whatever the treatment. Clone K produced male offspring only in HFLD and HFHD conditions. On the contrary, clone S produced newborn males in all treatments, with the highest percentage in crowded conditions (HFHD treatment); in these conditions clone S also produced ephippia, although in small quantities (2.67±0.58 per replicate) (Fig. 2.5). No ephippia were recorded for the other two clones. Growth was almost identical in all conditions within each clone until the 5^{th} day, then it started to decline similarly in both HFHD and LFLD treatments (Fig. 2.6). As a consequence, daphnid mean carapace length on the 11^{th} day was significantly lower ($p<0.001$) in these treatments than in HFLD (Fig. 2.7). Several necktoothed newborns were observed especially in HFHD.

Besides being genetically different, the three clones displayed diversified phenotypic responses to the various conditions tested. In particular, the clones differed in fecundity and in their tendency to produce ephippia and male individuals. Crowding and food shortage do not represent environmental stimuli able to induce male production in clone I while, in clones K and S male production occurred in conditions that can be regarded as optimal both in terms of food availability and population density. In addition, these two clones, differently from clone I, gave birth to males also at low temperatures and short photoperiod (16 °C and 12:12 L:D) (personal observation). Clone S was the only one that produced ephippia, which were observed in the most stressing tested condition characterized by a low volume of medium and, consequently, a per-capita low food availability despite the high algae and yeast density.

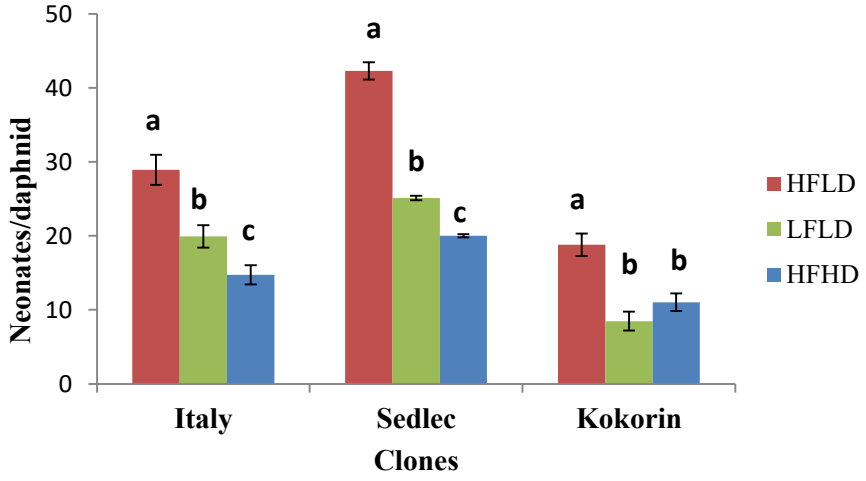


Figure 2.4: Fecundity of daphnids maintained in different conditions of population density and food availability. HFLD=high food availability ($1.5x10^5$ cells/ml of *P. subcapitata* and of *S. cerevisiae)* and low daphnid density (1 individual/50 ml); LFLD=low food availability ($0.3x10^5$ cells/ml of *P. subcapitata* and of *S. cerevisiae)* and low daphnid density (1 individual/50 ml) that constitute a situation of food shortage due to low algae and yeast density; HFHD=high food density ($1.5x10^5$ cells/ml of *P. subcapitata* and of *S. cerevisiae)* and high daphnid density (1 individual/10 ml), that constitute a situation of food shortage due to crowding. Different letters label significantly different values within each clone (mean ±SD) (ANOVA and Tukey's test; $p<0.01$).

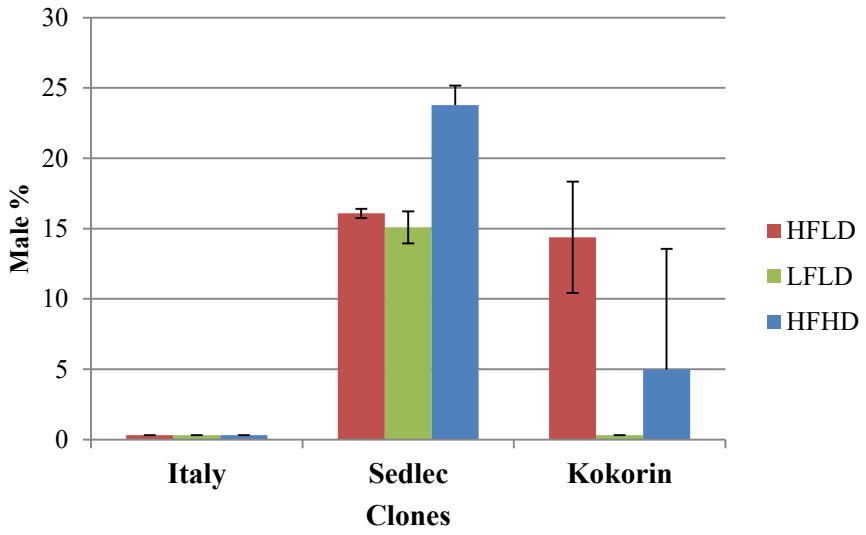


Figure 2.5: Percentage of male offspring in the progeny (mean ±SD) of daphnids maintained in different conditions of population density and food availability. HFLD=high food availability ($1.5x10^5$ cells/ml of *P. subcapitata* and of *S. cerevisiae)* and low daphnid density (1 individual/50 ml); LFLD=low food availability ($0.3x10^5$ cells/ml of *P. subcapitata* and of *S. cerevisiae)* and low daphnid density (1 individual/50 ml) that constitute a situation of food shortage due to low algae and yeast density; HFHD=high food density ($1.5x10^5$ cells/ml of *P. subcapitata* and of *S. cerevisiae)* and high daphnid density (1 individual/10 ml), that constitute a situation of food shortage due to crowding .

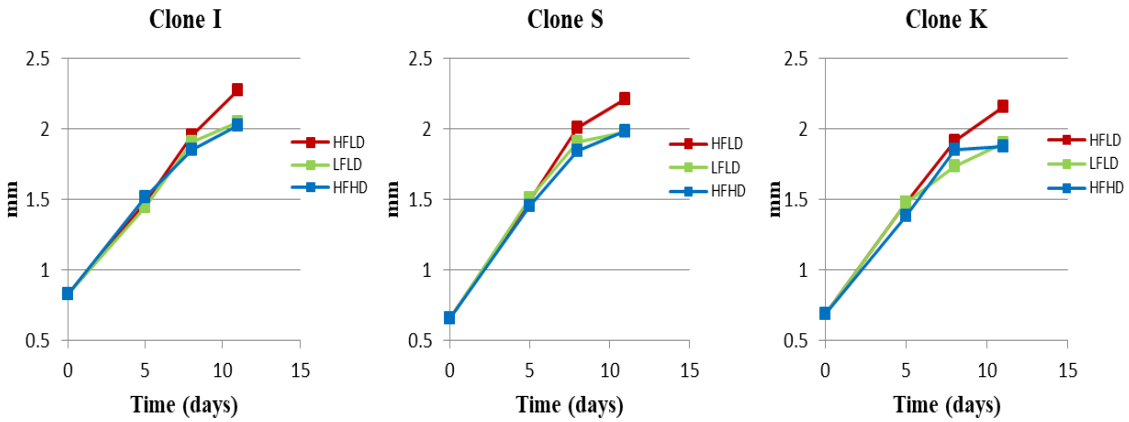


Figure 2.6: Growth of daphnids maintained in different conditions of population density and food availability. HFLD=high food availability ($1.5x10^5$ cells/ml of *P. subcapitata* and of *S. cerevisiae)* and low daphnid density (1 individual/50 ml); LFLD=low food availability ($0.3x10^5$ cells/ml of *P. subcapitata* and of *S. cerevisiae*) and low daphnid density (1 individual/50 ml) that constitute a situation of food shortage due to low algae and yeast density; HFHD=high food density ($1.5x10^5$ cells/ml of *P. subcapitata* and of *S. cerevisiae)* and high daphnid density (1 individual/10 ml), that constitute a situation of food shortage due to crowding.

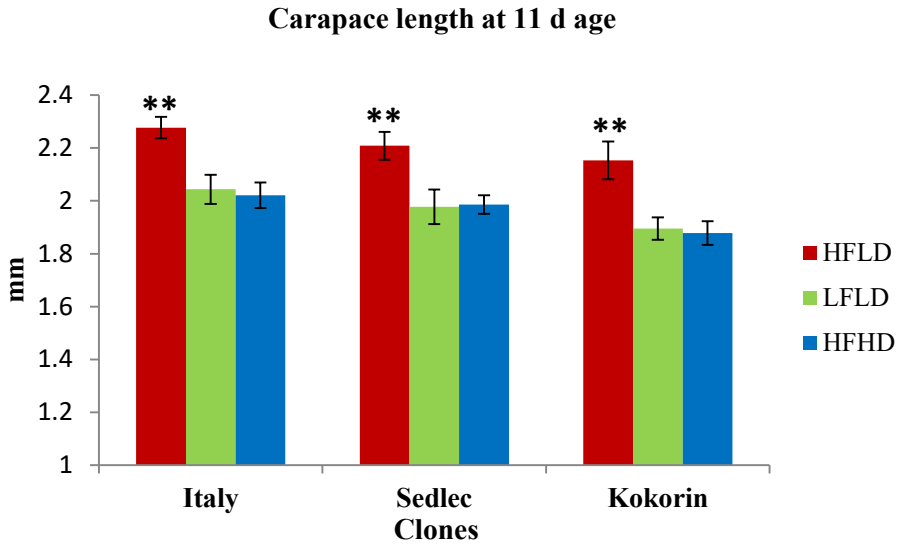


Figure 2.7: Carapace length (mean±SD) of daphnids maintained in different conditions of population density and food availability. HFLD=high food availability ($1.5x10^5$ cells/ml of *P. subcapitata* and of *S. cerevisiae)* and low daphnid density (1 individual/50 ml); LFLD=low food availability ($0.3x10^5$ cells/ml of *P. subcapitata* and of *S. cerevisiae)* and low daphnid density (1 individual/50 ml) that constitute a situation of food shortage due to low algae and yeast density; HFHD=high food density ($1.5x10^5$ cells/ml of *P. subcapitata* and of *S. cerevisiae)* and high daphnid density (1 individual/10 ml), that constitute a situation of food shortage due to crowding.**=significant difference ($p<0.001$) among treatments within each clone (ANOVA and Tukey's test).

Chapter 2.1: Maternal effects and predator-induced defences in *D. pulex*

Maternal environment may provide information about the probable progeny environment and a mother may adjust within-brood variability in an adaptive manner increasing the likelihood that at least some of her offspring will survive and reproduce (Mousseau and Fox, 1998). However, important role in phenotype development have also abiotic and biotic factors present in a mother's environment. For example, adaptive polyphenisms develop when individuals' phenotype is triggered by chemical cues released by predators (Gilbert, 2001).

Antipredator defences, well-documented in *Daphnia*, consist of several morphological and behavioural components and represent a typical example of how the environment affects phenotype and induces polyphenisms (Weider and Pijanowska, 1993; Kvam and Kleiven, 1995; David et al., 2014). However, also transgenerational transfer of defences from mother to offspring may be important part of predator-prey interaction (Agrawal et al., 1999).

To reduce the risk of predation, many species of *Daphnia* have evolved different life histories, and produce different morphological structures such as spines, neckteeth, and helmets in response to chemicals, kairomones, released by vertebrate and invertebrate predators (Krueger and Dodson, 1981; Weider and Pijanowska, 1993; Grant and Bayly, 1981; Tollrian, 1990; Sorensen and Sterner, 1992; Tollrian, 1995; Dzialowski et al., 2003; Gélinas et al., 2007; Petrusek et al., 2009; Walsh et al., 2015). Kairomones play an important role in prompting these phenotypic changes, while abiotic factors (e.g. temperature, photoperiod, water chemistry) are implicated in the modulation of these shifts (Krueger and Dodson, 1981; Havel and Dodson, 1985; Hebert and Grewe, 1985; Parejko and Dodson, 1990; Mirza and Pyle, 2009; Riessen et al., 2012). Conspecific populations often exhibit variation in the size of the structure, although they typically produce similarly-shaped helmets (with spines and/or crests) or neckteeth type (e.g. single to multiple teeth in a row or rosette) (Beaton and Hebert, 1997; Juračka et al., 2011). Following maternal exposure to kairomones, antipredator defences in juveniles can be transmitted from the mother to progeny either via the oocytes or the embryos that are held in the maternal brood chamber (Harris et al., 2012). The effect is cumulative: progeny born with a maternally-induced defence have larger helmets than offspring from mothers that have not been exposed to kairomones (Agrawal et al., 1999).

Neckteeth are characteristic small spines formed along the dorsal surface of the carapace in juveniles of *Daphnia* that efficiently increase their resistance to predation by the larvae of

Chaoborus (Diptera) (Havel and Dodson, 1984; Repka et al., 1995; Colbourne et al., 1997; Kotov et al., 2006). Within species, alternative phenotypes and predator-induced polyphenisms may be related to genetic diversity among strains, concentration of kairomones released by predators and to the effect of multifarious environmental cues (Parejko and Dodson, 1991; Weber and Declerck, 1997; Spitze, 1992; Boersma et al., 1998; Pauwels et al., 2005; Dzialowski et al., 2003; Laforsch and Tollrian, 2004b; Imai et al., 2009; Naraki et al., 2013; Weiss et al. 2015, a;b). In *D. pulex,* predator-induced polyphenisms are regulated mostly through embryonic and postembryonic development when the exposure to kairomones is required for neckteeth formation (Imai et al., 2009). According to Miyakawa et al. (2013a), individuals that develop in the absence of kairomones, seldom express defensive morphs, while low neckteeth induction (less than 10%) was recorded in newborns of mothers not exposed to *Chaoborus'* kairomones and reared in long day and crowded conditions (Schwarzenberger et al., 2014). Furthermore, methyl farnesoate (MF, juvenile hormone in Crustacea) induces the expression of genes involved in neckteeth formation in the first instar juveniles of *D. pulex* (Miyakawa et al., 2010; 2013a).

We described cyclomorphosis in coexisting natural populations of *D. pulex* and *D. longispina* from the pond Bodrio del pastore III and recorded six different defensive morphotypes, varying in the numbers, location and thickness of neckteeth (Rossi et al., 2014; Maurone et al., 2018). The percentage of predator-induced morphotypes, for both *D. pulex* and *D. longispina*, was not related to the density of *Chaoborus flavicans* larvae. Instead, the direct or transgenerational effects of kairomones and genetic differences among *Daphnia* strains, along with other factors or environmental cues such as maternal environmental conditions (e.g. long-day photoperiod, temperature, crowding), might act as proxies which signal predation risk, and might be involved in polyphenisms.

Laboratory experiments were conducted to examine the maternal impact on neckteeth formation in newborns from *D. pulex* mothers that have not been exposed to *Chaoborus* kairomones. Clonal females were reared under different experimental conditions that simulated variable seasonal risk of predation and/or environmental stressors (e.g., temperature, photoperiod, crowding). The aim of this study was to test if the transgenerational effect, due to transmission of altered maternal epigenome to the progeny via the oocytes, can induce neckteeth's formation in the F_1 generation. To examine this hyphotesis/assumption, we exposed three clonal lineages of *D. pulex* to different treatments. Three treatments, high temperature and long day photoperiod (20 °C 14:10 L:D), low temperature and short day photoperiod (16 °C 12:12 L:D) and crowding were

chosen to simulate the seasonal conditions recorded in the pond Bodrio del pastore III, when the density of *D. pulex* is high and percentage of individuals with neckteeth fluctuates. One treatment with juvenile hormone MF was used as a reference for neckteeth induction.

Materials and methods

In the laboratory life-table experiments, we used a clonal lineage of *D. pulex* (clone I) originated from the pond Bodrio del pastore III because the *D. pulex* population of this pond showed a distinct seasonal cyclomorphosis and polyphenisms in natural conditions (Rossi et al., 2014; Maurone et al., 2018). The pond Bodrio del pastore III pond is a shallow fishless pond where *Chaoborus* larvae do not perform diel vertical migration (Rossi personal observation, see also Rossi et al., 2014; Maurone et al., 2018). For comparison, we used two clonal lineages of *D. pulex* (clone K and clone S) from two village ponds in Czech Republic.

All three clonal cultures were acclimated and synchronized for 10 generations at 20 °C and 14:10 L:D photoperiod. In breeding cultures, 20 females per clone were maintained in 600 ml of natural commercial water (San Benedetto) and fed twice a week with equal amounts of the unicellular green alga (*Pseudokirchneriella subcapitata*) and the yeast (*Saccharomyces cerevisiae*) (the final concentration of food was $1.5x10^5$ cells/ml). The culture medium was renewed twice a week.

Fifty female juveniles (less than 24 hours old) per clone, produced in the third clutch, were isolated and used to start five life-table experimental treatments (10 individuals of each clone randomly assigned to each treatment).

To test the effect of environmental factors (temperature and photoperiod) on the induction of neckteeth in the F_1 progeny, we used two experimental treatments: 1) 20 LD treatment: the laboratory conditions were set up at 20 °C and 14:10 L:D photoperiod; 2) 16 SD treatment: the laboratory conditions were set up at 16 °C and 12:12 L:D photoperiod. This setting of conditions was chosen to simulate the seasonal succession recorded in the natural conditions of the pond Bodrio del pastore III, when the density of *D. pulex* is high, but percentage of the individuals with neckteeth can fluctuate (Maurone et al., 2018). To acclimate to 16 SD treatment, all clonal strains were reared at 16 °C and 12:12 L:D photoperiod for at least two generations. Natural commercial water was used as culture medium.

The crowded water (CW) treatment was used to test the effect of crowded conditions on neckteeth formation in the F_1 progeny, in which *Daphnia* were exposed to long-day

photoperiod conditions (20 °C, 14:10 L:D). In this treatment, we used medium conditioned by a crowded *Daphnia* population (i.e. in 600 ml jars with natural commercial water about 200 *Daphnia* individuals were reared for 15 days at 20 °C, 14:10 L:D photoperiod). The conditioned water was filtered through a 50 μm mesh size net. Each clone was exposed to medium conditioned by its own crowded clonal population.

The Methyl Farneosate (MF) is a juvenile hormone that is known to induce neckteeth development in *Daphnia* (Miyakawa et al. 2010, 2013a). A stock solution of 1 mg/ml MF (Echelon Bioscience) was dissolved in dimethylformamide (DMF) (Sigma-Aldrich), a solvent commonly applied in aquatic toxicity tests, and kept at -20 °C (stock solution) until used. For experiments, 0.8 μM solution of MF (Tatarazako et al., 2003; Miyakawa et al., 2013a) was used. In this treatment, *Daphnia* individuals were reared at 20 °C and 14:10 L:D photoperiod conditions. A treatment with dimethylformamide (DMF), at the same conditions, was used as a control for the MF treatment. The final concentration of DMF in the medium was below 0.01% (v/v) .

Ten juveniles per clone per treatment were individually reared in a six-well culture-plate (one juvenile per well in 10 ml of culture medium). These experimental conditions were set up based on preliminary breeding conditions of *Daphnia* showing differences in male's and ephippia's production among clonal lineages (see above). In each treatment, the medium was renewed three times a week and *Daphnia* was fed with a mixture of *P. subcapitata* and *S. cerevisiae*, each at a final concentration of $1.5x10^5$ cells/ml. All experimental treatments were replicated 10 times using individuals from the three clones (5 treatments x 3 clones x 10 replicates = 150 females). The experiment lasted for 15 days until the release of the 3rd clutch, except the 16 SD treatment that took 21 days because the development time at 16 °C was longer than at 20 °C and the MF treatment where most of females released only the 1st clutch. The number of neonates per clutch per female was recorded three times a week. Juveniles were removed every 24 hours and moulted carapaces were monitored. First-instar juveniles were sorted using a Leica CLS 50x microscope (Leica, Mannheim, Germany) into four different morphotype groups according to the number of neckteeth: M0 - no teeth, M1 - one tooth, M2 - two teeth and M3 - three teeth (for details see Fig. 2.1.1). The percentage of neckteethed juveniles per clutch and per female for each clonal lineage and treatment was calculated. All neckteethed neonates were maintained in culture medium at 20 °C and 14:10 L:D photoperiod conditions. A subset of neckteethed juveniles from each treatment and clonal lineage (about 30%) was checked daily for neckteeth maintenance.

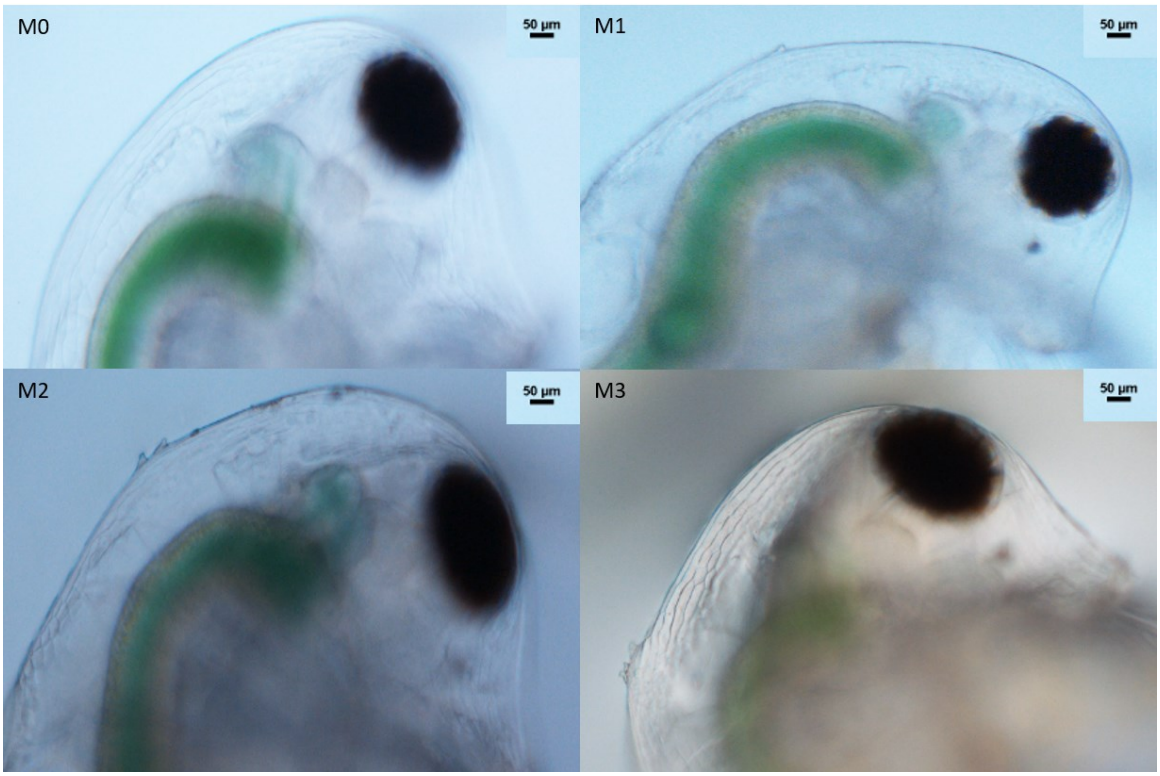


Figure 2.1.1: Four different morphotypes of *D. pulex* according to the number of neckteeth induced numbers: M0 - no teeth, M1 - one tooth, M2 - two teeth, M3 - three teeth.

A two way analysis of variance (ANOVA) was used to test for significant differences in number of clutches, in number of newborns and in percentage of neckteethed juveniles per female among treatments and clones. When variances were not homogeneous among treatments and clones and when data transformation did not solve the problem of variance heterogeneity, the median non-parametric test was conducted. For pairwise comparisons, Sheffè's test was applied and for nonhomogeneous variances between treatments or between clones, the Dunnett's test was used. Pearson χ^2 was performed to evaluate the relationship between percentages of individuals with neckteeth and fecundity and between percentages of neckteethed juveniles and morphotype frequencies. All statistical tests were performed using SPSS 25.0 software (IBM Corp., 2017).

Results

Altogether 329 clutches and 1484 juveniles were recorded. Significantly fewer clutches per female were observed in the MF (1.30 +/- 0.119) than in the DMF (2.73 +/- 0.179) treatment ($p < 0.05$). Considering all treatments, clone K produced a higher number of clutches per female (2.38 +/- 0.127) than clones S (2.22 +/- 0.167) and I (1.98 +/- 0.119), but differences were not statistically significant. Moreover, a total of 27 ephippial clutches were produced. Clone S produced 1, 7, and 11 ephippia in the MF, CW, and 16 SD treatments, respectively. Clone K produced 8 ephippial clutches in the 16 SD treatment. Clone I did not produce any ephippium. The mean number of newborns per female was affected by treatment (median test $p < 0.001$) but did not vary significantly among clones (Fig. 2.1.2). The mean number of juveniles per female was lower in MF, CW and 20 LD treatments than in 16 LD and DMF treatments ($p < 0.05$). Generally, males were produced by clones I (in the MF treatment), K (in MF, CW and 16SD treatments) and S (in all treatments), but hereafter, gender differences among juveniles were not recorded.

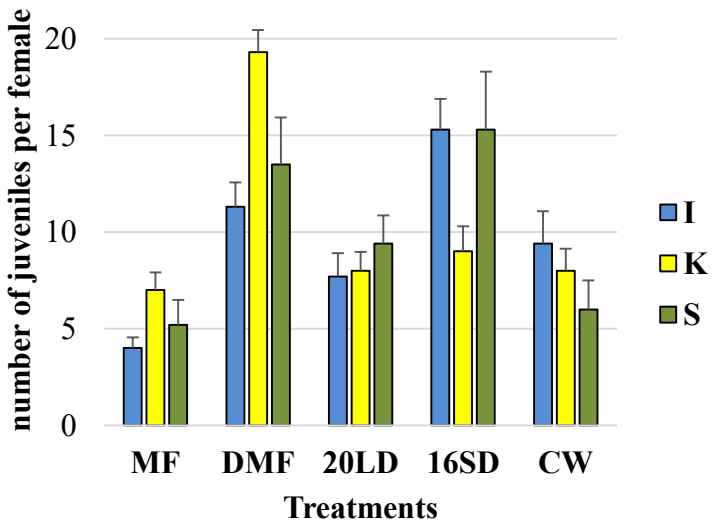


Figure 2.1.2: Mean cumulative number of juveniles per female. reported in three *D. pulex* clonal lineages (see Materials and Methods for details on the origin of each of the clones) and five different treatments: MF - methyl farnesoate, DMF - dimethylformamide, 20 LD - 20 °C and 14:10 L:D photoperiod, 16 SD - 16 °C and 12:12 L: D photoperiod, CW - crowding. Bars indicate standard errors and, for each treatment, different letters indicate significant differences between clones in pairwise comparisons ($p < 0.05$).

In all five treatment groups, 78% of first-instar juveniles showed neckteeth formation and 82% of all induced juveniles formed only one necktooth (M1 morphotype). Morphotypes M2 and M3 were produced in very low frequencies, 16% and 2%, respectively. The percentage of induced juveniles increased in the order of 1st (76%), 2nd (77%), 3rd (84%)

and 4th (85%) clutch. However, because only a small percentage of females produced the 3rd and 4th clutches, those results were not statistically compared. Significant differences in the percentage of induced juveniles per female were observed among treatments and clones (Table 2.1.1 and Fig. 2.1.3).

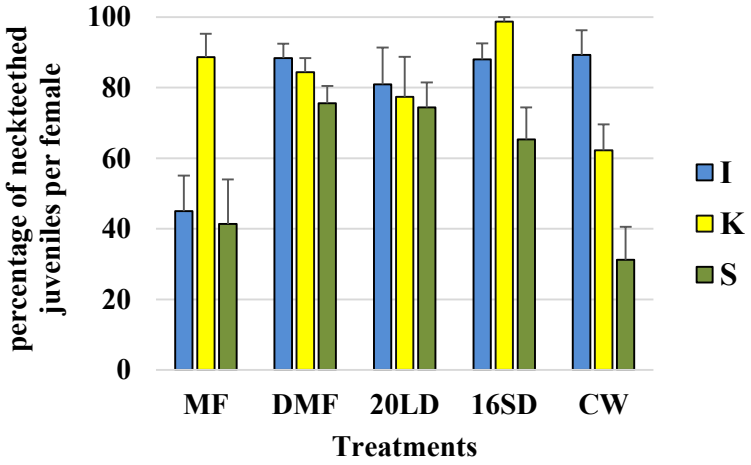


Figure 2.1.3: Percentage of induced juveniles per female in three *D. pulex* clonal lineages (see Materials and Methods for details on the origin of each of the clones) and five different treatments: MF - methyl farnesoate, DMF - dimethylformamide, 20 LD - 20 °C and 14:10 L:D photoperiod, 16 SD - 16 °C and 12:12 L: D photoperiod, CW - crowding. Bars indicate standard errors and, for each treatment, different letters indicate significant differences between clones in pairwise comparisons ($p < 0.05$).

Table 2.1.1: ANOVA analysis: the percentage of neckteeth-induced first instar juveniles per female was the dependent variable, Treatment (MF- Methyl Farnesoate, DMF- Dimethylformamide, 20 LD- 20 °C and 14:10 L:D photoperiod, 16 SD- 16 °C and 12:12 L:D photoperiod and CW- Crowded Conditions) and clone (I, K and S) were used as fixed factors. Original data were arcsin transformed.

Source	Sum of square	df	F	P
Model	15.390	14	6.270	< 0.001
Intercept	136.428	1	776.900	< 0.001
Treatment	3.322	4	4.980	0.001
Clone	5.604	2	16.803	< 0.001
Treatment x Clone	6.569	8	4.924	< 0.001
Error	21.679	130		
Total	176.966	145		

The highest percentage of induced juveniles per female was recorded in the 16 SD treatment (84%) while the lowest was observed in the MF and CW treatments (60% in both treatments) ($p < 0.05$). Clones I and K produced a higher percentage of induced

individuals than clone S ($p < 0.05$). A significant interaction between treatment and clone was recorded due to the high percentage of induced juveniles (> 80%) produced by I and K clones from the CW and MF treatments, respectively (Table 2.1.1 and Fig. 2.1.3). In general, the percentage of induced juveniles was positively related to the total number of juveniles per female (Pearson = 0.919, $p < 0.001$). In particular, an S-shaped curve was observed in a plot of percentages of neckteeth and all juveniles per female of clones I and S ($R2 = 0.307$, $F_1,91 = 40.222$, $p < 0.001$) (Fig. 2.1.4).

The frequency of M1 morphotype juveniles per female was depended on treatment (median test $p < 0.006$) and clone (median test $p < 0.001$) (Fig. 2.1.5).

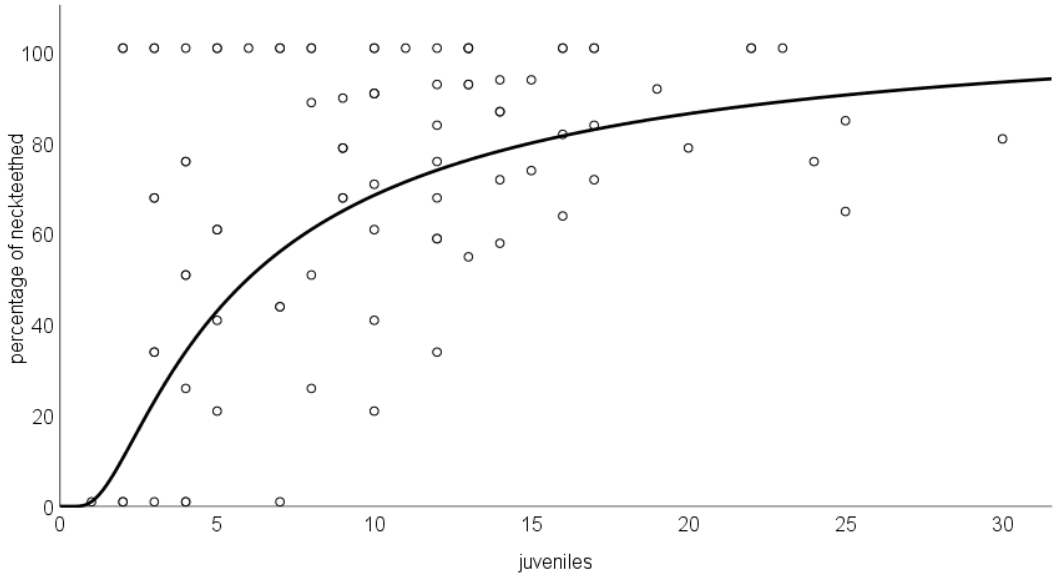


Figure 2.1.4: Relationship between the percentages of neckteeth-induced juveniles and all juveniles per female in clones I and S (combined for all 5 treatments).

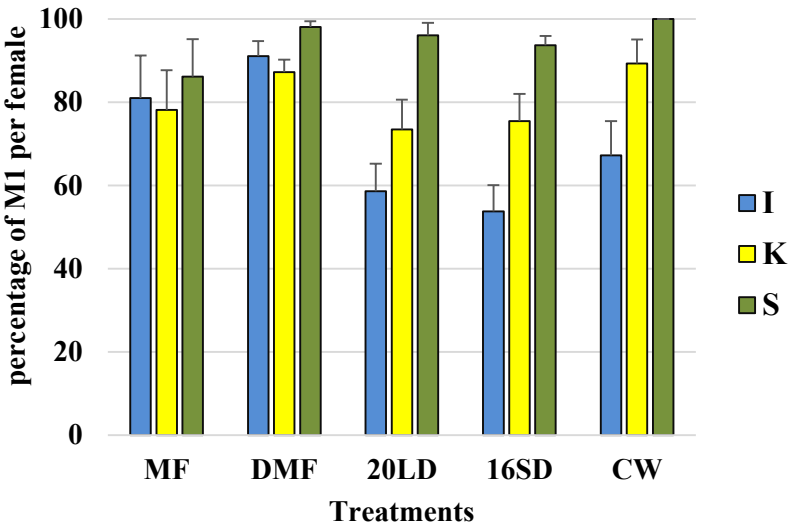


Figure 2.1.5: Percentage of M1 morphotype reported in three *D. pulex* clonal lineages (see Materials and Methods for details on the origin of each of the clones) and five different treatments: MF - methyl farnesoate, DMF - dimethylformamide, 20 LD - 20 °C and 14:10 L:D photoperiod, 16 SD - 16 °C and 12:12 L: D photoperiod, CW - crowding. Bars indicate standard errors and, for each treatment, different letters indicate significant differences between clones in pairwise comparisons ($p < 0.05$).

The percentage of the M1 morphotype was higher in DMF than in 16 SD and 20 LD treatments ($p < 0.05$). Considering all treatments, clone S produced only M1 morphotype juveniles (100%) ($p < 0.05$). The frequency of the M1 morphotype was inversely related to the percentage of induced juveniles per female (Pearson = -0.170, $p = 0.048$).

All induced juveniles from all treatments and clones lost the neckteeth after the first moult.

Discussion

We have recorded almost 80% of neckteethed neonates produced by mothers that were not directly exposed to kairomones. We found significant differences in the percentage of neckteeth induction in first-instar juveniles from different treatments and clones. Our results provide strong evidence that the development of neckteeth is not accidental and may be related to genetic (i.e. difference among clonal lineages) and to epigenetic and maternal effects in the formation of antipredator defences. We described polymorphisms in antipredator defences, according to the number of neckteeth produced, and recorded three different defensive morphotypes (M1 - M3). A very similar variability in neckteeth types (e.g. single and multiple teeth) has been reported in natural population of *D. pulex* from the

pond Bodrio del pastore III (Maurone et al., 2018) and in conspecific populations of *D. pulex* from different habitats and localities (Juračka et al., 2011).

We showed that the neckteeth formation in *D. pulex* can be induced early in the embryonic development by maternal transgenerational effects, but is limited to the first juvenile instar. This result is in accordance with previous studies that emphasized the importance of juvenile exposure to kairomones for neckteeth maintenance in the second and third instars (Laforsch and Tollrian, 2004 a;b; Imai et al., 2009; Naraki et al., 2013). The evolutionary experiences with the invertebrate predation regime and epigenetic changes can be transferred to the progeny even when parental generations are no longer exposed to kairomones (Vandegehuchte and Janssen, 2011). We showed that, in the absence of chemicals released by predators, newborns lost their antipredator defences after the first moult. This result is in accordance with previous studies that emphasized the importance of kairomones exposure of juveniles for neckteeth maintenance in the second and third instars (Imai et al., 2009).

Abiotic environmental factors (e.g. temperature and photoperiod) are involved in the modulation of plasticity and, in some cases, can be more reliable cues of predation risk than kairomones alone. For example, the spiny water flea *Bythotrephes longimanus* reacts to the increase of water temperature by increasing spine and body length and by decreasing clutch size and reproductive age because the larger size of fish in midseason, when the temperature is higher, increase predation risk (Miehls et al., 2013). We showed that, percentage of induced juveniles varied between 77% and 84% in treatments 20 LD and 16 SD that mimicked the seasonal succession recorded in the Italian population of the pond Bodrio del pastore III. Although differences in the percentage of induced juveniles were not statistically significant, clone I produced higher numbers of juveniles and juveniles with neckteeth at 16 SD (88%) than 20 LD (81%) condition. The combination of environmental cues 16 °C temperature and 12:12 L:D photoperiod (16 SD), mimicked the conditions recorded in the pond Bodrio del pastore III in early spring or early autumn when *Daphnia* and *Chaoborus* larvae generally showed seasonal population peaks, the risk of predation by *Chaoborus* is high and *Daphnia* showed the highest percentages of neckteethed juveniles (almost 80%) (Rossi et al., 2014; 2015; Maurone et al., 2018). The combination of 20 °C temperature and 14:10 L:D photoperiod (20 LD) mimicked the conditions recorded in the pond Bodrio del pastore III during early summer, when both *Daphnia* abundance and the percentage of individuals with neckteeth (40-60%) are lower than in early spring and autumn (Rossi et al., 2015; Maurone et al., 2018). Moreover, experimental conditions (one juvenile per well in 10 ml of culture medium) we set up are

stressful *per se* (see above). Therefore, the organisms may respond to kairomones that are produced by predators, to environmental factors that act as proxies for signaling change in predation risk (Miehls et al., 2013) and to endogenous MF produced in response to stressful conditions (LeBlanc and Medlock, 2015; Toyota et al., 2016). This fact would explain why, in the pond Bodrio del pastore III, the frequencies of induced morphotypes of *D. pulex* was not significantly related to the density of *Chaoborus* larvae (Maurone et al., 2018).

A modulating effect of biotic factors (e.g. hormones melatonin and MF) has been described in neckteeth development (Miyakawa et al., 2013a; Schwarzenberger et al., 2014). In our study, the frequencies of induced defensive morphotypes (M1 - M3) exposed to MF varied between 40%, in clones I and S, and 89% in clone K and was the lowest (60%) among all treatments. MF, known as a juvenile hormone in crustaceans, plays important roles in the regulation of development (Laufer et al., 1993) and stimulates male production in daphnids (Olmstead and LeBlanc, 2007; Imai et al., 2009; LeBlanc et al., 2013; Miyakawa et al., 2013a; 2015; Toyota et al., 2015a). Actually, all three clonal lineages produced males in the MF treatment and, in general, the low number of clutches recorded in the MF treatment was due to a delayed maturation of clonal females (see Chapter 2.2). A similar percentage of induced juveniles (60%) was recorded in the CW treatment, where we tested the effects of catabolites or chemicals that might exhibit hormone-like activity on neckteeth formation (Sumpter, 2005).

According to our results, the relationship between the percentage of induced juveniles per female and fecundity, especially in clones I and S, was described by a characteristic S-shaped curve. Once a defence mechanism is subject to a trade-off, prey can reduce investment into antipredator mechanisms during high population density or low food availability (Tollrian et al., 2015). The observed pattern may be interpreted as a functional response (anti-predator adaptation) to predator satiation (Begon et al., 1996). Still, predators can consume only a certain amount of food, and because of this “satiation effect”, prey at high densities can benefit from a “safety-in-numbers” effect. As food supply begins to overwhelm the predator's ability to consume and process prey items, the predator decelerates its rate of predation and at high levels of prey density, saturation occurs. This strategy has evolved in a range of diverse prey, including many species of plants, insects, and fish (Williams and Simon, 1995; Espelta et al., 2008). On the other hand, the observed relationship might be ascribable to predator dilution or anti-predator vigilance (Courchamp et al., 1999).

Actually, *Daphnia* can produce morphological defences and/or altered life-history traits as a response to the presence of predators. For instance, there is evidence that *Daphnia* exposed to kairomones, can increase the rate of development and invest more heavily in reproduction (Stibor, 1992; Riessen, 1999; Walsh et al., 2015). *Daphnia* may use ephippial production as an escape strategy from predation pressure (Ruvinsky et al., 1986; Larsson, 1991; Spitze, 1992; Hairston, 1996). *D. magna* produces ephippia in the presence of kairomones and conspecific alarm chemicals originated from injured conspecific prey (Slusarczyk, 1995; 1999). In our experiments, particularly in clones S and K reared in the 16 SD treatment, we observed a switch/trade-off between neckteeth and ephippial production. The juvenile hormones produced in response to environmental stress stimuli such as changes in photoperiod, reduced food availability and crowding, might be associated with the activation of genes involved in neckteeth development, as well as in sex determination and ephippia production (Suzuki and Nijhout, 2008; Tollrian et al., 2015; Christjani et al., 2016; see Chapter 2.2). Interestingly, on average, clone I produced the highest percentage of induced juveniles and did not produce any ephippium, while clone S produced the lowest percentage of induced juveniles and the highest percentage of ephippial clutches.

We described three different defensive morphotypes, M1, M2 and M3, that are very similar to the most frequent neckteeth types described in spring population of *D. pulex* from the pond Bodrio del pastore III (Maurone et al., 2018). However, in our laboratory experiments, the frequencies of the most abundant morphotype, M1 (one tooth, total frequency about 82%), and the number of induced juveniles per female had an inverse relationship. This result might be linked to a dose-response relationship between stressor (kairomones) and prey response. Likewise, double-exposure to the stress factor (when both the mother and the neonates are exposed) will result in a higher percentage of individuals with neckteeth and a higher overall number of neckteeth (3-4) per individual than single-exposure (when only the mother is exposed) (Imai et al., 2009; Naraki et al., 2013; Weiss et al., 2015a). Our laboratory result would support our hypothesis about a mismatch between the kairomone-sensitive phase in embryonic development (maternal effect) and the post-embryonic phase of neckteeth formation to explain the low frequency of induced morphotypes during peak of *D. pulex* population density recorded in the pond Bodrio del pastore III in autumn 2014 (Rossi et al., 2015; Maurone et al., 2018). We suggested that, the unusual autumnal population density peak was caused by a recruitment from the ephippial egg bank. The lack of indirect maternal exposure to kairomones during embryogenesis should result in very few or none neckteethed juveniles (Rossi et al., 2015;

Maurone et al., 2018). Actually, in the pond Bodrio del pastore III, in spring 2013, all sampled juveniles of ex-ephippial generation were neckteethless (Rossi et al., 2014).

Differences in neckteeth formation have been found in different clones of single daphniid species exposed to various environmental cues (Parejko and Dodson, 1991; Luning, 1992; Spitze, 1992; Weber and Declerck, 1997; Boersma et al., 1998; Pauwels et al., 2005). Thus, polymorphisms in responses to environmental changes might be related to genetic diversity among strains and/or to local adaptation (Boeing, 2006; Suzuki and Nijhout, 2008; Fiorino and McAdams, 2017). Significant interactions between genotype and environment indicate that a genotype produces discrete alternative phenotypes in response to varying environmental conditions and involves evolution of plasticity. Additionally, phenotypic differences among clones might also reflect the so called 'anticipatory maternal effects', a maternal effect that acts differentially on offspring fitness under different environmental conditions; (Marshall and Uller, 2007). Quality of neonates varies according to either the environment experienced by the mother or the environment that the neonates will cope with, due to their mother's life strategy. The relationships we described between the percentage of induced individuals and fecundity might represent a further signal of maternal anticipatory effect that could improve and accelerate population response to environmental changes (Gorbi et al., 2011; Donelson et al., 2012; Vehmaa et al., 2012; Dey et al., 2016). By maternal epigenetic effects, temperature and photoperiod may serve as reliable cues of predation risk. In this framework, global change and mismatch between temperature and photoperiod could compromise a mechanism of rapid local adaptation such as transgenerational maternal effects (Dey et al., 2016; see Chapter 3.1).

Our results support the hypothesis that both exogenous MF and endogenous MF produced in stressful conditions may activate the expression of genes involved in neckteeth formation (e.g. morphogenetic genes: Hox3, exd and endocrine genes: JHAMT, Met, InR, IRS-1) (Miyakawa et al., 2010; Hales et al., 2017; see Chapter 2.2). Analyses of genes involved in the cellular signaling and reproduction (e.g. rhodopsin and vitellogenin) might explain the positive effect of DMF (solvent control for MF) we observed on *Daphnia* fecundity (Hutchinson et al., 2006; Hannas et al., 2011b).

Chapter 2.2: Maternal effect and hormonal regulation in methyl farnesoate signaling of sex determination and sexual reproduction in *D. pulex*

The ability of individual genotypes to produce different phenotypes when exposed to certain environmental cues, known as phenotypic plasticity, includes the possibility to change phenotypic state or activity (e.g. metabolism) (Garland and Kelly, 2006; Fusco and Minelli, 2010). Sex in animal biology is defined by the production of gametes; and the reaction norm concept is clearly applicable to cases of sex change and to environmental sex determination. Among metazoans, environmental sex determination is the process whereby sex is not determined by sex chromosomes but by environmental factors that influence the maternal organism or oocytes. Environmental sex determination provides population sex ratios that will maximize sustainability of the population under incipient environmental conditions (Bull, 1981) and represents a phenomenon that could allow to define the mechanistic of an environmental x genotype interaction. Environmental factors responsible for sex determination of offspring include temperature (Janzen and Phillips, 2006), nutrition (Warner et al., 2007), photoperiod (Korpelainen, 1990), and population density (Olmstead and LeBlanc, 2001). Generally, the environmental cue is considered to stimulate the release of a chemical signaling molecule (i.e. hormone) that orchestrates the sex programming of the neonate (Bowden et al., 2000).

Branchiopod crustaceans, such as *Daphnia* sp., are cyclic parthenogens that are subject to environmental sex determination (Hebert, 1978). Under suitable environmental conditions, *Daphnia* populations consist largely of females that reproduce asexually. This kind of clonal reproduction provides for the rapid expansion of the population. However, in response to specific environmental cues, that typically represent a limiting factor to population growth, *Daphnia* produces male offspring. Male sex determination is under endocrine control. The production of males and sexual eggs (ephippial resting eggs) in *Daphnia* is triggered by similar environmental stimuli such as changes in photoperiod, food quality and quantity, and infochemical traces associated with crowding and predators (Stross and Hill, 1965; Kleiven et al., 1992; Deng, 1996). Hobaek and Larsson (1990) showed that both a short-day photoperiod and density-dependent factors induced a sex ratio adjustment to 0.50. However, in their experiments with food fluctuations, no ephippia, or sexually produced resting eggs, were released. Starvation or a decline in food availability has long been suspected to be the density dependent factor required to induce

the production of ephippia in cladocerans. According to Kleiven et al. (1992) the simultaneous action of three stimuli (an inductive photoperiod, crowded water and limiting food concentrations) are required to elicit ephippial broods in *D. magna*. Any two-factor combination was completely ineffective in the development of sexual eggs, although the sex ratio of parthenogenetic offspring was strongly influenced.

Environmental sex determination provides genotype linkages between endocrinology and developmental response and several genes are candidate in sex determination (Toyota et al. 2015b). Olmstead and LeBlanc (2002) were the first to describe the effect of methyl farnesoate on male production in *D. magna*. Toyota et al. (2015a) demonstrated that de novo methyl farnesoate (MF) synthesis is necessary for male offspring production in *D. pulex*. These results indicate the key role of innate MF signaling as a conductor between external environmental stimuli and the endogenous male developmental pathway. Despite these findings, the molecular mechanisms underlying up- and downstream signaling of MF have not yet been well elucidated.

Juvenile hormones (JHs) (Methyl Farnesoate-MF in crustaceans) are sesquiterpenoid hormones involved in physiological and developmental processes of arthropods, including metamorphosis, moulting, growth, reproduction, sex and caste determination (Nijhout and Wheeler, 1982; Nijhout 1994; 1999b; Hartfelder and Emlen, 2005; Truman et al., 2006; Cornette et al., 2008; Riddiford, 2008). In the MF signaling pathway, in response to environmental stimuli (such as crowding, short photoperiod), the receptor N methyl D aspartate (NMDA) activates the MF synthesis and causes the triggering of male production (Toyota et al., 2015b) (Fig. 2.2.1). In the *Daphnia* genus, the MF is bio-synthetized through the mevalonate/squalene pathway, probably in the Mandibular Organ (MO, an agglomeration of endocrine glands located in the posterior region of the head), since the genes involved in this molecular pathway were found in this organ (Toyota et al., 2015a; Miyakawa et al., 2017). The molecular pathway of MF synthesis in *Daphnia* is similar to the biosynthesis of the Juvenile Hormone III (JH III) of insects and these hormones present similar protein structures (Noriega et al., 2014). In insects, the synthesis of JH III is carried out through 13 enzymatic reactions and in the last step MF is converted into JH III by the enzyme Methyl Farnesoate Epoxidase (MFE) (Noriega et al., 2014). In the genome of *Daphnia*, the presence of the MFE gene has not been evidenced, but the genes coding for the enzymes involved in the up-stream steps of MF pathway have been sequenced (Toyota et al., 2015a). In particular, the enzyme that catalyzes the formation of MF (the precursor of JH III in insects) was found; this enzyme is named Juvenile Hormone Acid O-Methyltransferase (JHAMT) and catalyzes the conversion of Farnesoic Acid (FA) in

Methyl Farneosate (MF) using S-Adenosil-Metionine (SAM) as methyl group donator (Hui et al., 2010; Miyakawa et al., 2010; Toyota et al., 2015a). SAM can control the epigenetic state of DNA because it donates a methyl group to a methyltransferase, an enzyme that catalyzes the addition of $–CH_3$ to DNA causing hypermethylation of DNA, the formation of heterochromatin and transcriptional repression (Fig. 2.2.1).

Similarly to insects, MF in *Daphnia* is released in the hemolymph and recognizes specific targets called "peripheral target cells" (Miyakawa et al., 2017). MF enters the target cell and binds to Methoprene-tolerant (Met), a receptor that is rhythmically produced and stored in inactive homodimers and is activated after the MF binding (Kakaley et al., 2017). The complex, composed by Met and MF, binds to the Steroid Receptor Coactivator (SRC) forming a heterodimer (Miyakawa et al., 2013b; LeBlanc et al., 2013). The SRC protein is known as a DNA binding protein that interacts with DNA in specific nucleotide sequences called Juvenile Hormone Response Element (JHRE) (Miyakawa et al., 2017). It is able to act as a transcription factor and activates the modulation of specific genes. The genes bound and activated by the complex SRC-Met-MF remain still unknown, but the effect caused by the MF production is clear and the information about the genes activated by the JH signal transduction are increasing (Jindra et al., 2015).

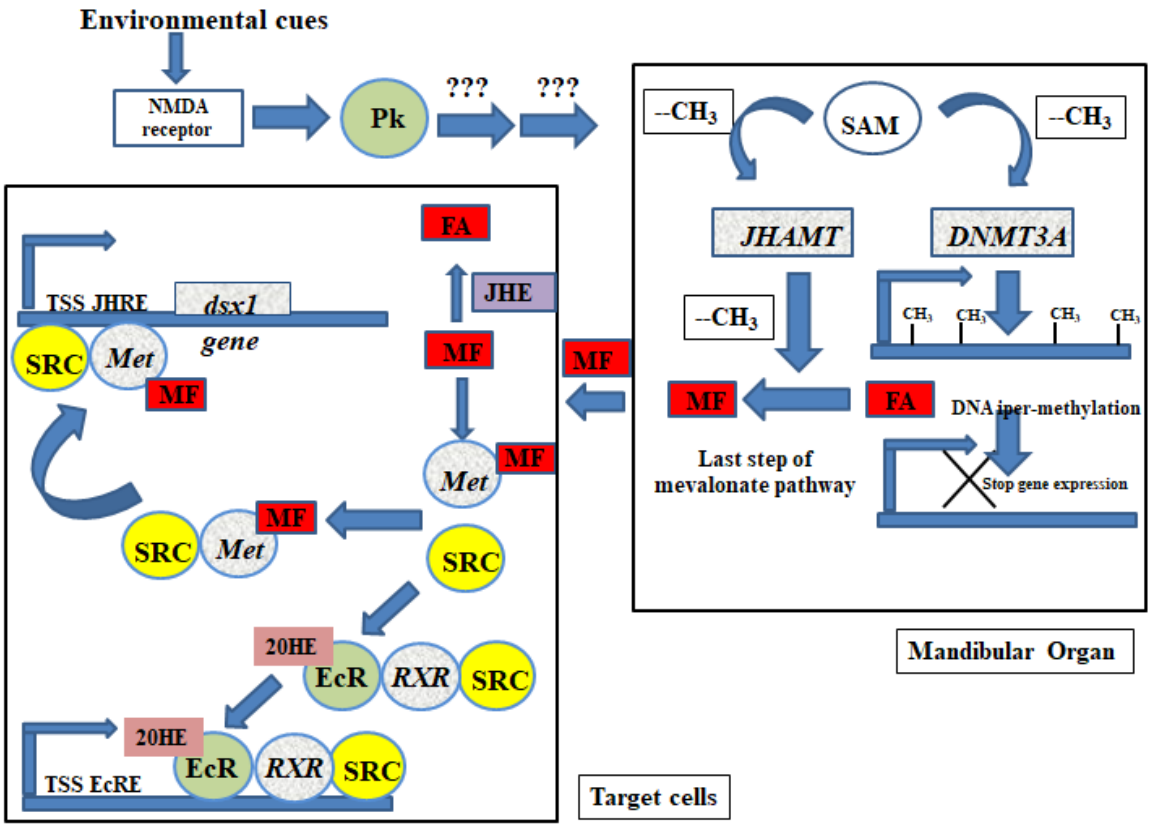


Figure 2.2.1: Molecular pathways of Methyl Farnesoate
Abbreviations: NMDA, N-Methyl D-Aspartate; Pk, Proteinase K; SAM, S-Adenosil Metionine; DNMT3A, DNA Methyltransferase *de novo* 3A; JHAMT, Juvenile Hormone Acid O-Methyltransferase; FA, Farnesoic Acid; JHE, Juvenile Hormone Esterase; MF, Methyl Farnesoate; Met, Methoprene-tolerant; SRC, Steroid Receptor Coactivator; JHRE, Juvenile Hormone Response Element; 20-HE, 20-hydroxyecdysone; EcR, Ecdisteroid Receptor; RXR Retinoid X Receptor; EcRE, Ecdisteroid Response Element.

In insects, there are evidences that the heterodimer Met-SRC-JH binds nucleotide sequences like the Krüppel homolog 1 gene (AaKr-h1) and the early trypsin gene (AaET) (Liu et al., 2015). Schwarzenberger and Von Elert (2016) have indicated the Hox gene antennapedia (ant) as the gene involved in the molecular pathway that induces the male phenotype in *Daphnia*. The Met-SRC-MF complex can cause the increase of the expression of hemoglobin genes as well as the down-regulation of vitellogenin and cuticle genes (Tokishita et al., 2006; Kim et al., 2011; Toyota et al., 2013). In response to environmental stimuli such as photoperiod change, crowding and exposure to some chemicals, *Daphnia* synthesizes MF that causes the oocytes, in their last stage of maturation, to develop into males (Olmstead and LeBlanc, 2002; Rider et al., 2005; LeBlanc et al., 2013; LeBlanc and Medlock 2015). Alternatively, the protein SRC acts in the ecdysteroid pathway through the binding to the Retinoid X Receptor (RXR, called

Ultraspitracle in insects) and activates the Ecdisteroid Response Element (EcRE) in ecdysteroids signaling connected to MF signaling (see Fig. 2.2.1).

Differences among clones in the propensity to produce males might be due to: i) differences in the responsiveness of the NMDA receptor and/or the MF signaling; ii) mutations of some enzymes involved in the bio-synthesis of MF with the consequent impairment of the mevalonate pathway; iii) a reduced affinity of the Met-receptor in binding MF or target genes involved in male production; iv) mutations in genes involved in sex determination downstream of the MF signaling pathway.

In the present chapter, the responses of three genetically different clonal lineages of *D. pulex,* to MF were analysed in terms of survivorship, fecundity and production of males and ephippia, that is in sex determination and sexual reproduction, and in terms of expression of genes involved in sex determination (dsx1), hormonal regulation (JHAMT, met, RXR) and epigenetic regulation (DNMT3A). Our aims were to further investigate the role of MF in sex determination and sexual reproduction and to evaluate if differences in life history traits among clonal lineages may be related to differences in the MF pathway and in the expression of the selected genes. A preliminary experiment was performed to select clones with a different propensity to produce males (and ephippia) in response to environmental stimuli such as population density, short photoperiod, temperature and food limitation (see Chapter 2). Clonal individuals were then tested for their response to MF in different experimental conditions: i) exposure to high MF concentration (0.8 µM) combined with conditions mimicking high population density, i.e. a stressful condition; ii) exposure for two subsequent generations to a lower MF concentration (0.15 µM) in optimal conditions of population density and food availability. It has to be underlined that, from the physiological/biochemical point of view, MF itself does not constitute the stressor, rather it represents the internal reaction to signals of the presence of external stressors. The use of clones with a different propensity to produce males in stress conditions and in response to the exposure to MF represents an approach to clarify the role of different steps of environmental sex determination in daphnids (NMDA receptor sensitivity to environmental stimuli, MF pathway activation and MF signaling).

Material and Methods

In the laboratory life-table experiments we used three clonal lineages of *D. pulex.* The clone I, from a pond located in northern Italy, the clone S and the clone K from two different ponds located in Czech Republic (Rossi et al. 2014, see Chapter 2).

All three clonal cultures were acclimated and synchronized at 20 °C and 14:10 L:D photoperiod, a condition that better discriminates male production among the three clonal lineages. In breeding cultures, 20 females per clone were maintained in 600 ml of natural commercial water (San Benedetto) and fed twice a week with an equal-part of the unicellular green algae *Pseudokirchneriella subcapitata* and the yeast *Saccharomyces cerevisiae* (the final concentration of food: $1.5x10^5$ cells/ml). The culture medium was renewed twice a week.

Experiment 1: life table and treatments for male production

After acclimation for ten generations to the conditions reported above, thirty female juveniles (less than 24 hours old) per clonewere isolated and used to initiate the life-table experiment to test the effect of MF (methyl farnesoate) on male production.

A stock solution of 1 mg/ml MF (Echelon Bioscience) was dissolved in dimethylformamide (Sigma-Aldrich), a solvent commonly applied in aquatic toxicity tests, and kept at -20 °C (stock solution) until used. Daphnids were exposed to MF at a concentration 0.8 µM that, according to Tatarazako et al. (2003) and Miyakawa et al. (2013) is able to induce production of high percentages of males. In addition to the canonical control, a treatment with DMF (dimethylformamide) was performed as a negative control. The final concentration of the DMF in the medium was below 0.01% (v/v) regarded as not toxic to daphnids. The experiment was performed at 20 ± 1 °C and 14:10 h L:D photoperiod.

Ten juveniles per clone per treatment (3 treatments x 3 clones x 10 replicates = 90 females) were individually reared in a six-well plate (one juvenile per well in 10 ml of culture medium, i.e. the same natural water as the cultures). Daphnids were fed with a mixture of *P. subcapitata* and S. *cerevisiae*, each at the final concentration of $1.5x10^5$ cells/ml. In each treatment, medium and food were renewed three times a week. Experimental conditions were considered as stressful, mimicking crowding and were chosen on the basis of the preliminary experiment carried out to maximize differences among clones in term of male production.

The whole experiment lasted for 15 days. Size (the distance between the head and the base of the tail spine) at the 8^{th} day of treatment, age at maturity (i.e. first time eggs were observed in the brood chamber), fecundity (total number of alive offspring per female), failed reproduction (total number of aborted eggs or dead offspring per female) were recorded three times a week. Juveniles were classified according to their gender using a

Leica CLS 50x microscope (Leica, Mannheim, Germany). The percentage of males per clutch and per female in each clone and in each treatment was calculated.

Experiment 2: transgenerational effect of MF

To test if Methyl Farnesoate (MF) can have transgenerational effects on the life cycle of *D. pulex*, the two clones that showed the most divergent responses in the preliminary and first experiments, clones I and S, were exposed to MF for two generations (F_0 and F_1) in a life table experiment.

For life table experiment of F_0, female juveniles (less than 24 hours old) of each clone, produced in the third clutch, were isolated from the cultures and randomly assigned to each treatment (10 females per treatment).

The stock solution of MF was prepared as described above. For this experiment, a 0.15 µM concentration was used to obtain a lower percentage of males in the progeny of F0 generation (Tatarazako et al., 2003; Miyakawa et al., 2013).Daphnids exposed to DMF were used as negative control for the MF treatment. The final concentration of the DMF in the medium was below 0.01% (v/v). The experiment was performed at the same condition as above.

Ten juveniles per treatment per clone (3 treatments x 2 clones x 10 replicates = 60 females) were individually reared in 50 ml of culture medium. Daphnids were fed and medium and food were renewed as described above. The conditions of this experiment were different from the previous one (0.15 µM final concentration of MF instead of 0.80 µM and 50 ml of culture medium per individual instead of 10 ml) and were considered less stressful thus assuring the production of a sufficient number of females for F1 testing .

In this experiment, carapace length at the 8^{th} day of treatment, age at maturity, fecundity and failed reproduction were monitored until the 21^{st} day. The percentage of males per clutch and per female in each clone and in each treatment was calculated.

Moreover, to check ephippia production, a batch culture of 40 individuals per clone per treatment was reared in 2 l and fed with a mixture of *P. subcapitata* and *S. cerevisiae*, each at the final concentration of $1.5x10^5$ cells/ml (40 individuals x 3 treatments x 2 clones = 240 females).

For the F_1 life-table experiment, female juveniles per each clone (less than 24 ours old), produced in the third clutch by F_0 females, were used to start the treatments. A total of 7 treatments were set up. A control from F_0 control females (CC). Two treatments for F1 females born from F_0 females in DMF negative control: DMNT (F_0 exposed to DMF and

F_1 not exposed) and DMDM (F_0 exposed to DMF and F_1 exposed to DMF). Three treatments for F1 females born from F_0 females exposed to MF: MFNT (F_0 exposed to MF and F_1 not exposed); MFDM (F_0 exposed to MF and F_1 exposed to DMF) and MFMF (F_0 exposed to MF and F_1 exposed to MF). Concentration of MF and DMF, when applied, was the same as for F0. Ten juveniles per treatment per clone (7 treatments x 2 clones x 10 replicates = 140 females) were individually reared in 50 ml of culture medium. The experimental conditions were the same as those previously described.

To check ephippia production, a batch culture of 40 individuals per clone per treatment was reared in 2 l as described above. (40 individuals x 7 treatments x 2 clones = 560 females).

Gene expression analysis

A transcriptional analysis of 5 candidate genes providing gene-trait association between genes and life history traits was performed for Experiment 1 and Experiment 2. The candidate genes were previously associated with hormonal regulation (JHAMT, met, RXR), epigenetic control (DNMT3A) and sex determination (dsx1). RT-qPCR was conducted to measure their relative mRNA expression (see Gene Boxes).

Fifty juveniles (less than 24 hours old) per treatment per clone were isolated and maintained in 2.5 l of natural water (NW, i.e. culture medium) (control), DMF solution in NW (final concentration below 0.01% (v/v) and MF solution in NW (final concentration: 0.15 µM) at 20 ± 1 °C and 14:10 h L:D photoperiod. After 48 hours, the treatment medium was renewed. The exposure lasted 72 h and, during the test, daphnids were fed *P. subcapitata* and *S. cerevisiae* (each at the final density of $1.5x10^5$ cells/ml).

After exposure, the organisms were laid onto a 50 µm mesh nylon net, positioned on blotting paper to adsorb excess water, rapidly picked up with a needle, carefully avoiding damage, and transferred into 1.5 ml tubes containing 200 µl of natural water. Water was then eliminated and tubes were frozen using liquid nitrogen and kept at -80 °C until mRNA extraction. For organism fragmentation, glass microbeads (Ø= 0.45-0.50 mm) were added to the tubes. After a further refrigeration in liquid nitrogen, the tubes were subjected to a 15 seconds shock with an amalgamator (TAC 200/S – Linea TAC s.r.l., Asti, Italy; oscillation frequency: 4200 strokes/min) and transferred to an ice-jacket.

For mRNA extraction, the GeneJET RNA Purification Column (Thermo Fisher Scientific) kit was used: 300 µl of lysis buffer (Lysis Buffer; Kit GeneJET RNA Purification) and 6 µl of β-mercaptoethanol were added to the tubes containing the homogenates, maintained in an ice-jacket. A second shock by the amalgamator was then applied to the tubes.

Afterwards, each homogenate was transferred into a 1 ml sterile syringe, connected to a Millipore Swinnex filter holder equipped with a nylon filter (50 µm mesh) previously sterilized and soaked for 2 minutes in the lysis buffer, filtered into a new RNAse-free tube, containing 590 µl of TE Buffer (10 mM Tris HCl, 1 M EDTA, pH=8.0) and 10 µl Proteinase K, and centrifuged (12.000 g for 5 min). Each supernatant was then transferred into new RNAse-free tube and the kit protocol for completing mRNA extraction was applied. RNA concentration and purity was measured by a NanoDrop spectrophotometer (Thermo Fisher Scientific). The A260/280 and A260/230 ratios were calculated to check for extract contamination by protein/phenol or carbohydrate, peptides or aromatic compounds, respectively. Synthesis of cDNA was performed by Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific) according to the instructions of the manufacturer.

The transcriptional activity of the four selected genes was evaluated: Juvenile Hormone Acid O-Methyltransferase (JHAMT-NCBI_GNO_2422013), Methoprene-tolerant (Met-NCBI_GNO_24374; BAM83853), Retinoid X Receptor (RXR-DQ530508) and doublesex1 (dsx1-AB569296.1). The primers used for RT-qPCR were the following: JHAMT 5'-ggctgcgcgagagctatcta-3' (Fw) and 3'-cggagccttcagtttttgga-5' (Rv), Met 5'-cttggtgcgcattcttcaaa-3' (Fw) and 3'-gcttgcatgatggtcaatgg-5' (Rv), RXR 5'-cttgccgtgaagatcgtcag-3' (Fw) and 3'-accgatttctctggcgtttg -5' (Rv), dsx1 5'-ccattcatcattaccaaatcccttc-3' (Fw) and 3'-aagtttggtgtaggggaggatgag-5' (Rv), (Eurofins Genomics) (Hang et al., 2007; Miyakawa et al., 2010; Kato et al., 2011; Toyota et al., 2015). The coding sequence within the full length cDNA of DNMT3A was identified based on the full sequence derived from NCBI's GenBank database. The cDNA sequence of DNMT3A was deduced using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) and primers obtained were 5'-atttaagggcccagtcggaa-3' (Fw) and 3'-agttacagcgttggggaaga-5' (Rv) (Eurofins Genomics).

Quantitative real time PCR was performed on a STEP One Plus RT PCR System (Thermo Fisher Scientific) and using SYBR Green PCR Master Mix (Thermo Fisher Scientific) as a fluorescence detector. The thermal profile consist of 30 minutes at 48 °C, 10 minutes at 95 °C, followed by 40 cycles, each cycle consisting of a temperature of 95 °C for 15 seconds followed by 57 °C for 1 minute. Afterwards, a melting curve analysis was performed to verify the authenticity of the amplified product by its specific melting curve analysis following the instructions of the manufacturer. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH-FJ668125) was used as a reference gene using the primers 5'-accactgtccatgccatcact-3' (Fw) and 3'-cacgccacaactttccagaa-5' (Rv) (Eurofins Genomics)

(Miyakawa et al., 2010). To evaluate the transcriptional activity of the selected genes in comparison to the target gene GAPDH, a comparative threshold cycle method (Livak and Schmittgen, 2001) was used.

To compare gene expression among controls and between different experiments, threshold cycle (Ct) and Fold Change (FC) parameters were used. The Ct is the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold, a fluorescent signal significantly above the background fluorescence. At the threshold cycle, a detectable amount of amplicon product has been generated during the early exponential phase of the reaction. The Ct is inversely proportional to the original relative expression level of the gene of interest (mRNA concentration). The FC describes how much a quantity changes going from an initial to a final value. This result is obtained considering:

i) ΔCt = Ct [Target] - Ct [Housekeeping]: the difference between the Ct of the target gene and the Ct of a reference gene (GAPDH in the present experiment);

ii) $\Delta\Delta Ct$ = (ΔCt Treatments) - (ΔCt Control): the difference between the ΔCt of the treatments and the ΔCt of control;

iii) the value of $2^{-\Delta\Delta Ct}$ is the Fold-Change (FC).

By common consent, FC values higher than 2 indicate~~s~~ up-regulation and FC values lower than 0.5 indicate down-regulation.

Statistical analysis

A two way ANOVA was used to test for significant differences in size, offspring and clutch number, percentage of failure in reproduction (aborted eggs and dead neonates), percentage of males and ephippia per females and gene-expression (fold change) among treatments and clones. Tukey's test was used for pairwise comparisons. When variances were not homogeneous among treatments and clones and data transformation did not solve the homogeneity problem, a median non parametric test was computed. and Dunnet test was used for pairwise comparisons. All statistical tests were performed using SPSS 25.0 software (IBM Corp. 2017).

Results

Experiment 1: life table and treatments for male production

Mortality was negligible: a total of 5 females died before reproduction: 2 females of I clone (1 in control and 1 in MF treatment), 2 females of K clone (1 in control and 1 in MF treatment) and 1 female of S clone in control.

The size of 8 d old females was not significantly different among treatments and clones (Fig. 2.2.2a). A significant delay in age at reproduction was observed in the MF treatment (9.37 +/- 0.152 days) (median test, $p < 0.001$): (Fig. 2.2.2b). In general, clone I females reached adulthood later (7.66 +/- 0.326 days) than the other clones (clone K = 6.41 +/- 0.335days; clone S = 6.64 +/- 0. 342 days) (median test $p = 0.006$).

Significant differences among treatments and clones were observed in the mean number of clutches per female. All clones showed a significant reduction of the mean clutch number in the MF treatment (Fig. 2.2.2c). Considering all treatments, clone K produced a higher number of clutches per female (3.24 +/- 0.137) than clone S (2.54 +/- 0.221), (Tukey test $p < 0.05$). The mean number of offspring per female was influenced by treatment and clone type. All clones showed a significant reduction of juveniles per female in MF treatment (Fig. 2.2.2d). Considering all treatments, clone K produced the highest number of juveniles per female (20.52 +/- 1.307) (clone S =15.41 +/- 1.308; clone I = 12.97 +/- 1.290) and clone I produced the lowest number of juveniles per clutch per female (Tukey test $p < 0.05$) (Fig. 2.2.2e).

The percentage of failure in embryo development per female was affected by treatment (median test, $p < 0.05$) and was significantly different among clones (median test, $p < 0.05$). The highest failure percentage, with a minimum of 7% in control and a maximum of 22% in the DMF treatment, was recorded in clone I, the lowest one, between 0 (in control and MF) and 0.5% (in DMF) in K clone.

Male percentage per female was significantly different among treatments (median test $p < 0.05$) and among clones (median test, $p < 0.05$) (Fig. 2.2.2f). The highest male production (37%) was recorded in daphnids exposed to MF. Clone S produced males in all treatments, while I and K clones produced males only in the MF treatment, with a higher percentage in clone I than in clone S ($p < 0.05$). There was no correlation between the proportion of males and the number of juveniles per female. One ephippium was produced by clone S in MF treatment.

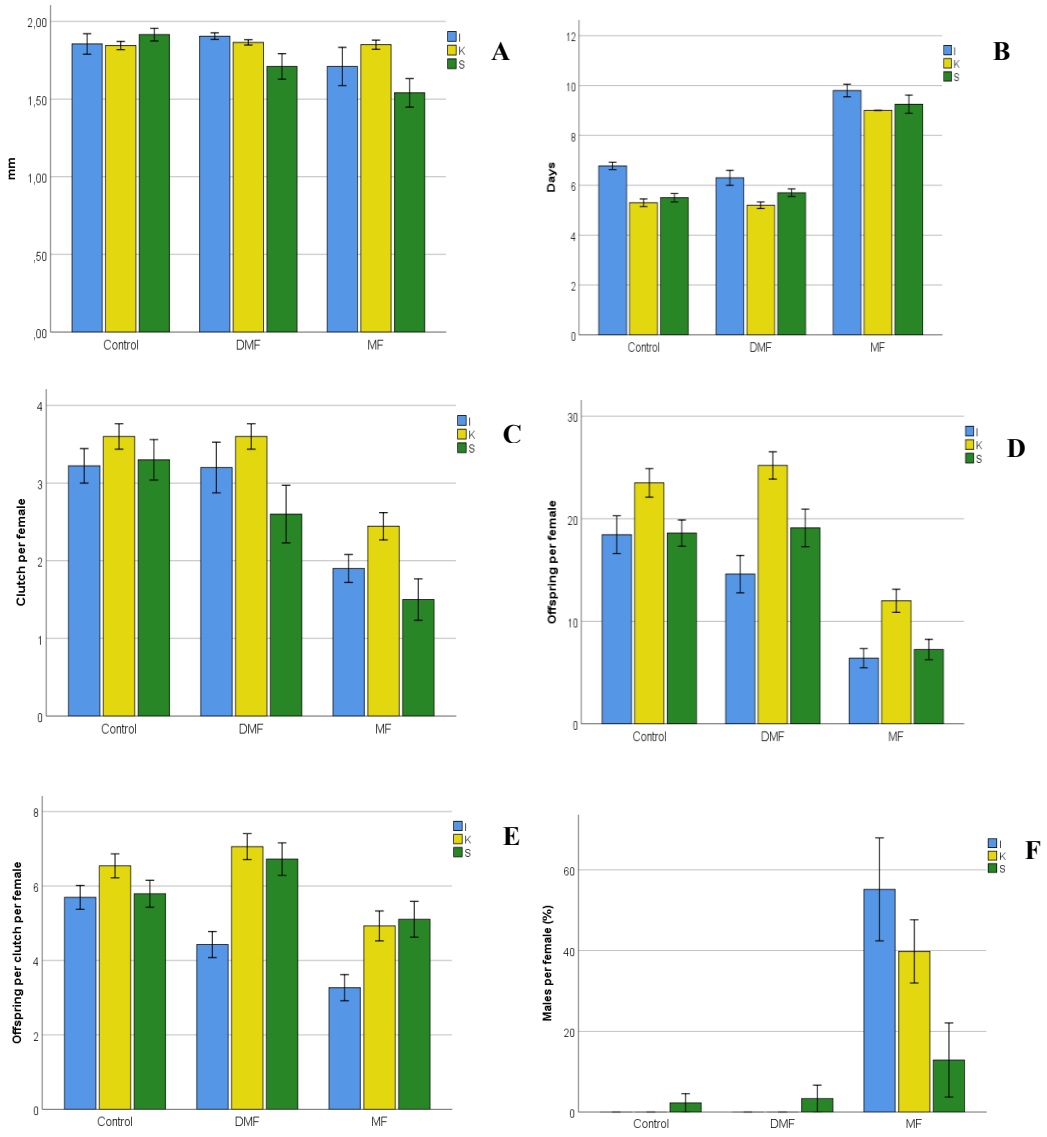


Figure 2.2.2: Experiment 1- Effect of the exposure to MF 0.8 µM in clones I, K and S on body size (A), age at maturity (B), number of clutches (C), number of offspring per female (D), number of offspring per clutch per female (E) and percentage of males per female (F) (mean ± SE).

Experiment 1: Gene expression

In the experiment 1, the expression of the RXR gene was not evaluated because of a low amount of mRNA available in the MF trated organisms. For this reason, we referred to the expression of JHAMT, Met, DNMT3A and dsx1 genes.

The exposure to DMF did not cause modulation of the expression of most genes in all clones, i.e. the fold change values were within the range 2.0 – 0.5 (Fig. 2.2.3). A slight down-regulation was observed for dsx1 and Met in K clone, while a slight up-regulation was observed for DNMT3A in S clone.

Considering the fold change respect to DMF-treated organisms, daphnids exposed to 0.8 µM MF showed a marked up-regulation in all clones with significantly higher values in I and K clones compared to S clone. Up-regulation was also observed for Met and JHAMT in I and K clones. A down-regulation was observed for DNMT3A in clone I.

In clone S, the Ct value of gene dsx1 in control daphnids was lower than that of clones K and I (Table 2.2.1). This means that in clone S these stressful conditions, the gene dsx1, involved in sex determination, was over-expressed in clone S with respect to I and K clones.

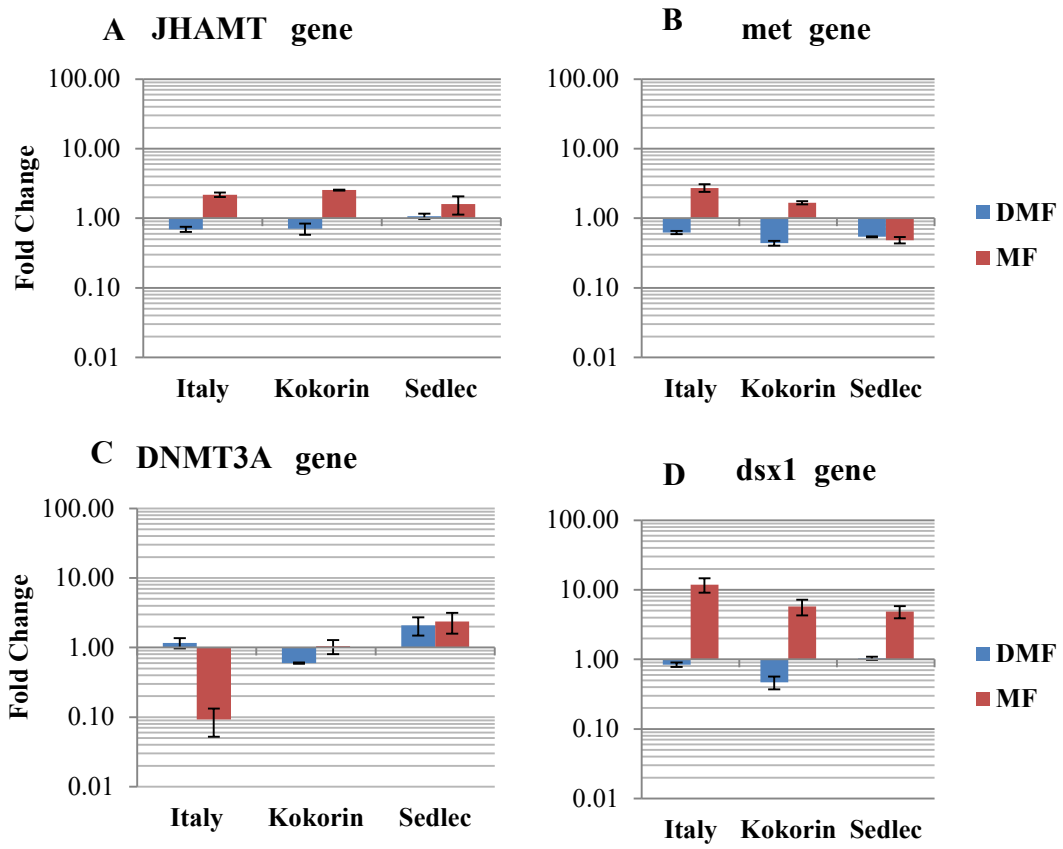


Figure 2.2.3: Gene expression of JHAMT (A), met (B), DNMT3A (C) and dsx1 (D) in *D. pulex* neonates (age <24 h) of clones I, K and S after exposure to dimethylformamide (DMF) (blue) and methyl farnesoate (MF) (orange) 0.80 µM for 72 h.

Table 2.2.1: Ct values of genes JHAMT, Met, DNMT3A and dsx1 in clones I, K and S in control and in organisms exposed to MF 0.8 μM.

Gene	Clone	Control	MF
JHAMT	**clone I**	26.557	24.213
	clone K	23.840	23.140
	clone S	23.183	22.447
Met	**clone I**	28.003	25.343
	clone K	28.467	28.375
	clone S	26.157	27.093
DNMT3A	**clone I**	28.483	30.780
	clone K	30.297	30.903
	clone S	28.630	27.340
dsx1	**clone I**	24.363	19.600
	clone K	23.183	21.337
	clone S	18.037	15.660

Experiment 2: transgenerational effect

In F_0, the size of the 8 d old females was not significantly different among treatments and between clones ($p = 0.531$). Mean age at maturity was not different between clones and was higher in MF than in the control and DMF treatment (Dunnet test, $p < 0.05$) (Fig. 2.2.4a). Exposure to MF caused a significant reduction in the mean number of clutches per female was observed the MF treatment and the negative effect of MF was higher in clone S than in clone I (Fig. 2.2.4b). The mean number of juveniles per clutch per female was not different between clones and was lower in MF than in control and in DMF-treated daphnids (Fig. 2.2.4c). Males were produced by both clones only in the MF treatment: the percentages of males per female, 54.7 (+/- 6.199) in clone I and 45.4 (+/- 6.736) in clone S, were not significantly different ($F_{1,18} = 1.032$; $p = 0.323$). Respectively, 4 (10% of total clutches) and 3 (9% of total clutches) ephippia were produced by 40 females of clone I and clone S treated with MF.

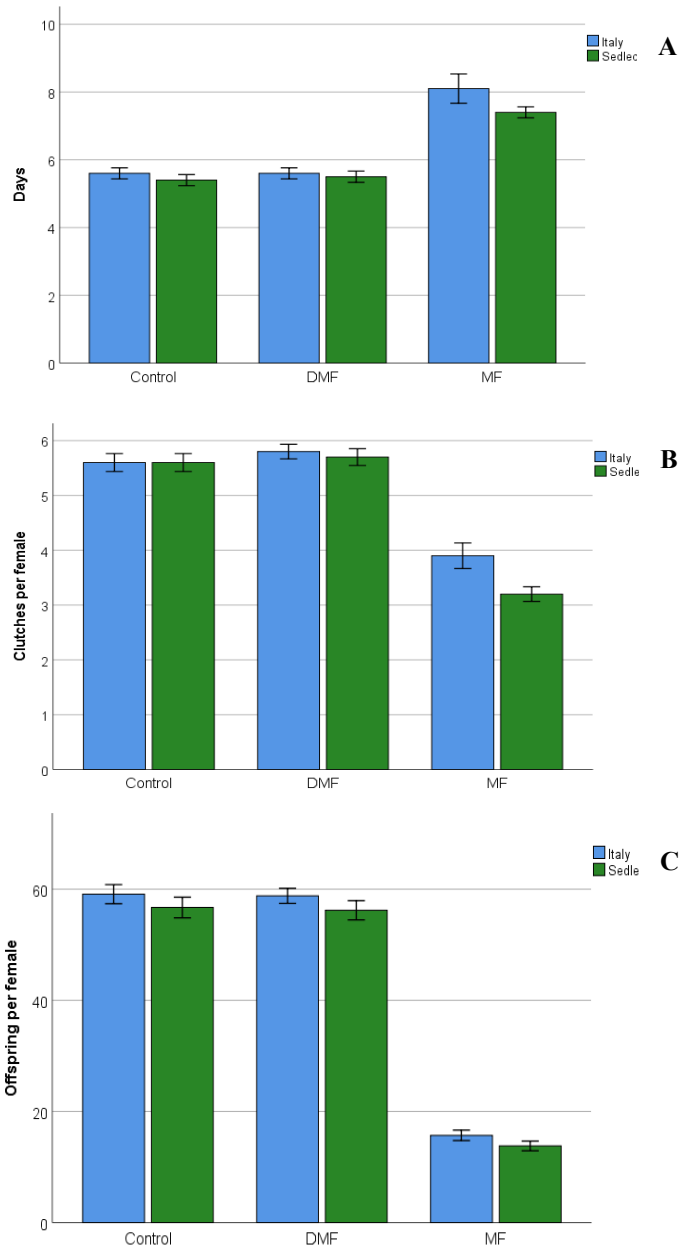


Figure 2.2.4: Experiment 2- Effect of exposure to MF 0.15 µM in clones I and S in age at maturity (A), number of clutches per female (B) and number of offspring per female (C) in generation F_0 (mean ± SE).

In F_1, the size of the 8 d old females was not significantly affected by MF and clone I was generally smaller than clone S ($p < 0.001$) (Fig. 2.2.5a).

The highest mean age at maturity was recorded in the MFMF treatment (Fig. 2.2.5b) and age at maturity was higher in clone I than in clone S. In treatment MFMF, females of both clones produced the lowest number of clutches ($p < 0.05$) (Fig. 2.2.5c) and neonates per clutch per female (Fig 2.2.5d). The mean number of neonates per clutch increased in non-

treated F1 females (MFNT), as well as in MFDM, whose mothers (F_0) were exposed to MF (Tukey's test $p < 0.05$), but remained significantly lower than in controls (CC, DMNT and DMDM) (Tukey's test $p < 0.05$). Males were produced only in the MFMF treatment by both clones: the percentages of males per female were significantly higher in clone I (32.7%) than in clone S (18.5%) ($F_{1,18} = 7.62$; $p = 0.013$).

Respectively, 18 (41% of total clutches) and 19 ephippia (35% of total clutches) were produced by clone I and clone S females maintained in the batch cultures (40 females) in treatment MFMF: ephippia were produced only in the first or second clutches.

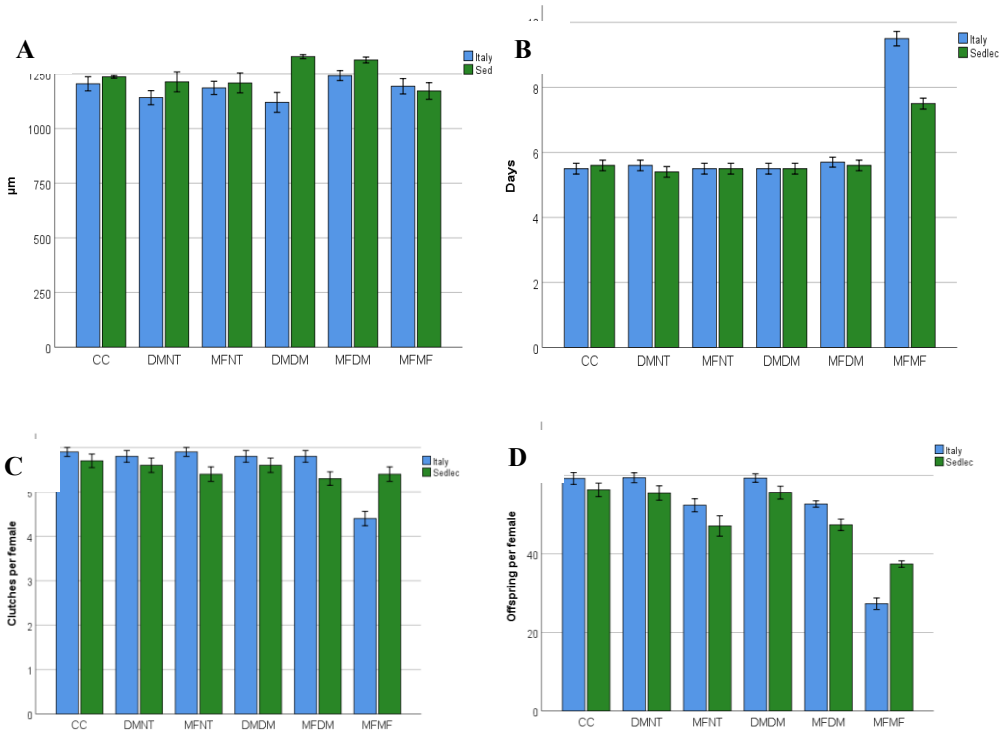


Figure 2.2.4: Experiment 2- Effect on body size (A), age at maturity (B), number of clutches per female (C) and number of offspring per female (D) in generation F_1 of I and S clones. The treatments were the following: DMNT (F_0 exposed to DMF and F_1 not exposed), DMDM (both F_0 and F_1 exposed to DMF), MFNT (F_0 exposed to MF and F_1 not exposed), MFDM (F_0 exposed to MF and F_1 exposed to DMF) and MFMF (both F_0 and F_1 exposed to MF 0.15 µM) (mean ± SE).

Experiment 2: Gene expression in generation F_0

In experiment 2, the expression of the DNMT3A gene was not detected because of technical problems due to DNMT3A primers. For this reason, we referred to the expression of JHAMT, Met, RXR and dsx1 genes.

After exposing the daphnids to 0.15 µM MF for 72 h, the RT-qPCR data showed distinct expression patterns for both clones in comparison to control and negative control DMF. After the exposure to the solvent DMF all genes maintained a basal transcriptional profile. After exposure to MF, JHAMT levels increased in both clones; for genotype I a higher up-regulation (FC = 20.53) was detected in comparison to genotype S (FC = 9.65) (Fig. 2.2.5a). Similar expression for mRNA Met was observed in the two clones (FC = 6.96 and 4.35 in clone I and S, respectively) (Fig. 2.2.5b). A strong up-regulation of gene RXR was detected in clone I (FC = 14.22), while only a slight modulation (FC=2.77) was evidenced in clone S (Fig. 2.2.5c). Both clones also showed up-regulation of gene dsx1 and, once again, the genotype I presented higher mRNA levels (FC = 26.91) in comparison to clone S (FC = 13.36) (Fig. 2.2.5d). Consequently, after exposure to MF 0.15 µM, all genes selected for transcriptomic analysis were up-regulated in both clones I and S and the genotype I showed an higher gene activation in comparison to genotype S.

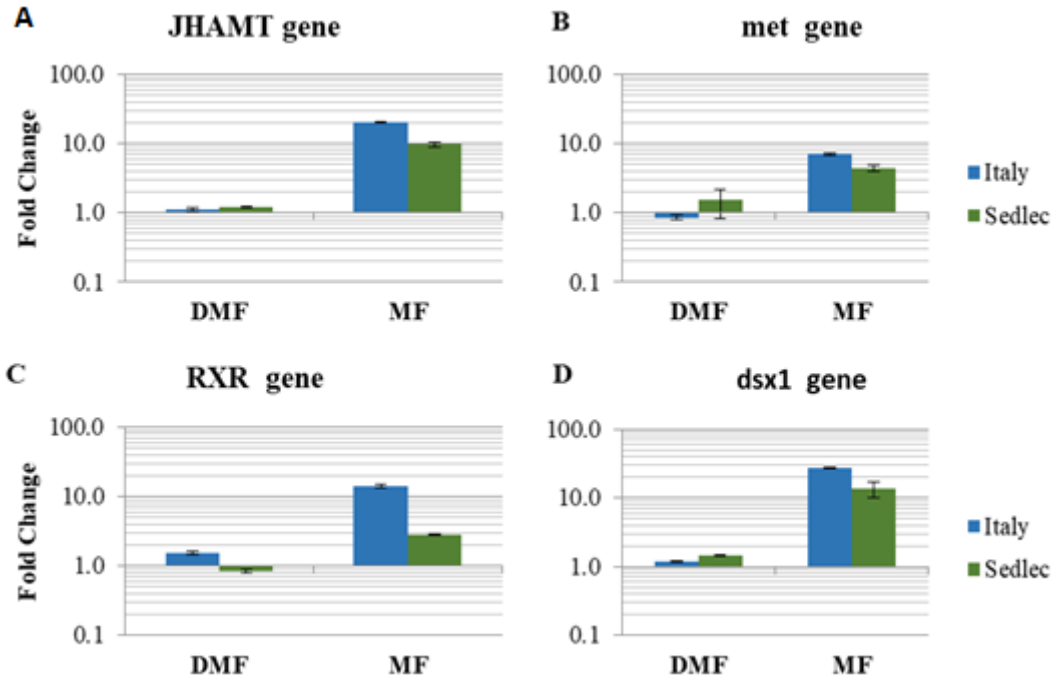


Figure 2.2.5: Gene expression of JHAMT (A), met (B), RXR (C) and dsx1 (D) after exposing *D. pulex* neonates (< 24 h) of clones I (blue) and S (green) to dimethylformamide (DMF) and methyl farnesoate (MF) 0.15 µM for 72 h.

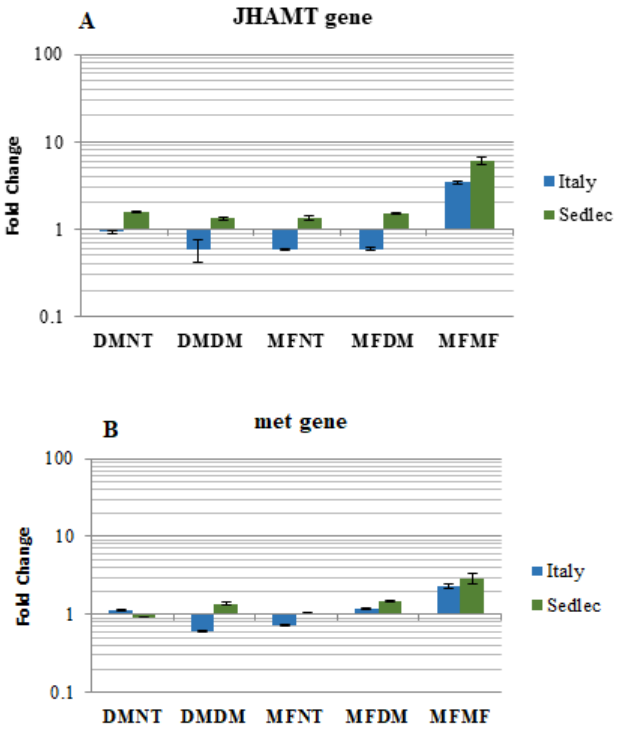
Experiment 2: Gene expression in generation F_1

The transcriptional activity of the genes selected for F_0 (JHAMT, met, RXR, dsx1) was also analyzed in F_1 daphnids and gene expression in CC organisms (F_0 and F_1 not exposed) was used to compute modulation in all the other treatments. No changes were observed in

the gene expression of clones I and S in treatments DMNT (F_0 exposed to DMF and F_1 not exposed), DMDM (F_0 exposed to DMF and F_1 exposed to DMF), MFNT (F_0 exposed to MF and F_1 not exposed) and MFDM (F_0 exposed to MF and F_1 exposed to DMF). For the tretment MFMF (F_0 exposed to MF and F_1 exposed to MF) a general up-regulation was detected in both clones for all genes.

In the MFMF treatment, the RT-qPCR revealed an increase in the expression of the JHAMT gene, with a lower up-regulation in clone I (FC = 3.46) in comparison to clone S (FC = 6.02) (Fig. 2.2.6a). The expression pattern of Met indicates a slight modulation in both clones (FC = 2.28 and 2.87 in clone I and S, respectively) (Fig. 2.2.6b). As far as RXR mRNA is concerned, once again the genotype I presented a lower up-regulation (FC = 2.41) in comparison to clone S (FC=3.39) (Fig. 2.2.6c) and a similar genotype-response was observed for gene dsx1, that was up-regulated in both clones (clone I FC = 3.1, clone S FC=4.26) (Fig. 2.2.6d).

Consequently, after exposure to MF 0.15 µM for two generations (F_0 and F_1), all the selected genes were up-regulated in both clones and the F_1.



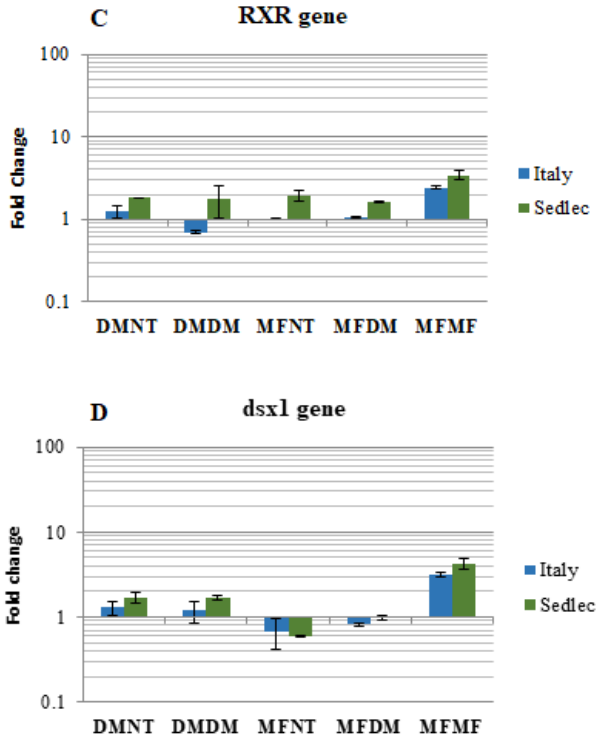


Figure 2.2.6: Expression of genes JHAMT (A), Met (B), RXR (C) and dsx1 (D) in generation F_1 after exposing *D. pulex* neonates (<24 h), obtained by F_0 of clone I (blue) and S (green), for 72 h. The treatments were the following: DMNT (F_0 exposed to DMF and F_1 not exposed), DMDM (F_0 exposed to DMF and F_1 exposed to DMF), MFNT (F_0 exposed to MF and F_1 not exposed), MFDM (F_0 exposed to MF and F_1 exposed to DMF) and MFMF (F_0 exposed to MF and F_1 exposed to MF).

Table 2.2.2: Ct values of genes JHAMT, Met, RXR and dsx1 in clones I and S in controls and organisms exposed to MF 0.15 µM in generation F_0 and F_1.

Gene	Clone	Control (F_0)	Control (F_1)	MF (F_0)	MFMF (F_1)
JHAMT	**clone I**	26.603	25.320	24.970	23.203
	clone S	25.757	24.277	23.693	21.983
Met	**clone I**	22.537	24.347	22.457	22.827
	clone S	22.337	25.757	21.423	24.527
RXR	**clone I**	24.850	25.530	23.750	23.937
	clone S	23.210	26.513	22.943	25.047
dsx1	**clone I**	25.923	26.333	23.900	24.373
	clone S	25.217	26.403	22.680	24.607

Life history traits: F_0 versus F_1

In both clones, the age at maturity was higher in MFMF and in MF than in controls (Dunnet test $p < 0.05$). In clone I, the age at maturity was higher in MFMF than in MF (median test $p < 0.05$). In both clones the lowest mean number of clutches per female was recorded in the MF treatment. In clone I, the mean number of clutches per female in the MFMF treatment remained significantly lower than in controls (CC DMDM MFNT) and was not different from the value recorded in MF (median test $p = 0.628$). In clone S the mean number of clutches per female in MFMF was significantly higher than in the MF treatment ($F_{1,18} = 108.900$; $p < 0.001$) and was not different from the values recorded in controls and in non-treated specimens (CC, DMDM, MFNT) (Tukey test $p < 0.05$). In both clones the lowest mean number of offspring per female and per clutch was recorded in the MF treatment (Tukey test $p < 0.05$). The mean number of offspring per females and per clutch was significantly higher in MFMF than in MF (Tukey test $p < 0.05$), in MFNT than in MFMF (Tukey test $p < 0.05$) and in controls (CC and DMDM) rather than in MFTN (Tukey test $p < 0.05$).

The percentage of males per females decreased from MF to MFMF in both clones, but in the MFMF treatment clone S produced less males than clone I (see above).

The percentage of ephippia increased significantly from MF to MFMF in both clones (I clone: df =1, $\chi^2 = 9.972$, p =0.001; S clone: df =1, $\chi^2 = 7.031$, p =0.006).

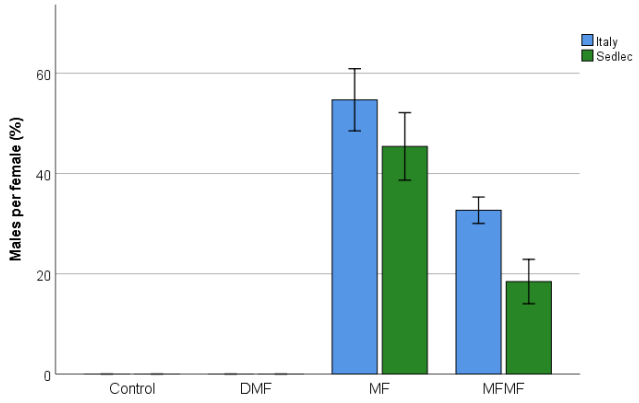


Figure 2.2.7: Comparison between the percentage of males after exposure of clones I and S to MF 0.15 µM in generation F_0 (MF) and F_1 (MFMF).

Effect of experimental conditions: 10 ml versus 50 ml

To evaluate the stressful effect of the experimental conditions, that is rearing single individuals in 10 ml (experiment 1) compared to rearing them in 50 ml (experiment 2), we compared life history traits recorded in the control treatment of Experiment 1 and in that of F_0 in Experiment 2. In both clones, age at maturity was higher in 10 ml than in 50 ml and differences were significant between clones, treatments as well interaction clone x treatment, that is, differences between treatments are higher for clone I than for clone S. Mean number of offspring per clutchper female in 50 ml was almost doubled the one in 10 ml and did not significantly differ between clones. Only clone S produced 2% of male offspring in 10 ml.

Threshold cycle (Ct) of JHAMT and dsx1 was higher in 50 ml than in 10 ml in both clones and differences between clones (the highest values were recorded in I clone), and among treatments and the interaction clone x treatment were significant: differences between treatments were higher for clone S than for clone I. The highest difference in Ct was observed in clone S for gene dsx1, in which the Ct value is 25.217 in 50 ml and decreased in 10 ml (Ct = 18.037). In other terms, mRNA concentration was higher in 10 ml than in 50 ml.

Effect of MF concentration: 0.80 µM versus 0.15 µM

To evaluate the effect of MF concentration we compared life history traits of I and S clones recorded in MF treatment of experiment 1 (0.80 µM concentration of MF) and in MF treatment of F_0 in experiment 2 (0.15 µM concentration of MF). In both clones age at maturity was significantly higher at 0.80 than at 0.15 µM concentration . Mean number of offspring per clutch did not change significantly between treatments but a significant difference between clones and a significant interaction clone x treatment were observed. Mean number of offspring per clutch increased from 0.80 to 0.15 µM concentration in clone I while it decreased in clone S. Percentage of males did not change significantly between treatments in clone I (55%) while it increased from 13% at 0.80 µM to 48% at 0.15 µM concentration in clone S ($F_{1,16} = 14.658$, $p = 0.001$).

Threshold cycle of dsx1 was higher at 0.15 µM than at 0.8 µM concentration in both clones and the difference between clones (the highest values were recorded in I clone), and treatments and the interaction clone x treatment were significant: differences between treatments was higher for clone S than for clone I. In other terms, mRNA concentration was higher in 0.8 µM than in 0.15 µM treated organisms.

Discussion

A significant effect of MF on male production was observed in all clones with significant differences among clones either in response to stressful conditions (crowding and food shortage) and to MF treatment. These findings are in accordance with previous results by Lampert et al. (2012) on *D. pulex*. Genetic response, that is differences among clones, is a well known phenomenon (Larsson, 1991; Innes and Dunbrack, 1993; Deng, 1996, Lampert et al. 2012). In experiment 1, only S clone produced males in controls where stressful conditions mimicking crowding should induce male production by endogenous MF. I and K clones produced males only when treated with MF (0.8 µM). This means that clone I, the only one that did not produce males in preliminary experiment (see Chapter 2), did not undergone mutations in genes involved in the sex determination downstream the MF signaling pathway and does not have a reduced sensitivity of receptor Met to bind MF and activate the transcription of its target genes. In fact, after exposure to MF, the up-regulation (high fold change) of dsx1 in all three clones, of Met in K and of Met and JHAMT in I clone was observed. In S, Met up-regulation is probably masked by its higher expression in control organisms that produced males. However, in control organisms of experiment 1, clone S showed a higher dsx1 mRNA expression (= a lower Ct value) than I and K clones. For this reason, the high fold change value observed in clone S for dsx1 gene in response to MF 0.8 µM is not ascribable only to the treatment but also to the high dsx1 mRNA expression already present in control organisms. Considering that, in *Daphnia*, the highest dsx1 mRNA concentration was observed in male specimen (Kato et al., 2011; Xu et al., 2014), the high dsx1 mRNA expression in untreated organisms of S clone explains the high male production in control conditions. This suggests that clone S has a higher NMDA receptor sensitivity to environmental stimuli (such as crowding and/or food shortage) in activating the MF signaling pathway than K and I clones, the last one showing the lowest sensitivity. K and I, that have a low and a very low propensity to produce males respectively, showed higher male production than clone S when exposed to MF. Exposure to MF 0.8 µM had indeed a lower, even opposite, effect on male production in clone S, that showed the highest propensity to male production in stressful conditions and the highest induction at the lower MF concentration (see F_0 in experiment 2). A similar result was reported by Oda et al. (2006; 2007) in *D. magna* clones that differed with respect to male production in response to fenoxycarb, an analog of juvenile hormone MF. LeBlanc (2007) and Lampert et al. (2012) reported that the multiple functions of MF in the endocrine gene regulation might explain the mechanism of this effect. In clone S, that should have a high production of endogenous juvenile hormone even in untreated organisms in stressful

conditions, the high concentration of exogenous MF might have caused an endocrine negative feedback in the target cells. Actually, in clone S, differently from I and K clones, the exposure to 0.8 µM MF did not induce up-regulation of JHAMT,the enzyme involved in MF synthesis. Our hypothesis might be strengthened considering several studies in which the negative feedback control of MF bio-synthesis pathway (mevalonate pathway) has been reported (Weinberger, 1996; Rauschenbach et al., 2004; Zhang et al., 2017). The negative feedback could occur via the differential modulation of the genes involved in both the synthesis and the degradation of MF. In the mevalonate pathway of crustaceans, the enzyme JHE hydrolases MF into its precursor FA and regulates the concentration of MF (Homola and Chang, 1997; Lee et al., 2011; Sin et al., 2015). According to Zhu et al. (2017) the nuclear receptor Met regulates the expression of JHE (Juvenile Hormone Esterase). In the lepidoptera *Helicoverpa armigera*, Zhang and collaborators (2017) observed that the exposure to methoprene (an analog of MF) caused up-regulation of genes involved in juvenile hormones degradation (e.g. JHE). The hypothesis of a negative feedback of MF signaling pathway might explain the lower male production recorded in clone S at high MF concentration (0.8 µM in experiment 1) with respect to the lower concentration (0.15 µM in F_0 experiment 2) and in F_1 generation of MF-treated S clone with respect to F_0 in experiment 2. In clone I, the one with less propensity to male production, the endocrine negative feedback seems not activated in function of exogenous MF concentration, it seems instead to act under multi-generation exposure, since in F_1 progeny a lower percentage of males than in F_0 was observed.

Interestingly, in clone I a down-regulation of DNMT3A gene after exposure to MF 0.8 µM was observed. The DNMT3A enzyme adds methyl groups to DNA causing iper-methylation, heterochromatin formation and silencing of transcription. Down-regulation of DNMT3A gene after exposure to MF 0.8 µM should mean that transcriptional activity, probably of genes induced by MF and involved in male production, was enhanced thus resulting in the highest males percentage. The molecular pathway of epigenetic control can be connected to the MF bio-synthesis, considering that both DNMT3A and JHAMT use the co-enzyme S-Adenosyl-Metionine (SAM) as $–CH_3$ donator. In clone I, the down-regulation of the gene DNMT3A, after exposure to MF, could be linked to a rapid activation of JHAMT enzyme by the addition of $–CH_3$ and, consequently, to the lowered availability of methyl groups for the enzyme DNMT3A. Accordingly, the genes JHAMT, Met and dsx1 showed up-regulation in comparison to control. The hypothesized competition between DNMT3A and JHAMT for methyl groups suggests that the

epigenetic control trough DNA methylation/de-methylation is involved in the control of male production.

Differently from experiment 1, the rearing conditions in the experiment 2 were not stressful and clone S, as well as clone I, did not produce males in controls. In MF-treated daphnids of F_0, age at maturity and offspring and male production were similar to the ones observed in experiment 1 (MF 0.8 μM).

The effect of MF on life history traits and gene expression (fold change) was unexpectedly higher in generation F_0 (treatment MF) than in generation F_1 (MFMF treatment) in both clones. In particular the exposure to MF for two subsequent generations (treatment MFMF) resulted in a lower percentage of males in the progeny in F_1 in respect to F_0. The effect of MF was lost by untreated individuals born from MF-treated parents (treatments MFNT and MFDM). Up-regulation of all selected genes was observed in F_1 females treated with MF (MFMF treatment), but the up-regulation decreased from F_0 to F_1 in both clones (except RXR in S clone). In particular, the lowered up-regulation of gene dsx1 matches with the lower male production in accordance with the role of dsx1 in daphnid male determination.

the juvenile hormone MF has a role in regulating the ecdysteroid hormonal signaling pathway which induces up-regulation of Ecdysone Receptor (EcR) and Retinoid X Receptor (RXR) in *Daphnia* (Mu and LeBlanc, 2004). Actually, the effect of MF on RXR expression is controversial. Wang and LeBlanc (2009) did not detect modulation of RXR mRNA expression in *Daphnia* after exposure to MF and Gong et al. (2016) suggested no relationship between the exposure to MF and the activation of RXR in the mud crab *Scylla paramamosain*. A positive correlation between MF and RXR expression was instead observed in the crustacean *Homarus americanus* (Tiu et al., 2012). Joãrdao and colleagues (2016) suggested a possible interaction among the nuclear receptors and transcriptional factors Met, SRC, EcR and RXR mediated by MF (Joãrdao et al., 2016).

Interestingly, exposure to MF for two generations (MFMF treatment) significantly increased ephippium production in both clones. In our experiment, a very low number of ephippia was produced by F_0 females exposed to MF, production increased in the subsequent generation (F_1) subjected to a continued exposure to the hormone. In particular, ephippium production was also observed in clone I that, differently from the other clone, did not produce ephippia in any other tested stressing condition (crowding and food shortage). These results suggest that MF could be implied in the induction of ephippium production, but the effect could be evidenced by multi-generation experiments. In F_1 generation, ephippia were produced only in the first and second clutches. This behavior suggest that, after having assured population survival (and a reduction of population

density) by dormant egg production, daphnids turned to the less expensive (in term of energy cost) parthenogenetic reproduction even in the presence of the stressor signaling MF. The results of our multi-generation experiment thus suggest that the induction of male and ephippium production might have a common root in the MF pathway.

The effect of exposure to MF on life history traits may have an impact at population level. Actually, an effect of MF on the age at maturity, that underwent a delay depending on MF concentration, and on mean number of offspring per clutch and per female, that was reduced in all clones, was observed. Moreover MF did not influence body size after 8 day exposure that is, MF did not affect molting and body growth rate but retarded mean age at reproduction, a reasonable effect for a juvenile hormone.

It is also worthy to be highlighted the similarity between the responses displayed by organisms exposed to MF and the effects of population density and adult sex ratio described by Booksmythe et al. (2018) in *D. magna*. Actually, MF-treated daphnids showed responses that are in accordance with the "model" reported by Booksmythe and colleagues (2018). The authors showed that females produced more males when population density was high but a dampened response was observed with the increase of male proportion (high adult sex ratio). Moreover, the likelihood of ephippium production increased with the adult sex ratio. We have shown that, even in clones with low propensity to male production, exposure to MF decreased the offspring number and induced male production and that a prolonged (multi-generation) exposure to MF dampened the response (in term of male percentage) and induced ephippium production. The tested clones (I and S), despite their constitutive genetic difference in the propensity to male and ephippium production, responded in an almost identical manner to MF, suggesting that the response cascade became qualitatively identical (at both phenotypic and molecular levels) when the MF pathway was activated. The intraspecific difference of our clones seems thus to reside in the signaling steps upstream the MF biosynthesis, i.e. in the steps that lead from the stimulus reception to MF pathway activation (see Fig. 2.2.1). On these basis, MF seems to have a key role in regulating daphnid population dynamics. However Toyota et al. (2015a) observed that clones of *D. pulex* exposed to MF were not able to produce males because of possible defects in step(s) downstream MF production. In natural *D. pulex* populations, the co-occurrence of clones with different propensity to sexual reproduction (that is non-male-producing females and females which produce both males and sexual ephippial eggs) and intraspecific variability in response to stimuli for both male and ephippium formation have been reported (Larsson, 1991; Innes and Dunbrack, 1993). Laboratory data indicate that, in several clones, there is an uncoupling of investment in males and ephippial sexual eggs

(Hobaek and Larsson, 1990; Ferrari and Hebert, 1992). According to De Meester and Vanoverbeke (1999), this mismatch has obvious fitness cost due to reduction of likelihood of fertilization but the males mate with genetically different sexually receptive females reduce inbreeding depression and mutation load (De Meester and Vanoverbeke, 1999; Paland and Lynch, 2006). According to Kleiven et al. (1992), in *Daphnia*, offspring sex ratio is regulated to about 0.50 over a broad range of environmental conditions. When stimuli are of low intensity, a lower sex ratio occurs. Under adverse environmental conditions and high population density ephippium production occurs.

Our hypothesis is that clonal variability in the sensitivity to stressors (i.e. variability in stimulus reception) assures diversified responses in variable environments; once the stimulus has reached a specific threshold and the MF pathway has been activated, the similarity in clonal responses (i.e. production of both male and sexual female) reduces the risk of inbreeding.

Gene Boxes

Box 1: Juvenile Acid Hormone O-Methyltransferase (JHAMT-AB222845)

Juvenile Acid Hormone O-Methyltransferase (JHAMT) is a critical enzyme involved in one of the last steps in the biosynthesis of the Juvenile Hormone (JH) and was cloned for the first time in the silkworm *Bombyx mori* (Shinoda and Itoyama, 2003). The presence of this enzyme was also found in the fruit fly *Drosophila melanogaster*, in the beetle *Tribolium castaneum* and in the mosquito *Aedes aegypti* (Niwa et al., 2008; Minakuchi et al., 2008; Mayoral et al., 2009). In daphnids, an ortholog of this enzyme was found and it catalyzes the conversion of Farnesoic Acid (FA) to Methyl Farneosate (MF) using S-Adenosil-Metionine (SAM) as methyl group donor(Hui et al., 2010; Miyakawa et al., 2010; Toyota et al., 2015a). In *D. pulex*, JHAMT was detected in a single copy gene and its expression increases transiently during the MF-sensitive phase, inducing male production (Toyota et al., 2015a). Miyakawa and Iguchi (2017) hypothesized that JHAMT acts as switch in MF signaling activation (Miyakawa and Iguchi, 2017). This gene is also up-regulated in juveniles after exposure to kairomones released by the predator *Chaoborus* and following photoperiod changes (Miyakawa et al., 2010; Toyota et al., 2015b).

Box 2: Methoprene tolerant (Met-NCBI_GNO_24374; BAM83853)

Methoprene tolerant is a protein belonging to the basic helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) family of transcription factors that, in insects, is activated when linked by JH III (Miura et al., 2005; Konopova et al., 2007; Abdou et al., 2011). In its inactive form, in absence of JH, Met is present as a homodimer Met-Met, while the production of JH III induces the binding between Met and a basic helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) called Steroid Co-Repressor (SRC) (Godlewski et al., 2006). This heterodimer acts as transcription factor through its carboxy-terminal PAS domain and activates downstream elements called Juvenile Hormone Response Elements (JHRE) (Charles et al., 2011; Li et al., 2011; Zhang et al., 2011). In the JH signaling pathway, in insects Met binds JH III, while in *Daphnia*, Met recognizes the hormone MF, a precursor of JH III (Toyota et al., 2015b). Daphnids are capable of binding JH III due to an amino acid substitution in position 297 from valine to threonine; valine makes it possible to bind JH III, while threonine enables the binding of MF (Miyakawa et al., 2013b). Furthermore, the amino acidic sequence of Met is important to recognize SCR in a short time and mutations may affect ligand selectivity (Miyakawa and Iguchi, 2017). In response to MF, the gene expression of Met increases and mRNA Met changes rhythmically with peaks during the 36 h that follow moulting, in the period of oocyte susceptibility to MF (Kakaley et al., 2017). The activation of Met and the formation of the Met-SCR complex is observed after exposures to MF in the dose range of 3 to 10 µM (Kakaley et al., 2017).

Box 3: DNA methyltransferase 3A (DNMT3A)

DNA methyltransferases (DNMT) are enzymes that mediate the addition of a methyl group to specific positions in CpG islands on DNA in position 5 on cytosine (Bird, 2002; Clark et al., 1994; Oakeley, 1999; Watson and Goodman, 2002). This process causes an epigenetic control based on silencing or reduction of transcriptional activity of genes and allows the regulation of phenotypic traits (Roberts and Gavery, 2012; Sarda et al., 2012). DNA methyltransferase 3A (DNMT3A) is responsible for *de novo* methylation (to distinguish DNMT1 that is involved in the maintenance of metilome status) (Okano et al., 1998; Pradhan et al., 1999). DNMT3A is implicated in epigenetic changes transferred from parents to offspring that are essential for processes such as cellular differentiation and embryonic development (Jia et al., 2016). In arthropods, the role of DNMT3A is known in Hymenoptera (ants, bees, wasps and sawflies), where it is involved in behavioural plasticity and social behaviour (Yan et al., 2014; 2015; Bewick et al., 2017). The gene methyltransferase DNMT3A was sequenced in the genome of *Daphnia* and it is a homolog of human DNMT3A (Vandegehuchte et al., 2009). However, no information is currently available about the role of this enzyme in the phenotypic plasticity of *Daphnia* and other crustaceans.

Box 4: Doublesex 1 (dsx1- AB569296.1)

Doublesex 1 (dsx1) is a gene acquired from a duplication event, which plays a key role in the sexual differentiation of *Daphnia* (Kato et al., 2011). Dsx1 was sequenced in various cladocera species such as *D. pulex*, *D. magna*, *D. galeata* and *Ceriodaphnia dubia* (Toyota et al., 2013). The gene dsx1 is translated into a protein characterized by 330 amino acids obtained by two mRNA sequences that present differences only in their 5'-UTR (DapmaDsx1- α and DapmaDsx1-β) (Kato et al., 2011). This gene is highly conserved in cladocerans in the DM and oligomerization domains and it is usually transcribed during male embryo development, in particular during the gastrulation stage (Kato et al., 2011; Toyota et al., 2013; Nong et al., 2017). The role of dsx1 is different in insects and in *Daphnia*. In insects, splice variants originated by pre-mRNA maturation produce females, while a non-functional dsx1 splice variant induces male production (LeBlanc and Medlock, 2015). In *Daphnia,* the microinjection of dsx1 mRNA into parthenogenetic eggs determined offspring masculinization (Kato et al., 2011). However, dsx1 concentration decreased in the following order: males, parthenogenetic females and resting eggs (Xu et al., 2014). No more evidence was obtained about the control of dsx1 gene expression. However, Ishak and collaborators (2017) showed that the activation of this gene is mediated by Vrille (Vri), a bZIP transcription factor ortholog of *Drosophila* circadian clock genes (Ishak et al., 2017).

Box 4: Retinoid X Receptor (RX- DQ530508)

Retinoid X Receptor (RXR) is a nuclear hormone receptor belonging to the retinoid/steroid family (Wang et al., 2007): The activation of RXR is mediated by the binding of JH (Davey, 2000; Xu et al., 2002). RXR acts as homodimer (Chawla et al., 2011). In *Daphnia* the gene RXR has 1888 bp coding for a protein composed of 400 amino acids and a molecular weight of 44, 292 Da (Wang et al., 2007). The RXR protein is made of five domains: a ligand independent A/B activation domain, two zinc finger DNA binding domains, a hinge region, a ligand binding domain and an F domain (Wang et al., 2007). RXR is important for embryo development and it is a key regulator for daphnid sex determination (Wang et al., 2007). The insect ortholog of RXR is the Ultraspiracle Protein (USP), that has been shown to bind MF with high affinity (Jones et al., 2006). In arthropods, RXR forms a heterodimer complex with another nuclear receptor, the Ecdysteroid Receptor (EcR) (Iwema et al., 2009; Techa and Chung, 2013; Qian et al., 2013). Exposure for 24 h of 5 days old *D. magna* individuals to pyriproxyfen (an analog of MF) causes a down-regulation of RXR expression (Wang et al., 2007), while the biocide tributyltin causes an increase of RXR mRNA (Wang et al., 2011; Joãrdao et al., 2015). Wang et al. (2009) demonstrated no relationship between the activation of RXR and the effect of MF on *Daphnia* sex determination (Wang and LeBlanc, 2009). However, Joãrdao and collaborators (2016) proposed a conceptual model in which RXR can interact with the proteins EcR, Methoprene-tolerant (Met) and Steroid Co-Repressor (SCR) in a molecular complex to activate genes involved in lipid storage after exposure to both single chemicals (20-hydroxyecdysone, methyl farnesoate, pyrirproxyfen, tributyltin) and mixtures of these (Joãrdao et al., 2016). Consequently, the RXR mode of action is probably based on a co-regulation in which EcR, Met and SCR are involved in a complex hormonal regulation.

Chapter 3: Transgenerational plasticity in response to earlier spring warming and effects of glyphosate on life history traits, transcriptome, microbiome and DNA integrity on four *D. magna* clones

Human activity and human-driven climate change have altered the natural environment quality and the timing of seasons, resulting primarily in warmer winters, especially at higher latitudes, and earlier spring warming (IPCC 2013; 2014). Unlike temperature, the latitudinal gradient of seasonal changes in day length is a stable, abiotic environmental factor that does not change with local or global climate. At seasonally appropriate times, light initiates a cascade of physiological events that ultimately determine whether an organism prepares to develop, reproduce, hibernate, enter dormancy, or migrate (Bradshaw and Holzapfel, 2010). The altered timing of seasons leads to warm temperature occurring at short photoperiods, affecting physiological and developmental processes (Bradshaw and Holzapfel, 2007; 2010; Grevstad and Coop, 2015).

Glyphosate (N-(phosphonomethyl) glycine) is a chemical that acts on 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme involved in the shikimate pathway (also called shikimic acid pathway). This molecular pathway is involved in the aromatic amino acids production the in plants, algae, fungi and bacteria (Duke and Powles, 2008). The use of glyphosate as herbicide was introduced in 1974, and its application quickly increased (Brenbrook, 2016). Roundup (Monsanto) is a non-selective herbicide composed by glyphosate and additives kept secret by manufacturer. (about glyphosate and its characteristics and effects see Van Bruggen et al., 2018). Considering the intensive use of this herbicide in last years, in 2012, about 127,000 tons of glyphosate were used in the USA and 700,000 tons worldwide, many institutions have investigated the possible impact of glyphosate on human health (US Geological Survey, 2012; Swanson et al., 2014). In 2015, the World Health Organization (WHO) classified the glyphosate as probably carcinogenic to humans (EFSA - European Food Safety Authority, 2015; IARC – International Agency for Research on Cancer, 2015). This herbicide has been frequently found in drinking water, seawater, groundwater, surface freshwater (Hanke et al., 2010; Peruzzo et al., 2008; Mercurio et al., 2014; Rendón-von Osten and Dzul-Caamal, 2017; Gunarathna et al., 2018) and represents a risk for aquatic non-target organisms (Annett et al., 2014).

Material and Methods

Clones selection and breeding

Our common garden experiments were conducted on thirty clonal lineages separated in time, resurrected from the sedimentary archive of Lake Ring, a shallow mixed lake (maximum depth is 5m) in Jutland, Denmark (55°57'51.83'' N, 9°35'46.87'' E) (Sayer et al., 2010) with a known history of increase in average temperature (Orsini et al., 2016). Lake Ring's history of anthropogenic impact is known from historical records (Berg et al., 1994) and paleolimnological analyses (Orsini et al., 2016; Cambronero and Orsini, 2017; Cambronero et al., 2017). The lake temperature experienced a steadily, even if modest (~1°C) increase in the last decades (Cambronero et al., 2017). Further, changes in water chemistry and transparency occurred over time due to an event of severe eutrophication triggered by sewage inflow from a nearby town in 1960 (Michels, 2007; Cambronero et al., 2018). This event was followed by high pesticides use due to agricultural intensification until 1985 (Michels, 2007). Finally, the lake partially recovered and returned to clear-water conditions in modern times (>1999). From each lake phase, hereafter referred to as eutrophic phase (EP), pesticide phase, (PP), and clear-water phase (CWP), (sub)populations of *D. magna* separated in time (hereafter referred to as populations) were resurrected from dormant embryos and maintained in the laboratory as monoclonal cultures as described in Cambronero et al., (2017). Between nine and eleven randomly selected genotypes from each lake phase (CWP, PP, and EP, respectively) were used in common garden experiments described below (Table 3.1).

To evaluate the males production propensity, the thirty clones resurrected from the sedimentary archive of Lake Ring were used in a common garden experiment. For comparison a reference laboratory strain P-IT; this clone, is reared in the ecotoxicological laboratory of Department of Chemistry, Life Science and Environmental Sustainability (previously Institute of Ecology) of the University of Parma since '80 of the last century and was originally obtained by the Institute of Ecosystem study, CNR (Verbania, Italy).

Clone P-IT has never been reported to produce male broods in any experimental condition (Gorbi et al., 2011) was used. Importantly, when exposed to methyl farnesoate, a juvenile hormone that has been shown to induce male production in daphnids (Toyota et al., 2015a), P-IT produces male offspring.

Prior to the experiment, all genotypes were acclimated for two generations in common garden conditions at 20 °C, 12:12 h light:dark regime, and fed daily with 0.8 mg C/l of *Chlorella vulgaris* (CCAP strain no. 211/11B) to reduce interference from maternal effect. The culture medium (borehole water) was refreshed every second day. After two

generations in these conditions, 1 individual juveniles for each clone of 24 hours from the second or following broods were randomly assigned to the experimental conditions, 20 °C, 14:10 h L:D photoperiod or 20 °C, 10:14 h L:D. We measured the percentage of male offspring across eight broods in these 30 genotypes and in the laboratory clone that never produced males (P-IT).

Results and Discussion: Validation of the genetic basis of male formation in *D. magna*

In the common garden experiment we observed variation in the propensity to form male offspring in response to long photoperiod (14 h light: 10 h dark) and short photoperiod (10 h light:14 h dark) (Fig. 3.1).

Several clones produced males broods both in long and short photoperiods: 0_1, 0_4, 1_2, 2_1, 2_5-9, 3_5-2, 3_6 (belonging to CWP population), 6_2, 6_3, 7_3, 7_5, 7_5-4, 8_5-3, 9_5-1, 9_5.3, 9_6, 9_20 (belonging to PP population), 12_4, 12_5-1, 14_5-1, 15_1-5 (belonging to EP population). Clones 12_3, 13_2, 13_3, 3_5-15 (belonging to EP population) produced males only in short photoperiod. Clones 0_2, 2_5-11, 3_5-15 (belonging to CWP population), 13_1, 13_5-1 (belonging to EP) produced male broods in long photoperiod. Clone P-IT did not produce males (Fig. 3.1).

Thirty genotypes resurrected from the sedimentary archive of Lake Ring were used in a transgenerational common garden experiment to evaluate the genetic and plastic responses to asynchrony between temperature and photoperiod (Chapter 3.1, CGE1 experiment) and to perform gene-trait association analysis between 15 candidate genes and the life history traits measured in the transgenerational common garden experiment CGE1 (Chapter 3.1).

Based on results of males propensity obtained in this experiment and from transgenerational common garden experiment (Chapter 3.1, CGE1 experiment) four clones (3.5_15, 13_5.1, 13_2 and P-IT) was selected to validate the genetic basis of male formation in *D. magna* (Chapter 3.1, CGE2 experiment) and to evaluate the role of microbiome and transcriptome in daphnids resistance to anthropogenic factors (Chapter 3.2). Clones 3_5-15 and 13_5-1 produced most male broods in long photoperiod but were from different lake phases (CWP and EP, respectively), clone 13_2 produced male broods in short photoperiod and clone P-IT did not produce males (Fig. 3.1). Clones selected were obtained by different phases: 13_2, which dates back to the 1960s with no exposure to pesticides; 13_5-1, that also dates back to the 1960s but is a resistant variant according to the results of a prior life history trait study and 3_5-15, a susceptible variant that had prior and recent exposure to common pesticides. All genotypes except for P-IT were obtained from the same population.

Table 3.1. List of clones used in this study

List of *Daphnia magna* clones used in the common garden experiments to identify life history trait response to photoperiod changes. All clones, except for P-IT, were resurrected from Lake Ring, Denmark. P-IT is a laboratory strain provided by Department of Chemistry, Life Science and Environmental Sustainability of the University of Parma (Italy). Clone ID and population ID are as in Cambronero et al. (2017). Population indicates each phase of Lake Ring from where each clone was resurrected from dormant embryos: EP - eutrophic population, PP - pesticide population, and CWP - clear-water population. CGE indicates experiment, 1 or 2 or both in which each clone was used in Chapter 3.1. An asterisk (*) marks the clones used in the experiments reported in Chapter 3.2.

Clone	Population	Genome-resequence	CGE
0_1	CWP	Available	1
0_2	CWP	Available	1
0_4	CWP	Available	1
1_2	CWP	Available	1
2_1	CWP	Available	1
2._-9	CWP	Available	1
2_5-11	CWP	Available	1
3_6	CWP	Available	1
3_5-1*	CWP	Available	1, 2
3_5-2	CWP	Available	1
3_5-15	CWP	Available	1
6_2	PP	Available	1
6_3	PP	Available	1
7_3	PP	Available	1
7_5	PP	Available	1
7_5-4	PP	Available	1
8_5-3	PP	Available	1
9_6	PP	Available	1
9_2	PP	Available	1
9_5-1	PP	Available	1
9_5-3	PP	Available	1
12_3	EP	Available	1
12_4	EP	Available	1
12_5-1	EP	Available	1
13_1	EP	Available	1
13_2*	EP	Available	1, 2
13_3	EP	Available	1
13_5-1*	EP	Available	1, 2
14_5-1	EP	Available	1
15_5-1	EP	Available	1
P-IT*	-	Available	2

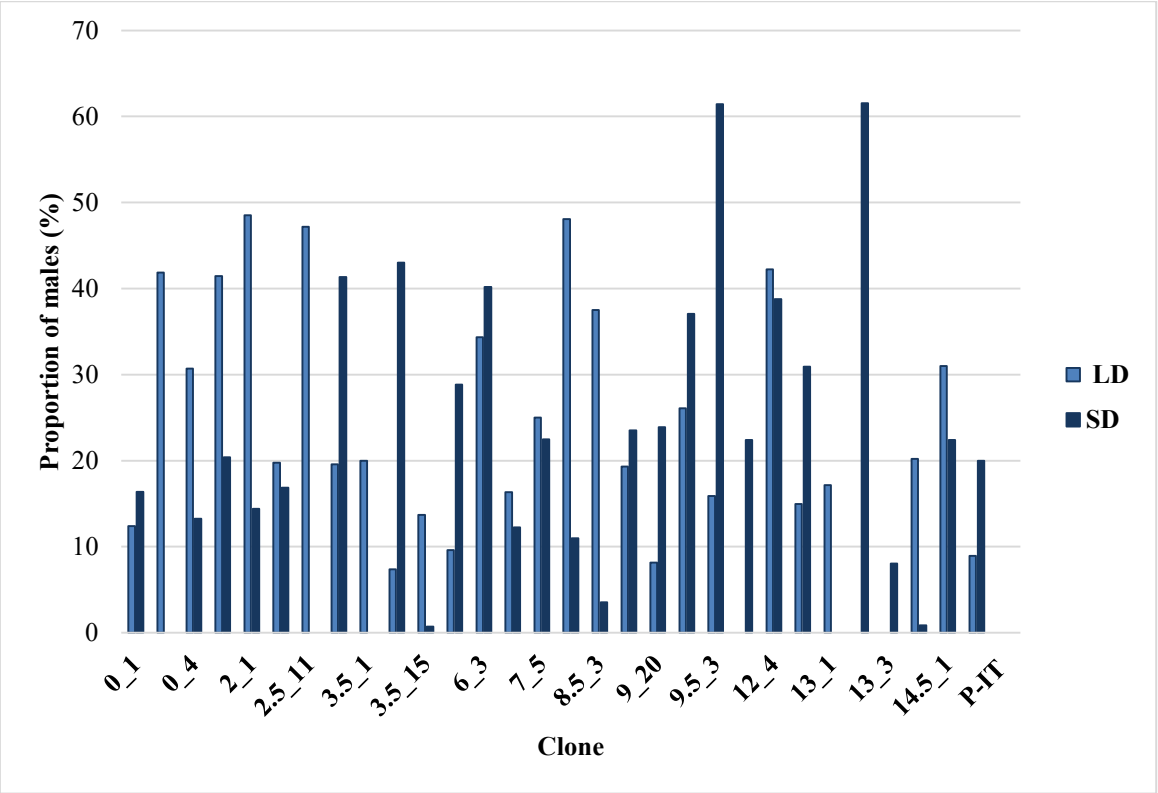


Figure 3.1. **Males produced in different photoperiods across 31 clones**.
The percentage of male offspring per clone in common garden experiment of 30 genotypes from Lake Ring exposed to warm temperature (20°C) combined with either long photoperiod (14:10 h L:D) (LD) or short photoperiod (10:14 h L:D) (SD). Genotype names are as in Table 3.1.

Chapter 3.1: Transgenerational plasticity in response to earlier spring warming in *D. magna*

Organisms respond to changing environments by shifting the distribution of fitness related traits, via genetic adaptation or through plasticity (Hoffmann and Sgrò, 2011). Plasticity is expected to play a key role in rapid-changing environments, especially when genetic adaptation may be constrained under predicted global change (Merila, 2012; Donelson et al., 2018;). So far, within-generation plasticity (WGP) is advocated as the main mechanism of response to environmental change, allowing for rapid adjustments to novel environmental conditions (Hendry et al., 2008; Merila and Hendry, 2014; Hendry; 2015). Yet this consensus is subject to a scarcity of studies on the mechanisms of genetic adaptation to environmental change, because of the significant challenges at identifying the genetic elements underpinning adaptive phenotypic trait variation in nature (Hoffmann and Sgrò, 2011; Merila, 2012; Jansen et al., 2017). Moreover, it is unclear how plasticity impacts long-term evolutionary responses to environmental change, as plasticity can either help (Ghalambor et al., 2007; Mitchell et al., 2011) or hinder (Hendry, 2016) evolutionary responses. Obtaining evidence that identifies the relative contribution of genetic adaptation and plasticity in the wild represents an ongoing challenge (Merila and Hendry, 2014).

Plasticity experienced within a generation may influence the response of following generations via non-genetic or epigenetic processes. This form of plasticity is known as transgenerational plasticity (TGP) and occurs when the environment that individuals experience influences the expression of traits in their offspring (Uller, 2008; Dubey et al., 2011; Uller et al., 2013). TGP is especially relevant to understand the impact of environmental change that persists across generations. Currently, the term TGP is loosely used to include any non-genetic effects that are observed in the offspring generation, which is associated with exposure of a previous generation to a new environmental condition (Salinas et al., 2013). However, TGP may also be adaptive, enhancing offspring performance, by buffering populations against the immediate effects of environmental change and providing time for genetic adaptation to happen in the longer term (Chevin et al., 2010). Adaptive transgenerational plasticity, mediated by parental effects, maximizes offspring fitness when the parental and the offspring environments are matched (Uller, 2008; Uller et al., 2013). Examples of adaptive transgenerational plasticity include the effect of maternal environment on the timing of seed germination (Herman and Sultan, 2011) and shade-avoidance in plants (Galloway and Etterson, 2007). Understanding the

role of adaptive transgenerational plasticity is key to predict the consequences of parental effects on population dynamics, and to inform our understanding of how species will respond to rapid environmental change (Bonduriansky et al., 2012). Presently, the prevalence and strength of TGP in natural systems remains controversial (Uller et al., 2013).

Temperature and photoperiod are two pivotal cues that regulate key fitness traits in plants and animals (e.g. flowering time and dormancy). However, whereas climate is changing, photoperiod is stable across latitudes (IPCC, 2013; 2014). The asynchronous change of temperature and photoperiod cues has disrupted the optimal seasonal timing of physiological, developmental and reproductive events in many species (Bradshaw and Holzapfel, 2007; 2010; Grevstad and Coop, 2015). Therefore, many organisms have experienced a decline in fitness when preparing to develop, reproduce, hibernate, enter dormancy, or migrate at seasonally inappropriate times (Bradshaw and Holzapfel, 2010). Parthenogenetic zooplankton species (e.g. daphniids, rotifers) use both temperature and photoperiod cues to time life history events such as male production and dormancy (Tessier and Caceres, 2004; Bradshaw and Holzapfel, 2007). Generally, short photoperiod and low temperatures induce a switch from the production of clonal (parthenogenetic) females to the parthenogenetic generation of males and to initiating sexual reproduction, which culminates in dormant embryos (Ebert, 2005). Conversely, long photoperiod and high temperatures are cues for the termination of dormancy in spring (Bradshaw and Holzapfel, 2007; 2010). Dormant embryos are early stage embryos that arrest their development to overwinter; dormant embryos at the water-sediment interface hatch during the following growing season contributing to the genetic diversity of the local population (Yampolsky, 1992; Hobaek and Larsson, 1990; Innes and Singleton, 2000). A proportion of these embryos becomes buried in the sediment missing their opportunity to hatch thereby creating biological archives, which represent an excellent resource to investigate population dynamics in natural systems over evolutionary time (Frisch et al., 2014; Orsini et al., 2012; 2016). Because of the changes in water temperature due to global warming, the temperature cue thresholds that are experienced by some parthenogenetic zooplankers are producing shorter photoperiods in population-level investments in dormancy and in male formation (Dupuis and Hann, 2009; Jones and Gilbert, 2016). However, the impact of persistent asynchrony between temperature and photoperiod across generations is poorly understood. Understanding the transgenerational impact of this asynchrony is helpful to predict how rapidly these species, and the community which they sustain, may track climatic change.

Here, we study the impact of the asynchrony between temperature and photoperiod on the fitness of the freshwater crustacean *Daphnia magna.* We study within-generation plastic and genetic responses, as well as the cross-generational responses of fitness-linked life history traits to environmental conditions that mimic early spring warming (warm temperature and short photoperiod) as compared to a typical spring environment (warm temperature and long photoperiod).

Studies that investigate mechanisms of response to environmental change face two main limitations. If genetically diverse species are studied, the same genotype or a diversity of genotypes cannot be replicated across environmental exposures and transgenerational responses to environmental change are confounded by genetic variation. Conversely, if clonal species are used and genotypes are replicated across experiments, genetic diversity is not representative of sexually reproducing species. *Daphnia*'s life cycle overcomes these limitations to reveal the relative contribution of plastic and genetic responses to environmental change across generations and over extended time periods (Stoks et al., 2015; Cambronero et al., 2017). *Daphnia* alternates sexual recombination with asexual (clonal) reproduction. Sexual recombination results in early stage embryos that can be 'resurrected' and propagated indefinitely as clonal lineages in the laboratory (Cambronero and Orsini, 2018). These properties and a short life cycle provide the advantages of clonal species while retaining the natural genetic diversity. Taking full advantage of these properties, we measured transgenerational fitness changes in three populations of dormant embryos previously resurrected from the biological archive of Lake Ring and spanning 50 years (Cambronero et al., 2018). We quantified WGP and TGP as changes in trait values of fitness-linked life history traits between environments and across generations. We quantified genetic responses to photoperiod as mean trait differences among populations and as genetic divergence at 15 candidate genes, previously linked to environmentally driven local adaptation (Roulin et al., 2016; Reisser et al., 2017). We also performed a gene-traits association analysis between the life history traits measured in the parental generation and the 15 candidate genes in an effort to identify the genetic basis of fitness-linked life history traits responding to photoperiod and temperature cues. Our study provides important insights into the transgenerational mechanisms of response to early spring warming in a freshwater keystone species.

Materials and Methods

Genetic and plastic responses to photoperiod length

In this experiment (CGE 1) the effect of short photoperiod (10:14 h light: dark regime) combined with spring temperature (20°C) was quantified on life history traits over two generations as compared to a typical spring environment, in which warm temperature co-occurred with long photoperiod (control, 14:10 h light: dark regime). The experimental conditions of the second generation were maintained identical to the first generation to assess trade-offs between within generation and across generations plasticity. Thirty clones (genotypes) used in this experiment were from the sedimentary archive of Lake Ring (Chapter 3, table 3.1).

After two generations in these conditions, individual juveniles of 24 hours from the second or following broods were randomly assigned to the experimental conditions in which life history traits were measured. A 24 hours old individual, randomly selected from the third brood of the first generation, was used to establish the second generation. On the experimental animals from the two generations, we measured: size at maturity (the distance between the head and the base of the tail spine), age at maturity (first time eggs were observed in the brood chamber), fecundity (total number of offspring across eight broods), mortality, and the percentage of males produced in each brood across eight broods. For size at maturity, all animals were measured after releasing their first brood in the brood pouch using image J software (https://imagej.nih.gov/ij/index.html).

The experimental design is not full factorial because of the number of genotypes and conditions tested in parallel. However, because genotypes were fixed across experimental conditions and generations, we were able to control for confounding factors e.g. genetic changes occurring from one generation to the next and genetic variation among experimental exposures, enabling WGP (within generation plasticity) and TGP (transgenerational plasticity) effects to be studied in isolation.

Life history traits: genetic and plastic responses to photoperiod and temperature cues

We assessed evolutionary mechanisms (genetic changes, WGP, TGP, and their interactions) of response to early spring warming on life history traits using linear mixed models (LMMs) in the “lme4” package in R v.3.3.3 (R, 2017). We quantified the effects of Generation, Population, Treatment and their interactions on individual life history traits. Genotype was fit as a random effect nested within population. Prior to the analyses, all variables were tested for normality. Except for male proportion (a binomial variable), all

life history trait measurements followed a Gaussian distribution. Because the populations separated in time originate from the same genetic pool and because genetic drift is negligible (Orsini et al., 2016), a significant population term indicates genetic differences among populations. Differences in mean trait values between photoperiods, after controlling for maternal effects, are the expression of WGP, an environmental effect. Differences in mean trait values between generations are the expression of TGP. If the effect of the treatment (photoperiod) differed significantly among populations (genetic effect), we would have evidence of a Pop (population) x Pht (photoperiod) interaction. Similarly, if the effect of the treatment (photoperiod) differed significantly among generations, we would have evidence of Gen (generation) x Pht (photoperiod) interaction. If population means varied by generation, we would have evidence of a Gen (generation) x Pop (population) interaction. We also measured the three-way interaction term (Gen x Pop x Pht). All evidence of interactions or main effects were assessed via Type II analysis of deviance tables using the Anova function in the "car" package (R v.3.3.3) (Fox and Weisberg, 2011). We visualized the main effects of population (P1, P2 and P3), treatment (SP and LP) and generation (G1 and G2) plus their interaction terms on individual life history traits through reaction norms, which describe the pattern of phenotypic expression of each population across treatments and generations (Roff, 1997).

We performed a principal component analysis (PCA) to quantify the principal modes of variation and covariation among life history traits within generation using the 'prcomp' function in the R "stats" package. The PCA plots were obtained using the "ggbiplot" (R v.3.3.3, (R, 2017)). Prior to the PCA analysis, the life history traits variables were log transformed.

Mortality rates per population were calculated with a survival model fit via the "psm" function in the rms R package V.3.3.3 (R, 2017). A separate model was fitted to each treatment and generation, in which the day of mortality and the mortality event were combined as the dependent variables (e.g. censoring) and population was treated as fixed effect. All mortality curves were plotted using the "survplot" function form "rms" package in R v.3.3.3 (R, 2017).

Gene-trait association

We performed gene-trait association analysis between 15 candidate genes and the life history traits measured in the transgenerational common garden experiment (CGE1). These genes were previously associated with environmental driven local adaptation, including sex induction and male formation (Roulin et al, 2016; Reisser et al., 2017). Population-

level gene polymorphism was obtained from genome resequencing of the 30 genotypes resurrected from Lake Ring mapped against the draft genome of *D. magna* 2.4. The whole genome resequencing will be published in NCBI repository. However, we report all SNP polymorphisms found in the 31 genotypes in Table 3.1.1.

To assess whether the candidate genes were under selection in the populations studied here, we used measures of genetic differentiation (F_{ST}) and neutrality tests. Previous results identified a small yet significant proportion of neutral genetic divergence among the *Daphnia* populations studied here (1%) (Orsini et al., 2016). Larger divergence among populations at the 15 candidate genes would identify genes under divergent selection, whereas a smaller divergence at the candidate loci would indicate balancing selection. In the former, different polymorphic sites at the same genes are found in different populations, resulting in higher divergence than at neutral loci. Conversely, balancing selection reduces population differentiation, because of moderate to intermediate frequencies of polymorphic sites (Lewontin and Krakauer, 1973).

We quantified pairwise population differentiation as F_{ST}, using MSA Analyser (Dieringer and Schlötterer, 2003), and the partitioning of genetic variance within and among populations (AMOVA) using Arlequin (Excoffier et al., 2005). The two hierarchical levels used in the analysis are (i) among populations and (ii) within populations. Statistically significant values ($P < 0.001$) were calculated with permutation tests (10,000 permutations). Further, we performed neutrality tests on the candidate genes to quantify departure from neutrality using DnaSP (Rozas et al., 2003). Typically, these tests are affected by demography. In our study, the three populations are snapshots of the same genetic pool at different time points across five decades. Therefore, demographic confounding factors are negligible.

Table 3.1.1. Neutrality tests

Statistics testing for departure from neutrality of 15 candidate genes linked environmental driven local adaptation and partial sex determination. For each candidate gene (GeneID), the scaffold location (ScaffoldID), start and end position of the gene, nucleotide diversity (S), theta per site (θ), gene function and bibliographic reference are shown. For each gene, the result of Tajima's D., Fu&Li D and F tests are shown. Significant values, calculated with a FDR = 0.05, are marked with asterisks (*).

GeneID	ScaffoldID	Start	End	S	Pi	θ	Gene function	Ref	Tajima's D	Fu&Li's D	Fu&Li's F
Dapma7bEVm001004t1	scaffold00027	2877	6078	23	0.36	0.21	Serine arginine-rich splicing factor 7	Reisser *et al.*, 2017	2.13 *	1.76 **	2.25 **
Dapma7bEVm005301t1	scaffold00848	96321	97283	5	0.29	0.21	Aldo-keto reductase family 1, member C4	Reisser *et al.*, 2017	0.8	1.08	1.16
Dapma7bEVm002245t1	scaffold02003	35289	35935	2	0.30	0.21	Poly-U-binding splicing factor Half Pint	Reisser *et al.*, 2017	0.71	0.73	0.84
Dapma7bEVm015923t3	scaffold02003	213333	214454	48	0.45	0.22	Cytochrome P450 314 family	Reisser *et al.*, 2017	3.62 ***	2.03 **	3.14 **
NA	scaffold02569	3227	4315	7	0.39	0.21	Zinc transporter zip11	Reisser *et al.*, 2017	2.14 *	1.23	1.79 *
Dapma7bEVm006598t1	scaffold02569	9179	10907	12	0.30	0.21	Zinc transporter zip9	Reisser *et al.*, 2017	1.19	1.48 *	1.63
Dapma7bEVm008171t1	scaffold02569	35151	44725	91	0.42	0.22	SOX-9-like transcription factor	Reisser *et al.*, 2017	3.07 **	2.18 **	3.02 **
Dapma7bEVm002217t1	scaffold02569	218892	220701	4	0.36	0.21	DnaJ homolog dnaj-5	Reisser *et al.*, 2017	1.44	0.99	1.32
Dapma7bEVm028519t1 /Dapma7bEVm010615t1	scaffold02569	334258	337000	79	0.21	0.22	Broad-complex	Reisser *et al.*,	-0.19	2.15 **	1.50

								2017			
Dapma7bEVm004407t1	scaffold02569	340469	342584	20	0.21	0.21	Transformer2	Reisser *et al.*, 2017	-0.01	1.70 **	1.30
Dapma7bEVm000710t1	scaffold02569	76814	79370	19	0.42	0.21	Protein SPT2 homolog	Reisser *et al.*, 2017	2.92 **	2.92**	2.52 **
Dapma7bEVm007919t1	scaffold02569	228772	229714	7	0.45	0.21	Histone deacetylase complex subunit sap18	Reisser *et al.*, 2017	2.83 **	1.23	2.06 **
Dapma7bEVm005463t1	scaffold02723	1124	6033	37	0.31	0.22	Epidermal growth factor receptor kinase	Reisser *et al.*, 2017	1.33	1.95 **	2.05 **
Dapma7bEVm001751t1	scaffold03156	4200	8559	40	0.27	0.21	Lysine-specific histone demethylase 1A	Reisser *et al.*, 2017	0.89	1.96 **	1.87 *
Dapma7bEVm015675t1	scaffold01036	708969	713276	64	0.37	0.22	Rhodopsin	Roulin *et al.*, 2016	2.32 *	2.10 **	2.61 **

To assess whether the candidate genes were associated with life history traits showing responses to photoperiod, we performed gene-trait association analysis using Mixed Linear Models in TASSEL (Bradbury et al., 2007) with the following criteria: minor allele frequency (MAF) > 0.05 and Hardy-Weinberg Equilibrium (HWE) > 0.001, applying a false discovery rate of FDR = 0.05 to correct for multiple testing. We investigated significant association between gene polymorphisms at the 15 candidate genes and the life history traits in long and short photoperiod, separately. We also assessed association between the gene polymorphisms and the plastic change in life history traits between long and short photoperiod (delta). Eight of the 15 candidate genes sit on the same *D. magna* scaffold (scaffold02569) of the draft reference genome. We calculated linkage disequilibrium among these genes.

Validation of the genetic basis of male formation in *D. magna*

In the transgenerational common garden experiment (Fig. 3.1.1, CGE1), we observed variation in the propensity to form male offspring in response to changes in photoperiod, both among genotypes and between generations. Among the 30 genotypes, we identified three that showed a consistent pattern between generations and photoperiods: clones 3_5-15 and 13_5-1 produced most male broods in long photoperiod but were from different lake phases (CWP and EP, respectively), clone 13_2 produced male broods in short photoperiod and clone P-IT did not produce males. In a follow up experiment, in which six clonal replicates per genotype were used, we measured the percentage of male offspring across eight broods in these three genotypes and in a reference laboratory strain (P-IT, Institute of Ecosystem study, CNR Verbania, Italy), which has never been reported to produce male broods in any experimental condition (Gorbi et al., 2011) (Fig. 3.1.1 – CGE2).

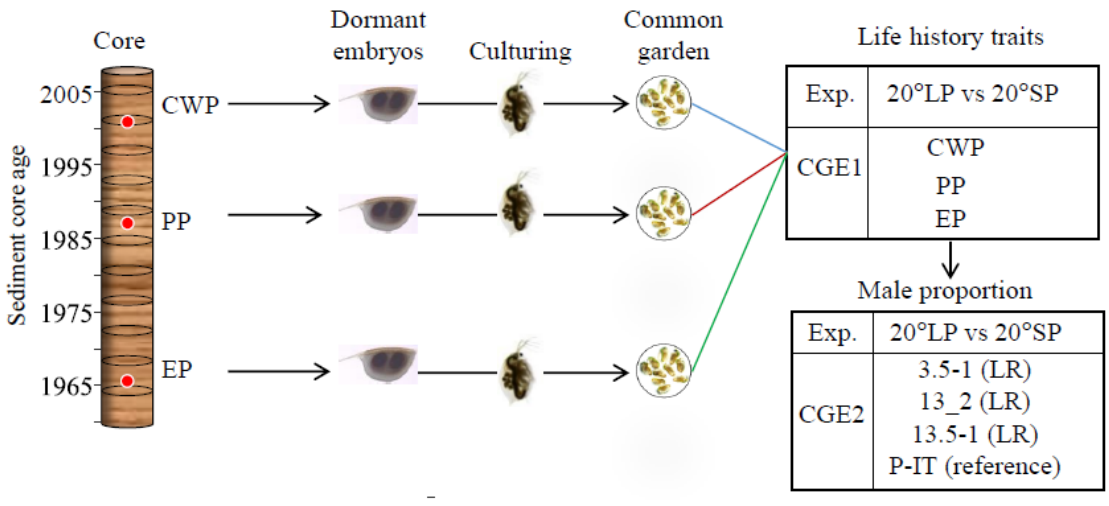


Figure 3.1.1. Sampling and experimental design.
D. magna dormant embryos were resurrected from three populations separated in time along the sedimentary archive of Lake Ring: CWP – clear water population; PP – pesticide population; EP – eutrophic population, described in (Cambronero et al., 2017). Between 9 and 11 genotypes per populations were used in common garden experiment one (CGE1), in which life history traits and male offspring proportion across eight broods were measured across 2 generations at two different photoperiods: long photoperiod (LP; 10:14 light: dark regime) and short photoperiod (SP; 14:10 light: dark regime). Three genotypes from CGE1 showing divergent patterns of male production in response to photoperiod changes and a reference laboratory genotype (P-IT) never reported to form male offspring were used in CGE2 to validate the pattern of male offspring formation in higher replicated design.

DNA extraction

To identify gene polymorphisms associated with the propensity to form male offspring, we studied gene polymorphism at the candidate genes in the four strains. The genome sequence of the reference laboratory strain P-IT will be available in NCBI repository (NCBI SRA database: SRX4042391), and was obtained as follows. Genomic DNA (gDNA) was extracted using Agencourt DNA Advance (Beckman Coulter - A48706) with minor modifications, and quantified using a ND-8000 Nanodrop (Thermo Fisher Scientific - ND-8000-GL). Up to 1µg of gDNA per genotype was sheared using a Bioruptor® Pico ultrasonicator with integrated cooling module (Diagenode - B01060010), following cooling on ice for 10 minutes. Sheared gDNA was assayed on a 2200 TapeStation (Agilent) with High Sensitivity DNA Screentapes to determine the distribution of sheared fragments. The sheared gDNA was then prepared into Illumina compatible DNA Sequencing 250bp paired-end libraries using KAPA HyperPrep Kit (Roche - KK8504), without amplification step. Following library construction, libraries were assayed and quantified on a 2200 TapeStation (Agilent) with High Sensitivity DNA Screentapes.

Libraries were normalized to an average concentration of 2 nM prior to pooling and sequenced on an Illumina4000 sequencer. Allelic variants and indels were identified by mapping against the low-recombining genomic region containing the 15 candidate genes of the reference *D. magna* genome (Reisser et al., 2017).

Results

Life history traits: genetic and plastic responses to photoperiod and temperature cues

We investigated the impact of photoperiod and temperature cues on fitness-linked life history traits in *Daphnia*. Short photoperiod at 20 °C mimicked early spring warming, whereas long photoperiod at 20 °C mimicked a typical spring environment, acting as reference.

We quantified the effects of Generation, Population, Treatment and their interactions on individual life history traits using an analysis of variance. The three-way interaction term (Generation x Population x Photoperiod) was significant for the proportion of male offspring (Table 3.1.2). The effect of photoperiod varied significantly between generations in four of the five life history traits (Table 3.1.2; Gen x Pht). The effect of photoperiod did not vary significantly by population, except for the proportion of male offspring (Table 3.1.2; Pop x Pht). A significant interaction term between generation and population was only observed for the proportion of male offspring (Table 3.1.2; Gen x Pop). We detected no difference among populations in trait means, except for fecundity (Table 3.1.2; Pop). All mean trait values differed significantly between generations (Table 3.1.2; Gen). The effect of photoperiod was significant on fecundity, size at maturity and time elapsing between broods (Table 3.1.2; Pht). Mortality was negligible in all experimental conditions (Table 3.1.2).

Table 3.1.2 Analysis of variance

Univariate ANOVAs per single life history traits (fecundity over the life span of the genotypes, size at maturity (mm), age at maturity (days), proportion of males offspring over the life span of the genotypes, the time elapsed between broods averaged over eight broods (Av. time interval between broods, days) and mortality are shown. Generation (Gen), Population (Pop), Photoperiod (PhT), and their interaction terms are shown. Significant *P-values* are in bold.

	Fecundity			Size at maturity			Age at maturity			Male proportion			Average interval between broods			Mortality		
	Df	Chisq	p-value	Df	Chisq	p-value	Df	Chisq	p-value	Df	Chisq	p-value	Df	Chisq	p-value	Df	Chisq	p-value
Generation (Gen)	1	0.48	**<0.001**	1	23.09	**<0.001**	1	13.33	**0.001**	1	114.60	**<0.001**	1	19.15	**0.001**	1	2.04	0.15
Population (Pop)	2	10.52	**0.005**	2	0.11	0.94	2	0.21	0.90	2	3.89	0.14	2	0.96	0.62	2	5.35	0.07
Photoperiod (Pht)	1	31.89	**<0.001**	1	20.71	**<0.001**	1	0.47	0.49	1	1.31	0.25	1	41.23	**0.001**	1	2.30	0.13
Pop x Pht	2	1.47	0.48	2	4.21	0.12	2	0.03	0.98	2	39.55	**<0.001**	2	2.41	0.30	2	5.27	0.07
Pop x Gen	2	2.86	0.24	2	0.83	0.66	2	4.32	0.11	2	18.82	**<0.001**	2	1.39	0.50	2	4.78	0.09
Pht x Gen	1	16.64	**<0.001**	1	12.25	**<0.001**	1	13.55	**0.001**	1	4.80	**0.028**	1	2.02	0.15	1	2.04	0.15
Pop x Pht x Gen	2	0.38	0.83	2	0.13	0.94	2	0.69	0.71	2	26.70	**<0.001**	2	2.00	0.37	2	4.75	0.09

Short photoperiod caused a decrease in size and in the time elapsing between parthenogenetic broods in the first experimental generation (Fig. 3.1.2; F_0). In the second generation (F_1), short photoperiod induced increase in fecundity, smaller size at maturation, and longer time elapsing between parthenogenetic broods (Fig. 3.1.2; F_1). Male proportion varies with generation and population, showing both genetic and plastic responses (Fig. 3.1.2).

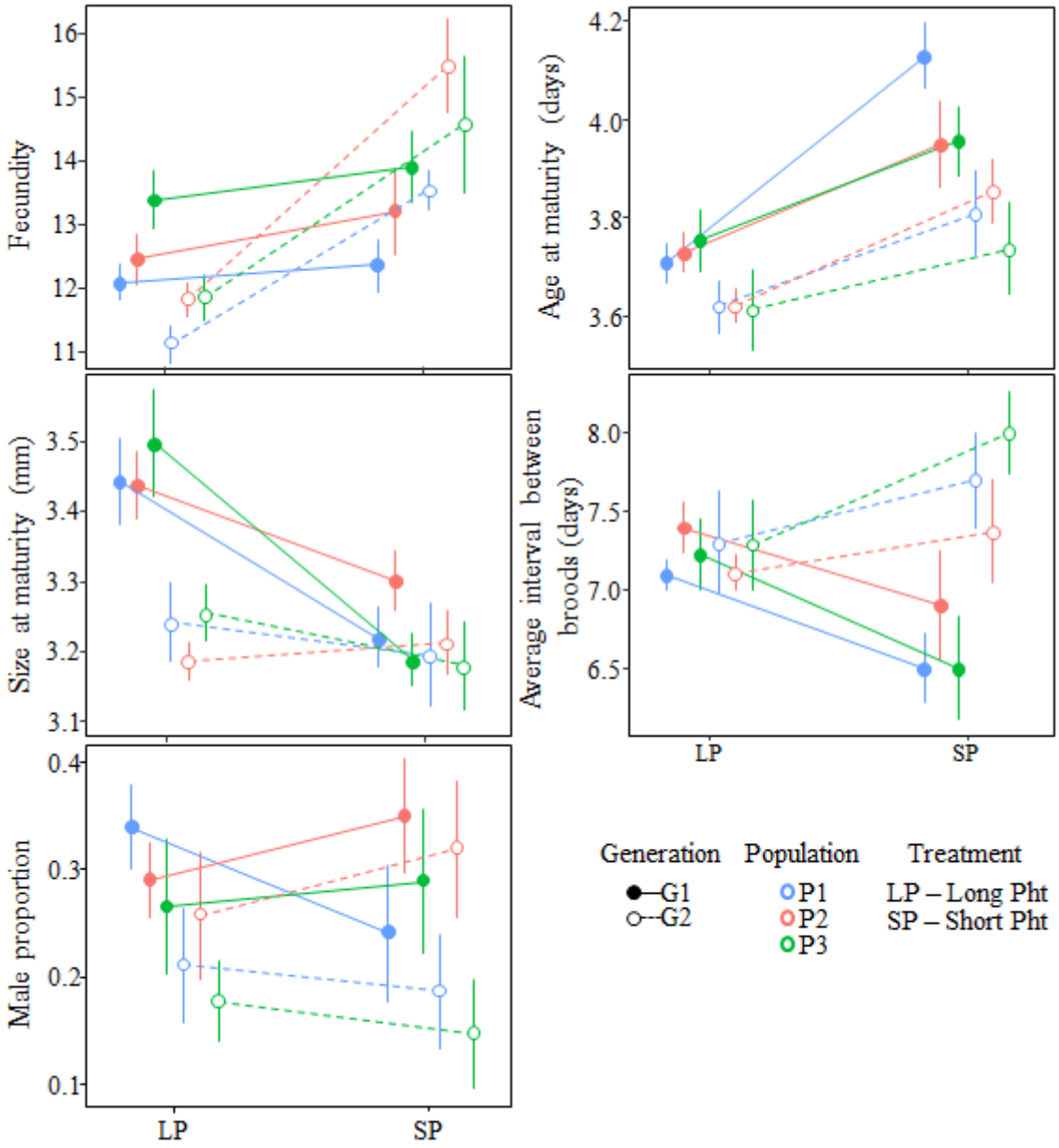


Figure 3.1.2. Life history traits response to changes in photoperiod
Population reaction norms based on population means (N = 9-11) and SE measured in long photoperiod (LP) and short photoperiod (SP) for generation F_0 (G1; continuous lines) and generation F_2 (G2; dotted lines). The life history traits measured are: fecundity, age at maturity (days), size at maturity (mm); proportion of male offspring, and average interval between broods (days). The populations are colour coded are: P1 – blue; P2 – red; P3 – green. Statistical analyses supporting the reaction norms are in Table 3.1.2.

We observed a clear difference in the trait space between generations and experimental conditions (Fig. 3.1.3). In F_0, PC1 was positively correlated with average time elapsing between broods (PC1: 52%) and negatively correlated with age and size at maturity ($PC1_{age}$: -53%; $PC1_{size}$: -62%). PC2 was positively correlated with fecundity (PC2: 48%) and negatively with the proportion of male offspring (PC2: -71%). PC3 was positively correlated with fecundity (PC3: 87%) and negatively with size and age at maturity ($PC3_{size}$: -16%; $PC3_{age}$: -30%) (Fig. 3.1.3A). In F_1, PC1 was positively correlated with age and size at maturity ($PC1_{age}$: 70%; $PC1_{size}$: 65%), whereas PC2 negatively correlated with fecundity, the proportion of male offspring and the time elapsing between broods ($PC2_{fecundity}$: -62%; $PC2_{male}$: -41%; $PC2_{av.\ interval}$: -58%). In this generation, PC3 was negatively correlated with the proportion of male offspring (PC3: 89%) and positively correlated with fecundity and average time elapsing between broods ($PC3_{fecundity}$: 21%; $PC3_{av.\ interval}$: 39%) (Fig. 3.1.3B).

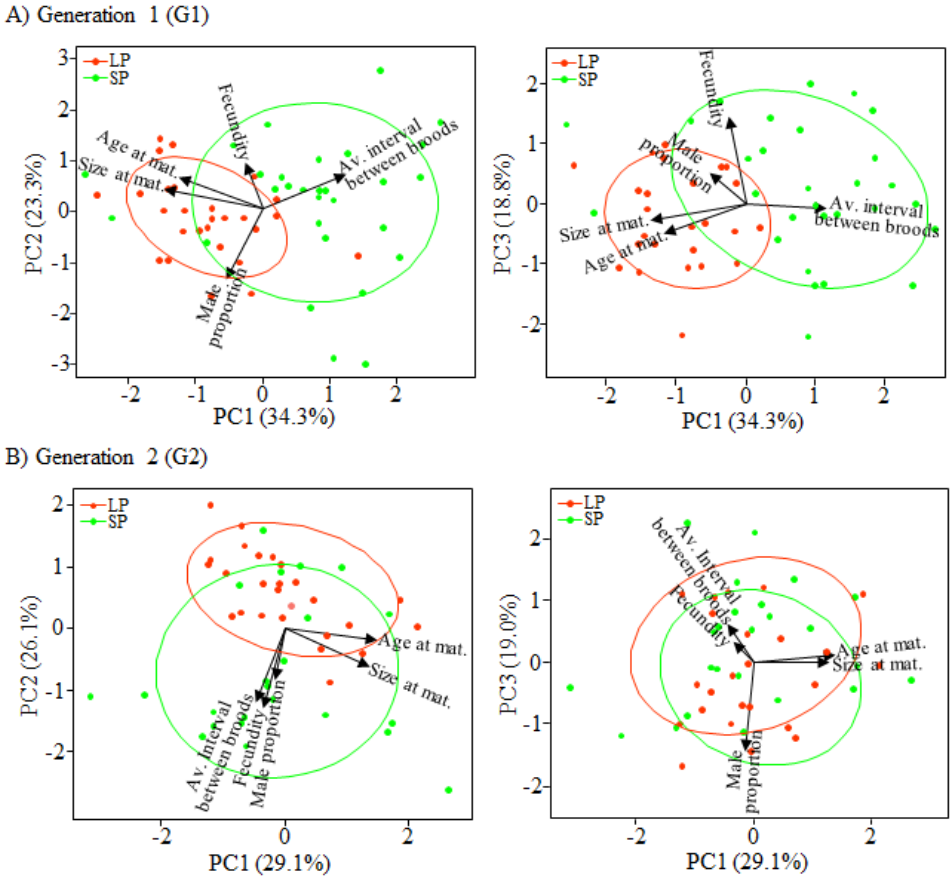


Figure 3.1.3: Principal component analysis

PCA plots showing phenotypic plasticity at five fitness-linked life history traits measured in A) generation 1 (F_0) and B) generation 2 (F_1) in long photoperiod (LP, 14:10 h light: dark regime; orange) and short photoperiod (SP, 10:14 h light: dark regime; green). Patterns are given for PC1 and PC2 and PC1 and PC3. The life history traits are the same as in Table 3.1.2: age at maturity, size at maturity, fecundity, male proportion and average interval between broods.

Candidate gene analysis: genetic responses to temperature and photoperiod cues

The genetic divergence among populations was higher at neutral loci than at the candidate genes in presence of negligible drift (Table 3.1.3). The neutrality test on the candidate genes identified nine out of fifteen genes as putatively under selection; at least two independent tests supported departure from neutrality in these genes (Table 3.1.1). The candidate genes showed significant association with fecundity and male offspring proportion both in short and long photoperiod (SP, LP). Fecundity showed significant association with the histone deacetylase complex subunit SAP18. The proportion of male offspring showed significant association with Amino-oxidase, SAP18, Phosphotyrosine (PTB), and Aldo/keto reductase. In addition, the candidate genes showed significant association with plastic changes in all traits. Plastic changes in age at maturity were significantly associated with the rhodopsin g-protein coupled receptor; plastic changes in fecundity were significantly associated with the Histone deacetylase complex subunit SAP18; variation in male offspring proportion was significantly associated with Amino-oxidase, SAP18, Phosphotyrosine (PTB), and Aldo/keto reductase; plastic change in size at maturity was significantly associated with Pyridine nucleotide-disulphide oxidoreductase, PTB, SAP18, and an RNA binding protein.

Table 3.1.3: Analysis of molecular variance

AMOVA analysis showing the partitioning of genetic variance within and among populations at neutral microsatellite loci and at the 15 candidate genes.. The two hierarchical levels used in the analysis are (i) among populations and (ii) within populations. Statistically significant values (*, $P < 0.001$) are based on permutation tests (10,000 permutations).

	Among populations	Within populations
Neutral μsat	1.08*	98.92*
Candidate genes	0	100*

Discussion

We studied the impact of earlier spring warming on the cyclical parthenogen *D. magna*, and investigated the genetics underpinning of environmentally induced fitness-linked life history traits at 15 candidate genes, previously associated with environmental driven local adaptation and partial sex determination (Roulin et al., 2016; Reisser et al., 2017). Because we used a resurrection ecology approach and exposed clonal replicates of the same genotype to different environmental conditions, we were able to identify both genetic and plastic mechanisms of response to earlier spring warming across generations.

In the parental generation, we identified genetic differences in fecundity among populations, in response to earlier spring warming (warm temperature and short photoperiod). The ANOVA results were supported by the reaction norms identifying the most recent population (CWP) as less fecund at both photoperiods as compared to the other two populations (EP and PP). We also identified significant plastic response to photoperiod change, resulting in earlier maturation and smaller size at maturity in the three populations examined. Moreover, shorter photoperiod prompted the majority of genotypes to invest in male offspring starting from the second brood.

The ANOVA also revealed that exposure of the offspring generation to the same environment of the parental generation resulted in population differences in the allocation of resources to male offspring production (genetic response). In addition to this genetic response, we also observed a significant plastic change in fecundity across the three populations, leading to higher fecundity in conditions mimicking earlier spring warming.

Cross generation exposure to earlier spring warming highlighted significant differences between generations in size and age at maturity, as well as in the propensity to form male offspring. These differences were driven by significant differences among populations (genetic responses) in size at maturation with the two most recent populations (PP and CWP) having opposite responses than the historical population. The analysis of within versus between generations plasticity revealed negative trade-offs in age at maturity, fecundity and propensity to invest in male offspring, indicating a decrease in plasticity in the F_1 generation.

Overall, our results are inconsistent with the hypothesis that selection will promote simultaneous or coupled increases in within- and across-generation plasticity (Kuijper and Hoyle, 2015; Leimar and McNamara, 2015). Conversely, they align with expectations of organisms living in fluctuating environments (Hoyle and Ezard, 2012). Simultaneous within and across generations plasticity is favoured in stable environments in which maternal effect minimizes variance around the optimum phenotype of the descendant generations and thus maximize population mean fitness (Hoyle and Ezard, 2012). A positive maternal effect or a positive correlation between within and across generation plasticity at a life history trait indicates accelerated rates of microevolution that can facilitate adaptation, whereas a negative maternal effect suggests that maternal effects slows (or even reverse) any response to selection in the offspring generation (Lande and Price, 1989). A negative maternal effect, therefore, can reverse phenotypic evolution from one generation to the next [birds (Schluter and Gustafsson, 1993) and insects (Janssen et al., 1988)]. The trade-offs between within and across generation plasticity observed here

can indicate that within-generation phenotypic plasticity has a much stronger influence on *Daphnia* response to environmental cues than transgenerational plasticity. This may be expected in a highly plastic species (Kuijper and Hoyle, 2015). Indeed, maternal or transgenerational effect of plasticity is expected to be negative in unpredictable environments to minimize the effect of genetic variance and facilitate rapid responses to changes (Uller, 2008).

The lake from which the populations of *D. magna* were sampled has a documented increase in average ambient temperature and recurrence of heat waves over time (Cambronero et al., 2017). Whereas we observe significant different responses among populations to earlier spring warming cues, there is no evidence that suggests evolution of adaptive responses of the most recent population to these conditions. This may be because the average temperature increase recorded for this lake is modest (~1°C) and/or because other environmental factors, such as water transparency and lake chemistry that occurred in this lake have affected micro-evolutionary responses of these populations. The impact of multiple stressor in water ecosystems can lead to unpredictable evolutionary trajectories (Jackson et al., 2016).

The genes-trait association analysis identified association with life history traits of genes previously linked to dormant stages induction in *Daphnia* (rhodopsin) (Roulin et al., 2016), embryonic development and environmental stress response in insects (SAP18) (Canudas et al., 2005; Costa et al., 2011). It also identified an epidermal growth factor receptor kinase, involved in sexual differentiation of insects and crustaceans (PTB) (Foronda et al., 2012; Corona et al., 2016). Furthermore, a catalyser the juvenile hormone MF was found to be significantly associated with the propensity to form male offspring (aldo-keto reductase: AKR) (Tatarazako et al., 2003; Toyota et al., 2015a). In the MF pathway, the AKR family catalyses the conversion of farnesal to farnesol, and mutations in the AKR gene have been shown to reduce MF production in favour of the juvenile hormone, resulting in highest male production (Toyota et al., 2015a; Rivera-Perez et al., 2013*)*. The neutrality analysis across multiple tests (FDR = 0.05), revealed that these genes depart from neutrality and appear to be under selection. The impact of demography as potential confounding factor in this analysis is negligible as the three populations analysed are the same genetic pool sampled over time across five decades. However, we cannot exclude that additional genes not investigated here are contributing to modulate the life history traits analysed. Indeed, all traits showed significant association with multiple candidate genes, as well as single candidate genes showed association with multiple traits, suggesting extensive pleiotropy. It is expected that multiple genes regulate complex fitness-linked life history traits. Further

evidence supporting pleiotropy is the lack of SNP mutations within the candidate genes that would distinguish genotypes with different propensity to form male offspring. A genome-wide association analysis is required to identify all genes responsible for male formation.

Chapter 3.2: Effects of Roundup and its active ingredient glyphosate on life history traits, transcriptome, microbiome and DNA integrity on four *D. magna* clones

The relationship between *Daphnia* and their microbiome is an aspect of interest because microbial metabolites can influence epigenetics by altering the pool of compounds used for modification or by directly inhibiting enzymes involved in epigenetic pathways (Hullar and Fu, 2014). Microbiota can affect epigenetic processes of the host because translates environmental signals and influences the host gene expression and, subsequently, its physiological responses (Pevsner-Fischer et al., 2017). It was clearly shown by Sison-Mangus et al. (2015) and further confirmed by Mushegian and Ebert (2017) that the absence of the microbiome resulted in significant adverse effects on fitness including slower growth, smaller size, lower reproducibility and higher mortality, all of which were preventable with the restoration of the microbiome. Additionally, Mushegian and collaborators (2018) demonstrated that the microbiome facilitates healthy hatching and embryonic development of the animal, whereas Macke et al. (2017) pointed to the adaptive importance of the gut microbiome, in the context of tolerance to cyanobacteria toxicity. This, and it has long been shown in *Drosophila* species, whose gut flora are comparable to that of *Daphnia*, that genes underlying a certain group of functions including immunity, metabolism and development are partially regulated by the gut microbiome (Broderick et al., 2014), where another study analyzing the transcriptional networks showed that the co-expression of those genes is also enhanced by the bacteria (Dobson et al., 2016).

While it is important to experiment on appropriate model organisms, perhaps the most important step would be to utilize advanced omics and multiomics tools that would leverage the available data to obtain the most informative results through more comprehensive and in-depth analyses. In this regard, integrated approach based on microbiome and host transcriptome analysis is helpful to understand the link between bacteria community composition and host gene modulation and their roles in organism responses to environmental cues and stress.

Chronic exposure of the estuarine crab *Neohelice granulata* to both glyphosate and Roundup had led to the impediment of ovarian growth, where glyphosate significantly inhibited ovarian protein synthesis (Canosa et al., 2018). In the Mediterranean mussels *Mytilus galloprovincialis,* exposure to environmentally relevant concentrations of glyphosate (10, 100, and 1000 µg/l) had adverse effects on key biological pathways including endoplasmic reticulum stress response, cell signaling, metabolism and Ca^{2+} homeostasis (Milan et al., 2018). A significant decrease in reproductive rates was reported for *Simocephalus vetulus*, a common wetland crustacean zooplankton, exposed to the

glyphosate-based herbicide Visionn (0.75 and 1.50 mg acid equivalent/l) (Chen et al., 2011) and for *Moina micrura*, exposed to 50, 250 and 325 µg/L of glyphosate (Iwai et al., 2011).

In *D. magna*, glyphosate and its commercial formulation Roundup have negative effects in phenotypic and behavioural traits such as survival, growth, fecundity, juvenile body size, also causing egg abortion and swimming velocity (Cuhra et al., 2013; Hansen and Roslev 2016). In *D. magna*, Papchenkova et al. (2009) reported the negative effect of Roundup on fecundity and the alteration in the activities of carbohydrases and proteinases. These effects, were transferred by exposed organisms to the subsequent generations. The decrease in the tolerance to the acute toxic action of Roundup observed in the fourth generation crustaceans assumes the development of a cumulative toxic effect in the lineage of exposed animals. In *D. carinata*, glyphosate can increase the negative effect of other chemicals in mixture such as cadmium (Zalizniak and Nugegoda 2006). It is worth recalling, negative phenotypic effects of glyphosate and Roundup were observed at high concentrations. For *D. magna*, Reno et al. (2018) reported a 48h LC_{50} value of 11.68 mg/l for a Roundup formulation (Roundup Ultramax®- Monsanto). After RT-qPCR, it was observed that this herbicide changes the transcriptional activity of genes involved in metabolism and detoxification processes such as haemoglobin (dhb), aryl hydrocarbon receptor nuclear translocator (Arnt) and cytochrome P450s (CYPs) (Le et al., 2010). Therefore, glyphosate causes negative effects in fitness of organisms and in transcriptional processes of genes; even if Le and collaborators (2010) exposed daphnids to very high concentrations of glyphosate (190, 202, 214, and 234 mg/l for 24 h). To understand the real impact of glyphosate, the exposure of organisms to lowest concentrations can clarify the effect of this herbicide at physiological and molecular levels. Furthermore, differences in sensitivity to glyphosate toxicity were observed among organisms at different ages and clones (Cuhra et al., 2013). Different effects of glyphosate was observed in LC_{50} values among *D. magna* genotypes: 13–24 mg/l (FAO, 2001) whereas others report values of 234 mg/l (Le et al., 2010), 780 mg/l (McAllister and Forbis, 1978), 930 mg/l (Forbis and Boudreau, 1981) or even above 2000 mg/l (Pereira et al., 2009). Consequently it is clear that this chemical can cause different effect among genotypes; however, the effect of glyphosate and Roundup on different *Daphnia* lineages whit all transcriptome analysis have not been evaluated.

The main objective of this study is to assess *D. magna* mechanisms of resistance to common pesticides, while analyzing the transcriptome data is done to specifically differentiate between *D. magna* organismal and microbiome-mediated responses to ecologically relevant concentrations of the toxicants. A multi-disciplinary approach, based on phenotypic and molecular studies, was used. Organisms were exposed to a mixture of antibiotics (streptomycin, ampicillin, tetracycline) to evaluate differences in response between non- and germ free daphnids and the effect of glyphosate

and Roundup exposure in four genotypes. The effects on life history traits of different genotypes, all gene expression (RNA-Seq), and DNA damage (comet assay) were considered. In this regard, four genotypes were used in this study, to understand if the response of organisms to pesticides is obtained via transcriptome or microbiome. For these experiments, four genotypes were used to detect the most resistant and most susceptible one, and to evaluate if the response to glyphosate/Roundup is genotype-dependent or similar for all clones of *D. magna*.

Material and Methods

Daphnia magna genotypes and cultures

In this experiment the 3_5-15, 13_2 and 13_5-1 clones, obtained from Lake Ring in Jutland, Denmark (55°57'51.83'' N, 9°35'46.87'' E), and the clone P-IT obtained from the Department of Chemistry, Life Sciences and Environmental Sustainability of Parma University (Italy) were used (Chapter 3).

The genotypes were acclimated and synchronized for two generations in High Hardness COMBO media (COMBO HH) water (see Appendix A for COMBO HH composition). Cultures (20 organisms in 1 litre of water) were maintained at 20 °C and 14 h light/10 h dark photoperiod and fed daily with 0.8 mg C/l of *Chlorella vulgaris* (CCAP strain no. 211/11B). The breeding medium was completely renewed three times a week transferring adult females into jars containing fresh water and food and neonates were removed every day.

Exposure of organisms

Individual juveniles obtained of mothers of cultures of 24-48 hours from the second or following broods were randomly assigned to the different treatments. For each genotype, neonates were exposed to the following treatments: antibiotics (streptomycin sulphate salt-S (Sigma-Aldrich), ampicillin-A (Sigma-Aldrich), tetracycline-T (Sigma-Aldrich) each at concentration of 20 mg/l, glyphosate (PESTANAL, Sigma-Aldrich), at 1 mg/l concentration, antibiotics 20 mg/l + glyphosate 1 mg/l, the herbicide Roundup Optima+ (containing 170 g/l glyphosate acid (present as 208 g/l potassium salt of glyphosate)) at 1 mg/l (Monsanto) and antibiotics 20 mg/l + Roundup 1 mg/l in 200 ml of COMBO HH water. Three replicates per treatment were realized. Medium was renewed every two days, while organisms were fed every day with 0.8 mg C/l of a C. vulgaris suspension. Experiments carried out for the four genotypes was the following (Fig. 3.2.1):

- Life history traits
- Microbiome

- Transcriptome
- DNA damage

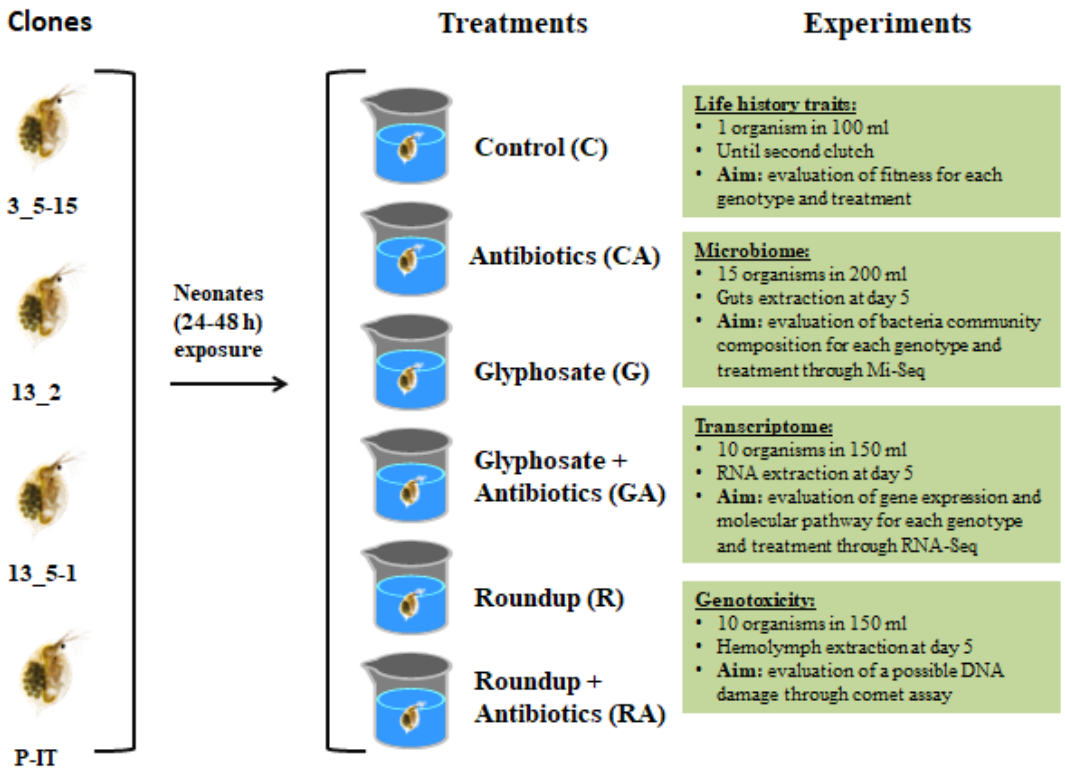


Figure 3.2.1: Experimental design. A scheme showing overview experiment in four *D. magna* clones (3_5-15, 13_2. 13_5-1 and P-IT). Organisms treatments have been the following: Control (C), Antibiotics (CA), Glyphosate (G), Gyphosate + Antibiotics (GA), Roundup (R) and Roundup + Antibiotics (RA). Endpoint evaluated life history traits, microbiome, transcriptome and genotoxicity.

Life history traits experiment

To evaluate the life history traits one neonate (24-48 hours) in 100 ml of COMBO HH water was maintained. Medium was changed every two days and daphnid was fed every day with 0.8 mg C/l of a C. *vulgaris* suspension. In this treatment, organisms were reared at 20 °C and 14:10 L:D photoperiod conditions. All experimental treatments were replicated 3 times using individuals from all four *D. magna* genotypes (6 treatments x 4 genotypes x 3 replicates = 72 females). Age at maturity, mortality, fecundity at first and second clutches (number of offspring), size at maturity (size after the first brood) and failed development were evaluated. For size at first brood, all animals were measured using image J software (https://imagej.nih.gov/ij/index.html). The whole experiment lasted for 15 days until the release of the second clutch.

A two-way ANOVA analysis was performed to assess the effect of treatment, genotype and their interaction on single life-history traits (*i.e.* size at maturity, age at maturity, fecundity). A linear

mixed-effects model with "replicate" as random effect was first fitted to the data by using *lme* function of R package *nlme* (Pinheiro et al., 2018). Since the variance associated to the "replicate" effect was never statistically significant, a more parsimonious linear model was used. All analyses were run in R by using the *aov* function, which allowed us to successively use a Tukey Test to assess whether differences in life history traits between all pairwise comparisons were statistically significant. Mortality rates per genotype and treatment were calculated with a survival exponential model fitting by using *psm* function of the R package *rms* (Frank and Harrell, 2018). Day of mortality and mortality event itself were the response variables, while genotype, treatment and their interaction were used as explanatory variables. Mortality curves were plotted using *survplot* function of the same package. A principal component analysis (PCA) was performed on scaled life-history traits (*i.e.* size at maturity, age at maturity, fecundity, failed development, and number of days between the first and second clutches). After that, PC scores were used as response variables in a MANOVA analysis, using genotype, treatment and their interaction as explanatory variables.

Microbiome analysis

Preliminary experiment

In a preliminary test, to evaluate the age at which gut microbiota formation occurs, guts of organisms aged 3, 4, 5 and 6 days maintained in 200 ml of borehole water were dissected and bacteria community composition analysed by DNA extraction and sequencing of specific regions (see paragraph *Extraction of microbial DNA*). In addition, to preliminarily evaluate the responses of the microbiota to daphnids exposure to pesticides, daphnids belonging to clone 3_5-15 were exposed to two treatments: the herbicide glyphosate (PESTANAL, Sigma-Aldrich), at 1 mg/l concentration, and the insecticide carbaryl (PESTANAL, Sigma-Aldrich), at 4 µg/l concentration, in 200 ml of borehole water. Fifteen neonates (<24 h) per replicate and three replicates per treatment were considered. Guts of treated organisms aged 3, 4, 5 and 6 days were dissected. Since carbaryl was prepared in ethanol, organisms were also exposed to EtOH 0.005% as negative control. Medium was renewed every two days, while organisms were fed daily with 0.8 mC/l of a *C. vulgaris* suspension until the 6^{th} day. Together with bacteria community of guts, borehole water and carapace communities were analysed.

Based on results obtained by preliminary experiment, guts of 5 d old daphnids were analysed to evaluate gut bacteria community composition in subsequent experiments on glyphosate and Roundup.

Effects of glyphosate/Roundup on microbiome

Fifteen neonates (24-48 hours) in 200 ml of COMBO HH water were exposed for 72 h to treatments reported above. Organisms were fed daily and water was renewed every two days. After the 5-d treatment, daphnid guts were extracted to evaluate guts bacteria community composition. To avoid contamination from carapace bacteria, before dissection, organisms were washed twice with sterilised distilled deionized water for 5 minutes and maintained in this water until gut dissection. Guts were dissected with sterilised dissection forceps under a stereo microscope; for each genotype, 15 guts per replicate per treatment were pooled into eppendorf tubes and frozen in liquid nitrogen. During dissection, the tubes were maintained in ice to avoid gut degradation. To avoid a possible contamination by carapace bacteria, EtOH 70% was used to clean the forceps and microscope slides were changed between organism dissection. After dissection, guts were frozen at -80 °C until extraction of microbial DNA.

Extraction of microbial DNA

The bacterial DNA was extracted by the QIAamp DNA Microbiome Kit (QIAGEN), modified in some steps. In this experiment, Pathogen Lysis Tube S (QIAGEN), that contain small glass beads that are suitable for bacteria lysis, were used. To obtain a sufficient amount of bacterial DNA, cells Precellys24 (Bertin Instruments) was applied, using a velocity of 6.5 m/s and changing the number of cycles and time of guts fragmentation. In this step, three methods were tried:

- 1 cycle of 5 seconds;
- 1 cycle of 10 seconds;
- 2 cycles of 5 seconds with a 30-seconds interval during which the samples were maintained at room temperature.

The highest DNA concentration was obtained by using 2 cycles of 5 seconds. Therefore, this step was used in the QIAamp DNA Microbiome Kit protocol for the subsequent experiments.

After extraction, bacterial DNA concentration and A260/230 was evaluated using NanoDrop 8-Sample Spectrophotometer-ND-8000 (Thermo Fischer Scientific) and Qubit Fluorometric Quantification (Thermo Fischer Scientific). The quality of DNA was evaluated by 2200 TapeStation (Agilent Technologies) using Genomic DNA Reagents (Agilent Technologies) and Genomic DNA ScreenTape (Agilent Technologies).

PCR on microbial DNA

To evaluate the bacteria community composition, a PCR was performed by using long primers (IDT- Integrated DNA Technologies). To distinguish the gut samples, different primes were used,

according to the method described by Caporaso et al. (2012). To obtain the primers working concentration, water nuclease free was added at master stock, following the volume of water reported by manufacturer. 5® High-Fidelity DNA Polymerase (New England BioLabs) was used to amplify the bacteria DNA in the regions V1 and V4 of 16S rRNA, using the following instructions.

Table 3.2.1: Components used to perform PCR on microbial DNA.

Components	**25 µl RXN**
5X Q5 Reaction Buffer	5 µl
10 mM dNTPs	0.5 µl
10 µM Forward Primer	1.25 µl
10 µM Reverse Primer	1.25 µl
Template DNA	Variable
High-Fidelity DNA Polymerase	0.25 µl
Nuclease-Free Water	Variable

The thermal profile consist of 30 seconds at 98 °C for the initial denaturation, followed by 25 cycles of PCR at 98°C for 10 seconds, 64 °C (for region V1 16S rRNA, while the annealing temperature for region V4 16S rRNA was at 66 °C) for 30 seconds, 72 °C for 30 seconds and a final extension of 2 minutes at 72 °C.

After PCR, amplified DNA concentration and A260/230 was evaluated according to the method described above. Agarose gel electrophoresis (1% in TAE Buffer) was performed to investigate the presence bacteria DNA and evaluate the DNA size. To evaluate the bacterial DNA amplification 5 µl of PCR product were added into agarose gel. Six µl of Midori Green (Nippon Genetics Europe GmbH) was used as fluorescence detector. To control 1 µl of Nuclease-Free water, 1 µl of DYE and 1 µl of DNA Ladder were used. 120 mV were used and after 45 minutes the presence of bands was evaluated. After, an equal amount of amplicon from each sample (240 ng) was transferred into a sterile tube and used for the subsequent steps, in which each amplicon pools was cleaned using MoBio UltraClean PCR Clean-Up Kit (Mo Bio Laboratories, Inc) and prepared for Mi-Seq run (Illumina).

Genotoxicity assessment: Comet assay

For genotixity evaluation 10 neonates (24-48 hours) in 150 ml of COMBO HH water were exposed for 72 h to treatments reported above. Genotoxicity assessment on juvenile *D. magna* individuals was performed via comet assay according to Pellegri et al (2014) with minor modifications. Briefly, *Daphnia* haemolymph obtained through heart puncturing adding 2 µl of Phosphate-buffered saline (PBS) for each daphnid and isolated into microtubes and centrifuged (45 × g, 5 min). The supernatant was discharged and the pellet was gently resuspended in 25 µl of Low Melting Point Agarose (LMA, 0.7%) and then transferred onto degreased microscope slides previously dipped in Normal Melting Point Agarose (NMA, 1%). After solidification at 4 °C for 15 min, a second layer of 25 µl of LMA was added. Slides were placed in a lysis solution overnight at 4 °C (2.5 M NaCl, 100 mM Na2 EDTA, 10 mM Tris–HCl, 1% Triton X-100 and 10% DMSO, pH 10) to digest both the plasma and the nuclear membranes. DNA unwinding and electrophoresis (0.78 V/cm, 300 mA) were performed in an alkaline buffer (1 mM Na2EDTA, 300 mM NaOH, pH > 13). After electrophoresis, each slide was neutralized with 2 ml of neutralization buffer (0.4 M Tris–HCl, pH 7.5), fixed in ethanol at −20 °C and left to dry for at least 4h prior to staining. DNA was stained overnight with 75 µl SYBR™ Gold Nucleic Acid Gel Stain (Thermo Scientific, Waltham, MA, US) before the examination at 200× magnification under a Leica DMLS fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) (excitation filter BP 515–560 nm, barrier filter LP 580 nm), using an automatic image analysis system (Comet Assay III–Perceptive Instruments Ltd, Stone, Staffordshire, UK). DNA damage is evaluated through the percentage of Tail Intensity (TI%), that is calculated considering the ratio between the total intensity of the tail and total intensity of the comet (head and tail together) and is directly proportional to DNA damage.

Transcriptomic assessment

For RNA extraction 10 neonates (24-48 hours) in 150 ml of COMBO HH water were exposed for 72 h to treatments reported above. Medium was changed every two days and daphnid was fed every day with 0.8 mg C/l of a *C. vulgaris* suspension. In this treatment, organisms were reared at 20 °C and 14:10 L:D photoperiod conditions. All experimental treatments were replicated 3 times using individuals from all four *D. magna* genotypes (6 treatments x 4 genotypes x 3 replicates = 72 samples). After 72 h of exposure, organisms were checked and daphnids with ovaries in brood chamber were discarded. Females with no ovaries in brood chamber were collected in tubes, frozen in liquid nitrogen and kept at -80 °C until RNA extraction.

RNA-seq

Total RNA was extracted using the RNA advance kit (Beckman Coulter) following the manufacturer instructions. Extracted RNA was quantified using a Nanodrop-8000 Spectrophotometer (ThermoFisher ND-8000-GL) and integrity assessed on the Agilent TapeStation 2200 (Agilent G2964AA) with High Sensitivity RNA screentapes (Agilent 5067- 5579).

Total-RNA samples (1µg) was poly(A) selected using the NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs E7490L) and then converted in mRNA libraries using a NEBNext Ultra Directional RNA Library Prep Kit (New England Biolab E7420L) and NEBnext Multiplex Oligos for Illumina Dual Index Primers (New England Biolabs E7600S), following the manufacturer guidelines. Handling was performed with the Biomek FxP work station (Beckman Coulter A31842). Constructed libraries were assessed for quality using the TapeStation 2200 (Agilent G2964AA) with High Sensitivity D1000 DNA ScreenTape (Agilent 5067-5584). Multiplexed libraries (100-bp paired end) were sequenced on a HiSeq4000 at the University of Birmingham Environmental Omics Sequencing Facility (5M reads per samples).

Transcriptomic analysis

Whole transcriptome (RNA-seq) studies can be classified in differential gene expression studies, where the overall transcriptional output of each gene is compared between conditions and differential transcript usage studies, where the composition of a gene's isoform abundance spectrum is compared between conditions. Consequently, in the transcriptome experiment, comparison between control and treatments for transcript level and gene level were reported.

The raw fastq files of the transcriptomics data consisted of 100 pairs of read files spanning 50 samples sequenced in two separate runs of a HiSeq Illumina system. First, the quality of the reads was assessed using `fastqc` (v0.11.5) followed by `multiqc` (v1.5) for an overview of the individual reports. The reads were then trimmed accordingly using Trimmomatic 0.36 with the following parameters: cutting out Illumina adapter sequences with 2 seed mismatches, a palindrome clip threshold of 30 and a simple clip threshold of 10; a headcrop of 5 bps, removal of bases from both ends of a read with a quality score lower than 30 and finally retaining trimmed reads with a minimum length of 50 bp. Corresponding sample reads from their two separate pools were then merged into single files, after which Salmon 0.9.1, an alignment-independent RNA-Seq quantification tool, was run with the specification of the `seqBias` and `gcBias` flags to correct for both sequence-specific and fragment-level GC biases. The `--incompatPrior` parameter was additionally set to 0.0 to discard any incompatibly mapped fragments and the library type was set to

be automatically inferred by Salmon using `-l A` (Patro et al., 2017). The final count matrix was then created from the Salmon output file and consisted of 100,501 transcripts across 50 samples. Differential transcript expression was assessed from the count matrix using the DESeq2 1.20.0 package in R in a pairwise manner, where the contrast argument of the `results` function specified each of the 6 pairwise comparisons of interest:

- Control vs. Glyphosate/Roundup without any antibiotic exposure
- Control vs. Glyphosate/Roundup all with antibiotic exposure
- Glyphosate/Roundup with and without antibiotics

A list of differentially expressed transcripts with a log fold change above 2 or below -2 was thus deduced from each of the 6 analyses above, all from which a separate list of common transcripts was extracted, reducing the length of each list to be unique to their specific treatment. Additionally, an 8th list was created of common transcripts using the reduced comparison data that only considered the presence versus the absence of antibiotics, including that of all controlled samples. Enrichment analysis of all 8 lists was done using DAVID 6.8 (Huang et al., 2008), to assess affected pathways inflicted by each treatment condition. To do so, *Daphnia* transcript IDs were first converted to *Drosophila* FlyBase gene IDs using the homolog tag information in the attributes section (9th column) of the provided gff3 reference file. For data exploration and further analyses, the count matrix was first transformed by DESeq's variance stabilizing transformation, which also normalizes by library size (Love et al., 2014), followed by the creation of PCA plots and sample correlation matrices, once for the whole transcriptome and once using only the significantly differentially expressed transcripts, i.e. | logFC | > 2. PCA of the differentially expressed transcripts was performed in a pairwise manner for the 6 comparisons of interest mentioned above, in addition to controlled samples that compare the effects of antibiotics.

MODA (MOdule Differential Analysis) is an R package for gene co-expression network differential analysis used to identify specific modules associated with relevant biological processes (Li et al., 2016). MODA 1.2.0 was run as a BlueBEAR application to return the modules of weighted co-expressed genes for each of the 4 available genotypes, each of the 6 treatment conditions as well as a list of modules for the background count matrix which consisted of all experimental conditions. Here, each genotype or treatment specific matrix was constructed by excluding the samples of interest from the background matrix to give a total of 11 matrices, with rows and columns specifying samples and genes respectively. For each matrix, the `WeightedModulePartitionHierarchical` function was run to construct gene co-expression networks and to detect modules via hierarchical clustering of the genes. This was followed by

MODA's differential analyses of the co-expression networks using the `CompareAllNets` function twice, once including the genotype specific results of the earlier function and once for the treatment specific results. In each case, this returned lists of which of the reference modules were conserved and which were specific to a certain genotype or treatment. These modules of interest were then used for DAVID enrichment after converting the gene IDs to its *Drosophila* homologs. Usage of MODA functions is provided in the supplementary information.

To adhere to the maximum number of genes accepted by MODA since it utilizes the `hclust` function, the transcripts were first converted to genes by reducing the transcript IDs to be gene specific and then, for each sample, summing up the counts of all transcripts originating from the same gene. This reduced the number of columns in the MODA input matrix from 100,501 transcripts to only 24,682 genes. Moreover, since MODA and any subsequent DAVID enrichment and biological interpretation was performed on the gene level rather than the transcript level, all previous pairwise DESeq and data exploration analyses were repeated using the gene level count matrix in order to assess the differences in results when analyzing gene expression data using gene versus transcript level counts.

Results

Life history traits

The exposure of daphnids to antibiotics caused detrimental effect on survival of organisms all clones in all treatments (Fig. 3.2.2). There were no significant differences among genotypes and treatments in survival rates after exposure without antibiotics (Fig. 3.2.3). Mortality rates per genotype × treatment were calculated with a survival model fit: the day of death and the mortality event itself were treated as dependent variables, whereas genotype and treatment were treated as fixed effects. When the antibiotic treatments were included in the analysis, the treatment factor was statistically significant, with a negative effect on survival probability associated to all antibiotics treatments. When the antibiotics treatments were not considered, neither treatment nor genotype had a significant effect on mortality. Due to high mortality, antibiotic treatments were excluded in further statistical analyses.

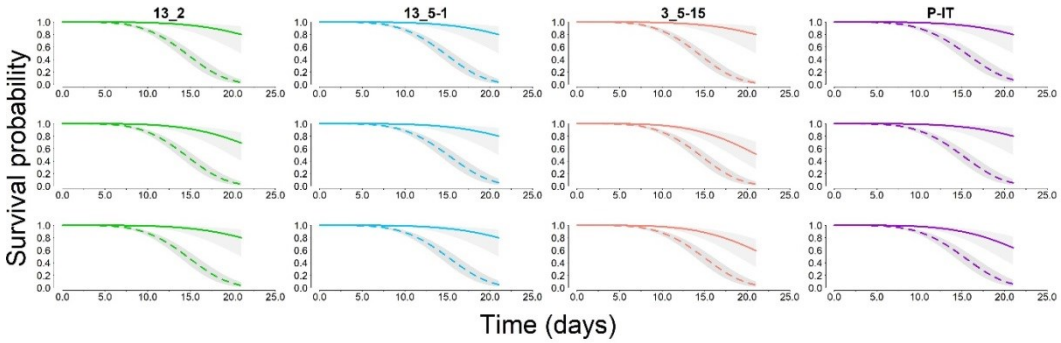


Figure 3.2.2: Survival of daphnids clones 13_2, 13_5-1, 3_5-15 and P-IT in Control (up), glyphosate 1 mg/l (middle) and Roundup 1 mg/l (down) with antibiotics (continuous line) and without antibiotics (broken line).

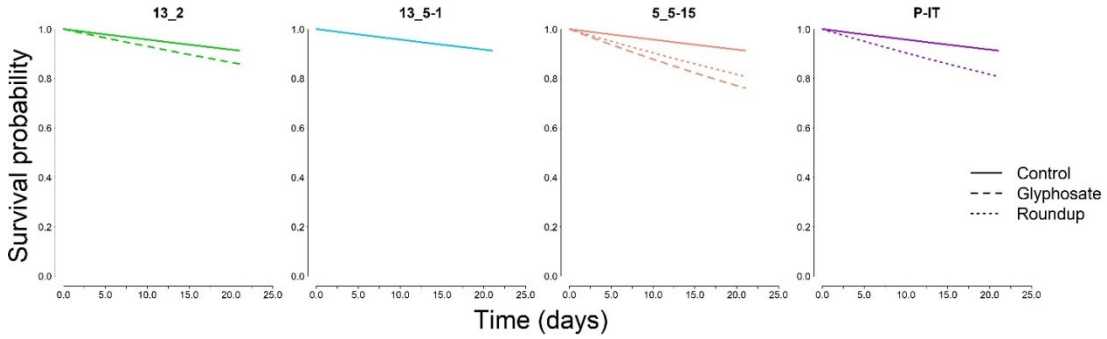


Figure 3.2.3: Survival of daphnids clones 13_2, 13_5-1, 3_5-15 and P-IT in Control, glyphosate 1 mg/l and Roundup 1 mg/l.

The effects of glyphosate and Roundup on age at maturity, size at maturity, fecundity and failed development were reported in Fig. 3.2.4. The clone 13_2 delayed the age at maturity after exposure to glyphosate both and Roundup; the size at maturity, fecundity and development were negatively affected by Roundup (Figg. 3.2.6, 3.2.7 and 3.2.8). In clones 3_5-15 and P-IT, age at maturity and size were not affected by glyphosate and by Roundup, while fecundity failed development of both clones were negatively affected by glyphosate and Roundup (Figg. 3.2.6, 3.2.7 and 3.2.8). In clone 13_5-1, age at maturity, size, fecundity and failed development were not significantly affected by exposure to glyphosate and Roundup in comparison to control (Figg. 3.2.6, 3.2.7 and 3.2.8). 13_5-1 did never show any negative effect, while 13_2 turned out to be the most sensitive strain to the negative effects of glyphosate/Roundup. P-IT and 3_5-15 showed intermediate behaviours, experiencing negative effects only for some life-history traits. About the Failed Development an increase of died neonates and aborted eggs were observed in clones 3_5-15 and P-IT after exposure both to glyphosate and Roundup. Consequently, after exposure for 15 days to glyphosate and

Roundup for 72 h clones showed differences in life history traits: the clone 13_2 was the most susceptibility while the clone 13_5-1 was the most resistant.

PCA clearly discriminates among clones according to their life history traits and treatments. PCA2 splits the clone from the other three clones due to size at maturity and failed development; both PCA1 and PCA2 split the clone 3_5-15 from the other three clones in the Control (Fig. 3.2.5).

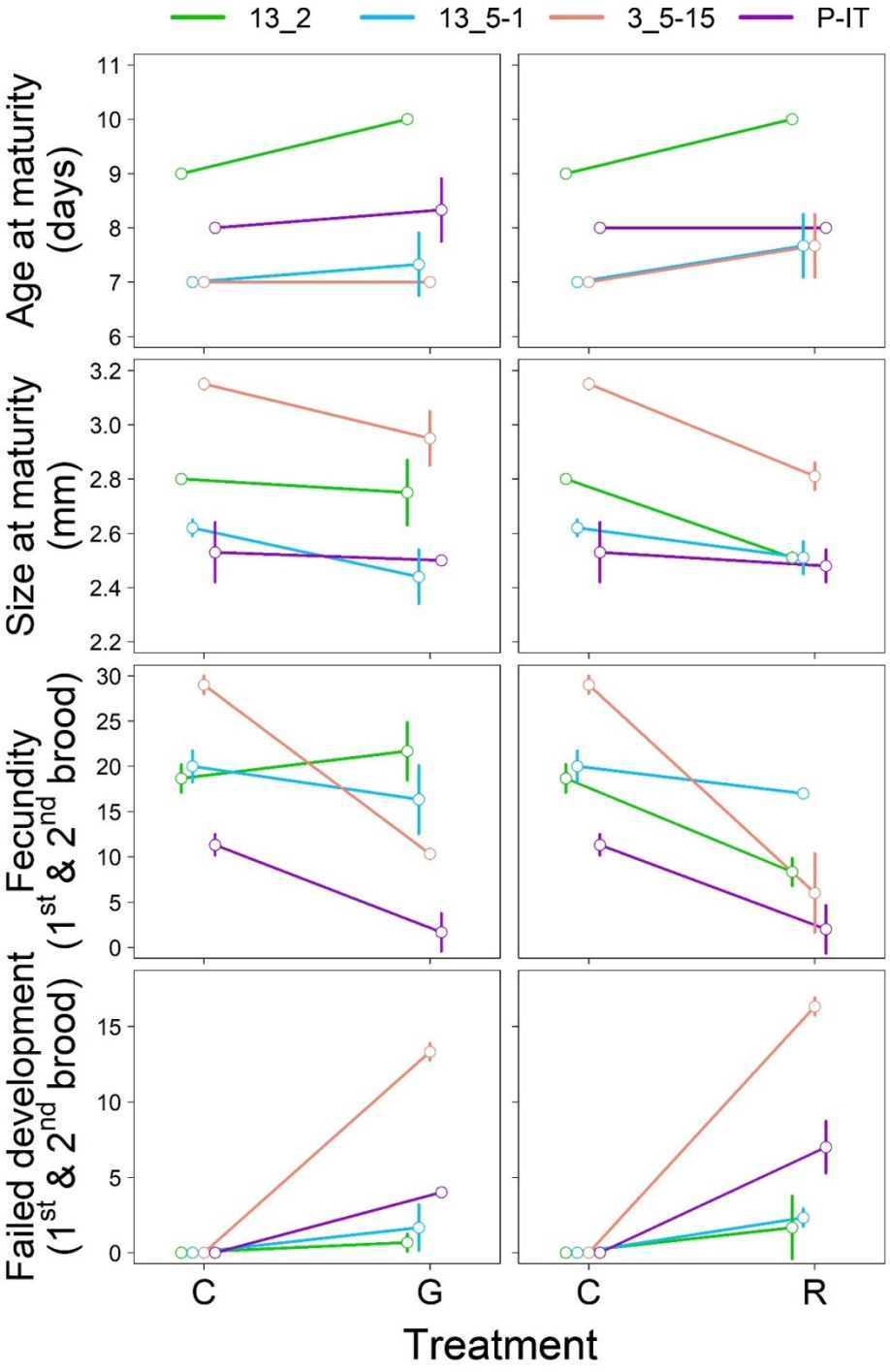


Figure 3.2.4: Age and size at maturity, fecundity and failed development after exposure of daphnids 13_2, 13_5-1, 3_5-15 and P-IT to glyphosate 1 mg/l (left) and Roundup 1 mg/l (right) for 72 h.

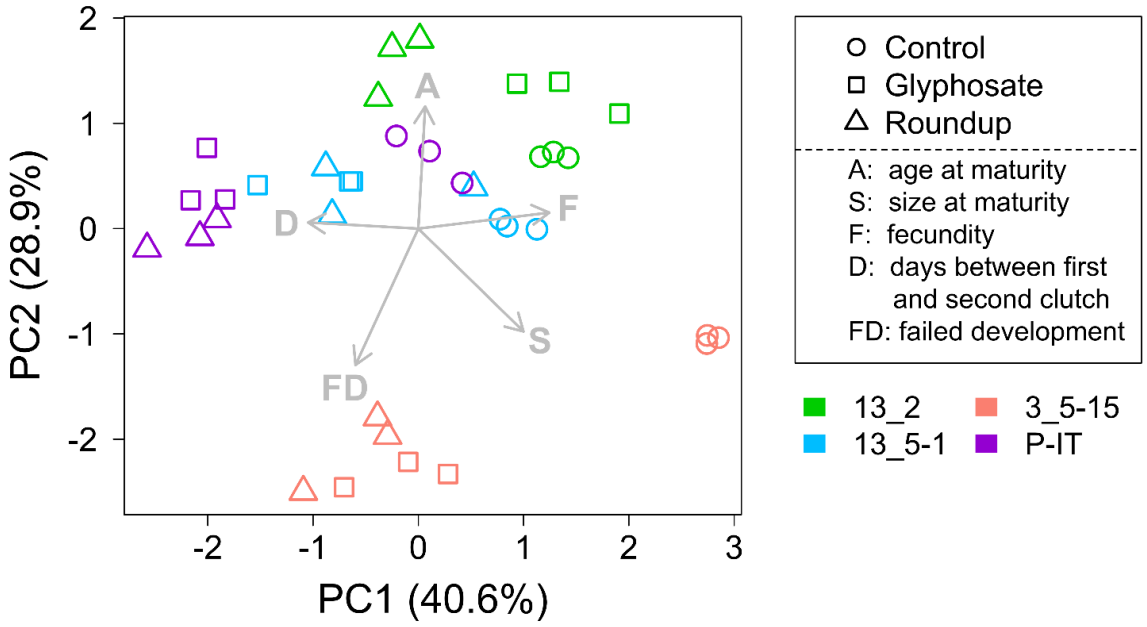


Figure 3.2.5: Principal component analysis (PCA) on scaled life-history traits for age at maturity(A), size at maturity (S), fecundity (F), number of days between the first and second clutches (D) failed development (FD) after exposure of daphnids 13_2, 13_5-1, 3_5-15 and P-IT to glyphosate 1 mg/l(left) and Roundup 1 mg/L (right) for 72 h.

According to MANOVA (Table 3.2.2) all explanatory variables were statistically significant. The interaction term (genotype × treatment) was significant, meaning that the effect of the treatment was different among genotypes.

Table 3.2.2: The effects of genotype (Gen), treatment (Exp) and their interaction (Gen:Exp) on PC scores investigated through MANOVA analysis are reported. All the considered variables were significant ($P < 0.001$).

	Df	**Pillai**	**approx. F**	**num Df**	**den Df**	**Pr(>F)**	
Gen	3	2.7782	55.122	15	66	< 2.2e-16	***
Exp	2	1.2902	7.635	10	42	8.648e-07	***
gen:exp	6	2.6803	4.622	30	120	8.905e-10	***
Residuals	24						

All explanatory variables were statistically significant. Both glyphosate and Roundup reduced *Daphnia* clones' size, but the effect of the latter was stronger. However, the interaction term (genotype × treatment) was significant, suggesting that the effect of treatment was different among genotypes (Fig. 3.2.6). In particular: i) P-IT and 13_5-1 sizes were not affected nor by glyphosate

neither by Roundup, *ii*) 13_2 size was negatively affected just by Roundup and *iii*) both glyphosate and Roundup had a significantly negative effect on 3_5-15 size.

In *size_Tukey.txt* are reported results of the Tukey Test for all the pairwise comparisons.

Table 3.2.3: The effects of genotype (Gen), treatment (Exp) and their interaction (Gen:Exp) on size at maturity investigated through ANOVA analysis are reported. All the considered variables were significant (P < 0.001).

	Sum Sq	**Df**	**F value**	**Pr(>F)**	
Gen	1.23997	3	88.3953	4.134e-13	***
Exp	0.24429	2	26.1223	9.463e-07	***
gen:exp	0.14070	6	5.0151	0.001852	**
Residuals	0.11222	24			

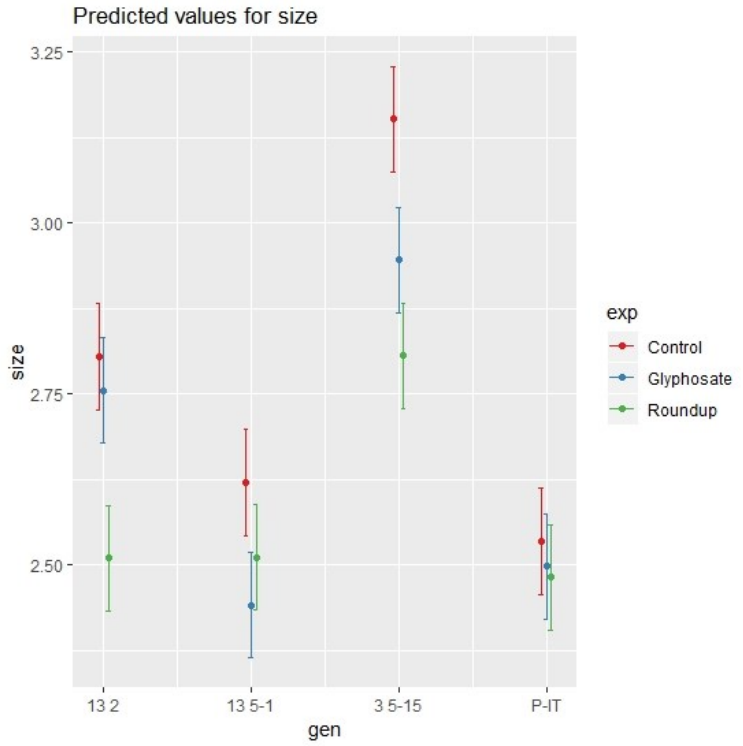


Figure 3.2.6: Predicted values for size of daphnids clones 13_2, 13_5-1, 3_5-15 and P-IT in Control (red), glyphosate 1 mg/l (blue) and Roundup 1 mg/l (green).

Both the effects of genotype and treatment were statistically significant (P <0.001), while the interaction term (genotype × treatment) was only marginally significant (P = 0.057). Both glyphosate and Roundup had a similar positive effect on age, making *Daphnia* genotypes reaching maturity later. The effect of treatment was different among genotypes (Fig. 3.2.7). In particular: *i*)

P-IT, 13_5-1 and 3_5-15 ages were not affected nor by glyphosate neither by Round-up, *ii*) 13_2 age was positively affected by both glyphosate and Roundup.

In *maturity_Tukey.txt* are reported results of the Tukey Test for all the pairwise comparisons.

Table 3.2.4: The effects of genotype (Gen), treatment (Exp) and their interaction (Gen:Exp) on age at maturity investigated through ANOVA analysis are reported. All the considered variables were significant ($P < 0.001$).

	Sum Sq	**Df**	**F value**	**Pr(>F)**	
Gen	34.306	3	102.9167	7.716e-14	***
Exp	2.167	2	9.7500	0.0007955	***
gen:exp	1.611	6	2.4167	0.0571057	.
Residuals	2.667	24			

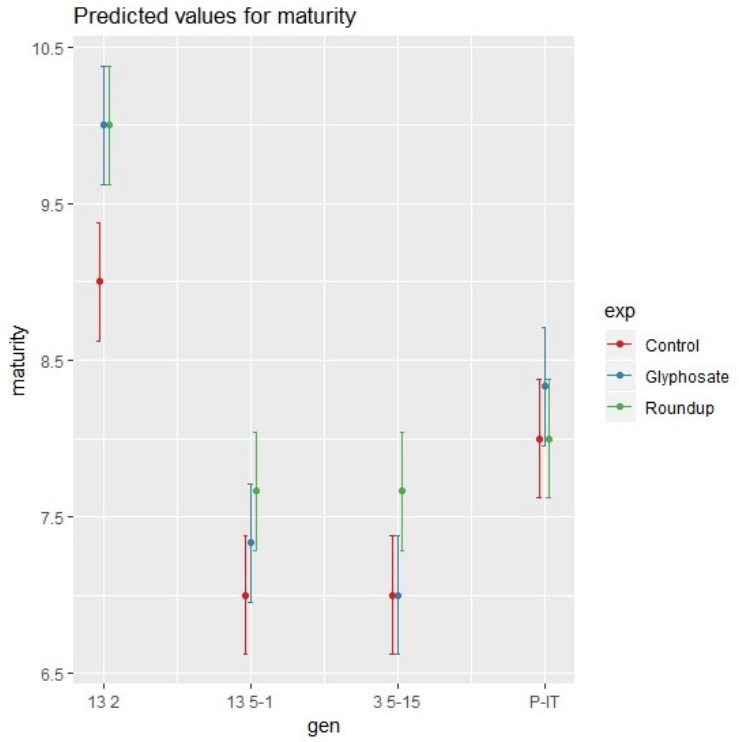


Figure 3.2.7: Predicted values for age at maturity of daphnids clones 13_2, 13_5-1, 3_5-15 and P-IT in Control (red), glyphosate 1 mg/l (blue) and Roundup 1 mg/l (green).

All explanatory variables were statistically significant. Both glyphosate and Roundup reduced *Daphnia* clones' fecundity, but the effect of the latter was stronger. However, the interaction term (genotype × treatment) was significant, suggesting that the effect of treatment was different among genotypes (Fig. 3.2.8). In particular: *i*) 13_5-1 fecundity was not affected nor by glyphosate neither

by Roundup, *ii*) 13_2 fecundity was negatively affected by just Roundup, and *iii*) P-IT and 3_5-15 fecundities were negatively affected by both glyphosate and Roundup.

In *fecundity_Tukey.txt* are reported results of the Tukey Test for all the pairwise comparisons.

Table 3.2.5: The effects of genotype (Gen), treatment (Exp) and their interaction (Gen:Exp) on fecundity investigated through ANOVA analysis are reported. All the considered variables were significant (P < 0.001).

	Sum Sq	**Df**	**F value**	**Pr(>F)**	
Gen	**904.97**	3	**55.407**	**6.164e-11**	***
Exp	**801.06**	2	**73.566**	**5.788e-11**	***
gen:exp	**592.28**	6	**18.131**	**7.679e-08**	***
Residuals	**130.67**	24			

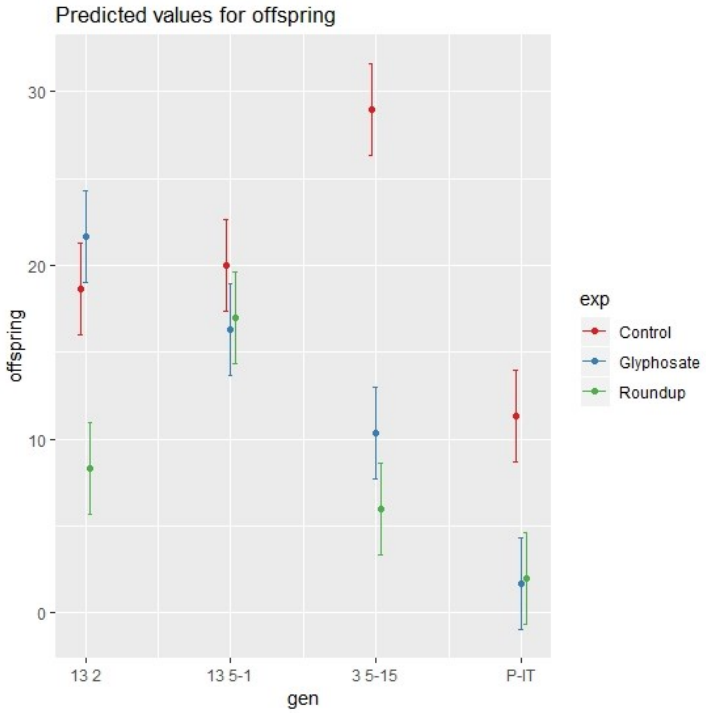


Figure 3.2.8: Predicted values for offspring of daphnids clones 13_2, 13_5-1, 3_5-15 and P-IT in Control (red), glyphosate 1 mg/l (blue) and Roundup 1 mg/l (green).

Gut microbiome analysis: preliminary results

In a preliminary test, performed on clone 3_5-15, the bacterial community composition at different ages was analysed. Data showed that the community structure, in term of bacterial families, was quite similar in daphnids at different ages (Fig. 3.2.9a). Carbaryl (0.4 µg/l) did not seem to heavily influence microbiome composition (Fig 3.2.9b), while glyphosate (1 mg/l) seems to influence the percentage of bacterial family Burkholderiaceae (Fig 3.2.9c).

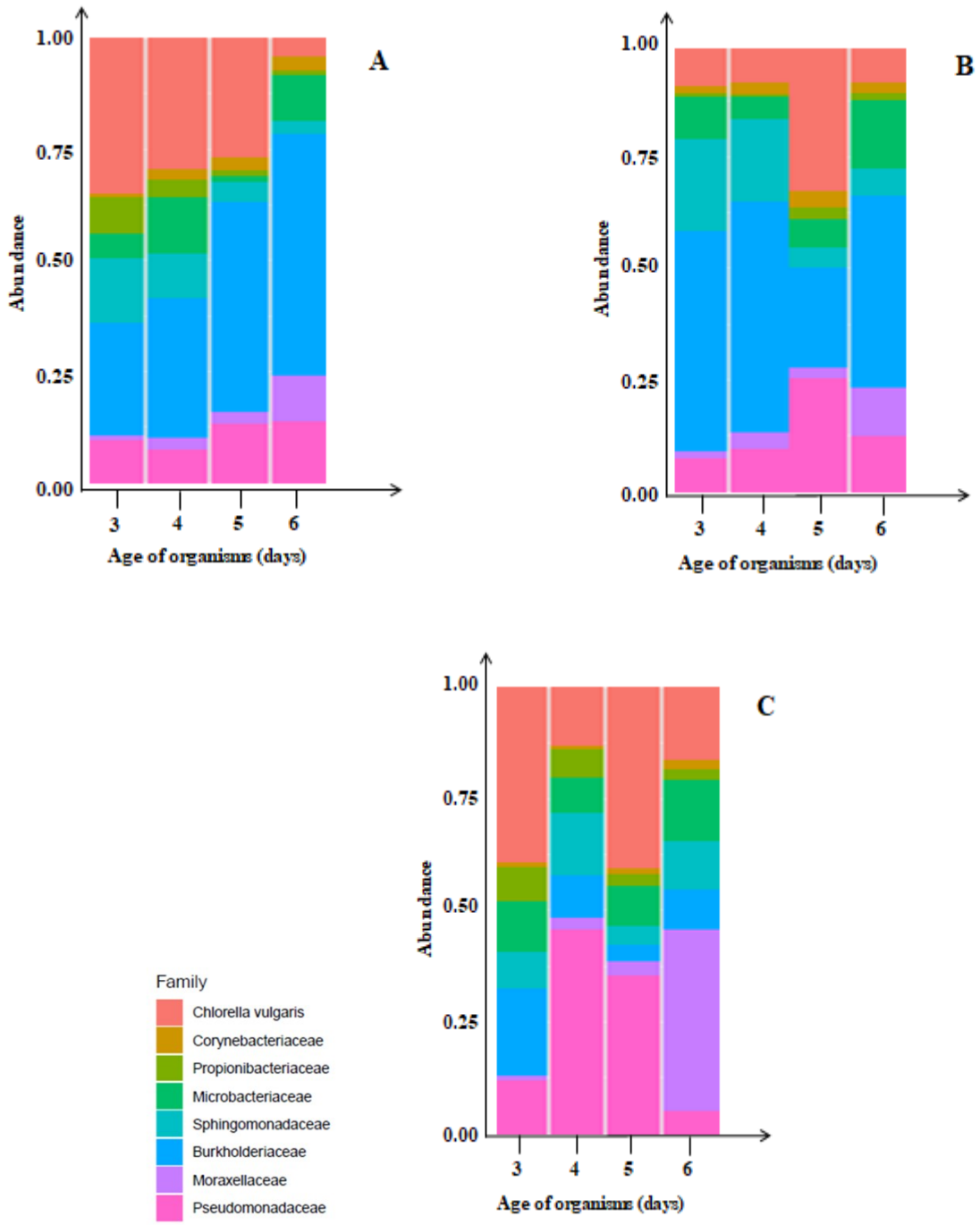


Figure 3.2.9: Bacteria community composition in gut of clone 3_5-15 in control (A) and organisms treated with carbaryl 0.4 µg/l (B) and glyphosate 1 mg/l (C). Guts were extracted from daphnids aged 3, 4, 5 and 6.

In borehole water, the most abundant bacteria were Microbacteriaceae (Fig 3.2.10). This family was instead scarcely present in carapace samples and microbiome extracted from guts of organisms maintained in borehole and artificial (COMBO HH) water (Fig 3.2.10).

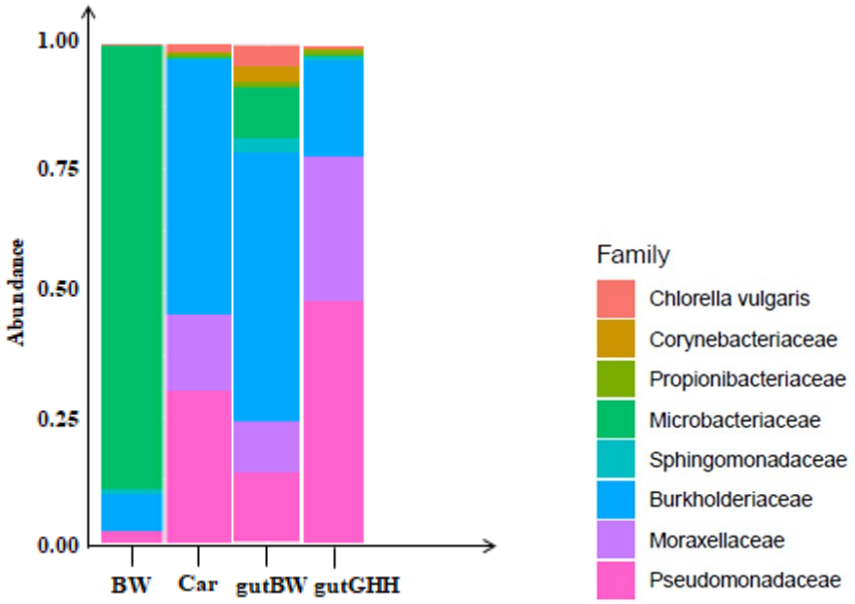


Figure 3.2.10: Bacteria community composition in borehole water (BW), carapace (Car) samples and in guts extracted from organisms maintained in borehole (gutBW) and COMBO HH (gutHH) water.

In Fig. 3.2.11 the abundance of bacterial families in controls and organisms treated with glyphosate 1 mg/l and Roundup 1 mg/l is shown. In clone 13_2 glyphosate and Roundup seems to have a similar and limited impact, while in clone 13_5-1 they seem to have different influence on microbiome composition (Fig. 3.2.11). In Fig. 3.2.12 the abundance of bacterial families in different clones is shown.

Data analyses are still in progress and have to be completed to acquire a clear picture of the effects of glyphosate and Roundup on microbiome of different clones.

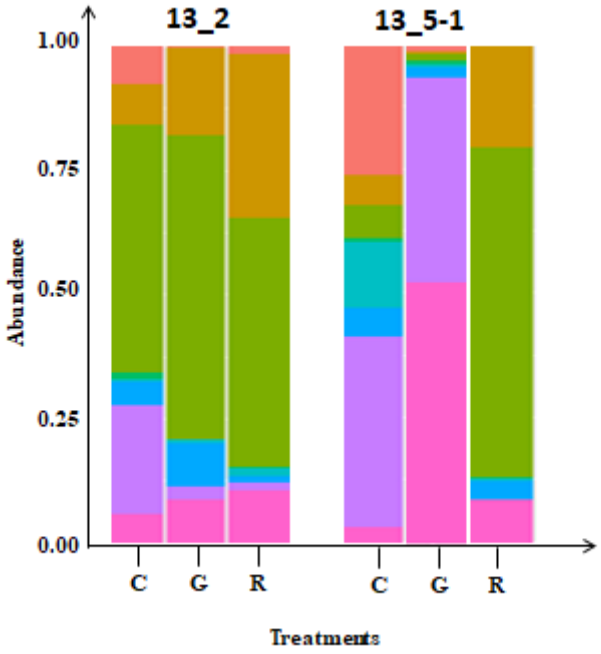


Figure 3.2.11: Bacteria community composition in clones 13_2 and 13_5-1 after exposure for 72 h to glyphosate (G) and Roundup (R). C=Control.

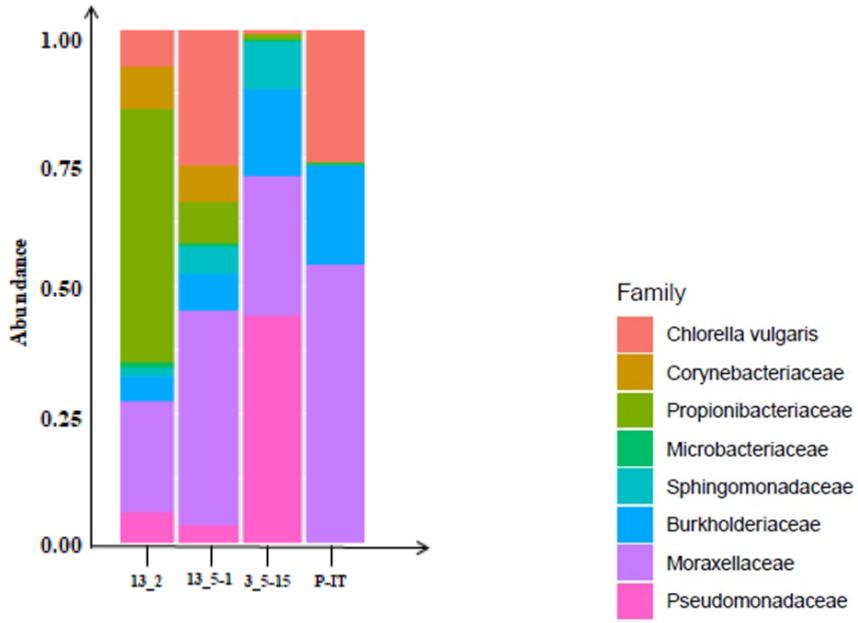


Figure 3.2.12: Bacteria community composition in clones 13_2, 13_5-1, 3_5-15 and P.IT in COMBO HH water. Guts were extracted at day 5.

Genotoxicity assessment: Comet assay

In Fig. 3.2.13 the level of genotoxic damage of selected clones after exposure to glyphosate 1 mg/l and Roundup 1 mg/l and control was reported. The genotoxic damage is calculated using Tail Intensity percentage (TI%); the TI% value is directly proportional to DNA damage. In clone 13_2 no significantly difference was observed after exposure to Roundup. Clone 13_5-1 showed Tail Intensity% (TI) values similar to control both after exposure to glyphosate and Roundup. Significant differences was observed in clone 3_5-15 and P-IT after exposure to both glyphosate and Roundup.

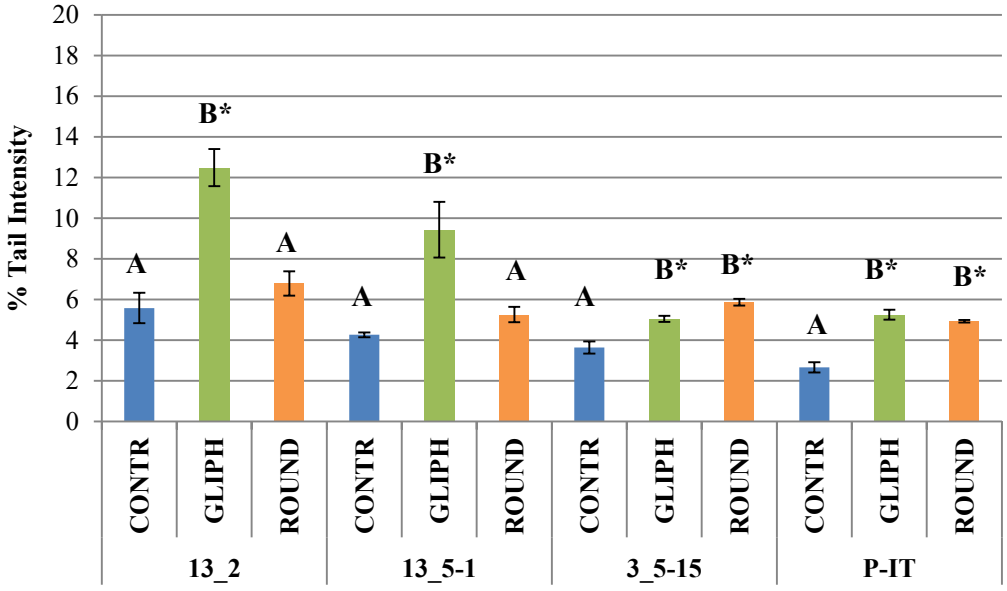


Figure 3.2.13: Tail Intensity percentage in Control, glyphosate 1 mg/l and Roundup 1 mg/l in daphnids clones 13_2, 13_5-1, 3_5-15 and P-IT.

Transcriptomic assessment

The transcriptomics analyses aimed at discerning the microbiome response from the animal response to ecologically relevant concentrations of common pesticides, where the data consisted of 50 samples of 4 genotypes and 6 treatment conditions.

In trascriptomics analysis 96 h old organisms were used and mortality due to antibiotics was negligible. For this reason, all clones, 3 from Lake Ring (from two different lake phases) and one from Italian laboratory (Chapter 3), exposed to 6 treatment conditions (Control, Roundup, glyphosate, Control with antibiotics, Roundup with antibiotics and glyphosate with antibiotics) were analysed.

The whole transcriptome sample correlation matrix shows that the samples separate by the four genotypes rather than by treatments (Fig. 3.2.14a). Reconstruction of the whole transcriptome correlation matrix shows that the influence of the genotypes at segregating the samples are diminished as the resolution of the data was reduced (Fig 3.2.14b). The correlation matrix using only | logFC | > 2 transcripts on the other hand, clearly separates between samples treated and untreated with antibiotics for only three of the four genotypes (Fig. 3.2.15a), while the 13_5-1 genotype seems to be the exception, where all of its samples, whether exposed to antibiotics or not, groups with the untreated genotypes. On average, four transcripts were aggregated per gene, a process by which the impact of alternative splicing was discounted. However, when including only the differentially expressed genes (Fig. 3.2.15b), the main segregation pattern is retained as all of the antibiotics exposed samples separates from the unexposed samples, except for most of the 13_5-1 genotype replicates.

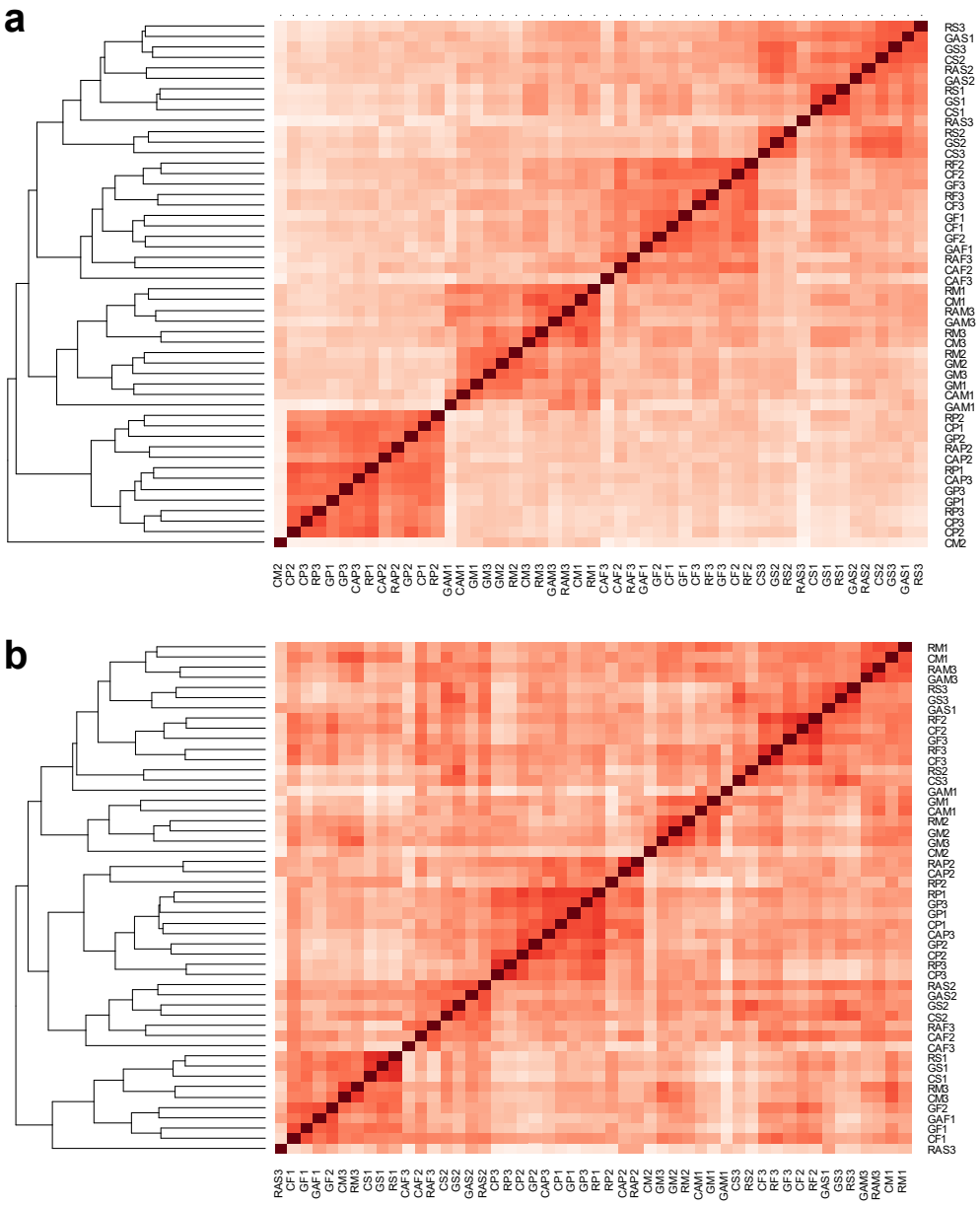


Figure 3.2.14: Whole transcriptome sample correlation matrix. Sample correlation is plotted on the transcript level (a) and on the gene level (b). Labels describe *Daphnia* clones (P= P-IT ; M= 13_2; F= 13_5-1; S= 3_5-15); treatments (C= Controls; G= glyphosate; R= Roundup; A= Antibiotics) and replicate number. Darker shades represent higher similarity.

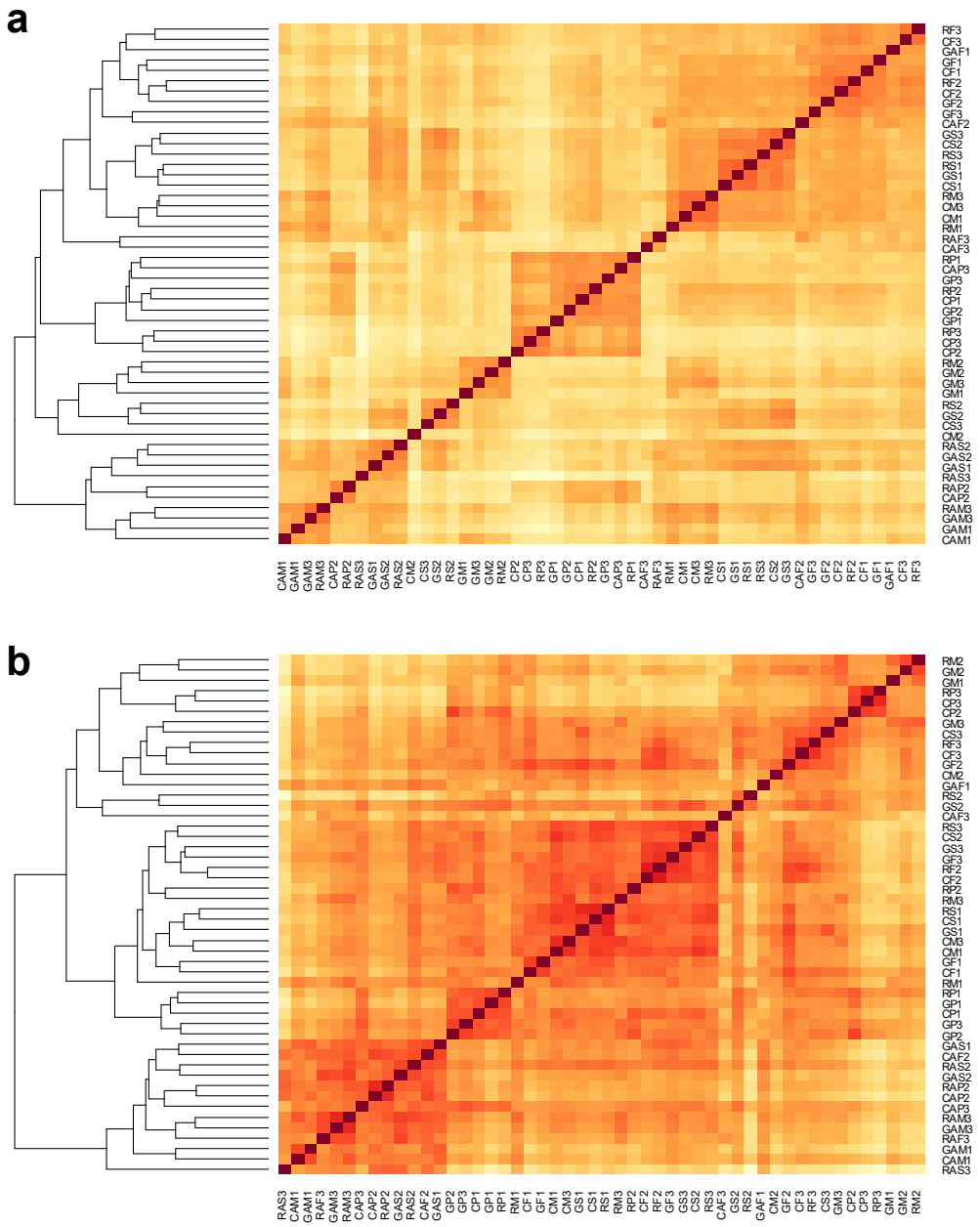


Figure 3.2.15: **Differential expression sample correlation matrix**. Sample correlation is plotted on the transcript level (a) and on the gene level (b) for only the differentially expressed transcripts/genes, i.e. | logFC | > 2. Labels describe *Daphnia* clones (P= P-IT ; M= 13_2; F=13_5-1; S=3_5-15); treatments (C= Controls; G= glyphosate; R= Roundup; A= Antibiotics) and replicate number. Darker shades represent higher similarity.

Principle Component Analysis (PCA), of the whole transcriptome clearly divides the samples according to their genotypes, where PC1 also splits the clone P-IT from the other three (Fig 3.2.16a). The whole transcriptome PCA plot on the other hand, which previously showed a marked separation by genotype, has lost almost all segregation with only the clone P-IT exhibiting a partial separation from the other three genotypes (Fig 3.2.16b).

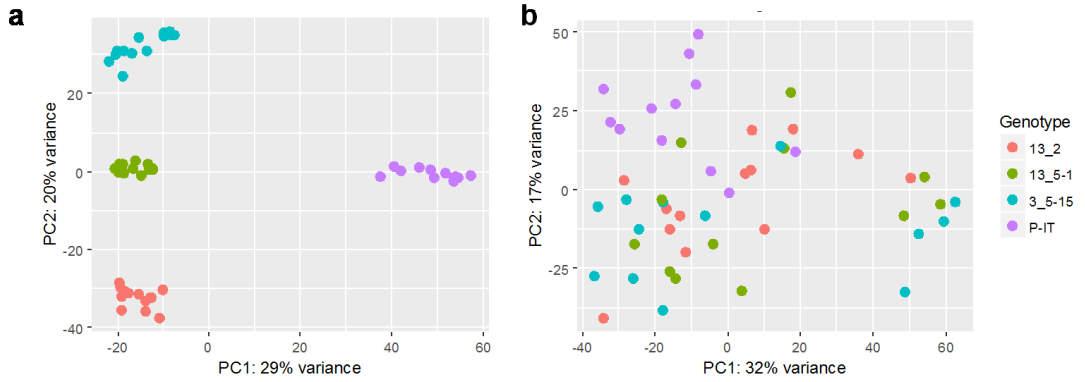


Figure 3.2.16: Whole transcriptome PCA. PC analysis is illustrated for the whole transcriptome *Daphnia* data on the transcript level (a) and on the gene level (b).

Comparisons of controls against either glyphosate or roundup samples do not show any separation according to the different conditions for any of the genotypes (Fig. 3.2.17, left). PCA plots comparing significantly up or down regulated transcripts in the presence of antibiotics for controlled samples against either glyphosate or roundup exposures do not separate the samples by treatment either (Fig. 3.2.18, left). There is, however, a noticeable lack of sample replicates due to the harsh effect of the antibiotics that either directly affected the *Daphnia* individuals or left them vulnerable by suppressing their microbiome. RNA extracted from surviving *Daphnia* was also poor in quality such that library construction was not feasible, so data points in the plots remain scarce. PCA plots of the differentially expressed genes in the pairwise analyses shows results that could draw similar conclusions to those of the transcript level, as the comparisons of control versus glyphosate or roundup, either with or without antibiotics (Figg. 3.2.17 and 3.2.18, right), showed no separation between samples of different treatments, suggesting that the toxicants do not significantly affect the animal's gene expressions.

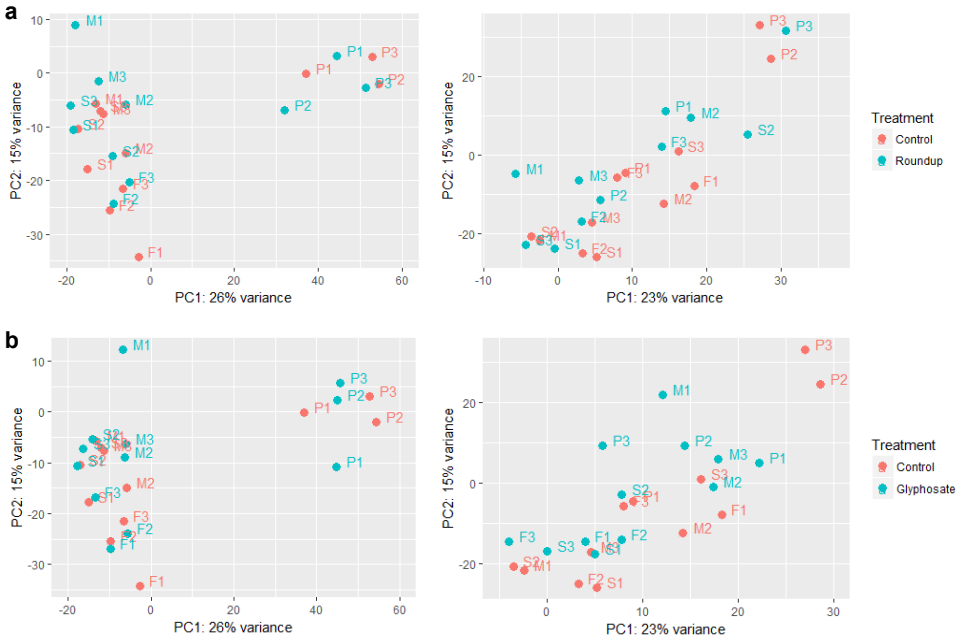


Figure 3.2.17: **Pairwise PCA analyses in the absence of antibiotics**. PCA of the | logFC | > 2 differential expression is shown on the transcript level (left plots) and gene level (right plots) between controls and either Roundup (a) or glyphosate (b) in the absence of antibiotics. Samples are labeled by genotypes (P= P-IT; M= 13_2; F= 13_5-1; S= 3_5-15) and replicate number.

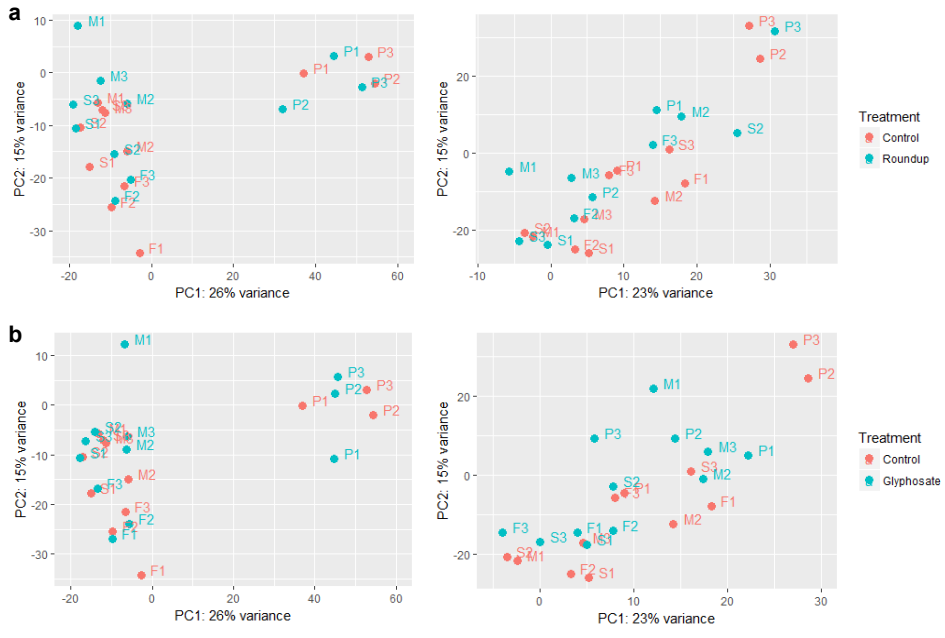


Figure 3.2.18: Pairwise PCA analyses in the presence of antibiotics. PCA of the | logFC | > 2 differential expression on the transcript level (left plots) and gene level (right plots) between controls and either Roundup (a) or glyphosate (b) are shown in the presence of antibiotics. Note that a considerable amount of replicates is unavailable due to the damaging effects of the antibiotics, and so a definitive conclusion would be dubious. Samples are labeled by genotypes (P= P-IT; M= 13_2; F= 13_5-1; S= 3_5-15) and replicate number.

Comparing the gene expression of samples exposed to roundup with and without antibiotics (Fig. 3.2.19a, left), some separation can be seen according to antibiotics exposure, again, with some replicates unavailable for the antibiotics samples. Moreover, some variations in the technical replicates of the samples are observed which can be an effect of biological variations in the samples, batch effects, or due to inconsistent antibiotic efficiencies. Similarly, comparing between glyphosate exposed samples with and without antibiotics show some separation between the samples, with the exception of the single antibiotic exposed replicate of the 13_5-1 genotype which clearly groups with all glyphosate samples that were not treated with antibiotics (Fig. 3.2.19b, left). This could point to a distinct response to the treatment but the lack of replicates would render conclusions dubious. Comparing between the presence and absence of antibiotics on the gene level, whether exposed to roundup, glyphosate or as a control (Fig. 3.2.19, right); a more clear-cut separation between samples is seen compared to the transcript level counterparts for all four genotypes, where varying biological effects of the different replicates were probably diminished by the lower resolution of the gene level data.

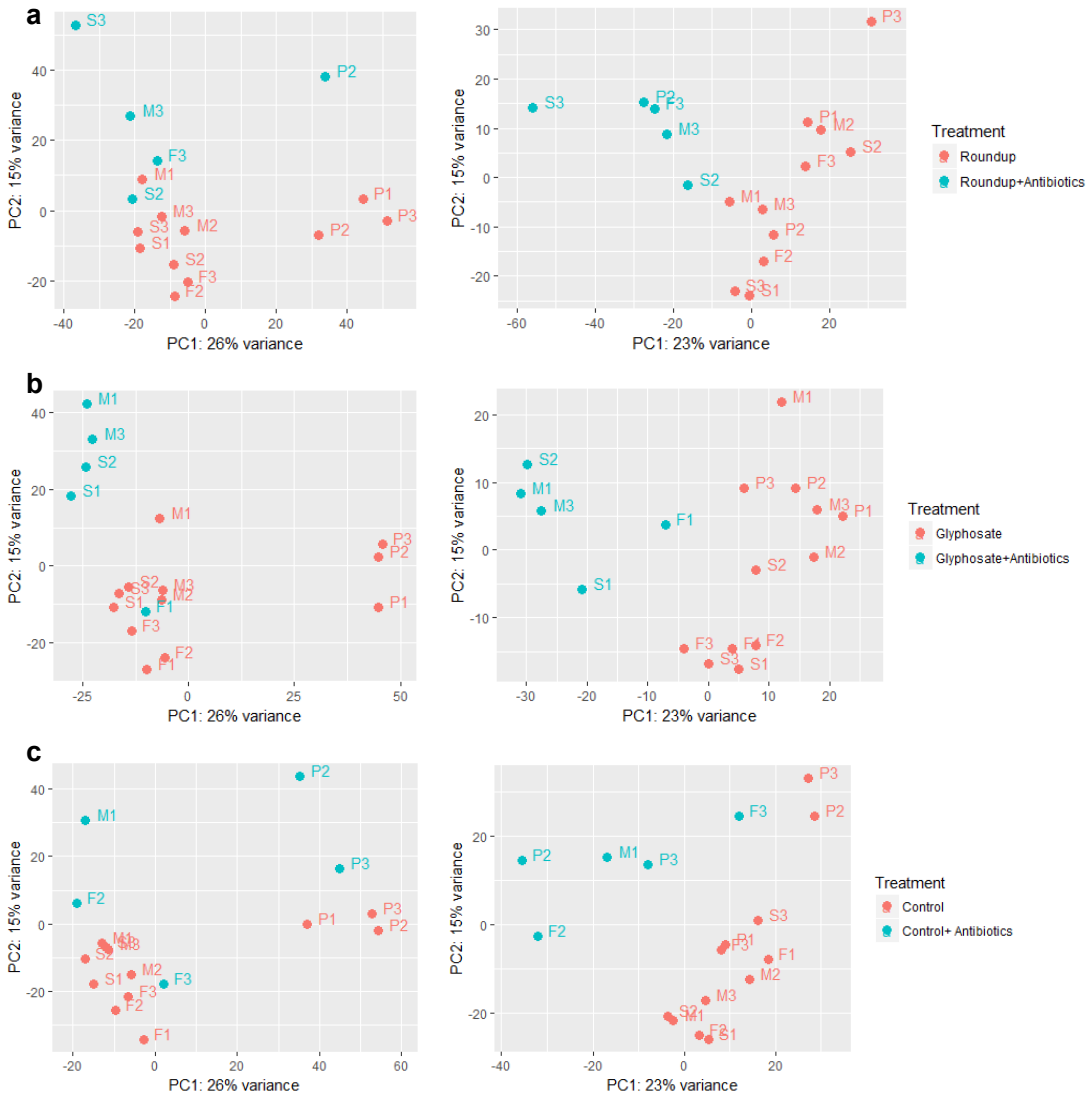


Figure 3.2.19: Pairwise PCA analyses comparing the effects of antibiotics. PCA of the | logFC | > 2 differential expression on the transcript level (left plots) and gene level (right plots) comparing the effects of antibiotics on samples exposed to roundup (a), glyphosate (b) or controls (c). Some antibiotics exposed samples are missing due to its damaging effects on the genetic material. Samples are labeled by clones (P= P-IT; M= 13_2; F= 13_5-1; S= 3_5-15) and replicate number.

MODA results

MODA's network differential analysis was run twice, once to investigate the differences among genotypes and once among treatments. In both cases, module 14 of the background matrix is commonly conserved across all 4 genotypes and all 6 treatment conditions (Figg. 3.2.20 and 3.2.21). This module contains 84.3% (20,810 genes) of all *Daphnia* genes and is considered to represent general stress response genes that are activated regardless of the genetic background of the animal or the conditions they were exposed. This module is enriched for a wide range of functions including mitochondrial activities, response to oxidative stress, whole organism growth

regulation, DNA repair, nitrogen and amino acid starvation, neurogenesis, adult life span determination, cellular and organism morphogenesis and various effects on gametes among many other responses.

On the genotype level (Fig. 3.2.20) the 13_2 and 3_5-15 genotypes are assigned more condition-specific modules than the others, with three modules assigned for 13_5-1, only one for P-IT and with none shared between any of the genotypes. Again, it is worth mentioning that only 13 of all 20 MODA modules of interest had sufficient information to proceed to DAVID enrichment analysis with presentable results, with each of those 13 modules containing only 7% of genes that are annotated with *Drosophila* homologs. Hence, the P-IT specific module remains unannotated. The module frequency plot for the per-treatment MODA analysis (Fig. 3.2.21) illustrates that samples pertaining to antibiotics exposure are the ones more likely to share modules, and are assigned more condition-specific modules than the other samples.

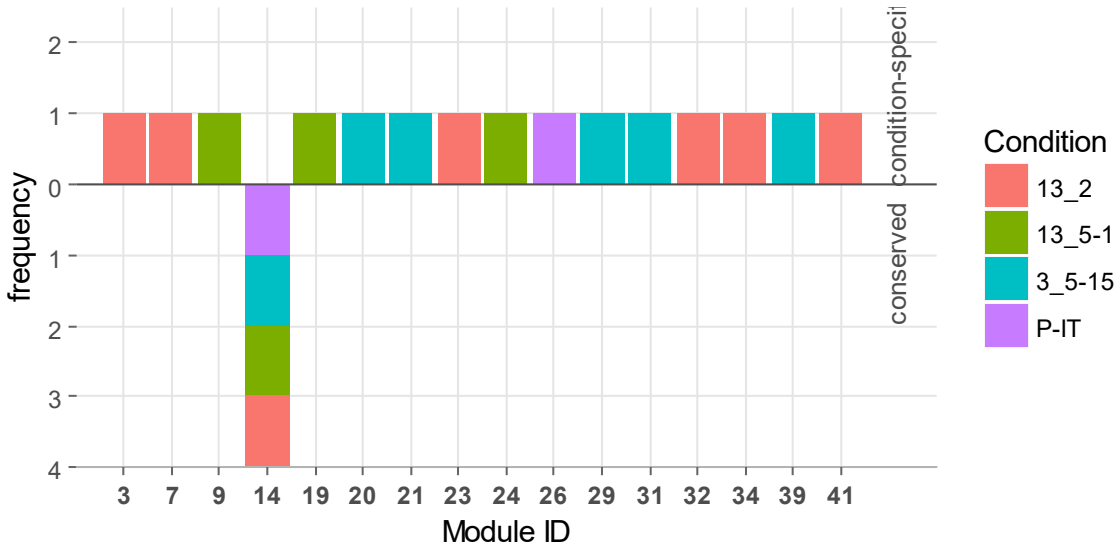


Figure 3.2.20: Gene co-expression modules per genotype. Illustrated above are the frequencies of the gene co-expression modules that are either genotype-specific, or conserved with the background co-expression network; where conserved modules would more likely represent general stress response genes. Module IDs on the *x*-axis pertain to those of the background co-expression network.

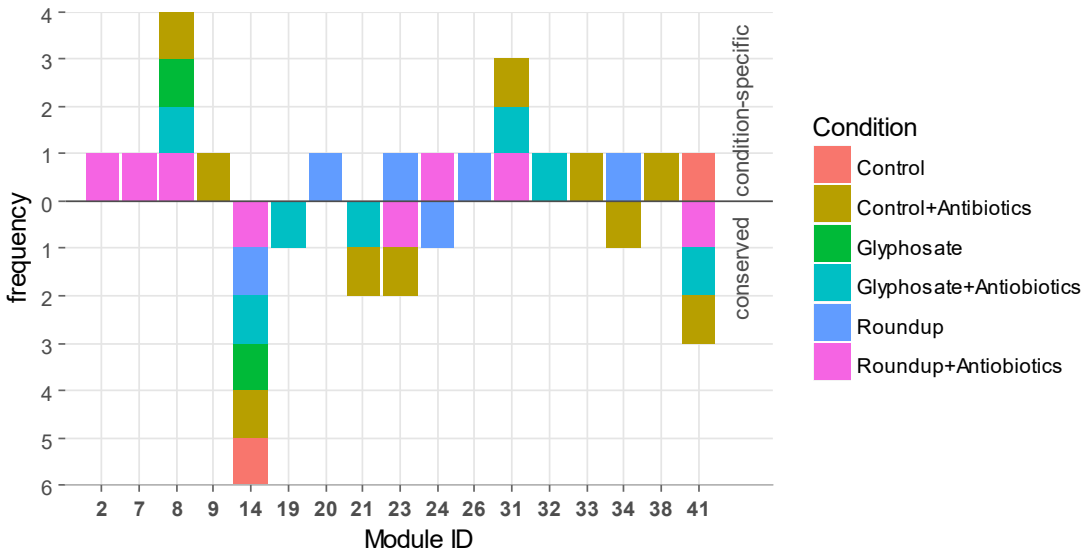


Figure 3.2.21: Gene co-expression modules per treatment. Frequencies of the background gene co-expression modules that are specific to a treatment are conveyed above point 0 on the y-axis, whereas those of the conserved modules are conveyed below it. Conserved modules would more likely represent general stress response genes. Module IDs on the *x*-axis pertain to those of the background co-expression network.

Discussion

The results obtained showed that the exposure of daphnids to antibiotics (with and without glyphosate and Roundup) have adverse effect on survival and gene expression. Gorokhova and collaborators (2015) reported that antibiotics changes the microbiome of *D. magna* compromising the fitness of organisms. The microbiome obtained from *D. magna* in the present experiment was sequenced through Mi-seq and is currently being analysed by bioinformatics approach. The negative effect on survival due to exposure of daphnids to antibiotics prevented to evaluated the role of microbiome role in the resistance to common pesticides. However, incoming data on microbiome diversity might be helpful to understand its variation role in ecologically different conditions.

About life history traits, glyphosate and Roundup did have no effect on *Daphnia* survival but reduced size and fecundity and delayed the reaching of sexual maturity. It is worth noting that these effects were not consistent among genotypes. The exposure of *D. magna* to glyphosate and Roundup caused varying susceptibility among different clones of *D. magna* and chronic exposure (15 days) induced adverse effect on organisms life history traits (survival, growth, fecundity, abortion rate and juvenile body size). Furthermore, Roundup caused more negative effects at same concentration (1 mg/l) of its active ingredient glyphosate and an effect clone-depending was

observed after exposure to glyphosate and Roundup, while the exposure to antibiotics (with and without chemicals) caused adverse effect in all clones.

Bacteria community of guts showed marked differences in composition (in term of abundance of bacteria families) among the analysed clones. These differences are quite interesting, because, for the first times, clones maintained for a long time of acclimation in identical conditions, medium and feeding are compared. The comparative analysis of microbiome and transcriptome is currently under way to elucidate the role of microbiome in *Daphnia* resistance to chemicals. Actually, the analysed clones showed different sensitivity, in term of phenotypic responses, to the tested toxicants. However, the results of transcriptional analysis suggest that the co-exposure of daphnids to glyphosate or Roundup and antibiotics changes the gene expression in all clones.

Differential expression sample correlation matrix showed that the resistant 13_5-1 genotype distribution of the samples is more influenced by the genetic background more so than by pesticide exposure. These matrices show that gene expression of the given samples is mainly governed by the genetic background of the critters but when differentially expressed, are more affected by the removal of the microbiome than by the pesticide exposure. They also suggest that the behavior of the 13_5-1 genotype in response to antibiotics introduction might be distinct from the others, which could consequently affect response to common pesticides.

Whole transcriptome PCA showed that P-IT genotype is splits from the other three clones; this because genotype P-IT variant was created in a laboratory, whereas the other three originated naturally and from the same population. PCA including only the significantly differentially expressed transcripts, i.e. $|logFC| > 2$, produced in a pairwise manner, on the other hand, illustrate how the genotypes respond to various treatment conditions.

PCA analyses in the absence of antibiotics could indicate that exposure to the toxicants do not elicit a significant response from the animal. DAVID analysis regarding the effects of roundup reports changes in the patterns of ecdysis and carbohydrate metabolism, a significant effect on normal cell growth and apoptosis, response to oxidative stress as well as negative regulation of the c-Jun N-terminal kinase (JNK) signaling pathway that plays an important role in apoptosis, inflammation, cytokine production, metabolism and which could affect embryonic development (Xiao et al., 2013). Comparison of control against glyphosate is enriched for haemopoiesis and DNA catabolism, along with alternative splicing, metal and Ca^{2+} binding and transport for both cases. The PCA analyses in presence of antibiotics suggests that no animal response to the toxicants are directly elicited in the absence of the microbiome. According to DAVID, Roundup and glyphosate commonly affect alternative splicing, glucose metabolism, amino acid transport and cell adhesion. Transcripts that are differentially expressed between control and Roundup samples treated with

antibiotics are particularly enriched for neuronal activities; positive regulation of the MAPK cascade which, in turn, could affect transcription, translation, cell cycle and proliferation (Plotnikov et al., 2011); as well as for the paired box genes, which code for tissue specific transcription factors that are important during early developmental stages (Chi and Epstein, 2002). On the other hand, differentially expressed transcripts between control and glyphosate antibiotic samples are enriched for positive regulation of hippo signaling, a pathway that helps regulate organ size (Zhao et al., 2011); several developmental processes; oocyte differentiation and fate determination as well as the regulation of the innate immune system. About the samples exposed to Roundup with and without antibiotics, Enrichment analysis reports significant alterations in carbohydrate metabolism, neuronal processes, peroxidase activities and developmental components, such as the homeobox, a DNA sequence found in genes that help regulate anatomical development and morphogenesis. DAVID enrichment reports the involvement of alternative splicing as the most significant outcome, followed by sugar metabolism, polarity specification, oocyte fate determination, melanotic encapsulation of foreign target and the open tracheal system among others. Enrichment analysis for the influence of antibiotics on organismal gene response include changes in cell shape regulation, glucose metabolism and tracheal and cuticle development among others.

Enrichment analysis of control against roundup reported phospholipid binding, whereas that of control against glyphosate reported several stress, neural and developmental responses including DNA repair, negative regulation of cell death, and oxidative stress. Results comparing control to either roundup or glyphosate with antibiotics are related to chitin activities. Roundup exposure with and without antibiotics show the same results as the transcript level, which involves the homeobox and neural activities. Glyphosate exposure with and without antibiotics are enriched for protoporphyrinogen IX biosynthetic process, endopeptidase activity and sensory organ precursor cell division. Comparing the effect of antibiotics on a controlled sample was enriched for carbon metabolism, chitin activities, organism growth and neuron remodeling; where as effects of the antibiotics in all comparisons are enriched for negative regulation of Notch signaling pathway and sensory organ precursor cell division.

Finally, transcripts that are commonly differentially expressed in all pairwise comparisons were also extracted in order to define the general stress responses, which were reported to include chitin catabolism; lateral inhibition, a process that regulates cell fate during development; and positive regulation of the JNK cascade, a promoter of cell apoptosis. Transcripts common in with-and-without antibiotics comparisons, on the other hand, are enriched for magnesium, ATP binding, transmembrane transport and metabolic pathways. These pairwise PC analyses suggest that *Daphnia* response to common pesticides is mediated by their microbiome instead of direct animal

response via gene expression regulation. This is congruous with studies mentioned earlier that demonstrate the microbiome's capability of regulating host genes which, is this context, would suggest a role in modulating *Daphnia* response to environmental toxicants. Furthermore, some of the enrichment analysis results concerning the effects of roundup and glyphosate are consistent with what was found in the literature, as mentioned above, regarding reports of oxidative stress and possible teratogenic effects that was found with aquatic vertebrates, invertebrates and zooplankton, indicating that potentially targeted pathways could be conserved across species. Effects of the toxicants are suggested by the enrichment reports to be more harmful in the absence of the microbiome, with adverse effects on development, immunity, metabolism and, in the case of glyphosate, reproduction. In the light of recent news pertaining on going debates involving scientific publications that investigate the carcinogenicity of roundup and its active ingredient (Levin, 2018), genes relating to particular assigned functional annotations such as the regulation of cell growth, cell apoptosis, the MAPK cascade, the JNK signaling pathway and hippo signaling as mentioned above, as well as the presence of certain domains like the p53-like transcription factors along with others were further analyzed. Indeed, fly homologs for human cancer-related genes were found such as NF2 (Neurofibromin 2), KSR2 (Kinase Suppressor of Ras 2), RUNX1 (Runt-related transcription factor 1), ALK (Anaplastic Lymphoma Kinase), RBL1 (Retinoblastoma-like 1), TNK2 (Tyrosine Kinase Non Receptor 2), MAP4K3 (Mitogen-activated protein kinase kinase kinase kinase 3) among others. These also include samples containing antibiotics, and numerous additional genes were found that were either targets for cancer treatments or indicators of prognoses. Since enrichment analyses are being discussed, it is worth noting that only around 7% of the entire assembly of transcripts has a corresponding *Drosophila* homolog gene ID, which was also assigned solely by sequence similarity. The majority of the transcripts have unknown functions and cannot be used directly in DAVID due to the lack of a *Daphnia* identifier database. This is a major dilemma for the biological interpretation of *Daphnia* responses, as possibly important genes and biological pathways might be missed due to the lack of sufficient annotations. Moreover, the definition of differential expression in this context was limited to having an absolute log Fold Change of above 2 regardless of the p-values; in order to avoid possible false negative results. In order to bridge these gaps, MODA is applied to first group genes of interest with their co-expressed entities and by doing so, provide some functional identity to unknown genes, since all genes in a module would share the same biological functions or pathways.

Gene co-expression modules per genotype firstly suggests that the two former genotypes exert a greater effect on the animal's gene expression and secondly that each genotype affect the gene expression of *Daphnia* in a unique manner. According to DAVID, 3_1-15 particular modules are

enriched for Wnt signaling pathway, splicing, development, neural activities, glucose metabolism, and cell shape regulation, while 13_2 genes are enriched for glycosyltransferase activities in the Golgi apparatus which indicate post-translational modifications and 13_5-1 genes for transferase. Again, it is worth mentioning that only 13 of all 20 MODA modules of interest had sufficient information to proceed to DAVID enrichment analysis and the P-IT specific module remains unannotated. The module frequency plot for the per-treatment MODA analysis shown that the number of unique, condition-specific modules relating to roundup, whether treated with antibiotics or not, is much greater than that of glyphosate, similarly signifying the contrast between their effects. Moreover, in the absence of antibiotics, glyphosate and roundup do not share any modules, suggesting that the mechanisms by which they affect the animal vary. According to the literature, roundup should, in theory, have a more potent effect since it contains other chemicals like surfactants besides its active ingredient glyphosate. One study conducted on several species, including the waterflea *Ceriodaphnia dubia*, reported that the roundup surfactant polyethoxylated tallow amine contributed to at least 46% of the herbicide's toxicity (Tsui and Chu, 2003). Of the shared condition-specific modules between antibiotic treatments, module 31 is enriched for mRNA surveillance pathway, spliceosome, snoRNA binding and translation initiation factor activity; whereas module 8, which also includes the only glyphosate module, is enriched for beta-Alanine metabolism and sacromere organization. Of the three modules specific to roundup with antibiotics, module 2 is significantly enriched for mitotic activities, response to oxidative stress, apoptosis, germ cell development, metal-binding and oocyte related responses. The four modules specific to roundup without antibiotics, however, remains unannotated. The conserved modules shared between some or all antibiotic samples include module 21, enriched for the Wnt signaling pathway, and module 41, enriched for protein stability and binding, anterior/posterior pattern specification, larval lymph gland haemopoiesis, neurotransmitter transporter activity, G-protein coupled receptor signaling pathway and regulation of transcription by RNA polymerase II as reported by FlyBase. The latter module is also shared with the control in a condition-specific manner. Modules specific to controlled samples in the presence of antibiotics, on the other hand, are significantly enriched for proteolysis as reported from DAVID analysis of module 33, in addition to ATP binding, mitotic cell cycle, protein phosphorylation and cellulose\chitin binding protein as inferred from FlyBase for module 38.

Response to ecologically relevant concentrations of both Roundup and its active ingredient seems to be mediated by the microbiome and, from the enrichment reports, could be affecting a wide range of biological functions including oxidative stress response, development, reproduction, metabolism, neural activities and ecdysis among others. In any case, the microbiome exerts a regulatory effect

on their host's gene expression affecting processes responsible for metabolism and development. Performing these analyses on the transcript rather than on the gene level offers a higher resolution via alternative splicing and would give more informative results. MODA further suggests that roundup is more potent than glyphosate alone, and each could be affecting the animal via distinct pathways. Since MODA was able to assign presumed functions to unannotated genes, future studies could further characterize genes of interest, since the amount of *Daphnia* genes of unknown functions constitutes a hindrance for a more accurate biological interpretation. Additionally, examining the microbiome data would be a very beneficial extension to the study in order to further understand the mechanisms of action, where the potentials of a more multi-omics approach would offer a greater insight regarding these contaminants.

Chapter 4: Ecotoxicological applications

The highly eco-responsive genome (Colbourne et al., 2011) and the molecular characteristics of *Daphnia* are a good premise for using these organisms to study the molecular basis of the mechanism of action (MOA) and the toxicogenomic effects of chemicals (Tautz et al., 2011; Kim et al., 2015). The omics-based technologies (i.e., transcriptomics, proteomics, metabolomics, and epigenomics) can facilitate the investigation of adverse effects caused by environmental stressors such as heavy metals and persistent pollutants (Boverhof and Gollapudi, 2011). The term "toxicogenomic" was used for the first time by Nuwaysir and collaborators (1999) and describes the application of genomic into the toxicology. In freshwater monitoring, the ecotoxicogenomic approach seems a promising tool as an alternative of analysing phenotypic responses. By daphnids bioassays and the use of specific biomarkers allow to detect environmental risk due to very low pollutants concentration in shorter time than by long term (chronic) exposure (Kim et al., 2015).

However, some critical aspects still exist and standardization methods are need to reach a suitable validation of bioassay in water quality monitoring and environmental risk assessment

i) It is important to establish the most suitable age at which evaluate gene expression (critical window of development) (Watanabe and Iguchi, 2006; Iguchi et al., 2006). As an example, Toyota et al. (2017) reported that haemoglobin genes are responsive to juvenile hormone and its analog fenoxycab (an insecticide), but gene regulation is influenced by organism life-stage.

ii) By considering the high eco-responsiveness of *Daphnia*' genome, a broader analysis of the responses to changes in water chemistry is necessary. Actually, in freshwater monitoring, daphnids are exposed to waters that might have a background composition quite different from the breeding water, independently of the presence of contaminants.

iii) The resulting molecular response to mixtures of contaminants that elicit opposite modulation of the same genes (up- versus down-regulation) has to be deeply investigated to evaluate the sensitivity to contaminant mixtures in aquatic environments.

In the following chapters we describe the application of *D. magna* in ecotoxicogenomic field. In particular, we report the modulation of 4 different genes (see Genes Box) after Daphnids exposure to waters with different chemical composition, to different pollutants, to an ecdysteroid and to binary mixtures of them.

In order to evaluate the critical aspect ii) and to develop a standardised method, organisms were exposed to different artificial and natural waters (Chapter 4.1). To evaluate the effects of the herbicide glufosinate ammonium (Chapter 4.2) and to evaluate the effect in mixtures of chemicals with pseudo-hormonal activity (20-hydroxyecdysone) and heavy metal (cadmium) frequently found in surface waters. In all experiments exposure the clone of *D. magna* P-IT was used. After exposure

of organisms, RT-qPCR was performed to evaluate a possibly modulation of specific genes. The genes selected for transcriptomic analysis, vtg1, cut12, cpa1 and dhb1 (see Genes Box) were indicated as biomarker of environmental risk. All these genes are involved in the expression of phenotypic traits (vtg1 in reproduction, cut12, cpa1 in growth and development, dhb1in red colour).

Materials and Methods

Daphnia magna cultures

Parthenogenetic females of strain P-IT were cultured in natural water, previously aerated for 24 h, commercially available and suitable for rearing daphnids. The strain of *D. magna* P-IT is reared in the ecotoxicological laboratory of Department of Chemistry, Life Science and Environmental Sustainability (previously Institute of Ecology) of the University of Parma since '80 of the last century and was originally obtained by the Institute of Ecosystem study, CNR (Verbania, Italy).

The cultures were maintained in a climate controlled chamber (temperature = 20±1°C; photoperiod 14h light: 10h dark provided by 800 lux cool-white fluorescent lamps) at a population density of 3 individuals/100 ml and fed, at each renewal, with aliquots of stock cultures of the unicellular green alga *Pseudokirchneriella subcapitata* (to reach a final density of $6x10^5$ cells/ml) in exponential growth phase, cultured according to the method by US-EPA (1978) and *Saccharomyces cerevisiae* (to reach a final density of $6x10^5$ cells/ml) according to the method by APAT and IRSA-CNR (APAT IRSA-CNR, 2003). The breeding medium was completely renewed twice a week (i.e. Tuesday and Friday) transferring adult females into crystallizing dishes containing fresh NW and food. After a 7-week period, cultures were discarded and started again with newborn organisms. The appropriate sensitivity of daphnids has been periodically checked by assays with potassium dichromate as reference chemical.

RNA extraction and quantification, cDNA synthesis and RT-qPCR

For information about method for RNA extraction and quantification see Chapter 2.2.

Synthesis of cDNA was performed by QuantiTect Reverse Transcription Kit (Qiagen) according to the instructions of the manufacturer.

The transcriptional activity of four selected genes was evaluated: vitellogenin 1 (vtg1- DY037239), cuticle 12 (cut12- DW985490), precursor of carboxypeptidase A1 (cpa1- DW724601) and hemoglobin (dhb1- DW724693). The primers used were the following: for vtg1 5'-ccagcgaatcctacacc-3' and 5'-cgcacagaccacagag-3', for cut12 5'-agccagtggaactacg-3' and 5'-tccagcatcatcagcg-3', for cpa1 5'-ccgacattcacgtcagt-3' and 5'-cctcgtaggtgtgatagtt-3', for dhb1 5'-

ggaagcggattcactg-3' and 5'-tgtcacccatagccga-3'(Primm srl, Milano, Italia). For each primer a concentration of 10 µM was used. qRT-PCR was conducted using SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts) as fluorescence detector, using STEP One Plus RT-PCR System (Thermo Fisher Scientific) following the manufacturer's instructions. The thermal profile consist of 30 minutes at 48 °C, 10 minutes of polymerase activation at 95°C, followed by 40 cycles of PCR at 95°C for 15 seconds and 55 °C for 1 minute. Afterwards, a melting curve analysis was performed to verify the authenticity of the amplified product by its specific melting curve analysis following the instructions of the manufacturer. Prohibitin 2 (phbr2- DW724510) was used as reference gene using the primers 5'-aattgttcaagccgagg-3' and 5'-cgtcaaaggaaacgtcac-3' (Primm srl, Milano, Italia). The comparative cycle method was used to evaluate the relative levels of target gene mRNAs (gene expression profile), normalized with the transcriptional levels from the housekeeping gene phbr2 using a comparative threshold cycle method (Livak e Schmittgen, 2001) was used. To compare gene expression among controls and between different experiments, Fold Change (FC) parameter was used. The FC describes how much a quantity changes going from an initial to a final value. This result is obtained considering:

i) ΔCt = Ct [Target] - Ct [Housekeeping]: the difference between the Ct (threshold cycle) of target gene and the Ct of a reference gene (phbr2 in the present experiment);

ii) ΔΔCt = (ΔCt Treatments) - (ΔCt Control): the difference between the ΔCt of treatments and the ΔCt of control;

iii) the value of $2^{-\Delta\Delta Ct}$ is the Fold-Change (FC).

By common consent, FC values higher than 2 indicates up-regulation and FC values lower than 0.5 indicates down-regulation.

Gene Boxes

Box1: vtg1 (DY037239)

Vitellogenin serves to store amino acids, carbohydrates, lipids and phosphates for the embryo development (Byrne et al., 1989). This protein is the most abundant polypeptide in *D. magna* parthenogenetic eggs (Kato et al., 2004). *D. magna* has two vtg genes oriented in opposite directions which might enable a coordinated hormonal regulation of their transcription (Tokishita et al., 2006). For the present study, vtg1 gene, as noted how "vitellogenin fused with superoxide dismutase", was selected (Kim et al., 2011). The transcriptional silencing and/or activation of vtg genes are regulated by cis-regulatory elements, located in the intergenic region between vtg1 and vtg2 (Tokishita et al., 2006). These cis-elements are the Juvenile Hormone Response Element (JHRE), the Ecdysteroid Responsive Element (EcRE) and the binding sites E75, E74 and GATA (Tokishita et al., 2006). Changes in vtg transcription are physiological during the molting and reproduction cycles or can be due to exogenous compounds (Soetaert et al., 2007a; b). Vtg1 gene is up-regulated in response to chemicals with ecdysteroidal activity (Tokishita et al., 2006) The up-regulation of this gene was observed in response to toxic stress caused by the insecticide methomyl, the herbicides propanil (Pereira et al., 2010) and the insecticide piperonyl butoxide (Hannas et al., 2011b). The insecticides fenoxycarb and pyriproxyfen, analogues of juvenile hormone, cause instead a down-regulation (Kim et al., 2011). The down-regulation of vtg1 was found to be associated with low fecundity and embryo development (Kato et al., 2004).

Box2: cut12 (DW985490)

The cuticle is a protein of arthropods that strengthens the exoskeleton to protect the inner tissues (Andersen et al., 1995). In *Daphnia pulex* 53 genes were annotated as cuticle (Colbourne et al., 2007). Modulation of cuticle genes is regulated by juvenile hormone and ecdysteroids, which respectively inhibits and induce molting (Charles et al., 2010). In daphnids, changes of cuticular mRNA expression are related with molting processes and reproduction (De Schamphelaere et al., 2008; Poynton et al., 2008). For the present study, cut12 gene was selected because it has been demonstrated that various xenobiotics can change its transcriptional activity. Actually, this gene is up-regulated by bisphenol A (Jeong et al., 2013) and down-regulated by insecticides with anti-ecdysteroidal activity such as the fungicide fenarimol (Soetaert et al., 2007a) and the insecticide fenoxycarb (Kim et al., 2011). In the state of the art, cut12 is regarded as biomarker of various stresses (Noji et al., 2003), but it can be used as a biomarker of exposure to chemicals with pseudo-hormonal activity (Kim et al., 2011).

Box3: cpa1 (DW724601)

Carboxypeptidase is a proteolytic enzyme that cleaves the amino acids in the C-terminal portion. In mammals these proteases act during digestion. In the arthropod *Bombyx mori*, this protein is secreted in the molting liquid, where it is activated accumulated between the new and old cuticle and serves to complete the ecdysis (Ote et al., 2004). In *D. magna*, it acts during ecdysis in breaking down daphnid exoskeleton and completing the exuviation process (Soetaert et al., 2007a). The large number of this gene (30 carboxypeptidase-like loci) suggests a function in adaptation to environmental variation (Schwerin et al., 2009). For the present study, cpa1, which encode for the precursor A1 of carboxypeptidase, was selected as changes in its expression is regarded as a biomarker for alterations in molting and growth. Cpa1 is up-regulated by the fungicides with anty-ecdysteroidal activity such as fenarimol (Soetaert et al., 2007a), the polycyclic aromatic hydrocarbon pyrene (Vandenbrouck et al., 2010), the insecticide methomyl and the herbicide propanil (Pereira et al., 2010). This gene is down-regulated by the heavy metal cadmium (Taylor, 2010) and by bisphenol A (Jeong et al., 2013).

Box4: dhb1 (DW724693)

In *D. magna*, hemoglobin (dhb) proteins have 330 aa residues composed by two domains (domain 1 and domain 2) which have low similarity each other (Tokishita et al., 1997). Daphnia has 11 genes coding for hemoglobin protein (Gerke et al., 2011). The Hb concentration increases at low oxygen concentration (Kobayashi et al., 1990). This effect depends on a modulation of responsive elements present on dhb genes, called Hypoxia Inducible Factor (HIF)/Hypoxya Response Element (HRE) (Gorr et al., 2004). In *D. magna*, dhb genes are considered biomarkers of exposure to environmental contaminants (Ha and Choi, 2009). In our study, the dhb1 gene, that is not regulated by hypoxic conditions, was considered (Zeis et al., 2003a/b; Gorr et al., 2004; Paul et al., 2004). One of the promoters, that regulate the trascriptional activity of dhb1, is CCAAT Box (Kato et al., 2001), that is under the control of a protein called C/EBP (CCAAT Enhancer Binding Protein) responsive to hormones (Ramji and Foka, 2002). The juvenile hormone methyl farnesoate and its analogues such as pyriproxyfen, fenoxycarb and methoprene up-regulate the dhb genes (Rider et al., 2005). Dhb is also up-regulated by the fungicide fenarimol (Soetaert et al., 2007a). The heavy metals Cd and Ni cause a down-regulation of this gene (Soetaert et al., 2007b; Vandenbrouck et al., 2009), as well as the herbicides glyphosate and methidathion (Le et al., 2010).

Chapter 4.1: Transcription profile after exposure to artificial and natural waters in *D. magna*

Introduction

As reported in the Introduction of the present thesis, *Daphnia* species are regarded as highly sensitive organisms to environmental contaminants and, in an ecotoxicological context, they can be used to evaluate the potential impact of contaminants in aquatic ecosystems (Piña and Barata, 2011).

However, in ecotoxicogenomic field, the gene expression could be influenced by the chemical composition of water (cationic and anionic ions, pH, conductivity) used as reference control, the age of organisms (juveniles and adults could have differences in transcriptional activity) and the time of daphnids' exposure. As evidenced in table 4.1.1, in the experimental designs of ecotoxicogenomic studies there are many differences, that could lead to results difficult to be compared. In an ecotoxicogenomic context and in environmental monitoring, it is instead important to adopt uniform and standardized procedures to compare data obtained by different laboratories.

The aim of the study was to develop and implement a procedure to standardise a method of ecotoxicogenomic approach based on *D. magna* responses. To this aim the transcriptional profile of genes involved in reproduction (vitellogenin fused with superoxide dismutase-vtg1) (Box 1), development (cuticle 12-cut12 and precursor of carboxypeptidase A1-cpa1) (Box 2 and Box 3) and energy allocation (di-domain hemoglobin precursor-dhb1) (Box 4) (see Soetaert et al., 2007a) was analysed. Afterwards, alteration in gene expression profile was analysed in daphnids exposed to commercial natural waters (drinking waters), having different chemical characteristics (such as conductivity, cationic and anionic ions concentration), and artificial waters reconstituted according to the recipes of international organizations (ISO, OECD and ASTM).

Table 4.1.1: Experiments performed using *Daphnia magna* and a ecotoxicogenomic approach. Differences in water used as reference control, age of organisms and time of exposure were reported.

Water used as reference control	**Age of organisms**	**Time of exposure**	**Bibliography**
Reconstituted artificial water	Neonates < 24 h	24 hours	Heckmann et al., 2006
COMBO medium	Adults (16-18 days)	24 hours	Poynton et al., 2007
Water faucet filtered	Neonates < 24 h	24 hours	Watanabe et al., 2007
MHRW (Moderately hard reconstituted water)	Adults (10 days)	24 hours	Poynton et al., 2012
OECD medium	Age not reported	96 hours	Vandenbrouck et al., 2009
OECD medium	Age not reported	48 hours 4 days 8 days	Soetaert et al., 2006
ADaM (Aachener Daphnien Medium)	Neonates < 24h	48 hours 96 hours 144 hours	Jansen et al., 2013

Materials and Methods

D. magna cultures

For information about maintenance of daphnid culture see Chapter 4.

Exposure to natural and artificial waters

Fifty juvenile daphnids (4 days old) of clone P-IT were transferred to crystallizing dishes containing 200 ml of commercial natural water (4 different bottled waters) or artificial water reconstituted according to the recipes of international organizations (ISO, OECD Elend M4 medium and ASTM hard water). The exposure time was 48 h: no food was added. The chemical characteristics of the various natural and artificial waters are reported in table 4.1.2, together with the characteristics of the breeding water considered as control. The selection of natural waters was carried out considering the cationic and anionic ions concentration and conductivity reported on the labels. As reported in table 4.1.2, the natural waters evaluated in this experiment present differences in pH values (from 7.2 of EV to 7.9 of NV), in HCO_3^- concentration (from 57.4 mg/l of LE to 360 mg/l of EV) and in Ca^{2+} concentration (from 20.4 mg/l of LE to 91.2 mg/l of VI). After 48 h exposure, organisms from each treatment were collected and subjected to extraction and gene expression analysis as reported above.

Table 4.1.2: Chemical characteristics of artificial (OECD, ISO, ASTM hard water) semi-artificial (SB+O+Fe) and natural waters (SB, EV, VE, LE, VI): pH, conductivity (µS cm^{-1}), cationic and anionic ions (mg/l), ratios Ca/Mg and Na/K were reported.

	pH	Conductivity (µS cm -1)	mg/l									Ca/Mg	Na/K
			Calcium (Ca^{2+})	Magnesium (Mg^{2+})	Sodium (Na^{2+})	Potassium (K^{+})	Silica (SiO_2)	Bicarbonates (HCO_3^-)	Sulphates (SO_4^-)	Chlorides (Cl^-)	Nitrates (NO_3^-)		
OECD	7.8	602	80.1	12.2	19.4	3.2	2.1	47.1	48.4	144.9	0.2	6.6	6.1
ISO	8.1	660	80.9	0	54.7	11.7	0	145.3	14.3	143.1	0		4.7
ASTM hard water	8.2	517	27.9	24.2	52.6	4.2	0	139.5	162.8	3.8	0	1.2	12.5
SB+O+Fe	8.1	415	51.4	29.7	6.0	0.97	16.7	296.0	4.5	3.0	9.0	1.7	6.2
SB	7.5	417	51.4	29.7	6.0	0.97	16.7	296	4.2	2.6	9	1.7	6.2
EV	7.2	509	80	26	6.5	1	15	360	14	10	3.8	3.1	6.5
VE	7.9	251	36.9	12.6	2	0.5	nr	158	19.3	2.4	4.4	2.9	4
LE	7.8	119	20.4	1.8	1.9	1.6	5.4	57.4	16.1	nr	1.5	11.3	1.2
VI	7.6	399	91.2	10.4	2.9	0.89	8.6	232	50	4.9	2.3	8.8	3.3

nr=not reported

RNA extraction and quantification, cDNA synthesis and RT-qPCR

For information about RNA extraction and quantification, cDNA synthesis and RT-qPCR see Chapter 4.

Results

In the figures reported below, the fold change of selected genes (vtg1, cut12, cpa1 and dhb1) after daphnid exposure to artificial and natural waters is shown. Compared to SB (breeding water), regarded as reference medium, all artificial and natural waters, excluding NV and EV, caused down-regulation of vtg1 (Fig. 4.1.1). This gene show the greatest down-regulation after LE exposure.

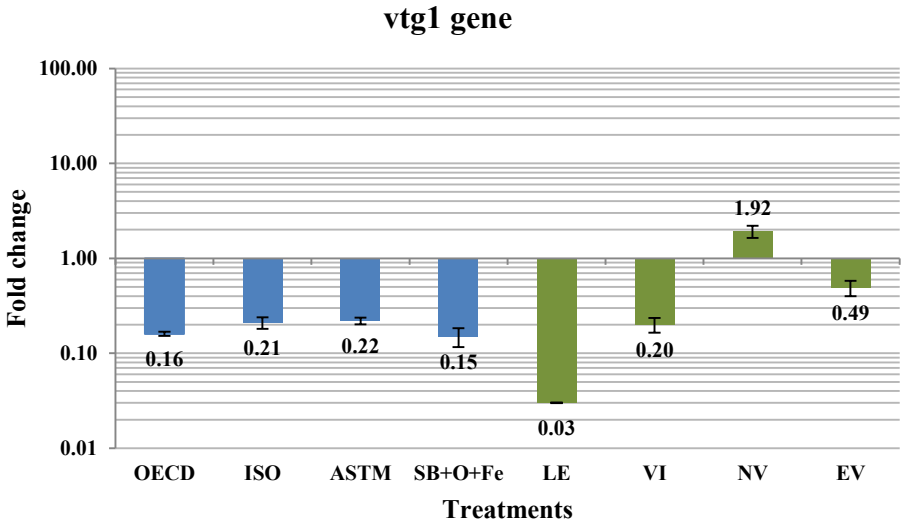


Figure 4.1.1: Transcriptional activity of gene vtg1 after exposure for 48 hours to artificial waters (blue) and natural waters (green).

The genes involved in organism growth (cut12 and cpa1) showed no change in transcriptional activity after daphnid exposure to OECD and ISO media (Figg. 4.1.2 and 4.1.3). Both genes were down-regulated by the exposure to SB+O+Fe^{2+} and LE and up-regulated by NV. The exposure to ASTM medium, VI and EV caused contrasting responses of cut12 and cpa1, i.e. up- versus down-regulation depending on the medium.

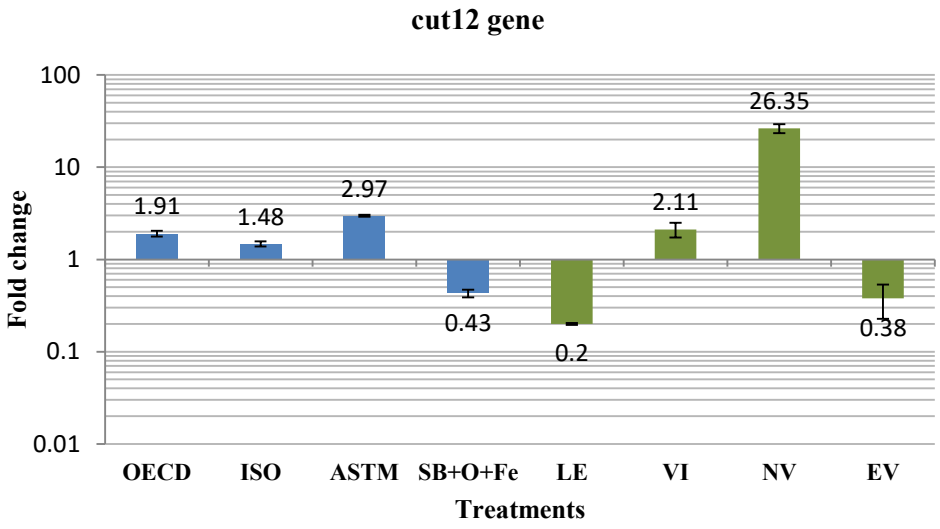


Figure 4.1.2: Transcriptional activity of gene cut12 after exposure for 48 hours to artificial waters (blue) and natural waters (green).

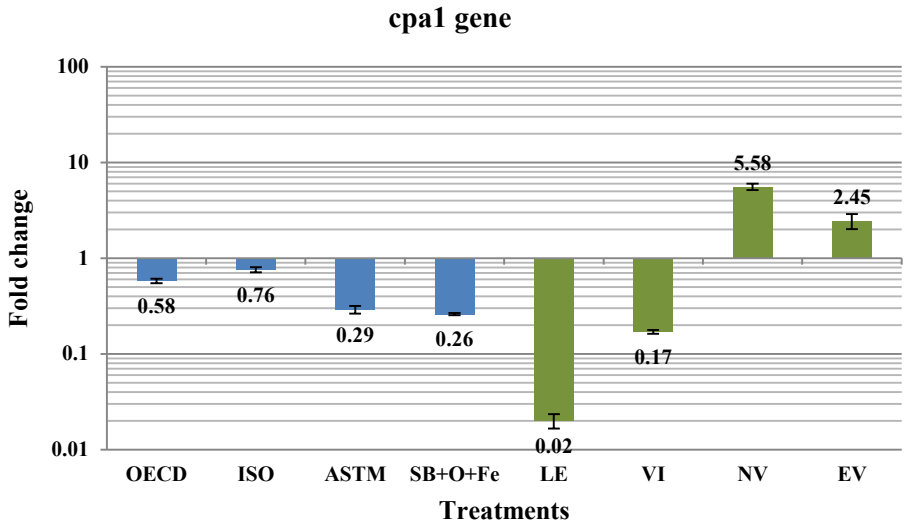


Figure 4.1.3: Transcriptional activity of gene cpa1 after exposure for 48 hours to artificial waters (blue) and natural waters (green).

The haemoglobin gene (dhb1) showed a basal expression in daphnids exposed to ASTM, SB+O+Fe^{2+}, LE, VI and up-regulation after all the other treatments. None of the artificial and natural waters caused down-regulation of the gene dhb1 (Fig. 4.1.4).

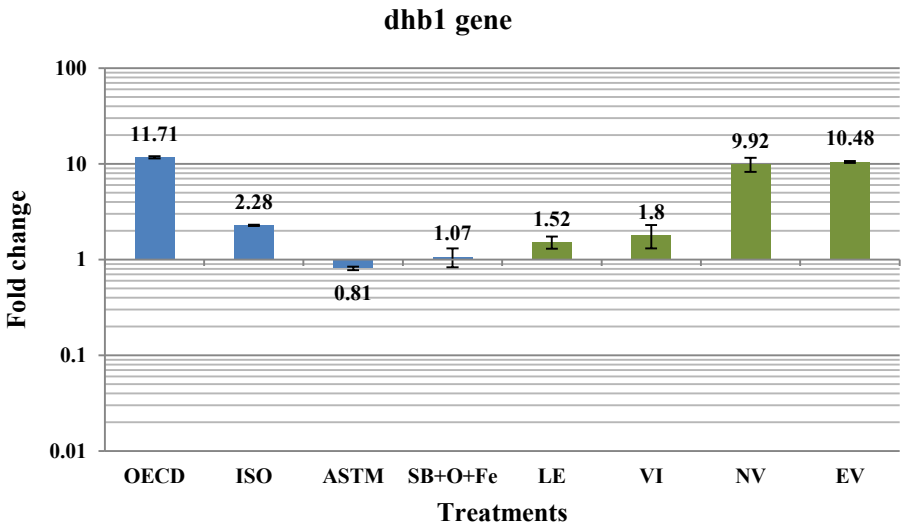


Figure 4.1.4: Transcriptional activity of gene dhb1 after exposure for 48 hours to artificial waters (blue) and natural waters (green).

Statistical analysis did not reveal any significant correlation between gene modulation and chemical characteristics of waters, either artificial or natural. Significant correlation was instead observed among vtg1, cut12 and cpa1 fold change ($p<0.01$) and between cpa1 and dhb1 ($p<0.05$).

Discussion

As shown in table 4.1.2, the selected natural waters and the artificial media present different chemical composition. The transferring and maintenance, for a 48 h period, into a water (natural, artificial or semi-artificial) with chemical characteristics different from the breeding water induced modulations of the selected genes in daphnids. This means that a difference in water chemistry can influence daphnid gene expression. It is well known that water chemistry (e.g. concentration of specific anionic and cationic ions, pH, conductivity) can change phenotypic traits such as reproduction and carapace formation in daphnids (Tan and Wang, 2009; Mažuran et al., 2015). Few studies are available in the literature about the effect of the single ions on *Daphnia*'s life history traits and gene expression. Some studies evaluated the effect of calcium concentration on daphnids life cycle. Changes in water calcium concentration influences the effects of temperature and toxic cyanobacteria *Microcystis aeruginosa* (Betini et al., 2016; Akbar et al., 2017). Furthermore, calcium concentration can also influence the possibility to produce morphological defences caused by predation risk (Riessen et al., 2012). Consequently, calcium has a role in overcoming

the exposure to stressors and calcium excess can also have negative effects. Bogart and collaborators (2016) reported a 98% mortality, in *D. magna* after 96 h exposure to a concentration of 600 mg Ca^{2+}/l (as $CaCO_3$). In the present work, the calcium concentrations were very lower (from 27.9 mg/l of the artificial water ASTM to 91.2 mg/l of the natural water VI) in comparison to the concentration used by Bogart and collaborators and none of daphnids exposed for 48 h to natural and artificial waters died, but only effects at sub-cellular level were observed.

In the present study, a down-regulation of gene vtg1 after exposure to all artificial and natural waters (except NV) was observed. As reported above, vtg1 regulates reproduction and embryogenesis in *Daphnia* (Kato et al., 2004). Giardini and collaborators (2015) showed that a calcium concentration of 25 mg/l induces defect in embryo development in comparison with a concentration of 5 mg/l. High concentrations of Ca^{2+} causes a decrease in reproduction (concentrations ≥240 mg Ca^{2+}/l) and an increase of aborted eggs in daphnids (concentrations ≥553 mg Ca^{2+}/l) (Mažuran et al., 2015); on the other hand low level of Ca^{2+} (≤0.5 mg/l) impairs carapace calcification (Tan and Wang, 2009). The modulation of vtg1 could be linked with the negative effects of calcium shift on reproduction and embryo development observed by Mažuran et al. (2015) and Giardini et al. (2015). In the artificial media, there are differences in calcium concentration: the OECD and ISO medium have a similar Ca^{2+} concentrations (80.1 mg/l and 80.9 mg/l, respectively). These concentrations are higher than the one of ASTM medium (27.9 mg/l) and of the natural water used for the maintenance of daphnid cultures and used in this experiment as reference control (51.4 mg/l).

As shown in table 4.1.2, there are differences in Ca^{2+} and other ions concentrations among the tested artificial and natural waters. However, the absence of correlation between fold change values and any of the available chemical characteristics of the tested waters did not allow us to formulate hypothesis about the possible factors implied in the modulation.

The natural waters selected for the present study were commercial bottled waters in PET (Polyethylene Terephthalate) which is suspected to release pseudo-hormonal substances (e.g. estradiol, bisphenol A, nonylphenol) even if at very low amounts (i.e. picogram equivalens per liter bottled water) (Pinto and Reali, 2009; Barrett, 2009; Wagner and Oehlmann, 2009; 2011; Wagner et al., 2013; Plotan et al., 2013; Maggioni et al., 2013). In 2011, a study carried out in Italy, Germany and France on 18 plastic bottles reported that 61,1% of these (11 plastic bottles) release chemicals with estrogenic activity (Wagner and Oehlmann, 2011). The chemicals released by plastic bottles can modulate gene expression in daphnids (Ha and Choi, 2009, Hannas et al., 2011b; Campos et al., 2013; Jeong et al.,

2013). However, in the present work, gene modulation (both up- and down-regulation) was also observed in artificial waters, in which contamination by pseudo-hormonal substances has to be absolutely excluded, and in the semi-artificial water SB+O+Fe^{2+}, whose background composition is identical to the water used as control and for daphnid breeding. It seems therefore that gene expression in daphnids can rapidly vary in response to variations in water characteristics not necessarily related to the presence of contaminants. The observed modulation of gene expression in response to the transferring from the breeding water to natural or artificial water with different background composition, in absence of toxic substances, constitutes an important item in the case of an ecotoxicogenomic approach in freshwater monitoring. Actually, in bioassays on environmental samples, daphnids have to be necessarily transferred to waters that can differ from the home water in the background chemical composition, which can also differ among sampling stations independently from the presence of contaminants. However, looking at the single genes, vtg1 was almost always down-regulated especially in artificial waters, while cut12 and cpa1 showed a different modulation depending on the tested water. The gene dhb1 showed instead a basal expression or up-regulation; none water caused down-regulation. Despite the number of the different waters was limited, these observations suggest that vtg1, cut12 and cpa1 expression may undergo a wider modulation than dhb1 in response to water chemical composition. The data obtained in these experiments showed the effects of rapid shifts (effects after 48 hours of waters exposure) in waters with a different chemical composition on transcriptional activity. In future, inter-laboratory calibration could allow to select a reference water to use in ecotoxicogenomic studies for freshwater monitoring.

Chapter 4.2: Glufosinate ammonium effect in *D. magna*

Introduction

Glufosinate ammonium [ammonium (3-amino-3-carboxypropyl) methyl phosphinate] (Fig. 4.2.1) is the active ingredient of non-selective broad spectrum herbicides (Zhang et al., 2014). This chemical is used to control a broad range of weeds in vegetables, vineyards, ornamental trees and bushes (Nagatomi et al., 2013). Glufosinate ammonium has the chemical formula $C_5H_{12}NO_4P$ and a glutamate-similar chemical structure (Park et al., 2018) and by inhibiting glutamine synthetase enzyme involved in the amino acid glutamine synthesis and ammonia detoxification (Wendler et al., 1990; Donn and Köcher, 2012), causes defect in production of glutamine , the most abundant non-essential amino acid that provides nitrogen for protein and nucleotide synthesis (Reitzer et al., 1979). In this way, glufosinate ammonium prevents photosynthesis and causes the death of plants (Koureas et al., 2014). Furthermore, it can activate glutamate receptors, causing negative effects on neuronal network activity (Park et al., 2018). For this reason, scientific community has interest in understanding the impact of glufosinate ammonium on human and environment health. In agricultural areas, humans exposed to glufosinate ammonium presents high induction of oxidative damage to DNA and risk to undergo neurotoxic effect (Koureas et al., 2014; Moon and Chun, 2016; Cha et al., 2018). For these reasons, the European Community has included glufosinate ammonium in the Directive 91/414/EEC [Commission Directive (CD) 2007/25/EC] as possible risk factor for mammals.

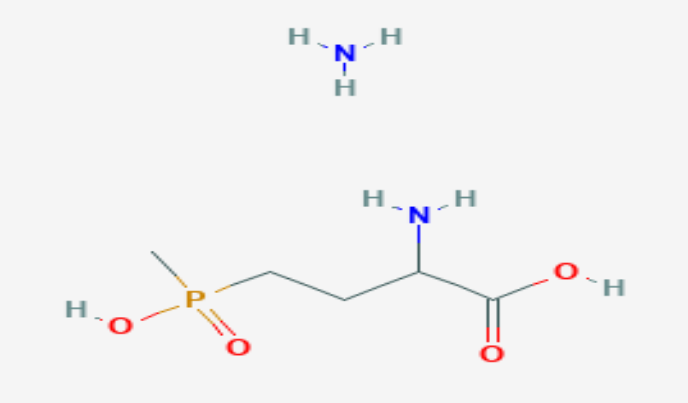


Figure 4.2.1: Chemical structure of glufosinate ammonium (https://pubchem.ncbi.nlm.nih.gov/compound/glufosinate-ammonium#section=Top).

This herbicide have attracted scientific interest, considering that its residues have been found in plants, soil, water and food (EFSA-European Food Safety Authority, 2015). Residues at concentration of 9.8 µg/l (Liao et al., 2018) of glufosinate ammonium were found in food samples, a concentration that could have effects on organisms health. Few studies are available on the real impact of glufosinate ammonium in arthropods and further investigation are needed to understand the effect on aquatic organisms. This herbicide is soluble in water (solubility ~ 1370 g/l) and has a half-life range of 3 to 42 days (Siimes et al., 2006; Dinehart et al., 2009; Carpenter and Boutin, 2010) In Northern Italy, the average concentration of glufosinate ammonium detected in surface waters is 0.10 µg/l and the higher annual concentration is 0.72 µg/l (Masiol et al., 2018). These are low concentration, but exposure for a long time period could have negative effect for human and environmental health. In toxicological studies, chronic effect at low doses is unclear (Calas et al., 2008) and unfortunately, there are not many papers that evaluate the impact of this herbicide at low concentration and in aquatic organisms. A study carried out by European Food Safety Authority reported contrasting results about the effects of glufosinate ammonium on arthropods (European Food Safety Authority 2015). Khan et al. (2015) found that glufosinate ammonium increases mortality in *Trichogramma pretiosum* (Hymenoptera, Trichogrammatidae), and Ahn and collaborators (2001) observed the same negative effect on *Tetranychus urticae* (Acari, Tetranychidae). However, Khan and collaborators (2015) observed effects at a concentration 4 fold higher than the recommended field concentration (RCF). It is instead obtain more information through the evaluation using environmentally relevant concentrations. *Daphnia magna* can represents a good model organism to evaluate the effect of chemicals, considering the characteristic of its eco-responsive genome (see Introduction about information of *Daphnia*'s application in ecotoxigenomic field).

The aim of the present study was to study the negative effect posed by glufosinate ammonium for aquatic organisms. *D. magna* was used as model species and exposed to low concentrations (0.6 µg/l, 6 µg/l and 60 µg/l) in chronic bioassays (21 days). Modulation of the transcriptional activity of specific genes (vtg1, cut12, cpa1 and dhb1- see Gene Boxes for a detailed information about these genes) was evaluated in organisms exposed to the lowest concentration (0.6 µg/l) for 24h and 48h. The molecular approach based on gene modulation can help to highlight responses at sub-cellular level and the possible early negative effect that can lead to the impact at phenotypic level.

Material and Methods

D. magna cultures

For information about maintenance of daphnid culture see Chapter 4.

Exposure to glufosinate ammonium: chronic test

The chronic test was performed using the Test Guideline 211 developed by OECD (2012), with some modifications according to Pellegri (2015). Briefly, neonates less than 24 hours old were randomly assigned to treatments. In this test, three glufosinate ammonium concentrations (0.6 µg/l, 6 µg/l and 60 µg/l) and ten replicates per treatment were used. Each organism was maintained in 100 ml of natural water; the medium was renewed and food (the unicellular green alga *P. subcapitata* and the yeast *S. cerevisiae*, each one at the density of $6x10^5$ cells/ml) was added three times a week. To avoid the possible influence of the algal medium, the algae suspension was transferred into 50 ml tubes and centrifuged for 10 minutes at 2000 g. The supernatant was discarded and the pellet was resuspended in the natural water used to breed daphnids and centrifuged for 15 minutes at 2000 g. The supernatant was again discarded and the pellet was resuspended in 10 ml of natural water and used to feed daphnids at each medium renewal. The survival of daphnids, size at 8 day age, age at first reproduction, fecundity, number of aborted eggs and died neonates was recorded until 21^{st} day. To perform the chronic bioassay, aliquots of trace elements solutions for ELENDT M4 artificial medium (OECD, 2012) were added (see table B in Apeendix) to controls and treatments. Neonates released by mothers during the test were counted and removed from the medium each other day. Mortality of parents in laboratory control (less than 20% after 21 days) and mean number of offspring released by each mother (more than 60% after 21 days) were used as validity criteria according to OECD 211 Guideline.

According to Pellegri (2015), the Failed Development (FD) was calculated considering the Aborted Eggs (AE), Dead Offspring (DO) and the Life Offspring (LO) for each mother:
Failed Development (FD) % = $(AE+DO)_{day\ 21}/(AE+DO+LO)_{day\ 21}$ x 100
The average of FD % was calculated for each treatment.

Exposure to glufosinate ammonium

For the herbicide BASTA (Roussel-Hoechst Agrovet S.p.A.), whose active ingredient is glufosinate ammonium (120 g/l), the concentration 0.5 µg/l, expressed as glufosinate ammonium, far below the environmental expected concentration (1 mg/l) for waterbodies subjected to overspray or drift and the EC_{20} for total Cladocera abundance (0.16 mg/l) reported by Faber et al. (1998).

RNA extraction and quantification, cDNA synthesis and RT-qPCR

For information about RNA extraction and quantification, cDNA synthesis and RT-qPCR see Chapter 4.

Statistical analysis

One way-ANOVA was performed to assess the effect of treatments on life istory traits (fecundity, age at maturity, size, failed development). Tukey's test was used for pairwise comparisons and Dunnet was used when variances were not homogeneous between treatments.

Results

Chronic test

After exposure to *D. magna* for 21 days to three concentrations of glufosinate ammonium (0.6 µg/l, 6 µg/l and 60 µg/l) no significant differences were observed in terms of age at maturity, size at day 8 (data not shown) and reproduction (Fig. 4.2.2). Interestingly, significant differences were observed in failed development (aborted eggs and died neonates) (Fig. 4.2.3).

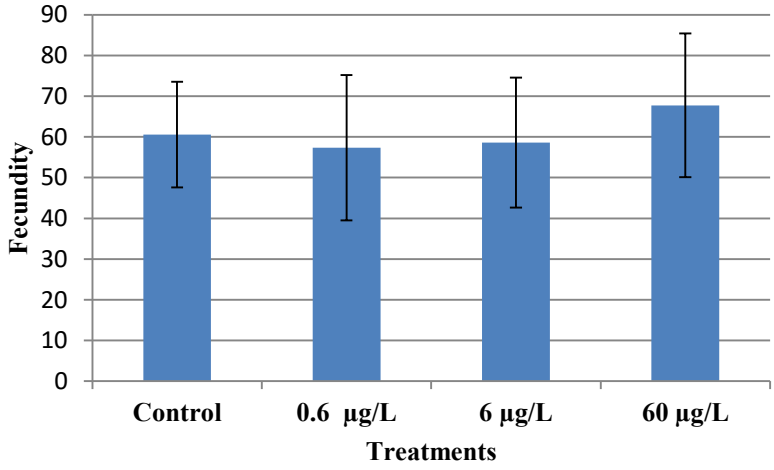


Figure 4.2.2: Fecundity (mean cumulative number of live offspring) after exposure of *Daphnia magna* for 21 days to glufosinate ammonium 0.6 µg/l, 6 µg/l and 60 µg/l.

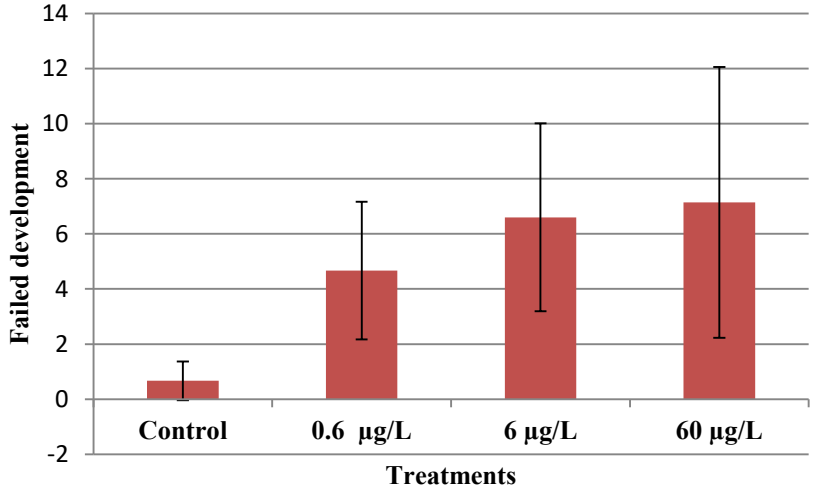


Figure 4.2.3: Mean number of FD % after exposure of *Daphnia magna* for 21 days to glufosinate ammonium 0.6 µg/l, 6 µg/l and 60 µg/l.

Genes modulation

After 24 h exposure, vtg1 and cut12 (FC = 0.57 for both gene) showed maintained the basal expression. A slight down-regulation was observed for cpa1 and dhb1 (FC = 0.44 and FC = 0.34, respectively) (Fig. 4.2.4).

After 48 h exposure, cut12 maintained the basal expression (FC = 0.52), while vtg1, cpa1 and dhb1 genes underwent a relevant down-regulation (FC = 0.17, FC = 0.19 and FC = 0.02, respectively), the fold change of dhb1 indicating a repression of this gene. Significative differences were observed comparing the FC values after 24 h and 48 h of exposure for genes vtg1, cpa1 and dhb1 ($p<0.001$).

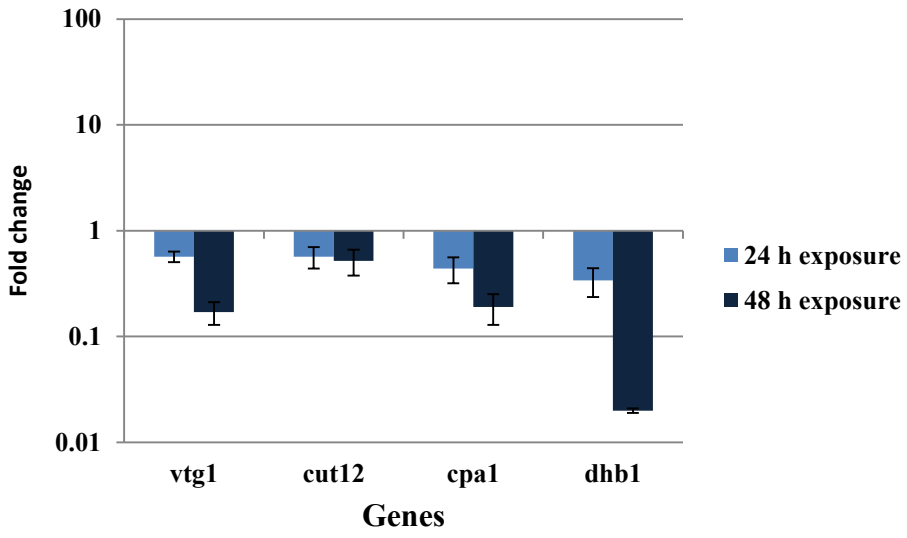


Figure 4.2.4: Comparison of gene expression of all genes after 24 and 48 hours of exposure to glufosinate ammonium 0.5 µg/l.

Discussion

Glufosinate ammonium has been found in surface water at low concentrations but with high frequency (Mottes et al., 2017; Masiol et al., 2018). Therefore, this herbicide can represent a risk also for aquatic environments. In the present study, for the first time, the effect of glufosinate ammonium on the model aquatic species *D. magna* was evaluated through chronic test at environmental relevant concentrations and transcriptional (RT-qPCR) approach.

The exposure of daphnids for 21 days did not cause negative effects in reproduction at the three concentrations of glufosinate ammonium (0.6 µg/l, 6 µg/l, 60 µg/l). However, organisms underwent increase of the failed development (aborted eggs and died neonates). No significant differences in age at maturity and size after exposure of daphnids to glufosinate ammonium with respect to control organisms. However, this concentration caused down-regulation of two genes (cpa1 and dhb1) after 24 h exposure and of three genes (vtg1, cpa1 and dhb1) after 48 h exposure, demonstrating a time-dependent action. Vtg1 is a gene coding for vitellogenin, a protein with the function of storing amino acids, carbohydrates, lipids and phosphates for the embryo development (Byrne et al., 1989). A down-regulation of this gene can cause a reduction of vitellogenin in daphnids' eggs during reproduction and consequently a lack of nutrients during embryo development. This is even more evident during the chronic test, in which the concentration of 60 µg/l caused

the development failure of a significant percentage of eggs/embryos. The gene mostly modulated was dhb1, the precursor of haemoglobin. cut12 was the only gene that kept a basal expression after both 24 and 48 hours of exposure of daphnids. However, a slight gradual decrease of expression (fold change from 0.57 to 0.52) was observed also in this gene. The gene cpa1, involved in growth and ecdysis (Ote et al., 2004), was down-regulated suggesting a negative effect in moulting process during daphnid life cycle. Dhb1 (haemoglobin) was the gene mostly down-regulated by glufosinate ammonium. In the literature, no information is available about the possible negative effect that glufosinate ammonium on haemoglobin transcription and translation. In a report published by World Health Organization (WHO) and Environmental Protection Agency (EPA) in 2012, a slight reduction of haemoglobin concentration after exposure of rats for 52 weeks was reported (EPA, 2012). This is the only report in which evidences of glufosinate ammonium effect on haemoglobin levels are described. Data obtained for dhb1 in *D. magna*, and in particular the high down-regulation, suggest that this chemical can influence the energy allocation and oxygen transport in a short exposure time (24 and 48 hours) and at low concentration. The genes used in the present work were never analysed after glufosinate ammonium exposure in daphnids or other aquatic organisms. However, it is important to remember that this chemical acts inhibiting glutamine synthetase, and consequently causing a reduction of glutamine synthesis (Wendlel et al., 1990; Donn and Köcher, 2012). Glutamine regulates the transcriptional activity of genes involved in important cellular pathways such as metabolism, signal transduction, cell defence and repair (Corless et al., 2006; Curi et al., 2007; Kan et al., 2015). The data of the present work suggest that glufosinate ammonium could influence the regulation of genes involved in reproduction, development and energy allocation.

Glufosinate ammonium is also able to bind the N-methyl-D aspartate (NMDA) receptor and stimulate neuronal network activity (Matsumura et al., 2001; Lantz et al., 2014). NMDA is a type of ionotropic glutamate receptor involved in *Daphnia* upstream the methyl farnesoate signaling (a juvenile hormone) and male offspring production (Toyota et al., 2015a;b). The genotype P-IT used for the present experiment, which has never reported to produce males in any experimental conditions (Gorbi et al., 2011). In future, the use of genotypes that produce males can represents a possibility to obtain more information about the environmental risk and the molecular pathways modulates by glufosinate ammonium.

It is worth recalling that in the present study a commercial herbicide containing glufosinate ammonium (BASTA) was used. The commercial herbicide contains additives and

surfactants (not revealed by the manufacturer) that could increase the negative effect caused by the single active ingredient.

These results suggest that glufosinate ammonium can represents a risk factor for aquatic invertebrates, such as *D. magna* and that further studies on other aquatic species are needed to comprehend the risk posed by this chemical for the aquatic biota. The evaluation of gene expression can give information about the early negative effects at low concentrations. In this context, public organizations can improve directives about the use and concentration limit of glufosinate ammonium in the aquatic compartment.

Chapter 4.3: Effect of binary mixture of glufosinate ammonium, Cadmium and 20-hydroxyecdysone in *D. magna*

Introduction

Effects and toxicity of 20-HE and cadmium, as single substances and mixtures, on *Daphnia* have been reported in various studies (Attar and Maly, 1982; Mu and LeBlanc, 2002; Baldwin et al., 2009; Wang et al., 2011; Pérez and Hoang, 2017). The effects of glufosinate ammonium on *D. magna* have been described in the previous chapter 4.2. To our knowledge these chemicals were never tested in binary mixtures.

20-HE, named also moulting hormone, is an ecdysteroid involved in embryo development and metamorphosis in arthropods such as *Drosophila* and *Bombyx mori* (Riddiford, 1993; Subramoniam, 2000; Sekimoto et al., 2006). In *D. magna*, this hormone is essential in embryogenesis processes, reproduction (Subramoniam, 2000; Mu and LeBlanc, 2002) and molting (LeBlanc et al., 1999; Martin-Creuzburg et al., 2007). 20-HE acts through the binding of the nuclear receptor Ecdysone Receptor (EcR), that recognizes the Ultraspiracle protein (USP) (Yao et al., 1993). Conversely, at concentrations ≥125 µg/l, this hormone has negative effects on moulting, fecundity and survivorship (Baldwin et al., 2001). At molecular level, in *D. magna* 20-HE is known to up-regulate specific genes including vtg1 and cut12 (Tokishita et al., 2006; Soetaert et al., 2007a), while Hannas et al. (2011b) observed a down-regulation for gene vtg1 (Hannas et al., 2011b). Ecdysteroids can be released into the water by aquatic plants and invertebrates and can be detected in aquatic environment and can represent a risk because of their hormonal activity (Nakanishi,1992). For this reason, the ecdysteroid 20-HE was selected as a reference compound for substances displaying endocrine interference and was used in mixtures to evaluate the synergistic effect of biocide tributyltin and the antagonistic effects of the insecticides pyriproxyfen and fenoxycarb and the hormone testosterone (Mu and Leblanc, 2002; 2004; Wang et al., 2011)

Information about effects and MOA of glufosinate ammonium have been reported in the introduction to the chapter 4.2 of the present thesis.

Cadmium (Cd) is a non-essential metal without biological role and is toxic for aquatic organisms (US EPA 2001), also known to have mutagenic and pseudo-hormonal effects (Henson and Chedrese, 2004; Thompson and Bannigan, 2008). The effects of cadmium on *D. magna* was widely studied by acute and chronic test (Elnabaraway et al., 1986; Cui et

al., 2018; Sadeq and Beckerman, 2018) and transcriptomic approach (Connon et al., 2008; Poynton et al., 2007; 2008; 2011; Vandenbrouck et al., 2009; Altshuler et al., 2011; Lyu et al., 2014). In *D. magna*, the exposure to cadmium causes negative effects on development, survival, reproduction and embryo development (Soetaert et al., 2007b; Altshuler et al., 2011). This heavy metal has proven to down-regulate vtg1, dhb1 and cuticular genes (Soetaert et al., 2006; Connon et al., 2008; Poynton et al., 2008). The negative effects on reproduction, embryo development and survival was also evaluated on cadmium in combination with zinc, copper and nickel (Meyer et al., 2015; Traudt et al., 2016; Lari et al., 2017; Pérez and Hoang, 2017; 2018). However, effects of contaminant mixtures are still largely unexplored, especially at molecular level, even if it is known that, beside additive effects, they can elicit synergism and antagonism at physiological level.

The exposure of organisms to mixtures can be used to understand the real impact that freshwater samples can cause on aquatic organisms. It is well known that contaminated waters most often represent a mixture, in which various compounds such as pesticides, heavy metals and drugs are present (Moschet et al., 2014). Nørgaard and Cedergreen (2010) and Kretschmann et al. (2015) reported that some water contaminants such as organophosphate/carbamate insecticides in combination with the triazole fungicides prochloraz, epoxiconazole and propiconazole have synergistic effects in *D. magna*.

The molecular responses to the single substances (20-HE, glufosinate ammonium and cadmium) and to their binary mixtures were analysed. The effects of DMSO (dimethyl sulfoxide, solvent used to solubilise 20-HE) were also taken into account.

Material and Method

D. magna cultures

For information about maintenance of daphnid culture see Chapter 4.

Exposure to Cadmium, 20-Hydroxyecdysone and binary mixture of glufosinate ammonium, Cadmium and 20-hydroxyecdysone

The exposure of organisms was carried out using the following concentrations of the single chemicals:

- for cadmium a concentration (2.5 µg Cd/l supplied as $CdCl_2$) slightly higher than the recommended freshwater quality criteria by US-EPA in case of a brief exposure (1.8 µg Cd/l; 1 h exposure);
- for 20-HE (Sigma-Aldrich) the concentration 1 µM, corresponding to 464.63 µg/l,

that induces negative effects under chronic exposure (Baldwin et al., 2001);

- for the herbicide BASTA (Roussel-Hoechst Agrovet S.p.A.), whose active ingredient is glufosinate ammonium (120 g/l), the concentration 0.5 µg/l, expressed as glufosinate ammonium, far below the environmental expected concentration (1 mg/l) for waterbodies subjected to overspray or drift and the EC_{20} for total Cladocera abundance (0.16 mg/l) reported by Faber et al. (1998).

The stock solution of 20-HE was made in dimethyl sulfoxide (DMSO), consequently negative controls with DMSO ware also carried out, both in tests with single substances and in the ones with mixtures. The tested mixtures were as follows:

- 2.5 µg Cd/L + 20-HE 1 µM (in DMSO; <0.01% v/v)
- 2.5 µg Cd/L + DMSO (<0.01% v/v)
- 0.5 µg/L glufosinate ammonium + 20-HE 1 µM (in DMSO; <0.01% v/v)
- 0.5 µg/L glufosinate ammonium + DMSO (<0.01% v/v).

The natural water (NW), used for culturing daphnids, was used as dilution water and control. Fifty juvenile daphnids (4 days old) per treatment and control in 200 ml of solution were used. The exposure time was 24 h. Daphnids were not fed during the test. After treatment, organisms were collected and subjected to extraction and gene expression analysis as reported above. All experiments were conducted at the same ambient conditions as for daphnid cultures.

RNA extraction and quantification, cDNA synthesis and RT-qPCR

For information about RNA extraction and quantification, cDNA synthesis and RT-qPCR see Chapter 4.

Statistical analysis

One way-ANOVA was performed to assess the effect of treatments gene expression. Tukey's test was used for pairwise comparisons and Dunnet was used when variances were not homogeneous between treatments.

Results

In Fig. 4.3.1 the fold change of vtg1 after daphnid exposure to the single substances and their mixtures are reported. DMSO and GA did not modulate the transcription of this gene, while Cd and 20-HE caused down-regulation. The binary mixture Cd+DMSO did not alter the expression compared to Cd alone. A deeper down-regulation was instead observed after exposure to 20-HE+Cd ($p<0.01$) and 20-HE+GA ($p<0.05$) in comparison to Cd and

GA respectively. It seems therefore that the exposure to Cd or GA in presence of the hormone 20-HE causes a more pronounced reduction of vtg1 mRNA.

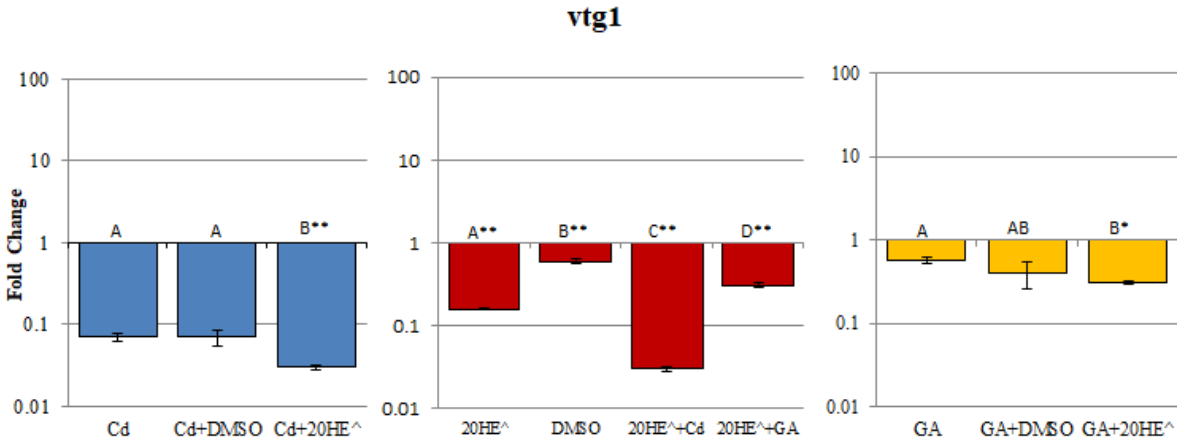


Figure 4.3.1: Transcriptional activity of gene vtg1 after exposure of *D. magna* for 24 hours to cadmium (blue), 20-hydroxyecdysone (red) and glufosinate ammonium (yellow) in single and mixtures.

Cut12 maintained a transcriptional activity comparable to control in daphnids exposed to all the single chemicals and their mixtures (Fig. 4.3.2). Only the solvent DMSO decreased the transcriptional activity of this gene.

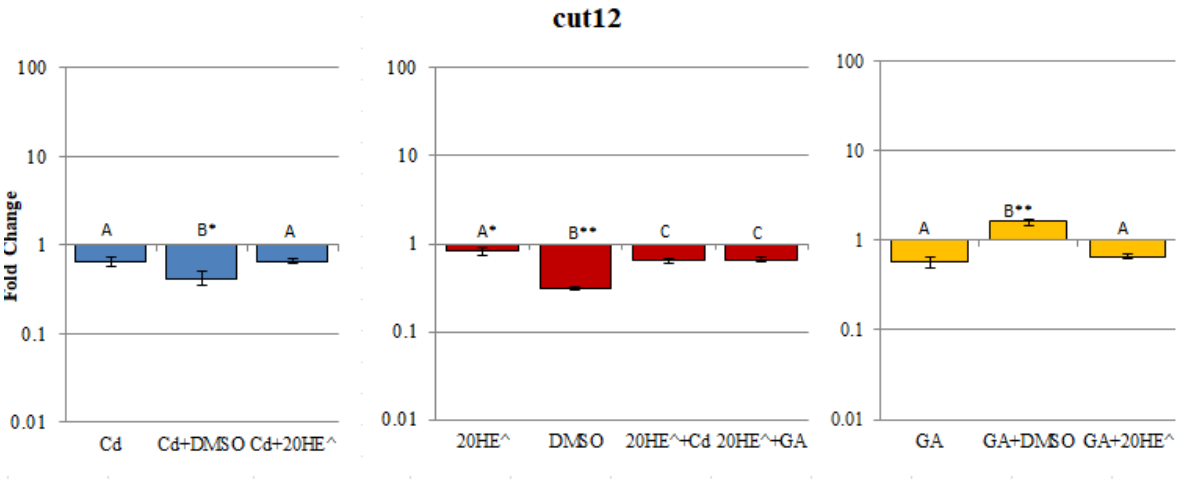


Figure 4.3.2: Transcriptional activity of gene cut12 after exposure of *D. magna* for 24 hours to cadmium (blue), 20-hydroxyecdysone (red) and glufosinate ammonium (yellow) in single and mixtures.

The exposure to Cd or GA caused down-regulation of cpa1 gene (Fig. 4.3.3), however GA had only a slight effect. The down-regulation caused by treatment with 20-HE seems attributable to the solvent DMSO. A deeper down-regulation was observed after Cd+20-HE and GA+20-HE exposure in comparison to Cd and GA alone. As observed for vtg1 expression, the exposure to Cd in presence of the hormone 20-HE caused a more pronounced reduction of cpa1 mRNA. A similar effect was caused by the mixture GA+20-HE compared to GA alone. However, this mixture seems less effective than DMSO/20-HE in down regulating cpa1 expression, suggesting an antagonist effect.

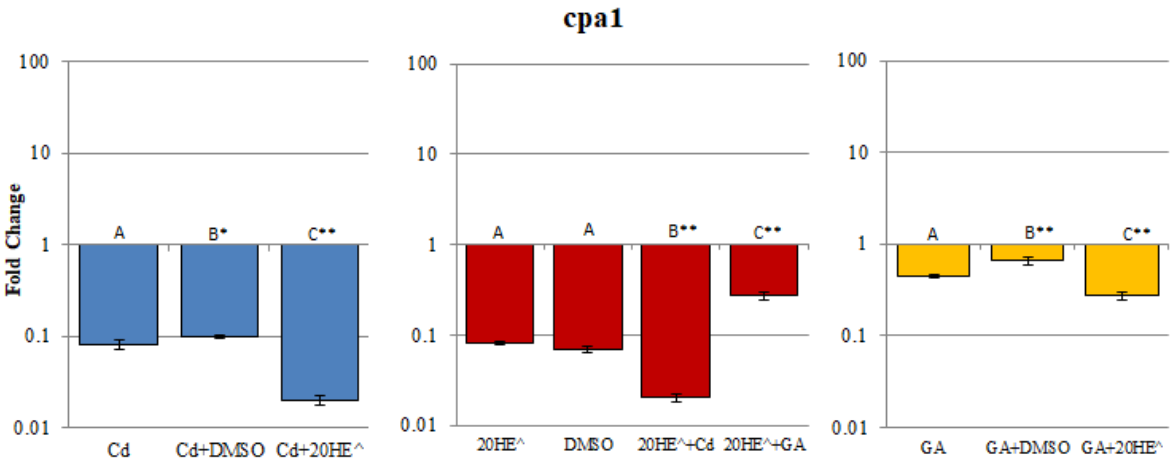


Figure 4.3.3: Transcriptional activity of gene cpa1 after exposure of *D. magna* for 24 hours to cadmium (blue), 20-hydroxyecdysone (red) and glufosinate ammonium (yellow) in single and mixtures.

After exposure to DMSO, Cd or 20-HE, Daphnids showed a dhb1 expression similar to control and a slight down-regulation after exposure to GA (Fig. 4.3.4). All the mixtures caused a deeper down-regulation, with the only exception of GA+DMSO.

No treatment caused up-regulation of the selected genes. Indeed, a basal level of expression or a down-regulation was observed for all genes in daphnids treated with the selected substances, single or in mixtures.

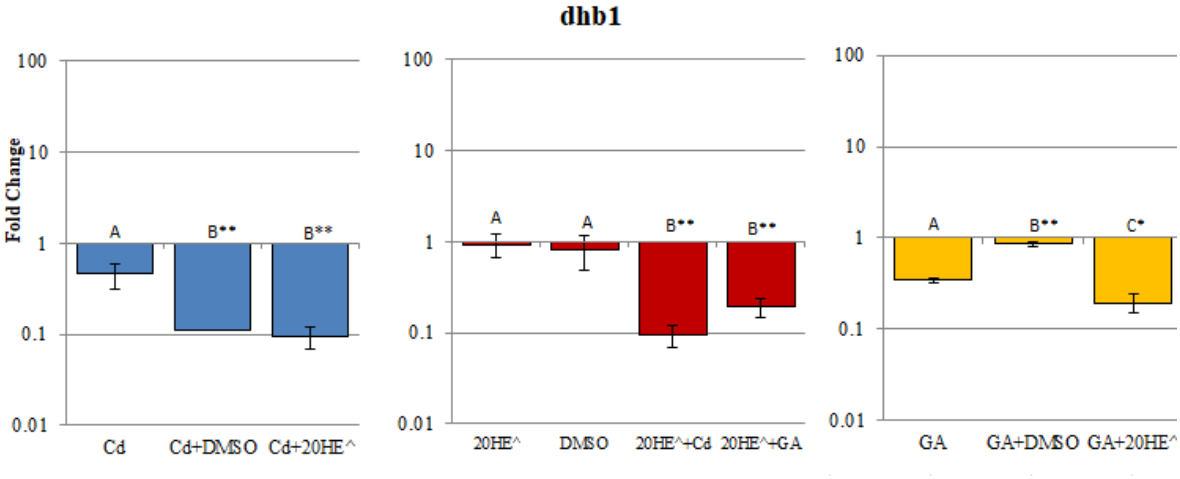


Figure 4.3.4: Transcriptional activity of gene dhb1 after exposure of *D. magna* for 24 hours to cadmium (blue), 20-hydroxyecdysone (red) and glufosinate ammonium (yellow) in single and mixtures.

Discussion

As reported above, in ecotoxicological and ecotoxicogenomic fields, the effects of both single chemicals and their mixtures have to be analysed to understand the real impact and risk due to environmental contamination, because of possible synergistic or antagonistic effects that are difficult to predict on the basis of the effect of the single chemicals (Folt et al., 1999; Chrinstensen et al., 2006; Connon et al., 2012; Petrie et al., 2015). However few studies are available in the literature about gene modulation by mixtures of chemicals.

The exposure of daphnids to the various substances and their mixtures caused a down-regulation or the maintenance of the basal expression of the studied genes. Gene modulation in organisms exposed to the mixtures was greater than that caused by the single substances, with the only exception of the mixture 20-HE^+GA respect to 20-HE^ for the genes vtg1 and cpa1. The gene cut12 was the one less influenced by both the single substances and the mixtures.

Cervera et al. (2005) reported that cadmium reduces vitellogenin production in arthropods. The exposure of *D. magna* to 2.5 µg Cd/l caused the down-regulation of vtg1. This finding is accordance with literature data on phenotypic and molecular responses. The same concentration was reported to induce a reduction of fecundity after 14 days of exposure (Elnabaraway et al., 1986). Vandenbrouck et al., (2009) reported that daphnid exposure to cadmium concentration of 10, 50 and 100 µg/l for 96 hours induces down-regulation of vtg1. In the present study the negative effect of cadmium on vtg1 expression was observed at a lower concentration (2.5 µg/l) and after a shorter exposure time (24 h). At molecular

level, the gene vtg1 is activated by the nucleotide promoters GATA-1 and EcR (Tokishita et al., 2006). Cadmium interfere with both nucleotide sequences (Ermentrout et al., 2006; Shaw et al., 2007); the down-regulation could thus be caused by this molecular control of vtg1 expression. The down-regulation of vtg1 after exposure to 20-HE and cadmium as singles substances was detected in previously studies on *Daphnia* (Soetaert et al., 2007b; Vandenbrouck et al., 2009; Hannas et al., 2011b); however, the detection of gene modulation after exposure to mixtures of 20-HE, cadmium and GA are still unknown. Interestingly, after exposure to Cd or GA in mixtures with 20-HE^, a deeper down-regulation was observed in comparison to Cd and GA alone. Since vtg1 was not significantly modulated by DMSO, this greater modulation can be attributable to the combined effect of Cd and 20-HE, while is questionable in the case of GA+ 20-HE because of the response observed in the treatment GA+DMSO. Considering the negative effect of 20-HE observed in mixtures and that ecdysteroids (such as 20-HE) can be release in aquatic environments (Nakanishi, 1992), the presence of these substances can be regarded as able to increase the environmental risk of other chemicals in surface waters.

The genes cut12 and cpa1 are involved in development of organisms and moulting process (see Gene Boxes). cut12 kept a basal gene expression whatever the substance and the mixture tested, with the only exception of DMSO and Cd+DMSO which caused down-regulation. cpa1 instead underwent deep down-regulation even in daphnids exposed to DMSO. In this regard, similar fold change values were observed after exposure to DMSO (FC=0.06) and 20-HE^ (FC=0.08); therefore it seems that the observed down-regulation in cpa1 is caused by the solvent used to dissolve 20-HE rather than by the hormone itself. It is interesting to observe that daphnids showed quite similar responses of down-regulation between the genes vtg1 and cpa1 (except for DMSO, that causes down-regulation in cpa1 and no effect in vtg1) after exposure to the chemicals in single and mixtures. Unfortunately no information is available about the promoters and transcriptional control of cut12 and cpa1 expression, consequently it is difficult to understand the molecular pathways involved in modulation of these genes by the tested substances.

In *Daphnia*, negative effects on the expression of dhb1 were observed at cadmium concentration of 10, 50 and 100 µg/l after 96 h exposure by Soetaert et al. (2007b) and Vandenbrouck et al. (2009). In the present work a concentration of 2.5 µg/l down-regulated this gene. Consequently, low cadmium concentration and 24 h of daphnids exposure are enough to induce negative effect in dhb1 expression. This effect can be due to the transcriptional control of dhb1, that is regulated by the promoter CCAAT (Kato et al., 2001). In human cells was reported that cadmium can reduce the activity of the protein

C/EBP (CCAAT Enhancer Binding Protein). The down-regulation of dhb1 observed could be caused by the inhibition of C/EBP protein, that do not bind the CCAAT promoter and do not activated the transcription of dhb1. 20-HE did not modulate the expression of dhb1, while interestingly the co-exposure of daphnids to 20-HE and cadmium (or GA) causes down-regulation of this gene. However, it is important to remember that the co-exposure of daphnids to DMSO and cadmium caused a down-regulation of dhb1. Consequently, the effect of down-regulation observed after the exposure to mixture 20-HE and cadmium can be to due to the solvent DMSO.

Interestingly, the gene dhb1 was the only one to show down-regulation after exposure to chemicals, while up-regulation or basal expression were observed after exposure to artificial and natural waters. These results constitute a good premise for the use of dhb1 gene as a potential biomarker for freshwater toxicity monitoring. Ha and Choi (2009) suggested that this gene can be efficiently applied to get information about the health of surface waters.

It is worthy to underline that also DMSO was active in modulating gene expression thus the solvent can not be regarded as a "neutral" substance but rather a further component of the mixtures. Beside down-regulating cut12 and cpa1, it enhanced down-regulation of cut12 and dhb1 in combination with cadmium. It can also act by reducing the effect of the single substance as in the case of the mixture GA+DMSO and, probably, Cd+DMSO for the gene cpa1, since the down-regulation caused by this mixture was well limited respect to the expected effect on the basis of the responses to the single substances. According to Haap et al. (2008) and David et al. (2012), DMSO is a solvent that can act by changing the toxicity and the availability of chemicals and can have a negative effect on reproduction of daphnids reproduction (Zhang and Baer, 2000). In future, studies on organisms after exposure to solvents can improve the choices on their use in ecotoxicology and ecotoxicogenomics.

Chapter 5: Discussion and Conclusion

Here we evaluated several aspects of phenotypic plasticity (e.g. morphological changes, male and ephippia production, fecundity) in different clones of 2 species of *Daphnia* (*D. pulex* and *D. magna*) and we tried to bridge the gap between molecular and ecological aspects of *Daphnia*'s life cycle. The involvement of genetic and epigenetic factors (maternal effect via hormonal signals and microbiome) in the modulation of phenotypic and transgenerational plasticity in response to specific environmental cues was investigated. The use of molecular tools linked with the study of *Daphnia* life history features allowed to increase our knowledge about molecular and cellular genetics, recombination and sex, hormonal regulation, gene control and transcription, gene co-expression networks, mutation, intron evolution, aging, epigenetics, sex determination and development (Shiga et al., 2002; Omilian et al., 2006; Li et al., 2009; Kato et al., 2011; Colbourne et al., 2011; Eads et al., 2012; Harris et al., 2012; LeBlanc et al., 2013; Mahato et al., 2014; Schumpert et al., 2014; 2015a; Toyota et al., 2015a; LeBlanc and Medlock, 2015; Keith et al., 2016; Spanier et al., 2017; Ye et al., 2017; Orsini et al., 2018). In this context, *Daphnia* represents an ideal model organism for phenotypic plasticity and by molecular tools (e.g. analysis of gene expression and SNPs, NGS next-generation sequencing technologies) we tried to link variations in gene expression with specific plastic traits such as morphological defences (neckteeth), males and ephippia production propensity. In the end, we showed how our results and our approaches might be used to establish and to improve biological assay systems for screening the hormonal activities of chemicals and endocrine disruptors in surficial waters.

Seasonal rhythms, natural or anthropogenic environmental changes and stimuli, but also endogenous signals induce/modulate gene transcription that leads to adaptive changes (in neuroendocrine function). Seasonal change in photoperiod provides a predictive strategic cue and induces a wide variety of hormonal signals that regulate the timing of gene transcription. One challenge for the delineation of the mechanisms that govern genomes and plasticity include disentangling the relative contribution of environmental cues and internal hormonal signaling pathways (Stevenson, 2017). Changes in embryo programming due to alterations of the hormonal environment both from maternal influences, or due to maternal exposure to environmental chemicals and drugs, are generally considered to be caused by disruptions or alterations in hormonal regulation of epigenetic programming events (LeBlanc et al., 2013). Various components of the epigenetic mechanism are under the control of hormones and embryo exposure to hormones or their mimics may affect epigenetic modifications of several genes (Zhang and Ho, 2011). Although genetics

determine endocrine phenotypes, evidence now suggests that epigenetics (i.e. heritable but reversible changes in gene function without changes in nucleotide sequence) links genetics and environment in shaping endocrine function (Zhang and Ho, 2011). Mechanisms that regulate developmental hormones can mask genetic variation and *vice versa*. Last but not least, microbiome and microbial metabolites can influence epigenetics by altering, directly or indirectly, the activity of enzymes involved in epigenetic pathways (Hullar and Fu, 2014).

In Chapter 2, we showed how both exogenous and endogenous Methyl Farnesoate (a juvenile hormone), produced in stressful conditions, are involved in the regulation of the morphogenesis of the defensive form of *D. pulex* and in male and ephippia production. Variation in the frequencies of morphotypes with neckteeth and in the propensity to produce males are reasonably linked to flexibility in developmental responses of genetically different *D. pulex* clones to experimental conditions. However, in clonal lineages of *D. pulex,* transgenerational maternal (epigenetic) effects are involved in neckteeth development by the endogenous production of MF under environmental stimuli that may act as proxies of seasonal variations in the predation risk from Chaoborus, even without the presence of kairomones. Actually, D. pulex can produce morphological defences and/or altered life-history traits as a response to the presence of predators. For instance, there is evidence that *Daphnia* exposed to kairomones, can increase the rate of development and invest more heavily in reproduction (Stibor, 1992; Riessen, 1999; Walsh et al., 2015). *Daphnia* may use ephippial production as an escape strategy from predation pressure (Ruvinsky et al., 1986; Larsson, 1991; Spitze, 1992; Hairston, 1996). *D. magna* produces ephippia in the presence of kairomones and alarm chemicals originated from injured conspecific prey (Slusarczyk, 1995; 1999). In our experiments, particularly in clones S and K reared at 16 °C and 12:12 L: D photoperiod (see Chapter 2.1), we observed a switch/trade-off between the production of neckteeth and ephippia. The juvenile hormones produced in response to environmental stress stimuli, such as changes in photoperiod, food shortage and crowding, might be associated with the activation of genes involved in neckteeth development, as well as in sex determination and ephippia production (Suzuki and Nijhout, 2008; Tollrian et al., 2015; Christjani et al., 2016). Juvenile hormones are key factors in the regulation of development; however, little is known about other functions related to physiological regulation. To our knowledge, no direct or indirect effects on ephippial production have been reported yet (Miyakawa et al., 2010). Interestingly, on average, clone I produced the highest percentage of neckteethed juveniles and did not produce any ephippium, while clone S produced the lowest

percentage of induced juveniles and the highest percentage of ephippial clutches. Thus, individual clones seem to be specialized either in the production of juveniles with neckteeth or ephippia.

In Chapter 2.2, we showed a down-regulation of the DNMT3A gene after exposure to MF 0.8 µM in clone I, the one with the lowest inclination to produce males. DNMTs are key epigenetic modifiers of the genome; DNMT3A enzyme adds methyl groups to DNA causing hyper-methylation, heterochromatin formation and silencing of transcription (Chen and Riggs, 2011; Moore et al., 2013). Down-regulation of the DNMT3A gene after exposure to MF should confirm that transcriptional activity, probably of genes induced by MF and involved in male production (JHAMT, Met, dsx1 and, probably RXR), is in working progress. Actually, no information is available on the transcriptional activity of DNMT3A in response to changes in environmental conditions. The down-regulation of DNMT3A we observed is in accordance with the idea that epigenetic control through DNA methylation/de-methylation is involved in the control of male production in *D. pulex*. Studies that try to evaluate the maternal effect by the transfer of specific mRNAs or proteins to oocytes are still only a few. The presence of RXR mRNA in the ovaries of the mud crabs *Scylla serrata* and *S. paramamosa* was proven (Girish et al., 2015; Gong et al., 2016). Nong et al. (2017) suggested that, in daphnids, dsx1 mRNA is probably transferred in the developing oocytes by the mother. Actually, the maternal effect of the transfer of specific mRNAs or proteins to oocytes could explain the lower responsiveness and gene up-regulation in F_1 than in F_0 juveniles of *D. pulex* to MF.

Our results support the hypothesis that both exogenous and endogenous MF produced in stressful conditions may activate the expression of genes involved in the developmental processes and might contribute to the plasticity of various life-history traits. Maternal effect might activate the expression of endocrine genes (Insuline-like receptor-InR, Insuline Receptor Substrate 1-IRS-1) and others involved in the development of neckteeth (e.g. morphogenetic genes (Hox3, extradenticle-exd). The analyses of genes involved in reproduction such as vitellogenin might explain the positive effect of DMF (solvent control for MF) that we observed in the fecundity of *D. pulex* (Hutchinson et al., 2006; Hannas et al., 2011b). Again, Roulin et al. (2016) showed that, in *D. magna*, ephippial production is initiated by the activation of a G protein-coupled receptor (rhodopsin), which is part of cascading gene regulatory networks involved in switches from asexual to sexual reproduction. Roulin et al. (2016) were unable to elucidate functional aspects of the link between genotype and phenotype; however, the finding of a photoreceptor gene influencing a trait induced by a photoperiod cue provides a functional hint and fits

prediction. Expression analysis of these candidate genes awaits further exploration (Miyakawa et al., 2010; Hales et al., 2017). Last but not least, the integration of transcriptome studies on methyltransferase *de novo* activity (DNMT3A) and the modulation of all genes mentioned above can bring further information on the role of epigenetic modifications in phenotypic plasticity.

Our study on the multigeneration exposure to MF provides further insight into the involvement and manner in which MF might regulate daphnid population dynamics, an involvement also suggested by Olmstead and LeBlanc (2001) and LeBlanc et al. (2013). Crowding, as well as food restriction and maternal exposure to MF cause a delay in sexual maturity, a decrease in fecundity and an increase in the percentage of males in offspring. According to Booksmythe et al (2018), *D. magna* females produce more males when population density is high, but when male proportion increases (high adult sex ratio) the response dampens and the likelihood of producing ephippia increases. We showed that, in *D. pulex* clones with a low propensity to generate males, multigenerational exposure to MF caused a reduction of male percentage in the progeny and induced ephippium production. Intriguingly, the effect of MF on male production, age at maturation and fecundity might give a meaning, in molecular terms, to the general framework of the cost of sex and to the linkage between male production and population density in *Daphnia*.

In Chapter 3, we analysed genetic and plastic responses to photoperiod length in different lineages, we tried to validate the genetic basis of male formation in *D. magna* and to evaluate the role of microbiome in resistance to glyphosate, a largely used herbicide. Moreover, by an integrated approach based on microbiome and host transcriptome analysis we tried to understand the link between bacteria community composition and host gene modulation and its role in organism responses to environmental stressors.

In Chapter 3.1 we performed gene-trait association analysis (GWAS) between 15 candidate genes associated with environmental driven local adaptations, including sex induction, and the life history traits measured in 31 clonal lineages of *D. magna* during a transgenerational plasticity experiment. Clonal lineages were resurrected from the sediment archive of a shallow mixed Danish lake, Lake Ring, with a history known for an increase in the average temperature and with a historical record of anthropogenic impact (Orsini et al., 2016). We showed differences in fecundity among clones, in response to early spring warming (warm temperature and short photoperiod) and significant plastic response to photoperiod change. Shorter photoperiod prompted the majority of clones to invest in male offspring but differences in male production were recorded among clones. In addition to this genetic response, we observed a significant plastic change in fecundity

across clones and trade-offs between, within and across generation plasticity. This result can indicate that within-generation phenotypic plasticity has a much stronger influence on the response of *Daphnia* to environmental cues than transgenerational plasticity, as suggested also by Kuijper and Hoyle (2015). Among 31 clones with different life history traits and different propensity in giving birth to males, we identified 7 genes that showed SNPs (see Table 3.1.1 in Chapter 3.1). According to Roulin et al. (2016) and Reisser et al. (2017) these 7 genes code for proteins like the zinc transporter (zip11A) and are involved in signal transduction (EGFR-epidermal growth factor receptor kinase and rhodopsin), in detoxification and hormonal regulation (AKR1-aldo-keto reductase family 1, member C4), in transcriptional activity and epigenetic control (histone deacetylase complex subunit sap18 and lysine-specific histone demethylase 1A) and in splicing processes (serine arginine-rich splicing factor 7). By GWAS we tried to identify associations between genetic regions (loci) and phenotypic traits, including male production propensity. We identified 2 genes (histone deacetylase complex subunit sap18 and zip11A) on a chromosomic region (scaffold02569) that Reisser et al. (2017) named supergene LG6, that includes 8 genes involved in the production of males, and co-evolved in *D. magna* at population level. According to Roulin et al., (2013), the production of males and ephippia are controlled by different QTL regions: LG6 for male production and LG10 for ephippia production. Tatarazako et al. (2003) and Toyota et al. (2015a) suggested that aldo-keto reductase AKR1, that catalyse the conversion of farnesal into farnesol (a precursor of MF), was significantly associated with the propensity to produce male offspring in *Daphnia.* Mutations in the AKR1 gene have been shown to cause an increase in MF levels (via the block of MF degradation pathway) resulting in the highest male production (Rivera-Perez et al., 2013; Toyota et al., 2015a). Expression analysis (RT-qPCR) of AKR1 and of the other two genes involved in epigenetic control (histone deacetylase complex subunit sap18 and lysine-specific histone demethylase 1A) in clonal lineages, could explain the relationship between SNPs on these genes and differences in sex determination among clones.

In Chapter 3.2, we aimed at discerning the role of the microbiome in the response of daphnids to common pesticides with the analysis of 4 clones in 6 treatment conditions including exposure to environmentally relevant concentrations of both roundup and glyphosate, along with a control, all with and without the treatment with antibiotics. The detrimental effects of exposure to antibiotics observed in all clones confirmed the importance of the microbiome in the survival and fitness of *Daphnia* (Gorokhova et al., 2015; Mushegian and Ebert, 2017) and it did not allow the evaluation of the direct effect of

the microbiome on survival and fecundity in response to glyphosate. However, we showed significant differences in the tolerance to the herbicide among genetically different clones: clone 13_5-1 was the most resistant while the clone 13_2 was the most sensitive. Both clones were awoken from dormant embryos which date back to the 1960s with no exposure to pesticides (Cambronero et al., 2017). With NGS (Next-Generation Sequencing technologies), we showed that gene expression of different clones in different treatments was mainly governed by the genetic background and by the removal of the microbiome than by exposure to the pesticide. The clone 13_5-1 showed responses quite different from the other clones, suggesting that microbiome might affect its resistance to glyphosate and Roundup. According to the MODA analysis, different clones in different treatments showed different gene co-expression pathways. For instance, in response to glyphosate, the most susceptible clone 13_2 showed the activation of the glycosyltransferase activities in the Golgi apparatus while the most resistant clone 13_5-1 showed the activation of genes for a transferase. As a consequence of the differences in gene co-expression pathways among clones in different treatments, clonal differences in life history traits and in responses to antibiotics or to glyphosate might be linked with the expression of both known and unknown genes.

The exposure to glyphosate induced the modulation of several genes homologous to human oncogenes (e.g. NF2, KSR2 RUNX1, ALK, RBL1, TNK2, MAP4K3) involved in leukemia, linfoma, large-cell lymphomas, myelomas and neuroblastomas (Asou, 2003; Webb et al., 2009; Siveen et al., 2018). The gene RBL1 codes for a cancer suppressor protein involved in cell cycle regulation (Ewen et al., 1991). This result should highlight and confirm the carcinogenic effect of glyphosate (EFSA - European Food Safety Authority, 2015; IARC – International Agency for Research on Cancer, 2015).

In Chapter 4 we showed how a transcriptomic approach may be employed in ecotoxicological studies and in screening assays to evaluate endocrine disrupting potential and the environmental risk of chemicals and a combination of them. We evaluated the expression of 4 genes involved in the expression of phenotypic traits such as reproduction (vtg1), growth and development (cut12, cpa1) and in the red colour of *Daphnia* due to hemoglobin production (dhb1) (Poynton et al., 2007; Watanabe et al., 2007; 2008; Connon et al., 2008). We showed that differences in water chemistry can influence gene expression and that gene expression can rapidly vary in response to variations in water characteristics. This result suggests the use of a reference water in future, inter-laboratory calibration in ecotoxicogenomic approaches for freshwater monitoring.

The exposure of daphnids to glufosinate ammonium at three different concentrations (0.6, 6 and 60 µg/l) did not cause negative effects in reproduction but exposure to the lowest concentration caused down-regulation of genes cpa1 and dhb1. These results suggest that glufosinate ammonium can represents a risk factor for aquatic invertebrates and that the transcriptomic approach can bring information about the early negative effects at low chemical concentrations. The negative effect of exposure to chemical mixtures was higher than an exposure to the single substances. In particular, the down-regulation due to the exposure to Cd or glufosinate ammonium in mixtures with 20-hydroxyecdysone (20-HE) was increased. Ecdysteroids, such as 20-HE, are natural hormones released into the water by aquatic plants and invertebrates and they represent a risk because of their endocrine disrupting properties (Nakanishi, 1992). Our results confirmed the synergistic negative effect of 20-HE (Mu and Leblanc, 2002; 2004; Wang et al., 2011). Among the 4 genes analysed by RT-qPCR, dhb1, coding for hemoglobin, was the only one that showed up-regulation or basal expression after exposure to artificial and natural waters and down-regulation after exposure to chemicals (glufosinate ammonium, Cd and 20-HE). In accordance with Ha and Choi (2009) which suggest hemoglobin as a potential biomarker, our result indicated that dhb1 has the most valuable potential biomarker for screening assays in freshwater toxicity monitoring.

In the present study we used both the analysis of single gene expression (RT-qPCR) of genes directly involved in MF signaling (JHAMT, Met, dsx1, RXR) and epigenetic control (DNMT3A) (Chapter 2.2) and involved in reproduction, growth (vtg1, cut12, cpa1, dhb1) (Chapter 4) and whole transcriptome analysis (RNA-seq) (Chapter 3.2). The RT-qPCR allows a more specific and sensitive evaluation of gene expression of specific molecular pathways than RNA-seq. On the other hand, the RNA-seq allows to evaluate the overall transcriptional activity (Wang et al., 2009) and provides some functional identity to still unknown genes which, in a pathway, would share the same biological functions. Recently, Montes and collaborators (2017) developed a simple method to detect the endogenous MF concentration through chromatography – mass spectrometry in arthropods. The application of this method, along with whole transcriptome analyses, (RNA-Seq) represents a future goal to understand the effect of juvenile hormones on the life history traits of *Daphnia*. The rapid advances in technology for DNA analysis and miRNA profiling (Kong et al., 2009) and the advent of next-generation sequencing technologies (Metzker, 2010) will deepen our understanding of the interplay of genetics and epigenetics with the environment, enhancing our knowledge at molecular, individual and population levels (Zhang and Ho, 2011).

The effect of MF on the production of ephippia has not been yet reported, but our results suggest that both sex ratio regulation and sexual egg (ephippia) production may be modified by this hormone (Kaupinis et al., 2017; Navis et al., 2018). Actually, Navis and colleagues (2018) reported a null effect of fenoxycarb (an analog of MF) on the number of ephippia produced by *D. magna*. However, in the experiment of Navis et al. (2018), daphnids produced a high number of ephippia in the control too. In general, we want to emphasize that, the use and comparison of clonal lineages that showed (basal) differences in life history traits (e.g. propensity to produce males and ephippia) are essential to disentangle the genetic and epigenetic effects of environmental factors (e.g. hormones) and to evaluate the genetic x environment interaction.

Toyota et al. (2017) suggested that the regulation of gene expression might depend on the life stage and tissue. Actually, none of the genes up-regulated in the present study matched the up-regulation levels detected by Toyota and collaborators (2017) after exposure to MF in 42 modulated transcripts (32 genes down-regulated and 9 genes up-regulated). The different life-stages in which the mRNA extraction was performed, along with the ovary of *Daphnia* by Toyota et al. (2017) and all the 96 hours old juveniles in the present study could explain the discrepancy between the results. MF caused up-regulation of the haemoglobin gene in adults of *D. pulex* and *D. magna*, while the same concentration (200 ppb) caused down-regulation of the same gene in the ovaries of *D. pulex* (Hannas et al., 2011b; Toyota et al., 2015a; 2017). These observations demand standardization but, at the same time, they suggest that new different insights might come for the analysis of different tissues and organisms of different ages. As well, the analyses of differential gene expressions in different tissues in individuals with different ages from the same clonal lineages will shed more light on anticipatory gene regulation (Nespolo et al., 2015).

Regarding our initial questions, we showed that different clones use different molecular pathways to cope with environmental changes: we observed differences in transciptomics pathways among *D. magna* clones exposed to glyphosate and Roundup (Chapter 3.2). Moreover, we showed differences in hormonal regulation and gene expression, involved in phenotypic plasticity, among clones: in *D. pulex* we reported differences in gene expression in response to juvenile hormone (Chapter 2.2) and in *D. magna* we recorded differences in resistance to glyphosate and Roundup (Chapter 3.2).

Appendix A – Components of High Hardness COMBO medium

Table 1A-Compounds which make up High Hardness COMBO media (adapted from Baer and Goulden, 1998).

No.	Compound	Chemical Formula	Stock (g/L)
1	Calcium chloride dihydrate	$CaCl_2 \cdot 2H_2O$	110.28
2	Magnesium sulphate heptahydrate	$MgSO_4 \cdot 7H_2O$	113.5
3	Potassium phosphate dibasic	K_2HPO_4	1.742
4	Sodium nitrate	$NaNO_3$	17
5	Sodium metasilicate nonahydrate	$Na_2SiO_3 \cdot 9H_2O$	13.27
6	Boric acid	HBO_3	24
7	Sodium bicarbonate	$NaHCO_3$	63

Table 2A- Compounds which make up the animal trace elements (ANIMATE) solution (adapted from Baer and Goulden, 1998).

Compound	Chemical Formula	Stock (mg/L)
Lithium chloride	LiCl	0.31
Rubidium chloride	RbCl	0.07
Strontium chloride hexahydrate	$SrCl_2 \cdot 6H_2O$	0.15
Sodium bromide	NaBr	0.016
Potassium iodide	KI	0.0033

Table 3A- Compounds which make up the vitamin stock solution (VIM) solution (adapted from Baer and Goulden, 1998).

Compound	Concentration
Biotin (d-biotin)	10 mg into 96 ml dH_2O
B12 (cyanocobalamin)	10 mg into 89 ml dH_2O

Appendix B – Trace elements solutions for ELENDT M4 artificial medium (OECD, 2012)

No.	Salt	g in 50 mL of bidistilled H_2O	µl/L of NW
1	H_3BO_3	0.286	500 µL
2	$MnCl_2$	0.360	50 µL
3	LiCl	0.306	50 µL
4	RbCl	0.071	50 µL
5	$SrCl_2 \cdot 6H_2O$	0.152	50 µL
6	NaBr	0.016	50 µL
7	$NaMoO_4 \cdot 2\ H_2O$	0.063	50 µL
8	$CuCl_2\ 2 \cdot H_2O$	0.017	50 µL
9	$ZnCl_2$	0.013	50 µL
10	$CoCl_2\ 6 \cdot H_2O$	0.010	50 µL
11	KI	0.325	Dilute 1:100, 50 µL
12	Na_2SeO_3	0.219	Dilute 1:100, 50 µL
13	NH_4VO_3	0.0575	Dilute 1:100, 50 µL
14	$FeSO_4 \cdot 7\ H_2O$	0.0995	500 µL (only for Control)

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Scientific communications

Journal articles

Suppa A., Pellegri V., Montalbano S., Gorbi G., Buschini A., 2017. Metodi ecotossicogenomici nel monitoraggio degli ambienti acquatici: modulazione dell'attività trascrizionale in *Daphnia magna*. *Biologia Ambientale*, 31:211-217.

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