Multigenerational effects of diet and temperature on size and fitness associated traits in *Daphnia*

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Abstract

This thesis presents a synthesis of experimental studies with *Daphnia*, in a multigenerational perspective. The main goal of the thesis was to investigate the role of diet (phosphorous (P) limitation) and temperature (10 ˚C, 20 ˚C) on various fitness-related traits, as well as diploid DNA content (2C-value). For a wide range of ectotherms, it has been demonstrated that individuals reared under reduced temperatures obtain larger body size. *Daphnia* may have a mixed strategy for this, i.e. they may increase their body size both by increasing cell size and cell numbers. It is further hypothesized that temperature and phosphorus limitation can drive changes in genome size, which in many cases scale with cell size and body size.

To explore these ideas, 2C-value was estimated in two *Daphnia* species using flow cytometry. Significant genome size differences were found between the two species. *D. pulex* has a smaller genome compared to *D. magna*, and the estimates align well with previous studies. Additionally, differences were found in 2C-value between the two temperature regimes. However, this came along with increased coefficients of variance (CV), which is further discussed in the thesis. This study did not find evidence for the hypothesis that diet in respect to C:P ratio can induce changes in 2C-value over a modest number of generations.

The growth rate hypothesis predicts positive relationships among growth rate body RNA and body P (% of dry mass). This study tested this within- and across-species by investigating the experimental lines reared in 20 ˚C under a “common garden” experiment with reciprocal transplantations. Additionally, the level of the enzyme alkaline phosphatase (AP) was measured in the daphnids, since it is hypothesized to be involved in P-metabolism in *Daphnia*. The present study find strong support for the growth rate hypothesis, and could reveal maternal and line effects for *D. magna* in some of the measured traits. Further, this study finds evidence for a significant decrease in AP activity in P-stressed daphnids.

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Contents

1 Introduction

Size in one of the key metrics of life, and has bearings on major issues in ecology and evolution, both at the inter- and intraspecific level. Within a life span, organisms can grow either by increasing their cell volume or cell number – or a combination of both (Kozlowski et al., 2003). Cell size scales with fundamental properties such as genome size, growth rate, protein synthesis and metabolic activity, although the causal direction of these correlations is not settled (Gregory, 2005).

Intraspecific variation in ecologically important traits can be transmitted both on gene level as well as via maternal environmental effects, where differences in the environmental conditions experienced by previous generations consistently affect the phenotype of their progeny (Roach &Wulff, 1987). Flexible traits come at some costs, but for organisms living in variable environments, the ability to make rapid adjustments is vital (Hessen et al., 2012). In this study both the effects of temperature and diet in terms of its nutritional quality were investigated using the clonal model organism *Daphnia*. One aim was to reveal variation within the clones after multiple generations of divergent environmental conditions. In fact, studies that implement this longer time perspectives are scarce. This thesis also aims to shed some light on the nature of a character at the bottom of an organism's organization – the genome. And boldly dear to test one hypothesis suggested to account for variation in the size of this character.

Growth rate

Growth rate (GR) is a good predictor of response to changing environment. GR defines the relationship between growth and age, is a key life-history trait that correlates to other vital traits (e.g. metabolic rate, fecundity, life-span) and is an important predictor of fitness (Arendt, 1997). While being a complex and inheritable trait, typically affected by many genes, plasticity in GRs is an apparently universal feature of animal life histories (Abrams &Rowe, 1996), and in most organisms also a dynamic trait that rapidly can change in response to environmental variation (Dmitriew, 2011). The major ecological factors influencing GRs include temperature, food quantity and/or quality, predation (Roff, 1992, Stearns, 1992) and time constraints (Werner &Anholt, 1993, Abrams et al., 1996). Typically, animals mature both earlier and at a lager size at high food levels, while when resource level

declines, animals tend to grow slower, reaching maturity later and at smaller size (Dmitriew, 2011).

Biological stoichiometry, which is the study of the balance of multiple chemical elements in living systems (Sterner &Elser, 2002), established that GR is tightly coupled to the carbon (C), nitrogen (N) and phosphorus (P) content of an organism, as they are the main constituents in macromolecules (Sterner &Elser, 2002, Weider et al., 2004). C typically constitute close to 50 % of dry mass of organisms, N typically 5 - 10 %, while P often constitute 1 % or less of dry mass (Sterner &Elser, 2002). Both N and P are crucial for protein synthesis (N- as amino acids or peptides, P as ribosomes) and thus GR, and herbivorous consumers generally have higher mass-specific N- and P-contents than that of their plant or algal food creating the potential of food quality limitation (elemental imbalance) with negative effect on consumer growth (Hessen, 1992, White, 1993, Sterner &Elser, 2002). In ecosystems, food with unbalanced elemental composition can further influence the community dominance of consumer taxa with different characteristic C: N: P (Main et al., 1997, Elser &Urabe, 1999), emphasizing the fundamental coupling of energy and material in food webs (Sterner et al., 1998).

The mechanistic basis for variation in stoichiometry in living systems is well understood (Elser, 2002). A number of studies on prokaryotes to invertebrates have demonstrated that GR is positively related to RNA and P concentration (in various *Daphnia* species (Demott et al., 1998, Vrede et al., 1999, Acharya et al., 2004), the bacterium *E. coli* (Makino et al., 2003), cyanobacteria (Lepp &Schmidt, 1998), yeast (Aiking &Tempest, 1976), algae (Rhee, 1978) and other biota (Elser et al., 2003)). Nucleic acids are very P rich, with the highest % P value of the major classes of compounds that contribute significantly to biomass (Sterner &Elser, 2002). Ribosomes, the macromolecular machines that synthesize proteins in all living organisms are composed of ribosomal RNA (rRNA) and ribosomal proteins. Ribosomes are the most P rich and lowest N: P organelles in cells (Sterner &Elser, 2002). As much as 85 % of the bulk RNA can be ascribed to rRNA (Alberts, 1983). It is estimated that approximately 60 % of all cellular transcriptional activities in rapidly growing yeast cells can be attributed to the synthesis of rRNA (Warner, 1999), which is not only costly in terms of energy used to create them but also in demands of P, especially when P is limited. This strong coupling between specific GR, RNA and P, well documented in a number of organisms, has been formalized as the growth rate hypothesis (GRH). It predicts that low C:P

and N: P ratios in rapidly growing organisms reflect increased allocation to P-rich rRNA, as rapid protein synthesis by ribosomes is required to support fast growth (Hessen &Lyche, 1991, Elser et al., 1996, Elser et al., 2000).

There is also evidence for GR regulation at the genome level by increasing the rDNA copy number (Prokopowich et al., 2003) and regulating rDNA intergenic spacer (IGS) length variation (Gorokhova et al., 2002). These two mechanisms have been shown to be potential targets of both artificial and natural selection (Weider et al., 2005), highlighting the potential of linking subcellular and genetic processes with the evolution of major life-history traits. The fact that changes in IGS-length was induced after only 5 generations in Daphnia (Gorokhova et al., 2002) also suggest hat such traits with bearings on GR can be rapidly selected for.

Daphnia experiences growth retardation as a direct effect of P-limitation whenever molar C:P in their diet crosses the threshold 300 (Hessen, 1992, Urabe et al., 1997). Particularly, the susceptibility to poor food quality, as evident in many rapidly growing invertebrate taxa, is linked to their lack of mineral storage of P (as in vacuoles, crystals or bones) (Elser et al., 2000). Experimental evidence has shown that P release by *Daphnia* continues at a significant rate even when P is severely depleted in the diet (Demott et al., 1998), implying a role of nutrient elements in maintenance. In an environment where access to P can constrain growth, obtaining sufficient quantities of this element and prevent rapid loss is critical, and one would predict strong evolutionary drivers to maximize P-acquisition. Alkaline phosphatase (AP) is an enzyme that cleaves the phosphate group from organic P monoester substrates, and is commonly expressed by microbes in response to P limitation (Perry, 1972, Jansson et al., 1988, Thingstad &Mantoura, 2005). The enzyme is also produced and excreted by metazoans (Wojewodzic et al., 2011), and the AP expression can thus serve as an indicator on P-limitation across phyla. AP has been found to be over-expressed in both *Daphnia magna* and *Daphnia pulex* when feeding on algae of high C:P ratio (Mccarthy et al., 2010, Wojewodzic et al., 2011, Wagner &Frost, 2012), supporting the hypothesis that this enzyme is involved in P-metabolism in *Daphnia*. However, the physiological function and regulation mechanism of AP within *Daphnia* bodies over a longer time of stressful conditions in terms of P-limitation remain unclear.

The fact that organisms with fast growth like *Daphnia* may suffer chronic P-limitation should pose an evolutionary adaptation towards an efficient P-household or P-economy. More precisely, the organisms should maximize the P-allocation to RNA to boost protein synthesis

(and thus growth), and one potential source of P could in fact be the non-coding elements of DNA. It is thus interesting to look into patterns and regulators of genome size, and how this may relate to GR.

Genome size in organisms

The first broad-scale survey of animal genome sizes (Mirsky &Ris, 1951) came with the surprising news that there was no simple correlation between organism complexity and the genome size (total amount of nuclear DNA). Many apparently simple organisms could have over a thousand times more DNA than presumed more complex multicellular organisms. The total decoupling of phenotypic complexity and genome size was coined the "C-value paradox" (Thomas, 1971). This paradox was partly explained by the discovery that the great majority of eukaryotic DNA content variation was due to variation in the amount of non-coding DNA. The genome sequencing projects also conclusively confirm that differences in genome size are not due to variation in numbers of genes. Overall, across eukaryotes the number of genes varies by approximately 10-fold, in sharp contrast to a greater then 200,000-fold variation in total genome size (Gregory, 2005). Still, this striking variation in genome size and its proximate and ultimate causes for the variable portions of non-coding DNA are largely unresolved, and genome size evolution is now more accurately portrayed as a "C-value enigma" (Gregory, 2001). Several new and independent component questions arise (Gregory, 2001, Gregory, 2002, Gregory, 2004). Which type of non-coding sequences predominates in genomes? Which evolutionary forces are responsible for the extraordinary variation in its amount? What impacts, if any, does this non-coding majority have on the cellular and organism phenotype? Does the non-coding DNA have any function? Why do some groups have so much non-coding DNA, while others have remarkably streamlined chromosomes?

Although, the large diversity in genome size often is considered to be adaptive, population-genetic theory suggests that non-adaptive parameters is sufficient to explain the evolutionary diversification of many aspects of genome sizes found among phylogenetic lineages (Lynch et al., 2011). Under the mutation-hazard model variation in genome size and contents of genomes have been considered to emerge as a consequence of the differential degree of genetic drift. Under this model, species with small effective population sizes have larger genomes because they can tolerate the slightly deleterious accumulation of extra DNA

in the form of transposable elements, multiple introns, and gene duplications (Lynch, 2002, Lynch &Conery, 2003).

Under the traditional selfish DNA theories, the relationship between genome size and cell size is considered purely coincidental (Charlesworth et al., 1994). But this type of explanation has considerable difficulty explaining why the relationship between genome size and cell size should persist across such a wide range of variation (Gregory, 2005). In regard to vertebrates this correlation holds within each of the major classes i.e., fishes, amphibians, reptiles, birds and mammals (Gregory, 2005). A general correlation between cell size and nuclear size appear nearly universal. Additionally, haploid and polyploid cells are typically smaller and larger, respectively, compared with their diploid counterparts. There are several theories proposed to explain the relationship by applying a causative link. *The nucleotypic theory*, postulate a causative link at the nuclear level (Commoner, 1964), where bulk DNA (besides from DNAs obvious coding function) exerts an influence on the cell's size and metabolism. In this view one can hypothesize that nuclear size is a function of DNA content.

The early study by E.G. Conklin (1912) run counter to this hypothesis. He found that the size of organelles including the nucleus depends on the volume of cytoplasm rather than the physical dimensions of the cell (Conklin, 1912). *The Nucleoskeletal theory* proposed by Cavalier-Smith (Cavaliersmith, 1982) suggest that the correlation between nucleus size and cell size arise thorough a process of coevolution in which nuclear size is adjusted to match alterations in cell size. There are examples of experimental studies in support of the hypothesis that the nucleus is a function of cell size (Gurdon, 1976, Jorgensen et al., 2007). However, the mechanisms behind nuclear scaling remain largely unknown (Goehring &Hyman, 2012).

As cells become larger, the ratio of surface area to volume decreases, this has potential influence on key physiological parameters. The cell size of phytoplankton affects both physiological and ecological function (growth, photosynthesis, respiration etc.) (Finkel et al., 2010) and nutrient uptake (Tambi et al., 2009). If genome size and cell size are tightly coupled, we can predict that genome size may be linked to one or more features at the organismal level (e.g., metabolic rate, body size and organ complexity). For multicellular organisms, body size is determined either by the number of cells, their size, or both (mixed strategies) (Timofeev, 2001, Arendt, 2007). A positive correlation between genome size and body size has been found in a variety of invertebrates (Gregory, 2005). In copepods growth is

observed to be determinate with cell number constancy, interestingly genome size increase in this group exert a particularly notable influence on body size (Gregory, 2005, Hessen &Persson, 2009). In the order Cladocera there is also a positive correlation between genome size and body size.

Observed pattern of variation in genome size at the haploid or diploid level is primarily related to accumulation of various introns; i.e. repetitive non-coding sequences, transposons and retrotransposons (Gregory, 2001, Lynch, 2007). Genome size and the lengths of individual introns appear to be positively correlated between species of Drosophila (Moriyama et al., 1998) and within the class of mammals (Ogata et al., 1996). Transposable elements (TEs) are repetitive and mobile DNA sequences, with the ability to move in and invade genomes. Differences in TE abundance play a major part in differences in genome size among eukaryotes (Floudas et al., 2012). TEs or their derivatives often constitute a significant proportion of the genome (e.g., 80 % of maize, 45 % of human, 15 % of the fruit fly)(Chenais et al., 2012). They are of great evolutionary significance, as they contribute to the mutation rate, the source of genetic variation upon which natural selection acts. Interestingly, the spread of transposon insertions is associated with obligate asexuality in *Daphnia* (Eads et al., 2012). Several studies in plants report TE over-expression following abiotic or biotic stress conditions such as temperature, nitrate starvation, wounding, etc (Chenais et al., 2012). Suggesting a TE-mediated stress response and genetic adaptation to environmental changes. In drosophila, significant differences of transposition rates are detectible according to developmental temperature (Giraud &Capy, 1996).

The *Daphnia pulex* sequencing and annotation (Colbourne et al., 2011) revealed a "streamlined" genome of only 200 Mb, but it contains surprisingly many genes (~ 31.000). About 13.000 of the genes are identified as paralogues related by duplication events. Many of these duplicated genes and genes without homologs, are demonstrated to be responsive to specific ecological conditions. *Daphnia* species are in the lower range in the scale of variability in genome size for Crustaceans (Hessen &Persson, 2009). Still closely related species within this clade might provide good models to assess the evolutionary drivers and consequences of changes in nuclear DNA content (Vergilino et al., 2009). Diploid clones in the *Daphnia tenebrosa* group have the largest genomes in the whole genus *Daphnia* (Dufresne &Hebert, 1995). There are indications that the genome size increase of 22 % in this

particular group within the *Daphnia pulex* complex may be due to a greater amount of repetitive DNA or to the proliferation of transposons (Vergilino et al., 2009).

It is known that invertebrates often tend to be larger in cold environments, reflecting an evolutionary adaptation (although the benefits of being large in the cold are disputed). It is, however, also commonly observed that animals raised at low temperatures gain increased adult body size compared with those raised at high temperature, despite lower growth rates. This phenomenon is called temperature-size-rules (Atkinson, 1994), and seem also to hold for unicellular eukaryotes (Finkel et al., 2010), suggesting that this could be a cellular response. The flip-side of this argument is that elevated temperatures could yield smaller organisms (Daufresne et al., 2009). This point out that it is critical to assess the evolutionary nature of the observed changes linking temperature and size across the different biological scales to understanding the impacts of global warming on biota (Winder et al., 2009, Daufresne et al., 2009). The original Bergmann's rule dealt specifically with endotherms at the intraspecific level (Watt et al., 2010). This rule linked body size to the thermal environment in which the organisms lived (Bergmann, 1847), and argued for a mass specific heat loss being dependent on body volume to surface ratio, were large body size was thus favoured in could environments. James' rule consider the relevance of Bergmann's rule at the intera-specific scale (James, 1970). Since then, enlarge body size in colder areas has been documented by several studies on ectothermal metazoans (Atkinson, 1994, Angilletta et al., 2004, Timofeev, 2001). The causation of such clines in ectotherms obviously differs fundamentally from those originally explaining the rule in endotherms. There are also contradictory findings to these rules, many which should be seen in the light of life history traits and adaptations (Chown &Gaston, 2010). Genome size is important in this context as a potential covariate and potential determinant of cell size– and thus potentially body size. The strong coupling between low temperature and large genome- and body size typically found in many marine invertebrates is to note in this context (Atkinson, 1994, Timofeev, 2001, Rees et al., 2007, Hessen &Persson, 2009). A fundamental question is whether such differences in thermal sensitivity reflect ultimate or proximate causations; i.e. is it an innate property of the processes, or a mechanism reflecting a favoured thermal response on cell size? It is worth stressing that any genome size increase at low temperature may not necessarily be an adaptive trait, but could reflect a passive response accumulation by "selfish DNA", e.g. by transposon proliferation simply because there is a low counter selective pressure at low temperatures (Hessen et al. 2012).

It has been hypothesized (Hessen et al., 2008, Hessen et al., 2010) that another route to changes in genome size (and thus presumably cell size) has to do with phosphorus (P) availability. Recently, Hessen et al. (2008) explore growth rate (GR) as a potential evolutionary driver for reduced genome size in eukaryotic genomes, with a particular focus on the material costs of rapid growth in terms of P demands for ribosomes. Their hypothesis predicts a causal relationship, on evolutionary scale, between GR, cell specific RNA, genome size and cell size in some taxa. Suggesting that maintaining high GR under nutrient limitation can promote an evolutionary pressure for reduced genome size due to the trade-off between material cost in terms of phosphorus (P) allocation from DNA (storage information molecule) to RNA (growth promoting molecule). Underlying this is also the observation that meiosis and cell division often is negatively correlated with genome size across plants and animal taxa (Bennett, 1987, Gregory, 2005). Accordingly, a smaller genome size may be liked to faster cell division and developmental rate.

In my study, I wanted to address whether high and low temperature (20 ˚C and 10 ˚C) as well food quality (indexed by the C:P ratio) do affect estimates of diploid DNA content and nuclei size over successive generations, as assessed by flow cytometry. The experimental organism used is *Daphnia*, a much-used model organism for aquatic ecologists which also have been a key model organisms for genetic, genomic and proteomic works (cf. Colbourne et al. 2011).

Hypothesis and goals of the work

Part I:

To test whether observable changes in genome size could be induced over a modest number of generations, I analysed genome size by use of flow cytometry over a range of generations (depending on temperature and diet) at two temperatures (10 and 20 ^{o}C) and with high and low P-diets. My null hypothesises for the flowcytometry experiments were as following, $H0_1$:Diet, in respect to P, do not cause any changes in estimated 2C DNA content over short time. $H0_2$: Temperature does not cause any changes in estimated 2C DNA content . $H0_3$: There are no correlations between 2C-value, nuclei size and body size.

Part II:

Further, to asses potential effects induced by the different parental environments with a focus on the diet, samples of animals reared at 20 ˚C underwent a "common garden" experiment with reciprocal transplantations. For two following generations, I analysed the individuals for mass specific growth rate (MSGR) to see if the long term exposure to different food quality resulted in different growth rates, somatic C, N and P content, somatic RNA content and somatic alkaline phosphatase activity (APA). These parameters are counter linked with growth, P acquisition and P effects in general. The transplant design could help to reveal maternal effects from mothers consuming both P-sufficient as well as P-limited food. Further, it can assess effects caused by food quality on the measured traits after many generations of exposure. My null hypothesises for the reciprocal transplant experiment was as following: $H0_4$: There is no effect of diet on any of the measured variables [RNA], [APA], MSGR, % P. $H0_5$:There are no maternal effects. $H0_6$: The long-term exposure to different food quality did not result in differences. $H0_7$: There are no correlations between the measured variables. Any differences found in this experiment have the potential to reveal if the daphnids can undergo selection pressure for P demands, in this relatively short time period.

2 Materials and Methods

2.1 Algal Food Culturing

A strain of the unicellular chlorophyte *Selenastrum capricornutum* (obtained from the culture collection at the Norwegian Institute for Water Research, Oslo) was used as the food source for the cultivated populations of *Daphnia* in all experiments and stock cultures. This green algae was grown in continuous cultures in chemostats (Hessen et al., 2002) in a climate controlled room with a constant temperature of 20 ^{0}C. Four sterile 2-L glass vessels sealed with silicone stoppers were inoculated with 100 mL of *S. capricornutum* and 1.8 L sterile COMBO medium (Kilham et al., 1998) with a P-concentration of either 50 µmol PL^{-1} or 2 µmol PL^{-1}, for producing P-sufficient or P-deficient algae respectively. The COMBO medium was supplied at a flow corresponding to a dilution rate of 0.2 day $^{-1}$ provided by two peristaltic pumps (Schego, M2K3, 5W), one for input of the COMBO medium the other for outlet of the algae suspension. The peristaltic pumps worked in a 36:360 sec on-off cycle. Irradiance was provided by 25-W blue-white fluorescent tubes (OSRAM FQ) and the light level was nominally at 70 µmol quanta $m^{-2}s^{-1}$. Sterile filtered air (Millipore, 0.2 µm) was supplied through sinters to ensure continues access of CO_2 for algal photosynthesis, while magnetic stirrers mixed the cultures to reduce sedimentation and aggregation of algae. All equipment used in the chemostat setup was acid washed and autoclaved prior to use.

2.1.1 P-sufficient and P-limited algal growth

The COMBO medium was modified by adding different total amount of P to result in P-sufficient or P-limited algal growth. According to the chemostat theory, and which is also well confirmed by previous experiments with the same set-up (Hessen et al., 2002, Wojewodzic et al., 2011), 50 µmol PL^{-1} in the COMBO medium produces high quality algal food with no nutrient limitation (molar C:P ratio approximately 100). While, 2 µmol PL^{-1} in the medium, was expected to yield molar C:P ratios of approximately 600-900 (Hessen et al., 2002). Thus, in this experiment the medium for P-sufficient algae (high quality) contained 50 µmol L^{-1}P, and the medium for P-limited algae (low quality) contained 2 µmol L^{-1}P with an addition of 48 µmol KCl L^{-1} to maintain the same ionic strength as in the P-sufficient

medium. All COMBO media were adjusted to pH 7.80 ± 0.05 with 1 M HCl (Kilham et al. 1998) before use.

The algal outflows from the continuous cultures were analysed for its optical density (OD) at 633 nm. By the use of a 1 cm path cuvetts and baseline set on NP free COMBO in a spectrophotometer (Shimatzo), a reliable estimate of food concentration for the feeding of Daphnia. The carbon (C) - concentration was calculated from the OD by a previously established calibration curve between OD and measured C -concentration where blanks were made on NP free COMBO medium (Wojewodzic et al., 2011):

$$For\ P\ sufficient\ algae:\ \ mgC\ ml^{-1} = OD\ at\ 633\ nm/7.6444$$
$$For\ P\ limited\ \ algae:\ \ \ \ mgC\ ml^{-1} = OD\ at\ 633\ nm/5.6497$$

After approximately 2 weeks, algae grown in the chemostat reached a stable density and were used as food source for the animals. Backups of P limited chemostats were always kept to avoid problems related to random crashing of the cultures.

2.1.2 High and low food quality treatments

While manipulating the food into two quality treatments, indexed by algal C:P ratio, the quantity or the amount of algal cells was held equal in both treatments in the experiment. This was achieved as following. After reading the OD and calculating the C -concentration of the algal outflow (based on equations), the algae were diluted, with N- and P free COMBO medium, to a standardized particulate carbon concentration of 2.0 mg CL^{-1}. Thus, we were creating two types of diets of different algal quality but of the same quantity. A third type of diet was also included, hereafter named "spiked" (S). This diet was prepared from the low quality diet but included an additional step, where the algae cells were spiked with dissolved inorganic P (K_2HPO_4) (as in; Persson et al. 2011). The spiking procedure is based on the observation that P-depleted phytoplankton assimilates such inorganic P within few minutes, yielding a shift in C: P (Rothhaupt, 1995, Plath &Boersma, 2001). The assumption is therefore that by spiking the algal cells their C:P ratio decrease, while other food quality parameters are kept unchanged (e.g., N, sterols, and fatty acids). There might still be changes in the macromolecular makeup of the algae in this diet during the course of the experiment,

yet there are good reasons to assume that they should be minor compared with the direct P effects (Persson et al., 2011).

We saved samples of food mixtures for post-experimental determination of food P:C and N:C ratios, collected on pre-combusted (530 °C, 3h) GF/F filters (Whatman, Kent, UK). C and N content was analyzed on a Thermo Finnigan FlashEA 1112 elemental analyzer. Samples for particulate P were analyzed using a modified molybdate blue method (Menzel and Corwin., 1965) after persulphate digestion.

2.2 Daphnia

2.2.1 Model organism

Daphnia are opportunistic species, which respond quickly to environmental changes and by asexual reproduction may rapidly build up large populations. Also, *Daphnia* are an important link between the laboratory and the field in estimating the effects of environmental changes. Their fast reproduction is due to direct development without free larval stages, which results in short generation time. Although *Daphnia* are small (1 to 5 mm), they are large enough to be handled individually. The most common mode of reproduction in *Daphnia* is parthenogenesis; accordingly it is easy to produce clonal lines that can be maintained in the laboratory for extended periods. However, although most daphnia clones use parthenogenetic mode of reproduction, they can reproduce sexually under certain conditions.

The *Daphnia magna* clone used in this study was obtained from a stock collection kept at the laboratory of Dr. K. Pulkkinen at the University of Jyväskylä (Finland) before arriving at our laboratory at the University of Oslo (Norway). This clone originating from a pond in North Germany near Gaarzerfeldt was collected in 1997, and it has been maintained in the laboratory since then (Pulkkinen &Ebert, 2004). The *Daphnia pulex* clone (clone LL4-15) used in this study was isolated from a small pond in North-Western Iowa and was obtained from a stock collection in the laboratory of Dr. L.J. Weider at the University of Oklahoma Biological Station.

The *Daphnia* stock cultures of both *D. magna* and *D. pulex* were kept at a climate controlled room in jars (> 800 ml) of N- and P free COMBO medium (Kilham et al. 1998) and fed P-sufficient algae *ad libitum*. Animals were fed every other day and transferred to

new jars with fresh medium weekly. Culture plastic containers with a high edge and plastic transfer-pipettes (Sarsted, 86.1171.000) were used to manually transfer animals to new jars. The edge of the pipette was cut to create an optimal tip size in order to avoid any harm to the animals during handling. The cultures were always kept at low densities to minimize stress and ensure clonal reproduction. All jars and equipment used handling the animals at the laboratory were soda ($NaHCO_3$) and acid (0.1 M HCl) washed and rinsed in distilled water.

2.2.2 Establishment of experimental lines

Both clones were exposed to a factorial design with two temperatures (20^0C and 10^0C) and three diets (P-sufficient, P-limited and Spiked), giving a total of 12 experimental units. Each unit started off with one jar (400 ml) of 5-10 juveniles. For *Daphnia magna* all experimental units started 18.05.2011 from the same synchronized mothers raised in a stock culture at 20 ^{0}C. For *Daphnia pulex* the experimental units in 20 ^{0}C started 23.03.2011 while the experimental units in 10 ^{0}C started 02.05.2011. Thus, the juvenile individuals of *D. pulex* used to establish the units within the same temperature originated from the same synchronized mothers, whereas the units established in 10 ^{0}C were sampled from the same stock culture as the units in 20 ^{0}C, but from synchronized mothers of a later cohort. Each experimental unit was kept in time as a clonal line intended to run for several generations. The date when the animals reached first reproduction was recorded. When the next generation reached a number of 10 - 20 animals, the mothers were taken out and used for analysis. This course was repeated to the end of the experiment.

2.3 **Flow cytometry**

Flow cytometry (FCM) is the measure of particles such as cells, nuclei or beads in a flow system, which delivers the particles singly past a point of measurement (Fig. 1). Additionally, FCM refers to instruments in which light is focused at the point of measurement. The scatted light and fluorescence of different wavelengths are recorded. Typically, light scatter at two different angles (Fig. 2). Forward scatter (FSC, ~2 - 10˚), which increases with the size of the particle, but not monotonously, and side scatter (SSC, ~50 - 130˚) which is more sensitive to internal structures and granularity. Further, FCM is well suited to DNA analysis because dyes, such as propidium iodide (PI), bind DNA in a proportional and linear fashion. These dyes are only weakly fluorescent in aqueous solution but fluoresce strongly when bound to DNA due to the hydrophobic nature of their environment. This allows the quantification of DNA content. PI has red fluorescence and can be excited at 488nm, and the fluorescence is recorded in FL2 (585/42 BP) detector.

Figure 1. BD FACS Calibur flow cytometer system is an automated bench top flow cytometry system. The optical assembly is fixed with air-cooled argon-ion laser, 15 mW 488 nm Ar ion laser. The data acquisition computer is interfaced to a MacIntosh computer system.
(http://depts.washington.edu/imgcore/facs/bdFACS.html)

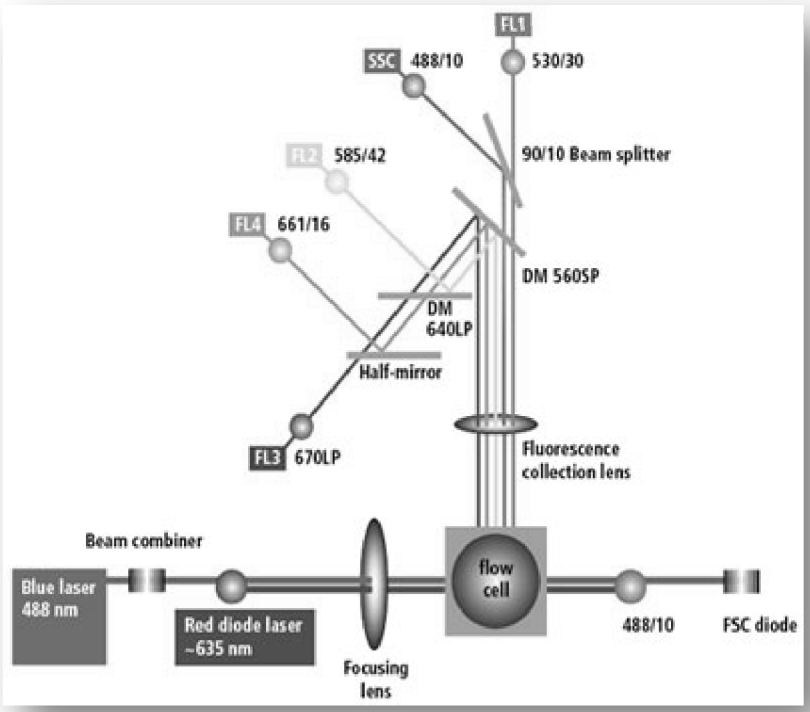

Figure 2. FACS Calibur optical system; the figure shows a diagram of optical path design of the FACS Calibur, with detectors (FSC, SSC and FL1-4). http://www.bdbiosciences.com/instruments/facscalibur/features/index.jsp

The value of the FCM technique lies in the ability to make measurements on large number of single particles within a short period of time. The heterogeneity of populations can be revealed and different subsets of particles identified and quantified. In this thesis only the diploid (2C) nuclei were further analyzed in the result section.

FCM Software

Adjacent populations may overlap each other so a modeling program is required to de-convolute the populations and assign percentage values to each population. ModFit LT, is a automatic cell cycle analysis software, used to define the gating pattern to enabling the identification of normal diploid cells at rest, those that are actively synthesizing DNA, and those that are either premitotic or actually in mitosis. With FCM peak detection, the system identifies ploidy patterns and dynamically creates models to match. Improvements like AutoDebris™, AutoAggregate™, and AutoLinearity™ make analysis more robust and consistent. The use of *Gallus gallus domesticus* blood cells (CRBC) as standard allow to determine which cycle is classified as diploid, which further guide to tetra- and octoploids.

The automated gating from ModFit make it easy to define gating and further statistic analysis using FCS express software, where FCM data is further processed and introduced as figures. The use of FCS express was also important for FSC measurements of nucleus size, since ModFit is made for cell cycle analysis.

Excluding aggregates and debris

Two 2N nuclei stuck together will have the same DNA content as a 4N single cell and the two should be distinguished if the DNA histogram is to reflect accurately the state of the cell cycle. In instruments designed for DNA analysis, the laser beam is focused to give an elliptical cross-section whose width is close to the diameter of a typical nucleus. As a particle crosses the beam, the integrated fluorescence will be proportional to the DNA content; the width of the signal in time will be the addition of the width of the particle and that of the laser beam. Because of the flow system, clumps of cells will tend to align along the direction of flow and will give a wider signal than single cells. 4C Cells will give a higher peak signal compared to two clumped 2C cells but a narrower width (Fig.3A). Fig. 3B shows pulse width versus area, and this is the plot used to distinguish between single cells and aggregates. Single cells (2C, 4C, and 8C) will have similar pulse width (transit time) values. Aggregates will have larger width values and can be easily discriminated by gating the single cells, which are further displayed on the area histogram (Fig 3C).

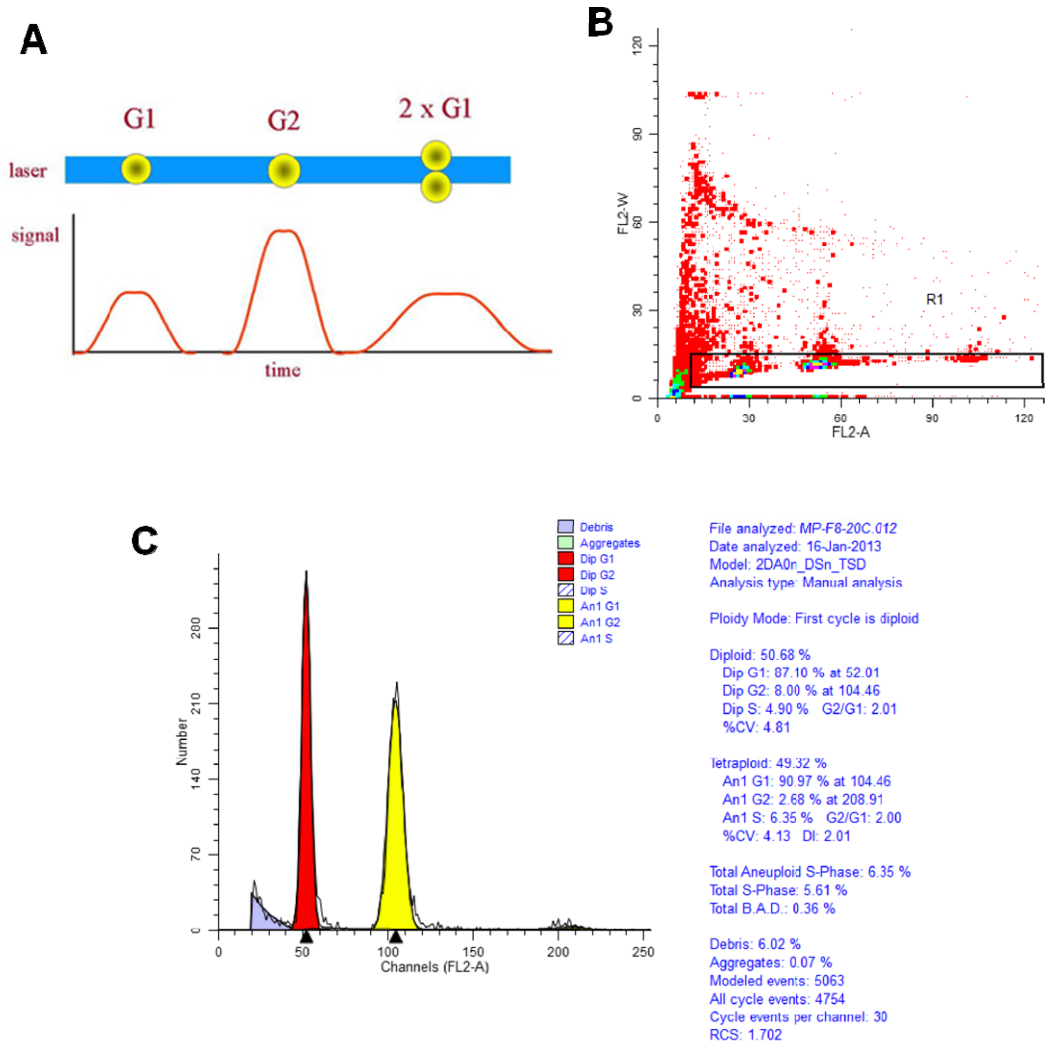

Figure 3. Random sample of gating method used in our experiment with ModFit FCM analysis program. Signal (pulse) width versus time course change according to size of particle intersects the laser beam (A). Aggregates and debris are removed from final DNA analysis by gating single nuclei/cells (R1) on FL2-W versus FL2-A cytogram (B). The final DNA content (2C, 4C, and 8C) is measured on FL2-A histogram using ModFit auto analysis tools (C).

Descriptive statistics for defined populations of cells

In each run approximately 10,000 counts is recorded by the FL2 detector (primary threshold), these counts are distributed into 2C, 4C, 8C, debris and aggregates, displayed on a linear scale. After gating for single nuclei population on FL2-W vs. FL2-A, we further sub gated for 2C, 4C, and 8C population (ModFit LT and FCS express). These gates were used for complete automated descriptive statistics of the arithmetic mean, event count and CV.

Since all G_1 cells have the same DNA content, the same fluorescence should (in theory) be detected, and only a single channel of the histogram should be filled. In practice, however, instrumental error and biological variability in DNA dye binding result in a Gaussian (normally distributed) fluorescence distribution from G_1 cells. Greater variation in measurement result in broader DNA content peaks, and the term coefficient of variation (CV) is used to describe the with of the peaks;

CV = 100 x SD / (peak channel) [%],

In theory, acceptable CV value for DNA estimation have (in several articles) been defined as an indicator for purity of stoichiometric DNA amount measurement, where CV equal to 6 % or less defined as suitable histogram measure of DNA amount estimations (Baretton et al. 1994; Vilhar et al. 2001; Darzynkiewicz 2010) and CV below 3 % fully acceptable (Marie &Brown, 1993a). But regardless of these definitions, there exist no universal rule of acceptable CV value.

Flow cytometry protocol for Daphnia

For FCM analysis, 6 – 10 adult female *Daphnia* were collected from each cohort after first brood release. Four randomly selected animals were placed in a sterile Petri dish (cat. no. 150318, Nunc™, Denmark) and photographed (Dino-Lite Digital Microscopes, Dino-Lite, The Netherlands), prior to extraction of nuclei. The body size estimates are standardized at first reproduction. Body size, top of head to base of spine (cf. Galbraith Jr 1967), was directly measured from photographs using Adobe Photoshop CS4. Furthermore, animals of each clone were placed in microcentrifuge tube and COMBO medium was gently removed. Further steps from here were performed on ice. The animals were washed once with 500 µL cold grinding buffer (10 mM Tris-HCL, 10 mM $CaCl_2$, 3 mM $MgCl_2$, 0.5 % Nonidet P-40, pH=7.4) following the protocol of Korpelainen et al. (1997). The buffer was removed after 5 minutes incubation and a new portion of 350 µL grinding buffer was added before grinding with straight fine tip teasing needle for approximately 25 strokes. After grinding, 1 mg RNase A (cat. no. 12091-021, Invitrogen Life Science, CA, USA) was added, and samples were then stained with 50 µg of propidium iodide (PI, emission max 617 nm, P3566, Invitrogen Life Science, CA, USA). Fresh (24-48 h old in heparin) *Gallus gallus domesticus* blood cells (CRBC) at a density of 5.0 x 10^5 cells ml^{-1} in grinding buffer were both used as internal and external standard (Galbraith et al. 1983). CRBC was used as external standard because 8C *D.*

magna and *D .pulex* overlap with 2C CRBC nuclei population during FCM analysis. Thus, for pure 8C *Daphnia* nuclei estimations, CRBC samples were used as external standard, stained and FCM run in parallel with same conditions as *Daphnia* samples (Dressler 1990). CRBC nuclei were also added as internal standard for quality control of staining variability to some *Daphnia* samples. These samples were prepared by splitting each sample into two after the grinding step, where one part received CRBC before the fluorochrome staining while the other part was directly stained. At the same time, a clear CRBC sample (external standard) was also prepared, with the same cell density similar to CRBC that was added directly to the *Daphnia* sample (internal standard). All samples were dark-incubated for 1 h. All suspensions were clarified by filtering directly in to the test tubes through BD cell strainer cap with mesh size of 35 μm (cat. no. 352235, BD Biosciences, New Jersey, USA). In addition to CRBC nuclei, 2.5 μm alignment beads (P-14831, Invitrogen Corporation, USA) were used to keep instrument settings (amplification and sample rate) constant throughout the experiment, and to confirm low coefficient of variation (CV) alignment. The PI stained nuclear DNA content (both diploid genome size and incidence of endopolyploidy) of each clone was determined using FACS Calibur flow cytometer (Becton Dickinson, San Jose, USA) equipped with a 15 mW 488 nm air-cooled argon-ion laser and a standard filter set-up (Galbraith et al. 1983). PI fluorescence emission signal was measured in FL2 detector with 585/42 bandpass filter setup. Doublets and cell aggregates were discriminated from the analysis by gating around the singlet population in the fluorescence pulse width (FL2-W) versus pulse area (FL2-A) dual parameter cytogram (Shapiro 2003). FL2-A measured in linear scale, was used as a measure of cellular DNA content. Nuclei population was assessed from forward (FSC) and side (SSC) light scatter dual parameter cytogram on a four-decade logarithmic scale. FSC indicates nuclei size and SSC is an indicator of the granularity of nuclei. The flow rate was set up to 12 μl min^{-1} during each FCM acquisition and 10 000 events were recorded per sample. Both beads and CRBC were included during each FCM analysis. Beads were measured on both FL2-A and FSC and were added to some samples at a density of 5.0×10^4 particle ml^{-1} or run separately before each acquisition. All FCM instrument control and data acquisition were recorded using Becton Dickinson CellQuest Pro software package (Becton Dickinson, San Jose, USA). Acquisition setup and intensities were identical for all Daphnia, CRBC, and bead samples. The FCM acquisition setup for both temperatures was identical, and for some of the analysis, animals from both temperatures were measured during same FCM run.

The recorded fluorescent signal of fluorochrome is directly proportional to the amount of DNA in the nucleus (Shapiro 2003). The Daphnia 2C nuclei peak from 20 and 10 °C was determined and gated by comparing it with CRBC 2C nuclei peak on FL2-A histogram. These 2C gates (Daphnia and CRBC nuclei) were limited by measuring arithmetic mean value of Gaussian distribution, taking the highest peak channel value into consideration. The peak channel value is measurement of the fluorescence emitted from a fluorochrome at the highest amount or numbers of events present (Shapiro 2003). Furthermore, Daphnia 4C and 8C gates were determined by multiplying 2C values with 2 and 4, respectively. The CV was measured from same gates as arithmetic mean value of 2C to 8C peaks in FL2-A histogram. C-value = pg DNA nucleus^{-1} was calculated following standardized formula of Galbraith et al. (2001):

Sample 2C DNA content (pg nucleus^{-1}) = [(Sample 2C peak mean) / (Standard 2C peak mean)] * Standard DNA content (pg nucleus^{-1}).

The standard DNA content used was CRBC = 2.5 pg nucleus^{-1} (Vergilino et al. 2009). Nuclei size (FSC; arbitrary units) of 2C to 8C nuclei was estimated as geometric mean values from FSC versus FL-2 cytogram. All FCM results were analyzed using FCS express 3 software (De Novo, USA) and Modfit LT (Verity, USA).

Comparison between the experimental groups in FCM parameters as well as body size (mm) were done by one-way ANOVA after testing for homogeneity and normal distribution. Tukey's honestly significant difference (HSD) test was used to test pairs of means.

2.4 Reciprocal transplant experiment

To test whether the different food quality treatments offered to the daphnids over multiple generations could manifest differences, a reciprocal transplant experiment was conducted. If all treatment combinations (6 units per species) were to be reciprocally transplanted, it would make 36 groups per clone, which was not manageable. Since the main concern was to study the effect of P-limitation over several generations compared to the more acute response, a limited subset of treatment combinations was chosen. The reciprocal transplant included both daphnia species, but only treatment groups cultured in 20 ^{0}C under the two main diets (P-sufficient (F) and P-limited algae (P)). This made four treatment combinations per species and the experiment was performed over two generations (Fig. 4).

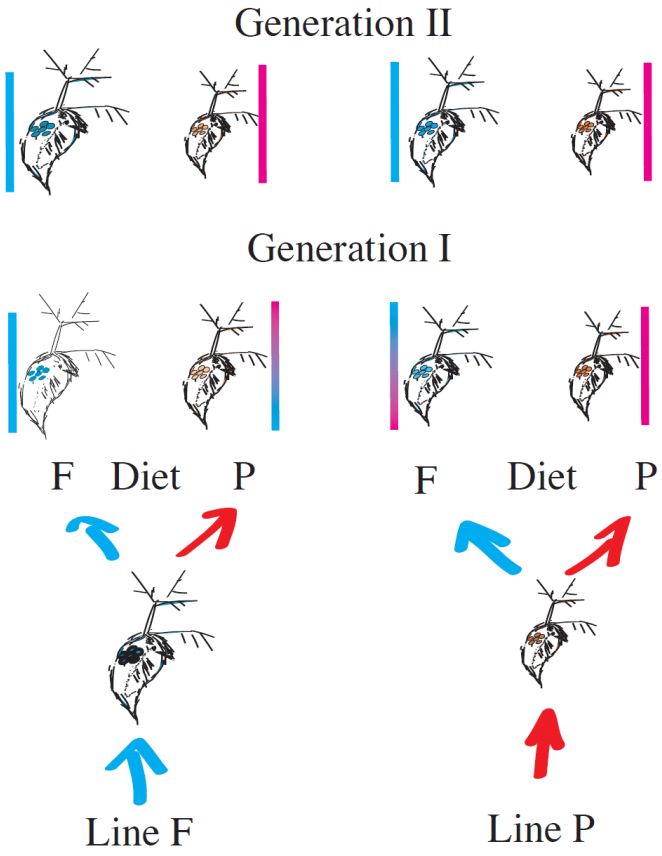

Figure 4. Illustration of the reciprocal transplant, with *Daphnia* of the multigenerational lines fed P-limited (P) diet or P-sufficient (F) . In the reciprocal transplant, daphnids originating from the Line P and Line F were fed both P - and F-diet over two generations.

The animals were grown for 6 days after which their responses -mass specific growth rate, somatic RNA content, % C , % N and % P body content and somatic APA were analyzed (see descriptions below).

The animals were cultured in plastic, single use, culture bottles and a foil was used to reduce light levels to avoid photo-damage (Fig. 5). Each bottle, containing several animals, was defined as one replication. All treatment groups were performed in several replicates. All units in the experiment were inspected every day, and fresh media and algae were replaced every other day (25 mL per individual animal). Neonates (< 24-h-old) were separated from their mothers and pooled into new bottles representing replicates of the next generation.

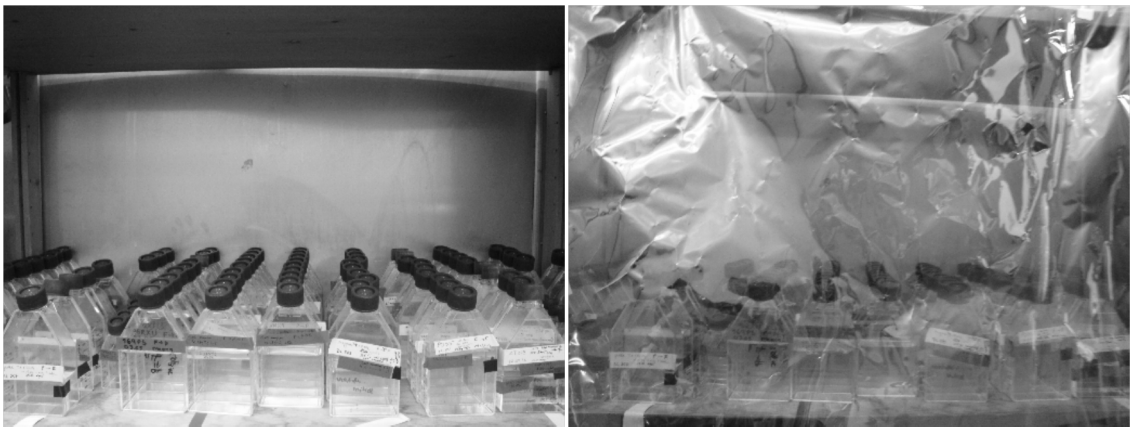

Figure. 5 Cultures of Daphnia during the reciprocal experiment. Animals were kept in the culture bottles that were randomly distributed and covered by foil (right). Animals were placed in a temperature-controlled room, at 20 ^{o}C.

2.4.1 Mass specific growth rate (MSGR)

Specific growth rate μ ($units\ of\ time^{-1}$) is given as the rate of change of biomass of the organism normalised to its biomass ($\mu = (dM/dt)/M$ where M is biomass). In discrete time, μ is given by $[ln(M_t/M_0)]/t$ (Sterner &Elser, 2002). Where M_t is the biomass at time t, and M_0 is the biomass at the start of the interval. In this study animals were allowed to grow for 6 days, thus time is number of days were M_6 is the biomass after 6 $days$ of growth and M_0 is the average biomass of neonates (<24h old) at the start of the growth period.

Animals, of both ages from all groups, were carefully placed under a stereo microscope (Leica) and photographed, transferred with a thin brush to a pre-weighted tin cup, dried for 24 h at 60 ˚C, and weighed using a microbalance (Mettler ME 30; Mettler-Toledo GmbH, Greifensee, Switzerland). The tin cups with the dried daphnieds were stored in an exicator. These samples were later on used to quantify the elements C, N and P.

Mass - length regression

The pictures taken of the six-day-old animals from the growth rate experiment were used to establish the relationship between body dry weight and body length. Individual average dry mass and individual average body length were determined per sample. The body length was measured using ImageJ software. A regression analysis was performed for the average log-transformed dry mass and body length (r^2= 0.95). The resulting regression equation:

$$\text{Dry weight} = 8.5 \cdot 10^{-3} \cdot \text{Size}^{2.86}$$

was later applied to convert body length to body mass of the individuals photographed and sampled to RNA and APA analysis, in the interest of normalizing total body RNA content and alkaline phosphatase activity to dry weight.

2.4.2 Elemental C, N and P content

The phosphorus (P) content of daphnids, grown under different diets and histories in the reciprocal transplant experiment, was quantified with the purpose of estimating the amount of P incorporated into the body relative to C and N, and thus verify that we produced the desired food quality for the consumers. *Daphnia* generally maintain a homeostatic element regulation (Andersen and Hessen 1991) and stoichiometric changes was expected to be far lower that that of the algae. Body C and N content were analyzed using an element analyzer and performed by Berit Kaasa (UiO).

Phosphorus

Body P content in both < 24-hours-old and 6-days-old animals, for both species, were analysed spectrophotometrically using a modified molybdate blue method (Menzel and Corwing 1965) after persulphate digestion. The protocol was modified for low volume extraction. The calibration curve was in the range of 7.8 µg P L^{-1} to 1000 µg P L^{-1} and replicated. Blanks were randomly included in the run set with nine replicates, and in addition internal controls were added (apple leaf, reference material no 1515, National Institute of Standards and Technology)

Each of the tin cups (samples) containing the dried daphnids was carefully emptied on a piece of aluminium foil and the mass collected weighed on a microbalance (Mettler ME 30; Mettler-Toledo GmbH, Greifensee, Switzerland) before transferred into a pyrophosphate free microcentrifuge tubes. MilliQ-water (0.25 ml) was added to each tube and the samples were sonified in a cup horn for 2 minutes (Branson Sonifier®, S450A) at constant duty cycle and output 80. All samples were diluted with an additional of 2.25 mL of MilliQ-water, before adding 0.5 mL potasium persulphate (1%) and autoclaved (120 ˚C, 1 hour, program no 6, liquid and cooling cycle).

Phosphate content was determined after persulphate digestion by a modification of the molybdate blue method. The reagent solution contained sulphuric acid (1 M), ammonium molybdate (4 %) and potassium tartrate (1 mg Sb ml^{-1}) in the following proportions: 100, 30 and 10 ml, respectively. Seventy ml of this reagent solution was mixed with 30 ml of ascorbic acid (0.1 M) immediately before use. Finally, 0.50 mL of the final reagent mixture was added to the samples (2.5 mL) and mixed thoroughly. All samples were incubated for 30 minutes at room temperature before measure of the absorbance at 882 nm. Calculation of the total P content in the sample was estimated using the calibration curve between µM P and blank-corrected OD. Internal controls (apple leaves) were included to assess quality of the performed analysis.

2.4.3 RNA quantification

Total RNA was isolated from the whole body of 6-day-old individuals, from all experimental groups examined under the reciprocal transplant experiment. The RNA isolation was performed using a RiboGreen fluorescence protocol developed by Gorokhova & Kyle et al. (2002), based on methods for extraction and quantification of nucleic acids in insects although considerably modified for the use on *Daphnia*. Commercial standards of ribosomal RNA (16S and 23S rRNA from *E.coli*, Molecular Probes) were used to create a standard curve and to control for proper RNA digestion during the assay.

Prior to analysis samples (one sample containing a single daphnia) were photographed and placed in a nuclease free micro-centrifuge tube, snap frozen in liquid nitrogen and stored in - 80 ^{o}C. On the day of analysis samples were placed on ice and extraction buffer (1 % sarcosyl, Sigma) was added immediately, while the samples were still in the frozen state. The samples were then homogenized by ice-cold sonification (Branson Sonifier®, S450A) in a cuphorn (Brandson 101147048) for 2 minutes at constant duty cycle and output 80. Up to six samples were homogenized at a time. Right after sonification the samples were placed on ice and diluted 1:5 with ice cold TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.5). Duplicates of 75 µl of each sample were dispensed into individual wells in a 96-well plate (655076, Greiner Bio-One, USA). Each sample was dispensed into two wells, one for total reading, the other for digestion by RNase. This included also the blank, which is the Standard buffer (75 µl of sarcosil + TE, 1:5), and all standards and samples. In the well for total reading 20 µl of RNase-free water (GibcoBRL 1071) was added, into the other duplicated well 20µl of 0.1 µg RNase A (A7973, Promega) was added, followed by 25 minutes on a shaking table (200 rpm) incubated at 37 ^{0}C on a shaking table (200 rpm) to digest RNA. Afterwards, samples were incubated for 5 minutes with 75 µl of 100 x diluted RiboGreen dye (R-11490, Molecular Probes, USA) added with an automatic 8 channel pipette. Immediately after, the 96-well plate was analysed using a BioTek FL x 800 plate reader (BioTek, USA) with excitation wavelength of 480/20 nm and an emission wavelength of 525/20 nm (sensitivity 80). Subtraction of the fluorescent signal measured for the digested sample from the total signal allowed RNA to be quantified. To assess the quality of measurements, the internal controls in each of the plate were included.

2.4.4 Alkaline Phosphatase Activity

D.magna and *D.pulex,* representing all experimental groups examined under the reciprocal transplant experiment were analysed for body alkaline phosphatase activity (APA) using the CDP-Star chemiluminescence method (Wojewodzic et al., in review). Total body APA was normalized to dry weight.

One possible confounding factor for the quantification of zooplankton AP activity is the AP activity contributed by the gut content at the time of extraction. Caution must be taken for animals fed P-deficient algae with possibility of elevated AP activity. In such cases, animal gut contents can be purged by a feeding period on P-sufficient algae prior to sample preservation. Accordingly, we fed the all daphnids P-sufficient algae for 30 min prior to each sampling (Wojewodzic et al. 2010).

Standards were prepared using AP type VII-S from bovine intestinal mucosa (Sigma Aldrich, P5521). Our target standard curve was 2 to 100 μU of AP, diluted by using 1% (v/v) Triton X-100 (Sigma Aldrich, 93443; here after called Triton). Traditionally one unit $[U]$ is defined as the amount of enzyme required to hydrolyse 1 μmole of 4-nitrophenyl phosphate per minute at pH 9.8 and 37 ^{o}C.

Daphnids were processed by adding 300 μL of 1% (v/v) Triton to the microcentrifuge tube containing the frozen animal. Tubes were placed in a floating holder on a cuphorn and sonified ice-cold for 2 minutes (Branson Sonifier®, S450A) which allows for rapid processing of samples, temperature control to maintain enzyme activity, and elimination of cross contamination.

Samples and standards (20 μL per well) were dispensed into a 96-well plate (pyrophosphate free-plates, Nunc, 236105) standing on ice. Two hundred μl of ready-to-use 0.4 mM CDP-Star was dispensed with an automatic 8-channel pipette into all wells. After this step the plate is ready and immediately placed into the BioTek FL x 800 plate reader (BioTek, USA). During the kinetic reading, the sensitivity of the photomultiplier was set at 10 minutes, using a 6 second signal acquisition, for a period of 50 minutes. Measurement was carried out at ambient temperature using the luminescence mode where the lamp is turned off and the emitted light was filtered through a 460/40 nm filter. Gen5 software (version 1.0014, BioTek™) was used both for acquisition and data reduction.

3 Results

This part is divided in to three sections.

I) First, results from the experimental lines and their generational succession over time are presented with focus on the variable age at 1st reproduction as an indicator of population growth and fitness. These results are of importance as they portray the background of the study material for the subsequent results.

II) Second, 2C DNA content (pg nucleus^{-1}) and nucleus size was analyzed by flow cytometry during the long term study to assess eventual multigenerational effects of the food and temperature treatments. Measurement of the body size (at first reproduction) of the daphnids from the same treatments was also analyzed.

III) Finally, individuals from the last generation from the long-term treatments were subjected to reciprocal transplant experiment were animals were grown for 6 days after which their responses (MSGR, body C, N and P, RNA content and body APA) to food quality was measured. This was done for two consecutive generations (for both species) to test for maternal "carry-over", or the degree of long-lasting effects of the treatments.

3.1 Experimental lines

Most of the experimental lines were successfully kept for several generations. Age of first reproduction was scored, in the time period 23.03.2011 - 12.03.2012, for consecutive generations for all treatments, but with somewhat different number of generations represented due to both different growth rates, and unequal starting dates (see material and methods).

Age of first reproduction

The distribution of the onset of first reproduction was not normal distributed, and hence a generalized linear model (GLM) of the Gamma family with a logarithmic link was applied rather than a standard linear model. The three predictors Temperature, Diet and Generation were tested for additivity and pairwise interactions. Significance was judged from p-values. Model selection was performed using the "step" function in R (R core development team

2.11.11), reducing the full model (additive plus interaction) to a more parsimonious one by minimizing the Akaike Information Criterion (AIC). The two species were significantly different and analyzed separately. Differences in generation times unbalanced the sample sizes between treatments, such that the data set contained more generations from e.g. animals consuming high quality food at 20 ˚C. In order to try to identify a robust model with interactive terms between the predictors, a subset of data consisting of a more balanced number of observations between the experimental lines was tested additionally to the complete set of data. Finally, an analysis including both species but only from the high temperature treatment (20 ˚C) was conducted. When the Spiked (S) diet was not different from Full (F) diet, F and S were pooled to one factor level representing the good diet. This was the case for all analyses, except the one including both species at only 20 ˚C, where all the three diet levels were kept separately in the model.

Daphnia magna

In the additive model, for the full dataset (14 generations, Fig. 6 left), all three predictors, temperature, diet and generation gave significant contributions to onset of first reproduction ($p < 0.001$, $p= 0.0025$, $p=0.0158$, respectively). Moreover, when including interactions, diet interacts significantly with generation ($p=0.04$). The differences in onset of reproduction between high and low food quality increased with generation, i.e. the predicted slope of the generation effect was significantly higher in the P-limited group than for P-sufficient animals, suggesting an additive stress due to P-limitation over time under the low food quality treatment.

When the number of observations was harmonized among treatments to maximally 7 generations (Fig. 6 right), the minimal adequate model included all three predictors, with Diet and Generation both significant on < 5 % level, while Temperature was not. Moreover, the interaction between Diet and Generation was highly significant ($p << 0.001$). Additionally, there was a weaker, but still significant interaction term between Temperature and Generation ($p=0.0057$). The contrast between high and low temperature treatments increased over the generations in this model, with the predicted slope significantly less for the animals growing in 20 ˚C.

Daphnia pulex

In an additive model for the complete dataset for *D. pulex* (maximal 27 generations, Fig. 7 left), both Temperature and Diet contributed significantly to the age of first reproduction ($p < 0.001$, $p= 0.02$, respectively). Generation was not significant, however. Unsurprisingly, high temperature had an overall positive effect on growth (and thus reduced time to maturation), relative to animals grown at low temperature. Similarly, good food (high P) also reduced time from hatching until maturation. Interestingly, diet interacted significantly with generation ($p= 0.001$), suggesting an accumulated stress from P-deficiency (as for *D. magna*).

For the more balanced subset (10 generations, Fig. 7 right), temperature was the sole, significant predictor of time to maturation ($P< 0.001$). For this more limited number of generations, the interaction between diet and generation was not significant, suggesting that the accumulative, negative effect of poor diet only became manifest when a larger time span (more generations) were included.

Both species grown under three diet conditions at 20 ˚C

In this analysis, both *Daphnia species* from the 20 ˚C are included for a direct interspecific species comparison (Fig. 8). Species, diet and generation is included in the model, again with interactions. The best model yielded three significant interactions. First, diet interacted with species ($p = 0.04$), in that the cumulative delay in onset of reproduction on P-limited diet was less for *D. pulex* than for *D. magna*. Secondly, a positive interaction between diet and generation was revealed for both species ($p = 0.006$). Last, Species interacted significantly with generation ($p = 0.04$), the overall cumulative increase in onset of reproduction was less for *D. pulex* than for *D. magna*.

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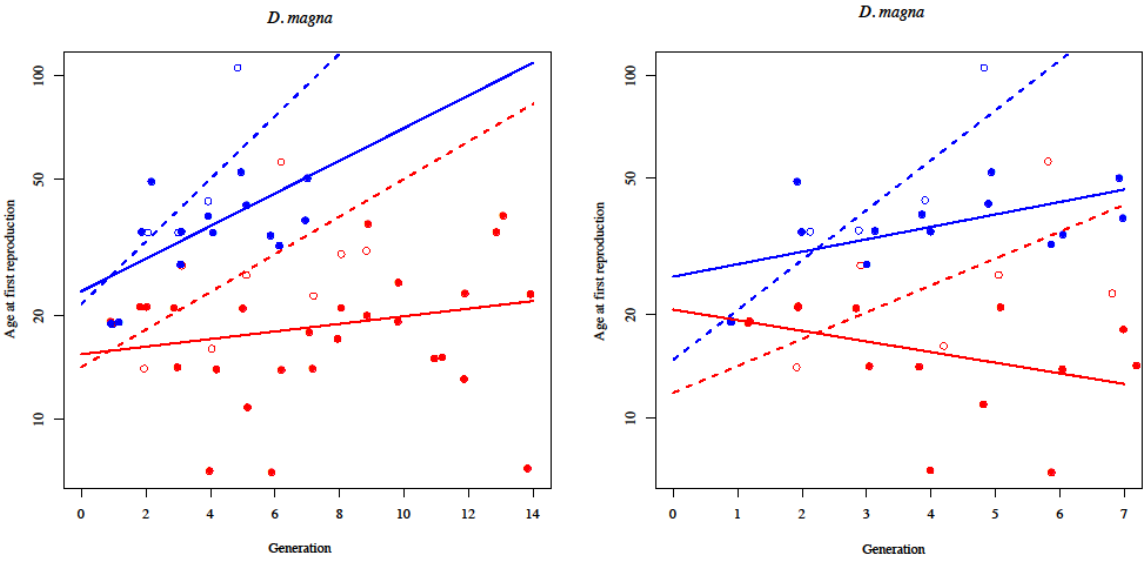

Figure 6. Joint effect of generation, temperature (20 and 10 ˚C) and dietary quality treatment (P-sufficient and P-limited diet) on Age of 1st reproduction for *Daphnia magna*. Each data point represents one observation. Left graph, the complete dataset, that include up to14 generations; right graph, the subset which include up to 7 generations. Red points and lines represent observations in 20˚C, whereas blue points and lines represent observations in 10˚C. Filled points and continuous line represent animals consuming high food quality, whereas hollow points and dashed line represent animals consuming low quality food.

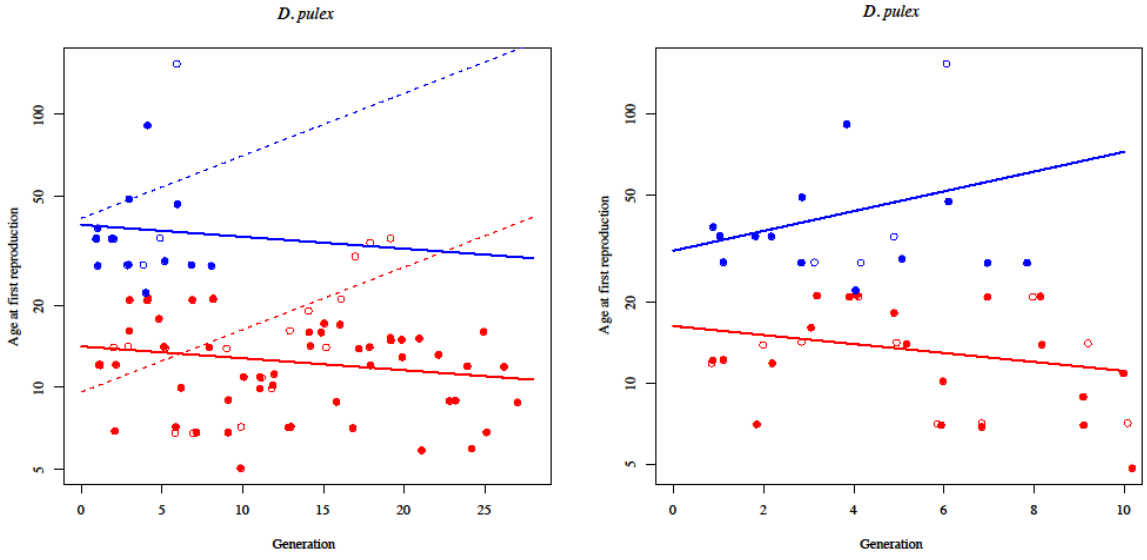

Figure 7. Joint effect of generation, temperature (20 and 10 ˚C) and dietary quality treatment (P-sufficient and P-limited) on Age of 1st reproduction for *Daphnia pulex*. Each data point represents one observation. Left figure, the full dataset, that include up to 27 generations. Right figure, the subset which include up to 10 generations. Red points and lines represent observations in 20˚C, whereas blue points and lines represent observations in 10˚C. Filled points and continuous line represent animals consuming high food quality, whereas hollow points and dotted line represent animals consuming low quality food.

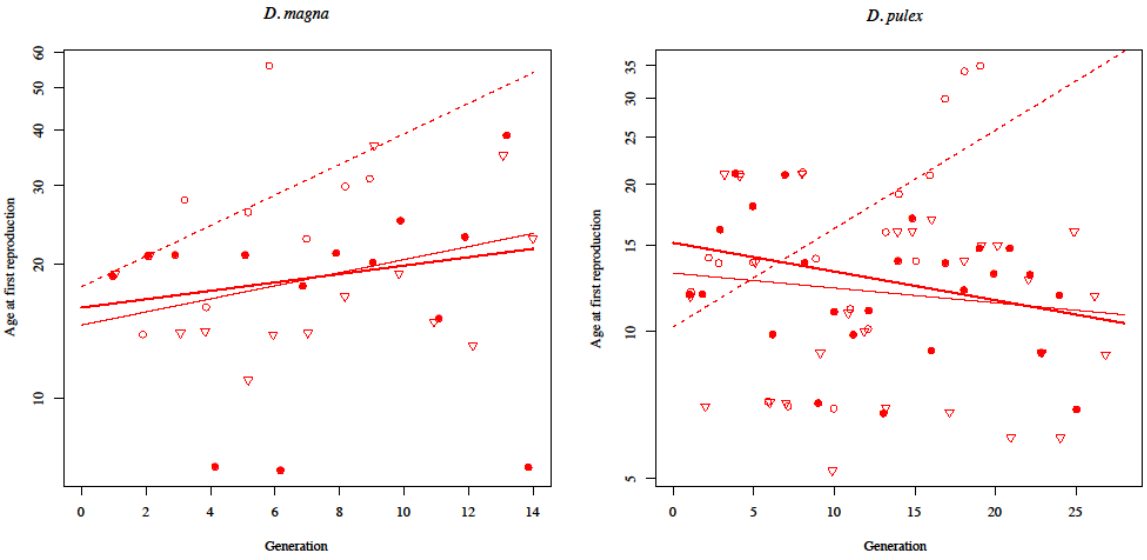

Figure 8. Joint effect of generation and dietary quality treatment (P-sufficient, spiked and P-limited) on Age of 1st reproduction for *Daphnia magna* (left figure) and *Daphnia pulex* (right figure). Each data point represents one observation. Filled points and thick continuous line represent animals fed P-sufficient diet, triangles and thin continuous line represent animals fed spiked diet, whereas hollow points and dashed line represent animals consuming low quality food.

3.2 Flow cytometry analysis

The somatic ploidy levels (2C, 4C and 8C) appeared as periodic doubling of the genome, the nuclei were grouped into distinct peaks on PI-fluorescence FL2-A histograms (Fig. 9). Number of FCM measured nuclei per ploidy level expressed as percentage, indicates that the majority of nuclei remains as 2C under both temperature regimes and diet treatments, while 8C nuclei remained as the smallest population. Further inspection of the data from the 2C populations, reveal that the coefficient of variance (%), was highly temperature dependant (p-value <0.001, r: 0.53, d.f = 91). The predictors Species, Temperature and Diet were tested with additive and pare wise interactions, and reduced to the most parsimonious model (Tab. 1). The predictor Diet did not contribute to the variation in the best model. The average CV at 10 °C was 9.20 % (SD = 2.015, n = 33) compared to an average of 5.99 % (SD = 0.941, n = 60) at 20 °C. Additionally, the interaction term between species and temperature was significant in explaining the variation in CV. All combinations between species and temperature, except for one: the comparison between *D.pulex* and *D. magna* at 20 °C, were found to be statistically different in their CV by the Tukey HSD test (not shown).

Table 1. Anova table for the CV for the 2C populations of nuclei isolated from *Daphnia*. The model explained 63.3 % of the variance in CV.

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Species	1.23	1	1.23	0.75	0.39
Temperature	233.17	1	233.17	142.74	<0.001***
Species : Temperature	24.09	1	24.09	14.74	<0.001***
Total	258.49	3			
Residuals	142.12	87	1.6		

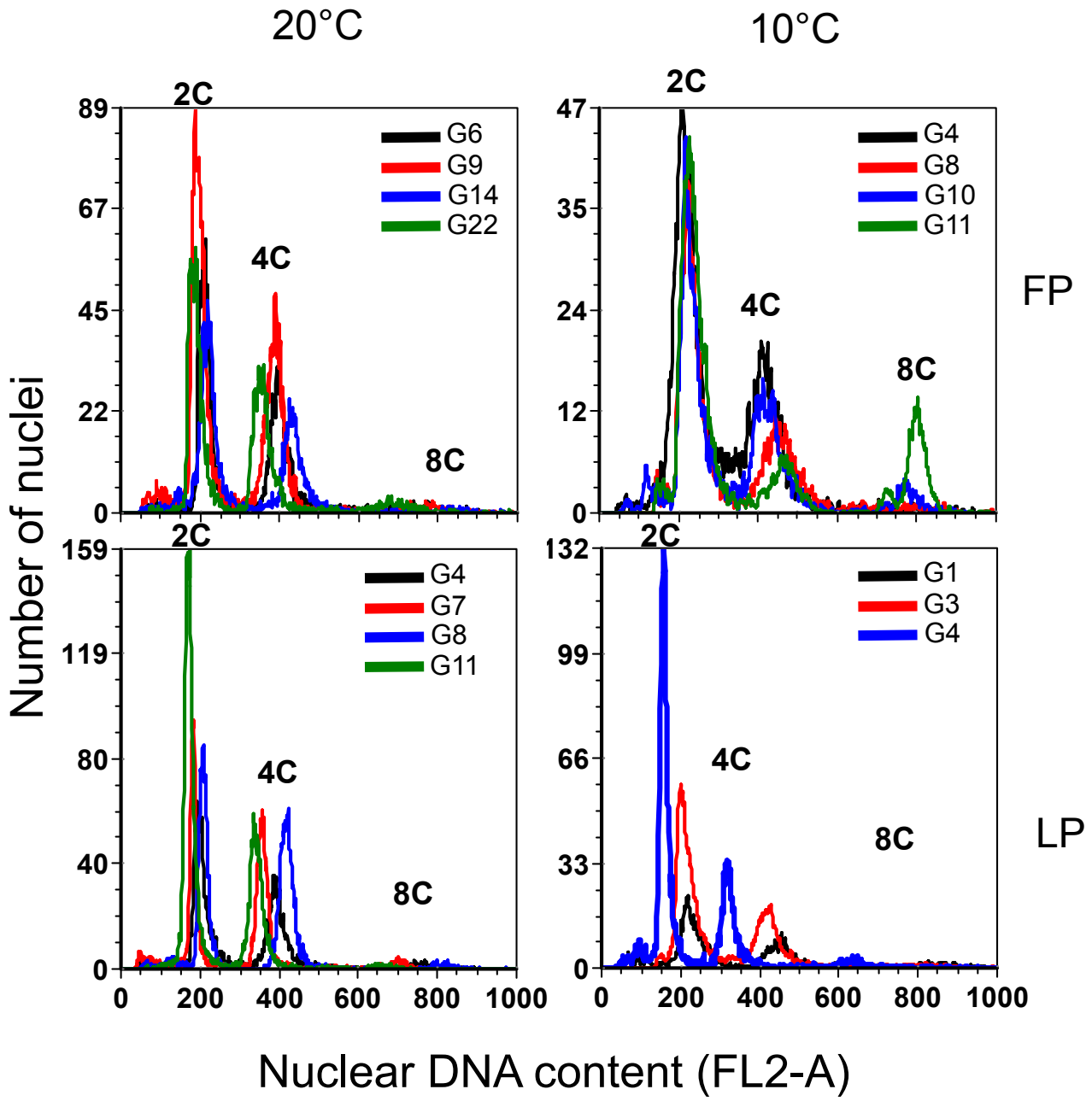

Figure 9: Representative FCM histograms (colour code for different generations) of PI stained nuclear DNA content (horizontal axis, linear scale) of *D. magna,* reared at 20 ˚C (left) and 10 ˚C (right) fed P-sufficient food - F (upper panels) and P-limited food- P (lower panels). Alignment beads and CRBC nuclei were used as external standards (not shown).

Diploid DNA content in *Daphnia magna* and *Daphnia pulex*

Diploid (2C) DNA content (Fig. 10, Tab. 4) was investigated as a dependent variable in a linear model. The predictors Species, Temperature and Diet were tested with additive and pair-wise interactions, and reduced to the most parsimonious model. Two observations were manually removed from the dataset, for causing heteroscedastity as revealed by the diagnostic plot of the residuals (observation 17 and 58, data not shown). The F test and P-values from the ANOVA analysis show that there are differences between groups (Tab. 2).

Table 2. Anova table for the Diploid (2C) DNA content in Daphnia. The model explained 85 % of the variation.

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Species	0.49	1	0.49	394.11	<0.001***
Temperature	0.076	1	0.076	61.23	<0.001***
Diet	5.5×10^{-4}	2	2.8×10^{-4}	0.22	0.8018
Species : Temperature	0.045	2	0.045	36.0	<0.001***
Species : Diet	0.030	2	1.25×10^{-4}	11.88	<0.001***
Total	0.64	4			
Residual	0.10	83	1.25×10^{-3}		

The Tukey's honestly significant difference (HSD) test was used to test all pairs of means. First, the two *Daphnia* species are significantly different, *D. magna* has a larger genome than *D. pulex* (Tukey HSD for *D. Pulex* vs. *D. magna* = [-0.16 , -0.13]). Second, the combined mean of all observations from 20 ˚C is significantly lower than the combined mean for all observations from 10 ˚C "(Tukey HSD for 20 ˚C vs. 10 ˚C = [-0.08 , -0.04]).

As already revealed from the ANOVA analysis there are no significant combined comparisons between the diets. Third, the significant interaction term between species and temperature, reveal that all combinations between the two species are significantly different. *D. magna* have a higher combined mean in all comparisons with *D. pulex* (not listed). Additionally, the differences between the observations at 20 ˚C and 10 ˚C for the two species separately, reveal that *D. magna* individuals reared at 10 ˚C have significant ($p<0.001$) higher combined mean than those reared at 20 ˚C (Tukey HSD for *D. magna* 20 ˚C vs. *D. magna* 10 ˚C = [-0.14 , -0.08]). For *D. pulex* there is not a significant ($p=0.6$) difference between the observations in 10 ˚C compared to 20 ˚C (Tukey HSD for *D. pulex* 20 ˚C vs. *D. pulex* 10 ˚C = [-0.04 , -0.02]).

Finally, the significant interaction term between species and diet, reveal that all combinations between the two species and diets are significantly different. *D. magna* have in all comparisons a higher overall mean than *D. pulex* (not listed). Additionally, there is a significant ($p<0.001$) but weak difference within *D. magna*. Animals fed the P-limited diet have a lower overall mean than those fed the P-sufficient diet. (Tukey HSD for *D. magna* :P vs. *D. magna* :F = [-0.09 , -0.01]). Within the species there are no other significant comparisons between the diets.

In order to reveal if the statistical difference in estimated 2C-value between individuals of *D. magna* fed the P vs. F diet, came along with a different CVs for the two treatments a linear model with CV as the dependent variable was tested (restricted to a sub-set with observations from only *D. magna*). The most parsimonious model (Tab. 3) revealed that the predictor Diet did contribute to the variation in CV.

Table 3. Anova table for the CV for the 2C populations, from *D. magna* samples only. The model explains 63 % of the variance.

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Temperature	194.0	1	194.0	172.3	<0.001***
Diet	19.1	2	9.5	8.5	<0.001***
Total	213.1	3			
Residuals	46.2	41	1.1		

There is a significant decrease in the CV for the samples from the *D. magna* individuals fed P limited food compared to P sufficient (Tukey HSD for P vs. :F = [-1.76 , -2.81]), and compared to those fed the diet spiked with inorganic P (Tukey HSD for S vs. P = [0.24 , 2.37]). There was no statistical difference between the two high quality diets (S vs. F)

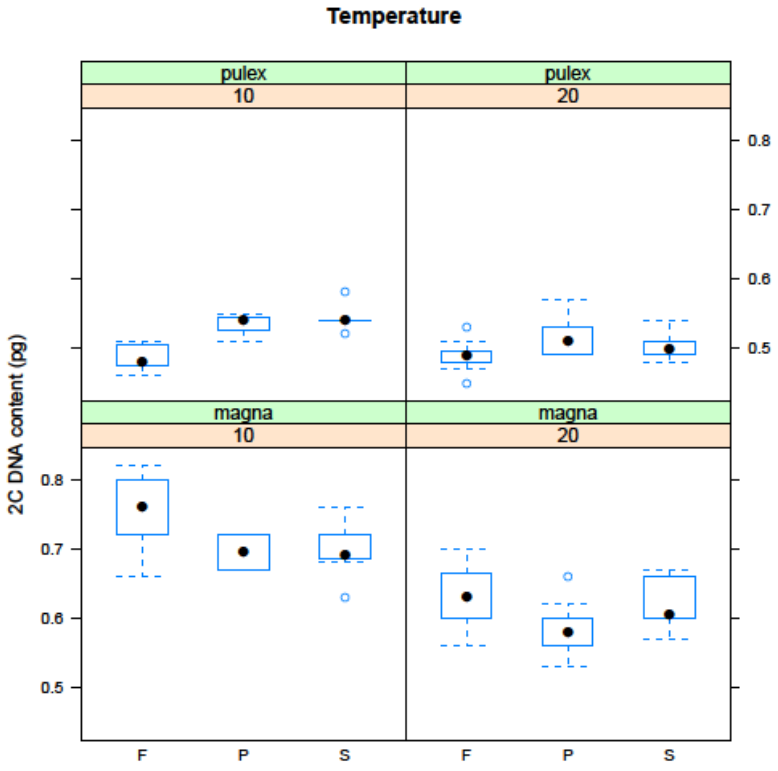

Figure 10. Box plots of 2C DNA content (pg nucleus^{-1}) for *D. pulex* (upper panel) and *D. magna* (lower panel), reared in 10 ˚C (left) and 20 ˚C (right) fed three different diets F (P-sufficient diet), P (P-limited diet) and S (spiked with inorganic P).

Table 4. Descriptive statistic of 2C DNA content (pg nucleus^{-1}) samples for all treatment groups for both Daphnia species. Diet: F = P-sufficient diet, P= P-limited diet, S= spiked diet.

Species	Diet	°C	N	Mean	Median	Min	Max	Std.Dev.
D. magna	F	10	7	0.75	0.76	0.66	0.82	0.06
	P		2	0.70	0.70	0.67	0.72	0.04
	S		7	0.70	0.69	0.63	0.76	0.04
	F	20	12	0.63	0.63	0.60	0.70	0.05
	P		7	0.58	0.58	0.53	0.66	0.04
	S		10	0.62	0.62	0.57	0.67	0.03
D. pulex	F	10	7	0.49	0.48	0.46	0.51	0.02
	P		3	0.53	0.54	0.51	0.55	0.02
	S		5	0.56	0.54	0.52	0.58	0.04
	F	20	12	0.49	0.49	0.45	0.53	0.02
	P		9	0.52	0.41	0.49	0.57	0.03
	S		10	0.50	0.50	0.48	0.54	0.02

Adult body size

For each species separately, adult body size at first reproduction was compared with a significance test, between same diets at different temperatures (Tab. 5), and between diet combinations within each temperature (Tab. 6). First, for *D. magna* there were no significant contrasts between equal diets (F or P) at different temperatures. For *D .pulex* the contrast between the low quality diets at different temperatures was significant. At 20 ˚C *D. pulex* was 0.58 mm smaller on average. Secondly, comparing the effect of different food quality treatments (F vs. P) within the same temperature reveal no significant comparisons between the diet types for *D. magna*. For *D .pulex* there was one significant comparison at 20 ˚C, animals fed high quality diet were on average 0.60 mm bigger than those fed the low quality diet.

Table 5. Tukey HSD test for body size (mm) between equal diets (F= P-sufficient diet, or P= P-limited diet) at different temperatures (10 °C and 20 °C) for *D. magna* and *D. pulex* separately. Significance code is the adjusted p-value. * significant combinations.

Body size (mm)	***D. magna***	***D. pulex***
Same diets at different temperature	significant	significant
F (20 ^{o}C) vs F (10 ^{o}C)	0.99	0.55
P (20 ^{o}C) vs P (10 ^{o}C)	0.29	0.024*

Table 6. Tukey HSD test for body size (mm) for body size between diet combinations (F= P-sufficient diet, and P= P-limited diet) within each temperature for *D. magna* and *D. pulex*. Significance code is the adjusted p-value. * significant combinations.

Body size (mm)	***D. magna***	***D. pulex***
Different diets at the same temperature	significant	significant
F vs P at 20 ˚C	0.21	<0.001*
F vs P at 10 ˚C	0.99	0.86

The relationship between 2C DNA content and nucleus size

The correlation between 2C DNA content (pg nucleus^{-1}) and nucleus size (FSC) was assessed by simple log-log regression analysis for each species separately. The two variables were positively correlated ($p<0.001$) for both species (Fig. 11).

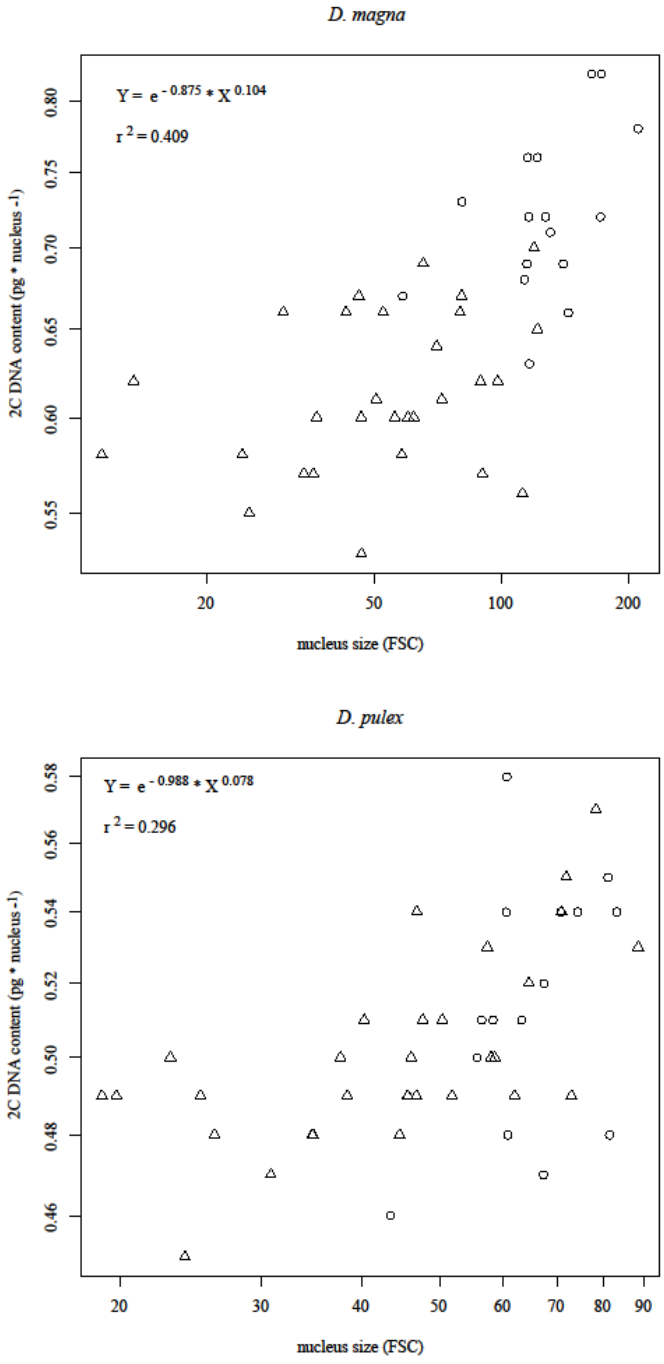

Figure 11. Scatter plot in logarithmic scale of 2C DNA content (pg nucleus $^{-1}$) and nucleus size (FSC). Upper: *D. magna*, lower: *D. pulex*. Triangles represent samples from 20 ˚C, whereas circles represent samples from 10 ˚C.

3.3 Reciprocal transplant experiment

The reciprocal transplant experiment was conducted with the last generations of the experimental lines raised at high and low food at 20 ^{o}C. The reason for choosing only the 20 ^{o}C treatments was partly because these has reached the highest number of generations (high quality : low quality, G20 : G11, and G31 : G23, for *D. magna* and *D. pulex* respectively), and also for practical reasons since handling more treatments were beyond the reach of this study. Within the experimental time-span (~ 70 days), samples for MSGR, RNA content and body APA were collected randomly. In the hope of repeating this experiment, four replicates were seen as enough for first assessment. Laborious and time consuming as it was, there was simply not enough time for repeating the experiment. The decision was therefore made to collect more replicates in first assessment when possible. Thus, a unit arriving later in the experiment and second generation is generally represented with more replicates than first part or first generation. Samples of food mixtures were saved for post-experimental determination of food C:P ratios over this entire experimental period, as also within-treatment variation of food C:P could potentially affect the results. Episodic changes in the chemostat occurred, which again affected algal C:P. Thus, despite a strong difference in C:P between the high and low P treatments (Fig. 12 right), algal C:P was also treated as a continuous variable rather that a treatment factor with two levels (i.e. Good food and Bad food) in the statistical analysis. This is taken in to account when analyzing the responses MSGR, RNA content and body APA in *Daphnia*.

3.3.1 C, N and P composition

Algal C:P-ratios

Algal C:P displayed a substantial difference between treatments over the entire experimental period (Fig. 12 left). The high and low P-treatments had mean C:P of 237.9 (SD = 37.5) and 1115.8 (SD = 249.8), respectively. C:P in the P-limited algae increased gradually over the course of the experiment, while the P-sufficient diet remained stable (Fig. 12 right). This suboptimal experimental conditions regarding the P-limited diet calls for caution in the biological interpretation of the results.

Percentage of C, N, and P in Daphnia

The percentage of C, N and P incorporated in the body of animals grown in the reciprocal transplant experiment, was analysed to test dietary effect on somatic stoichiometry. The specific P-content (as % of DW) of both *D. magna* and *D. pulex* was significantly affected by their diets ($p<0.001$ and $p= 0.015$, respectively) (Fig. 13, upper left). For both species food quality also significantly affected their specific N-content ($p < 0.001$, $p = 0.012$, respectively) (Fig. 13, upper right). The two species did not differ in their average specific P- or N-content. Animals feeding on P-limited algae experienced on the average a ~ 11 % decrease in specific N-content, and ~ 31 % decrease in P. On the contrary, food quality did not affect the specific C-content in neither of the species, but *D. pulex* had a significantly higher C-content ($p = 0.001$) than *D. magna* (Fig. 13, lower left).

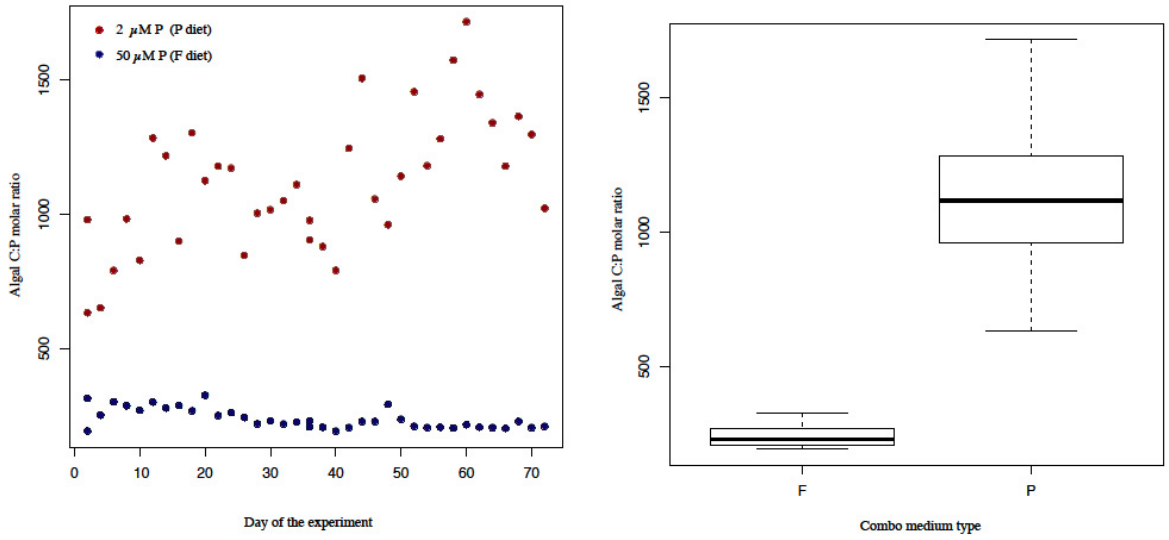

Figure 12. Algae food quality treatment. Upper figure; Algal C:P molar ratio (y-axis) over time (x-axis), P-sufficient treatments in blue (n = 38), P-limited treatments in red (n = 38). Bottom figure; box plot of algal C:P molar ratio of algae grown in P-sufficient Combo medium (F) (n = 38, mean = 238) and P-limited Combo medium (P) (n = 38, mean = 1115). Boxes with whiskers show 1st and 3rd quartiles, horizontal line mark the median.

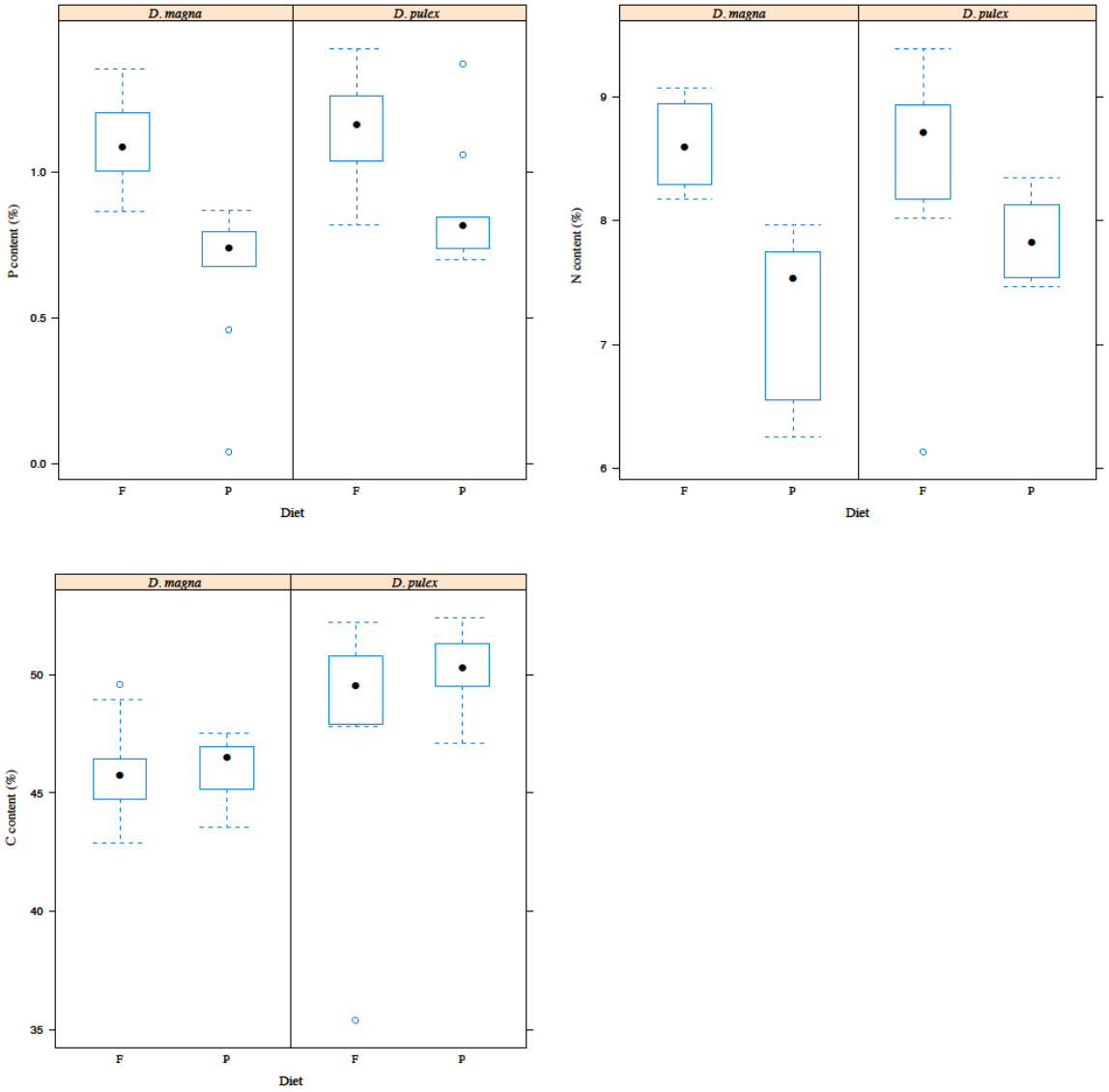

Figure 13. Box plot of the percent of the elements phosphorus (P) (upper left), nitrogen (N) (upper right), and carbon (C) (lower left) in the body of 6 day old *Daphnia magna* (left) and *Dapnia pulex* (right) in response to the two different diet treatments (F= P-sufficient diet, P= P-limited diet). Boxes show 1st and 3rd quartiles, black dot mark the median.

3.3.2 Mass specific growth rate

One of the goals in this thesis was to monitor the specific growth rate of the two daphnia species grown under conditions with high or low P over several generations, to see if heritable changes in this trait value occur. A linear model was used to investigate the data set following test for homoscedasticity and normality. The two daphnia species were statistically different in their distribution of MSGR, and their interaction with diet (Tab. 7). Further inspections were done with the species separately. The explanatory variables Line, Diet and Generation were included with interactions in the models. Additionally a second model (Model 2) was investigated replacing the factor version of Diet (in Model 1) with a continuous Diet variable equal to the C:P ratio of the algae (consumed by the daphnids in their 6 day period of growth).

Table 7. ANOVA table of mass specific growth rate explained by the predictors Species, Diet and their interaction. $R^2 = 0.70$. Diet is here a factor of two levels (good or bad diet). Replacing the Diet predictor with a continuous C:P ratio variable yielded the same results.

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Species	0.093	1	0.093	29.89	<0.001
Diet (treatment)	0.66	1	0.66	212.42	<0.001
Species: Diet	0.080	1	0.08	25.76	<0.001
Total	1.18				
Residuals	0.34	109	0.003		

Daphnia magna

Model 1: The best model included all the three predictors (Tab 8, Fig. 14). Diet was highly significant. There was a negative effect on MSGR for *D. magna* individuals consuming low food quality. There were also two significant interaction effects. First, an additional reduction in MSGR is seen in daphnids originating from the line P fed low food quality. Second, there is an additional significant decrease in MSGR for all daphnids eating low food quality in the second generation. This model explains as much as 86 % of the variation in MSGR with 57 degrees of freedom and can not be simplified any further.

Table 8. Anova table for Model 1 of mass specific growth rate for *D. magna* explained by the predictors Line, Diet and Generation. Diet is here a factor of two levels (high quality vs. low quality).

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Line	0.024	1	0.024	11.73	<0.001 **
Diet	0.70	1	0.70	339.87	<0.001***
Generation	0.020	1	0.020	9.66	<0.001 **
Line : Diet	0.024	1	0.024	11.91	0.001 **
Diet : Generation	9.9×10^{-3}	1	9.9×10^{-3}	4.85	0.032 *
Total	0.78	5			
Residuals	0.12	57	0.0021		

Model 2: Investigating the same model but with the variable C:P algae instead of Diet of two factor levels, all there predictors remained (Tab. 9). Diet was highly significant as a predictor. There is a decrease in MSGR in generation 2. Further, there is a significant interaction term in the model between Line and Diet, with an additional decrease in MSGR for animals originating form the P line fed low quality food. This model explain the variation equally well as the former model, revealing a R^2 =0.85 on 58 degrees of freedom.

Table 9. ANOVA table for Model 2 of mass specific growth rate for *D.magna* explained by the predictors Line, Diet and Generation. Diet is here a continuous variable of the C:P ratio of the algae.

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Line	0.024	1	0.024	10.96	0.001 **
Diet	0.710	1	0.710	322.81	<0.001***
Generation	0.010	1	0.010	4.65	<0.035 *
Line : Diet	0.022	1	0.022	10.10	0.002 **
Total	0.77	4			
Residuals	0.13	58	0.0022		

Daphnia pulex

Model 1: Only Diet and Generation remained as explanatory variables (Tab. 10). Individuals of *D. pulex* consuming low food quality show a significant negative effect on MSGR. There was also a significant interaction effects between Diet and Generation. Daphnids consuming low food quality had a reduced MSGR in the second generation. This model explains 51 % of the variation in MSGR on 46 degrees of freedom

Table 10. Anova tabel of Model 1 of mass specific growth rate for *D. pulex* explained by the predictors Diet and Generation. Diet is here a factor of two levels (high quality vs. low quality).

Source of variation	Sum of squares	df	Mean squares	F-ratio	P
Diet	0.11	1	0.11	49.64	<0.001*
Generation	1×10^{-6}	1	1×10^{-6}	2.5 x 10-3	0.96
Diet: Generation	9.2×10^{-3}	1	9.1×10^{-3}	4.25	0.045 *
Total	0.12	3			
Residuals	0.099	46	2.1×10^{-3}		

Model 2: This model was simplified equally to Model 1 (Tab. 11). Diet is highly significant as a main predictor with negative effect on MSGR. The model also suggest a non-significant interaction between the Diet and Generation, pointing out a decrease in MSGR in the second generation (Fig. 15). This model explained 52 % of the variation on 46 degrees of freedom. The two models still almost conclude the same. There is significance for an interaction between Diet and generation in the former but not in the latter.

Table 11. Anova table for Mode 2 of mass specific growth rate for *D.pulex* explained by the predictors Diet and Generation. Diet is here a continuous variable of the C:P ratio of the algae.

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Diet	0.11	1	0.11	54.27	<0.001***
Generation	1×10^{-5}	1	1×10^{-5}	4.6×10^{-3}	0.95
Diet: Generation	5.7×10^{-3}	1	5.7×10^{-3}	2.71	0.11
Total	0.12	3			
Residuals	0.097	46	2.1×10^{-3}		

Finally, to more closely examine the association among MSGR and the elemental ratio P:C in the daphnids, a simple linear regression analysis was performed between MSGR and P:C. There was a significant positive correlation between MSGR and P:C ($p < 0.001$, $r = 0.966$) for *D. magna* (Fig. 16, upper), and for *D. pulex* ($p = 0.022$, $r = 0.614$) (Fig. 16, lower).

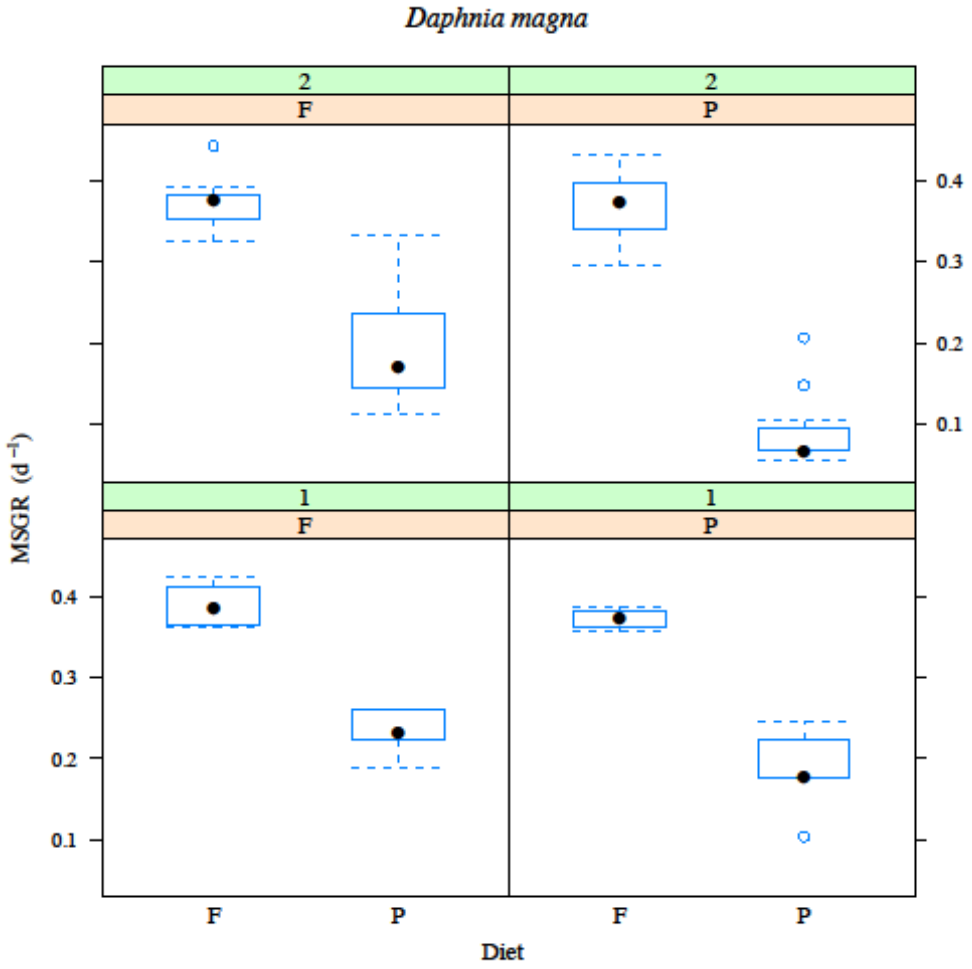

Figure 14. Responses in mass specific growth rate (MSGR, d^{-1}) in *Daphnia magna*. Food quality type F or P (Diet). Letter in the pink frame indicate the Line (the dietary history of the previous generations before the ones examined in this experiment) F (multiple generations on high food quality) or P (multiple generations on low food quality). Number in the green frame indicate the Generation, 1 (lower panel) or 2 (upper panel), mothers and daughters in this experiment. See figure.4 (section 2.4) for illustration of the reciprocal transplant.

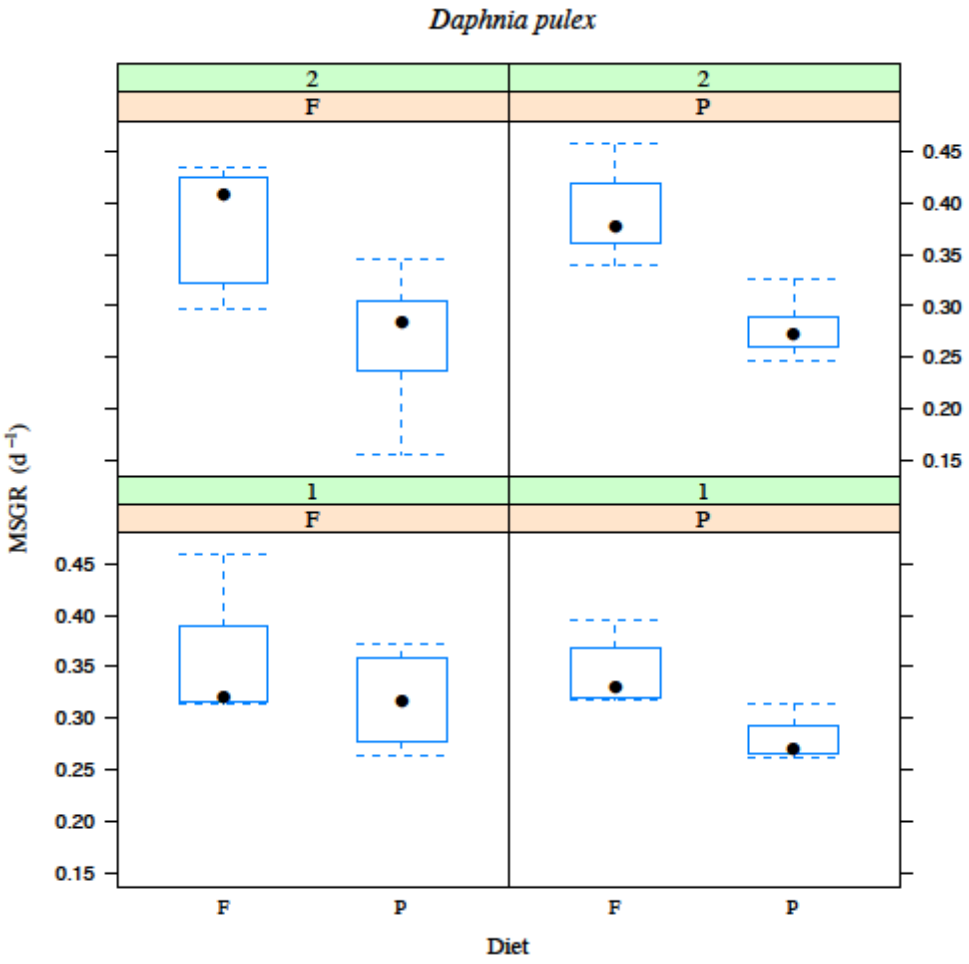

Figure 15. *Daphnia pulex* MSGR. Food quality type F or P (Diet). Letter in the pink frame indicate the Line (the dietary history of the previous generations before the ones examined in this experiment) F (multiple generations on high food quality) or P (multiple generations on low food quality). Number in the green frame indicate the Generation, 1(lower panel) or 2 (upper panel), mothers and daughters in this experiment. See figure.4 (section 2.4) for illustration of the reciprocal transplant.

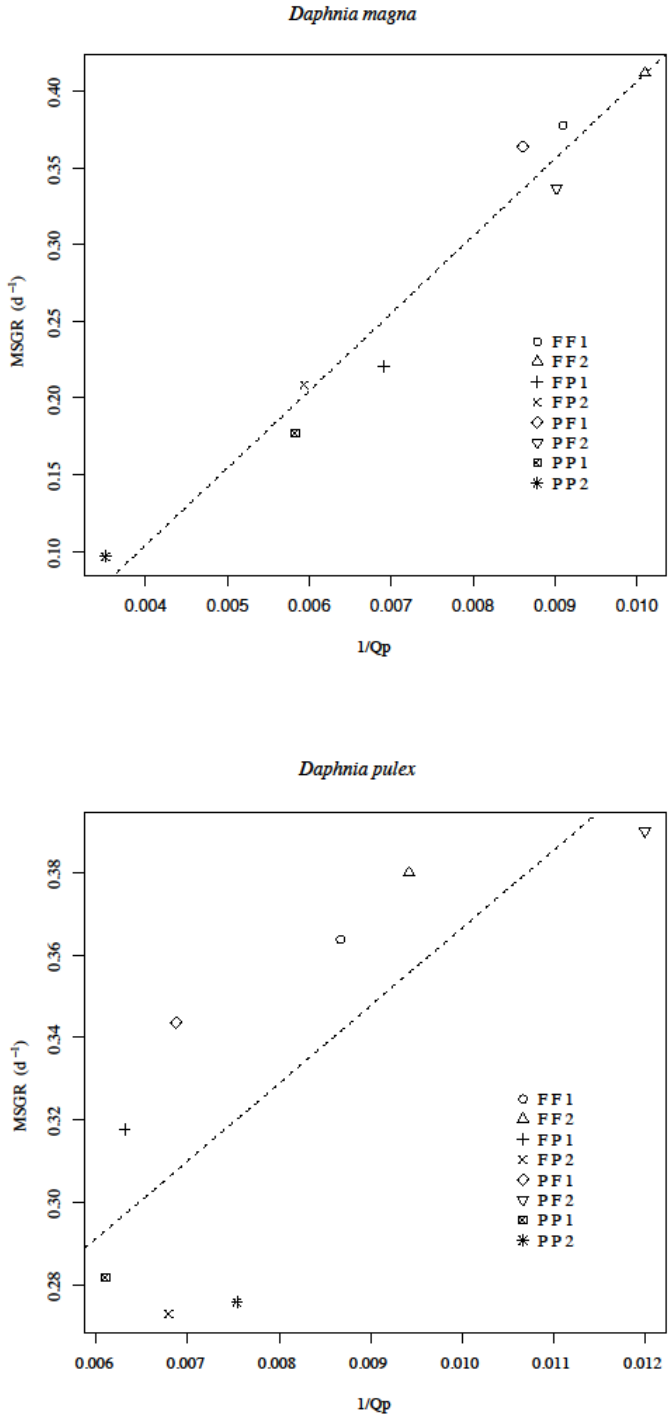

Figure 16. Relationships between MSGR and body P:C ($1/Q_P$). Upper panel: Daphnia magna (Equation: MSGR= -0.097 + 50.35 * P:C , r: 0.96). Lower panel: Daphnia pulex (Equation: MSGR = 0.178 + 18.84 * P:C , r: 0.55). Symbols: First abbreviation represent - Line, second - Diet, last number specify – Generation, as assessed under the reciprocal transplant.

3.3.3 RNA content

The specific RNA content (total RNA per dry weight) was used as a response variable (Fig. 17 and Fig. 18, *D.magna* and *D.pulex*, respectively). Effects of food quality (Diet), the multigenerational history (Line) and generation assessed (1^{st} and 2^{nd}) in the reciprocal transplant were tested for in the models. First the interspecific differences between the two species were tested. Further investigations were done for each species separately. Linear models are used in all analysis following test for homoscedasticity and normality.

Model 1: There was a significant interaction between food type and daphnia species affecting RNA content, the level of RNA content decreased more in individual of *D. magna* consuming low food quality than seen for *D. pulex* (Tab. 12). Additionally, there was an interaction between line and *Daphnia* species, the level of RNA content was higher in *D. pulex* originating from the P line compared to *D.magna* originating from the P line. The model explains 15 % of the variance on 177 d.f.

Table 12. Anova table for Model 1 of RNA content explained by the predictors Species, Line and Diet. Diet is here a factor of two levels (high quality vs. low quality).

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Species	1.65×10^{-5}	1	1.65×10^{-5}	0.22	0.64
Line	5.60×10^{-5}	1	5.60×10^{-5}	0.75	0.39
Diet	2.06×10^{-5}	1	2.06×10^{-5}	27.59	>0.001*
Species : Line	3.17×10^{-4}	1	3.17×10^{-4}	4.25	0.041 *
Species : Diet	3.00×10^{-4}	1	3.00×10^{-4}	4.03	0.046 *
Total	2.74×10^{-3}				
Residuals	0.013	177	7.45×10^{-5}		

Model 2: This model explained 13.5 % of the variance on 177 d.f. and is in concordance with Model 1.. The interaction terms between species and diet is no longer significant though (p=0.066), but indicate a different response for the species over the C:P gradient (Tab. 13).

Table 13. Anova table for Model 2 of RNA content explained by the predictors Species, Line and Diet. Diet is here a continuous variable of the C:P ratio of the algae.

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Species	1.65×10^{-5}	1	1.65×10^{-5}	0.22	0.64
Line	5.60×10^{-5}	1	5.60×10^{-5}	0.74	0.39
Diet	1.84×10^{-3}	1	1.84×10^{-3}	24.30	0.001***
Species : Line	3.50×10^{-4}	1	3.50×10^{-4}	4.62	0.033 *
Species : Diet	2.60×10^{-4}	1	2.60×10^{-4}	3.43	0.065
Total	2.52×10^{-4}				
Residuals	0.013	177	7.58×10^{-5}		

Daphnia magna

Model 1: After model selection only Diet remained as a significant explanatory variable. Individuals consuming low food quality show a highly significant main negative effect on RNA content. Line also remained with a non-significant reduction in RNA content for the animals in the P-line. This model explains 22.4 % on 80 d.f. (Tab. 14).

Table 14. Anova table for Model 1 of RNA content for *D. magna* explained by the predictors Line and Diet. Diet is here a factor of two levels (high quality vs. low quality).

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Line	2.98×10^{-4}	1	2.98×10^{-4}	3.47	0.066
Diet	1.91×10^{-3}	1	1.91×10^{-3}	22.21	<0.001*
Total	2.21×10^{-3}	2			
Residuals	6.88×10^{-3}	80	8.60×10^{-5}		

Model 2: The model output do not change drastically with a continuous Diet variable. By model selection only Diet remained as a significant predictor. This model explain somewhat less of the variation 20.1 % on 81d.f. (Tab. 15).

Table 15. Anova table for Model 2 of RNA content for *D. magna* explained by the predictors Line and Diet. Diet is here a continuous variable of the C:P ratio of the algae.

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Diet	4.50×10^{-4}	1	4.50×10^{-4}	6.90	0.009*
Residuals	6.38×10^{-3}	98	6.51×10^{-5}		

Daphnia pulex

Model 1: After model selection only Diet remained as a significant explanatory variable. There is a significant negative effect on RNA content in individuals consuming low food quality (r = 0.056, d.f: 98 ; Tab. 16).

Table 16. Anova table for Model 1 of RNA content for *D. pulex* explained by the predictor Diet. Diet is here a factor of two levels (high quality vs. low quality).

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Diet	1.9×10^{-3}	1	1.9×10^{-3}	21.57	<0.001***
Residuals	7.2×10^{-3}	81	8.8×10^{-5}		

Model 2: Replacing the factor Diet with the continuous variable of Diet, the resulting model is similar to the previous one (Tab. 17). Only Diet is significant as a predictor. In addition it suggest a non-significant interaction between Line and Diet, suggesting that animals originating from the line P do not decrease their RNA content as much as animals originating from the line F when consuming food with increased C:P ratio (r^2: 0.057, d.f:96; Tab. 17)..

Table 17. Anova table for Model 2 of RNA content for *D. pulex* explained by the predictors Line and Diet. Diet is here a continuous variable of the C:P ratio of the algae.

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Line	3.2×10^{-5}	1	3.2×10^{-5}	0.48	0.49
Diet	3.9×10^{-4}	1	3.9×10^{-4}	5.95	0.016 *
Line : Diet	1.4×10^{-4}	1	1.4×10^{-4}	2.45	0.12
Total	5.9×10^{-4}	3			
Residuals	6.2×10^{-3}	96	6.6×10^{-5}		

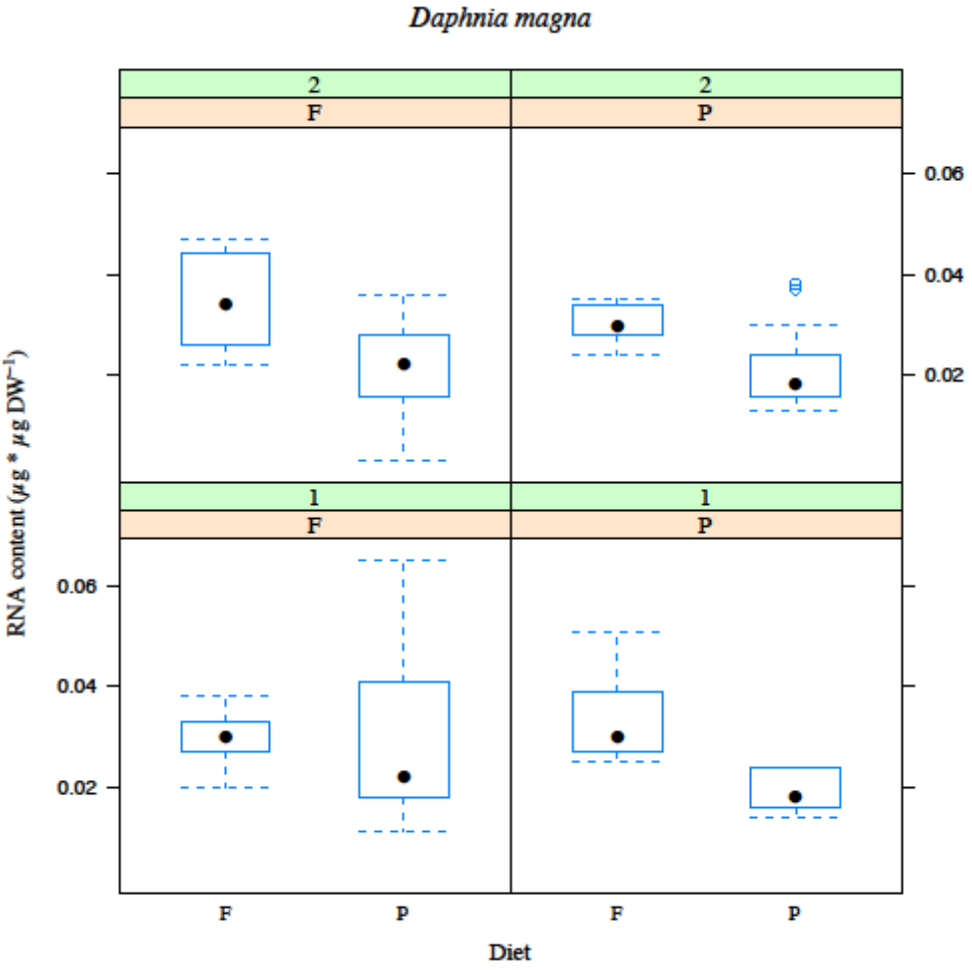

Figure 17. *Daphnia magna* RNA content. Food quality type F or P (Diet). Letter in the pink frame indicate the Line (the dietary history of the previous generations before the ones examined in this experiment) F (multiple generations on high food quality) or P (multiple generations on low food quality). Number in the green frame indicate the Generation, 1(lower panel) or 2 (upper panel), mothers and daughters in this experiment. See figure.4 (section 2.4) for illustration of the reciprocal transplant.

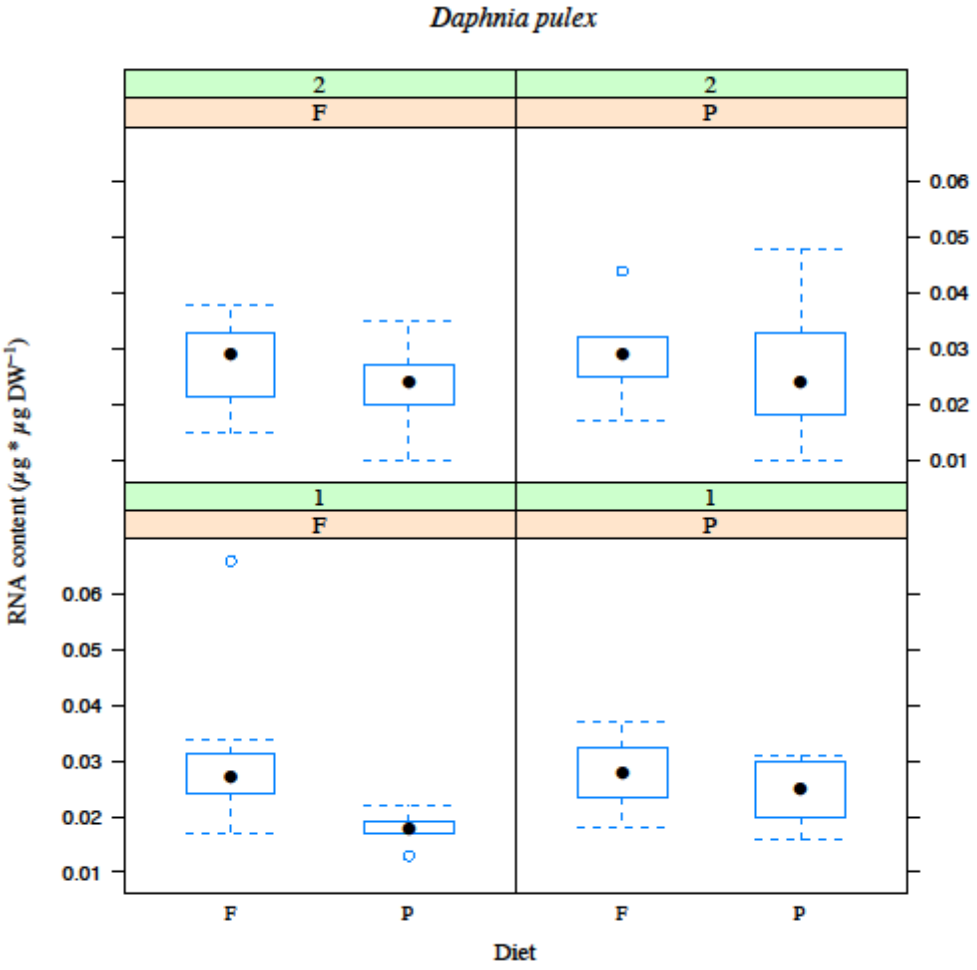

Figure 18. Box plot of *Daphnia pulex* RNA content. Food quality type F or P (Diet). Letter in the pink frame indicate the Line (the dietary history of the previous generations before the ones examined in this experiment) F (multiple generations on high food quality) or P (multiple generations on low food quality). Number in the green frame indicate the Generation, 1(lower panel) or 2 (upper panel), mothers and daughters in this experiment. See figure.4 (section 2.4) for illustration of the reciprocal transplant.

3.3.4 Body alkaline phosphatase activity

There was a significant main effect between the two daphnids species in body APA with higher average for *D. magna* (Tab 18).

Table 18. Anova table for body APA explained by the predictor Species.

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Species	4.46	1	4.46	10.66	0.001**
Residuals	67.81	72	0.42		

Daphnia magna

Model 1: *D. magna* did not increase body APA in response to low food-quality (Fig. 19). Diet is not significant as an explanatory variable, and there is no significant interaction effect with food quality. The complicated model with all potential explanatory variables and their interactions could be reduced to only "Line" being highly significant ($p < 0.001$) (Tab. 19). Body APA is significantly lower in animals consuming both high and low quality food originating from the line P, compared to higher levels of APA in animals consuming both high and low quality food originating from the line F. Model 2 resulted in the exact same result, with both model explaining 19 % of the variation on 72 d.f. (Tab. 19)

Table 19. Anova table for the best model of body APA for *D. magna.*

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Line	7.70	1	7.70	18.18	<0.001*
Residuals	30.50	72	0.42		

Daphnia pulex

Model 1: *D. pulex* did not increase body APA in response to low food-quality (Fig. 20). There were no significant main effects or interactions in the model. There was a non-significant indication of an increase in body APA in generation 2 compared to generation 1 (Tab. 20). Model 2 resulted in the exact same result, with both model explaining 1.1 % of the variation on 88 d.f.

Table 20. Anova table for the best model of body APA for *D. pulex*.

Source of variation	Sum of squares	Df	Mean squares	F-ratio	P
Generation	0.65	1	0.65	1.99	0.16
Residuals	28.97	88	0.33		

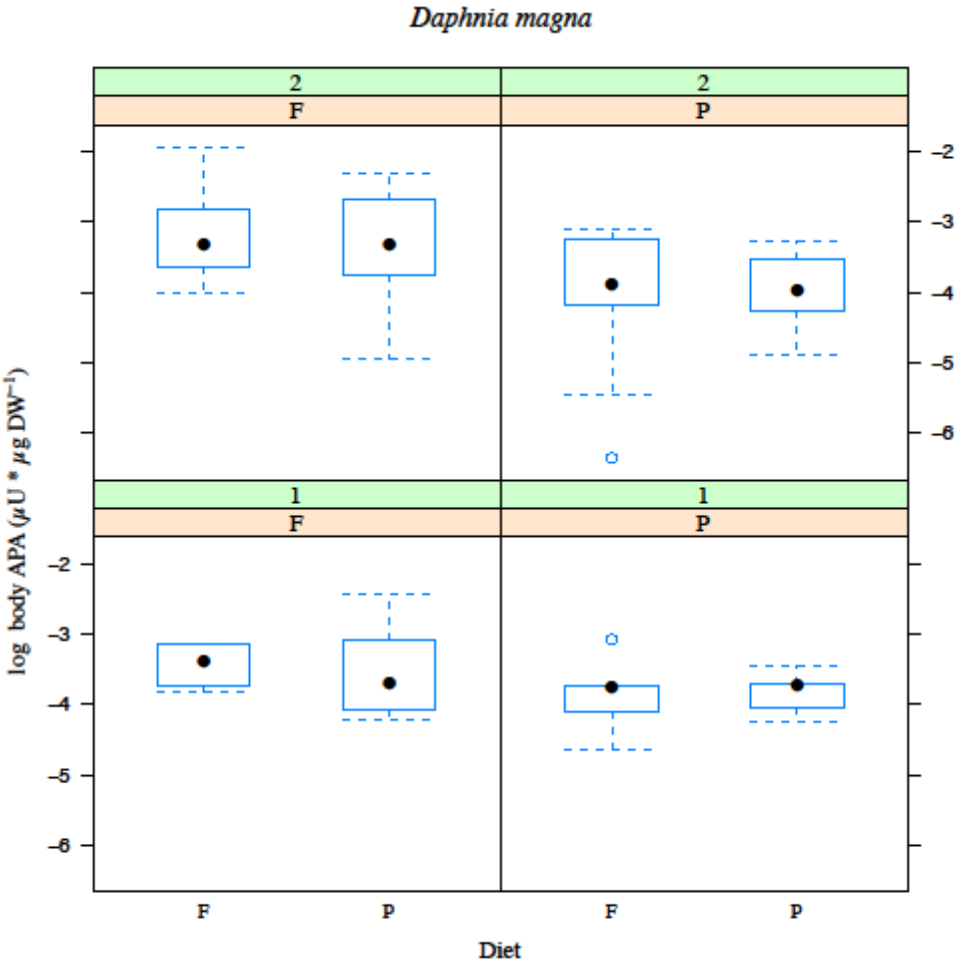

Figure 19. Box plot of *Daphnia magna* body APA. Food quality type F or P (Diet). Letter in the pink frame indicate the Line (the dietary history of the previous generations before the ones examined in this experiment) F (multiple generations on high food quality) or P (multiple generations on low food quality). Number in the green frame indicate the Generation, 1 (lower panel) or 2 (upper panel), mothers and daughters in this experiment. See figure.4 (section 2.4) for illustration of the reciprocal transplant.

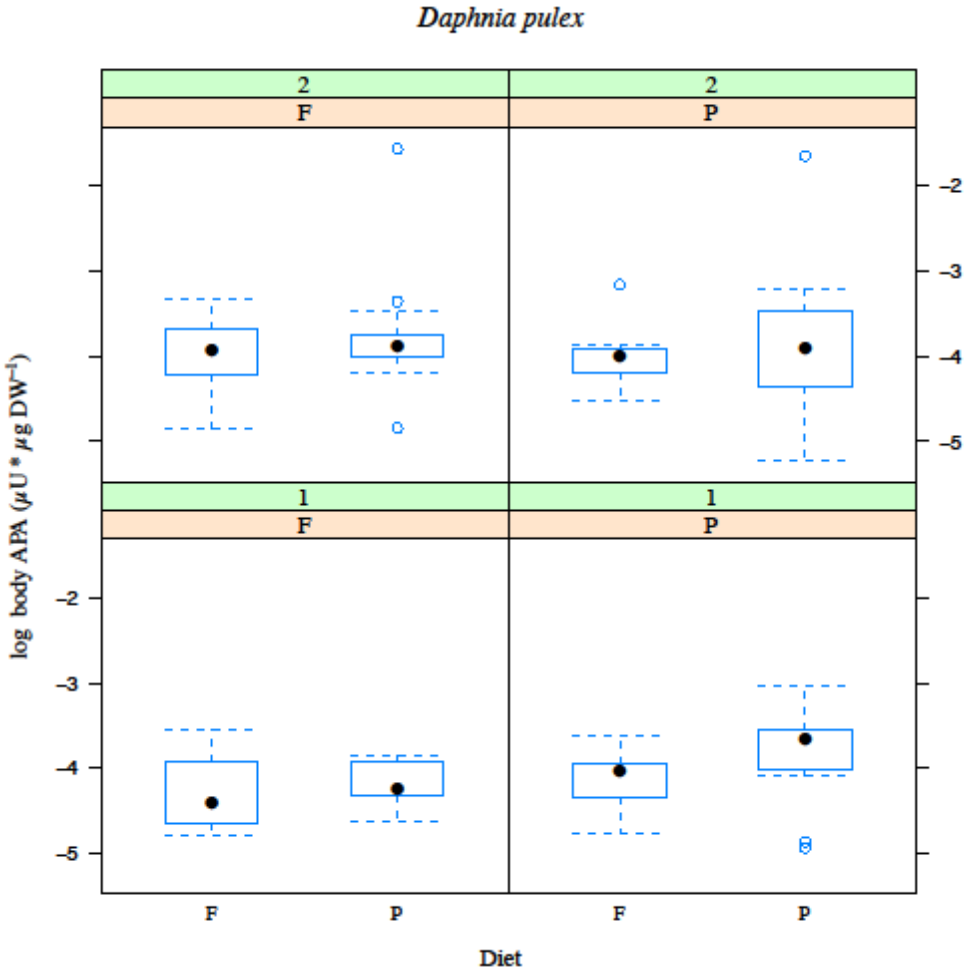

Figure 20. Box plot of *Daphnia pulex* body APA. Food quality type F or P (Diet). Letter in the pink frame indicate the Line (the dietary history of the previous generations before the ones examined in this experiment) F (multiple generations on high food quality) or P (multiple generations on low food quality). Number in the green frame indicate the Generation, 1(lower panel) or 2 (upper panel), mothers and daughters in this experiment. See figure.4 (section 2.4) for illustration of the reciprocal transplant.

4 Discussion

4.1 Decreased fitness and age at first reproduction under prolonged P-stress

For both *Daphnia* species in this study the generation time was shortest for the animals fed P-sufficient algae at 20 ˚C (*D. magna* ~18 days, *D. pulex* ~ 13 days). Age at first reproduction increased significantly in response to both low temperature and low food quality, confirming general expectations. For *D. pulex* the delayed time to reproduction became apparent after a larger time span at 20 ˚C, compared to *D. magna*. The shifts from food quantity (or carbon) limitation to limitation by another element (in our case P) are called the threshold elemental ratio (TER; Sterner and Hessen 1994). TER will depend on the relative assimilation efficiencies of C versus P, as well as food quality (Anderson &Hessen, 2005). The observed difference between *D. magna* and *D. pulex* could perhaps be ascribed to species difference in elemental composition that is mirrored in resource thresholds for elemental limitations (Andersen &Hessen, 1991, Sterner &Hessen, 1994). The results from the elemental analysis show a higher percentage of C in the body of *D. pulex* compared to *D. magna*, but equal percentage of P. This cause higher C:P-ratios in the former species, and indicates that *D. pulex* has a higher threshold C:P elemental ratio than *D. magna*. Also, apparently other differences such as size may affect resource allocation and cause species-specific differences in response to P stress.

The increase in time to first reproduction when the animals were reared at 10 ˚C was significant and already evident in the earliest generations for both species. Compared with animals fed P-sufficient food in 20 ˚C, the reduced temperature had a stronger effect on maturation (delayed time to reproduction) than P-limitation. Temperature is a principal regulator of metabolism because it directly influences all physiological and biochemical functions (Somero &Hochachk.Pw, 1971). Thus, growth, element acquisition, and enzymatic activity are all highly dependent on temperature. In two other studies with daphnia, temperature likewise affected somatic growth more than food composition at low temperatures (Orcutt and Porter 1984; Persson et al 2010). Possibly enzyme kinetics or other aspects of food quality overrides or confound the demands for P to RNA and protein synthesis at low temperatures. On the other hand, a recent life history study with *D. magna*, using stage specific fecundity as a proxy for population growth, found an increased risk of P limitation at lower temperatures (Rosnes et al, master thesis), where differences in cumulative fecundity

increased as temperatures decreased. Yet, the results in this study could not reveal whether *Daphnia* are more or less sensitive to P limitation at lower temperatures. The interaction between temperature and diet was not significant, suggesting that food stoichiometry do not influence the temperature reaction norm for the studied trait (age of first reproduction). Ideally, further generations should have been included in the low temperature treatments, but because of the slow generation time this was not manageable within the time span of this study.

The increasing delay in the onset of first reproduction revealed for daughters born to P-stressed mothers found in the present study, is indicative of a reduced fitness and strongly constrained population growth rate (Sibly &Hone, 2002). The results suggest that *Daphnia* indeed allocate more energy to growth and maintenance than reproduction under P stress. This can be paralleled to what have been found in a recent study by Frost et al. (2012). They also assessed transgenerational effects of P limitation indexed by the C:P ratio in the diet, and found that offspring from P stressed mothers when fed P-poor food grew more slowly and experienced delayed reproduction compared to their sisters born to control mothers. At least temporarily during their life-spans, cladocerans typically experience resource limitation (Sterner &Elser, 2002), which would favor a strategy of maintenance until resources are available for reproduction without reduced survival. Under low food conditions the probability of juvenile survivorship can be increased if egg/neonate size increases, but this is typically achieved at the expense of delayed maturation (Arendt, 1997). Under increased P-stress, it could be adaptive to reproduce later/or at a smaller size, which might imply earlier maturation, or reduced growth rates, or both.

In addition to the response in this fitness trait in response to temperature and P-limitation, I also analyzed the response at the cellular level by use of flow cytometry.

4.2 Responses at the cellular and genomic level

The purpose of the flow cytometry (FCM) analysis was to test whether high and low temperature and high and low P in the diet could impact genome size, and thus have bearings also for cell size and body size since low temperature often increase adult body size of invertebrates (cf. Angilletta et al., 2004), and low P could cause a reallocation from DNA to RNA (Hessen et al. 2009). The FCM revealed higher propidium iodine (PI) fluorescence intensity between nuclei of *D. magna* individuals, raised at low temperature relative to high

temperature. Since this fluorochrome is expected to bind stoichiometrically to DNA, this would in general be interpreted as increased amounts of DNA. However the increased DNA-fluorescence at 10 °C came along with increased variation in histograms and thus high CVs, suggesting structural changes in DNA as a response to low temperature. The higher CV at 10 °C was also significant for *D. pulex*. The level of "acceptable" CVs for DNA estimates have been set to 6 % (Vilhar et al., 2001, Baretton et al., 1994, Darzynkiewicz, 2010), with CVs < 3 % as ideal (Marie &Brown, 1993b). While such low CVs may easily be obtained on monocultures or single-cell samples, it is harder to achieve in metazoans like *Daphnia*, where each measurement include different cell types from different organs isolated from up to 10 individuals. Also some cytoplasmic constituents may be auto-fluorescent, or contain DNA (e.g., in mitochondria). This background cytoplasmic "noise" reduce the accuracy of nuclear DNA determination (Darzynkiewicz, 2010). Also, the fluorochrome concentrations and buffer may clearly impact the CVs. Hence, we adopted (with few modifications) the method of Korpelainen et al. (1997) both for isolating *Daphnia* nuclei and measuring the amount of DNA.

Estimate of the genome size of the *D. pulex* clone used in this study (C-value ~ 0.24 pg, when fed P-sufficient food), is close to the value of ~ 0.20 pg resulting through the *D. pulex* genome sequencing project (Colbourne et al., 2011). Additionally, Vergilino et al.2008, also using flow cytometry, found the average genome size from different *D. pulex* clones to be 0.23 ± 0.002 pg DNA nucleus $^{-1}$. These comparisons indicate the consistency of flowcytometric genome size estimates, making it useful in the study of interspecific differences. For *D. magna*, other studies report C-value estimates in the range 0.24 – 0.53 pg (method, Feulgen Densitometry) and 0.40 pg with FCM (Korpelainen et al., 1997), these estimates can reflect clonal differences, but also methodology. In this study *D. magna* fed P-sufficient food had C-value estimates of ~ 0.32 pg at 20 ˚C, and ~ 37 pg at 10 ˚C.

It is important to determine whether the variation in the histograms is due to instrument or particle variation or both. The FCM instrument was verified and tested for alignment before every measurements of the samples, and the magnitude of instrument variation was assessed by calculating a CV on uniform beads which always yielded a good CV. Further, the results show that the DNA measurements of the *Daphnids* reared at 20 °C, came along with acceptable CVs (average 6 %). The phenomenon with broader histograms for animals reared at 10 °C is likely to be genuine. The higher variation in fluorochrome

fluorescence intensity within populations of diploid nuclei can be due to non-stoichiometric DNA-labelling by PI. Despite the recommended use of PI for absolute DNA measurements, it has limitations. The sensitivity of PI to chromatin structure implies that alterations in chromatin condensation as a function of growth state or tissue type might affect DNA content estimation (Dolezel et al., 2003). It has been recommended that the target and standard nuclei should be isolated from tissue of similar metabolic and developmental state (Galbraith et al., 2001). Since an accumulation of nucleotides and thus a genome expansion is highly unlikely within the lifespan, the observed increased fluorescence at low temperature likely represent structural changes due to increased condensation (Lui et al., 2012). Since nuclei from both growth temperatures were subject to identical treatment (standard protocol) prior to FCM analysis, one can presumably exclude artefact effect of preparation of nuclear extraction. hence two hypothesis could be put forward: (1) chromatin is more condensed in *Daphnia* at 10 ˚C; and (2) chromatin in *D. magna* nuclei is structurally more sensitive to temperature than *D. pulex*.

No statistical difference in 2C-value for *D. pulex* between the food treatments groups was revealed. For D. magna, a significant decrease in estimates of 2C-value for individuals fed P- limited food (P diet) compared to individuals fed P-sufficient food (F diet) was found. But again this came along with confounding effects of unequal distribution of CVs between the diet treatments. The mean differences between the F and P diet at 20 ˚C were found to be very high: 0.05 pg or ~ 50Mb. It is not very likely that such dramatic changes in 2C DNA content could happen over relatively few generations. Hence we can not conclude that this really reflect a genome size response to dietary P.

The results of the FCM analysis are in support of the first null hypothesis $H0_1$: Diet, in respect to C:P ratio, does not cause any changes in measured 2C-value over short time. It holds for *D. pulex* and also *D. magna*. The spiked diet, which was to act as an intermediate diet, with possible low N content but enough P, was not found different from the P limited diet or the P-sufficient diet. Thus the differences between the F and P diet can not be attributed to P limitation alone. The second null hypothesis $H0_2$: Temperature does not cause any changes in measured 2C-value, is on the other hand not supported. The mechanisms behind the apparent increase in size at lower temperatures is however not clear.

In flow cytometry, forward light scatter (FSC) increase with the size of the particle, but not monotonously. The regression analysis between 2C DNA content and nucleus size (FSC) reflect that the relationship is not proportional. Although there was a positive trend, and support for a correlation with 2C DNA content, the analysis conducted cannot resolve the true relationship between the two variables. For more precise observation of the nuclei they should have been assessed by fluorescence microscopy and quantitative determination of nuclear size. This was however beyond the scope of this thesis.

This study could not broadly reveal that daphnids raised at low temperatures ended with a larger body size at first reproduction. Thus, there is no strong support for the temperature-size-rules in this study, or correlation between the higher 2C-values and body size. There were no contrasts for *D. magna*. For *D. pulex* there was one significant contrast, between the P-deficient diets at different temperatures, which imply that *D. pulex* is able to mature at a smaller size when raised at higher temperatures under P-deficiency. A flexible size at maturity has the potential to increase fitness if there is an increased senescence with age (Angilletta et al., 2004). If there is an increased risk of P-limitation at higher temperature and thus mortality, one can predict optimal sizes at maturity that are below the sizes that maximize production (Perrin &Sibly, 1993, Sebens, 2002). But one cannot resolve if this is the case here, more investigation would have been needed.

4.3 Transgenerational effects of low food quality on growth rate and body stoichiometry in *Daphnia*

All daphnids fed the low quality treatment decreased their growth rate in the reciprocal transplant experiment. Although the distribution of mass specific growth rate (MSGR) between the two species when fed high quality food was not found to be different, the distribution between the two species when fed low quality food was on the other hand statistically different. The overall mean percent reduction in MSGR for *D.magna* and *D. pulex* fed low quality food, relative to the mean for the individuals fed high quality food, was ~ 56 % and 25 % respectively. Additionally, the significant difference in somatic RNA content between the two quality food types was greater for *D. magna* than seen for *D. pulex*. Again, this provides evidence that the effect of P limitation is more pronounced for *D. magna*. Along with a significantly decrease in % P in daphnids fed P limited food, the general strong intraspecific couplings among MSGR, somatic RNA content and % P observed in this study

provide strong support for the growth rate hypothesis. However, the relationship between MSGR and body P:C as revealed in the regression analysis showed that *D.magna* has a lower intercept and a higher slope than *D. pulex*. Under P-stress, D. magna experienced a higher drop in MSGR, P content and also levels of RNA. This suggest that the maintenance level of RNA and P is different between the two species, and thus their physiological coupling of RNA and P to growth rate (Kyle et al., 2006).

For *D. pulex* the variation in MSGR when investigated with model.1 (factor diet) resulted in a significant interaction between the low quality diet and the 2nd generation. However, this interaction term was not significant in model.2 (continuous diet variable), suggesting that the decrease in the 2nd generation is confounded by the higher C:P ratio in the low quality food over time, and the potential maternal effect can not easily be recognized. The emerging pattern for *D.pulex* is that only food quality can explain the variation seen in MSGR over the reciprocal transplant experiment. There were in addition no maternal effects or line effects revealed for *D. pulex* in somatic RNA content.

The comparative results for *D. magna,* when considering both model 1 and 2 is however more interesting. Both models suggest a maternal effect on daughters (2nd generation) from P-stressed mothers (1st generation) originating from both the maternal lines- F and P, when fed P-deficient food. Additionally, the results strongly confirms a manifested maternal line effect, with a substantial decrease in growth rate, restricted to the animals fed P-poor food originating from the line P. The emerging pattern from the paralleled result for D. magna is; after multiple generations of P-stress the high growth phenotype is immediately induced when offspring is reintroduced to optimal food conditions (high food quality). However, P-stress is transferred across generations. This is evident in the short term experiment, but even more so in the long term when comparing animals originating from the two maternal lines fed P-poor food. This phenotypic difference is even more obvious in 2nd generation where the low P treatment became more P-deficient. The condition with chronic P-stress over multiple generations has caused a shift in the growth structure of *D. magna*, reveled by a broader reaction norm. Thus if this response represent epigenetic modifications, it is indeed interesting to see that the effect is manifest over 2 generations. This can only be tested by analyzing mRNA or transcripts under the various treatments however.

The cumulative increase in the age at first reproduction under P-deficiency, as indicated by this study, provide a further hint that chronic P- stress do not cause earlier

reproduction/maturation. Thus reduced growth rates might be the most adaptive in this case. Accordingly, the significant difference in growth rate under short term P limitation versus long term, suggest differences in efficient nutrient use and thus a trade-off with rapid growth. The combined results suggest that higher growth rate under P-limitation will eventually result in a growth penalty. It has been suggested that slow growth is not itself adaptive (in terms of increased efficiency) but is merely the consequence of morphological traits that improve nutrient retention and incidentally limit growth, reflecting that energy and nutrients must be allocated among numerous competing functions in an organism (Arendt, 1997). Slow growth might allow organisms that experience nutrient stress to use what nutrients are available most efficiently (Chapin, 1980). And because energy and nutrients allocated to growth cannot be used for other functions, a tradeoff exists between allocation for rapid growth and for maintenance or defense (including an immune system) (Arendt 1997). Interestingly, the offspring from P stressed mothers transplanted to high quality food reestablished the high growth rate phenotype already in the 1st generation.

4.4 Alkaline phosphatase activity related to P-limitation

The study involving a reciprocal transplant of the two maternal lines experiencing high or low food quality over multiple generations, provided an opportunity to further explore the significance of alkaline phosphatase activity (APA) in P stressed animals. An instant response in body APA to food quality in *Daphnia* was not supported by this study. Surprisingly, our results did not support previous studies on *Daphnia*, where an increase in AP expression within the body, as an acute response to nutritional P limitation, has been reported (Frost et al. 2010, Wojewodzic et al. 2011). Further, the results of the study with *D.pulex* did not reveal any significant maternal or line effects on body APA. The enzyme activity for this clone was homogenous over all groups investigated in the reciprocal transplant experiment. The results for *D. magna* however, showed a marked response in both 1st and 2nd generation. Body APA was significantly lower in animals originating from the line P, compared to higher levels of APA in animals originating from the line F. This is quite surprising, as it did not support the hypothesis that AP activity increases in P-stressed animals.

Although an overall significant positive relationship between body APA and C:P ratio has been found (Wojewodzic et al 2011), these authors reported this relationship to be non-

linear. Indeed, they found a striking decrease of the AP activity when C:P ratio in the offered food exceeded 500. The mean C:P ratio in this presented study, for the low food quality treatment was ~1000, thus the lack of difference between animals fed high and low food quality might be due to reduced APA at very high C:P, perhaps due to poor performance in individuals raised at this food.

The manifested decrease in body APA observed in *D. magna* individuals originating from the P-line indicate that *Daphnia* under strong and long lasting P-stress do not maintain or increase AP activity. It has been suggested that down-regulation of AP activity within the body of highly P-stressed daphnids could be due to a decrease in RNA concentration followed by a general decrease in the efficiency of transcription processes, consequently causing not only a smaller body size phenotype but also impairment of protein synthesis, including the AP enzyme (Wojewodzic et al., 2011). In this study, somatic RNA concentration was only explained by food quality, there was not any significant additional reduction in RNA content for the animals originating from the line P. More importantly, the decrease in AP activity in *D. magna* originating from the line P was apparent despite elevated body RNA and growth rate in the individuals reintroduced to high food quality. Thus a general decrease in the efficiency of transcription is not a sufficient explanation for the manifested decrease in body APA revealed in the line P.

As suggested above, some of these responses may actually represent epigenetic responses. It is unclear whether P-stress can alter the genome or epigenome of invertebrates and thereby their offspring, and potential molecular and physiological mechanisms for this phenomenon in *Daphnia* is largely unknown (Jeyasingh et al., 2011). The methylation level in *Daphnia* is found to vary between 0.13 % in 7-day-old animals to 0.26 % for older animals suggesting that 2,6 to 5,2 MB of the *Daphnia* genome is methylated and potentially carries regulatory properties (Vandegehuchte et al., 2009). Homologues of the major vertebrate DNA methyltransferase (Dnmt1, Dnmt2, and Dnmt3A) and CpG methylation are also present in the *Daphnia* genome (Colbourne et al., 2011). It is likely that there is an epigenetic cause behind the enzymatic down regulation in APA found in this study, perhaps affecting a bigger physiological pathway or specifically the AP genes. To conclude if this is a case of true epigenetic inheritance it would have been necessary to monitor the 3rd generation (Harris et al., 2012), but this was not conducted in this study.

Conclusion

These experiments clearly demonstrated that both low temperature and low-quality diet strongly reduced juvenile growth and increased age at maturity. However, it could not reveal if or how, this affected cell- or genome size. In order to address these responses in depth, full sequencing of DNA and mRNA must be performed, which was beyond the goals of this thesis. The main finding from the reciprocal transplant experiment was that *D. magna* demonstrated a slower growth phenotype after multiple generations of P-stress, with implications on enzymatic activity (APA).

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