Polyploidy in *Daphnia*

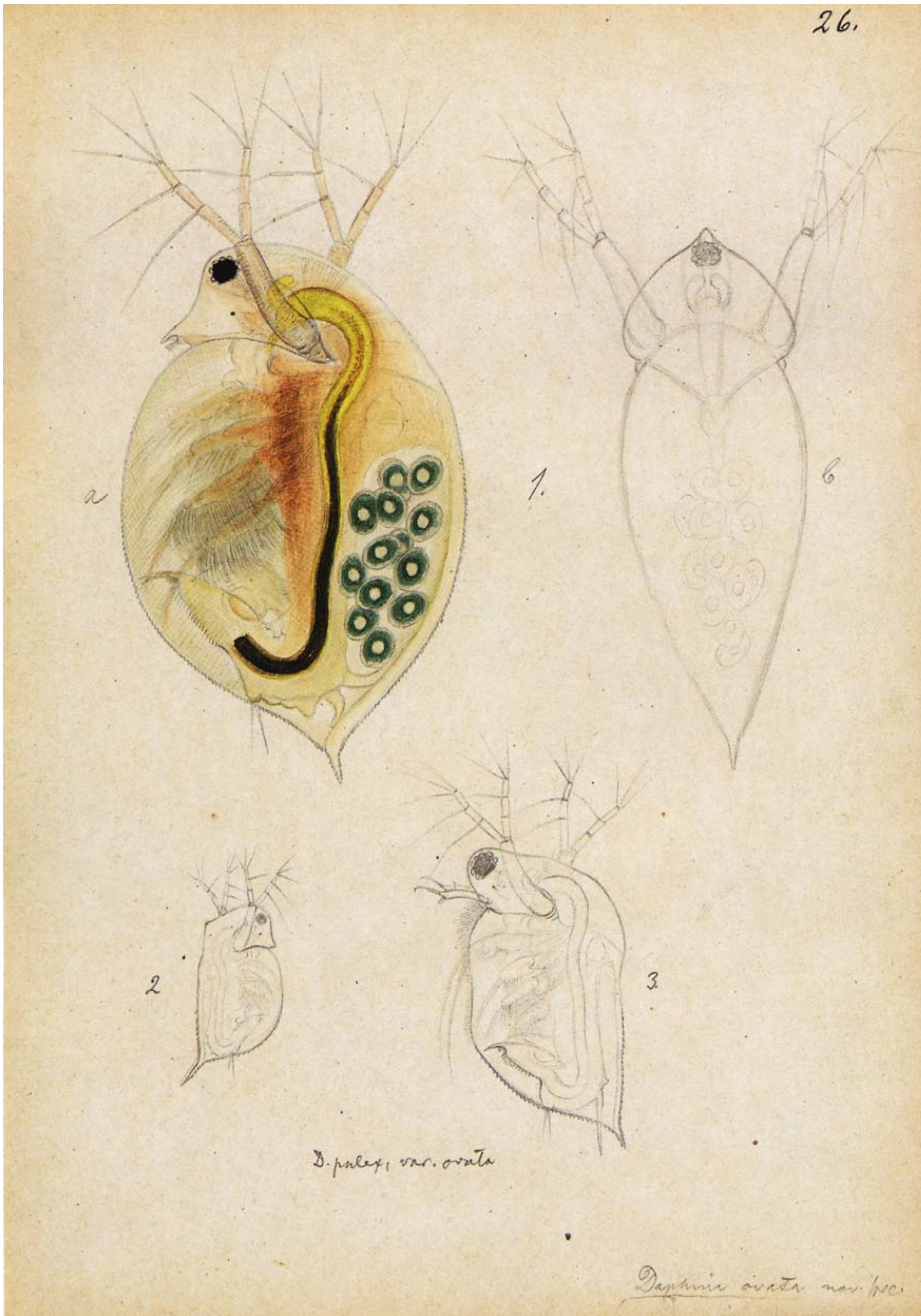
Methodology and effects on life history traits

Kristian Alfsnes

Master of Science Thesis

University of Oslo
Department of Biology
Center for Evolutionary and Ecological Synthesis

2007



***Daphnia pulex* taken from "On the freshwater crustaceans occurring in the vicinity of Christiania"**

by Georg Ossian Sars, 1861

Preface

The study was conducted at the University of Oslo, Norway, as a part of my master thesis. Ideas and initial proposals came from the ever-so-helpful professor Dag O. Hessen, which has been obliging in structuring and correcting the thesis, connecting me with people outside UiO and always being available for discussions. Countless hours with microscopy was done by professor Morten M. Laane, helping me with the cytogenetic analyses and lending great tips and hints for the other analyses. Great thanks to PhD-student Marcin Wojewodzic, who was working with me for the majority of this study, lending help whenever I needed, coming up with good solutions and reading through my manuscripts many times. Dziękuję! Per Færøvig patiently helped me with the flowcytometry and other laboratory work, Tom Andersen has lent great help with analyzing the data and the statistics and Jens Petter Nilssen for help with taxonomy and identification of the *Daphnia*.

Thanks to Morten Skage at the University of Bergen for running microsatellite and mtDNA analyses and correcting my interpretation of the data, Anders Hobæk at NIVA in Bergen, for helping me with the microsatellite analysis, and compiling the phylogenetic tree for *Daphnia* with sequences gratefully received from John K. Colbourne at the University of Guelph. Thanks to Gerben van Geest at Nederlands Instituut voor Ecologie which accompanied me at Svalbard, helping with sampling of *Daphnia* and later sharing data from the lakes and ponds of Ny-Ålesund. Also thanks to the staff at the research station at Ny-Ålesund, Kings Bay and the staff and professors at UiO, especially thanks to the lunch-group at the 4th floor.

Special thanks to family, friends and girlfriend which have endured me the last months, and giving me financial, moral and emotional support.

Kristian

Blindern, May 31st 2007

TABLE OF CONTENTS

PREFACE 3

CONTENTS 4

ABSTRACT 5

INTRODUCTION 6

MATERIALS & METHODS 13

1.1. SAMPLING & CULTIVATION 13
1.2. CYTOGENETIC ANALYSES 21
1.3. LIFE HISTORY EXPERIMENT 28
1.4. GENETIC ANALYSES 33

RESULTS 42

2.2. CYTOGENETIC ANALYSES 42
2.3. LIFE HISTORY EXPERIMENT 53
2.4. GENETIC ANALYSES 67

DISCUSSION 78

3.2. CYTOGENETIC ANALYSES 78
3.3. LIFE HISTORY EXPERIMENT 85
3.4. GENETIC ANALYSES 95

CONCLUSION 106

REFERENCES 108

APPENDIX 114

Abstract

Four arctic, obligate parthenogenetic populations assumed to be polyploid and one temperate, cyclic parthenogenetic, diploid population were used to compare life history traits, growth rates and content of nucleic acids for polyploidy versus diploidy. Cytogenetic studies were done to evaluate the level of ploidy optically or with the aid of optical instruments. A life history experiment was run to identify variations in fitness parameters, fertility, maturation, growth and survival, identifying the adaptive effects and costs of polyploidy. Variations in nucleic acids in polyploids and diploids were accessed by quantifying DNA, RNA and protein. A microsatellite analysis was run to verify the assumed ploidy levels, and species identification was done by sequencing mtDNA and comparing with previously published sequences. The study has a strong focus on assessment of different methods and is in structure affected by this.

Microsatellite analysis positively identified the arctic populations as polyploids, and the temperate as diploid. No assessment of the chromosome numbers was reached using cytogenetic analyses. Statistical difference was noted between the nuclei size in the intestine and connective tissue between the diploid and polyploid populations. Polyploid populations were found to have lower population growth, lower fertility, smaller size and delayed maturation, lower growth rate and lower survival than the diploid population. Ploidy level and quantity of nucleic levels and ratios were not found to be correlated, but RNA and DNA per dry weight and RNA/DNA ratio was found to be weakly correlated with specific growth rate when controlled for the ploidy levels.

Introduction

Polyploidy in *Daphnia* has repeatedly been reported in the Arctic where it seems more wide-spread than in temperate and tropical latitudes (Adamowicz *et al.*, 2002). It is occasionally found north of 58° N in North America and Europe, and become common at 70° N (Beaton & Hebert, 1988; Ward *et al.*, 1994). Observations of polyploid *Daphnia* has also been reported in alpine locations in Argentine at 46° S (Adamowicz *et al.*, 2002). Polyploidy in *Daphnia* is suggested to have arisen during the glacial-cycles in the Pleistocene; glacial expansion producing isolated refugia (selecting for parthenogenesis), glacial retreats then allowing for secondary contact and hybridization between closely allied species (Dufresne & Hebert, 1994; Dufresne & Hebert, 1997, Weider & Hobæk, 2003; see also Fig. 43). Distribution of polyploidy could thus be more based on ecological and historical contingencies than direct selection for polyploidy (Adamowicz *et al.*, 2002).

Polyploidy has been found to be correlated with increase in cell size, delay of maturity, increased size at maturation, lower fertility and increase in offspring size (Levin, 1983; Weider, 1987). Genetically, polyploidy increase the DNA-template, increasing production of RNA and protein, and the genetic variation (as each individual carries several copies of the DNA-template). However differences in life history traits are easily confounded with the concurrence of parthenogenesis breeding mode (Weider, 1987; Otto & Whitton, 2000). The advantage of increased genetic variation (heterozygosity) by increasing the genetic template available for mutations, silencing and reshuffling of duplicated genes (Bailey *et al.*, 1978; Otto & Whitton, 2000) should be evident in obligately asexual populations, as polyploidy increases plasticity to environmental variations in populations otherwise unable to shift in response to changes (Dufresne & Hebert, 1998). Cell division is found to be impeded at low temperatures, increasing the initial genetic template would hence allow for increased metabolic activity in the early stages which otherwise would be limited by the genetic template for production of RNA and proteins (Grime & Mowforth, 1982). Polyploidy in arctic *Daphnia* may also be the result of hybridization events between isolated populations during the Pleistocene glaciations (Dufresne & Hebert, 1994; Dufresne & Hebert, 1997).

The study aims to determine the ploidy level in temperate and arctic populations. Comparing life history traits and nucleic contents of these populations to examine the effect of increased ploidy levels and draw some conclusions of possible causations of the observed increase in polyploidy in arctic *Daphnia*.

Daphnia

The common water-flea, *Daphnia* Müller 1785 (Crustacea, Cladocera), is a much used model organism; both in evolutionary (i.e. Pijanowska *et al.*, 2006) and ecological (i.e. Kyle *et al.*, 2006) studies. Previous studies also include molecular (i.e. Weider & Hobæk, 1994), cytogenetical (Beaton & Hebert, 1994a), morphological (i.e. Zaffagnini, 1987) and taxonomical work (i.e. Colbourne & Hebert, 1996). The *Daphnia pulex* species complex (*sensu* Colbourne & Hebert, 1996) includes temperate, alpine and arctic species. The natural distribution range of the complex ranges from North America, the Arctic, Europe and Siberia (Weider *et al.*, 1999). The species complex can be split into three groups; *pulex*, *pulicaria* and *tenebrosa*, all of which were included in this study. The groups are not conclusive with the subgenera, as *D. pulex* Linné 1758 is found as a variety in both the *pulex* and *pulicaria* group, and *D. pulicaria* Forbes is found in both the *pulicaria* and *tenebrosa* group (Colbourne & Hebert, 1996, Markova *et al.*, 2007). *D. tenebrosa* (Weider *et al.*, 1999) is the only monophyletic group in this complex. Other subgenera such as *D. middendorffiana* and *D. melanica* are also found in the *D. pulex* species complex, but are restricted to locations in North America. Overlap of distribution range for the different subgenera have been reported in the Arctic and in the alpine Europe (Weider *et al.*, 1999). In this study, *D. tenebrosa* and two varieties of *D. pulicaria* were encountered within an area of a few square kilometres at Svalbard.

Daphnia forage on both pelagic and benthic algae, bacteria, other heterotrophic organisms and detritus (Rautio & Vincent, 2006). They are non-specific feeders (DeMott, 1988), limited by maximum size according to the individual size (length of carapace, Burns, 1968) and minimum size to the setae distance in the filtering apparatus (Hessen, 1985). *Daphnia* is commonly regulated by foraging of planktivorous fish (Winder *et al.*, 2003), notostracans such as *Lepidurus arcticus* (pers. obs.) and other invertebrates such

as fly larva and hydra (Winder *et al.*, 2003). *Daphnia* is shown to have diurnal migrations, to avoid predation and UV-radiation (Rhode *et al.*, 2001).

The organisms are easily reared in laboratory and sampled *in vivo*, they have a relatively quick reproduction and short generation time and much of the genetics and morphologic traits have been described. The adaptation to polyploidy seem an unique feature of cold climate, with increased focus on the worldwide climate change, studies of the ecology and evolution of the circumarctic *Daphnia* may provide interesting clues of the past, the present and also forecast the evolutionary future for the ponds and lakes these organisms dwell in.

Pigmentation

Daphnia has been found to produce melanin as well as some other pigment such as carotenoids (Markova *et al.*, 2007). Melanization of the carapace is an adaptation to increased UV radiation in shallow ponds and lakes in the Arctic and high alpine (Hessen, 1996). In deeper and more turbid waters, hyaline, or transparent, morphotypes are dominating, and in localities with fish, the dark coloration is counter selected. The different adaptations to UV radiation allow for the two morphotypes inhabiting the same waters, as the melanic morphotypes usually occupy the top layers of the water, while the hyaline morphotypes inhabit the deeper portions where the UV radiation is lower. Such an interaction between two morphotypes was not recorded for the arctic ponds/lakes in this study, but has been reported in the North American Arctic (Rhode *et al.*, 2001). It has been shown that the hyaline morphotype is the superior competitor and will suppress the melanic morphotypes when raised together exposed to low-UV stress (Hessen, 1996).

All *Daphnia* possess the ability to produce melanin, evident in the melanized ephippial eggs from hyaline morphotypes (Hebert & McWalter, 1983). The complete genetic make-up of the melanization proteins in *Daphnia* remain unresolved (Anders Hobæk, pers. com.), but has been studied in other pigmented organisms such as salmon (Haugarvoll *et al.*, 2006). Increased production of protein may be involved with the difference in ploidy levels (germ-line or somatic). Beaton & Herbert (1988) found that all melanic clones in their study were polyploid, while most of the hyaline clones were diploid, however they suggested that polyploidy and melanization were selected for

independently. It has been proposed that melanization evolved after the loss of sexual reproduction (Hebert & McWalter, 1983).

In this study one arctic, hyaline population (Øvrevatn) was included, surprisingly from the lake found to have the lowest UV-absorbance (hence highest stress) (Hessen, 1996). However this lake also had the greatest depth, >2 m, which allows for vertical migration to avoid UV radiation (Rhode *et al.*, 2001), the population was later assumed to be polyploid (see **Microsatellite analysis**).

Reproduction

Parthenogenesis, asexual reproduction, in *Daphnia* was first described by Lubbock in 1857 (*sited in* Zaffagnini, 1987). Two variations of this reproductive mode have been recorded; cyclic and obligate parthenogenetic (Edmondson, 1955; Zaffagnini, 1987). Parthenogenetic eggs are produced in the ovaries (during the 5th instar in *Daphnia*, Dunham & Banta, 1940), and then transferred to the brood chamber, between the soft body and the carapace. They are carried by the mother until fully developed when they swim out of the chamber shortly before mother moults (Zaffagnini, 1987). The process is repeated for consecutive 12-16 instars until death, interrupted by production of ephippial eggs governed by the environment (Dunham & Banta, 1940; Zaffagnini, 1987; Innes, 1997). Parthenogenetic breeding mode is generally found in disturbed habitats, higher latitudes and elevations, xeric rather than mesic sites, and on islands rather than on mainland (Beaton & Hebert, 1988). Parthenogenetic breeding mode is commonly found in polyploid animals (Lokki & Saura, 1979; Beaton & Hebert, 1988), Otto & Whitton (2000) reported that as many as two thirds of all polyploid animals reproduced parthenogenetically.

Cyclic parthenogenesis is the dominant and ancestral mode of reproduction and widespread in temperate North America and Europe, while obligate parthenogenesis have evolved much more recent and is confined to the polar range of North America (Ĉerný and Herbert 1993; Hebert *et al.* 1993), the Arctic and alpine Europe (Ĉerný and Hebert 1993, Hebert *et al.*, 1993, Weider *et al.*, 1999). In intermediate regions between temperate and polar, lake populations is dominated by cyclic parthenogenesis, while pond populations by obligate parthenogenesis (Ĉerný and Hebert, 1993). In Norway a gradual

shift from solely cyclic parthenogenesis in the south to increasingly frequent obligate parthenogenesis in the north, Svalbard being entirely devoid of males (Ward *et al.*, 1994). Despite being obligately parthenogenetic most populations at Svalbard have been found to be composed of more than one clone (1.8 ± 1.1 for 31 ponds and lakes, Weider & Hobæk, 1994), whether due to sympatric divergence or dispersal, several clones have been found to coexist for an extended period of time (Hobæk *et al.*, 1993).

Cyclic parthenogenetic *Daphnia* produce males by parthenogenesis, usually triggered by some environmental cues such as decrease in food quality or chemical cues caused by increased population density (Innes, 1997). Haploid, amphigonic eggs are produced in the ovaries which these males consequently fertilize. The eggs are encased by a protective coating, termed the ephippium (a modified carapace), which protects against freezing and desiccation (Innes, 1997). Females are hatched from the ephippial eggs in the beginning of the next growth season, or whenever the conditions are favourable for new recruitment (Innes, 1997). Obligate parthenogenetic *Daphnia* never produce males, and parthenogenetically produce diploid ephippial (resting eggs with ephippia coating) eggs asexually at the end of the growth season or if conditions get worse (Ĉerný & Hebert, 1993). Parthenogenetic produced eggs dominate the recruitment during the beginning of the growth season for both modes of reproduction, ephippial eggs produced predominately in late summer and fall before the end of the growth season (Winder *et al.*, 2003). Production of ephippia seems to be a weight of two goods, immediate fitness (parthenogenetic eggs) or delayed reproduction (ephippia) when conditions are less stressful (Epp, 1996).

Asexuality in form of parthenogenesis has the advantage that individuals do not have to invest in males, mate seeking and recognition. A major disadvantage would be that asexuals may suffer from mutational load and low evolutionary potential (Maynard Smith, 1978). In predictable, but marginal environments such as the high Arctic, advantages of sexual reproduction may be reduced, as adaptations to these environments may require co-adapted gene complexes, which are broken up by sexual recombination (Aguilera *et al.*, 2006).

The arctic populations in this study were all *a priori* assumed to be obligately parthenogenetic, while the temperate population was assumed cyclic parthenogenetic (Ward *et al.*, 1994).

Polyploidy

The ploidy level, i.e. the number of, is determined by the variations of the original numbers of copies of the genetic motif, the chromosomes. Most eukaryotic organisms are under normal circumstances 2n (diploid), while sexual cells are haploid (n) with only one copy of all chromosomes. Two haploid cells fuse together to form a new diploid progeny. Chromosome doubling may occur during meiosis, producing germ-line cells with more than two copies of the chromosomes, called polyploidy. In sexual reproducing organisms this could result in either an evolutionary dead-end or sympatric speciation, depending on the presence of other individuals in the population with correspondingly altered number of chromosomes copies. For asexually reproducing organisms increased chromosome numbers will be a source for increased genetic diversity (Soltis & Soltis, 1995). Polyploidy has been recorded in several groups of organisms, in most plant groups (Grime & Mowforth, 1982; Weber *et al.*, 2005), many invertebrates (Lokki & Saura, 1979), some fishes (Bailey *et al.*, 1978) and amphibians (Batistic, 1975; Mahony & Robinson, 1980) and even a few mammals (Gallardo *et al.*, 1999).

Change of ploidy level in *somatic cells* during growth and development of an organism, when a mitotic DNA replication is not followed by division, is termed endopolyploidy (Gregory & Hebert, 1999). Alternatively, not all chromosomes may be replicated producing an unbalanced increase in number of chromatids per chromosomes; termed polyteny (Korpelainen *et al.*, 1997). Increases in the genetic template is usually correlated with increased demand for RNA or protein in certain cells or tissues in the organism, it may allow for higher plasticity of life histories and miniaturization of the genome by making copies where activity requires more genetic template (Korpelainen *et al.*, 1997). Somatic polyploidy has been reported in every animal species studied to date (including human liver cells, Epstein, 1967), and has been shown to be especially common in arthropods (Gregory & Hebert, 1999). For *D. pulex* it has been shown that up to ¼ of the adult cells were endopolyploid (mainly tetraploid, 4n) (Korpelainen *et al.*,

1997). The reported degree of endopolyploidy in *Daphnia* varies from tetraploidy (4n) to more than thousand copies in some tissues (Beaton & Hebert, 1989). Tissues more prone to endopolyploidy are those involved in production of substances needed in large quantities (such as melanin, see below) or excretion of waste-products (such as excess carbon) (Korpelainen *et al.*, 1997). However Beaton & Hebert (1989) did not find that tissues with high level of endopolyploidy had secratory functions, and cells in the digestive tract have been reported to be stabile at a low ploidy level by Korpelainen *et al.* (1997). Somatic polyploidy has the advantage over germ-line polyploidy that it is more flexible, additionally there are no difficulties concerning sexual reproduction (Korpelainen *et al.*, 1997). The obligate parthenogenetic *Daphnia* in this study would not affect sexual reproduction, but would benefit from the flexibility offered by endopolyploidy. "Choice" of polyploidy, either germ-line or somatic, would be an evolutionary compromise between selection for miniature genomes and requirements for minimal DNA-template (Korpelainen *et al.*, 1997).

Materials & Methods

1.1. Sampling & Cultivation

To establish populations of estimated polyploid clones, individuals of *Daphnia* were collected during a field trip in June 2006. The station at Ny-Ålesund 78°55′ N, 11°56′ E (Fig. 1) was selected for collection of populations, as it is located in the high Arctic and is able to provide all the infrastructure and equipment needed for scientific field work. A temperate population was collected from St. Hanshaugen park in urban Oslo 59°56′ N, 10°45′ E (Fig. 1) for comparison of the arctic populations. Species identification was done *a priori* with help from Jens Petter Nilssen and various identification guides (e.g. Flössner, 2000; Benzie, 2005) and *a posteriori* by sequencing of mtDNA. The parthenogenetic *Daphnia* can be cultivated from one individual, which reduce the effects of multiple clones and lineages. Live individuals allowed for growth rate and life history experiments, and fresh stocks for genetic analyses.

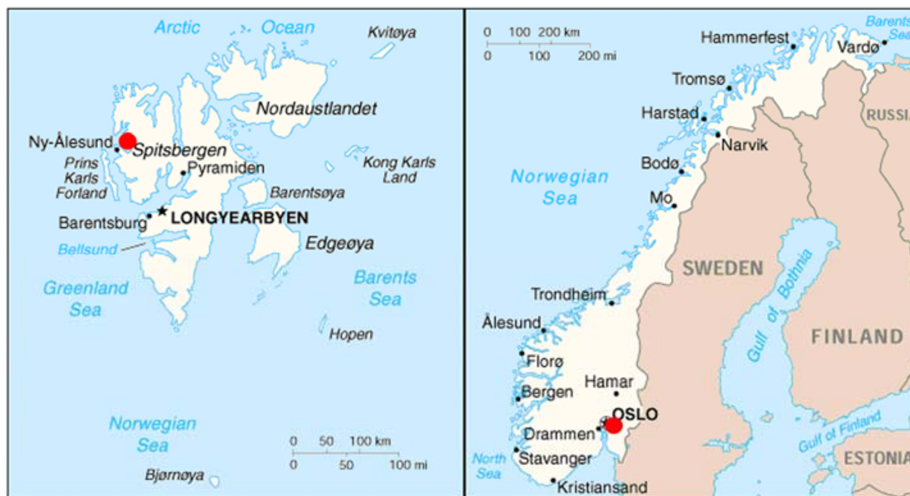


Fig. 1 Map of Svalbard (left) and Norway (right). Sample locations are indicated by red dots, Ny-Ålesund is situated at 78° 55′ N, 11° 56′ E, Oslo at 59° 56′ N, 10° 45′ E (Maps from U.S. Central Intelligence Agency).

Sample locations

Note: Letter code for the different populations was assigned arbitrary, and done to ensure objective analysis from external help (Morten M. Laane, UiO; Tove Bakar, UiO; Morten Skage, UiB).

A) Solvatn (Fig. 2) was sampled the 21.07.06. The lake[1] is located close to the Ny-Ålesund settlement, between the main building and the dock; it is compared to the undisturbed, pristine environment on Svalbard (and the other locations) heavily influenced by human activity because of its proximity to the settlements. It is frequently used by several research groups. There are also large resident flocks of geese, terns and other polar seabirds, adding nutrients to the lake. This eutrophic (76 μg P/L), Gerben van Geest, *unpublished*) lake is approximately 90 meters across and 0.5 m deep with muddy, soft bottom. A previous study has shown that UV absorbance from this lake is in the high range (Hessen, 1996), due to high nutrient content and consequently algal density and dissolved organic matter. The sampled area (indicated with an arrow and red lines) were 1 meter from shore and a total of 3 tows were made. Two clones have previously been recorded from the lake, both of which indicated polyploidy judged from unbalanced electromorphs in allozyme analysis (Location E in Hobæk *et al*., 1993). The heavily melanized clone sampled was sequenced (see **Species identification by mtDNA sequencing**), suggesting this clone to be *Daphnia tenebrosa* (Fig. 43).

B) Storevatn (Fig. 2) was sampled the 22.07.06. The oligotrophic (5 μg P/L), Gerben van Geest, *unpublished*) lake is located 100 meters south of the airport, stretching more than 200 meters across, with an average depth of 0.5 meter and loose gravel substratum. Flocks of geese and transient reindeers were observed in the eastern side of the lake. The entire western shore was towed 1 meter from land. Two clones have previously been recorded from this lake (Location H in Hobæk *et al.*, 1993), both of

[1] A lake is characterized by wind-induced mixing of the water column, while in a pond the mixing of the water column is due to temperature gradients (Wetzel, 1983; Brönmark & Hansson, 2005). All water-masses included in this study showed an intermediate between these two definitions, as strong winds prevail at the barren landscape, and huge day/night temperature differences are observed during the summer months.

which showed polyploidy judged from unbalanced electromorphs in allozyme analysis. mtDNA sequencing identified the melanic clone used in this study as a Polar *Daphnia pulicaria* (same as clone D, Fig. 43).

C) Øvrevatn (Fig. 2), was sampled the 20.07.06. The lake is situated on the Blomststrand Island; the lake is positioned roughly 1 km from the shore at approximately 100 meter elevation. Some ducks and terns were observed, but not as numerous as in Solvatn and Storevatn. P concentrations was 11 µg P/L (Gerben van Geest, *unpublished*) indicating the lake to be somewhere in the intermediate between an oligotrophic and mesotrophic classification (Wetzel, 1983, Brönmark & Hansson, 2005). UV absorbance has been shown to be low (Hessen, 1996) for this lake. The lake measures approximately 150 meter across, depth of sampling area ranged from 0.5 – 1.2 meter, estimated to be more than 2 meters at lake center (Hobæk *et al.*, 1993). Several tows were necessary as the *Daphnia* were observed in aggregations, and high densities of *Volvox* algae clogged the collection mesh-net. The predacious notostracan *Lepidurus arcticus* Pallas was observed close to the shore. One clone has previously been recorded from this pond, showing polyploidy judged from unbalanced electromorphs in allozyme analysis (Location M in Hobæk *et al.*, 1993). The hyaline population was identified as Eastern Nearctic *Daphnia pulicaria* (see **Species identification by mtDNA sequencing**, Fig. 43), an uncommon species at Svalbard where Polar *D. pulicaria* and *D. tenebrosa* (Dag Hessen & Anders Hobæk, pers. com.).

D) Nedrevatn (Fig. 2) was sampled the 20.07.06. This small pond is found another 100 meters towards Kongsfjorden from Øvrevatn, at around 75 meter elevation. No birds were noted during the sampling period, but both reindeer droppings and bird guano was observed close to the pond. The nutrient values were recorded at some intermediate level (27 µg P/L, Gerben van Geest, *unpublished*), classifying the pond as mesotrophic (Wetzel, 1983, Brönmark & Hansson, 2005). UV absorbance has previously been found to be intermediate (higher than Øvrevatn, but lower than Nedrevatn, Hessen, 1996). The pond measures less then 50 meters across, and depth was less then 0.50 m, with an extensive muddy bottom ranging at least another 0.50 m

deeper. The population density of *Daphnia* was extremely high. High numbers of the omnivorous *L. arcticus* was found in this pond, though no measures of density were done. Two clones have previously been recorded at this pond (Location N in Hobæk *et al.*, 1993), both of which showed polyploidy judged from unbalanced electromorphs in allozyme analysis. Like clone B, samples from this pond were found to be Polar *Daphnia pulicaria* (see **Species identification by mtDNA sequencing**, Fig. 43) and were highly melanized.

E) “Trehjørningen” (Fig. 2) was sampled the 22.07.06. The lake is located 2-3 km west of the Ny-Ålesund airstrip. The surrounding vegetation was much scarce here then at the other lakes, also the algal growth seemed to be lower. The assumed nutrient-poor lake (clear water, but no analysis of total P) is roughly 30 meters across, with a depth ranging from 0.1-1 meter. Large aggregations of melanic *Daphnia* were observed being predated by *L. arcticus* in the water column. *Volvox* algae were found in great density, equal to that of Øvrevatn. The population was only kept in laboratory for a few weeks before it collapsed.

G) The small pond at St. Hanshaugen Park (Fig. 2) was sampled 15.09.06. The pond contained two species of *Daphnia*, *D. rosea* (*D. longispina*) and *D. pulex* (Jens Petter Nilssen, pers. com.). The two species was kept at a high nutrient regime for 1 month successfully allowing *D. pulex* to out-compete *D. rosea*, the latter which is inferior competitor in eutrophic conditions (Jens Petter Nilssen, pers. com.). A thorough morphologic taxonomic check was executed at the end of the competition, positively identifying the remaining hyaline species to be European *D. pulex*, this was confirmed by mtDNA sequencing analysis done (see **Species identification by mtDNA sequencing**, Fig. 43). The artificial pond is 25 meters across and 0.5 meter deep, there is only a thin layer of substrate (mainly composed of rotting tree leaves at the time of collection) as the bottom is manmade concrete and the pond dries up every winter. Great number of ducks is resident, contributing to high nutrient content in the pond.

M) *Daphnia magna* Straus 1820, cultivated for several years at UiO. The clone has been used in prior analyses at the university and has been shown to be very sturdy and relatively well-reproducing (see **Life history experiment**). The population was excessively used as a test organism for staining, mitotic inhibition and flowcytometry. It was also used in the life history experiment and DNA/RNA and protein quantification for comparison with the *D. pulex* species, it was included in the microsatellite analysis, but the primers accessed didn't work for this species.

An additional search was executed around Stavanger, fall 2006. Samples taken from assumed "rock pools" (coastal ponds above splash-zone) where *D. pulex* were assumed to be present (Jens Petter Nilssen, pers. com.) at locations around Tungenes Fyr, Kvitsøy (North coast) and at Ølberg. Findings included cladocerans like; *D. longispina*, *D. rosea* and *Chydoridae* spp. and the copepode, *Cyclops* spp. at Kvitsøy and various ostracodes at Tungenes Fyr and Ølberg (all identifications by Jens Petter Nilssen). As no *D. pulex* was found these samples were not used in any later analyses.

Cultivation

Populations were initially set up in two temperature regimes, 7^oC and 20^oC, the latter was used for the subsequent analyses due to higher growth rate and survival (no estimations done). Populations at 7^oC were kept as back-up stock for most of the duration of the study, but not used. The colonies were introduced to the laboratory conditions at UiO after 3-4 days at the research station in Ny-Ålesund. Populations were kept in 1L beakers with COMBO medium (Kilham et al., 1998) and fed the green algae *Selenastrum capriconutum* Printz *ad libitum* (by demand), beakers were emptied and washed approximately once per month.

Population E collapsed within the first weeks due to unknown reasons, being greatly reduced during the transfer from Svalbard. The other populations were kept at intermediate densities (~50 individuals per L), European *D. pulex* and Eastern *D. pulicaria* were observed to reach higher densities than the melanized clones (see **Life history experiment**). All populations survived the duration of the study, high population

fluctuations were however observed, especially for the *D. tenebrosa* (population A) and Polar *D. pulicaria* (population B & D).

Some of the melanized clones were observed to reduce their pigmentation during the 10 months kept in cultivation, but were still distinguishable from the non-melanized clones at the end of the experiments.

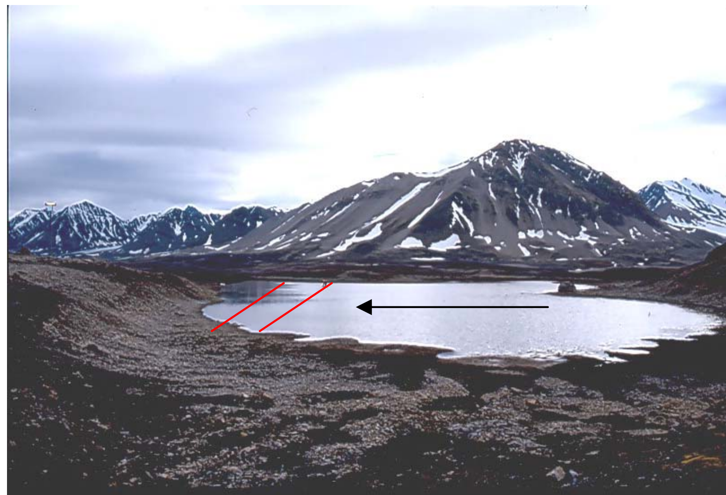


Fig. 2 Sample sites, from top to bottom; Solvatn (A) (composite picture using PanaVue v. 3.0) (Looking towards east), Storevatn (B) (Looking towards west) (Picture provided by Gerben van Geest) Øvrevatn (C) (composite picture using PanaVue v 3.0) (Looking towards east), Nedrevatn (D) (Looking towards east), – "Trehjørningen" (E) (Looking towards south-east), St. Hanshaugen park pond (G) (Looking towards east). Arrows and red lines indicate sampled area. All pictures taken by author unless otherwise stated.

1.2. Cytogenetic Analyses

Cytogenetic analyses were assessed to quantify the number of chromosomes in the different populations to determine level of polyploidy. Chromosome quantification analysis is traditionally done by microscopy and staining (Leuchtenberger, 1958; Trentini, 1980; Laane & Lie, 2001). Naturally this was the first assessment of the actual ploidy-level for *Daphnia*. Nuclear measurements were made from thin embedded samples (see below), relative ploidy-levels assessed by comparing nuclear sizes of the different populations, as DNA content (and consequently chromosomes) and nuclear size are found to be correlated (Stebbins, 1960). Relative ploidy-levels from nucleus size can also be established by running stained isolated nuclei from the different populations through a flowcytometer, which record emitted fluorescence from fluorochromes bound to the nuclear DNA and thus lack nuclear DNA, as a proxy of nucleus size and consequently DNA content.

Feulgen staining

A preliminary studies of the incidence of polyploidy for the different populations was assessed by staining using Feulgen reagent (or Schiffs reagent), a DNA specific reagent that binds after hydrochloric acid exposure and heat treatment (Leuchtenberger, 1958; Laane & Lie, 2001). The staining of the DNA should allow for quantification of number of chromosomes in the *Daphnia* using microscopy. Different protocols were tried; first, the methods described in Laane & Lie (2001) with variations, then method described in Beaton & Hebert (1988). Organisms used were *D. pulex*, D. *magna* and onion root (*Allium*). Onion root tips (*Allium*) were included as a control for the dye and the methods, as mitosis is readily accessible and is easily stained.

Samples of *Daphnia* were transferred to distilled water for 1 hour prior to all analysis to allow for digestion of algae in the intestines (Dag O. Hessen, pers. com.). Following the method described by Laane & Lie (2001), samples were fixed in 1:3 acetic acid/ethanol for 10 minutes, followed by hydrolysis in 1N HCl at 60°C for approximately 10 minutes. After heating treatment samples were washed in SO_2 water (following

protocol in Laane & Lie, 2001) and stained for approximately 10 minutes in Feulgen reagent[2] solution. Variations of hydrolysis time (10 ± 5 minutes), variations of staining time (10-60 minutes), different heating sources (heat block, water bath and heating cabinet), papain extract (a cysteine protease, to digest peptide bonds, prepared from papaya, added to sample prior to fixation) and chloroform (to clear the cytoplasm, added to the fixative) were all tried to improve the results, however the staining remain unspecific and the protocol was discarded..

The next method for staining using Feulgen reagent, described for staining of *Daphnia* in Beaton & Hebert (1988), done without heat-treatment[3], can be summarized as follows: sample fixated for 1 hour in 1:3 acetic acid/ethanol, washed for 30 minutes in distilled water, transferred to 5 M HCl in room temperature for 30 minutes, washed for 1 minute in 0.1 M HCl, and then stained in Feulgen reagent for 100 minutes. Finally the samples were washed three times in sulfite solutions (SO_2 water, see above) for 5 minutes each, and rinsed in distilled water for 10 minutes. The final sample was put on 20% ethanol for storage. Samples were examined in a microscope (Zeiss Photomicroscope III with Zeiss Planapochromate 63x/1.40) with an attached webcamera (Logitech 5000 Pro, 1.3 Mp) (Laane, 2007).

Stained samples were dissected (carapace removed) in a drop of acetic acid and examined under a microscope. Samples of interest were made permanent using technique by Laane & Lie (2001), first remove all access water by squeezing the preparation, cool sample to 4°C, then remove cover-glass from object-glass and put both parts in 96% ethanol for 1 minute to further remove access water. 3-4 drops of camphor was added to each glass, a clean cover-glass was added to the sample-containing object-glass, and *vice versa* for the sample-containing cover-glass. Samples were dried for 3 days, and then left with brass weight for 3 months to complete the embedding process.

---

[2] Feulgen or Schiff's reagent; 0.5 g basic Fuchsin dissolved in boiling water, cool to 50°C and add 10 mL 1N HCl, cool to 25°C and add 0.5 g K-metabisulphite. Keep at 4°C for 24 hours and filtrate.
[3] Similar method, staining without heat-treatment, works well for staining nuclei in many fungi and other organisms where usual Feulgen procedure does not work well (Laane, 1968).

Mitotic inhibitors

Mitosis in *Daphnia* appears to be nearly synchronized occurring at brief intervals in the intermoult stages, mitotic inhibitors was thus used to arrest mitosis and allow for microscopic analysis and quantification of the chromosomal bodies. Assessed chemicals for this purpose were colchicine (~95% powder, SIGMA, cat. # C9754) (Sharma, 1990; Laane & Lie, 2001; Sullivan & Castro, 2005) and 8-hydroxyquinoline (crystalline, SIGMA, cat. # H6878) (Mills, 1978; Sharma, 1990; Laane & Lie, 2001). Colchicine (an alkaloid from the plant *Colchicum*) is a chemical that directly inhibits mitosis by depolymerizing spindle microtubuli resulting in "metaphase arrest" (Sharma, 1990). A concentration of 0.1% was used which has been reported as non-lethal to *D. pulex* (Beaton & Hebert, 1994a; Morrow, 2001), however in this study many individuals died after only 1 hour. The concentration was therefore reduced to 0.01%, this allowed for colchicine treatment for more than 6 hours prior to analysis, increasing the chance to find and "arrest" synchronous mitosis. After 1 hour fixation samples were dissected (exoskeleton removed) and stained with non-fluorescent aceto orcein (Rabinovitch & Plaut, 1962; Wolstenholme, 1965) for 1-2 minutes by moving the cover glass up-and-down with a pincher. Aceto orcein dye was used because of the short preparation time (~10 minutes); no heat or acid treatment. Because of more detailed staining it should be possible to observe overlapping chromosomes (Barry & Perkins, online protocol). However, the stain quickly fades and samples can not be stored for more than a few days unless made permanent by rapid freezing of the coverglass and transfer via 96% ethanol to Euparal.

8-hydroxyquinoline (also known as hydroxyquinoline sulfate, a heterocyclic aromatic organic compound), inhibits pre-meiotic DNA replication (Mills, 1978). Hence, it does not directly block the mitosis steps, but aids in chromosome spreading and enhance structural details. Samples were added to a 0.02% 8-hydroxyquinoline solution up to 6 hours prior to fixation (1:3 acetic acid-ethanol), and stained with aceto orcein as described for colchicine (see above). Variations of exposure time, temperature and size of individuals were tried.

The microscopic slides were examined in the microscope (same as in **Feulgen staining**) with attached webcamera (same as in **Feulgen staining**), and whenever

necessary compound pictures were made from stacks of pictures using computer software (CombineZM by Alan Hadley). The digital image quality is thus equal to professional microscopic cameras (Laane, 2007).

Embedding & nuclear measurements

To assess the question of polyploidy, embedding of stained Daphnia would allow for measurements of nuclear diameter. As with flowcytometry, variations of nuclear "sizes" indicate various content of DNA, as increased polyploidy level cause an increase in the nuclei and cell size (Otto & Whitton, 2000). The epoxy embedding was done by Tove Bakar (IMBV, UiO), 5 juveniles (>24hrs old) and 5 adults (post-maturity) from population B, C, D and G were fixed in 2% Glutaraldehyde in 0.1M Phosphate buffer for >24 hours at $4^{o}C$. Entire individuals of *Daphnia* were prepared using Tove Bakar's Protocol: samples were rinsed for 2x10 minutes in 0.1 M Sodium cacodylate buffer at room temperature, fixated again in 2% OsO_4 in 0.1 M Sodium cacodylate buffer for 1 hour in darkness and rinsed twice in distilled water for 10 minutes. Samples were then stained with 1.5% Uranyl acetate ($(CH_3COO)_2UO_2$ x $2H_2O$) in distilled water for 30 minutes in darkness. The stained samples were dehydrated with increasing concentration of Ethanol (70-100%) for 10 minutes for the lower concentrations (70-96%) and 4 times 15 minutes for the higher (100%). Embedding with evaporation was done with rotation over-night using a 1:1 epoxy/propylene oxide solution. The samples were polymerized on a drop of epoxy in a mould, left for a day at $60^{o}C$, then mould was filled with more epoxy and left for another three days at $60^{o}C$ to harden. The finished epoxy capsules were sectioned on an LKB-Ultramicrotome into 1 µm thin slides.

The finished slides (consisting of 3-4 individual juveniles on average) were examined in microscope (at 100x magnification 1.30, Optovar 2x2, Zeiss Photomicroscope III with Zeiss Planapochromate 63x/1.40) and pictures made with an attached webcamera (Logitech 5000 Pro, 1.3 Mp). Two easily distinguishable tissue types were selected; intestine, "connective tissue type A". Intestine tissue is easily distinguishable by a single cell layer with a brush border (microvilli) towards the lumen, and their "pearls-on-a-string" like formation around the intestine tract. Connective tissue type A was always found in the proximity of muscle fibres, and assumed to have some

connection to the muscle tissue. Another type of assumed connective tissue, named “B”, was also found, distinguishable by their relatively large size and small nucleoli; this tissue type was found randomly throughout the organism, seemingly unrelated to any organ. Assumed chitin producing cells were found along the 1-2 cell layers thick carapace encrusting the organism, neural tissue was identified around the eyes; neither of these tissue types was included in the analysis.

Pictures were analysed using ImageTool v. 3.0 (UTHSCSA, University of Texas Health Science Center in San Antonio), measurements were done on the area of the nuclear envelope and nucleolus (see Fig. 11). Pixels were converted to μm^2 using standardized microscopic ruler as a scale. Comparisons of populations and nuclei sizes were assessed using ANOVA one-way analysis, when violated homogeneity of variance or normality, non-parametric Kruskal-Wallis was used with *a priori* Mann-Whitney test.

Flowcytometry

Flowcytometry allows for studies of the total ploidy level of an organism, as stained nuclei are quantified and qualitatively measured as a fluorescent signal collected from a light sensor. The method has previously been applied to studies of polyploidy in *Daphnia* (Korpelainen *et al.*, 1997). It has been shown to be especially applicable for endopolyploidy studies, and studies of different tissues. In this study however whole individuals from various age classes were included, as the main goal was to determine overall level of polyploidy.

Manual measurements from optical nuclear measurements produced normal distributed nuclear sizes and no pattern of polyploidy was evident (only one peak was observed). While the workload with manual measurement is high, flowcytometry should theoretically be simple, fast and produce immense amount of data (nuclear counts of 10 000 and more). A flowcytometer consists of a lasers and a focused stream of fluid containing the sample, sensors with various wavelengths detect reflection of the laser beam. The forward scatter sensor measure the size of the fragments in the fluid, the side scatter has a better resolution for the separation of the fragments, and fluorescence is measured by various fluorescence sensors. Coupling of the different sensors allow for discrimination, or “gating”, of fragments of a certain size or fluorescence.

The initial method for preparation prior to flowcytometry was that of Obermayer (2000), originally produced for preparations of plant tissue. The samples (>30 individuals) were kept alive in COMBO-water at 20°C for at least an hour prior to analysis to allow complete digestion of algae in intestine (Dag O. Hessen, pers. com.). Samples were chopped in 0.55 mL Isolation buffer[4] on a Petri dish using a razorblade, another 0.55 mL Isolation Buffer was used to wash the sample from the Petri dish onto a 10- or 20 µm nylon-filters. The filtrated sample was collected in a 5 mL polystyrene tube (BD Falcon™) and added 0.05 mL RNase (Promega, cat. # A7973, 1:200 v/v). The tube was kept in a water bath at 37°C for 30 minutes. Samples were stained using 2 mL Staining solution[5]. Propidium iodide (PI) is membrane impermeant and generally excluded from viable cells, the maximum absorption of PI bound to nucleic acids is 535 nm and the emission maximum is 617 nm (Fig. 3) (Product information, Molecular Probes). Microscopic analysis was done during every step of the preparation method by Obermayer (2000). As no or few nuclei were found, adjustments had to be made. Fluorescent acridine orange was used as a dye instead of Feulgen for analysis of the samples, as the prior is found to more sensitive than the latter, positively staining when the Feulgen reaction is negative, and more appropriate when working with small quantities of DNA (Wolstenholme, 1965).

Two additional nucleic extraction techniques were tried, sonification (Branson Sonifier 450, U.S.) and mashing of the sample using an RNase/DNase-free Eppendorf mortar. Both techniques improved the quality of the samples, but the Eppendorf mortar excelled both in increased number of nuclei and decreased noise-fragments.

It was further assumed that the high ionic concentration of the Isolation buffer could shrink the nuclei (Morten M. Laane, pers. com.). A buffer with a lower ionic concentration would leave the nuclei intact or slightly inflate them. The Isolation buffer was thus replaced with a 0.45 g/100 mL (57×10^{-4} Mm, a common invertebrate ionic concentration, Morten M. Laane, pers. com.) Sodium citrate solution. This resulted in a many-fold increase of observed number of nuclei and decrease of noise in the filtrated samples.

---

[4] 0.1 M Citric acid 1-hydrate and 11:100 v/v Triton X-100 (SIGMA, Cat. # 9002-93-1), diluted with dH_2O.
[5] 1:10 v/v of 10x Stock solution (100 mM Sodium citrate and 250 mM Sodium sulphate), 0.4 M Na_2HPO_4 and 0.06 v/v Propidium iodide (≥95 %, SIGMA, Cat. # 25535-16-4), diluted with dH_2O.

Flowcytometer analysis was conducted on a FACSCalibur (Becton & Dickinson, U.S.) and protocol for running flowcytometer apparatus was developed together with Per Færøvig. The flowcytometer apparatus uses low ionic miliq-H_2O by default, it was proposed (Per Færøvig, pers. com.) that the low ionic sheat water could rupture the nuclei, and was therefore replaced by FACSFlow water with a higher ionic strength. Fluorescence was measured using the Fluorescence Sensor 2 (FL2) (585±42 nm), Forward Scatter (FS), which identify size of the fragments, was initially used together with FL2 to produce a combined graph with both size and fluorescence intensity allowing for "gating" (similar to detection threshold levels) of clusters (if present). Increasing voltage and AMP for the selected sensor will increase the sensitivity at the cost of range, and had to be adjusted for each set of sample. Detection threshold limit remove all events with lower fluorescence than the set limit, assumed to be back ground noise, fluorescent non-chromosomal fragments. Speed of the flowcytometer may also influence the resolution; fast runs will decrease details, and may measure clusters of nuclei due to clogging, hence slow speed was used for this study to avoid these problems.

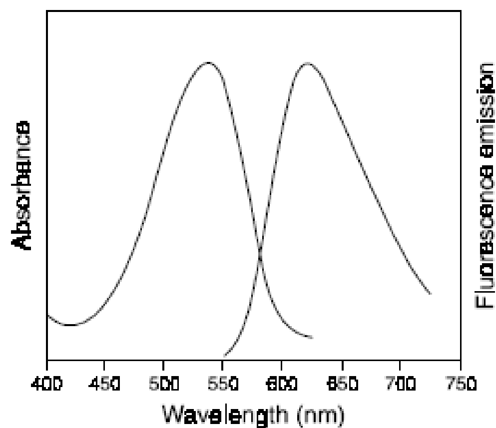


Fig. 3 Absorbance and fluorescence emission for PI (taken from PI Product information, Molecular Probes)

1.3. Life History Experiment

To examine the effects of different ploidy level on life cycle characteristics, a life history experiment was done. The measured growth rate, notes of fertility and mortality, and estimates of demography for the different populations, comparison with the results from the genetic analyses. Data were analysed using ANOVA one-way analysis with *post hoc* Tukey HSD test for comparisons of the populations for the different fitness parameters. Non-parametric Kruskal-Wallis test was used if the data had violations of normality or homogeneity of variance required for the ANOVA, Mann-Whitney test for statistical difference between the populations was run *post hoc*. Survival plots and Kaplan-Meier statistics run for age at maturity and survival (days of survival). A principle components analysis (PCA) was run using the PAST software (Hammer *et al.*, 2001) on the variables from the life history experiment to examine which variable makes up for most of the differences, and how the populations are clustered when comparing all variables in one plot. Principal components analysis (PCA) allows for projection of a multivariable data set containing linear measurements, down to two dimensions in a way that preserves as much variance as possible (Wold, 1978).

Length-weight calibration

In order to make optical, non-invasive measurements of the size of living *Daphnia* in the life history experiment, a calibration of the length to weight relationship was needed. Dry weight, or biomass, is assumed to be a better estimate of the body size of an individual, as it reflects the actual condition much more accurately. An estimation of weight from length measurements was also needed to express quantified DNA, RNA and protein per unit of biomass. Length, measured as the major axis from base of tail to tip of carapace above the pigment-eyes (like in Edmondson, 1955), is easily assessed when the individuals are alive (or semi-seduced with some drops of alcohol) by using a digital ocular camera (MD-300 3.0 Mp, Bresser) attached to a microscope (Wild M7A, Heerbrugg). A separate 1 mm scale was used for size calibration before each run. The carapace was noted to bulge outwards in gravid females, and weight was also suggested to increase non-corresponding with egg production. Mature egg-bearing females were

excluded. However females with small eggs or mature females with empty hatches were included. Samples were put into pre-weighted tin cups (Universal Tin container “light”, Thermo Finnigan, Milan, Italy), dried in a heating cabinet at 60°C for at least 24 hours, and weighted again for measurement of dry-weight. A sensitive balance weight (Mettler ME30; Mettler Toledo, GmbH, Greifensee, Switzerland) was used, as the weight of the animals was <0.1 grams. Data were analysed using statistical software (SPSS, v.15), correlations and best-fit equations obtained.

Chemostat set-up

A chemostat is a device to ensure stabile food concentration for the cultivated populations of *Daphnia*. The chemostat for this study consisted initially of 4 flasks (1.8 L) with *Selenastrum capriconutum* (clone NIVA CHL 10; Norwegian Institute of Water Reasearch, Oslo, Norway) and COMBO medium (50 µM P/L) (prepared as described in Kilham *et al.* 1998, stored in 20 L bottles) (later reduced to 3 due to contamination problems) placed on top of magnetic stirrers, sealed with air input (filtered through cotton and distilled water) and output, and connected to two peristaltic pumps (Cole Parmer 1-100 rpm # 7553-87 w/MasterFlex ® L/S modular controller, Chicago, U.S.) (Fig. 4). One was used for the input of COMBO the other for the outlet of the algae/COMBO solution. All equipment (flasks, tubes) was properly washed and autoclaved at 150°C for a minimum of 4 hours prior to use. The output fluid was led through a cuvette with a LED light source at one end and a Lux-meter (PASport, Light Sensor, PS-2106A, PASCO, Roseville, U.S.) (assembled by Morten M. Laane) connected to a laptop computer at the other end. The algae solution from the cuvette was led into a collection flask which was used for the cultures in the life history table experiment (see below). The peristaltic pumps and magnetic stirrers were connected to an on/off timer (30/566 seconds active time per cycle length) diluting the chemostat 0.2x each 24 hours. The chemostat was kept in good light conditions (70 µE/m^{2}×2, QSL-100, Biospherical Instruments, San Diego, USA) in a 20°C climate room. The chemostat was allowed to run for 2 weeks prior to the start of the life history experiment, Lux values was collected at 1 minute intervals for rough real-time estimates of the food concentration, OD (Absorbance) values was checked using a spectrophotometer, and 1ml samples fixated with 1% v/v Glutaldehyde

and Paraformaldehyde for *a priori* cell-counts using flowcytometry. 100ml of the algae solution was filtrated through precombusted (at 450°C) glass microfibre filters (GF/C 25mm Ø, Whatman®) for a posterior carbon measurement in element analysis (Flash EA 1112 Series, Thermo Finnigan, Milan, Italy).

Fig. 4 Chemostat set-up: 1. Sealed flasks with algae, 2. Laptop computer connected to Lux-meter, peristaltic pumps (left), power supply and controllers to peristaltic pumps (bottom), 3. Overview, computer (left) controls the on/off cycles through a relé (not depicted) of the peristaltic pumps, 4. One of the two sets of peristaltic pumps, 5. COMBO-water with inlet tubes to the chemostat, 6. Lux-meter (left), cuvette (middle, with tubes) and LED light (inside box, right), 7. Outlet-water from the chemostat.

Life history experiment

To define the different evolutionary parameters, demography and growth rates for the populations, a life history experiment was initiated. The experimental set up is described below.

Preparations of the life history table experiment included use of 200 ml beakers with 100 ml algae solution from the chemostat with 10-20 assumed gravid adults (visually picking out the biggest individuals in the stocks) from the different. The algae solution in these beakers was changed every other day to ensure stabile food concentrations. Once juveniles were hatched they entered the experiment, and were dispensed into 12.5 ml plastic beakers (NUNC A/S Denmark) with 10 ml algae solution kept in 24 hours light at 18°C. The beakers were arranged in 5 rows with 14 holes in each on a corkboard table (Fig. 5). These were left uncovered (which led to some precipitation; roughly 2 ml/day), which allowed a certain amount of light and air to the beakers. Measurements were performed every other day; starting the day the individual entered the experiment. Corkboards were then transferred to another room (~25 ^{o}C) with a digital ocular camera (MD-300 3.0 Mp, Bresser, Germany) attached to a microscope (Wild M7A, Heerbrugg AG, Switzerland). The *Daphnia* were transferred to a microscope slide with a central concavity using disposable 2 mL liquipettes, and pictures taken at 18x magnification. The digital ocular camera was operated with WebCamCompanion 1 (ArcSoft) and pictures analysed using ImageTool Version 3.00 (UTHSCSA, University of Texas Health Science Center in San Antonio, U.S.). The major axis length (from the base of the spine to the top of the eye) was measured using the digital images. Observations of maturity, released exoskeletons (moults) and counts of offspring were estimated visually in the beaker with a bright lamp and white background. The individuals were subsequently transferred to a new 12.5 ml beaker with fresh algae solution. Both the transfer out of the culture room and the transfer from the beakers to the microscopic slide and back to a new beaker, were executed with uttermost care to reduce possible stress on the animals.

Initially, a laboratory population of *D. magna* was included, but excluded from the comparisons since *D. magna* actually is genetically rather distant to the other *Daphnia* species, and would thus probably not reflect life history characteristics that

could be attributed to ploidy level or melanization. Statistical analysis showed that *D. magna* differed from the *D. pulex* spp. in almost all measured characteristics; they had high fitness, reached maturity late, grew fast and large, and produced resting eggs unlike any of the *D. pulex* populations. The idea of *D. magna* as an outgroup was dismissed, as the temperate population of *D. pulex* proved phylogenetically more appropriate and *D. magna* to distantly related to the *D. pulex* species complex to be assigned as an outgroup.

Fig. 5 Cardboard tables with 5x14, 12.5 mL beakers, extra table (left bottom) for handling of juveniles.

1.4 – Genetic Analyses

The preliminary trials were done to study polyploidy from direct observations of the cells by DNA-specific staining, microscopy and flowcytometry (see **Cytogenetic Analyses**). Despite numerous trials and different methods it never succeeded to quantify degrees of polyploidy in the different populations of *Daphnia*. The problem was then accessed not by direct observation, but by assumed by-products of polyploidy, such as number of alleles per loci using microsatellite analysis or RNA/DNA ratio and quantitative DNA estimations using a fluorescence microplate reader.

Microsatellite analysis

A diploid organism only have one homozygote or two heterozygote alleles, where as a polyploid organism have more than 2 alleles either as homozygotes or heterozygotes. Microsatellite analysis allows for counts of the number of alleles and hence allow for assumptions of levels of polyploidy.

Microsatellites are tandemly repeated motif of 1-6 bp, a type of fragment length polymorphism caused by slippage of the DNA enzyme during DNA replication (Schlötterer & Tautz, 1992). Nowadays, the base pair differences (alleles) are identified with the help of capillary electrophoresis systems. Allele identification or "genotyping" is done with software like GenoTyper, PeakScanner or GeneMapper (all from Applied Biosystems). Based on the genotyping, alleles frequencies are calculated and estimates of allelic heterogeneity and/or polyploidy can be made. Since non-coding DNA, such as microsatellites, has a high mutation rate differences between closely related individuals/clones are detectable (Markova *et al.*, 2007). Because we would expect more than two alleles for a given locus in polyploids the interpretation of allele number is not straight forward (Markwith *et al.*, 2004). Alleles with one locus will make distinct bands in the electrophoresis, interpreted as peaks in software (PeakScanner v1.0, Applied Biosystems) (Fig. 6); a homozygote allele will appear as one such peak, a heterozygote

allele as two peaks (with multiple of the repeated motif difference[6]). For three or more peaks one can assume polyploidy, as there are more than 2 alleles. However to differentiate a triploid and a tetraploid one need to identify four peaks, or successfully identify a homozygote in addition to two heterozygote alleles (compare peak heights, if <2 one can assume homozygosity). 8 primers (Table 1) reported to work for *Daphnia* (Colbourne *et al.*, 2004) were obtained to be investigated in a pseudo-multiplex, running all primers separately in a PCR machine, pooling them together before analysis in electrophoresis. Microsatellite analysis was performed in the DNA-lab at the Department of Biology (UiB) under the guidance of Morten Skage. All samples were sun on an ABI 3730xl capillary DNA analyser at the SARS sequencing facility, UiB.

Table 1 Primer pairs used in the microsatellite experiment, fragment size in bp, TM as suggested by Morten Skage, sequence of upper and lower primer, and motif of repeat (motif 2 indicate alterative repeat sequence) (Asterisk indicate trinucleotide repeat observed as a dinucleotide repeat). Primers from Colbourne *et al.* (2004).

Name	Locus	Accession #	Size	TM	Upper primer (UP)	Lower primer (LP)	Motif 1	Motif 2
Dp512	Dpu6	WFms0000529	138	54	TTTCGTTCTACCCAGGGAAG	TTTGCTCGTCTGTGATACGC	(GT)4N4(GT)7	
Dp513	Dpu7	WFms0000530	115	53	ATGATCTGCATTCGTCTGCG	AAGGGAAACGGATAAACGGG	(CA)4N3(AC)6	
Dp514	Dpu12/1	WFms0000531	102	54	GTTATAACCAATGGGAAGGC	TTCAGTCGCGTTGGTTTCG	(GAA)5*	
Dp514alt	Dpu12/2	WFms0000532	133	55	GCAGTTTGTGCTGTTCAATG	GCCTTCCCATTGGTTATAAC	(TTC)9	(GTT)3GT(GTT)2
Dp522	Dpu40	WFms0000540	120	55	ACGCGTTTCATCCTGACCC	GCCTTGTTGTTTCTTGCCTC	(AC)8	
Dp523	Dpu45	WFms0000541	134	53	GATGATTACTGTATTTTACG	GAGTTTTATTGCTTTCATAC	(AC)10	
Dp524	Dpu46	WFms0000542	128	53	GGGGAAATAAAGAAGAACCGC	ACAGCTAACACAAGTTGATAC	(AC)9	
Dp525	Dpu47/1	WFms0000543	123	54	GCACCAGATTGTCATGGAG	AATAGGCTCGGCTATATGGG	(TTC)8	

Preparations

Juveniles (<24 hrs) and adults (approximately 50% w/eggs) from population A, B, C, D, G and *D. magna* were sampled from cultures in 100mL beakers fed *ad libitum* two weeks prior to collection, and put to 1mL Eppendorf tubes with 96% ethanol. Samples were dried and transferred to a standard multiwell plate. 60 µL and 100 µL chelator (Chelex,

[6] E.g. for dinucleotide repeats the smallest difference between two peaks will be 2 bp, accordingly will the smallest difference for a trinucleotide repeat be 3 bp, peaks with less than repeated motif length will be due to other causes (see discussion).

InstaGene™ Matrix, BioRad) was dispensed to respectively juveniles and adults. A small disposable pipette tip was used to crush the samples. The multiwell plate was kept on a heating block at 55°C for 30 minutes followed by ~100°C for 10 minutes, before cooling the plate to room temperature. The supernatant DNA was diluted with dH_2O, respectively 5 fold (20 µL supernatant + 80 µL dH_2O) for juveniles and 10 fold (10 µL supernatant + 90 µL dH_2O) for adults before use in the PCR (polymerase chain reaction). 2.5 µL diluted DNA, 6 µL MasterMix (Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers, ProMega, cat. # M7501), 0.5µL Primer Upper, 0.5µL Primer Lower and 3µL dH_2O was added to each well. Remaining diluted DNA was used in the mtDNA sequencing (see below).

PCR

Polymerase chain reaction is a method for increasing the number of the targeted DNA site in the sample by inducing DNA replications through temperature cycles (Beebee & Rowe, 2004). Cycling parameters involved denaturing at 94°C for 3 minutes, followed by 30 cycles at 93°C for 30 seconds, 53-55°C (see TM in Table 1) for 30 seconds, and 72°C elongation for 40 seconds before one final 20 minutes elongation at 72°C. See Table 1 for primers.

Post-PCR

The samples for the capillary electrophoresis were ordinated as pseudo-multiplex samples, compiling 4 and 4 primer pairs together posterior to the PCR (as opposed to real multiplex where all primers are run together in the PCR).

A priori tests run showed above limit values when diluting finished PCR-products by 10x, hence a subsample of the pseudo-multiplex was diluted 20x, 40x and 80x and ran in the capillary electrophoresis. The PCR products diluted 20x was found the most promising. No primers were above detection limit, but some primers were found to have below detection values. Data were analysed in PeakScanner v.1.0 (Applied Biosystems) [7], and polymorphic fragment lengths presented visually as peaks. Alleles were scored

[7] Following settings were used in the PeakScanner software: "GS500(-250) LIZ" as "Size standard" (added during elecrophoresis), and "Sizing Default, PP" (primer in sample) as the "Analysis method".

according to the number of peaks observed, taken into account various artefacts/effects (such as “+A effect”, “stutters-bands” and true repeats differing with the bp length of the repeat motif). Two alternative ways of scoring the assumed allele peaks were developed: one “non-restrictive”; differentiating homozygotes from heterozygotes (2 for a homozygote peak and 1 for each heterozygote peak), scoring actual number of alleles, and one “restrictive”; no differentiation between homozygotes and heterozygotes (both scored as 1), only counting number of observed allele peaks.

Species identification by mtDNA sequencing

As morphological species identification of the *Daphnia pulex* complex is difficult, mtDNA sequencing was run to determine the genetic relationships of the populations.

The diluted (10x), supernatant DNA (prepared in the microsatellite study) was sequenced by Morten Skage, UiB, using mtDNA primer DpuND5a and DpuND5b for a fragment (~850 bp) with parts of the gene coding for NADH dehydrogenase subunit 5 (ND5) according to protocol by Colbourne *et al.* (1998). A 25 µL reaction-mix was prepared, consisting of 1x MasterMix PCR buffer (0.625 Units Taq DNA polymerase, ProMega), 1.0 µL of each primer (10 µM), 8 µL dH_2O and 2.5 µL supernatant DNA. PCR was run with the following steps; samples denatured for 2 minutes at 94°C, then 30 cycles consisting of: 1 minute denaturation at 92°C, 1 minute annihilation at 50°C (48°C for population A and G), 72°C for 1 minute. After 30 cycles the samples were elongated at 72°C for 7 minutes and incubated at 9°C for storage. PCR products were cleaned with ExoSap-IT (USB Corporation, U.S.) and sequenced with Big Dye v. 3.1 terminator (Applied BioSystems) on an ABI3730 DNA analyser (Applied BioSystems).

Quantification of total DNA, RNA and protein

DNA was quantified to indicate polyploidy, whereas RNA and protein was used to compare growth rates. Comparisons of ratio between the different nucleic acids and protein were done to check for differences between the diploid and polyploid populations.

Fluorescence techniques were used for DNA, RNA and total protein quantification work. A fluorescence microplate reader allows for quick and reliable

analysis of multiple samples. Sensitivity of this techniques permitted quantifying three components from the same sample: DNA, RNA and protein content. In addition to the commercial standards, DNA and RNA standards were isolated from *D. pulex* (population G), calibrated and diluted to the same concentrations as the commercial. Hence two different set of standards were achieved.

Data were analysed using ANOVA one-way analysis with *post hoc* Tukey HSD test for comparisons of the populations for the different fitness parameters. Non-parametric Kruskal-Wallis test was used if the data had violations of normality or homogeneity of variance required for the ANOVA, Mann-Whitney test for statistical difference between the populations was run *post hoc*.

DNA isolation for using as standards

About 50-100 individuals of *D. pulex* (population G) (30 mg wet sample) were kept in COMBO medium (Kilham *et al.*, 1998) for 2 hours prior to isolation of nucleic acids. Animals were filtrated through a nylon mesh (40 µm) and rinsed several times with distilled water. DNA isolation was done by cTAB protocol modified after Hombergen & Bachmann (1995) by Gabrielsen *et al.* (1997) in Steen (1999). To the sample it was added 600 µL cTAB extraction buffer[8] and mashed using RNase/DNase-free Eppendorf mortar, kept at 65°C for one hour and vortexed every 15 minutes. To extract proteins 500 µL chloroform/isoamylalkohol (24:1 v/v) was added, and then the sample was centrifuged for 5 minutes at 12,000 rpm. The supernatant was taken from the three phased sample, and last step repeated. To the resulting 10 µg of RNase (DNase free, Promega, cat. # A7973) per mL supernatant (0.5 µL) supernatant was added, incubated at 37°C for 30 minutes for digestion of RNA. Precipitation of DNA from the solution was obtained by mixing with cold isopropanol (1:3 v/v RNase and DNase free water, Invitrogen). After keeping it at -20°C for 10 minutes sample was centrifuged for 10 minutes (maximal speed) and supernatant was discarded. Pellet was washed twice with 400 µL of 70% ethanol. The supernatant was discarded and white pellet was dried and dissolved in 50-500 µl of RNase and DNase free water (Invitrogen) and kept at -20°C

[8] cTAB extraction buffer: 4g cTAB, 16,5g NaCl, 2,4g Tris-HCl, 1,2g $Na2EDTA$ per 200 mL DNA free water, autoclaved.

until next day. Purity and stock concentrations were measured spectrophotometrically (ND-1000, NanoDrop Technologies).

RNA isolation for using as standards

For RNA isolation E.Z.N.A. Total RNA kit II (R6934-01, Omega Bio-Tek, U.S.) was used with following protocol included. Samples were prepared as described for DNA isolation. Cells and tissues were lysed with 1mL 'RNA- Solv® Reagent/Phenol' solution and mashed with nuclease-free Eppendorf mortar. The sample was incubated at room temperature for 5 minutes before adding 200 µL chloroform and vortexed for 20 seconds. Separation of aqueous and organic phase was done by centrifuging sample for 10 minutes at 12,000 rpm, aqueous phase - supernatant (~ 600 µL) was transferred to a new tube, added 600 µL of 70 % ethanol and vortexed. 700 µL of the sample was transferred to a "HiBind® RNA spin column" (inserted in a 2 mL collection tube) and centrifuged at 10,000 rpm for 15 seconds, this step was repeated with the remaining sample. 300 µL "RNA Wash Buffer I" was added to the column and centrifuged at 10,000 rpm for 1 minute, 75 µL DNase I digestion reaction mix (73.5 µL OBI DNase I Digestion Buffer, 1.5 µL RNase-free DNase I (20 Kunitz units/µL, Gibco BRL, cat. # 18047-019) was then added directly to the membrane in the column and left for incubation at room temperature for 15 minutes. Another 300 µL RNA Wash Buffer I was added followed by 1 minute centrifuge at 10,000 rpm. 500 µL "RNA Wash Buffer II" was added and sample centrifuged at 10,000 rpm for 1 minute, step was repeated with a second addition of 500 µL RNA Wash Buffer II and centrifuge at full speed for 1 minute to dry the column. RNA in column was eluted with 50 µL DEPC-treated water and centrifuged for 1 minute at full speed. The RNA sample was run in spectrophotometer to determine concentration and its purity.

DNA & RNA quantification

Individuals of Daphnia representing all populations (A-D, G and M), both juveniles (<24 hours) and adults (~7 days) were included for this part of the study. A total of 73 samples was included in the study, whereas juveniles was pooled 2-3 together into one tube, number of individuals from the different populations varied, from 6 (population D) to 17

(population G) replications. To estimate body weight, pictures of individuals were taken before snap-friezing in liquid nitrogen. Animals were stored in RNase/DNase-free Eppendorf tubes at -80°C until analysis (one month). Protocol used for DNA and RNA quantification follows from modifications of Wagner *et al*. (1998) sited in Jones *et al*. (1998), later modified by Kyle *et al.* (2006).

Snap frozen samples kept at -80°C were added 60 µL extraction buffer (see above), centrifuged and sonified for 2.5 minutes at 100 % output (Branson Sonifier 450, U.S.). Samples were diluted with 300 µL TE buffer (Molecular Probes, T-11493). 75 µL from each sample was dispensed into two 4 wells (2 well for total reading, the others for digestion by RNase, and both DNase and RNase) in a multiwell slate (Nunc) for both DNA and RNA (remaining sample was later used for protein quantification and stored at -20°C until analysis). 10 µL RNase (1:200 v/v, Promega, cat. # A7973) and 10 µL DNase (GibcoBRL, 18047-019, in Mg/Ca buffer, 1,87 U in the well) was added to DNA-quantification slate, 10 µL RNase was added to the RNA-quantification slate, Invitrogen water was used to dilute all wells to a total of 95 µL of sample. After digestion of RNase and DNase (30 minutes at 37°C on the shaking table 12 x/minute, Grant Boekel HIR10M, U.S.) the slates were run in the microplate reader (Nunc™, Denmark). 75 µL Ribogreen dye (1:200 v/v dilution) was added by automatic dispenser to the wells in the microplate reader (ELx800™ Absorbance Microplate Reader, BioTek, U.S.) and excitation and emission wavelength was measured at respectively 485/20 nm and 528/20 nm after mixing on board and 5 minutes incubation with fluorescent dye (acquisition and analysis using Gen5™, BioTek, U.S.). Total signal obtained is the sum of DNA, RNA and any background coming from the sample. Subtracting the value obtained after RNase treatment from total signal gave the estimation of RNA content in the sample. From this value signal after RNase plus DNase digestion was subtracted for DNA estimation.

Standards were treated the same way as samples, kept in the same buffer and sonified for 2.5 min at maximal output following concentrations of DNA were prepared in sample buffer[9]: 1, 0.8, 0.4 and 0.1 µg/mL[10] using purified standards (from *D. pulex*)

---

[9] Sample buffer: 6 mL extraction buffer (5 g N-lauroysarcosine (sarcosyl SIGMA, cat.# L-5125) in 500 mL water (Invitrogen)) diluted with 30 mL TE buffer (Molecular Probes, T-11493)

[10] Concentration 5, 3 and 2 µg/mL was only used for commercial standard as material obtained from the DNA isolation using *D. pulex* was too little to make all the standards.

and commercial standards (Calf thymus, Sigma cat. # D-1501). Two replications of each standard concentration were used, additionally digestion using RNase and both RNase and DNase (background noise). The following concentrations of RNA were prepared in sample buffer: 3, 2, 1.5, 1, 0.5 and 0.25 μg/mL by using purified standards (from *D.pulex*) and commercial standards (RNA type III from Bakers Yeast, Sigma, cat. # R7125). Two replications of each concentration were used, and additionally digestion using RNase and both RNase and DNase (for quantification of background noise). Samples were stored on ice before using for quantification with RiboGreen.

Protein quantification

Bovine serum albumin (BSAI, Molecular Probes) was used as a protein standard (NanoOrange®, Molecular Probes) during protein quantification. Standards were prepared as described in NanoOrange® Protein Quantitation Kit (Invitrogen, Molecular Probes™, cat. # N6666), 2 mg/mL BSAI (Bovine serum albumin) was diluted with water to working concentration of 10 μg/mL solution and diluted with1x NanoOrange® working solution (diluted 10x with Invitrogen water) to make the standard concentrations (10, 5, 1, 0.6, 0.3, 0.01, 0.06, 0.03, 0.01, 0.006, 0.003 and 0.001 μg/sample) for a final volume of 245 μL for each replication (two were used in this study). Standards were incubated at 95°C (PCR incubator) for 10 minutes and cooled to room temperature before running in plate reader (BioTech800). 50 μL sample remaining after DNA/RNA quantification was diluted with 150 μL RNase/DNase-free water (Invitrogen). 120 μL from this solution was transferred into new tubes, added 125 μL 1x NanoOrange® working solution and incubated at 95°C for 10 minutes. Slates were run in microplate reader (BioTech800) once they had cooled to room temperature and excitation and emission wavelength was measured at respectively 485/20 nm and 528/20 nm (see Fig. 6).

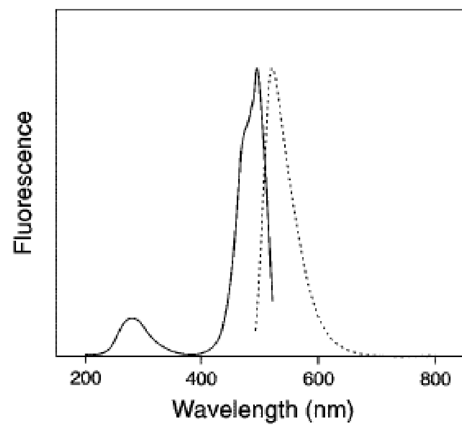


Fig. 6 Fluorescence excitation (solid line) and emission (dotted line) for the RiboGreen dye (Taken from Jones *et al.*, 1998)

Results

2.2. Cytogenetic Analyses

Feulgen staining

In contrast to most other organisms, chromosome and nuclear staining in *Daphnia* is quite difficult. Procedures that work well in mammals and for most plants do for technical reasons not work well for *Daphnia*.

The method described by Laane & Lie (2001) consists of a short heat treatment; the duration of this treatment has been noted to vary between different organisms and types of samples (Laane & Lie, 2001). Variations of 10 ± 5 minutes were tried. Best results were obtained at 12 minutes. With more than 12 minutes and the samples did not get properly stained, with less than 12 minutes and the sample was unspecific stained (staining non-chromosomal cell parts). The temperature when using a commercial heat block was found to be very unstable (varying up to ± 5°C), hence alternatives such as water bath and heating cabinet were tried. The latter gave the best results, and was used thereafter. Varying staining time produced results with similar variations, but less sensitive. A gradual increase in staining (including unspecific) was observed when staining time approached 60 minutes (whereat the staining rate was drastically reduced, staining continued for another 24 hours until all cell components were stained). Papain, used to digest peptide bonds and believed to enhance separation of chromosomes, gave somewhat better results (especially for the *Allium* root sample, Fig. 7). The thick solution (the papain was extracted from squashed papayas) seemed however to cause disturbances with the established digestion/staining times for the *Daphnia* and was abandoned. Addition of chloroform, a strong solvent, to the fixative was abandoned as no improvement was observed. The Feulgen reagent was found to be staining unspecific cell parts in addition to chromosomes when using a heat treatment for hydrolysis, the effect was sometimes gradual, but could also appear instantaneously. Replications of the staining experiments using established times and temperature still produced highly

variable results. The method by Laane & Lie (2001) was abandoned as it appears to be unsuited without modifications for *Daphnia*.

The procedure by Beaton & Hebert (1988) was found to be more replicable, Feulgen staining was more specific and better preservation of the samples (due to slower unspecific staining). However the method was more time-consuming (see methods). "Chromosome bodies" found were mainly in prophase, identified as "condensed chromosomes" (Fig. 8). It was noted that large clusters of cells seemed to be in the same mitotic stage. It was proposed that *Daphnia* could have synchronous mitosis (Beaton & Hebert, 1994a). Individuals were sampled within 3 hours after moulting, before mid-instar, the most mitotic active period (Beaton & Hebert, 1994a), to increase the chance of finding individuals with chromosomes in metaphase. A fair number of cell divisions were found in samples from individuals at the suggested most mitotic active stage, but no with extensive synchronous mitosis (nor any in metaphase). Single mitotic active cells were observed, but no accurate estimate of a base chromosome number was possible due incomplete chromosomal differentiation (see Fig. 8). The observed bigger chromosomal clusters could suggest polyploid cells, but any quantification at this stage was not possible (see **Embedding & nuclear measurements**).

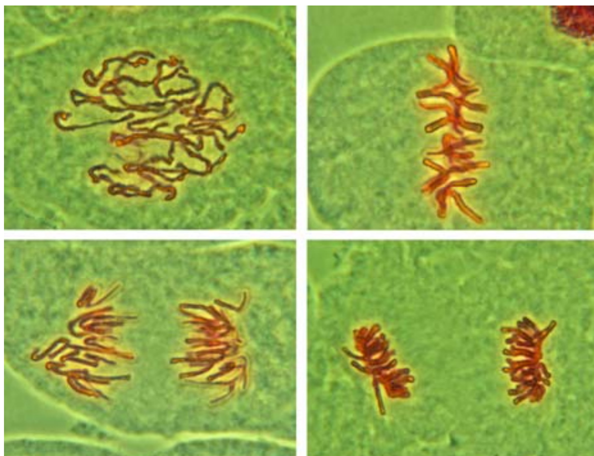


Fig. 7 Various stages of mitosis shown in an onion (*Allium*) root stained with Feulgen following method by Laane & Lie (2001), showing from upper left mid prophase, metaphase, anaphase and telophase (100x magnification).

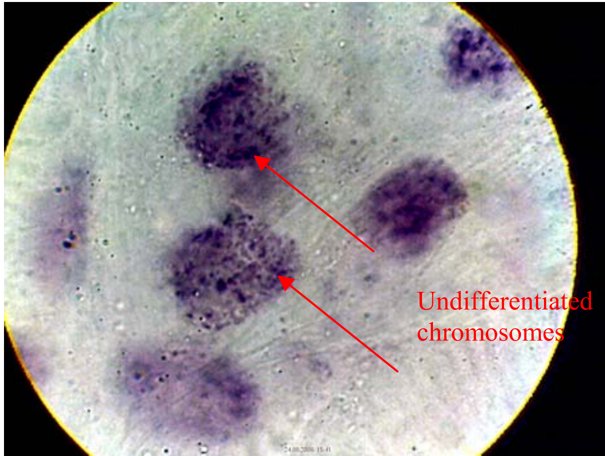


Fig. 8 Typical *D. magna* Feulgen stained nuclei (protocol by Beaton & Hebert 1988) showing undifferentiated (condensed) chromosomes appearing as "clouds" (~700x magnification).

Mitotic inhibitors

Colchicine in a 1% concentration was found to be lethal to the *Daphnia* (reported non-lethal in Beaton, 1994; Morrow, 2001). By lowering the concentration mortality decreased, and after staining clustered chromosomes could be observed (see Fig. 9). The clustering was thought to be caused by spindle depolymerization and chromosome condensation properties of the colchicine (Morten M. Laane, pers. com.). Hence no precise chromosome counts could be made.

Despite some preliminary good results with chromosomes halted in division, the difficulties with standardizing the method caused variations in the results using 8-hydroxyquinoline. Some samples were prepared reasonably well (see Fig. 10) showing 8 or 18 bodies identified as chromosomes.

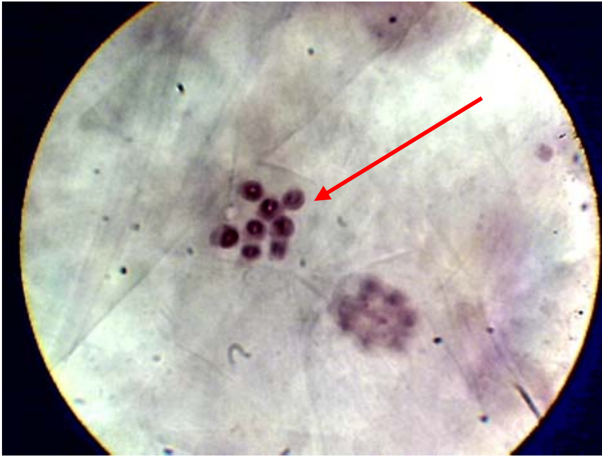


Fig. 9 ***D. magna*** **treated with colchicine and stained with aceto orcein, 8 bodies (red arrow) could possibly be identified as condensed chromosomes (but the structure of the bodies differ from that seen using 8-hydroxyquinoline, Fig. 1) (100x magnification).**

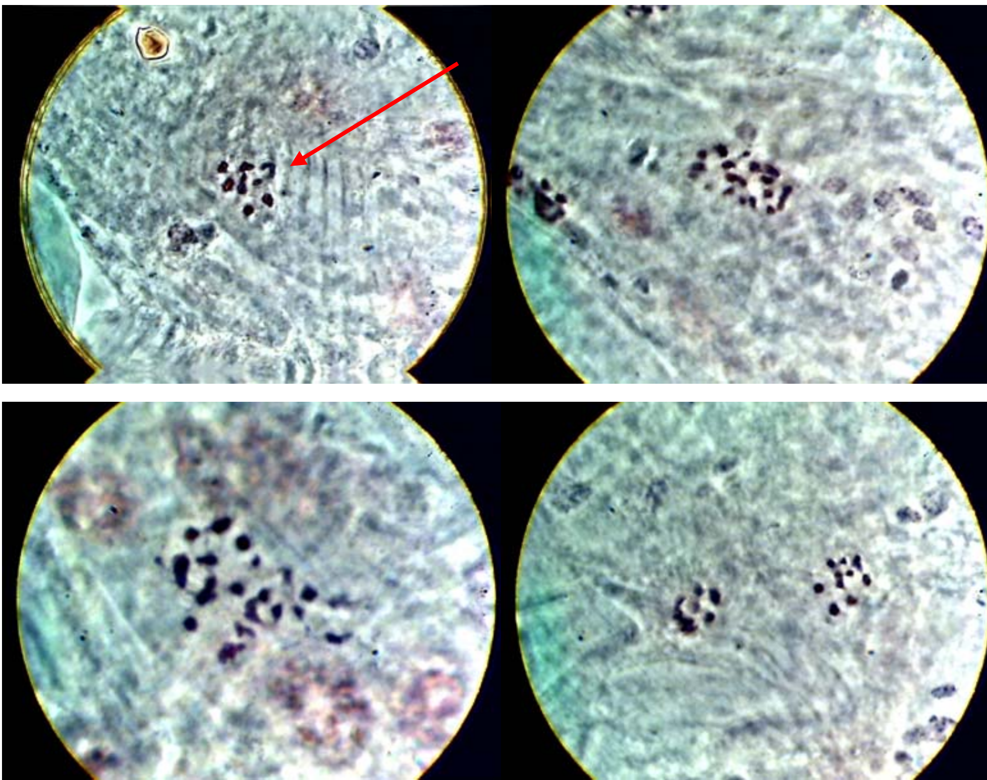


Fig. 10 ***D. magna*** **treated with 8-hydroxyquinoline exposure and stained with aceto orcein, top left; composite picture using CombineZM, showing 8 bodies possibly identified as condensed chromosomes (red arrow), top right and bottom left; 18 bodies possibly identified as condensed chromosomes.**

Embedding & nuclear measurements

The identification of the different tissue types was done after discussions with Morten Laane. The various tissue types in *Daphnia* appear to be only described on basis of rather rough histological techniques. The plastic thin sections revealed a wealth of details and a preliminary distinction was made between the tissues observed. Intestine and connective tissue type A were the most distinguishable tissue types in the preparations (Fig. 11), whereas the connective tissue type B (Fig. 12), chitin producing tissue (Fig. 13) and neural tissue in the eye region (Fig. 14) were less distinguishable and less present in the sections. Nuclei are more spherical and sections may occur at any angle. Tracing the periphery of numerous nuclei is an expression of its average volume and also its DNA content.

The populations were statistically different for the size of nucleus assumed to be from the connective tissue type A (violation of homogeneity for both normal and log transformed data ($P<0.001$), non-parametric Kruskal-Wallis, $P<0.001$, chi=155.528, d.f.=3, Fig. 15, Table 4), all populations except B and D were found to be statistically different from each other (Mann-Whitney test, $P<0.05$, Table 5). Statistical difference between the populations was also recorded for cells from the intestine (Fig. 16) (violation of homogeneity for both normal and log transformed data ($P<0.001$), non-parametric Kruskal-Wallis, $P<0.001$, $\chi^2=234.686$, d.f.=3, Table 6), whereas all populations were statistically different from each other (Mann-Whitney test, $P<0.05$, Table 7). The ratio between the average nuclear size of the intestine (Fig. 9) and connective tissue type A (Fig. 15 & 16) for the arctic populations (B, C & D) and the temperate (G) was ~2x, indicating probable doubling of genetic content, hence doubling of chromosome numbers.

The two tissues irrespectively of populations were found to be statistically different (violation of ANOVA, homogeneity for both normal and log transformed ($P<0.05$), Kruskal-Wallis test, $P<0.001$, H=130.786, d.f.=1, Table 8). Comparisons of the average nucleus size for tissue vs. tissue (1.38-1.96x) does not produce the same clear cut indication of doubling of genetic material.

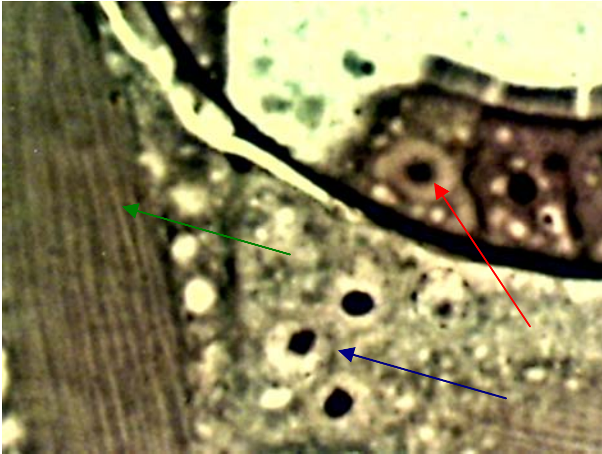


Fig. 11 Juvenile *D. pulex*, red arrow indicate intestine nucleus, blue arrow indicate connective tissue type A (note adjacent muscle tissue (green arrow) and lamellas on intestine cells). The nucleolus is visible as a small, black dot, while the nuclear envelope encompasses the paler halo seen around the nucleolus (Picture taken with Logitech 5000 Pro 1.3 Mp at 100x digital magnification, note that difference in colour between Fig. 1 and Fig. 2, 3 & 4 is due to variation in staining and minute differences in microscopic settings).

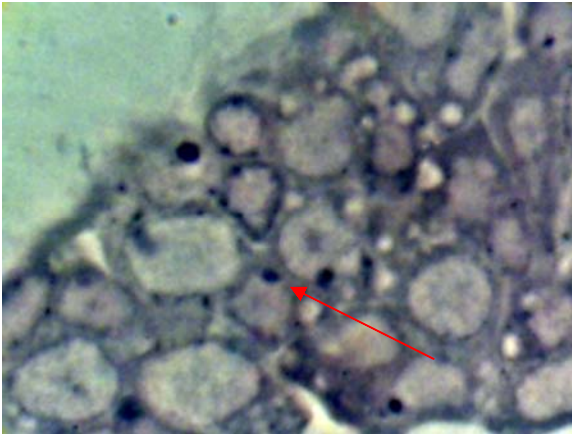


Fig. 12 Juvenile Polar *D. pulicaria*, unspecified region, connective tissue type B (note the small size of nucleoli and orientation to the nuclear membrane, red arrow) (Picture taken with Logitech 5000 Pro 1.3 Mp at 100x digital magnification) (tissue not included in study).

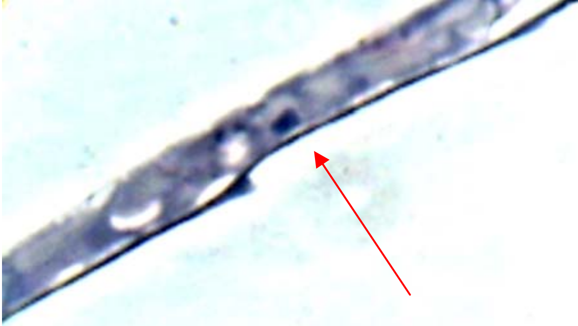


Fig. 13 Juvenile *D. pulex*, showing carapace (epidermis), red arrow indicate nucleus (Pictures taken with Logitech 5000 Pro 1.3 Mp at 100x digital magnification) (tissue not included in study).

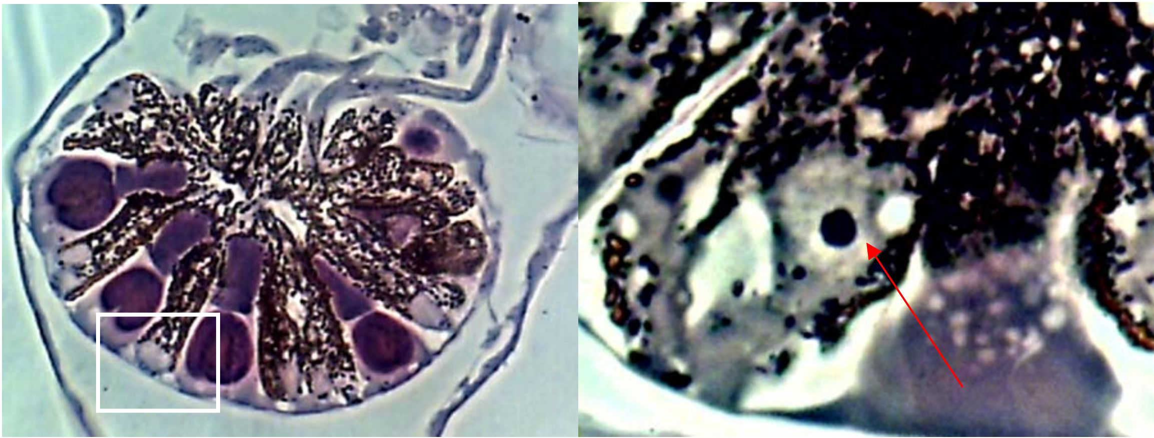


Fig. 14 Juvenile *D. pulex*, left: composite picture of complete eye (using AutoStich™ v. 2.184) (note neural cord at the top of the picture), right: subset of the eye region showing neural tissue cell with nucleus (red arrow) (Pictures taken with Logitech 5000 Pro 1.3 Mp at 100x digital magnification) (tissue not included in study).

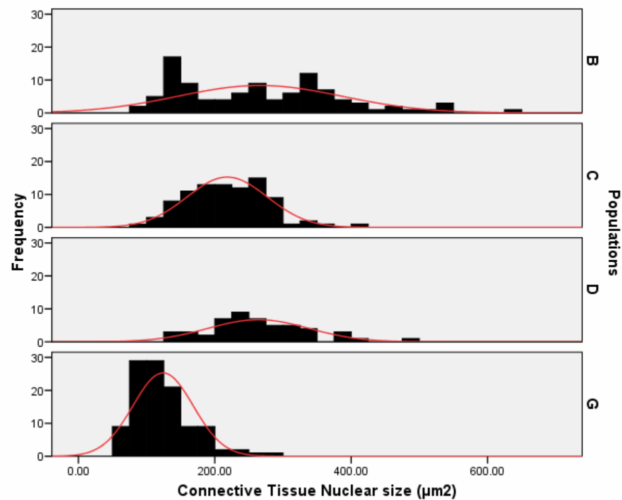


Fig. 15 Histograms for nuclear sizes (µm²) of connective tissue type A for population B, C, D and G (number of measurements per population N_B=101, N_C=90, N_D=50, N_G=112) (ratio between average size; B/G=2.03, C/G=1.73, D/G=2.13).

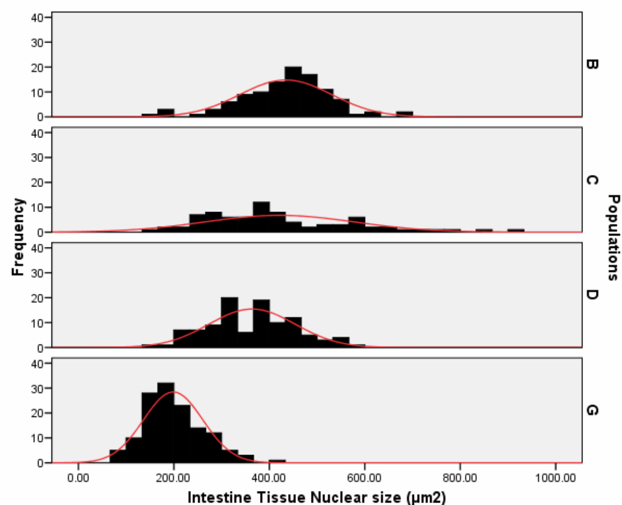


Fig. 16 Histograms for nuclear sizes (µm²) of intestine nucleus for population B, C, D and G (number of measurements per population N_B=107, N_C=80, N_D=105, N_G=133) (ratio between average size; B/G=2.19, C/G=2.12, D/G=1.83).

Flowcytometer

By using the protocol by Obermayer (2000) no difference between noise and possible nuclei was found and few or no nuclei were visible in microscopic inspection. However with the application of Sodium citrate solution a separate band from the noise was observed in the flowcytometer and also clearly visible nuclei were seen in microscope. Application of FS (Forward Scatter) together with FL2 (Fluorescence Sensor 2) did not

produce clusters of fragments, thus no "gating" could be made, and detection threshold limits had to be applied to a simple FL2 histogram.

Results from running *D. magna* (Fig. 17 & 18) and population C (Fig. 19) could indicate diploidy, as only one peak was evident when plotting the fluorescent response to number of counts. Events or counts refer to the number of positively identified fluorescent fragments (events below detection limits not included), low density of stained nucleus in the samples allowed for a total of 5000 events/counts for the test runs with *D. magna* and population C. Settings included adjustment of voltage, AMP, detection threshold limits for the FL2 sensor.

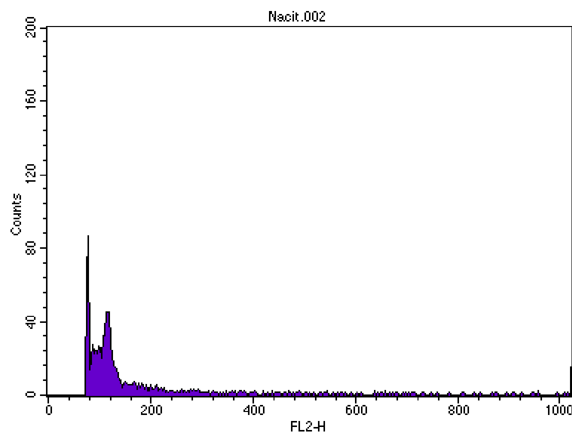


Fig. 17 – Histogram showing test run using *D. magna* for the FL2 sensor, showing pronounced peak at ~100 (relative values on x-axis) (~5000 events).

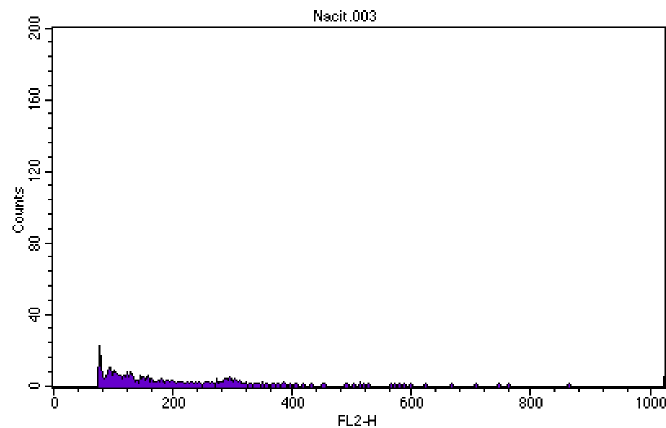


Fig. 18 – Histogram showing test run using *D. magna* for the FL2 sensor, with modified settings (showing evenly distributed fragments) (<500 events).

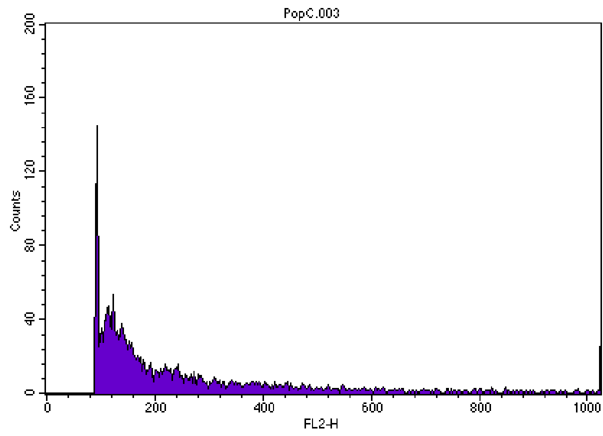


Fig. 19 – Histogram showing population C, assumed polyploid, for the FL2 sensor (~5000 events).

2.3. Life History Experiment

Length-weight calibration

The resulting length and weight values were compared by using a statistical software (SPSS, v.15) which produce scatter diagrams with selected best fit lines (Fig. 20 and Table 9). The measured major axis is a 2 dimensional parameter of the size of the animal, while weight is referring to the volume or the 3 dimensional properties of the animal; hence the linear equation will not fit our dataset (also low R square values; 0.69-0.89, Table 7). Logarithmic Power, S and exponential equations will consequently be more appropriate for the animal growth when comparing length and weight. The Power exponential trend line (below) had the highest R square values of the different logarithmic equations (except for population A, Table 9) and was used in the subsequent analyses (see **DNA, RNA & protein quantification** and **Life history experiment**).

$$\mathrm{Y} = ax^{b}$$

For the individual populations, the relationship was as follow:

$$\mathrm{Y}_{A} = 0.0051x^{3.2526}$$
$$\mathrm{Y}_{B} = 0.012x^{2.2333}$$
$$\mathrm{Y}_{C} = 0.0071x^{2.81}$$
$$\mathrm{Y}_{D} = 0.0077x^{2.9221}$$
$$\mathrm{Y}_{G} = 0.0079x^{3.277}$$
$$\mathrm{Y}_{M} = 0.0124x^{2.2249}$$

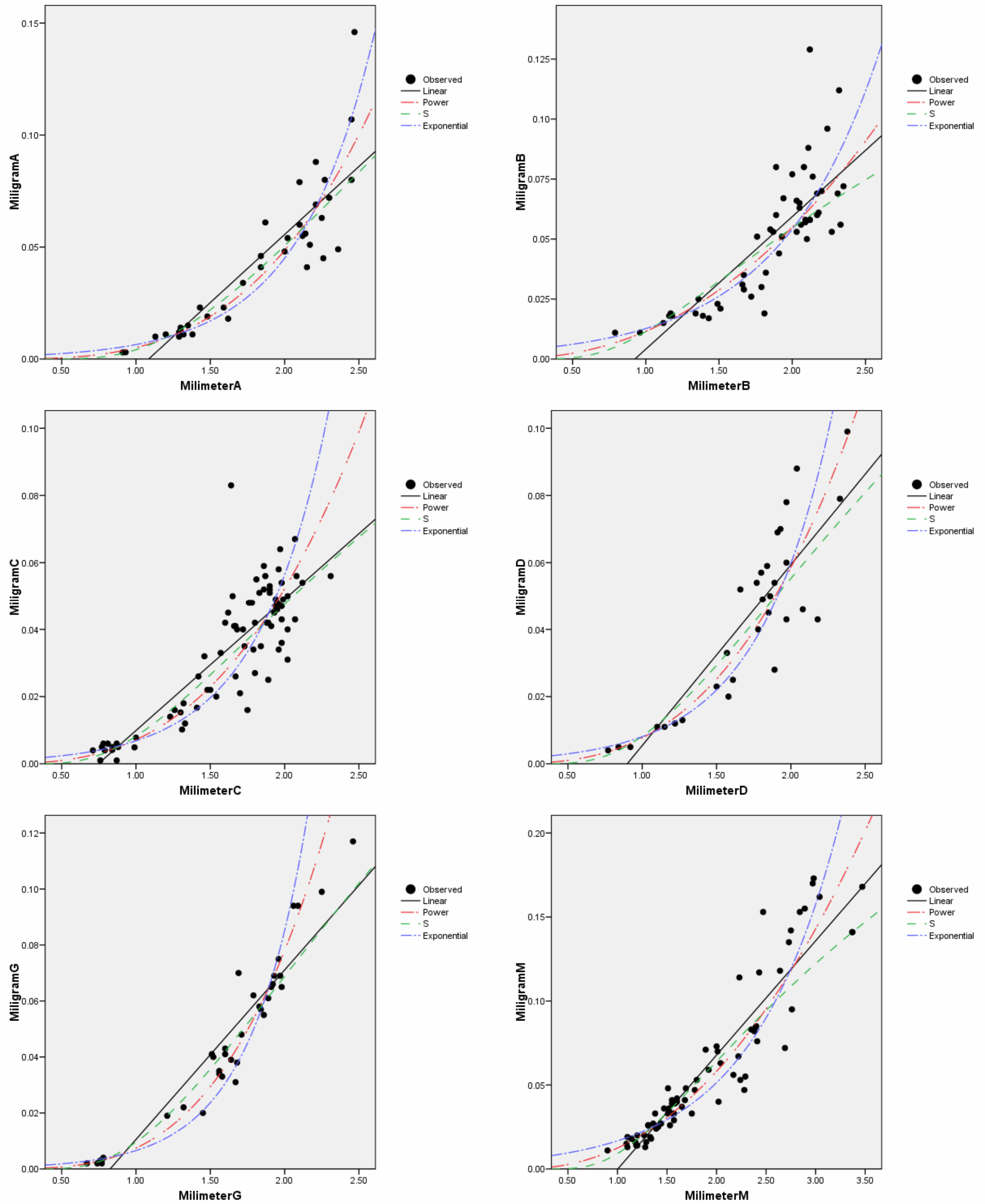


Fig. 20 Selected best-fit equations for length-weight correlations for the different populations, Power (red) equation had the highest R square values and was subsequently used.

Chemostat set-up

Changes in the nutrient concentrations were observed, but none that exceeded the assumed optimal range 2-6 mg C/L (Dag O. Hessen, pers. com.) (Fig. 21 and 22). The cuvette connected to the outlet water was found to accumulate algae over time. As the fourth flask (due to contamination) was disconnected water flow was reduced, and observed algae accumulation increased. This was countered by washing the cuvette every 3rd-4th day with lukewarm sink-water and rinsed with distilled water.

The dried micro-fibre filters with algae were run in the element analyser (Flash EA 1112 series, Thermo Finnigan, Milan, Italy) and as a direct measurement it could be assumed that this analysis of carbon content is the most accurate (Fig. 21). Mean C:N ratio was also calculated from the element analyser, found to be 6.79 (±0.88 SE). Absorbance values (OD) were obtained using a spectrophotometer, and converted to mg C/ L using equation by Thomas Correll Jensen (*unpublished*). Values were applied as a check versus Lux values for continuous monitoring during the experiment length. The Lux values were used as a proxy to monitor differences in carbon concentration, but the high variability caused by accumulation of algae forbade any useful application of the data (also note that the laptop computer connected to the Lux-meter crashed at several occasions due to immense amounts of data, leaving gaps in the data set). Lux values were converted to cell numbers using an equation developed by Marcin Wojewodzic (*unpublished*), and from cell numbers to mg C/L using Per Færøvig's equation (based on 10 µL P COMBO, *unpublished*). Cell number counts using flowcytometry (and Per Færøvig's equation) were also done *a priori* to the experiment duration, and added as an additional measurement (see Fig. 22 for comparison of the results from all the different analyses).

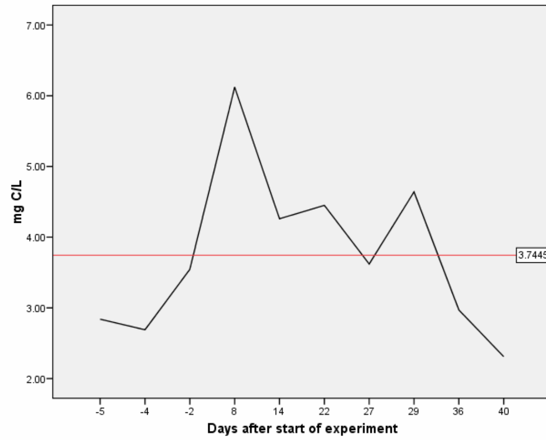


Fig. 21 Measurements of algal density as mgC/L using element analysis for the majority of the experiment duration (ended at day 53) (red line indicate mean value for the duration of the experiment).

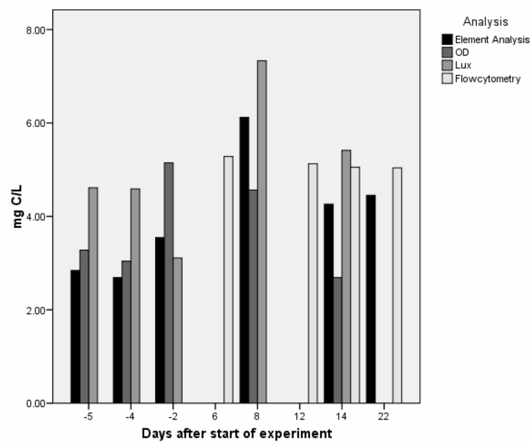


Fig. 22 Comparisons of the different approaches to carbon measurement; OD and Lux measurements were done continuously, while element analysis and flowcytometry were done *a priori* (Note that represented values only account for a part of the experiment lapse, Lux measurements were excluded as the cuvette accumulated algae and proved to be less accurate, while OD measurements was done irregularly to check that the values were stabile, but not all were recorded).

Life History Experiment

Qualitative descriptions

Population A and G were found to host epizooic ciliates (*Vorticella* sp.). This epibiont covered the carapaces of the *Daphnia*, and were most abundant on old individuals with less frequent moulting. Strands of algae were also observed on some old, large individuals, especially for *D. magna*, how these influenced growth and survival can only be speculated.

Release of neonates co-occurred with the moults, readily developed juveniles were observed several hours before a moult in the carapace of the mother. It was observed that the eggs often were not fully developed when released; these were not included in the observations and composed for the majority post-matural moults (>50 % of the moults). Subsamples of these "aborted" eggs were examined for more than 2 weeks, but none hatched into viable neonates. Resting eggs was only released by population M (*D. magna*), these were also excluded from the study as they do not include in the immediate fitness.

Fitness

The fitness parameter r, intrinsic growth rate, is found using the Euler-Lotka equation (Stearns, 1992). It is calculated for each breeding individual as it is not applicable to the population as a whole:

$$1 = \sum_{x=\alpha}^{x=\omega} e^{-rx} l_x m_x$$

α is the age of first reproduction (successful hatch) and ω is the age at last reproduction (which in our case usually coincided with death (some old individuals were found to die after successful hatchings) or the termination of the experiment), l_x is defined as the survival rate to age x, m_x is defined as the birth rate at age x. Positive r values indicate population growth, whereas negative r values show a population failing to reproduce and hence trailing for extinction. Population growth rate calculated for the whole population

produced negative *r* values for population B, C and M, whereas A and G had positive values (Fig. 23) (note: population D didn't produce any offspring hence no *r* value could be estimated).

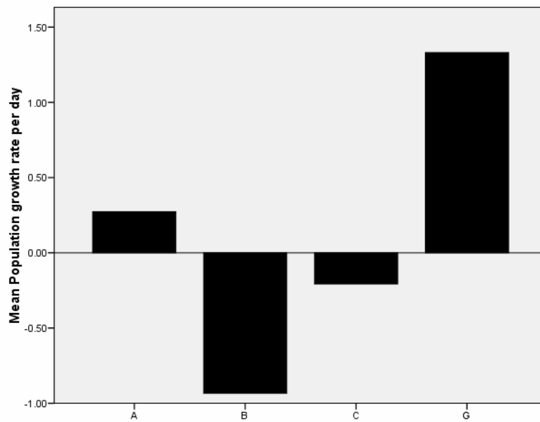


Fig. 23 Population growth rate *r*, for 5 populations, calculated from all individuals.

Fertility

Fertility, as opposed to fecundity measures the actual number of offspring, whereas fecundity measures the potential number of offspring (for *Daphnia* eggs in brooding chamber).

Many of the individuals never produced any live offspring (only undeveloped eggs or resting eggs). These individuals were not included in the average lifetime fertility estimate, as numbers of released undeveloped eggs were hard to quantify due to their small size and noise in beaker (empty moults, detritus and algae). Lifetime fertility or the number of offspring per individual (of reproducing females) during its lifecycle was calculated for the different populations (Fig. 24). The populations were statistically different (no violation of homogeneity of variables once log transformed (P=0.573, Levene Stat.=38.025, d.f.1=3, d.f.2=25), ANOVA one-way, P=0.014, F=4.31, d.f.=3, Table 10), only population G and C was found to be statistically different (Tukey HSD, P=0.016, Table 10). Population A and B included many females with no reproductive output, and when log transformed these zero values are removed, artificially enhancing their fertility.

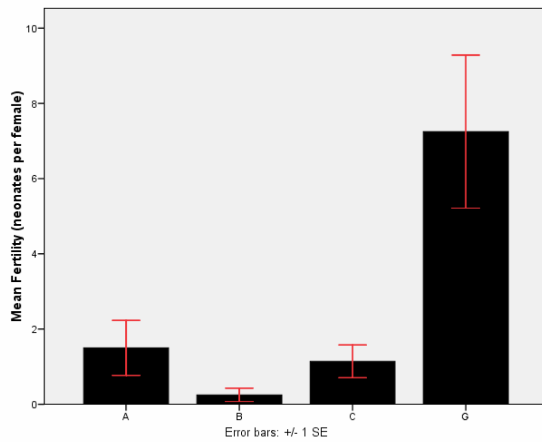


Fig. 24 Mean fertility for the different populations.

Generation time

Generation time was measured as average age of the mothers of neonates in the population, following equation in Stearns (1992):

$$T = \left(\sum_{x=\alpha}^{x=\omega} x l_x m_x \right) R_0^{-1}$$

R_0^{-1} is the net reproductive rate, l_x is defined as the survival rate to age x, m_x is defined as the birth rate at age x. All *D. pulex* populations (including the temperate) were found to have similar generation time (24-26 days, Fig. 25).

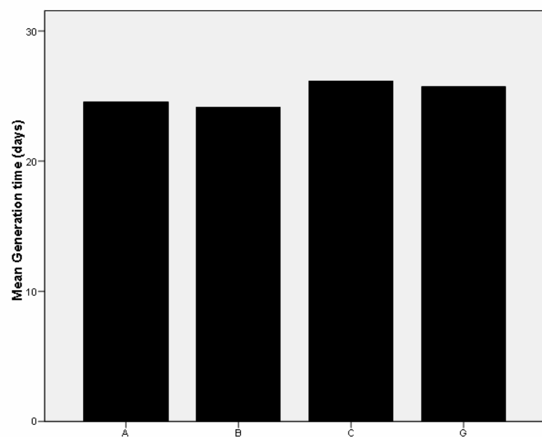


Fig. 25 Mean generation time (T) for the different populations.

Maturation data

Age at maturity, measured as the first day with visible eggs in the brood chamber, is like generation time (T) descriptive of the duration of the life cycle. Patterns observed between the different populations are also much like what observed for T. The 6 populations were significantly different (no violation to normality or homogeneity (P=0.152, Levene stat.=1.749, d.f.1=4, d.f.2=57), ANOVA one-way, P=0.004, F=4.242, d.f.=4, Fig. 26 left, Table 11), population G was statistically different from B and C (ANOVA one-way Tukey HSD, P=0.009, Table 11), while the remaining populations showed no difference (ANOVA one-way Tukey HSD, P>0.05, Table 11). Population D, which only produced undeveloped eggs, was included since the measurement only deals with maturity (and not fertility). By plotting the maturation time in a survival plot, there is also a statistical difference between the populations (Kaplan-Meier, Log Rank (Mantel-Cox) P=0.001, χ^2=18.029, d.f.=4, Fig. 26 right, Table 12).

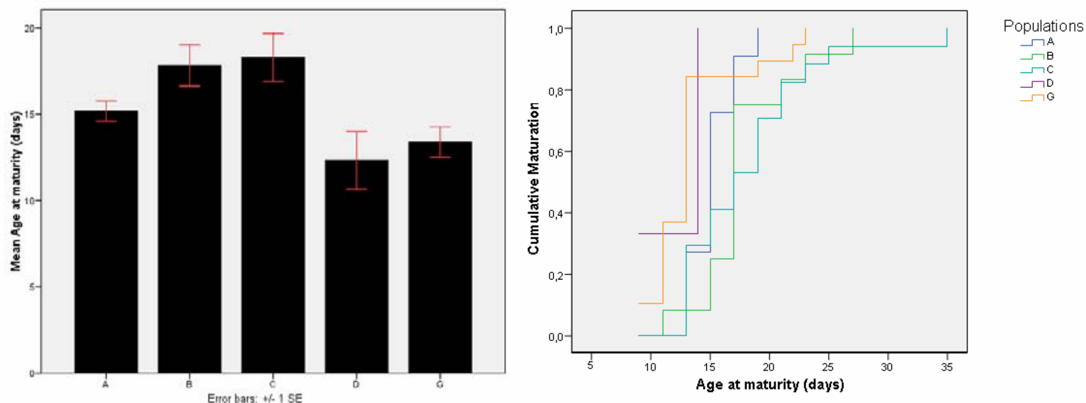


Fig. 26 Mean age (days) at maturity for the different populations, displayed in a bar chart (left) and a survival plot (right).

Size or biomass (recalculated from millimetres using equations in **Length-weight relationship**) at maturity measured as the size the first day eggs were observed in the brood chamber, is also an indication of the duration of a generation. It also shows the allocation of nutrient to growth/reproduction. All populations were statistically different (violation of homogeneity (P<0.001), thus log scaled size (µg) at maturity, no violation (P=0.928, Levene stat.=0.216, d.f.1=4, d.f.2=57), ANOVA one-way, P<0.001 F=32.770, d.f.=4, Fig. 27 left, Table 13). Population G was statistically higher than all other

populations (ANOVA one-way Tukey HSD, P<0.05, Table 13). No statistical difference was recorded between population A and B (ANOVA one-way Tukey HSD, P=1.00, Table 13), and C and D (ANOVA one-way Tukey HSD, P=0.37, Table 13). Population D, which only produced undeveloped eggs, could also be in this measurement as it only deals with maturity (and not fertility). Size at maturity and days of survival was compared to see if there was any positive fitness effect for larger size at maturation, however correlation not statistical significant (Pearson correlation=0.208, P=0.105, N=62, Table 14).

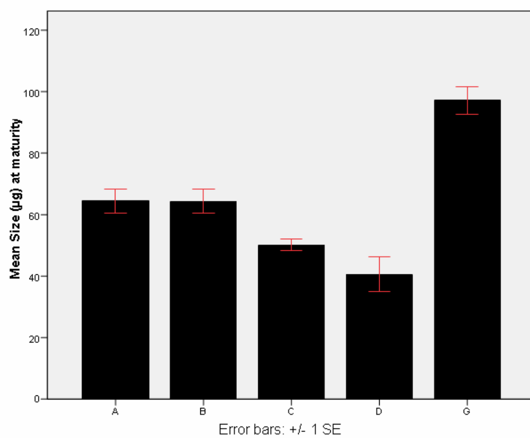


Fig. 27 Mean size (µg) at maturity for the different populations.

Prematural growth

Cladocerans invested most of their energy to the eggs once reached maturity (Perrin, 1989). Hence length measurements subsided once reached maturity. Growth was calculated from measured millimetres to dry weight (biomass) using equations from length-weight relationship analysis (see **Length-weight calibration**). The biomass proved to produce more marked differences and a better estimation of growth (as millimetres only cover the 2-dimentional growth) (Fig. 28). Statistical difference was found between the pre-matural growth rate (µg) for the different populations (Fig. 29, left) (violation of homogeneity of variances for normal data (P<0.05) and log scaled (P<0.05), thus the non-parametric Kruskal-Wallis test was used; P=0.019, χ^2=11.800, d.f.=4, Table 15). Difference between growth rate (µg) in population B and C, B and D,

and in population C and G was found to be statistically different (Mann-Whitney test, P<0.05, Table 16).

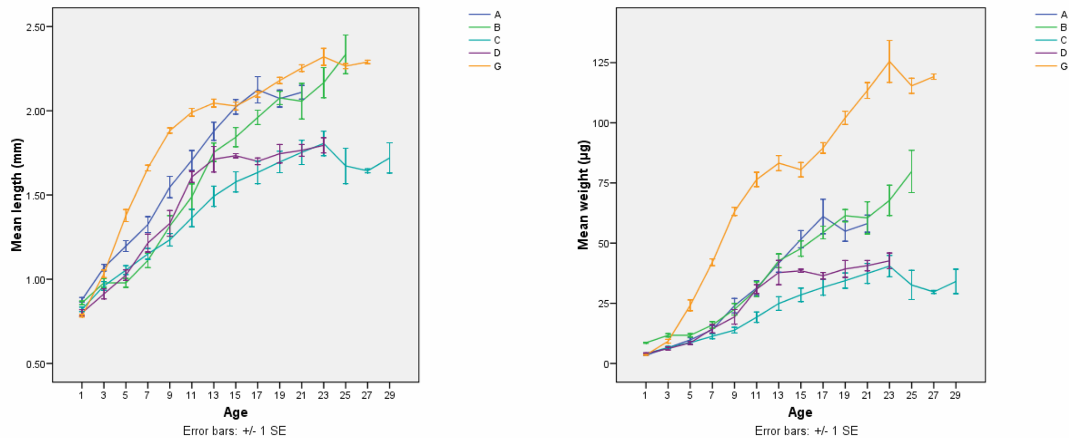


Fig. 28 Mean pre-matural length (milimetres) (left) and weight (µg) (right) for the different populations during the course of the experiment.

Since prematural growth rate had such high variance, especially during late development, the growth rate for the first 10 days was examined (Fig. 29, right). A statistical difference between the populations was found (no violation of normality or homogeneity (P=0.111, Levene stat.=4.764, d.f.1=4, d.f.2=15), ANOVA one-way, P=0.001, F=7.848, d.f.=4, Table 17). Population G was found to be statistically different from all other populations (ANOVA one-way Tukey HSD, P<0.05, Table 17). Difference between the other populations was not statistically significant. Average growth the first 10 days of development was plotted, additionally growth was log transformed to further examine linearity in growth rate (Fig. 30). Population A, B, C and D seem to have linear growth the first 10 days of development, while growth rate for population G seem to be weakly exponential.

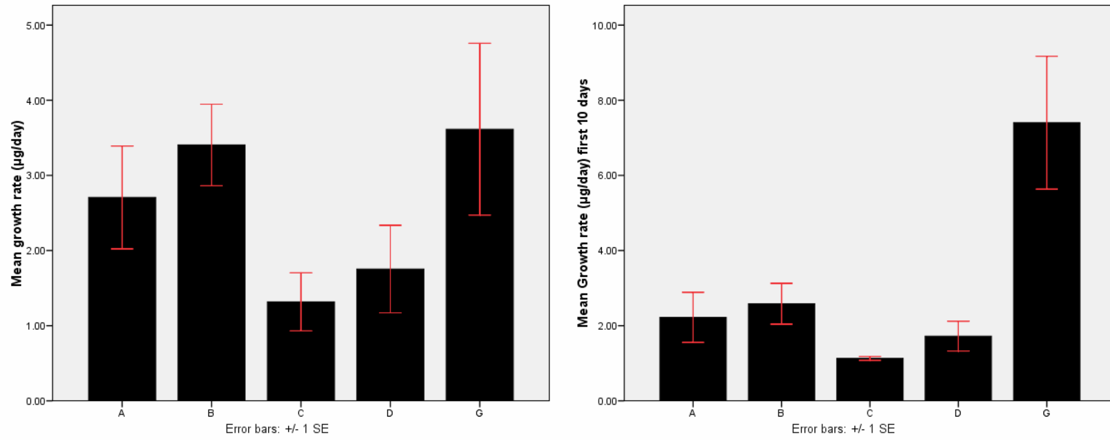


Fig. 29 Mean total prematural growth rate (µg/day) for the different populations (left), growth rate (µg/day) for the first 10 days of development for the different populations (right).

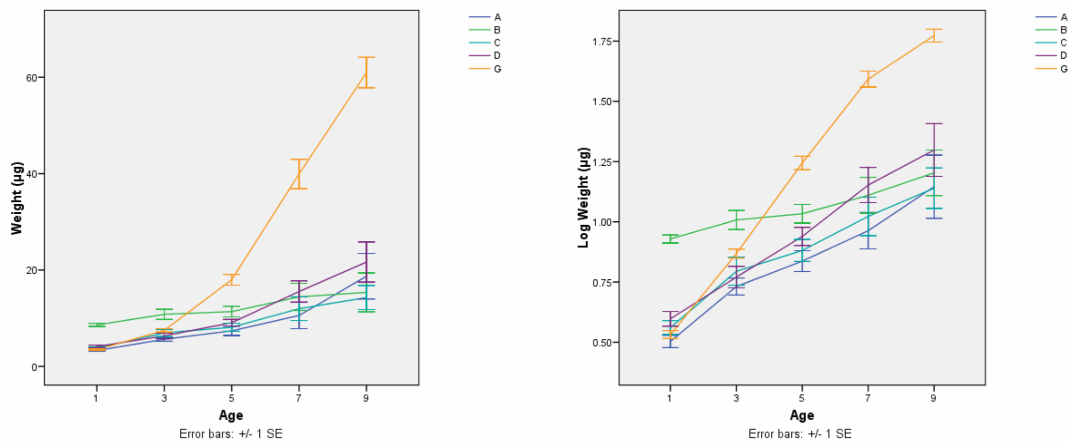


Fig. 30 Mean pre-matural dry weight (µg) for the different populations during the first 10 days of development and inverse log transformed weight (µg) for the first 10 days of development.

Standardized growth rate, termed specific growth rate, µ, was calculated using below (from Kyle et al., 2006).

$$\mu = \ln(m_e / m_i) / t$$

Where m_e is dry weight at the end of the growth period, m_i is initial dry weight and t is the time interval. Statistical difference between the populations was established (violation of homogeneity of variance (P=0.008, Levene stat.=5.228, d.f.1=4, d.f.2=15), no violation (P=0.385, Levene stat.=1.117, d.f.1=4, d.f.2=15) for log transformed specific growth rate, ANOVA one-way, P=0.006, F=5.591, d.f.=4, Fig. 31, Table 18), only

statistical difference was found between population G and B, and C (Tukey HSD, $P<0.05$, Table 18).

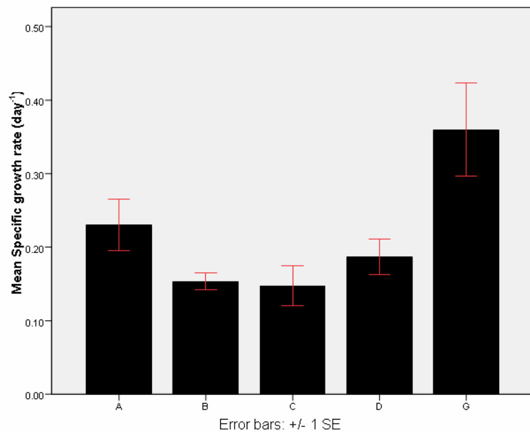


Fig. 31 Mean specific growth rate (μ) for the different populations for the first 10 days of development.

Survival

Days of survival for the whole populations was used as a proxy for mortality (Fig.32). Populations were found to be statistically different (Violation of homogeneity of variance ($P<0.001$) for normal and log transformed data, thus used non-parametric Kruskal-Wallis test, $P<0.001$, $\chi^2=34.421$, d.f.=4, Fig. 32 left, Table 19). Days of survival was statistical different between all populations except between A and B, B and D, and C and G (Mann-Whitney test, $P>0.05$, Table 20). A survival plot was also prepared showing statistical difference between the populations (Kaplan-Meier, Log Rank (Mantel-Cox) $P<0.001$, $\chi^2=36.117$, d.f.=4, Fig. 32 right, Table 21). Days of survival and days at maturity was found to be statistically correlated (Pearson correlation=0.497, $P<0.001$, N=52, Fig. 33, Table 22).

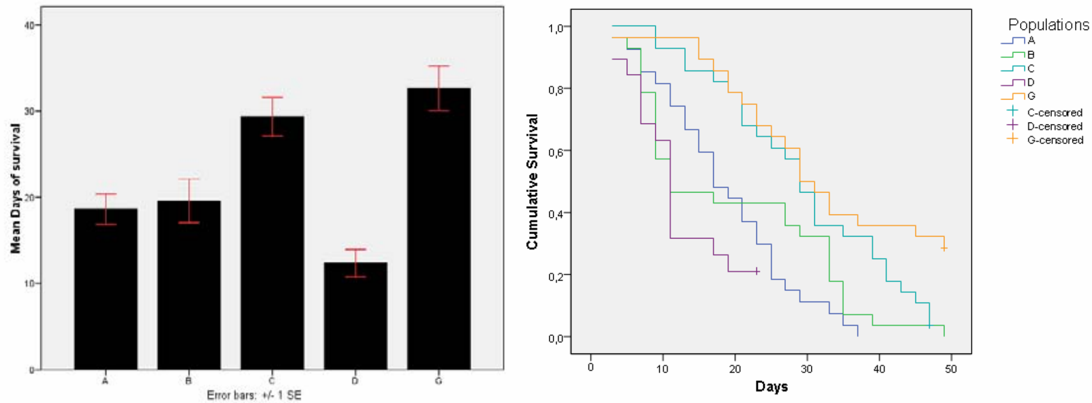


Fig. 32 Mean days of survival for individuals of the various populations, bar chart (left) and survival plot (right).

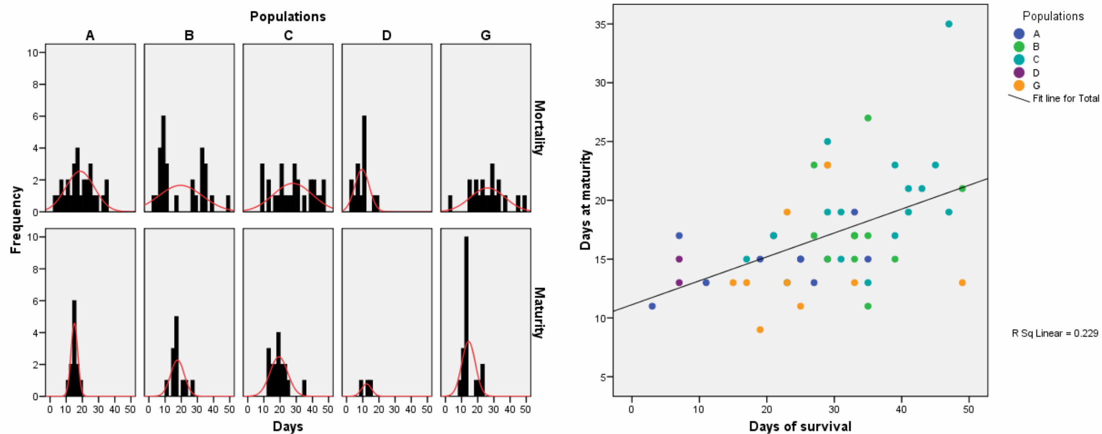


Fig. 33 Comparison of total mortality events (deaths) and maturity (day of first egg-production), histograms with added normal distribution (left) and scatter-plot (right).

Principal Components Analysis

The PCA analysis for this study using variables from the life history experiment is depicted in Fig. 34, the biplot shows the correspondence of the actual variables with the PCA components 1 and 2, which is further displayed in Fig. 3. Note the clustering of the assumed polyploid populations and the wide distribution of the assumed diploid population (G) (Fig. 34). Eigenvalues and percent of total variance for the different components is listed in Table 1 and depicted in Fig. 2.

Note from the biplot (in Fig. 34) that size at maturity (µg) corresponds with the principle component 1 (which account for ~75% of the variance, Table 2), also called the “loading” of the variables for the component (Fig. 36 left). Total fertility and survival

correspond (Fig. 36 right) with the principle component 2 (which account for ~17% of the variance, Table 2). Hence these three variables are the major cause of the clustering observed in Fig. 34, while the remaining life history traits to a lesser degree influenced the distribution/clustering for the first two components (Fig. 36).

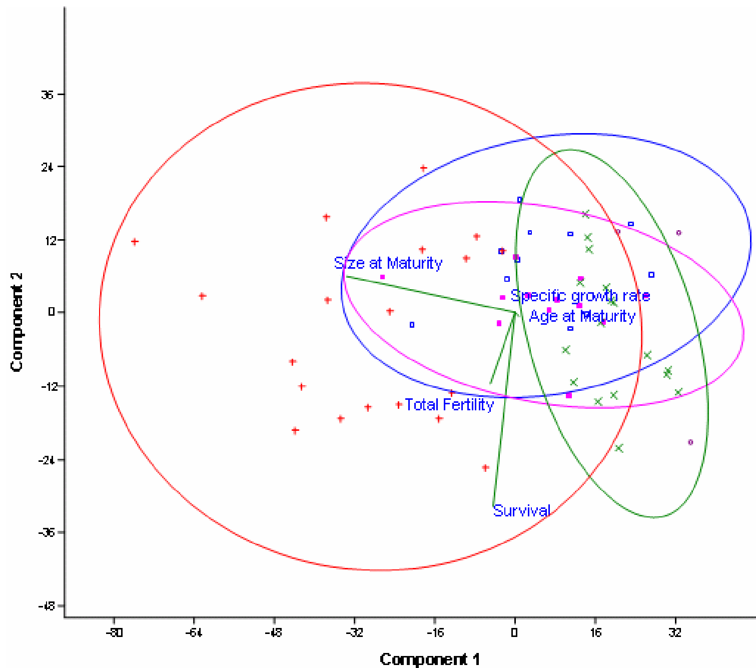


Fig. 34 Principal Components Analysis with variance-covariance matrix and drawn 95% ellipses of confidence (blue=A, pink=B, green=C, purple=D (only 3 points, 95% ellipses not available) and red=G), biplot showing the variables in relation to the principle components axis (Note that size at maturity corresponds well with component 1, while total fertility and survival correspond well with component 2 (Eigenvalues and percent of variance in Table 1) (included variables: total fertility, age and size at maturity, specific growth rate and survival).

Table 2 Principal components analysis for variance-covariance matrix showing the principle components and their respective Eigenvalues and percent of variance covered (PC=Principle component).

PC	Eigenvalue	% of variance
1	606.968	75.309
2	135.029	16.754
3	46.0515	5.7138
4	17.9192	2.2233
5	0.00282089	0.00035

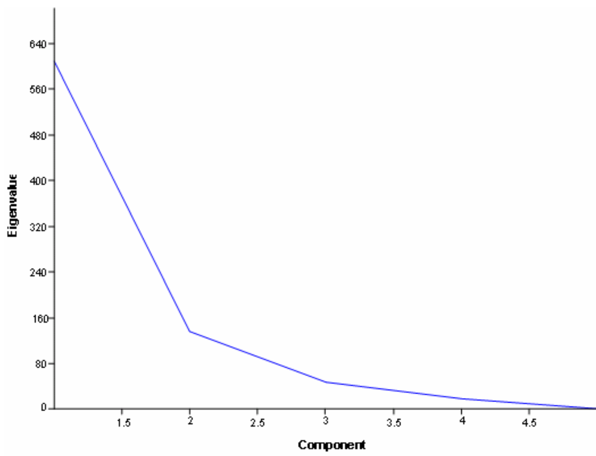


Fig. 35 PCA "scree plot", showing the Eigenvalues for the different components.

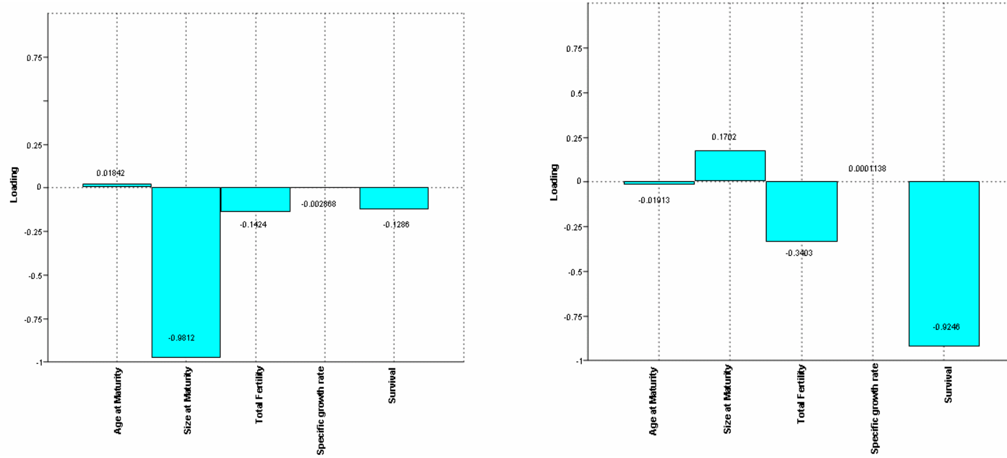


Fig. 36 PCA loadings for component 1 (left) & 2 (right).

2.4. Genetic Analyses

Microsatellite analysis

The two ways of scoring alleles produced different results, as the "non-restrictive" is less conservative than the "restrictive", positively identifying more alleles in the prior than in the latter (Table 3). Two primers (Dp523 & Dp524) were found to be below detection limit for all populations and samples, and no primer gave any response for *D. magna* (M). A total of 29 alleles were found for the 6 loci (Table 4), some alleles were common in all populations, other confined to only one.

Population A, B and D were found to have more than 2 average number of alleles per locus for both the "non-restrictive" and the "restrictive" method (Table 3 & 24), suggesting a strong indication of polyploidy. Population C had an average above 2 alleles per locus using the "non-restrictive" method. However using the "restrictive" method the average number of alleles per locus was found to be 2 (many polymorphic homozygotes were observed for this population). For all individuals from each population there was no difference in number of alleles per locus, neither was there any difference in number of alleles per locus in the age groups (but not all loci produced above limit values for both juveniles and adults, Table 24). Two primers (Dp523 & Dp524, Table 4) produced no signal for any samples (both reported successfully in Colbourne *et al.* (2004), and Dp523 in Markova *et al.* (2007)), and no signal was observed in any *D. magna* samples (opposed to Colbourne *et al.*, 2004).

The primer Dp514 has been noted (Colbourne *et al.*, 2004) to identify a trinucleotide repeat (Table 2). However in this study it was found to produce alleles with 2 bp differences (Table 4). Different artefacts were observed, such as the "+A effect" (caused by the addition of an extra adenine during PCR by the Taq enzyme, adding a +1 bp peak to the right of the true allele peak, Fig. 38) and "stutter-bands" (small peaks -2, -4 and -6 bp from the true peak, caused by slippage during PCR, Fig. 37). Various interpretations of results described in Fig. 37 – 42.

Table 3 Average number of alleles per locus for the different populations (Note: differences within adults and juveniles are due to different number of successful primers, no age specific differences observed), Blue numbers indicate possible polyploidy, green diploidy (see text).

Population	Average Allele # (non-restrictive)	Average Allele # (restrictive)
A Adult	3	2.5
A Juvenile	3	2.5
B Adult	3.167	2.333
B Juvenile	3.25	2.25
C Adult	2.4	2
C Juvenile	2.667	2
D Adult	2.833	2.167
D Juvenile	3	2.25
G Adult	2	1.5
G Juvenile	2	1.4

Table 4 Alleles (identified by different bp lengths) for the 5 working primers (this is the same as the restrictive scoring), note that Dp514 and Dp514alt are supposed to have a trinucleotide repeat motif, but Dp514 acts as a dinucleotide repeat and Dp514alt are also somewhat irregular.

Genotype	Dp512					Dp513				Dp514			
Population	130	134	136	140	142	104	111	113	115	94	96	98	100
A	x	x				x		x			x	x	
B	x		x	x				x		x	x	x	
C	x			x				x	x	x			x
D	x		x	x				x		x	x		
G					x		x	x			x		

Genotype	Dp514alt					Dp522					Dp525					
Population	106	122	130	136	143	120	122	124	126	128	114	117	120	123	125	128
A		x	x			x	x	x	x		x			x	x	
B		x		x				x	x			x	x			
C				x	x		x	x				x	x			
D		x		x				x	x			x	x			
G	x									x		x				x

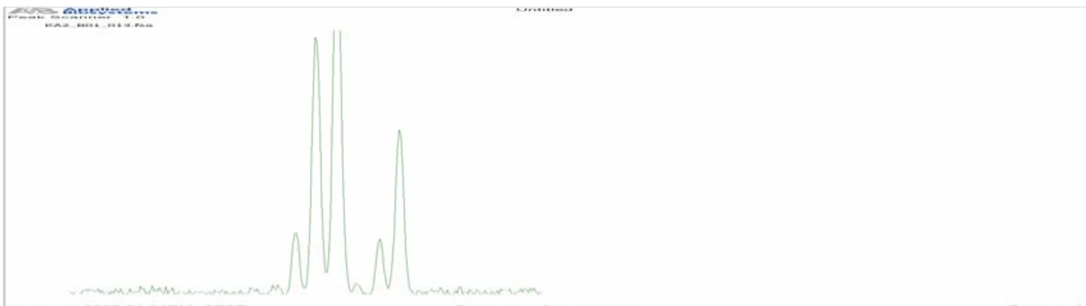


Fig. 37 Primer Dp512 (a dinucleotide repeat) for population A adult, scored as 2 alleles (first peaks in left cluster identified as "stutters", last peak in first cluster could be the "+A effect").

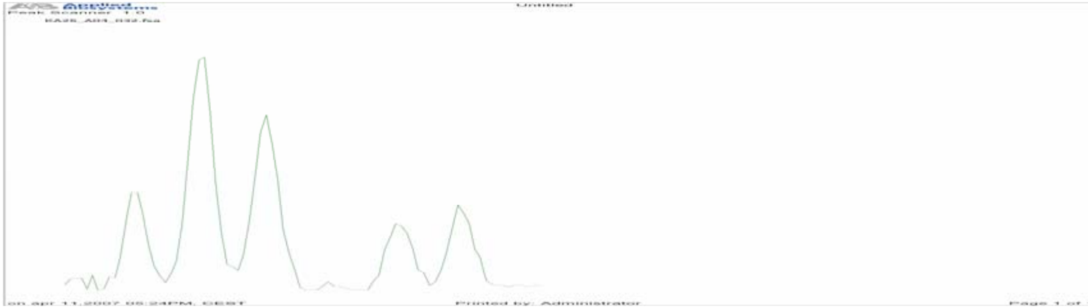


Fig. 38 Same as Fig. 1, Dp512 (a dinucleotide repeat), but for population A juvenile, scored as 2 alleles (middle peak in left cluster real, -2 bp "stutter" to the left, "+a effect" to the right).

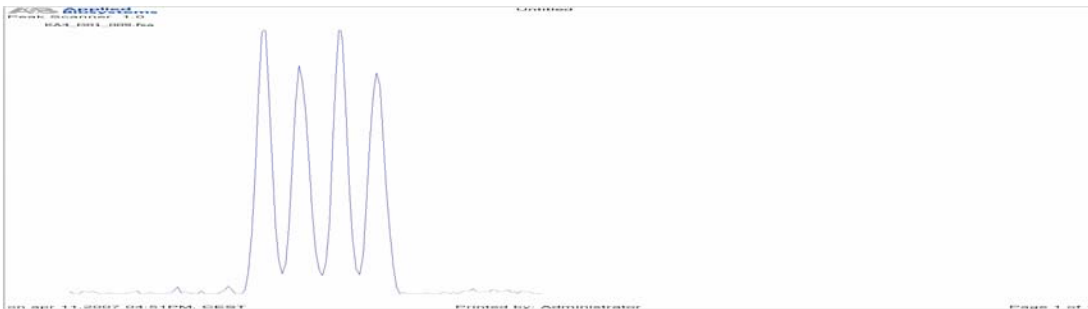


Fig. 39 Primer Dp514 (a trinucleotide repeat) for population A adult, peaks are 1 bp apart, scored as 2 alleles.

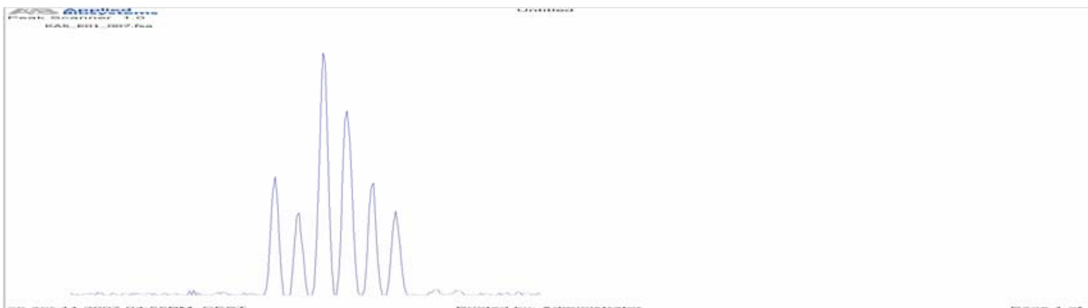


Fig. 40 Primer Dp514 (a trinucleotide repeat) for population B adult, peaks are 1 bp apart, scored as 2 alleles.

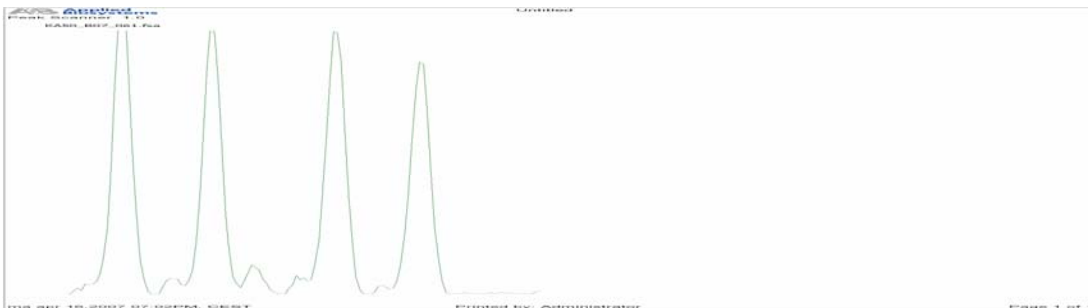


Fig. 41 Primer Dp522 (a dinucleotide repeat) population A adult, all peaks 2 bp apart, scored as 4 alleles.

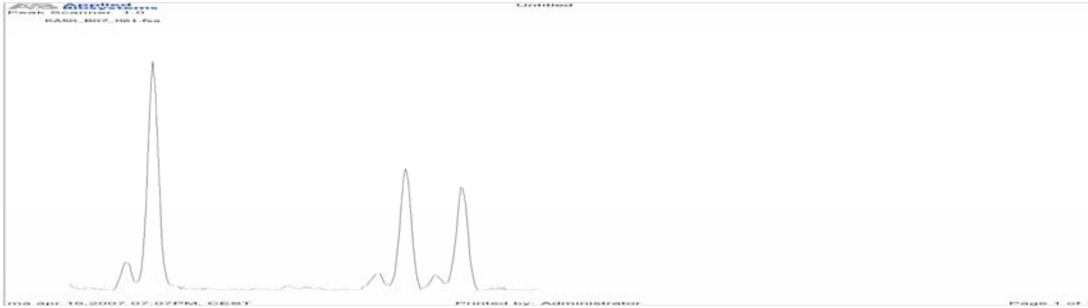


Fig. 42 Primer Dp525 (a trinucleotide repeat) population A adult, first peak 114 bp, second 123 bp, third 125 bp, scored as 3 alleles Can also be scored as 114/114 homozygote (because of the height of the peak) and 123/125 heterozygote.

Species identification by mtDNA sequencing

In addition to sequences from the populations in the study, sequence data used in Colbourne *et al.* (1998) were also obtained. A full phylogenetic tree of the *Daphnia pulex* complex could be made. A simple neighbour-joining tree with bootstrap replications (Fig. 43) was calculated by Anders Hobæk (NIVA), identifying the populations as Polar *D. pulicaria* (population B and D), Eastern Nearctic *D. pulicaria* (population C), *D. tenebrosa* (population A) and European *D. pulex* (population G).

The phylogeny obtained does not differ from recent analyses of the *D. pulex* species complex (Markova *et al.*, 2007). The *D. pulicaria* subgenera form a monophyletic group, like what has been reported elsewhere (Colbourne *et al.*, 1998, Markova *et al.*, 2007), European *D. pulicaria* group with *D. tenebrosa*, Polar *D. pulicaria*, Western *D. pulicaria*, Eastern *D. pulicaria* group with *D. middendorffiana* and Pan-arctic *D. pulex*.

Note that the Eastern *D. pulicaria* clones (two clones found, with a 2 bp difference) form a distinct branch within the Eastern *D. pulicaria* clade, different from clones from other Arctic locations. The groupings from the phylogeny were used in the remaining of the study.

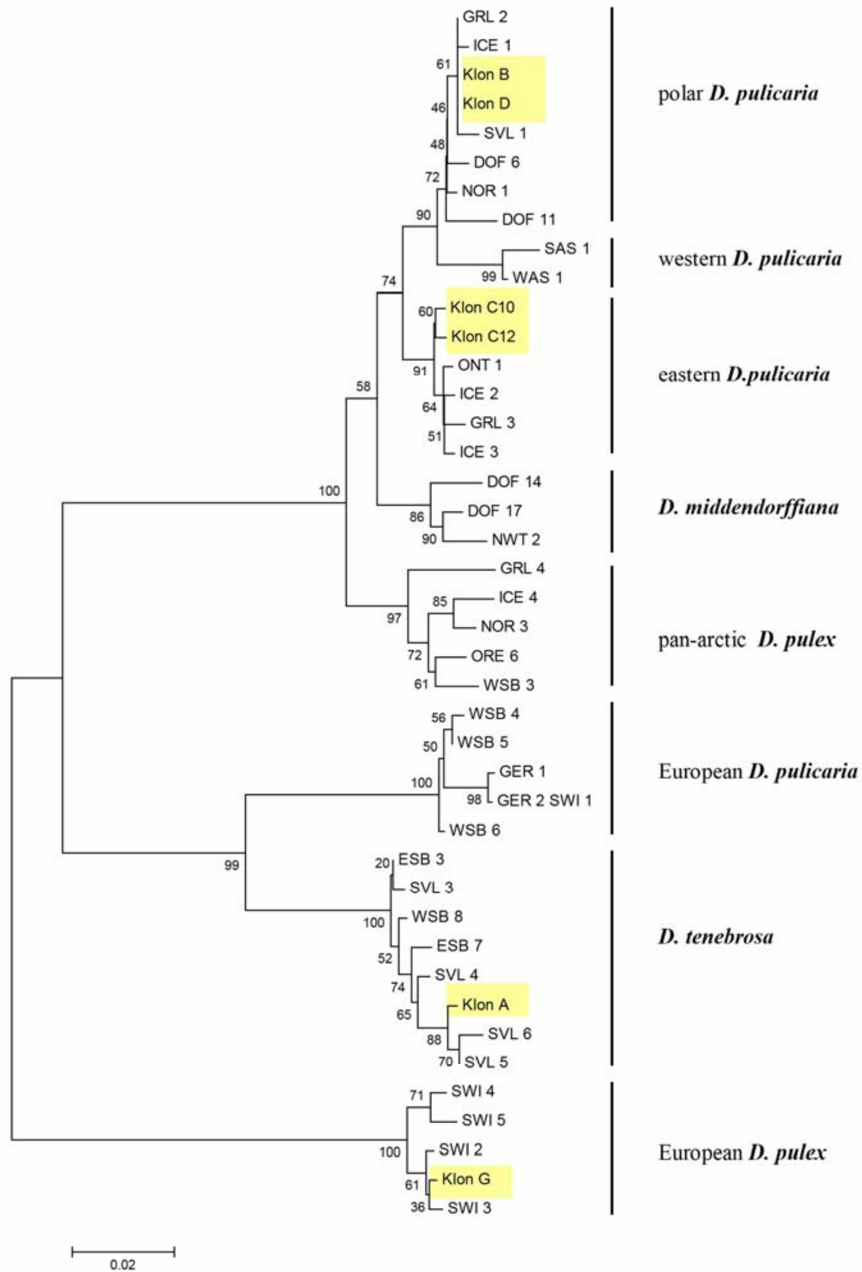


Fig. 43 Neighbour-joining tree with bootstrap replications, clones/populations in this study marked with yellow (sequencing by Morten Skage, UiB), clades and additional sequences taken from Colbourne *et al.* (1998) compiled and run by Anders Hobæk (at NIVA) (Legend in Table 25).

Quantification of total DNA, RNA and protein

Both commercial standards and purified from *D. pulex* were plotted. The RNA standard used was from purified *D. pulex*, while DNA standard used was from commercial standard (Fig. 44).

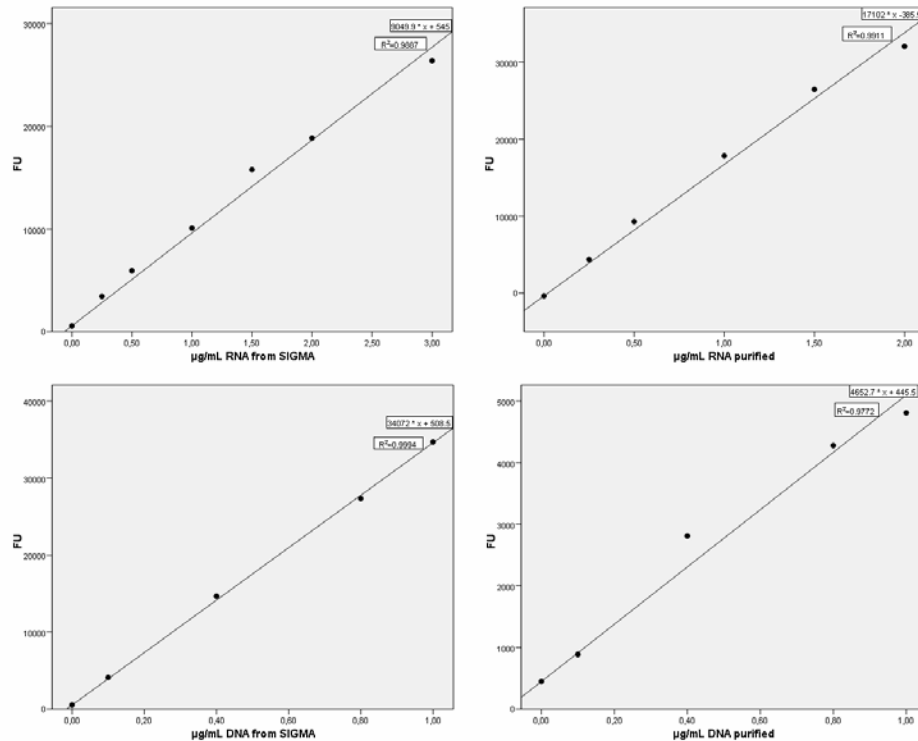


Fig. 44 Standard curves for RNA and DNA using commercial standard from <u>SIGMA</u> and <u>purified</u> from *D. pulex* (using method described above) (linear equation and R square values in boxes).

A higher background noise was generally observed in samples treated with DNase, as chemicals, such as magnesium and calcium, produce errors in the microplate reader. The total signal from the microplate reader minus background noise (assessed using RNase and DNase and both) indicate the total amount of nucleic acids found in the individual. It was found to be higher for juveniles then adults (Fig. 45). Statistical differences were found between all populations for total signal (pooled age classes) (violation homogeneity of variances ($P<0.05$ for both normal and log transformed) of ANOVA, non-parametric Kruskal-Wallis test, $P<0.001$, $\chi^2=20.605$, d.f.=4, Table 26). All populations were statistically different, expect between population C and D, and G, and between population D and G (Mann-Whitney test, $P>0.05$, Table 27).

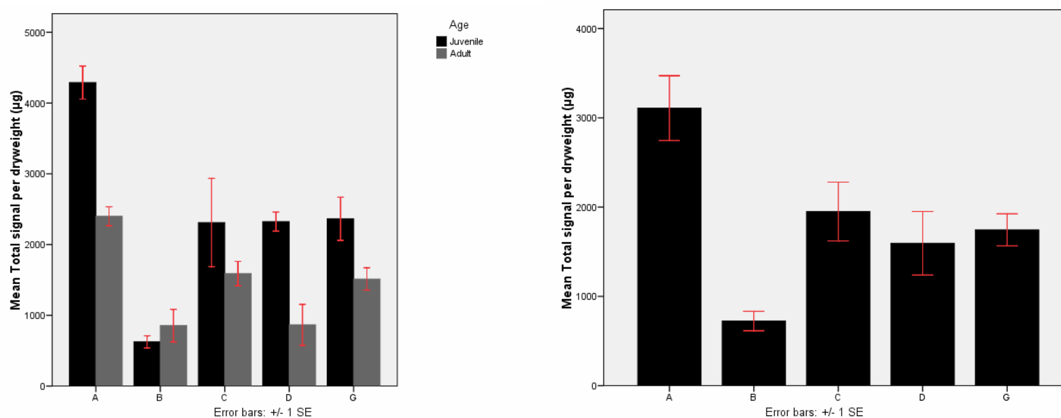


Fig. 45 Mean total signal (showing the relative amount of nucleic acids) for the different populations, split into two different age classes (Juvenile <24 hours, Adult >7 days) (left) and pooled age classes (right).

The amount of RNA, DNA and protein per dry weight (µg) was calculated and differences between populations examined. RNA per dry weight (µg) was found to be statistical different for the populations (violation of homogeneity of variances for normal and log transformed data ($P<0.05$), non-parametric Kruskal-Wallis test, $P<0.001$, $\chi^2=20.605$, d.f.=4, Fig. 46, Table 28), statistical difference was found between all populations, except between population D and B, C and G, and between C and G (Mann-Whitney test, $P>0.05$, Table 29). DNA per dry weight (µg) was also found to be statistical different for the populations (no violation of normality or homogeneity of variance ($P=0.10$, Levene stat.=2.107, d.f.1=4, d.f.2=36), ANOVA one-way, $P<0.001$, $F=10.485$, d.f.=4, Fig. 46, Table 30), population A statistically different from all but population C (ANOVA one-way Tukey HSD, $P=0.08$, Table 30), population C only statistically different from population B (ANOVA one-way Tukey HSD, $P=0.016$, Table 30). Protein per dry weight (µg) was not found to be statistical different for the populations (no violation of normality or homogeneity of variance ($P=0.593$, Levene stat.=0.704, d.f.1=4, d.f.2=43), ANOVA one-way, $P=0.471$, $F=0.902$, d.f.=4, Fig. 46, Table 31).

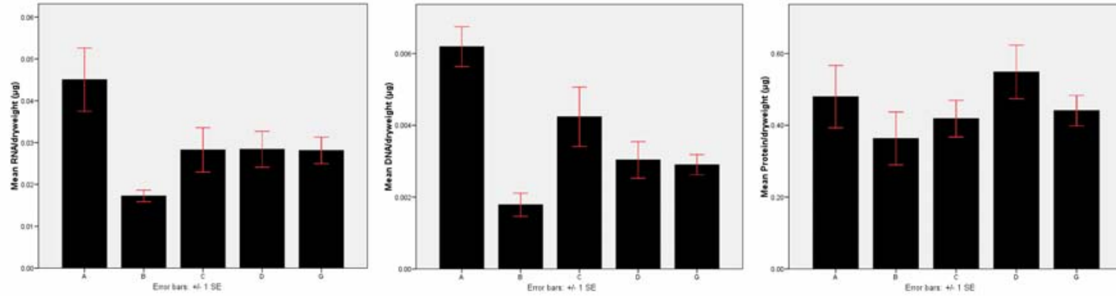


Fig. 46 Mean RNA (left), DNA (middle) and protein (right) per dry weight (µg) for the different populations.

Quantity of the different products was compared and examined for differences between the populations. No statistical difference was found for RNA per DNA between the populations (no violation of normality or homogeneity of variance (P=0.307, Levene stat.=1.252, d.f.1=4, d.f.2=35), ANOVA one-way, P=0.263, F=1.374, d.f.=4, Fig. 47, Table 32). The amount of RNA per protein was found to be statistically different between the populations (no violation of normality or homogeneity of variance (P=0.305, Levene stat.=1.259, d.f.1=4, d.f.2=34), ANOVA one-way, P=0.010, F=3.891, d.f.=4, Fig. 47, Table 33). Population A was found to be statistically different from population D and G (ANOVA one-way Tukey HSD, P<0.05, Table 33). Statistical differences was found for DNA per protein (violation of homogeneity for normal and log transformed data (P<0.05), non-parametric Kruskal-Wallis, P=0.008, χ^2=13.850, d.f.=4, Fig. 47, Table 34), population A was found to be statistical different from population B, D and G (Mann-Whitney test, P<0.05, Table 35).

RNA per DNA was found to be statistically correlated (Pearson Correlation=0.635, P<0.001, N=39, Fig. 47, Table 36), similar was RNA per protein (Pearson Correlation=0.599, P<0.001, N=39, Fig. 47, Table 37).

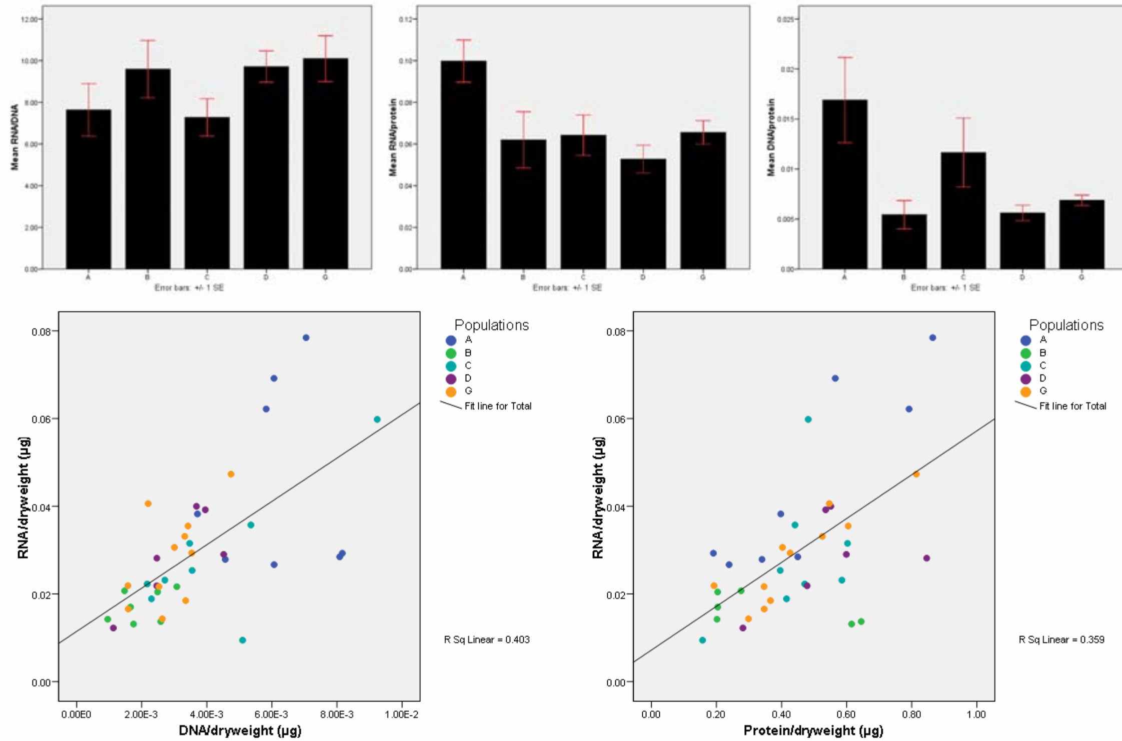


Fig. 47 Comparisons of relations between the different products; Mean RNA/DNA (upper left), RNA/protein (upper middle) and DNA/protein (upper right). Correlations between DNA per dry weight (µg) and RNA per dry weight (µg) (lower left), and protein per dry weight and RNA per dry weight (lower right).

For the comparisons of nucleic acids versus the growth rate, only the specific growth rate (SGR or µ) was used (see **Life history experiment**). RNA per dry weight (µg) and specific growth rate (µ) was not found to be statistically correlated (Pearson Correlation=0.513, P=0.129, N=10, Fig. 48, Table 38), when controlled for assumed ploidy level statistical correlation was found (Pearson Correlation=0.917, P=0.001, d.f.=7, Fig. 48, Table 39).

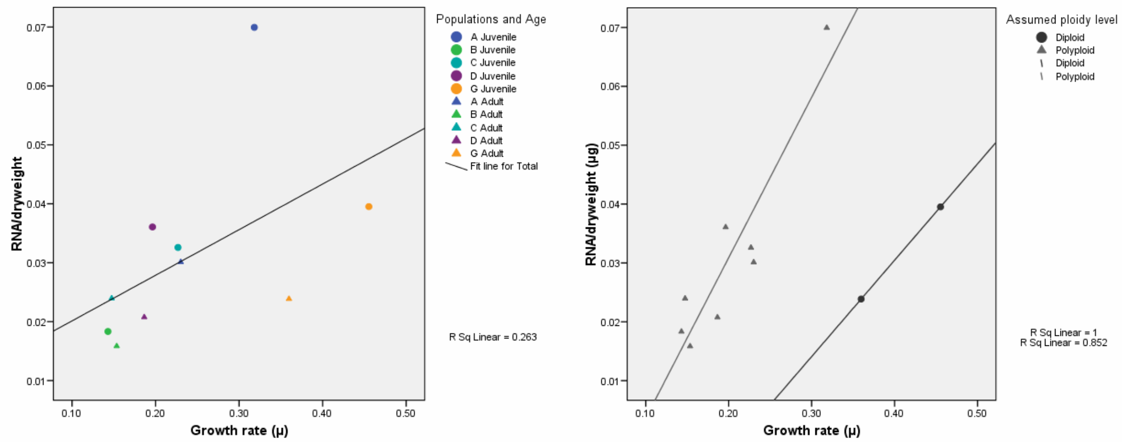


Fig. 48 Correlation between RNA/dry weight and specific growth rate (µ) for both age classes and all populations (left), correlation between RNA/dry weight and specific growth rate (µ) for the assumed ploidy levels (right).

DNA per dry weight (µg) and specific growth rate (µ) was not statistical correlated (Pearson Correlation=0.280, P=0.434, N=10, Fig. 49, Table 40), but statistically correlated when controlled for assumed ploidy level (Pearson Correlation=0.815, P=0.007, d.f.=7, Fig. 49, Table 41).

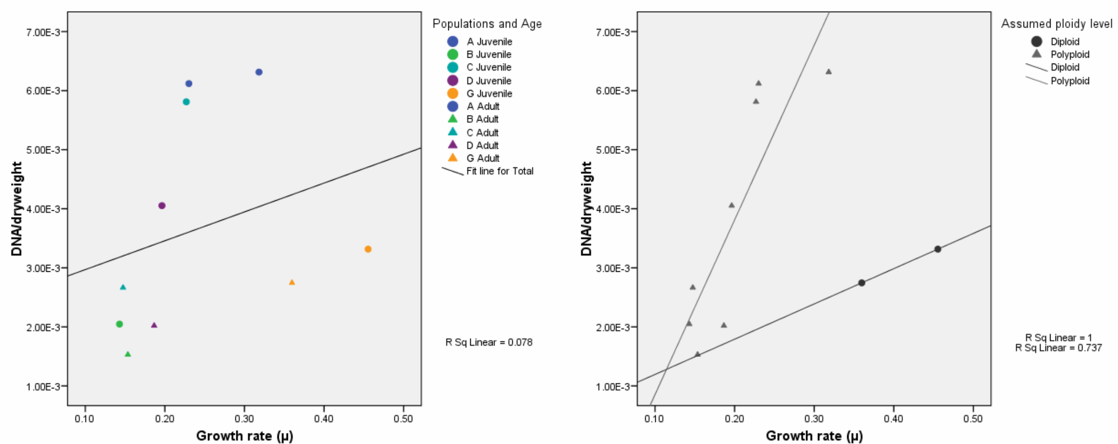


Fig. 49 Correlation between DNA/dry weight and specific growth rate (µ) for both age classes and all populations (left), correlation between DNA/dry weight and specific growth rate (µ) for the assumed ploidy level (right).

RNA per DNA and specific growth rate (µ) was not statistical correlated (Pearson Correlation, P=0.218, X=0.427, N=10, Fig. 50, Table 42). No statistical correlation was found when controlled for assumed ploidy level (Pearson Correlation=0.147, P=0.706, d.f.=7, Fig. 50, Table 43).

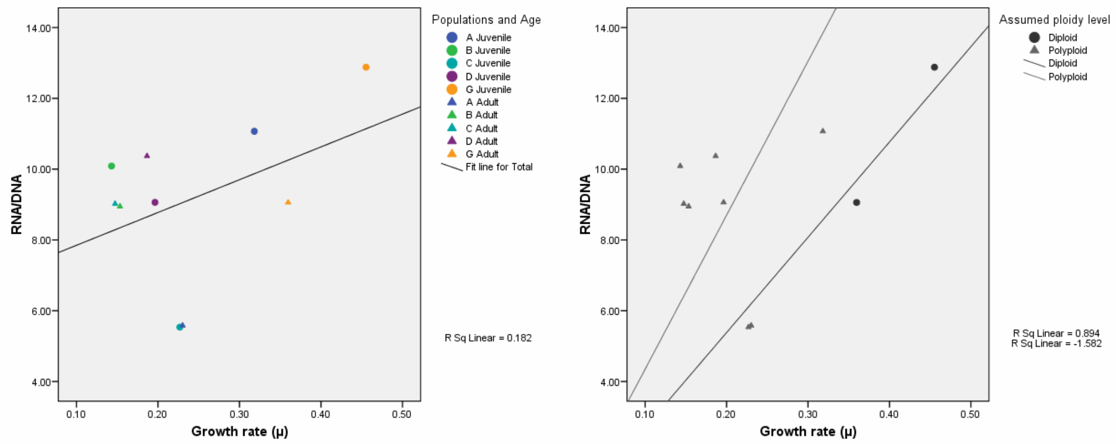


Fig. 50 Correlation between RNA/DNA and specific growth rate (µ) for both age classes and all populations (left), correlation between RNA/DNA and specific growth rate (µ) for the assumed ploidy levels, intercept suppressed (right).

Discussion

3.2. Cytogenetic Analyses

Few successful, cytogenetic studies have been done on *Daphnia* (Trentini, 1980), mostly due to relatively large number of chromosomes (2n=20-24) together with the prevalence of endopolyploidy and minute genome size (<2 μm) (Beaton & Hebert, 1994b). The small chromosome size is linked to the small genome size (1C=0.22-0.42 pg) (Beaton & Hebert, 1988; Beaton & Hebert, 1989; Korpelainen *et al.*, 1997; www.genomesize.com). To further complicate karyological observations, the chromosomes in *Daphnia* have been shown to be very condensed in the metaphase plate, as small spheroid or ellipsoidal bodies in groups of two or three (Zaffagnini, 1987). Uneven breakage of sister-chromatids during preparation may further obscure the results as differentiation between the small chromosomes and chromatids may be difficult (Dufresne & Hebert, 1998).

No marked difference in haploid chromosome number has been noted between *D. pulex* and *D. magna* (24 in prior, 20 in latter, Trentini, 1980) and these do not differ much in structure and morphology, the latter being somewhat larger (pers. obs.).

Feulgen staining

Nevertheless, staining of chromosomes by use of Feulgen reagent has been shown to work for *Daphnia* (Beaton & Hebert, 1988). Two different protocols were applied, but no chromosomal counts were possible in this study. In my study, despite several attempts with modification of the protocol for Feulgen staining, the method did not provide good results. Applying the method by Laane & Lie (2001) resulted in stained chromosomes, but also an extensive non-specific (non-chromosomal) staining. Variations of the protocol improved the resolution and less unspecific staining were observed. Variations of digestion time where found to correlate with the minimum required staining time, as more digested tissue allowed for faster, but also more unspecific staining, hence a compromise was reached that optimized both the digestion and staining time. Papain was chosen to digest the peptide bonds, and allow for better separation of the chromosomes. It worked exceptionally well in onion roots (*Allium*) (Fig. 7), but did not improve the results

for the *Daphnia*. Application of papain to digest peptide bonds would only improve the results in the case of a sample containing mitotic stages, which were not observed in my Feulgen experiments. Addition of chloroform to clear the cytoplasm produced no variation in the results and was discarded. Optimizing the methods did improve the staining, but the results were inconsistent and the protocol was discarded.

The modified Feulgen staining by Beaton & Hebert (1988) was found more reliable and with more consistent results. Digestion and staining of the samples were performed in room temperature for a long time period (respectively 30 and 100 minutes), hence lack of precision of the staining time caused negligible variations of the results. The nucleus and "clouds" of DNA was properly stained, but no mitosis or cells in metaphase were encountered, which led to the conclusion that the *Daphnia* may have synchronous mitosis or "mitotic waves" (Beaton & Hebert, 1994a). Efforts to collect samples within the most mitotic active period did not succeed, and hence applications of mitotic inhibitors to "arrest" the mitosis at the metaphase were tried (see below for details). In case of "mitotic waves" the whole individual will also be intoxicated (and usually die) and the tissue might permit staining of chromosomes in division. Feulgen staining was discarded as it was found to be too unspecific and time consuming within the time limits for a master thesis.

The protocol by Beaton & Hebert (1988) is actually just the first step for another method called Feulgen densitometry where measurements of light absorption of nuclear DNA (of stained tissue) using computer software on digital images, are used as a proxy of the DNA-content. A known diploid standard (e.g. temperate *D. pulex*) or cells with stabile size and known level of DNA (e.g. chicken red blood cells) is included and achieved IOD (Integrated optical densities) values are compared with this for determination of level of polyploidy or DNA quantity (Beaton & Hebert, 1988; Schulte *et al.*, 1991; Adamowicz *et al.*, 2000).

Mitotic inhibitors

Colchicine has been found to effectively preparing chromosomes in *Daphnia* allowing for quantification (Trentini, 1980), it has also been successfully applied to other organisms such as algae (Muravenko *et al.*, 2001). Mitosis in *Daphnia* has been shown to

be more sensitive to quinolines then to colchicines (Morrow, 2001), which could explain the difference in results. Aceto orcein has been reported to inadequately stain small chromosomes (Muravenko *et al.*, 2001), as described above *Daphnia* are known to have small chromosomes (Korpelainen *et al.*, 1997). Still aceto orcein worked much better than the Feulgen dye in this study.

The observed mortality of 0.1% colchicine, without arresting mitosis (or during metaphase), could indicate that it is easily absorbed in the body tissues. It is known to be among the strongest poisons that exists in nature, and probably kills the individuals prior to mitosis. Mortality was reduced by reduction of colchicine concentration (to 0.01%). No cells in metaphase were observed, and most likely the detrimental effect colchicine is also present at lower concentrations. Colchicine are also found to double the initial chromosome number upon treatment, producing artificial polyploidy or diploidization of haploids (Weber *et al.*, 2005). Despite previously reported success by applying colchicine, no mitotic effects were seen in this study, and the method discarded.

Application of 8-hydroxyquinoline did on the other hand not seem to cause any widespread mortality, but an anticipated gradual die-off of individuals through their cell-cycles which were "arrested" during mitosis. Despite numerous trials only a few of these supposed "arrested" individuals were found to have cells in metaphase. Some of these, presented in Fig. 10, seem to have 8 or 18 bodies that are identified as chromosomes. The observed numbers of bodies are close to what noted in Trentini (1980). A similar detrimental effect to the *Daphnia* as described for colchicine cannot be ruled out. The lack of reliable protocols for the accumulation of metaphase nuclei in *Daphnia* found in this study has previously been reported by Beaton & Hebert (1994b).

Embedding & nuclear measurements

Measurements of the nuclear membrane (in μm^2 of the sectioned individuals) of different tissues provide a measure of the amount of DNA in the specific cells. There is a correlation between cell size and DNA content (Brodsky & Uryvaeva, 1985).

Initially both juveniles (<24 hours, 1-2 instar) and mature adults (>7 days, >5 instar) were included for embedding, however due to time-constraint, only the juveniles were examined. Two tissue types were included in this study, intestine and connective

tissue type A. Contradictory reports of the ploidy level of the cells in the digestive tract have been published, Korpelainen *et al.* (1997) reported that the cells are stabilized at a relative low ploidy level, while Beaton & Hebert (1999) in contrast reported that the digestive tract was the only tissue which increases its endopolyploidy level beyond the fourth instar (maturation). My study could not judge which of these reports are most correct, as no adults were included. Other tissues described in this study, but not measured; include connective tissue type B (a smaller nucleolus oriented in one of the polar extremes of the nucleus), neural tissue in eye region and chitin producing cells in the carapace. Previous studies have been focusing on the thoracic limbs (Beaton & Hebert, 1988, 1989; Adamowicz *et al.*, 2002), labrum (parts of the mouth), rostrum (part of carapace extending from the eyes), epidermis and the intestine (Beaton & Hebert, 1999). Ploidy levels in the labrum and the epidermis was found to be affected by both age and food levels (Beaton & Hebert, 1999). The thoracic limbs are composed of a diploid exopodite, an endopolyploid epipodite and a central core containing both diploid and highly polyploid nuclei (Beaton & Hebert, 1989, 1999). The exopodite has previously been used as a diploid standard for cytogenetical studies of endopolyploidy and germ-line polyploidy (Adamowicz *et al.*, 2002). My study focused only on germ-line polyploidy, and an assumed diploid, temperate population (G) was used instead. The thoracic limb epipodites and the digestive tract jointly form the primary site for nutrient absorption and osmoregulation. These tissues represent together a secratory and highly metabolic active site, and are assumed to be highly polyploid (Beaton & Hebert, 1999).

Comparisons of the average size of the nuclear size in intestine and connective tissue type A for the arctic populations (B, C and D) with the temperate (G) showed a doubling in size, hence presumably a doubling of the genetic content, DNA and chromosomes. Whether this is the germ-line polyploidy or early endopolyploidy remains to be settled. But as the arctic population B, C and D were all found in the microsatellite analysis to have ~3 alleles per loci, it cannot be ruled out that these are germ-line triploids. No previous published data on endopolyploidy levels for intestine and connective tissue type A for the first instars exists, but endopolyploidy levels for other tissues have shown that tissues such as epidermis, epipodites, limb central core and rostrum are largely tetraploid during the first instars for an obligately asexual clone of *D.*

pulex (Beaton & Hebert, 1999). As all populations were sampled at the same stage of development, the difference in ploidy level seem more likely to be due to germ-line polyploidy. It is still a possibility that the size measured for the assumed diploid population (G) is in fact endopolyploid, 4n, and the arctic populations 8n.

Further analysis of the adults could shed some light on the degree of endopolyploidy in these populations, and analysis of other tissues such as the thoracic limbs, especially the diploid exopodites, could improve the results.

Flowcytometry

Results from flowcytometry should in theory confirm the nuclear measurements from embedded samples, regarding nuclear size. By flowcytometry one measures values of fluorescent emitted light from single particles. Using DNA specific staining techniques distribution curves that contain information about ploidy levels may be obtained. In other studies a standard have been used, commonly chicken red blood cells, with a known size. Due to time constraint no such standard was attained for this study, and the only application of the results would be the intra-specific relative difference in nucleus size for each population, thus giving an indication of endopolyploidy (Korpelainen *et al.*, 1997).

The protocol by Obermayer (2000) for plant tissues did not work well for *Daphnia*, probably due inappropriate isolation protocol for animal tissue and ionic differences in buffers used and sample. These were hence changed to more appropriate ionic concentrations for invertebrates (Morten M. Laane, pers. com.). Despite high back-ground noise a supposed diploid peak was observed (Fig. 17-19). No endopolyploidy can be deduced from these results, as settings for detection had to be changes for the different populations and the observation of only one distinct peak. No difference was observed between the assumed polyploid, arctic populations (C) and the assumed diploid *D. magna* population (M).

Endopolyploidy has been reported in all animals examined (including man) (Brodsky & Uryvaeva, 1985), studies on *Daphnia* have shown a high degree of endopolyploidy and it would be expected to be observed in all populations (including *D. magna* and temperate and hyaline *D. pulex*) when running flowcytometry. Juveniles and adults were pooled in this study due to the high biomass requirement (30-40 individuals).

Observed peaks would best reflect the pooled distribution of nuclei sizes. Less abundant types of tissues with more or less genetic content may be masked by the most abundant nuclei, probably digestive tissue, epidermis and muscle cells (pers. obs.). Settings had to be changed for the different populations as biomass varied, and the grinding method and filtering produced somewhat different concentrations of nucleus in the sample.

A general problem with flowcytometry is aggregation of cells and overestimation of polyploid cells (Korpelainen, 1997). Such aggregations were observed for the method by Obermayer (2000) when checked in microscopy, several aggregations of nuclei were attached together by "cemented" cytoplasm, but no aggregations were observed with the application of sodium citrate. Application of only exopodites (a part of the thoracic limb) assumed to have constant ploidy level throughout development (Korpelainen *et al.*, 1997) would allow for determination of the differences in germ-line polyploidy for the populations. However due to time-constraint and limited amount of stock samples, no such measurement could be made.

Due to the high level of back-ground noise, the contribution of small, unidentified, fluorescent fragments, a detection threshold was set in an intermediate level between the lowest values and the presence of the first peak. Additional settings included increased voltage of the selected sensor (detecting wavelength according to the dye) caused an increase in sensitivity (thus including more background noise), and increased resolution for sample with weak signal, cellular properties which could inhibit proper staining. Increased electric current (AMP) for the selected sensor (which detect wavelength according to the dye) also increased resolution, for the cost of overestimation of differences (the x-axis is stretched out). Counting only 5000 events (identified, fluorescent fragments within detection limits) is lower than what reported in other studies (Korpelainen et al., 1997). Due to low density in the stock populations a limited number of individuals could be spared for the experiments, and thus the total number of nuclei in the samples would be lower (even though measures were done to prevent this, such as limitation of the use of buffers). The membrane impermeability and separation from viable cell material of the PI (Product information, Molecular Probes) may be easier for plants for which it was described used (Obermayer, 2000), but not with *Daphnia* unless cells are degenerated or the membranes disrupted. Ethidium bromide (EtBr) has

previously been used (Korpelainen *et al.*, 1997) as a dye for flowcytometry of *Daphnia*, being easily absorbed at much lower wavelengths (~300 nm) than PI (535 nm) and it is permeable to the membranes. EtBr is known to minimize chromosomal condensation when a culture is exposed to mitotic arresting agents (such as colchicine or 8-hydroxyquinoline). It could alternatively replace Feulgen or aceto orcein dye in the microscopic analyses (see above). Mitotic inhibitors were not applied to the flowcytometry samples in this study. Future studies should include such, and trials should be run with alternative dyes, such as EtBr, acridine orange or RiboGreen. Alternatively, cell lines from specific tissues from the *Daphnia* may be cultured (as described in Robinson *et al.*, 2006) and run in the flowcytometer. This allows for histological studies of the different tissues and treatments with different nutrient concentrations, UV-stress and temperature.

Future studies should include a standard, such as chicken red blood cells, with known nuclei size, additional dyes should be tried, such as acridine orange or Feulgen reagent (Zubkova & Robaire, 2006). Cultivation of cell lines from different tissues, such as from the exopodites would allow for determination of germ-line polyploidy, while other tissues (e.g. from the epipodites or the intestine) would allow for assays of somatic polyploidy.

3.3. Life History Experiment

Length-weight calibration

Of the different curve fits, the Power equation gave the highest R square values for the different populations (>0.9). They were hence used for estimating mass from length in the life history experiment. Weight calibrated from length has been widely used (Færøvig *et al.*, 2002). It was however noted that the growth was not just in the tail to head direction, but also in width and height.

Alternatively weight has been calculated from an ellipsoid measurement of major axis (like in this study) and minor axis (width, not dorsal-ventral height), reported to have higher R square values and consequently found to better correlate with weight (Færøvig *et al.*, 2002). Minor axis, measured as width of the animal, is a better proxy when measurements are taken of swimming individuals (such as in Færøvig *et al.*, 2002), for microscopy; samples usually align on their sides, both increasing time used and stress on the animal.

Life history experiment

Life history experiments for *Daphnia* have been widely used to test for effects of ambient parameters such as food, temperature, presence of predators, toxins etc. (Epp, 1996; Dufresne & Hebert, 1998). Life history experiments are important to estimate whose parameters explain the most for fitness of the animals (Stearns, 1992). In this part of the study, cost and advantages of being polyploidy has been investigated and comparisons of fitness expressed by several parameters; r parameter, fertility, body growth rate and survival with a diploid population.

The experimental set-up was similar to that of Dufresne & Hebert (1998), with 28 replications from each population (25 in Dufresne & Hebert, 1998) and 12 mL beakers (30 mL in Dufresne & Hebert, 1998).

Chemostat set-up

Zooplankton, such as cladocerans, is considered energy limited (carbon, C), but elemental balance of other elements such as nitrogen (N) and phosphorous (P) are seemingly just as important (Sterner & Hessen, 1994). Energy content in algae was kept at a high level throughout the experiment (3.7 mg C/L) which is almost a 10 fold magnitude higher than what are recorded in some lakes (Gliwicz & Slusarczyk, 2001). The nitrogen content devised from the element analysis (6.79 C:N ratio) and base phosphorous content in the full-COMBO (50 μM C/L) should not be limiting to the growth and development in this study (Elser *et al.*, 2003; Færøvig & Hessen, 2003). Despite variation in the carbon level in the chemostat measured by element analysis (Fig. 22), the *Daphnia* was not assumed to be energy limited and fed *ad libitum* for the duration of the experiment. The advantage of a chemostat is to keep the concentration of algae (and nutrients) stable by continuously exchanging the growth medium (COMBO), however a chemostat has a limited duration as the algae seem to grow slower and accumulate less nutrients ("algal fatigue", Dag O. Hessen, pers. com.).

The applied methods for monitoring the carbon-levels in the chemostat were found to have huge difference both in applicability and in accuracy (Fig. 5), the element analysis done *a priori* is assumed to be most accurate, as actual carbon measures were taken, and compared with the results from the other methods as a standard. Absorbance, or OD, measured in a spectrophotometer, is not very accurate; both over- and underestimation are recorded. The method is not very time consuming, and give the results immediately. A Lux-meter or a light sensor, connected to a laptop computer, produced variations from the elemental analysis results similar to the "OD-method". The advantage of this method is its continuous monitoring, allowing monitoring temporal fluctuations, in this study the flow was too slow and the cuvette was too big, allowing for accumulation of algae in the chamber and hence gradually lowering the Lux values. The last method, cell counts using flowcytometry, was only applied for a short duration of the experiment. There is little differences between the measurements, neither is there much variation from the elemental analysis results. The method was however extremely time-consuming, and continuous measurements cannot be made.

Fitness & Fertility

The fitness parameter, measured as *r*, indicate positive or negative population growth. In this study two populations, arctic population A and temperate population G, were found to have positive population growth. Population B had low growth rates and trailing for extinction (like its sister population D, see **Species identification by mtDNA sequencing**). Population C also had negative population growth though not as pronounced as population B (Fig. 23). Total lifetime fertility show a similar pattern as *r*, and probably account for the majority of the differences observed in calculated *r*. The assumed diploid, temperate population G had higher fertility than all arctic, polyploid populations (although only statistically higher than C after log transformation) (Fig. 24). Note that population B and C are close to 1 neonate per female, indicating net reduction in population growth as shown in fitness, r. The low recorded fitness, *r*, and total lifetime fertility may not be that drastic, as 28 individuals (as used in this study) hardly mimic a population of several thousand, and great dichotomy in reproductive (asexual) success per female and during growth season has been recorded for *Daphnia in vivo* (e.g. Gliwicz & Slusarczyk, 2001). Individuals that successfully reproduced were observed to do so repeatedly, and may indicate individual differences in the beakers or differences within populations despite clonal structure. Pigmentation, either hyaline (population C and G) or melanic populations (A, B and D) showed no correlating for fitness or fertility, and pigmentation was assumed not to affect these life history characteristics. Manipulation will certainly have influenced the individuals as they were transferred from one beaker to another, especially during the pre-matural stage when they were transferred to and taken picture of in a microscope.

Fitness, *r*, has been shown to be statistically affected by food concentration (Epp, 1996; Pijanowska *et al.*, 2006). Energy should not be limiting in this study as the *Daphnia* was fed *ad libitum* (>3 mg C/L). The large number of “abortions” of parthenogenetic eggs (>50 % of moults) can be related to excess C (to N and P), as one way of excluding C is by allocating large quantities of C in eggs and consequently release them (Urabe & Sterner, 2001). This is however considered highly unlikely when fed *ad libitum*, as it drastically reduces the organisms’ fitness.

Negative *r* values have previously been reported for *D. pulicaria in vivo* during the course of their growth season, especially during mid-summer and winter (Hu & Tessier, 1995). The same trend has been observed for *D. galeata* under the ice-cover during the winter months (Winder *et al.*, 2003). It is possible that delayed recruitment from resting eggs can explain such seemingly negative fitness values; such egg banks have been shown to be quite formidable in size and may temporarily re-stock the populations when conditions get favourable (Cáceres, 1998; Mergeay *et al.*, 2006). Released resting eggs were only observed for the excluded population M (*D. magna*).

Intervals of hatching has been found to be temperature dependent, e.g. *D. magna* has been recorded to hatch neonates at intervals of 3-4 days when kept at 19°C (Zaffagnini, 1987). Average hatching intervals for the *D. magna* in this study was 5.54 days, and in the range of 4.03-4.86 days for *D. pulex* spp. (excluding population D), extended hatching intervals may indicate that temperature was not optimal in this study. In Sachse (2006) number of neonates in first two broods when reared at 18°C are in the range of 25-40 for temperate clones, and ~20 for arctic clones, both these values are far higher than what recorded in this study, see Fig. 25 (include all broods, maximum number of recorded broods = 9, most neonates were released within the first 4 broods). *Daphnia* are found to have a maximum of 12-16 reproductive instars before death (Dunham & Banta, 1940), however a maximum of 8 (population G) reproductive instars were recorded in this study, indicating an unknown detrimental effect decreasing number of late broods.

The assumed diploid population (G) was found to have higher fitness and fertility than the assumed polyploid for the life history experiment. The observed pattern is not due to temperature differences (such as described in Dufresne & Hebert, 1998), nor to nutrient limitations (excess C, and consequent exclusion of C in form of undeveloped eggs), possibly due to some unidentified difference (such as light intensity, microscopic parasites), but could also be the actual differences between the clones. Interestingly, the population A and G, showing the highest fertility values and positive population growth, were the only populations found to host epizooic ciliates (*Vorticella* sp.). Studies have shown that *Vorticella* sp. competes for food with their *Daphnia* hosts (Kankaala & Eloranta, 1987). Indicating that the positive correlation between fertility and population

growth is due to some other causes. It has previously been suggested that the ciliates compete for food with their hosts (25-33% of *Daphnia* intake, Kankaala & Eloranta, 1987); it is uncertain whether these epibionts may provide a positive effect to the host or chose to attach to these populations because of their (reproductive-) success. It is assumed that diploid population (G) is more successful at 20°C, as polyploidy is restricted to the Arctic with much lower temperatures. Polyploids *Daphnia* are generally found to produce larger, and fewer offspring than their diploid conspecifics (Dufresne & Hebert, 1998). Decreased predation and competition could favour such a "few large offspring strategy" (Yampolsky & Scheiner, 1996). Future studies should run life history experiment at different temperature, with and without UV radiation (for testing effect of melanin) and nutrient regimes (limitations to different elements, such as in Dufresne & Hebert, 1998) and efforts should be made to reduce parasitism and epibiosis which may cause random effects.

Generation time & age at maturity

No difference in generation time, T, was recorded in this study. In the short arctic growing seasons, one would anticipate a strong selective pressure for a short generation time, so as to leave as many offspring as possible. Polyploidy and the fact that most localities are inhibited by one (or two) clones could explain the relatively long generation time (as long as the diploids), as polyploidy structurally inhibit rapid growth and maturation. Absence of competition may select for higher pre-natal investments. On the other hand, invasions from neighbouring ponds should be expected to occur at high frequency, which would imply competition. The slow growth seems to be a negative trade-off trait for polyploids, and strongly suggest that some other trait should compensate for the low generation time.

Age at maturity, like generation time; indicate relative investment into reproduction. Age at maturation for two of the arctic populations (B and C) in this study (20°C) was statistically higher from the temperate population G. Thus slower maturation in assumed polyploids like in Dufresne & Hebert (1998). However population A and D (both assumed polyploid) did not differ statistically from the other populations and population D was even found to have shorter maturation time than population G.

Assumed polyploids were found to have longer maturation time than diploids in this study. No correlation was found between melanization and maturation time (hyaline populations C and G were statistically different), suggesting that whatever expenses melanization comes with it does not affect maturation time.

Alternatively age of maturity can also be measured as the first day with visible ovaries (Dufresne & Hebert, 1998). Observation of produced eggs in the brood chamber is however simpler. Actual maturity starts by production of parthenogenetic eggs in the ovaries in the fourth (adolescent) instar, and they are not transferred to the brood chamber until the beginning of the fifth (Dunham & Banta, 1940). Comparisons with other published maturation times are thus problematic (as the duration of the instars is unknown). The relative differences should not be that different, however. It has been shown that polyploid *Daphnia* mature slightly faster than diploids under cold conditions (1 day difference at 10°C, Dufresne & Hebert, 1998), and somewhat slower than diploids under intermediate or hot conditions (<1 day difference at 17°C and 24°C, Dufresne & Hebert, 1998). This could thus suggest that polyploidy could have some life-cycle benefits under the low temperature prevailing at high latitudes. The observed pattern in this study also show that the polyploid have a longer maturation time in elevated temperatures, probably due to enzymatic efficiency and reduced overall growth rate, no comparative data for low temperatures where obtained in this study.

Polyploidy are generally assumed to result in delayed maturation (Levin, 1983), as opposed to described in Dufresne & Hebert (1998). Whether this is a direct result of increased DNA (reduced development due to costly replication) or an independent adaptation remains to be concluded. The short reproduction season in the Arctic, where polyploidy is prevalent, would intuitive select for fast maturation time; however absence of predators and competition release due to asexual clones could explain such alternative life history adaptations.

Size at maturity

The diploid population G was found to have statistically more biomass than the polyploids at maturity, the unexpected low size at maturity could be due to some disadvantageous conditions for the arctic polyploids in this experiment, like with the

extended maturation time (above), as size at maturity is generally assumed to be higher for polyploids.

Like with age at maturity, size at maturity is assumed to be positively correlated with ploidy level at intermediate temperature (17°C in Dufresne & Hebert, 1998). Polyploidy is assumed to result in increased cell size, and slower development due to costly development, maturing at larger size resulting in larger, but fewer offspring (Dufresne & Hebert, 1998). In this study all assumed polyploid, arctic populations matured at smaller sizes than the assumed diploid population (G) (similar to 10°C and 24°C in Dufresne & Hebert, 1998). Future studies on polyploid and diploid populations should include different temperature treatment, one low (<10°C), one intermediate (~15°C) and one temperate (~20°C).

Size at maturity was found positively correlated with death rate in Winder *et al.* (2003) for a high-mountain lake with planktivorous fish. Selection for decreased size at maturity due to predation release in this study is not probable as all locations were assumed to be fish-free, and predation pressure minimal. Increased size at maturity was found to weakly correlate with decreasing mortality (or in this case, long survival) (Fig. 28). This pattern is not surprising as bigger size at maturity if not limited by nutrient allow for allocation of more energy to reproduction. It is possible that in the presence of medium sized predators, such as *L. arcticus*, there is a selection for rapid growth and high size at maturity to produce larger offspring (size of offspring not measured).

Growth rate

Growth rate has been shown to be statistical influenced by quality (C:P ratio) of the food (Kyle *et al.*, 2006), all populations were fed from the chemostat and effects should be even for all populations (however some clonal differences in their response to nutritional quality have previously been shown, Kyle *et al.*, 2006).

Growth rate in weight increase per day throughout the entire prematural growth period and during the first 10 days of development was compared for the different populations. For growth rate (µg) during the entire prematural growth period, only one of the arctic populations (C) differed from the temperate population (G), while all arctic populations differed from the temperate population for the growth rate (µg) during the

first 10 days of development (Fig. 30). Variation in the total prematural growth rate (µg) could be caused by unidentified maturation (ovaries matured, but no eggs visible) and delayed mortality (dying individuals showing decreased growth). Measuring growth rate (µg) only for the 10 first days of development decreased the variation (Fig. 30 right & Fig. 31) and allowed for comparisons of the populations. Growth rate (µg) the 10 first days for the temperate population was statistically different to all arctic populations, possibly due to adaptation to warmer environment (~20°C like in the experiment), but could also be a result of different ploidy levels, as the temperate population is assumed to be diploid (see **Microsatellite analysis**) and the arctic populations polyploid. Relative growth rate increment per day, or specific growth rate (µ) was calculated for populations for the 10 first days, and allowed for comparisons with other studies and organisms as specific growth rate is irrespective of scale. Specific growth rate of the assumed diploid, temperate population (G) was statistical different from two of the assumed polyploid, arctic populations (B and C), like with actual growth rate. It seems like differences in specific growth rate could be governed by difference in adaptation to temperature or some unidentified factor in the experiment, or to differences in ploidy level. Population A and G, had the highest specific growth rate, and it should be noted that both these populations were found to host epibiont *Vorticella* sp.. Whether symbiosis has induced increased growth, host selection of the protists (because of increased growth) or random contamination of the protists from some external source remain to be identified. No correlation between pigmentation and growth level was identified, as population C (arctic, hyaline) and G (temperate, hyaline) was statistically different for all estimates of growth.

Specific growth rate has previously been reported around ~0.17 for *D. magna* (fed 2.0 mg C/L, Pijanowska *et al.*, 2006) which is similar to what found for the arctic populations (Fig. 32). Other published specific growth rates for *D. pulex* and *D. pulicaria* fed *ad libitum* are somewhat higher than what found in this study (0.72±0.02 & 0.44±0.02 in Kyle *et al.*, 2006; 0.35-0.45 in Sachse, 2006). In Sachse (2006) it was shown that the arctic populations had higher specific growth rate than the temperate populations at ~20°C, which is opposite to findings in this study.

Alternatively to specific growth rate, Dagg & Littlepage (1972) applies equation by Winberg (1960), giving percent dry weight increase per day, which is found to be more appropriate when working with exponential growth. In this study however, all populations, except G (Fig. 31), showed linear growth the first 10 days of development, and the alternative discarded.

Body size is found to increase at low temperature and latitude for many ectotherms (Dufresne & Hebert, 1998), there could be a potential selective advantage of large size at low temperatures, but large size could also be because of slow development or increased cell sizes at low temperature (Dufresne & Hebert, 1998).

Survival

Survival or mortality was measured as the days of survival for the individuals in the study, which give an average longevity of the population (Fig. 33). The assumed diploid population G was found to have statistically higher survival than all arctic populations but population C. It can be assumed that the environment for the two hyaline populations C and G possibly was less disadvantageous than what it was for the other non-hyaline populations. The costly production of melanin after each moult could possibly decrease the longevity of the animal (Hebert & McWalter, 1983). However since no such dichotomy between the hyaline and melanic populations has been found for the other life history parameters, such as for fertility or growth, the expenditure cost of melanization can be assumed to be marginal.

Deaths were assumed due to old age for most of the individuals. Some seemed to halt their development and stop moulting (hence they were overgrown by algae and bacteria), other may have had problems during the moulting stage, and finally a few may have succumbed due to manipulation during transfer to new beakers and microscope (pers. obsv.). Mortality was not found to be normally distributed (Fig. 33), but rather stochastic, thus no life stage was found to be more critical, population A, C and G with evenly distributed mortality events, population B and D with increased mortality during the first stage, and final stage for population B.

Days of survival and days at maturity were found be positively correlated, indicating that these may be linked, whether delayed age at maturity caused increased longevity or *vice versa* remain to be concluded.

As the experiment was ended after 53 days, some individuals were still alive. The majority, however had already succumbed to death at this stage, but some were still alive and healthy looking (not covered by algae and swimming in normal manner), it has been noted that some *Daphnia* over-winter as females instead of/or in addition to ephippial eggs completing their life cycle after more than 1 year (Gliwicz & Slusarczyk, 2001). As all ponds and lakes in this study completely freezes (the shallow arctic lakes and ponds) or desiccate during winter. It was noted that recruitment the new growth season was mainly by ephippial resting eggs, and that a life cycle should typically be less than one growth season. In light of the unexpected slow reproduction and high mortality, the life history experiment should have been split into two parts; one for the prematural growth rate and one for the fertility and survival.

Principal Components Analysis

Running a PCA will produce several components (as many as the number of variables included) with various Eigenvalues or percent of total variance. The first two components in this study amounted for a total of ~90% of the variance (75%+17%, Table 2), hence most of the variance among the variables is explained in the scatter plot with these two components. Size at maturity was found to be accounting for most of the variance (loading) explained by component 1 (which accounted for ~75% of the total variance, Table 2), hence size at maturity is the best explanatory variable in this data set. Survival and total fertility accounted for most of the variance (loading) in component 2 (~17% of total variance, Table 2) thus being the second and third best explanatory variables in the data set. The other variables (age at maturity and specific growth rate) accounted for most of the variance (loading) in components 3, 4 and 5, but these were not used as they accounted for less than 10% of the total variance (Table 2), hence these variables were found to be less suited to observe differences between the populations in this study.

PCA lacks statistical significance measurements, and is mainly used as a descriptive and explorative method (Hammer *et al.*, 2001), hence the difference between

the assumed diploid population and the polyploid populations can not be said to be statistical. The 95% confidence ellipses in Fig. 34 indicate that the polyploid populations cluster into one group slightly overlapping with the diploid population. It also shows that the diploid population is more variable for the variables accounting for component 1 and 2.

3.4. Genetic Analyses

Microsatellite analysis

Whether judged by the conservative or non-restrictive estimate, population A, B and D were found to have more than 2 alleles on average per locus (Table 2), and a high degree of tissue polyploidy thus seem likely for these populations. No more than 4 alleles were identified at any locus, and hence ploidy level can not be assumed to be more than 4n (tetraploid). Application of more primers could reveal other loci with more than 4 alleles. Population C was found to have two heterozygote alleles for all loci (Table 2 & 3), however using the non-restrictive counting method many of these alleles were found to be homozygotes due to the difference in amplification of the different alleles (2x difference in peak height), resulting in several loci with possibly 3 alleles, whether this population can be identified as a triploid or a diploid remains to be concluded as more loci should be investigated. Population C is assumed to be polyploid in this study, possibly with a lower ploidy level than population A, B and D. Population G was found to have four homozygote loci, and two loci with heterozygotes (2 alleles per locus). Amplification of the alleles for the heterozygotes were equal (same heights of peaks; no homozygote heterozygote as identified in the arctic populations) and diploidy cannot be rejected.

Population B and D had the same phenotypic alleles (measured in bp, Table 3) for all loci, except for primer Dp514. Alleles with similar length may have different sequences, but for population B and D posterior mtDNA analysis grouped these two populations as the same species, namely Polar *D. pulicaria*, more neutral loci should be included to determine the true level of differentiation between these two clones. It can be

noted that the Dp514 primer assumed to have a trinucleotide repeat motif, produced invariable dinucleotide differences between alleles for all populations, and may possibly be unreliable. The two primers with below detection limit values (Dp523 and Dp524), could be due to a too high dilution rate of the finished PCR products or wrong TM (Table 1) for the PCR, or possibly not work at all due to absence of targeted sites in the populations in the study. However, PCR failure is probably the best explanation, as the primer has been successfully applied by Colbourne *et al.* (2004) & Markova *et al.* (2007).

The analysis of the bands of peaks of repeated fragments has many shortcomings and pitfalls, and hence various artefacts may distort the actual results. A typical dinucleotide repeat homozygote (Fig. 51) will show the peak according to the number of base pairs for the fragment length. Additionally small peaks with a few base-pairs missing (e.g. -2 bp, -4 bp and -6 bp of the actual allele length) will be seen as minor peaks called “stutters”. These are generally caused by polymerase slippage during the PCR (Schlötterer & Tautz, 1992). These “stutters” were easily recognized and relatively small (<5% of true allele height) in this analysis. However if alleles differ by only 2 bp (typical for dinucleotide repeats), the shortest allele will be artificially enhanced (increased height amplification) by underlying “stutter” peaks from the longest allele. “Stutters” found in this study were found to be relatively small and not assumed to cause any implications to the results. Additionally no difference in height relations of homozygotes and heterozygotes was noted (all 2x ± 0.2), no matter if the heterozygote alleles differed by 2 bp or 11 bp. An additionally artefact called the “+A form” is caused by the addition of an extra nucleotide (usually adenosine) to the 3´ end of the DNA fragment during the PCR. This will result in an extra peak +1 bp and with approximately the same height as the true allele peak (GeneScan® Reference guide). However, due to the 1 bp difference this artefact is easily controlled for, and did not cause any disruptions of the results in this study. Uneven allele amplification due to the exponential non-quantitative nature of the PCR, may increase amount of smaller fragments relative to longer fragments if difference is more than 10 bp and initial DNA amount is low (Welsh, 1992 *sited in* GeneScan® Reference guide; Markwith *et al.*, 2006). In this study few alleles differed more than 10 bp, and height differences between heterozygote alleles did not correlate with fragment length (most heterozygotes were either 1x or 2x, whereas a

homozygote could be both the longest or shortest allele), thus this effect can be discarded as a disturbance for this study.

Allozyme analysis of monomeric enzymes is an alternative method the microsatellite analysis, comparing the zones of activity in the enzymes to give an estimate of the number of functional gene codes for the different populations in question (Beaton & Hebert, 1988; Dufresne & Hebert, 1997; Ĉerný & Hebert, 1999; Aguilera *et al.*, 2007). In microsatellite analysis the actual DNA template is studied, whereas in products of increased DNA (and consequently chromosomes) are studied in allozyme analysis.

Difference between age groups may be due to different initial volume of DNA, which produce values below detection limits for some loci. The results did not differ between juveniles and adults (with a 10 fold difference in body size), however this explanation does not seem likely. Since there was no detected difference between juveniles and adults, germ-line polyploidy seem more likely as one would have expected increased number of alleles in the adults. However it remains to be concluded if microsatellites can positively identify endopolyploidy as somatic multiplication of chromosomes may not result in detectable variations of the alleles. Microsatellite analysis was found to give a rough estimate of degree of polyploidy. However since no calculations were found to correlate average number of alleles and heterozygosity with ploidy level (i.e. 2n, 4n, 8n, 16n etc.) no estimation other than differentiation between diploidy and polyploidy was possible. The analysis lacks the resolution of the cytogenetic analyses (e.g. Feulgen densitometry described in Beaton & Hebert, 1998) which allow for actual chromosomal counts. However for the sake of this study, the microsatellite analysis successfully confirmed that the arctic populations are polyploids and the temperate is diploid.

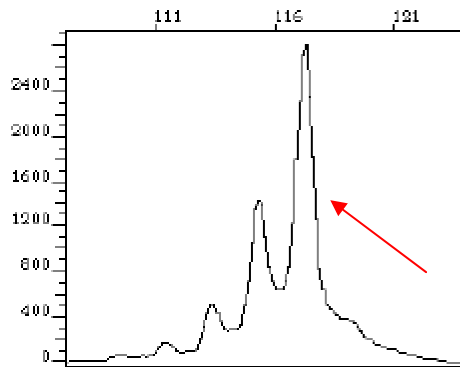


Fig. 51 – Typical dinucleotide repeat homozygote (Note the "stutters" at -2, -4 and -6 bp, see text) (Figure from GeneScan ® Reference Guide, Chemistry Reference for the ABI Prism ® 310 Genetic Analyzer) (Red arrow indicate true peak).

Species identification by mtDNA sequencing

The ND5 primer for mtDNA was selected for sequencing as this has previously been used in taxonomic work on the *Daphnia* complex (Dufresne & Hebert, 1997; Colbourne *et al.*, 1998, Markova *et al.*, 2007). Sequences were available through contact with J.K. Colbourne by Anders Hobæk, NIVA. Due to time-constraint Anders Hobæk compared all the sequences and prepared the phylogenetic tree (Fig. 43).

Samples in this study were positively identified in the Polar *D. pulicaria* clade (population B & D), Eastern *D. pulicaria* (population C), *D. tenebrosa* (population A) and European *D. pulex* (see Fig. 43). Note that the Eastern *D. pulicaria* clone (two clones found, with 2 bp difference) form a distinct clade within the Eastern *D. pulicaria* clade from other arctic locations. The observed phylogenetic tree is identical to the recent consensus (Colbourne et al., 1998, Markova, 2007). Grouping of the *D. pulicaria* and *D. pulex* did not form monophyletic groups (such as *D. tenebrosa*); *D. pulicaria* is found both in the *D. tenebrosa* branch and with Pan-arctic *D. pulex*, similar with *D. pulex* which also got a separate European clade from the root of the tree. Application of mtDNA clocks for arthropods indicating that the *D. pulicaria* and Pan-arctic *D. pulex* clade diversified 2.2 MA (Colbourne *et al.*, 1998) coincident with the onset of the Pleistocene. Despite the genetic variation in these subgenera, their morphological difference is negligible, and a great deal of taxonomic confusion has been dominating the last century.

Solvatn (location A) has been found to be rich in nutrients and thus eutrophied compared to the other ponds (3 times as high total P values, see **Sample locations**). It is

not surprising that clones from this pond was *D. tenebrosa*, which has been found to dominate eutrophic lakes (Gerben van Gest, *unpublished*). It was unexpected that the population B and D were so genetically similar, as they were found to differ both in the **Life history experiment** and **DNA, RNA and protein quantification**. All Eastern Nearctic *D. pulicaria* populations (including population C in this study) found at Svalbard, Iceland and Greenland are hyaline (except one weakly melanic population found on Iceland). However this clone has been found melanized in the Pyrenees and the High Tatra (Markova *et al.*, 2007). It seems as the hyaline morphotype are inferior to the melanized in shallow alpine or arctic ponds/lakes. The observation of the hyaline Eastern *D. pulicaria* in the deepest lake in the study further establish that the melanic clones are inferior competitors in deep (>2 meter) lakes and ponds. This dichotomy has been noted as a possible adaptation for coexistence of different species in one lake, as the hyaline individuals inhabit the deeper portion of the lake, and melanic roams just under the surface (Rhode *et al.*, 2001).

RNA, DNA & protein quantification

Choice of standards

Both standard sets prepared in this study showed good correlation between fluorescence and DNA/RNA concentration (see Fig. 44). The linear correlations did however differ. The best application was reached using the RNA standard purified from *D. pulex* and commercial DNA standard. The choice of standards were set by comparing the results with previous produced data, calculated RNA values did not differ using the two standards. The purified standard from *D. pulex* was selected as it is assumed that the purified standards are to behave more like the RNA in the organism in study. The DNA standards differed markedly, where the purified produced >10x higher values than expected, while the chosen commercial standard produced results within the expected range. DNA quantification had a low fluorescence response (low variability due to high background noise) and relative high response for RNA quantification (with above scale values for the biggest individuals). The two chosen standards were the best fitted in terms of the slope for the DNA and the intercept for the RNA. There are some implications of

comparing results obtained from different protocols and standards, such as different degree of RNA contamination and molecular weight (Buckley *et al.*, 1999). It has been reported to be no implications of choosing different standards (and consequently protocols for purifications), if RiboGreen dye is used and a similar slope for both commercial and purified RNA standard is achieved (Jones *et al.*, 1998). The high background noise observed when using DNase, is reported to be caused by Mg^{2+} and Ca^{2+} chemicals in the DNase that produce fluorescence (Buckley *et al.*, 1999).

Preparations

The application of an ultrasonicator worked well in this study, Vrede (2002) reported increased noise by application of an ultrasonicator, and used the whole *Daphnia* without any physical destructors. As the noise observed in this study was mainly from using DNase, no further investigation of different methods for destruction of the structural tissues was done.

Age classes

Two age classes was initially selected, juveniles (<24 hours) and adults (~7 days). However due to time constraints few replicates were included, and the age classes pooled as the inter-specific difference between juveniles and adults was negligible among the limited amount of replicates. It should however be kept in mind that RNA and protein are greatly influenced by the various life stages, and future studies should separate age.

Nucleic acids per dry weight

Amount of nucleic acids and protein were scaled for dry weight of the individuals, giving the amount of µg DNA, RNA or protein per µg biomass. Quantity of DNA and RNA were in the lower range of previously published results for adult *Daphnia hyalina* (0.46% DNA and 4.3% RNA, *in* Baudouin & Scoppa, 1973), for juvenile *Daphnia magna* (9.73% RNA, *in* McKee & Knowles, 1987) and recently published data for *Daphnia pulicaria* (0.25-0.72% DNA and 5.34-7.71% RNA, *in* Acharya *et al.*, 2004). It is assumed that the *Daphnia* will have more available RNA as juveniles than adults due to high growth rate (Elser *et al.*, 1996), but only weak correlation in DNA content and age

(McKee & Knowles, 1987). Thus RNA per dry weight in this study which includes both juveniles and adults should have been even higher than what reported for adults. It has been shown that limitation of P are correlated with decreasing DNA and RNA content (Acharya *et al.*, 2004), even though an *ad libitum* feeding regime was assumed in this study based on carbon measurements. It seems like there could have been limitations to P. The amount of protein per dry weight was within previously published range for *Daphnia* (52.6% protein, referred to in Peters, 1987) and juvenile *Daphnia magna* (47.7% protein, McKee & Knowles, 1987). No correlation between age and protein content has been found, however protein, like DNA and RNA, are closely correlated with P content (Elser *et al.*, 2003).

DNA per dry weight would be the best estimate for difference in ploidy level, as increased number of chromosomes invariably would increase the amount of DNA (Stebbins, 1960). Population A is the only assumed polyploid population in the study that shows statistical difference from the assumed population G. The polyploid populations C and D are not statistically different from G. This does however not imply they are not polyploid, as the amount of DNA does not have to be multiple of the increase in chromosome pairs. Additionally it has not been established whether these populations are triploid or tetraploid. Population B, also assumed to be polyploid, have a less DNA per dry weight than population G, it could however be noted that all individuals examined from population B were adult or adolescents.

RNA is not directly affected by ploidy level (and increased DNA template), the difference observed in Fig. 45 could rather be due to difference in growth rates. Quantity RNA per dry weight is correlated with growth rate as RNA is directly connected to protein production and which consequently are connected to growth (Elser *et al.*, 2003). Protein, like RNA, is coupled with growth and not ploidy level. This coupling seems weak in this study, both as there is no statistical difference between the groups, nor is there any significant correlation with growth rate. Zooplankton is known to have periodic changes in lipid content (Corner & Conwey, 1968 *sited in* Dagg & Littlepage, 1972) and some copepods digest bodily protein as an energy source during starvation (Martin, 1968 *sited in* Dagg & Littlepage, 1972). As individuals were fed *ad libitum* it is highly unlikely that protein has been digested. Changes in stored lipids (which also form a great portion

of the developing eggs in the ovaries, pers. obs.) could have influenced the organismal protein content, but mainly in the adults (as access protein in juveniles only cause increased growth, not storage). Triploids and tetraploids contain, as expected, three and four times the haploid amount of DNA (Batistic *et al.*, 1975). Comparisons of genome sizes in specific complexes have shown that polyploids do not possess simple multiplies of the haploid level, but rather lower DNA contents than expected, decline is not due to loss of individual chromosomes, disproportionate loss of repetitive DNA (Bachmann & Rheinsmith, 1973).

Nucleic acids compared

A low RNA/DNA ratio or a high DNA/protein ratio compared to the diploid population (G) will support polyploidy for the other populations. However statistical difference is only found between the DNA/protein ratios, where population A is statistical different from population B, D and G.

RNA/DNA ratio is correlated statistically, and no statistical difference was found between the populations, it can however be noted that population A and C have somewhat lower ratios than the diploid population G, supporting the assumption that these are polyploid. Population B and D, also assumed polyploids, did not differ from the diploid population G. Different age classes and different metabolic activity at the time of sampling could explain the observed pattern, further studies should focus on identifying this age-specific effect on the RNA.

RNA/protein ratios for the different populations are statistically different, population A statistically different from population D and G. RNA and protein were found to correlate statistically, increased RNA content will allow for increased protein content (however note that protein per dry weight, Fig. 46, do not differ markedly for the different populations). One hypothesis for lower RNA/protein ratio could be the production of melanin from protein. No pattern suggest this, as population A (with a high RNA/protein ratio) is just as melanic as population B and D (with an equal RNA/protein ratio as hyaline population C and G). Neither RNA nor protein is expected to substantially differ due to ploidy levels.

DNA/protein ratio indicates what RNA/DNA already shown since protein and RNA content seems to be correlated. Thus population A and C which are markedly higher than the other can be assumed to have a higher DNA content. Statistically only population A differ from the assumed diploid population G, assumed polyploid population B and D are actually found to have lower DNA/protein levels than the diploid. DNA/protein ratios are reported to be highest for juveniles, increasing until day 4 when it stabilizes (McKee & Knowles, 1987). Again age seem to influence data in this study, and more thorough delineation of the different age classes is needed. DNA/protein can also be a measure of the actual cell mass (McKee & Knowles, 1987).

Nuclei acids and growth rate

RNA per dry weight has been shown to correlate well with growth rate; increased growth requires increased cellular concentration of ribosomal RNA (which constitutes the majority of the organismal RNA, Elser *et al.*, 1996). It is also assumed that the RNA amount does not directly influence the increase in growth, but is a prerequisite for the maximum obtainable growth rate (Dagg & Littlepage, 1972). RNA content on the other hand has been shown to strongly correlate with the phosphorous body content, which in turn is strongly correlated with the C:P ratio in the food (Elser *et al.*, 2003). Increased growth causes an increased demand for rRNA which in turn increase the demand for phosphorous, this mechanism is termed the growth rate hypothesis (Elser *et al.*, 2003). Nitrogen limitation (>18 C:N) has been shown to induce limitation to protein production, disrupting specific growth rate and RNA content correlation (Elser *et al.*, 2003), in this study C:N ratio was low (~6) and not assumed to be limiting to the *Daphnia*, hence cannot explain any irregularities. Correlation between RNA/growth rate is most pronounced when growth rate is stabile, as growth rate decreases, RNA does not immediately break down, however as growth increases the production of RNA are quickly adjusted (Dagg and Littlepage, 1972). Correlations between RNA per dry weight (or percent) and growth rate (specific growth rate, μ) have been reported in organism like bacteria, algae, invertebrates and fish larvae (*references in* Dagg & Littlepage, 1972).

No statistical correlation was found between these two parameters in this study. But, a non-statistical pattern of positive correlation is observed. The slope and intercept

for the correlation curve does not differ markedly from what reported for *D. pulex* and *D. pulicaria* (Kyle *et al.*, 2006), except for the non-significance and low R^2 value. Even as it was assumed that individuals were fed *ad libitum* prior to snap-freezing one cannot rule out the possibility that the individuals were limited by some element (notably P). It is central in the growth rate hypothesis that the C:P ratio is limiting to RNA production and consequently growth rate, and with the limitation of P the RNA per growth rate correlation can be lost (Elser *et al.*, 2003). As observed in Fig. 45, juveniles have on average higher growth rate and RNA content. This is due to increased metabolic activity during the initial life stages, as most RNA produced are used for growth while in later life stages most RNA produced are for maintenance and reproduction (shown for copepods in Dagg & Littlepage, 1972).

If the samples are split into assumed polyploids and diploids, the correlation curves are statistically significant (note however that the diploids are only presented by two points and thus not applicable for any correlation curve. The clustering should be noted, which is different from the polyploids). The polyploid populations have a higher increase of RNA per dry weight per increase in specific growth rate, as expected as they have a more DNA available for RNA production. The observed increase in slope for the assumed polyploids indicates that for an increment increase in growth and more RNA per dry weight is produced. This makes sense in the light that the polyploids will have more DNA template. It could also be that the maintenance cost for the polyploids are higher than for the diploids. Hence the increase in RNA is possibly due to increased maintenance of e.g. pigments (melanin). If melanization is the due reason for the increased maintenance cost the hyaline population C assumed to be polyploid should be found in some intermediate between the hyaline diploid and the melanic polyploids, as it does not form any such pattern it is likely that some other protein requirements, then for production of melanin, are present.

Given constant amount of DNA per cell, DNA can also be used as a growth index (Dagg & Littlepage, 1972). Violations of DNA per growth rate could indicate endopolyploidy and an unusual increase in DNA content in the cells (possible polyploidy). No statistical correlation is found for all populations and age groups. Splitting the samples into the assumed ploidy level, indicate that the slope for the

polyploids may be much steeper than for the diploids. Unlike RNA per dry weight, DNA content cannot be said to be the direct prerequisite for growth rate, as available RNA is not limited by the template DNA. It might indicate something about the total amount of nucleic acids (like what mentioned above), that higher proportion of the organism is composed of nucleic acids (due to replications/mitosis) than of non-nucleic tissues when growth rate is high.

RNA/DNA ratio is assumed to be correlated with growth rate as more RNA for the constant (or near-constant) DNA level would be available for increased growth. RNA/DNA ratio was found to correlate weakly (not statistically) with growth rate. RNA/DNA ratio was generally higher for juveniles, possibly due to allocation of RNA to reproduction in adults. RNA/DNA and growth rate correlation has been shown to be species specific (Dagg & Littlepage, 1972) stage specific (Wagner, 1998) and temperature dependent (Saiz *et al.*, 1998), whereof the two first applies to the samples in this study. 4 different subspecies were used (Fig. 50), and even though the majority was collected within 24 hours or 7 days, intra- and interspecific differences in life stage were noted (see difference in relative nucleic acids in juveniles and adults). Strong linear relationship between somatic growth rate (specific growth rate) and RNA/DNA ratio (R^2=0.94, P=0.001 in Vrede *et al.*, 2002). RNA/DNA ratio has been shown to increase with increased algal C:P ratio (Vrede *et al.*, 2002).

Conclusions

Ploidy levels for the populations were successfully established using microsatellites. The method was unfortunately too rough to give any estimations of degree of polyploidy (i.e. 4n, 8n, 16n etc.). Cytogenetic analyses aimed to determine chromosome numbers and endopolyploidy did not succeed.

Statistical difference was observed between the diploid population and the polyploid populations for all measured life history traits; fitness and fertility, maturation size and age, growth rate and survival. Polyploid populations had typically lower fitness, lower fertility, later and smaller size at maturation, slower growth rate and lower survival. Hence, for the conditions which the experiment was run, diploid *Daphnia* do better than polyploid.

Quantities of DNA, RNA and protein in diploids and polyploids were not found to be statistically different in this study; DNA per dry weight was found to be somewhat higher for the polyploid populations. RNA per dry weight and growth rate were found to be correlated for the different ploidy levels, with higher increments of RNA per dry weight per growth rate increase for the polyploid populations. Similar trends were shown for DNA per dry weight and RNA per DNA against growth rate.

No statistical difference was found between arctic hyaline and melanic populations, thus for the experimental conditions no benefit (UV-protection) or cost (expenditure of nutrients) seem to affect the pigmented populations.

There must be a trade-off between the reduced effects observed in the life history experiment and some undefined parameter. One such parameter may be better adaptation to cold environment. The traits observed in the life history experiment may be the result of suboptimal conditions, or those traits may actually be advantageous in the Arctic. Another hypothesis is increased genetic variation due to doubling of chromosomes and the genetic template (allowing for genome rearrangement following polyploidization). This would increase the plasticity for the obligate asexual populations which otherwise would be vulnerable to biological, biochemical or chemical changes. A final hypothesis concerns growth; as established from the cytogenetic analyses *Daphnia* has synchronous mitosis in the early stage of each instar. Subsequently the most metabolic active tissues

are undergoing endopolyploidy, doubling of chromosome numbers and increase size of the cells. As the enzymatic and mitotic cycles are slower in the Arctic it can be hypothesised that increasing the germ-line ploidy level would give a selective advantage, increasing the initial size of the cells.

References

Acharya, K., Kyle, M. & Elser, J.J. (2004) Biological stoichiometry of *Daphnia* growth: An ecophysiological test of the growth rate hypothesis. *Limnology and Oceanography*, **49**, 656-665

Adamowicz, S.J., Gregory, T.R., Marinone, M.C. & Hebert, P.D.N. (2002) New insights into the distribution of polyploid *Daphnia*: the Holarctic revisited and Argentina explored. *Molecular Ecology*, **11**, 1209-1217

Aguilera, X., Crespo, G., Declerck, S. & Meester, L.D. (2006) Diel vertical migration of zooplankton in tropical high mountain lakes (Andes, Bolivia). *Polish Journal of Ecology*, **54**, 453-464

Bachmann, K. & Rheinsmith, E.L. (1973) Nuclear DNA amounts in Pacific *Crustacea*. *Chromosoma*, **43**, 225-236

Bailey, G.S., Poulter, R.T.M. & Stockwell, P.A. (1978) Gene duplication in tetraploid fish: model for genesilencing at unlinked duplicated loci. *Proceedings of the National Academy of Sciences of the United States of America*, **75**, 5575-5579

Barry, E.G. & Perkins, D.D. How to prepare acto-orcein squashes, especially for pachytene chromosomes. Available online from: http://www.fgsc.net/Neurospora/NeurosporaProtocolGuide.htm

Batistic, R.F., Soma, M., Becak, M.L. & Becak, W. (1975) Further studies on polyploid amphibians: a diploid population of *Phyllomedusa burmeisteri*. *Journal of Heredity*, **66**, 160-162

Baudouin, M.F. & Scoppa, P. (1973) The determination of nuclei acids in freshwater plankton and its ecological implications. *Freshwater Biology*, **5**, 115-120

Beaton, M.J. & Hebert, P.D.N. (1988) Geographic partenogenesis and polyploidity in *Daphnia pulex*. *The American Naturalist* **132**, 837-845

Beaton, M.J. & Hebert, P.D.N. (1989) Polyphyletic origins of asexuality in *Daphnia pulex*. I. Breeding-system variation and levels of clonal diversity. *Evolution*, **43**, 1004-1015

Beaton, M.J. & Hebert, P.D.N. (1994a) Patterns of DNA synthesis and mitotic activity during the intermoult of *Daphnia*. *Journal of Experimental Zoology*, **268**, 400-4009

Beaton, M.J. & Hebert, P.D.N. (1994b) Variations in chromosome numbers of *Daphnia* (Crustacea, Cladocera). *Hereditas*, **120**, 275-279

Beaton, M.J. & Hebert, P.D.N. (1999) Shifts in postembryonic somatic ploidy levels in *Daphnia pulex*. *Hydrobiologia*, **394**, 26-39

Beauchamp, D.A., Lariviere, M.G. & Thomas, G.L. (1995) Evaluation of competition and predation as limits to juvenile Kokanee and Sockeye salmon production in Lake Ozette, Washington. *North American Journal of Fisheries Management*, **15**, 193-207

Beebee, T.J.C. & Rowe, G. (2005) An introduction to molecular ecology. Oxford University Press, New York, U.S.

Benzie, J.A.H. 2005. Cladocera: the genus *Daphnia* (including *Daphniopsis*). *In* H. J. F. Dumont [ed.], Guides to the Identification of the Microinvertebrates of the Continental Waters of the World. Kenobi Productions & Backhuys.

Brodsky, V.Ya. & Uryvaeva, I.V. (1985) Genome multiplication in growth and development. Cambridge University Press, Cambridge, U.K.

Brönmark, C. and Hansson, L.A. (2005) The Biology of Lakes and Ponds. Second Edition. Oxford University Press, New York, U.S.

Buckley, L., Caldarone, E. & Ong, T.-L. (1999) RNA-DNA ratio and other nuclei acid-based indicators for growth and condition of marine fishes. *Hydrobiologia*, **401**, 265-277

Burns, C.W. (1968) The relationship between body size of filter-feeding Cladocera and the maximum size of particle ingested. *Limnology & Oceanography*, **13**, 675-678

Cáceres, C.E. (1998) Interspecific variation in the abundance, production, and emergence of *Daphnia* diapausing eggs. *Ecology*, **79**, 1699-1710

Ĉerný, M. & Hebert, P.D.N. (1993) Genetic diversity and breeding system variation in *Daphnia pulicaria* from North American lakes. *Heredity*, **71**, 497-507

Colbourne, J.K. & Hebert, P.D.N. (1996) The systematics of North American *Daphnia* (Crustacea: Anomopoda): a molecular phylogenetic approach. *Philosophical Transactions: Biological Sciences*, **351**, 349-360

Colbourne, J.K., Robison, B., Bogart, K. & Lynch, M. (2004) Five hundred and twenty eight microsatellite markers for ecological genomic investigations using *Daphnia*. *Molecular Ecology Notes*, **4**, 485-490

Colbourne, J.K., Crease, T.J., Weider, L.J., Hebert, P.D.N., Dufresne, F. & Hobæk, A. (1998) Phylogenetics and evolution of a circumarctic species complex (Cladocera: *Daphnia pulex*) *Biological Journal of the Linnean Society*, **65**, 347-365

Dagg, M.J. & Littlepage, J.L. (1972) Relationships between growth rate and RNA, DNA, protein and dryweight in *Artemia salina* and *Euchaeta elongate*. *Marine Biology*, **17**, 162-170

DeMott, W.R. (1988) Discrimination between algae and artificial particles by freshwater and marine copepods. *Limnology and oceanography*, **33**, 397-408

Dunham, H. & Banta, A.M. (1940) Some effects of colchicine on heredity in *Daphnia longispina*. *Genetics*, **25**, 310-325

Dufresne, F. & Hebert, P.D.N. (1997) Hybridization and origins of polyploidy. *Proceedings of the Royal Society of London*, *Biological Sciences*, **258**, 141-146

Dufresne, F. & Hebert, P.D.N. (1997) Pleistocene glaciations and polyphyletic origins of polyploidy in an Arctic cladoceran. *Proceedings of the Royal Society of London*, *Biological Sciences*, **264**, 201-206

Dufresne, F. & Hebert, P.D.N. (1998) Temperature-related differences in life-history characteristics between diploid and polyploid clones of the *Daphnia pulex* complex. *Écoscience*, **5**, 433-437

Edmondson, W.T. (1955) The seasonal life history of *Daphnia* in an Arctic lake. *Ecology*, **36**, 439-455

Elser, J.J., Dobberfuhl, D., MacKay, N.A. & Schampel, J.H. (1996) Organism size, life history, and N:P stoichiometry: towards a unified view of cellular and ecosystem processes. *Bioscience*, **46**, 674-684

Elser, J.J., Acharya, K., Kyle, M., Cotner, J., Makino, W., Markow, T., Watts, T., Hobbie, S., Fagan, W., Schade, J., Hood, J. and Sterner, R.W. (2003) Growth rate – stoichiometry couplings in diverse biota. *Ecology*, **6**, 936-943

Epp, G.T. (1996) Clonal variation in the survival and reproduction of *Daphnia pulicaria* under low-food stress. *Freshwater Biology*, **35**, 1-10

Epstein, C.J. (1967) Cell size, nuclear content, and the development of polyploidy in mammalian liver. *Proceedings of the National Academy of Sciences of the United States of America*, **57**, 327-334

Flössner, D. 2000. Die Haplopoda und Cladocera (ohne Bosminidae) Mitteleuropas. Backhuys.

Færøvig, P.J. & Hessen, D.O. (2003) Allocation stategies in crustacean stoichiometry: the potential role of phosphorus in the limitation of reproduction. *Freshwater Biology*, **48**, 1782-1792

Færøvig, P.J., Andersen, T., Hessen, D.O. (2002) Image analysis of *Daphnia* populations: non-destructive determination of demography and biomass in cultures. *Freshwater Biology*, **47**, 1956-1962

Gallardo, M.H., Bickham, J.W., Honeycutt, R.L., Ojeda, R.A. & Köler, N. (1999) Discovery of tetraploidy in a mammal. *Science*, **401**, 341

GeneScan® Reference Guide - Chemistry Reference for the ABI PRISM® 310 Genetic Analyzer (2000), Applied Biosystems

Gliwicz, Z.M. & Slusarczyk, A. (2001) Life history synchronization in long-lifespan single-cohort *Daphnia* population in a fishless alpine lake. *Oecologia*, **128**, 368-378

Gregory, T.R. & Hebert, P.D.N. (1999) The modulation of DNA content: proximate causes and ultimate consequences. *Genome Research*, **9**, 317-324

Grime, J.P. & Mowforth, M.A. (1982) Varitation in genome size – an ecological implication. *Nature*, **299**, 151-153

Hammer, Ø. Harper, D.A.T. & Ryan, P.D. (2001) PAST: Palaeontological statistics software package for education and data analysis. *Palaeontologia Electronica*, 4, 9 pp

Haugarvoll, E., Thonsen, J., Laane, M., Huang, Q. & Koppand, E.O. (2006) Melanogenesis and evidence for melanosome transpont to the plasma membrane in a CD83 teleost leukocyte cell line. *Pigment Cell Research*, **19**, 214-225

Hebert, P.D.N. & McWalter, D.B. (1983) Cuticular pigmentation in arctic *Daphnia*: adaptive diversification of asexual lineages? *The American Naturalist*, **122**, 286-291

Hebert, P.D.N., Schwartz, S.S., Ward, R.D. & Finston, T.L. (1993) Macrogeographic patterns of breeding system diversity in the *Daphnia pulex* group. 1. Breeding systems of Canadian populations. *Heredity*, **70**, 148-161

Hessen, D.O. (1985) Filtering structures and particle size selection in coexisting Cladocera. *Oecologia*, **66**, 368-372

Hessen, D.O. (1996) Competitive trade-off strategies in Arctic *Daphnia* linked to melanism and UV-B stress. *Polar Biology*, **16**, 573-579

Hobæk, A., Weider, L.J. and Wolf, H.G. (1993) Ecological genetics of Norwegian Daphnia. III Clonal richness in an Arctic apomictic complex. *Heredity*, **71**, 323-330

Hu, S.S. & Tessier, A.J. (1995) Seasonal succession and the strength of intra- and interspecific competition in a *Daphnia* assemblage. *Ecology*, **76**, 2278-2294

Innes, D.J. (1997) Sexual reproduction of *Daphnia pulex* in a temporary habitat. *Oecologia*, **111**, 53-60

Jones, L.J., Yue, S.T., Cheung, C.Y. and Singer, V.L. (1998) RNA quantitation by fluorescence-based solution assay: RiboGreen reagent characterization. *Analytical Biochemistry*, **265**, 368-374

Kankaala, P. & Eloranta, P. (1987) Epizooic ciliates (*Vorticella* sp.) compete for food with their host *Daphnia longispina* in a small polyhumic lake. *Oecologia*, **73**, 203-206

Kilham, S.S., Kreeger, D.A., Lynn, S.G., Goulden, C.E. & Herrera, L. (1998) COMBO: a defined freshwater culture medium for algae and zooplankton. *Hydrobiologia*, **377**, 147-159

Korpelainen, H., Ketola, M. & Hietala, J. (1997) Somatic polyploidy examined by flowcytometry in *Daphnia*. *Journal of Plankton Research*, **19**, 2031-2040

Kyle, M., Acharya, K., Weider, L.J., Looper, K. & Elser, J.J. (2006) Coupling of growth rate and body stoichiometry in *Daphnia*: a role for maintenance processes? *Freshwater Biology*, **51**, 2087-2095

Laane, M.M. (1968) A simple staining method for nuclear studies in filamentous fungi. *Zeitschrift für wissenschaftliche Mikroskopie und mikroskopische Technik*, **69**, 7-10

Laane, M.M. (2007) The use of web-cams in microscopy. *InFocus Magazine, Proceedings of the Royal Microscopical Society*, Issue 5

Laane, M.M. & Lie, T. (2001) Håndbok i Mikroskopi og fremstilling av preparater. Gyldendal Akademisk, Oslo, Norway

Leuchtenberger, C. (1958) Quantitative determination of DNA in cells by Feulgen microspectrophotometry. *General Cytochemical Methods*, **1**, 219-278

Levin, D.A. (1983) Polyploidy and novelty in flowering plants. *The American Naturalist*, **122**, 1-25

Lokki, J. & Saura, A. (1979) Polyploidy in insect evolution. *Basic Life Sciences*, **13**, 277-312

McKee, M.J. & Knowles, C.O. (1987) Levels of protein, RNA, DNA, glycogen and lipid during growth and development of *Daphnia magna* Straus (Crustacea: Cladocera). *Freshwater Biology*, **18**, 341-351

Mahony, M.J. & Robinson, E.S. (1980) Polyploidy in the Australian Leptodactylid frog genus *Neobatrachus*. *Chromosoma*, **81**, 199-212

Markova, S., Dufresne, F., Rees, D. J. Černý, M. & Kotlík, P. (2007) Cryptic intercontinental colonization in water fleas *Daphnia pulicaria* inferred from phylogenetic analysis of mitochondrial DNA variation. *Molecular Phylogenetics and Evolution*. In press

Markwith, S.H., Stewart, D.J. & Dyer, J.L. (2006) Program Note, Tetrasat: a program for the population analysis of allotetraploid microsatellite data. *Molecular Ecology Notes*, **6**, 586-589

Maynard Smith, J. (1978) The evolution of sex. Cambridge University Press, Cambridge, U.K.

Mergeay, J., Declerck, S., Vershuren D. & Meester L.D. (2006) *Daphnia* community analysis in shallow Kenyan lakes and ponds using dormant eggs in surface sediments. *Freshwater Biology*, **51**, 399-411

Mills, D. (1978) 8-hydroxyquinoline inhibition of DNA synthesis and intragenic combination during yeast meiosis. *Molecular and General Genetics*, **162**, 221-228

Morrow, D., Corrigan, D. & Waldren, S. (2001) Development of a bioassay for phytochemicals using *Daphnia pulex*. *Planta Medica*, **67**, 843-846

Muravenko, O.V., Selyakh, I.O., Kononenko, N.V. & Stadnichuk, I.N. (2001) Chromosome numbers and nuclear DNA contents in red microalgae *Cyanidium caldarium* and three *Galdieria* species. *European Journal of Phycology*, **36**, 227-232

Obermayer, R. (2000): Genome size variation in soybean (*Glycine max*). A flow cytometric analys with reference to the postulated correlation between maturation rate and nuclear DNA content. Diploma thesis. Wien.

Otto, S.P. & Whitton, J. (2000) Polyploidy incidence and evolution. *Annual Review Genetics*, **34**, 401-437

Perrin, N. (1989) Reproductive allocation and size constraints in the cladoceran *Simocephalus vetulus* Müller. *Functional Ecology*, **3**, 279-283

Peters, R.H. (1987) Metabolism in *Daphnia*. *Memorie dell'Instituto di Anatomia Comparata*, **45**, 193-243

Pijanowska, J., Dawidowicz, P., Howe, A. & Weider, L. J. (2006) Predator-induced shifts in *Daphnia* life-histories under different food regimes. *Archiv für Hydrobiologie*, **167**, 37-54

Rabinovitch, M. & Plaut, W., 1962. Cytoplasmic DNA synthesis in *Amoeba proteus*. I. On the particulate nature of the DNA-containing elements. *The Journal of Cell Biology*, **15**, 525-534

Rautio, M. & Vincent, W.F. (2006) Benthic and pelagic food resources for zooplankton in shallow high-latitude lakes and ponds. *Freshwater Biology*, **51**, 1038-1052

Rhode, S.C., Pawlowski, M. & Tollrian, R. (2001) The impact of ultraviolet radiation on the vertical distribution of zooplankton of the genus *Daphnia*. *Nature*, **412**, 69-72

Robinson, C.D., Lourido, S., Whelan, S.P., Dudycha, J.L., Lynch, M. & Isern, S. (2006) Viral transgenesis of embryonic cell cultures from the freshwater microcustacean *Daphnia*. *Journal of Experimental Zoology*, **305**, 62-67

Sachse, R. (2006) The effect of temperature and phosphorus-limitation on growth and reproduction and RNA-content of arctic and temperate *Daphnia*. Report about the student internship 13.02.06-28.04.06, Nederlands Instituut voor Ecologie (NIOO-KNAW), Maarssen, The Netherlands

Saiz, E., Calbet A., Fara, A. & Berdalet E. (1998) RNA content of copepods as a tool for determining adult growth rates in the field. *Limnology and Oceanography*, **43**, 465-470

Schlötterer, C. & Tautz, D. (1992) Slippage synthesis of simple sequence DNA. *Nucleic Acid Research*, **20**, 211-215

Schulte, E.K.W., Lyon, H. & Prento, P. (1991) Gallocyanin chromalum as a nuclear stain in cytology. I. A cytophotometric comparison of the Husain-Watts Gallocyanin chromalum staining protocol with the Feulgen procedure. *Histochemical Journal*, **23**, 241-245

Sharma, C.B.S.R., 1990, Chemical induced aneuploidy in higher plants, *Mutagenesis*, **5**, 105-125

Soltis, D.E. & Soltis P.S. (1995) The dynamic nature of polyploid genomes. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 8089-8091

Stearns, S.C. (1992) The evolution of life histories. Oxford University Press, Oxford, New York, Tokyo

Stebbins G.L. (1960) Variation and evolution in plants. Columbia University Press, New York, U.S.

Trentini, M., 1980. Chromosome Numbers of Nine Species of Dahniidae (Crustacea, Cladocera). *Genetica*, **58**, 221-223

Vrede, T., Persson, J. & Aronsen, G. (2002) The influence of food quality (P:C ratio) on RNA : DNA ratio and somatic growth rate of *Daphnia*. *Limnology and Oceanography*, **47**, 487-494

Wagner, M., Durbin, E. & Buckley, L. (1998) RNA:DNA ratios as indicators of nutritional condition in the copepod *Calanus finmarchicus*. *Marine Ecology*, **162**, 173-181

Ward, R.D., Bickerton, M.A., Finston, T. & Hebert, P.D.N. (1994) Geographical cline in breeding systems and ploidy levels in European populations of *Daphnia pulex*. *Heredity*, **73**, 532-543

Weber, S., Ünker, F. & Friedt, W. (2005) Improved double haploid production protocol for *Brassica napus* using microspore colchicines treatment in vitro and ploidy determination by flow cytometry. *Plant Breeding*, **124**, 511-513

Weider L.J. (1987) Life history variation among low-arctic clones of obligately parthenogenetic *Daphnia pulex*: a diploid-polyploid complex. *Oecologia*, **73**, 251-256

Weider, L.J. & Hobæk, A. (1994) Molecular biogeography of clonal lineages in a high-arctic apomictic *Daphnia* complex. *Molecular Ecology*, **3**, 497-506

Weider, L.J. & Hobæk, A. (2003) Glacial refugia, haplotype distribution, and clonal richness of the *Daphnia pulex* complex in arctic Canada. *Molecular Ecology*, **12**, 463-473

Weider, L.J., Hobæk, A., Colbourne, J.K., Crease, T.J., Dufresne F. & Hebert P.D.N. (1999) Holarctic Phylogeography of an Asexual Species Complex I. Mitochondrial DNA Variation in Arctic Daphnia. *Evolution*, **53**, 777-792

Wetzel, R.G. (1983) Limnology: Lake and River Ecosystems. Academic Press, U.S.

Winder, M., Bürgi H.R. & Spaak P. (2003) Mechanisms regulating zooplankton populations in a high-mountain lake. *Freshwater Biology*, **48**, 795-809

Wold, S. (1978) Cross-validatory estimation of the number of components in factor and principle components models. *Technometrics*, **20**, 397-405

Wolstenholme, D.R., 1965, The distribution of DNA and RNA in salivary gland chromosomes of chironomus tentans as revealed by fluorescence microscopy. *Chromosoma*, **17**, 219-229

Yampolsky, L.Y. & Scheiner, S.M. (1996) Why larger offspring at lower temperatures? A demographic approach. *The American Naturalist*, **147**, 86-100

Zaffagnini, F. (1987) Reproduction in *Daphnia*. *Memorie dell'Instituto di Anatomia Comparata*, **45**, 245-284

Zubkova, E.V. & Robaire B. (2006) Effects of ageing on spermatozoal chromatin and its sensitivity to *in vivo* and *in vitro* oxidative challenge in the Brown Norway rat. *Human Reproduction*, **21**, 2901-2910

Appendix

Table 5 Non-parametric Kruskal-Wallis test for differences between connective tissue type A nucleus size of for all populations.

Ranks

	Populations	N	Mean Rank
Connective Tissue Nuclear size (µm2)	B	58	221.03
	C	90	152.89
	G	112	65.63
	Total	260	

Test Statistics(a,b)

	Connective Tissue Nuclear size (µm2)
Chi-Square	175.374
df	2
Asymp. Sig.	.000

a Kruskal Wallis Test
b Grouping Variable: Populations

Table 6 Pairwise non-parametric Mann-Whitney test for differences of connective tissue nucleus size of the populations.

Ranks

	Populations	N	Mean Rank
Connective Tissue Nuclear size (µm2)	B	101	227.49
	C	90	203.33
	D	50	245.83
	G	112	79.58
	Total	353	

Test Statistics(a,b)

	Connective Tissue Nuclear size (µm2)
Chi-Square	155.528
df	3
Asymp. Sig.	.000

a Kruskal Wallis Test
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Connective Tissue Nuclear size (µm2)	B	101	104.75	10579.50
	C	90	86.18	7756.50
	Total	191		

Test Statistics(a)

	Connective Tissue Nuclear size (µm2)
Mann-Whitney U	3661.500
Wilcoxon W	7756.500
Z	-2.317
Asymp. Sig. (2-tailed)	.021

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Connective Tissue Nuclear size (µm2)	B	101	75.16	7591.00
	D	50	77.70	3885.00
	Total	151		

Test Statistics(a)

	Connective Tissue Nuclear size (µm2)
Mann-Whitney U	2440.000
Wilcoxon W	7591.000
Z	-.336
Asymp. Sig. (2-tailed)	.737

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Connective Tissue Nuclear size (µm2)	B	101	149.58	15108.00
	G	112	68.60	7683.00
	Total	213		

Test Statistics(a)

	Connective Tissue Nuclear size (µm2)
Mann-Whitney U	1355.000
Wilcoxon W	7683.000
Z	-9.576
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Connective Tissue Nuclear size (µm2)	C	90	61.44	5529.50
	D	50	86.81	4340.50
	Total	140		

Test Statistics(a)

	Connective Tissue Nuclear size (µm2)
Mann-Whitney U	1434.500
Wilcoxon W	5529.500
Z	-3.546
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Connective Tissue Nuclear size (µm2)	C	90	146.71	13203.50
	G	112	65.17	7299.50
	Total	202		

Test Statistics(a)

	Connective Tissue Nuclear size (µm2)
Mann-Whitney U	971.500
Wilcoxon W	7299.500
Z	-9.853
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Connective Tissue Nuclear size (µm2)	D	50	132.32	6616.00
	G	112	58.81	6587.00
	Total	162		

Test Statistics(a)

	Connective Tissue Nuclear size (µm2)
Mann-Whitney U	259.000
Wilcoxon W	6587.000
Z	-9.213
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: Populations

Table 7 Non-parametric Kruskal-Wallis test for difference between intestine nucleus size for all populations.

Ranks

	Populations	N	Mean Rank
Intestine	B	107	229.67

	C	80	209.62
	G	133	75.31
	Total	320	

Test Statistics(a,b)

	Intestine
Chi-Square	195.116
df	2
Asymp. Sig.	.000

a Kruskal Wallis Test
b Grouping Variable: Populations

Table 8 Pairwise non-parametric Mann-Whitney test for differences of intestine nucleus size of the populations.

Ranks

	Populations	N	Mean Rank
Intestine Tissue Nuclear size (μm2)	B	107	304.82
	C	80	271.03
	D	105	241.26
	G	133	81.91
	Total	425	

Test Statistics(a,b)

	Intestine Tissue Nuclear size (μm2)
Chi-Square	234.686
df	3
Asymp. Sig.	.000

a Kruskal Wallis Test
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Intestine Tissue Nuclear size (μm2)	B	107	100.87	10793.50
	C	80	84.81	6784.50
	Total	187		

Test Statistics(a)

	Intestine Tissue Nuclear size (μm2)
Mann-Whitney U	3544.500
Wilcoxon W	6784.500
Z	-2.008
Asymp. Sig. (2-tailed)	.045

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Intestine Tissue Nuclear size (μm2)	B	107	129.15	13819.50
	D	105	83.41	8758.50
	Total	212		

Test Statistics(a)

	Intestine Tissue Nuclear size (μm2)
Mann-Whitney U	3193.500
Wilcoxon W	8758.500
Z	-5.428
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Intestine Tissue Nuclear size (μm2)	B	107	182.79	19559.00
	G	133	70.38	9361.00
	Total	240		

Test Statistics(a)

	Intestine Tissue Nuclear size (μm2)
Mann-Whitney U	450.000
Wilcoxon W	9361.000
Z	-12.468
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Intestine Tissue Nuclear size (μm2)	C	80	101.91	8153.00
	D	105	86.21	9052.00
	Total	185		

Test Statistics(a)

	Intestine Tissue Nuclear size (μm2)
Mann-Whitney U	3487.000
Wilcoxon W	9052.000
Z	-1.976
Asymp. Sig. (2-tailed)	.048

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Intestine Tissue Nuclear size (μm2)	C	80	165.31	13225.00
	G	133	71.92	9566.00
	Total	213		

Test Statistics(a)

	Intestine Tissue Nuclear size (μm2)
Mann-Whitney U	655.000
Wilcoxon W	9566.000
Z	-10.709
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Intestine Tissue Nuclear size (μm2)	D	105	177.63	18651.50
	G	133	73.61	9789.50
	Total	238		

Test Statistics(a)

	Intestine Tissue Nuclear size (μm2)
Mann-Whitney U	878.500
Wilcoxon W	9789.500
Z	-11.574
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: Populations

Table 9 Non-parametric Kruskal-Wallis test of differences between the tissue types (irrespectively of the populations).

Ranks

	Tissue	N	Mean Rank
Nuclear size (μm2)	Intestine	260	378.78
	Connective	320	218.77
	Total	580	

Test Statistics(a,b)

	Nuclear size (μm2)
Chi-Square	130.786
df	1
Asymp. Sig.	.000

a Kruskal Wallis Test
b Grouping Variable: Tissue

Table 10 Model summaries of the different best-fit equations tried for the length and weight relationship.

Dependent Variable: MiligramA
N=37

Equation	Model Summary					Parameter Estimates	
	R Square	F	df1	df2	Sig.	Constant	b1
Linear	.770	117.136	1	35	.000	-.066	.061
Power	.937	524.585	1	35	.000	.005	3.251
S	.938	525.403	1	35	.000	-.483	-5.000
Exponential	.908	346.539	1	35	.000	.001	1.942

The independent variable is MilimeterA.

Dependent Variable: MiligramB
N=52

Equation	Model Summary					Parameter Estimates	
	R Square	F	df1	df2	Sig.	Constant	b1
Linear	.649	92.284	1	50	.000	-.051	.055
Power	.805	206.743	1	50	.000	.012	2.249
S	.742	143.813	1	50	.000	-1.343	-3.129
Exponential	.826	238.031	1	50	.000	.003	1.449

The independent variable is MilimeterB.

Dependent Variable: MiligramC
N=77

Equation	Model Summary					Parameter Estimates	
	R Square	F	df1	df2	Sig.	Constant	b1
Linear	.722	194.389	1	75	.000	-.029	.039
Power	.853	434.130	1	75	.000	.007	2.864
S	.850	424.791	1	75	.000	-1.289	-3.512
Exponential	.822	346.752	1	75	.000	.001	2.106

The independent variable is MilimeterC.

Dependent Variable: MiligramD
N=31

Equation	Model Summary					Parameter Estimates	
	R Square	F	df1	df2	Sig.	Constant	b1
Linear	.751	87.643	1	29	.000	-.048	.054
Power	.914	307.595	1	29	.000	.008	2.924
S	.895	248.212	1	29	.000	-.992	-3.807
Exponential	.886	226.069	1	29	.000	.001	2.001

The independent variable is MilimeterD.

Dependent Variable: MiligramG
N=37

Equation	Model Summary					Parameter Estimates	
	R Square	F	df1	df2	Sig.	Constant	b1
Linear	.891	284.956	1	35	.000	-.050	.061
Power	.978	1564.039	1	35	.000	.007	3.391

S	.976	1426.825	1	35	.000	-.707	-3.939
Exponential	.935	500.275	1	35	.000	.001	2.563

The independent variable is MilimeterG.

Dependent Variable: MiligramM
N=74

	Model Summary					Parameter Estimates	
Equation	R Square	F	df1	df2	Sig.	Constant	b1
Linear	.867	467.915	1	72	.000	-.068	.068
Power	.917	792.300	1	72	.000	.012	2.221
S	.901	654.076	1	72	.000	-.808	-3.882
Exponential	.883	541.122	1	72	.000	.005	1.121

The independent variable is MilimeterM.

Table 11 ANOVA one-way test of differences for log transformed lifetime fertility for all populations.

ANOVA
Log Fertility

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.084	3	.695	4.315	.014
Within Groups	4.025	25	.161		
Total	6.110	28			

Multiple Comparisons
Dependent Variable: Log Fertility
Tukey HSD

					95% Confidence Interval	
(I) Populations	(J) Populations	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
A	B	.08775	.32172	.993	-.7972	.9727
	C	.13527	.20767	.914	-.4360	.7065
	G	-.46130	.19084	.100	-.9862	.0636
B	A	-.08775	.32172	.993	-.9727	.7972
	C	.04753	.31722	.999	-.8250	.9201
	G	-.54904	.30647	.301	-1.3920	.2939
C	A	-.13527	.20767	.914	-.7065	.4360
	B	-.04753	.31722	.999	-.9201	.8250
	G	-.59657(*)	.18315	.016	-1.1003	-.0928
G	A	.46130	.19084	.100	-.0636	.9862
	B	.54904	.30647	.301	-.2939	1.3920
	C	.59657(*)	.18315	.016	.0928	1.1003

* The mean difference is significant at the .05 level.

Table 12 ANOVA one-way test of difference for age at maturity (days) for all populations.

ANOVA
Age at maturity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	307.773	4	76.943	4.242	.004

Within Groups	1033.920	57	18.139		
Total	1341.694	61			

Multiple Comparisons
Dependent Variable: Age at maturity
Tukey HSD

(I) Populations	(J) Populations	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	-2.65152	1.77780	.572	-7.6596	2.3566
	C	-3.11230	1.64803	.335	-7.7548	1.5302
	D	2.84848	2.77404	.842	-4.9660	10.6630
	G	1.81340	1.61359	.793	-2.7321	6.3589
B	A	2.65152	1.77780	.572	-2.3566	7.6596
	C	-.46078	1.60579	.998	-4.9843	4.0628
	D	5.50000	2.74916	.279	-2.2444	13.2444
	G	4.46491(*)	1.57043	.047	.0410	8.8888
C	A	3.11230	1.64803	.335	-1.5302	7.7548
	B	.46078	1.60579	.998	-4.0628	4.9843
	D	5.96078	2.66708	.182	-1.5524	13.4740
	G	4.92570(*)	1.42186	.009	.9203	8.9311
D	A	-2.84848	2.77404	.842	-10.6630	4.9660
	B	-5.50000	2.74916	.279	-13.2444	2.2444
	C	-5.96078	2.66708	.182	-13.4740	1.5524
	G	-1.03509	2.64594	.995	-8.4887	6.4186
G	A	-1.81340	1.61359	.793	-6.3589	2.7321
	B	-4.46491(*)	1.57043	.047	-8.8888	-.0410
	C	-4.92570(*)	1.42186	.009	-8.9311	-.9203
	D	1.03509	2.64594	.995	-6.4186	8.4887

* The mean difference is significant at the .05 level.

Table 13 Kaplan-Meier survival analysis using age at maturity as events.

Means and Medians for Survival Time

Populations	Mean(a)				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
	Lower Bound	Upper Bound	Lower Bound	Upper Bound	Lower Bound	Upper Bound	Lower Bound	Upper Bound
A	15.182	.569	14.066	16.297	15.000	.591	13.842	16.158
B	17.833	1.192	15.496	20.170	17.000	.500	16.020	17.980
C	18.294	1.393	15.565	21.024	17.000	1.646	13.773	20.227
D	12.333	1.667	9.067	15.600	14.000	.000	.	.
G	13.368	.883	11.638	15.098	13.000	.353	12.308	13.692
Overall	15.855	.596	14.687	17.022	15.000	.702	13.624	16.376

a Estimation is limited to the largest survival time if it is censored.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	18.029	4	.001
Breslow (Generalized Wilcoxon)	22.049	4	.000
Tarone-Ware	20.568	4	.000

Test of equality of survival distributions for the different levels of Populations.

Table 14 ANOVA one-way test of difference for log transformed size at maturity (μg) for all populations.

ANOVA

Log Size (μg) at maturity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.904	4	.226	32.770	.000
Within Groups	.393	57	.007		
Total	1.297	61			

Multiple Comparisons

Dependent Variable: Log Size (μg) at maturity

Tukey HSD

(I) Populations	(J) Populations	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	.00049	.03466	1.000	-.0971	.0981
	C	.10626(*)	.03213	.014	.0157	.1968
	D	.20048(*)	.05408	.004	.0481	.3528
	G	-.17869(*)	.03146	.000	-.2673	-.0901
B	A	-.00049	.03466	1.000	-.0981	.0971
	C	.10577(*)	.03131	.011	.0176	.1940
	D	.19999(*)	.05360	.004	.0490	.3510
	G	-.17918(*)	.03062	.000	-.2654	-.0929
C	A	-.10626(*)	.03213	.014	-.1968	-.0157
	B	-.10577(*)	.03131	.011	-.1940	-.0176
	D	.09423	.05200	.377	-.0522	.2407
	G	-.28495(*)	.02772	.000	-.3630	-.2069
D	A	-.20048(*)	.05408	.004	-.3528	-.0481
	B	-.19999(*)	.05360	.004	-.3510	-.0490
	C	-.09423	.05200	.377	-.2407	.0522
	G	-.37918(*)	.05158	.000	-.5245	-.2339
G	A	.17869(*)	.03146	.000	.0901	.2673
	B	.17918(*)	.03062	.000	.0929	.2654
	C	.28495(*)	.02772	.000	.2069	.3630
	D	.37918(*)	.05158	.000	.2339	.5245

* The mean difference is significant at the .05 level.

Table 15 Correlation between size at maturity and days of survival.

Correlations

		Size (μg) at maturity	Days of survival
Size (μg) at maturity	Pearson Correlation	1	.208
	Sig. (2-tailed)		.105
	N	62	62
Days of survival	Pearson Correlation	.208	1
	Sig. (2-tailed)	.105	

	N	62	62

Table 16 Non-parametric Kruskal-Wallis test of differences for growth rate (µg/day) for all populations.

Ranks

	Populations	N	Mean Rank
Growth rate (µg)	A	11	37.00
	B	13	43.15
	C	17	24.59
	D	11	24.64
	G	15	41.40
	Total	67	

Test Statistics(a,b)

	Growth rate (µg)
Chi-Square	11.800
df	4
Asymp. Sig.	.019

a Kruskal Wallis Test
b Grouping Variable: Populations

Table 17 Pairwise non-parametric Mann-Whitney test for differences between growth rate (µg/day) for all populations.

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Growth rate (µg)	A	11	11.55	127.00
	B	13	13.31	173.00
	Total	24		

Test Statistics(b)

	Growth rate (µg)
Mann-Whitney U	61.000
Wilcoxon W	127.000
Z	-.608
Asymp. Sig. (2-tailed)	.543
Exact Sig. [2*(1-tailed Sig.)]	.569(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Growth rate (µg)	A	11	18.27	201.00
	C	17	12.06	205.00
	Total	28		

Test Statistics(b)

	Growth rate (µg)
Mann-Whitney U	52.000
Wilcoxon W	205.000
Z	-1.952
Asymp. Sig. (2-tailed)	.051
Exact Sig. [2*(1-tailed Sig.)]	.053(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Growth rate (µg)	A	11	13.82	152.00
	D	11	9.18	101.00
	Total	22		

Test Statistics(b)

	Growth rate (µg)
Mann-Whitney U	35.000
Wilcoxon W	101.000
Z	-1.674
Asymp. Sig. (2-tailed)	.094
Exact Sig. [2*(1-tailed Sig.)]	.101(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Growth rate (µg)	A	11	11.36	125.00
	G	15	15.07	226.00
	Total	26		

Test Statistics(b)

	Growth rate (µg)
Mann-Whitney U	59.000
Wilcoxon W	125.000
Z	-1.220
Asymp. Sig. (2-tailed)	.223
Exact Sig. [2*(1-tailed Sig.)]	.237(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Growth rate (µg)	B	13	21.31	277.00
	C	17	11.06	188.00

	Populations	N	Mean Rank	Sum of Ranks
	Total	30		

Test Statistics(b)

	Growth rate (µg)
Mann-Whitney U	35.000
Wilcoxon W	188.000
Z	-3.160
Asymp. Sig. (2-tailed)	.002
Exact Sig. [2*(1-tailed Sig.)]	.001(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Growth rate (µg)	B	13	15.62	203.00
	D	11	8.82	97.00
	Total	24		

Test Statistics(b)

	Growth rate (µg)
Mann-Whitney U	31.000
Wilcoxon W	97.000
Z	-2.346
Asymp. Sig. (2-tailed)	.019
Exact Sig. [2*(1-tailed Sig.)]	.018(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Growth rate (µg)	B	13	13.92	181.00
	G	15	15.00	225.00
	Total	28		

Test Statistics(b)

	Growth rate (µg)
Mann-Whitney U	90.000
Wilcoxon W	181.000
Z	-.345
Asymp. Sig. (2-tailed)	.730
Exact Sig. [2*(1-tailed Sig.)]	.751(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks

		N	Mean Rank	Sum of Ranks
Growth rate (µg)	C	17	15.18	258.00
	D	11	13.45	148.00
	Total	28		

Test Statistics(b)

	Growth rate (µg)
Mann-Whitney U	82.000
Wilcoxon W	148.000
Z	-.541
Asymp. Sig. (2-tailed)	.589
Exact Sig. [2*(1-tailed Sig.)]	.611(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Growth rate (µg)	C	17	13.29	226.00
	G	15	20.13	302.00
	Total	32		

Test Statistics(b)

	Growth rate (µg)
Mann-Whitney U	73.000
Wilcoxon W	226.000
Z	-2.058
Asymp. Sig. (2-tailed)	.040
Exact Sig. [2*(1-tailed Sig.)]	.040(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Growth rate (µg)	D	11	11.18	123.00
	G	15	15.20	228.00
	Total	26		

Test Statistics(b)

	Growth rate (µg)
Mann-Whitney U	57.000
Wilcoxon W	123.000
Z	-1.323
Asymp. Sig. (2-tailed)	.186
Exact Sig. [2*(1-tailed Sig.)]	.198(a)

a Not corrected for ties.
b Grouping Variable: Populations

Table 18 ANOVA one-way test of differences for the growth rate (µg) the first 10 days for the different populations.

ANOVA

Growth rate (µg/day) first 10 days

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	101.176	4	25.294	7.848	.001
Within Groups	48.344	15	3.223		
Total	149.520	19			

Multiple Comparisons

Dependent Variable: Growth rate (µg/day) first 10 days

Tukey HSD

(I) Pop for 10 days	(J) Pop for 10 days	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	-.36194	1.26944	.998	-4.2819	3.5580
	C	1.09313	1.26944	.907	-2.8268	5.0130
	D	.49972	1.26944	.994	-3.4202	4.4196
	G	-5.18057(*)	1.26944	.007	-9.1005	-1.2606
B	A	.36194	1.26944	.998	-3.5580	4.2819
	C	1.45506	1.26944	.780	-2.4649	5.3750
	D	.86166	1.26944	.958	-3.0583	4.7816
	G	-4.81863(*)	1.26944	.013	-8.7386	-.8987
C	A	-1.09313	1.26944	.907	-5.0130	2.8268
	B	-1.45506	1.26944	.780	-5.3750	2.4649
	D	-.59341	1.26944	.989	-4.5133	3.3265
	G	-6.27369(*)	1.26944	.001	-10.1936	-2.3538
D	A	-.49972	1.26944	.994	-4.4196	3.4202
	B	-.86166	1.26944	.958	-4.7816	3.0583
	C	.59341	1.26944	.989	-3.3265	4.5133
	G	-5.68029(*)	1.26944	.003	-9.6002	-1.7604
G	A	5.18057(*)	1.26944	.007	1.2606	9.1005
	B	4.81863(*)	1.26944	.013	.8987	8.7386
	C	6.27369(*)	1.26944	.001	2.3538	10.1936
	D	5.68029(*)	1.26944	.003	1.7604	9.6002

* The mean difference is significant at the .05 level.

Table 19 ANOVA one-way test of differences for log transformed specific growth rate (SGR) for the first 10 days for all populations.

ANOVA

Log SGR

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.378	4	.095	5.551	.006
Within Groups	.256	15	.017		
Total	.634	19			

Multiple Comparisons
Dependent Variable: Log SGR
Tukey HSD

(I) Populations	(J) Populations	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	.16427	.09229	.419	-.1207	.4493
	C	.19857	.09229	.250	-.0864	.4836
	D	.08737	.09229	.874	-.1976	.3724
	G	-.18573	.09229	.307	-.4707	.0993
B	A	-.16427	.09229	.419	-.4493	.1207
	C	.03430	.09229	.995	-.2507	.3193
	D	-.07690	.09229	.916	-.3619	.2081
	G	-.35000(*)	.09229	.013	-.6350	-.0650
C	A	-.19857	.09229	.250	-.4836	.0864
	B	-.03430	.09229	.995	-.3193	.2507
	D	-.11121	.09229	.749	-.3962	.1738
	G	-.38430(*)	.09229	.006	-.6693	-.0993
D	A	-.08737	.09229	.874	-.3724	.1976
	B	.07690	.09229	.916	-.2081	.3619
	C	.11121	.09229	.749	-.1738	.3962
	G	-.27310	.09229	.063	-.5581	.0119
G	A	.18573	.09229	.307	-.0993	.4707
	B	.35000(*)	.09229	.013	.0650	.6350
	C	.38430(*)	.09229	.006	.0993	.6693
	D	.27310	.09229	.063	-.0119	.5581

* The mean difference is significant at the .05 level.

Table 20 Non-parametric Kruskal-Wallis test of differences for days of survival for all populations.

Ranks

	Populations	N	Mean Rank
Days	A	27	54.46
	B	28	55.11
	C	28	83.14
	D	19	34.45
	G	28	89.96
	Total	130	

Test Statistics(a,b)

	Days
Chi-Square	35.421
df	4
Asymp. Sig.	.000

a Kruskal Wallis Test
b Grouping Variable: Populations

Table 21 Pairwise non-parametric Mann-Whitney test for differences between days of survival for all populations.

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Days	A	27	28.35	765.50
	B	28	27.66	774.50
	Total	55		

Test Statistics(a)

	Days
Mann-Whitney U	368.500
Wilcoxon W	774.500
Z	-.160
Asymp. Sig. (2-tailed)	.873

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Days	A	27	20.81	562.00
	C	28	34.93	978.00
	Total	55		

Test Statistics(a)

	Days
Mann-Whitney U	184.000
Wilcoxon W	562.000
Z	-3.271
Asymp. Sig. (2-tailed)	.001

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Days	A	27	27.41	740.00
	D	19	17.95	341.00
	Total	46		

Test Statistics(a)

	Days
Mann-Whitney U	151.000
Wilcoxon W	341.000
Z	-2.366
Asymp. Sig. (2-tailed)	.018

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Days	A	27	19.89	537.00
	G	28	35.82	1003.00
	Total	55		

Test Statistics(a)

	Days
Mann-Whitney U	159.000
Wilcoxon W	537.000
Z	-3.700
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Days	B	28	22.66	634.50
	C	28	34.34	961.50
	Total	56		

Test Statistics(a)

	Days
Mann-Whitney U	228.500
Wilcoxon W	634.500
Z	-2.686
Asymp. Sig. (2-tailed)	.007

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Days	B	28	26.36	738.00
	D	19	20.53	390.00
	Total	47		

Test Statistics(a)

	Days
Mann-Whitney U	200.000
Wilcoxon W	390.000
Z	-1.442
Asymp. Sig. (2-tailed)	.149

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Days	B	28	21.93	614.00
	G	28	35.07	982.00
	Total	56		

Test Statistics(a)

	Days
Mann-Whitney U	208.000
Wilcoxon W	614.000
Z	-3.029
Asymp. Sig. (2-tailed)	.002

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Days	C	28	31.32	877.00
	D	19	13.21	251.00
	Total	47		

Test Statistics(a)

	Days
Mann-Whitney U	61.000
Wilcoxon W	251.000
Z	-4.455
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Days	C	28	26.05	729.50
	G	28	30.95	866.50
	Total	56		

Test Statistics(a)

	Days
Mann-Whitney U	323.500
Wilcoxon W	729.500
Z	-1.126
Asymp. Sig. (2-tailed)	.260

a Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Days	D	19	12.76	242.50
	G	28	31.63	885.50
	Total	47		

Test Statistics(a)

	Days
Mann-Whitney U	52.500
Wilcoxon W	242.500
Z	-4.656
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: Populations

Table 22 Kaplan-Meier survival analysis of cumulative survival for all populations.

Means and Medians for Survival Time

Populations	Mean(a)				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
	Lower Bound	Upper Bound	Lower Bound	Upper Bound	Lower Bound	Upper Bound	Lower Bound	Upper Bound
A	18.630	1.757	15.185	22.074	17.000	2.596	11.911	22.089

B	19.571	2.545	14.584	24.559	11.000	5.278	.655	21.345
C	29.357	2.232	24.983	33.731	29.000	1.759	25.552	32.448
D	12.368	1.540	9.350	15.387	11.000	.675	9.676	12.324
G	32.643	2.630	27.488	37.798	29.000	2.646	23.814	34.186
Overall	23.666	1.218	21.279	26.054	23.000	2.088	18.908	27.092

a Estimation is limited to the largest survival time if it is censored.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	36.117	4	.000
Breslow (Generalized Wilcoxon)	32.207	4	.000
Tarone-Ware	33.525	4	.000

Test of equality of survival distributions for the different levels of Populations.

Table 23 Correlation between days at maturity and days of survival.

Correlations

		Days of survival	Days of maturity
Days of survival	Pearson Correlation	1	.479(**)
	Sig. (2-tailed)		.000
	N	118	52
Days at maturity	Pearson Correlation	.479(**)	1
	Sig. (2-tailed)	.000	
	N	52	62

** Correlation is significant at the 0.01 level (2-tailed).

Table 24 Primers and respective peaks with size (bp), height (arbitrary values from capillary-electrophoresis), non-restrictive and restrictive allele counts, and height relations between possible homozygotes and heterozygotes (individuals from same age group or population were equal and one sample individual is represented below).

A adult

Primer	Sample File Primer	Size	Height		Non-restrictive	Restrictive	Non-restrictive #	Restrictive #	Height relations
512	KA1_A01_015.fsa	130.4397	1115	Homo	2	1			
512	KA1_A01_015.fsa	133.5794	506		1	1	3	2	2.20355731
513	KA1_A01_015.fsa	103.7788	507		1	1			2.1913215
513	KA1_A01_015.fsa	113.1343	1111	Homo	2	1	3	2	
514	KA1_A01_015.fsa	95.7624	290		1	1			
514	KA1_A01_015.fsa	97.7894	285		1	1	2	2	
514alt	KA2_B01_013.fsa	121.893	923		1	1			
514alt	KA2_B01_013.fsa	130.1354	663		1	1	2	2	
522	KA50_B07_061.fsa	119.3886	1710		1	1			
522	KA50_B07_061.fsa	121.4713	1585		1	1			
522	KA50_B07_061.fsa	124.349	1524		1	1			
522	KA50_B07_061.fsa	126.3646	1347		1	1	4	4	

525	KA50_B07_061.fsa	113.79	5978	Homo	2	1			
525	KA50_B07_061.fsa	123.1261	3173		1	1			2.04131808
525	KA50_B07_061.fsa	125.1367	2684		1	1	4	3	
							3	**2.5**	

A juvenile

Primer	Sample File Primer	Size	Height		Non-restrictive	Restrictive	Non-restrictive #	Restrictive #	Height relations
512	KA26_B04_030.fsa	130.3667	1290	Homo	2	1			1.94277108
512	KA26_B04_030.fsa	133.576	664		1	1	3	2	
513	KA26_B04_030.fsa	103.7012	1293		1	1			
513	KA26_B04_030.fsa	113.0107	2921	Homo	2	1	3	2	2.25908739
514	KA26_B04_030.fsa	95.7721	144		1	1			
514	KA26_B04_030.fsa	97.7986	129		1	1	2	2	
514alt	KA26_B04_030.fsa	121.7901	761		1	1			
514alt	KA26_B04_030.fsa	130.0209	503		1	1	2	2	
522	KA75_C10_076.fsa	119.4172	2614		1	1			
522	KA75_C10_076.fsa	121.5323	3086		1	1			
522	KA75_C10_076.fsa	124.4524	2873		1	1			
522	KA76_D10_074.fsa	126.4632	3524		1	1	4	4	
525	KA75_C10_076.fsa	113.8114	13207	Homo	2	1			2.28930491
525	KA75_C10_076.fsa	123.1233	6049		1	1			
525	KA75_C10_076.fsa	125.2512	5489		1	1	4	3	
							3	**2.5**	

B adult

Primer	Sample File Primer	Size	Height		Non-restrictive	Restrictive	Non-restrictive #	Restrictive #	Height relations
512	KA5_E01_007.fsa	130.4397	3455	Homo	2	1			2.02165009
512	KA5_E01_007.fsa	135.832	1709		1	1			
512	KA5_E01_007.fsa	140.2945	3412	Homo	2	1	5	3	1.99648917
513	KA5_E01_007.fsa	113.1343	8267		2	1	2	2	
514	KA5_E01_007.fsa	93.8313	444		1	1			
514	KA5_E01_007.fsa	95.8577	907	Homo	2	1			2.09953704
514	KA5_E01_007.fsa	97.9753	420		1	1	4	3	
514alt	KA5_E01_007.fsa	121.9072	2030		1	1			
514alt	KA5_E01_007.fsa	136.3739	1860		1	1	2	2	
522	KA53_E07_055.fsa	124.4648	2331	Homo	1	1			2.46927966
522	KA53_E07_055.fsa	126.5078	944		1	1	2	2	

525	KA53_E07_055.fsa	116.8829	2231		1	1			
525	KA53_E07_055.fsa	120.046	6633	Homox3	3	1	4	2	2.97310623
							3.167	**2.333**	

B juvenile

Primer	Sample File Primer	Size	Height		Non-restrictive	Restrictive	Non-restrictive #	Restrictive #	Height relations
512	KA29_E04_024.fsa	130.3452	613	Homo	2	1			2.47177419
512	KA29_E04_024.fsa	135.7179	248		1	1			
512	KA29_E04_024.fsa	140.1646	551	Homo	2	1	5	3	2.22177419
514alt	KA29_E04_024.fsa	121.8082	973		1	1			
514alt	KA29_E04_024.fsa	136.2489	773		1	1	2	2	
522	KA77_E10_072.fsa	124.4725	5602	Homo	2	1			1.98934659
522	KA77_E10_072.fsa	126.5124	2816		1	1	3	2	
525	KA77_E10_072.fsa	116.9072	5356		1	1			
525	KA77_E10_072.fsa	119.9746	13115	Homo	2	1	3	2	2.44865571
							3.25	**2.25**	

C adult

Primer	Sample File Primer	Size	Height		Non-restrictive	Restrictive	Non-restrictive #	Restrictive #	Height relations
512	KA9_A02_016.fsa	130.3797	622		1	1			
512	KA9_A02_016.fsa	141.415	1492		2	1	3	2	2.39871383
513	KA9_A02_016.fsa	112.9918	608		1	1			
513	KA9_A02_016.fsa	114.8312	398		1	1	2	2	
514 alt	KA9_A02_016.fsa	136.1721	309		1	1			
515 alt	KA9_A02_016.fsa	143.013	681	Homo	2	1	2	3	2.2038835
522	KA57_A08_064.fsa	122.4379	433		1	1			
522	KA57_A08_064.fsa	124.4295	555		1	1	2	2	
525	KA57_A08_064.fsa	116.9289	4764	Homo	2	1			2.31937683
525	KA57_A08_064.fsa	119.9358	2054		1	1	3	2	
							2.4	**2.2**	

C juvenile

Primer	Sample File Primer	Size	Height		Non-restrictive	Restrictive	Non-restrictive #	Restrictive #	Height relations
512	KA30_F04_022.fsa	130.4128	1005		1	1			
512	KA30_F04_022.fsa	141.3298	2024	Homo	2	1	3	2	2.01393035
513	KA30_F04_022.fsa	112.9476	1488		1	1			
513	KA30_F04_022.fsa	114.8392	1004		1	1	2	2	

514	KA30_F04_022.fsa	93.8823	369	Homo	2	1			2.06145251
514	KA30_F04_022.fsa	99.8202	179		1	1	3	2	
514alt	KA30_F04_022.fsa	136.1548	667		1	1			
514alt	KA30_F04_022.fsa	142.9684	1633	Homo	2	1	3	2	2.44827586
522	KA79_G10_068.fsa	122.564	3437		1	1			
522	KA79_G10_068.fsa	124.4396	4447		1	1	2	2	
525	KA79_G10_068.fsa	116.9675	16541		2	1			1.94371328
525	KA79_G10_068.fsa	119.9822	8510		1	1	3	2	
							2.666	**2**	

D adult

Primer	Sample File Primer	Size	Height		Non-restrictive	Restrictive	Non-restrictive #	Restrictive #	Height relations
512	KA14_F02_006.fsa	130.3797	1175		1	1			
512	KA14_F02_006.fsa	135.8189	1122		1	1			
512	KA14_F02_006.fsa	140.2659	2601	Homo	2	1	4	3	2.26469308
513	KA14_F02_006.fsa	112.9773	3751		1	1	2	2	
514	KA14_F02_006.fsa	94.7322	71		1	1			
514	KA14_F02_006.fsa	96.7869	191	Homo	2	1	3	2	2.69014085
514alt	KA14_F02_006.fsa	121.7927	2001		1	1			
514alt	KA14_F02_006.fsa	136.2597	1489		1	1	2	2	
522	KA61_E08_056.fsa	124.3898	1339	Homo	2	1			1.93777135
522	KA61_E08_056.fsa	126.3774	691		1	1	3	2	
525	KA61_E08_056.fsa	116.7671	4275		1	1			
525	KA61_E08_056.fsa	119.9232	10107		2	1	3	2	2.36421053
							2.833	**2.166**	

D juvenile

Primer	Sample File Primer	Size	Heigth		Non-restrictive	Restrictive	Non-restrictive #	Restrictive #	Height relations
512	KA34_B05_045.fsa	130.3964	1271	Homo	2	1			
512	KA34_B05_045.fsa	135.8263	953		1	1			
512	KA34_B05_045.fsa	140.2535	2324	Homo	2	1	5	3	2.08992806
514alt	KA34_B05_045.fsa	121.8155	661		1	1			
514alt	KA34_B05_045.fsa	136.2662	583		1	1	2	2	
522	KA82_B11_093.fsa	124.4516	1953	Homo	2	1			1.62885738
522	KA82_B11_093.fsa	126.4982	1199		1	1	3	2	
525	KA82_B11_093.fsa	116.9984	7011		1	1			
525	KA82_B11_093.fsa	120.0379	17070	Homo	2	1	3	2	2.4347454

							3.25	**2.25**	

G adult

Primer	Sample File Primer	Size	Height		Non-restrictive	Restrictive	Non-restrictive #	Restrictive #	Heigth relations
512	KA16_H02_002.fsa	142.775	4305	Homo	2	1	2	1	
513	KA16_H02_002.fsa	111.1612	1729		1	1			
513	KA16_H02_002.fsa	112.9528	1366		1	1	2	2	
514	KA16_H02_002.fsa	95.9803	548	Homo	2	1	2	1	
514alt	KA37_E05_039.fsa	106.5784	2907	Homo	2	1	2	1	
522	KA66_B09_077.fsa	128.4546	1991	Homo	2	1	2	2	
525	KA66_B09_077.fsa	117.0086	7133		1	1			
525	KA66_B09_077.fsa	128.2743	6665		1	1	2	2	
							2	**1.50**	

G juvenile

Primer	Sample File Primer	Size	Height		Non-restrictive	Restrictive	# Non-restrictive	# Restrictive	Height relations
512	KA37_E05_039.fsa	142.6069	2363	Homo	2	1	2	1	
513	KA37_E05_039.fsa	111.0833	1613		1	1			
513	KA37_E05_039.fsa	112.8573	1370		1	1	2	2	
514alt	KA39_G05_035.fsa	106.4873	7282	Homo	2	1	2	1	
522	KA85_E11_087.fsa	128.3324	4916	Homo	2	1	2	1	
523	KA85_E11_087.fsa	116.9631	879	Homo	2	1	2	1	
525	KA85_E11_087.fsa	116.9631	21153		1	1			
525	KA85_E11_087.fsa	128.2428	24273		1	1	2	2	
							2	**1.33**	

Table 25 Legend for Fig. 43 taken from Colbourne *et al.* (1998).

DOF	District of Franklin, Canada	ONT	Ontario, Canada
ESB	Eastern Siberia, Russia	ORE	Oregon, USA
GER	Germany	SAS	Saskatchewan, Canada
GRL	Greenland	SVL	Svalbard
ICE	Iceland	SWI	Switzerland
NOR	Norway	WSB	Western Siberia, Russia
NWT	Northwest Territories, Canada	WAS	Washington, USA

Table 26 Non-parametric Kruskal-Wallis test of difference for total signal per dry weight (µg) for pooled age classes for all populations.

Ranks

	Populations	N	Mean Rank
Total signal per dry weight (µg)	A	8	33.00
	B	7	5.71
	C	8	22.25
	D	6	19.17
	G	11	20.27
	Total	40	

Test Statistics(a,b)

	Total signal per dry weight (µg)
Chi-Square	20.605
df	4
Asymp. Sig.	.000

a Kruskal Wallis Test
b Grouping Variable: Populations

Table 27 Pairwise non-parametric Mann-Whitney test for differences between total signal per dry weight (µg) for all populations.

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Total signal per dry weight (µg)	A	8	11.50	92.00
	B	7	4.00	28.00
	Total	15		

Test Statistics(b)

	Total signal per dry weight (µg)
Mann-Whitney U	.000
Wilcoxon W	28.000
Z	-3.240
Asymp. Sig. (2-tailed)	.001
Exact Sig. [2*(1-tailed Sig.)]	.000(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Total signal per dry weight (µg)	A	8	11.13	89.00
	C	8	5.88	47.00
	Total	16		

Test Statistics(b)

	Total signal per dry weight (µg)
Mann-Whitney U	11.000
Wilcoxon W	47.000
Z	-2.205
Asymp. Sig. (2-tailed)	.027
Exact Sig. [2*(1-tailed Sig.)]	.028(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Total signal per dry weight (µg)	A	8	9.50	76.00
	D	6	4.83	29.00
	Total	14		

Test Statistics(b)

	Total signal per dry weight (µg)
Mann-Whitney U	8.000
Wilcoxon W	29.000
Z	-2.066
Asymp. Sig. (2-tailed)	.039
Exact Sig. [2*(1-tailed Sig.)]	.043(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Total signal per dry weight (µg)	A	8	14.38	115.00
	G	11	6.82	75.00
	Total	19		

Test Statistics(b)

	Total signal per dry weight (µg)
Mann-Whitney U	9.000

Wilcoxon W	75.000
Z	-2.890
Asymp. Sig. (2-tailed)	.004
Exact Sig. [2*(1-tailed Sig.)]	.003(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Total signal per dry weight (µg)	B	7	4.14	29.00
	C	8	11.38	91.00
	Total	15		

Test Statistics(b)

	Total signal per dry weight (µg)
Mann-Whitney U	1.000
Wilcoxon W	29.000
Z	-3.125
Asymp. Sig. (2-tailed)	.002
Exact Sig. [2*(1-tailed Sig.)]	.001(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Total signal per dry weight (µg)	B	7	5.29	37.00
	D	6	9.00	54.00
	Total	13		

Test Statistics(b)

	Total signal per dry weight (µg)
Mann-Whitney U	9.000
Wilcoxon W	37.000
Z	-1.714
Asymp. Sig. (2-tailed)	.086
Exact Sig. [2*(1-tailed Sig.)]	.101(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Total signal per dry weight (µg)	B	7	4.29	30.00
	G	11	12.82	141.00
	Total	18		

Test Statistics(b)

	Total signal per dry weight (µg)
Mann-Whitney U	2.000
Wilcoxon W	30.000
Z	-3.306
Asymp. Sig. (2-tailed)	.001
Exact Sig. [2*(1-tailed Sig.)]	.000(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Total signal per dry weight (µg)	C	8	8.00	64.00
	D	6	6.83	41.00
	Total	14		

Test Statistics(b)

	Total signal per dry weight (µg)
Mann-Whitney U	20.000
Wilcoxon W	41.000
Z	-.516
Asymp. Sig. (2-tailed)	.606
Exact Sig. [2*(1-tailed Sig.)]	.662(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Total signal per dry weight (µg)	C	8	10.50	84.00
	G	11	9.64	106.00
	Total	19		

Test Statistics(b)

	Total signal per dry weight (µg)
Mann-Whitney U	40.000
Wilcoxon W	106.000
Z	-.330
Asymp. Sig. (2-tailed)	.741
Exact Sig. [2*(1-tailed Sig.)]	.778(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
Total signal per dry weight (μg)	D	6	9.00	54.00
	G	11	9.00	99.00
	Total	17		

Test Statistics(b)

	Total signal per dry weight (μg)
Mann-Whitney U	33.000
Wilcoxon W	99.000
Z	.000
Asymp. Sig. (2-tailed)	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000(a)

a Not corrected for ties.
b Grouping Variable: Populations

Table 28 Non-parametric Kruskal-Wallis test of differences for RNA/dry weight (μg) for all populations.

Ranks

	Populations	N	Mean Rank
RNA/dry weight (μg)	A	8	29.75
	B	7	8.00
	C	8	20.13
	D	6	21.67
	G	11	21.36
	Total	40	

Test Statistics(a,b)

	RNA/dry weight (μg)
Chi-Square	13.140
df	4
Asymp. Sig.	.011

a Kruskal Wallis Test
b Grouping Variable: Populations

Table 29 Pairwise non-parametric Mann-Whitney test for differences between RNA/dry weight (μg) for all populations.

Ranks

	Populations	N	Mean Rank	Sum of Ranks
RNA/dry weight (μg)	A	8	11.50	92.00
	B	7	4.00	28.00
	Total	15		

Test Statistics(b)

	RNA/dry weight (μg)
Mann-Whitney U	.000
Wilcoxon W	28.000
Z	-3.240
Asymp. Sig. (2-tailed)	.001
Exact Sig. [2*(1-tailed Sig.)]	.000(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
RNA/dry weight (μg)	A	8	10.88	87.00
	C	8	6.13	49.00
	Total	16		

Test Statistics(b)

	RNA/dry weight (μg)
Mann-Whitney U	13.000
Wilcoxon W	49.000
Z	-1.995
Asymp. Sig. (2-tailed)	.046
Exact Sig. [2*(1-tailed Sig.)]	.050(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
RNA/dry weight (μg)	A	8	8.63	69.00
	D	6	6.00	36.00
	Total	14		

Test Statistics(b)

	RNA/dry weight (μg)
Mann-Whitney U	15.000
Wilcoxon W	36.000
Z	-1.162
Asymp. Sig. (2-tailed)	.245
Exact Sig. [2*(1-tailed Sig.)]	.282(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
RNA/dry weight (μg)	A	8	12.25	98.00

	Populations	N	Mean Rank	Sum of Ranks
	G	11	8.36	92.00
	Total	19		

Test Statistics(b)

	RNA/dry weight (μg)
Mann-Whitney U	26.000
Wilcoxon W	92.000
Z	-1.486
Asymp. Sig. (2-tailed)	.137
Exact Sig. [2*(1-tailed Sig.)]	.152(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
RNA/dry weight (μg)	B	7	5.43	38.00
	C	8	10.25	82.00
	Total	15		

Test Statistics(b)

	RNA/dry weight (μg)
Mann-Whitney U	10.000
Wilcoxon W	38.000
Z	-2.083
Asymp. Sig. (2-tailed)	.037
Exact Sig. [2*(1-tailed Sig.)]	.040(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
RNA/dry weight (μg)	B	7	5.00	35.00
	D	6	9.33	56.00
	Total	13		

Test Statistics(b)

	RNA/dry weight (μg)
Mann-Whitney U	7.000
Wilcoxon W	35.000
Z	-2.000
Asymp. Sig. (2-tailed)	.046
Exact Sig. [2*(1-tailed Sig.)]	.051(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
RNA/dry weight (µg)	B	7	5.57	39.00
	G	11	12.00	132.00
	Total	18		

Test Statistics(b)

	RNA/dry weight (µg)
Mann-Whitney U	11.000
Wilcoxon W	39.000
Z	-2.491
Asymp. Sig. (2-tailed)	.013
Exact Sig. [2*(1-tailed Sig.)]	.011(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
RNA/dry weight (µg)	C	8	7.13	57.00
	D	6	8.00	48.00
	Total	14		

Test Statistics(b)

	RNA/dry weight (µg)
Mann-Whitney U	21.000
Wilcoxon W	57.000
Z	-.387
Asymp. Sig. (2-tailed)	.699
Exact Sig. [2*(1-tailed Sig.)]	.755(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
RNA/dry weight (µg)	C	8	10.13	81.00
	G	11	9.91	109.00
	Total	19		

Test Statistics(b)

	RNA/dry weight (µg)
Mann-Whitney U	43.000
Wilcoxon W	109.000
Z	-.083
Asymp. Sig. (2-tailed)	.934
Exact Sig. [2*(1-tailed Sig.)]	.968(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
RNA/dry weight (μg)	D	6	8.83	53.00
	G	11	9.09	100.00
	Total	17		

Test Statistics(b)

	RNA/dry weight (μg)
Mann-Whitney U	32.000
Wilcoxon W	53.000
Z	-.101
Asymp. Sig. (2-tailed)	.920
Exact Sig. [2*(1-tailed Sig.)]	.961(a)

a Not corrected for ties.
b Grouping Variable: Populations

Table 30 ANOVA one-way test of differences for DNA/dry weight (μg) for the different populations.

ANOVA
DNA/dry weight (μg)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	4	.000	10.485	.000
Within Groups	.000	36	.000		
Total	.000	40			

Multiple Comparisons
Dependent Variable: DNA/dry weight (μg)
Tukey HSD

(I) Populations	(J) Populations	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	.00440(*)	.00073	.000	.0023	.0065
	C	.00195	.00073	.080	-.0002	.0041
	D	.00316(*)	.00079	.003	.0009	.0054
	G	.00329(*)	.00068	.000	.0013	.0053
B	A	-.00440(*)	.00073	.000	-.0065	-.0023
	C	-.00245(*)	.00073	.016	-.0046	-.0003
	D	-.00125	.00079	.524	-.0035	.0010
	G	-.00111	.00068	.489	-.0031	.0008
C	A	-.00195	.00073	.080	-.0041	.0002
	B	.00245(*)	.00073	.016	.0003	.0046
	D	.00120	.00079	.561	-.0011	.0035
	G	.00134	.00068	.307	-.0006	.0033
D	A	-.00316(*)	.00079	.003	-.0054	-.0009
	B	.00125	.00079	.524	-.0010	.0035
	C	-.00120	.00079	.561	-.0035	.0011
	G	.00014	.00075	1.000	-.0020	.0023

G	A	-.00329(*)	.00068	.000	-.0053	-.0013
	B	.00111	.00068	.489	-.0008	.0031
	C	-.00134	.00068	.307	-.0033	.0006
	D	-.00014	.00075	1.000	-.0023	.0020

* The mean difference is significant at the .05 level.

Table 31 ANOVA one-way test of differences for Protein/dry weight (μg) for the different populations.

ANOVA

Protein/dry weight (μg)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.130	4	.033	.902	.471
Within Groups	1.550	43	.036		
Total	1.680	47			

Multiple Comparisons

Dependent Variable: Protein/dry weight (ug)

Tukey HSD

(I) Populations	(J) Populations	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	.11646	.09826	.760	-.1633	.3962
	C	.06122	.08532	.951	-.1817	.3041
	D	-.06884	.10254	.962	-.3607	.2231
	G	.03882	.08415	.990	-.2007	.2784
B	A	-.11646	.09826	.760	-.3962	.1633
	C	-.05523	.08901	.971	-.3086	.1982
	D	-.18529	.10563	.413	-.4860	.1154
	G	-.07764	.08789	.901	-.3278	.1726
C	A	-.06122	.08532	.951	-.3041	.1817
	B	.05523	.08901	.971	-.1982	.3086
	D	-.13006	.09371	.639	-.3968	.1367
	G	-.02240	.07313	.998	-.2306	.1858
D	A	.06884	.10254	.962	-.2231	.3607
	B	.18529	.10563	.413	-.1154	.4860
	C	.13006	.09371	.639	-.1367	.3968
	G	.10766	.09264	.772	-.1561	.3714
G	A	-.03882	.08415	.990	-.2784	.2007
	B	.07764	.08789	.901	-.1726	.3278
	C	.02240	.07313	.998	-.1858	.2306
	D	-.10766	.09264	.772	-.3714	.1561

Table 32 ANOVA one-way test of differences for RNA/DNA for the different populations.

ANOVA

RNA/DNA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	56.994	4	14.248	1.374	.263
Within Groups	363.027	35	10.372		

Total	420.021	39			

Multiple Comparisons
Dependent Variable: RNA/DNA
Tukey HSD

(I) Populations	(J) Populations	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	-1.95936	1.66681	.765	-6.7516	2.8328
	C	.36055	1.61030	.999	-4.2692	4.9902
	D	-2.07639	1.73932	.755	-7.0770	2.9243
	G	-2.46080	1.49648	.480	-6.7633	1.8417
B	A	1.95936	1.66681	.765	-2.8328	6.7516
	C	2.31991	1.66681	.637	-2.4723	7.1121
	D	-.11703	1.79177	1.000	-5.2685	5.0344
	G	-.50143	1.55714	.998	-4.9783	3.9754
C	A	-.36055	1.61030	.999	-4.9902	4.2692
	B	-2.31991	1.66681	.637	-7.1121	2.4723
	D	-2.43694	1.73932	.631	-7.4376	2.5637
	G	-2.82134	1.49648	.344	-7.1238	1.4811
D	A	2.07639	1.73932	.755	-2.9243	7.0770
	B	.11703	1.79177	1.000	-5.0344	5.2685
	C	2.43694	1.73932	.631	-2.5637	7.4376
	G	-.38440	1.63451	.999	-5.0837	4.3149
G	A	2.46080	1.49648	.480	-1.8417	6.7633
	B	.50143	1.55714	.998	-3.9754	4.9783
	C	2.82134	1.49648	.344	-1.4811	7.1238
	D	.38440	1.63451	.999	-4.3149	5.0837

Table 33 ANOVA one-way test of differences for RNA/protein for the different populations.

ANOVA
RNA/protein

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.010	4	.002	3.891	.010
Within Groups	.021	34	.001		
Total	.031	38			

Multiple Comparisons
Dependent Variable: RNA/protein
Tukey HSD

(I) Populations	(J) Populations	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	.03781	.01352	.060	-.0011	.0767
	C	.03558	.01252	.054	-.0005	.0716
	D	.04704(*)	.01352	.011	.0081	.0860
	G	.03422(*)	.01163	.043	.0007	.0677
B	A	-.03781	.01352	.060	-.0767	.0011
	C	-.00223	.01352	1.000	-.0412	.0367
	D	.00922	.01445	.968	-.0324	.0508
	G	-.00360	.01270	.999	-.0402	.0330

C	A	-.03558	.01252	.054	-.0716	.0005
	B	.00223	.01352	1.000	-.0367	.0412
	D	.01146	.01352	.914	-.0275	.0504
	G	-.00136	.01163	1.000	-.0349	.0321
D	A	-.04704(*)	.01352	.011	-.0860	-.0081
	B	-.00922	.01445	.968	-.0508	.0324
	C	-.01146	.01352	.914	-.0504	.0275
	G	-.01282	.01270	.849	-.0494	.0238
G	A	-.03422(*)	.01163	.043	-.0677	-.0007
	B	.00360	.01270	.999	-.0330	.0402
	C	.00136	.01163	1.000	-.0321	.0349
	D	.01282	.01270	.849	-.0238	.0494

* The mean difference is significant at the .05 level.

Table 34 Non-parametric Kruskal-Wallis test of differences for DNA/protein for all populations.

Ranks

	Populations	N	Mean Rank
DNA/protein	A	8	32.13
	B	7	12.43
	C	8	22.88
	D	6	13.50
	G	11	19.27
	Total	40	

Test Statistics(a,b)

	DNA/protein
Chi-Square	13.850
df	4
Asymp. Sig.	.008

a Kruskal Wallis Test
b Grouping Variable: Populations

Table 35 Pairwise non-parametric Mann-Whitney test for differences between DNA/protein for all populations.

Ranks

	Populations	N	Mean Rank	Sum of Ranks
DNA/protein	A	8	10.88	87.00
	B	7	4.71	33.00
	Total	15		

Test Statistics(b)

	DNA/protein
Mann-Whitney U	5.000
Wilcoxon W	33.000
Z	-2.662
Asymp. Sig. (2-tailed)	.008

Exact Sig. [2*(1-tailed Sig.)]	.006(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
DNA/protein	A	8	10.13	81.00
	C	8	6.88	55.00
	Total	16		

Test Statistics(b)

	DNA/protein
Mann-Whitney U	19.000
Wilcoxon W	55.000
Z	-1.365
Asymp. Sig. (2-tailed)	.172
Exact Sig. [2*(1-tailed Sig.)]	.195(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
DNA/protein	A	8	10.25	82.00
	D	6	3.83	23.00
	Total	14		

Test Statistics(b)

	DNA/protein
Mann-Whitney U	2.000
Wilcoxon W	23.000
Z	-2.840
Asymp. Sig. (2-tailed)	.005
Exact Sig. [2*(1-tailed Sig.)]	.003(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
DNA/protein	A	8	14.38	115.00
	G	11	6.82	75.00
	Total	19		

Test Statistics(b)

	DNA/protein
Mann-Whitney U	9.000
Wilcoxon W	75.000
Z	-2.890
Asymp. Sig. (2-tailed)	.004

Exact Sig. [2*(1-tailed Sig.)]	.003(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
DNA/protein	B	7	6.00	42.00
	C	8	9.75	78.00
	Total	15		

Test Statistics(b)

	DNA/protein
Mann-Whitney U	14.000
Wilcoxon W	42.000
Z	-1.620
Asymp. Sig. (2-tailed)	.105
Exact Sig. [2*(1-tailed Sig.)]	.121(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
DNA/protein	B	7	6.57	46.00
	D	6	7.50	45.00
	Total	13		

Test Statistics(b)

	DNA/protein
Mann-Whitney U	18.000
Wilcoxon W	46.000
Z	-.429
Asymp. Sig. (2-tailed)	.668
Exact Sig. [2*(1-tailed Sig.)]	.731(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
DNA/protein	B	7	7.14	50.00
	G	11	11.00	121.00
	Total	18		

Test Statistics(b)

	DNA/protein
Mann-Whitney U	22.000
Wilcoxon W	50.000
Z	-1.494
Asymp. Sig. (2-tailed)	.135

Exact Sig. [2*(1-tailed Sig.)]	.151(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
DNA/protein	C	8	8.75	70.00
	D	6	5.83	35.00
	Total	14		

Test Statistics(b)

	DNA/protein
Mann-Whitney U	14.000
Wilcoxon W	35.000
Z	-1.291
Asymp. Sig. (2-tailed)	.197
Exact Sig. [2*(1-tailed Sig.)]	.228(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
DNA/protein	C	8	11.00	88.00
	G	11	9.27	102.00
	Total	19		

Test Statistics(b)

	DNA/protein
Mann-Whitney U	36.000
Wilcoxon W	102.000
Z	-.661
Asymp. Sig. (2-tailed)	.509
Exact Sig. [2*(1-tailed Sig.)]	.545(a)

a Not corrected for ties.
b Grouping Variable: Populations

Ranks

	Populations	N	Mean Rank	Sum of Ranks
DNA/protein	D	6	6.83	41.00
	G	11	10.18	112.00
	Total	17		

Test Statistics(b)

	DNA/protein
Mann-Whitney U	20.000
Wilcoxon W	41.000
Z	-1.307
Asymp. Sig. (2-tailed)	.191

Exact Sig. [2*(1-tailed Sig.)]	.216(a)

a Not corrected for ties.
b Grouping Variable: Populations

Table 36 Correlation between RNA/dry weight (µg) & DNA/dry weight (µg) for all populations.

Correlations

		RNA/dry weight (µg)	DNA/dry weight (µg)
RNA/dry weight (ug)	Pearson Correlation	1	.635(**)
	Sig. (2-tailed)		.000
	N	40	40
DNA/dry weight (ug)	Pearson Correlation	.635(**)	1
	Sig. (2-tailed)	.000	
	N	40	41

** Correlation is significant at the 0.01 level (2-tailed).

Table 37 Correlation between RNA/dry weight (µg) and protein/dry weight (µg) for all populations.

Correlations

		RNA/dry weight (ug)	Protein/dry weight (ug)
RNA/dry weight (ug)	Pearson Correlation	1	.599(**)
	Sig. (2-tailed)		.000
	N	40	39
Protein/dry weight (ug)	Pearson Correlation	.599(**)	1
	Sig. (2-tailed)	.000	
	N	39	48

** Correlation is significant at the 0.01 level (2-tailed).

Table 38 Correlation of Specific growth rate and RNA/dry weight (µg) for all populations.

Correlations

		RNA/dry weight (µg)	Specific growth rate, µ
RNA/dry weight (µg)	Pearson Correlation	1	.513
	Sig. (2-tailed)		.129
	N	10	10
Specific growth rate, µ	Pearson Correlation	.513	1
	Sig. (2-tailed)	.129	
	N	10	10

Table 39 Correlation of Specific growth rate and RNA/dry weight (µg) for assumed ploidy level.

Correlations

Control Variables	RNA/dry weight (µg)	Specific growth rate, µ

Assumed ploidy level	RNA/dry weight (µg)	Correlation	1.000	.917
		Significance (2-tailed)	.	.001
		df	0	7
	Specific growth rate, µ	Correlation	.917	1.000
		Significance (2-tailed)	.001	.
		df	7	0

Table 40 Correlation of Specific growth rate and DNA/dry weight (µg) for all populations.

Correlations

		Specific growth rate, µ	DNA/dry weight (µg)
Specific growth rate, µ	Pearson Correlation	1	.280
	Sig. (2-tailed)		.434
	N	10	10
DNA/dry weight (µg)	Pearson Correlation	.280	1
	Sig. (2-tailed)	.434	
	N	10	10

Table 41 Correlation of Specific growth rate and DNA/dry weight (µg) for assumed ploidy levels.

Correlations

Control Variables			Specific growth rate, µ	DNA/dry weight (µg)
Assumed ploidy level	Specific growth rate, µ	Correlation	1.000	.815
		Significance (2-tailed)	.	.007
		df	0	7
	DNA/dry weight (µg)	Correlation	.815	1.000
		Significance (2-tailed)	.007	.
		df	7	0

Table 42 Correlation of Specific growth rate and RNA/DNA for all populations.

Correlations

		Specific growth rate, µ	RNA/DNA
Specific growth rate, µ	Pearson Correlation	1	.427
	Sig. (2-tailed)		.218
	N	10	10
RNA/DNA	Pearson Correlation	.427	1
	Sig. (2-tailed)	.218	
	N	10	10

Table 43 Correlation of Specific growth rate and RNA/DNA for assumed ploidy levels.

Correlations

Control Variables			SpecG	RNA/DNA
Assumed ploidy level	SpecG	Correlation	1.000	.147
		Significance (2-tailed)	.	.706
		df	0	7
	RNA/DNA	Correlation	.147	1.000
		Significance (2-tailed)	.706	.
		df	7	0