UNIVERSITY OF NOVA GORICA
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ESTIMATION OF THERMAL SENSITIVITY OF DIFFERENT SPECIES WITHIN THE GENUS *DAPHNIA*

DISSERTATION

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ABSTRACT

Species of the genus *Daphnia* are a key element of zooplankton communities of many temperate lakes and ponds. Knowledge of their responses to temperature is therefore critical for understanding the effects climate change may have on these ecosystems. We measured metabolic activity and fitness of *Daphnia* collected from the field at different temperatures. There were significant differences between different species and between organisms of the same species, collected at different times in the season. Differences in thermal sensitivity of metabolism were reflected in growth and reproduction. Thermal sensitivity was connected with habitat preference. Lipid content of *Daphnia* depended mostly on the diet and was little affected by temperature acclimation or adaptation. We also examined the effects of an interaction between intergenerational changes in food quality and temperature on the fitness of two *Daphnia* clones. Maternal environment had a strong impact on the performance of offspring. The dietary requirements of *Daphnia* depended on temperature conditions. These interactions should therefore be taken into account when estimating productivity of natural populations.

Keywords: *Daphnia*, ETS activity, fitness, food quality, juvenile growth rate, lipids, maternal effects, respiration rate, reproduction, temperature acclimatization

POVZETEK

Ocena temperaturne občutljivosti različnih vrst iz rodu *Daphnia*

Vrste iz rodu *Daphnia* so ključni element zooplanktonskih združb mnogih jezer in mlak v zmernem klimatskem pasu. Poznavanje njihovega odziva na temperaturo je pomembno za razumevanje vpliva, ki ga utegnejo imeti klimatske spremembe na te ekosisteme. Pri različnih temperaturah smo merili presnovno aktivnost ter uspešnost (fitnes) različnih vrst iz rodu *Daphnia*, nabranih na terenu. Značilne razlike so bile tako med vrstami kot med osebki iste vrste, nabranimi v različnih časih med sezono. Razlike v temperaturni občutljivosti presnove so se odražale v rasti in razmnoževanju. Temperaturna občutljivost je bila povezana z življenjskim okoljem vrste. Vsebnost lipidov je bila odvisna predvsem od hrane; nanjo sta le malo vplivali temperaturna aklimacija ali adaptacija. Raziskovali smo tudi vpliv interakcije med medgeneracijskimi spremembami temperature okolja in kvalitete hrane na fitnes dveh klonov vodnih bolh. Maternalno okolje je imelo močan vpliv na uspešnost potomcev. Prehranske potrebe so bile odvisne od temperaturnih pogojev. Te interakcije je potrebno upoštevati pri ocenah produktivnosti naravnih populacij.

Ključne besede: aktivnost ETS, *Daphnia*, fitnes, hitrost dihanja, juvenilna rast, kvaliteta hrane, lipidi, maternalni vplivi, razmnoževanje, temperaturna aklimatizacija

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LIST OF SYMBOLS AND ABBREVIATIONS

A_{490nm}	Absorbance at the wavelength of 490 nm
AF	Acclimation food quality (maternal diet)
AICc	Akaike's information criterion corrected for small sample sizes
AT	Acclimation temperature
BAH	Beneficial acclimation hypothesis
Chl	*Chlamydomonas* sp.
CI	Confidence interval
Cry	*Cryptomonas* sp.
d.m.	Dry mass
E_a	Arrhenius activation energy
EF	Experimental food quality
ET	Experimental temperature
ETS	Electron transport system
ETS/R	The ratio of electron transport system activity to respiration rate
g_j	Juvenile growth rate
Hb	Haemoglobin
INT	2-(p-iodophenil)-3-(p-nitrophenil) tetrazolium chloride, a dye used in determination of electron transport system activity
k_{cat}	Catalytic rate constant in enzyme kinetics
K_m	The apparent Michaelis-Menten constant in enzyme kinetics
LT_{50}	Lethal temperature at which 50 % of experimental animals die
M	Body mass
Mr	Molecular mass
PC	Principal component
PCA	Principal component analysis
Po_2	Oxygen partial pressure
POC	Particulate organic carbon
Q	Metabolic rate
Q_{10}	The magnitude of the acceleration of metabolic rate if the temperature changes for 10 °C
R	Respiration rate
r	Intrinsic rate of population increase
r_{pot}	Potential rate of population increase – a measure of reproductive performance
SD	Standard deviation
T	Temperature

LIPID ABBREVIATIONS

ALA	α-linolenic acid – C18:3n3 fatty acid
ARA	Arachidonic acid – C20:4n6 fatty acid
Cho	Cholesterol content
DHA	Docosahexaenoic acid – C22:6n3 fatty acid
EPA	Eicosapentaenoic acid – C20:5n3 fatty acid
FA	Fatty acid
FAME	Fatty acid methyl ester
LIN	Linoleic acid – C18:2n6 fatty acid
MUFA	Monounsaturated fatty acids – fatty acids containing one double bond
PC	Phosphatidylcholine – a phospholipid species
PE	Phosphatidylethanolamine – a phospholipid species
PL	Phospholipid
PUFA	Polyunsaturated fatty acid – fatty acid containing more than one double bond
SAFA	Saturated fatty acid – fatty acid containing no double bonds
SDA	Stearidonic acid – C18:4n3 fatty acid
TAG	Triacylglycerols
UI	Unsaturation index – an index of the degree of unsaturation of fatty acids

1 INTRODUCTION

Temperature is an important environmental factor that affects the distribution of organisms. It affects the biochemical and physiological processes which are the basis of life. Global climate change, with the predicted rise of average environmental temperatures, more frequent extreme temperatures and greater fluctuations, can threaten the survival of some species in an ecosystem, and with it its stability and function. Due to climate change (Frantar, 2004), the ability of response of organisms to rising temperatures is of key importance. As lentic ecosystems (=lakes and ponds) are expected to respond directly to climate change, determination of their sensitivity to potential temperature change and the variability of their responses provides information necessary for the estimation of future aquatic ecosystems' functioning (Winder and Schindler, 2004a and b; Adrian *et al.*, 2006).

Among the key elements of zooplankton community of temperate freshwater lakes and ponds are water fleas (Crustacea: Cladocera), dominated by the species of the genus *Daphnia*. Due to their high numbers and feeding mode (filtration) they are the key link in nutrient circulation and energy flow from primary producers (phytoplankton) to higher trophic levels (invertebrate predators and planktivorus fish). That is why they are used as model organisms (Lampert, 2006).

Every organism of a given species has a specific temperature tolerance range, which enables its survival, growth, development and reproduction. The temperature tolerance range of a species (or population) is a sum of the tolerance ranges of its members and depends on evolutionary adaptations. It is determined by the general repertoire of adaptive mechanisms of the species. However, certain biochemical, physiological and behavioural mechanisms are available to individuals, which enable their phenotypic adaptation (acclimatization) to prevailing environmental temperatures (Kibby, 1971; Armitage and Lei, 1979; Venkataraman and Job, 1979; Galkovskaya and Morozov, 1981; McKee, 1995; Lamkemeyer *et al.*, 2003; Zeis *et al.*, 2004b). The scope of these mechanisms is species-specific. Animals maintain their metabolism on a certain level with adjustments in the structure and function of enzymes and structure and function of lipid membranes (Farkas and Herodek, 1964; Kasai *et al.*, 1976; Chapelle, 1978; Ohki *et al.*, 1979; Okuyama *et al.*, 1982; Rousch *et al.*, 2003; Schlechtriem *et al.*, 2006; Smirnov and Bogdan, 2006). All these adjustments enable individual organisms to respond to thermal stress. When individuals are unable to adjust to a changed environment, they do not survive and/or reproduce in it.

A population of an aquatic species can respond to temperature change in different ways: with a change in micro-distribution (e.g. diurnal vertical migrations), with a change in phenology (resting eggs formation in unfavourable conditions (Gyllstrom, 2004)), with acclimatization through phenotypic plasticity of individuals, with microevolution – acclimatization through a

change in the clonal (genetic) composition of the population, or, if all these mechanisms fail, with extinction (Demeester, 1996; Mitchell and Lampert, 2000; Van Doorslaer *et al.*, 2007). Clonal composition of *Daphnia* population changes during the season (Carvalho and Crisp, 1987). Summer clones have greater fitness at higher temperatures while winter clones perform better at lower temperatures (Carvalho, 1987; Pinkhaus *et al.*, 2007). By sampling at different times during the season, different clones are caught, which gives a good picture of the adaptive potential of a species. Ecological divergence for temperature tolerance is probably greater in *Daphnia* species from small and shallow habitats in comparison with species from deep, more thermally stable lakes (Carvalho, 1987), where temperature refuge (+4 °C) is always present (in the hypolimnion).

Many studies on temperature sensitivity of water fleas were conducted on individual clones (Sushchenya and Trubetskova, 1981; Korpelainen, 1986; Kessler and Lampert, 2004; Van Doorslaer *et al.*, 2007). The choice of a single genotype to represent a population is very risky without simultaneous knowledge of the clonal composition of the whole population. Thus the extrapolation of results to the entire population is limited. In our study we performed a part of experiments with organisms collected directly from natural populations, to reflect natural diversity. Because *Daphnia* individuals are relatively small, 30 or more individuals are joined into one replicate for most experiments. Higher number of specimens ensures a representative sample of the genetic variability of a population; which makes results ecologically relevant (effect of averaging; Lampert, 1984). Such an approach also has its flaws, because the width of the thermal tolerance range depends also on other environmental factors, such as food availability (Orcutt and Porter, 1984; Stich and Lampert, 1984; Sustchenya *et al.*, 1986; Xie *et al.*, 2000; Giebelhausen and Lampert, 2001; Kessler and Lampert, 2004; Rinke and Vijverberg, 2005), dissolved oxygen content (Laberge and Hann, 1990), presence of predators (Weetman and Atkinson, 2004), and the physiological status of the organism (age, energy reserves, reproductive cycle (Glazier, 1991), diseases and parasites). Results obtained in such way can therefore be difficult to compare between temporally distinct samples from the same population or between populations. While the interaction between food quantity and temperature is well explored (e.g. Orcutt and Porter, 1984; Giebelhausen and Lampert, 2001), the information on the potentially equally important interaction between food quality and temperature is scarce, and will therefore be investigated with the help of clones as a part of our study, along with maternal effects on offspring fitness.

The aim of our research is acquirement of new knowledge of thermal adaptations in freshwater invertebrates. With the help of our results, we will determine which *Daphnia* species are the most temperature sensitive and therefore appropriate for monitoring of the changes in freshwater ecosystems, caused by climate change. The research includes monitoring of

biochemical and physiological adaptations to temperature change and performance at different temperatures in pairs of closely related *Daphnia* species with different habitat preferences.

1.1 WORKING HYPOTHESES

(1) We presume that, since the chosen *Daphnia* species (*Daphnia pulex* Leydig, 1860, *D. pulicaria* Forbes, 1893, *D. rosea* G. O. Sars, 1862, and *D. hyalina* Leydig, 1860) represent a range of body sizes and thermally different habitats, they will have different thermal sensitivity, depending on their evolutionary adaptations (biochemical, physiological, behavioural and phenological) to the prevailing temperatures and temperature cycles in their environment.

(2) We expect that, after seasonal acclimatization or laboratory acclimation, cold-acclimatized/acclimated individuals will outperform warm-acclimatized/acclimated individuals at low temperatures, and the opposite to be true at high temperatures. We assume that this acclimatization/acclimation will be achieved through adjustments of membrane fluidity, adjustments on the enzyme level and adjustments in the metabolic rate.

(3) We assume that the species will differ in their ability to acclimate/acclimatize to different temperatures. Eurythermal (temperature tolerant) species, due to their effective adaptive mechanisms, will be able to elevate the relatively low metabolic activity at low temperatures and lower it at high temperatures. On the other hand, the stenothermal (narrow temperature adapted) species will not be able to adjust the intensity of their metabolism to changed temperatures. The capacity for acclimatization to certain environmental conditions will be reflected also in growth, reproduction and survival.

(4) We expect that food quality will interact with temperature conditions to determine the fitness of *Daphnia*.

(5) We presume that maternal conditions in terms of food quality and temperature interact with the offspring environment to affect offspring fitness.

2 THEORETICAL BACKGROUND

2.1 *DAPHNIA*

2.1.1 Phylogenetic relationships

The evolutionary history and phylogenetic relationships of *Daphnia* (Crustacea: Cladocera) are in a state of flux (Schwenk *et al.*, 1998). Uncertainties are caused by the high phenotypic plasticity of species, the occurrence of local races and natural interspecific hybridization (Schwenk *et al.*, 1998). Morphological work has traditionally divided species in the genus into two subgenera: *Ctenodaphnia* and *Daphnia*. Additional molecular evidence (12S rRNA), coupled with consistent divergence in several morphological attributes as well as chromosome number between the two long recognised lineages (*pulex*, *longispina*) of the *Daphnia* subgenus, has led Colburne and Hebert (1996) to suggest these lineages should also have a status of subgenera; *Daphnia* and *Hyalodaphnia*, respectively. Our chosen model species span all three proposed subgenera of the genus *Daphnia*; *D. magna* Straus, 1820, belongs to subgenus *Ctenodaphnia*, *D. pulex* and *D. pulicaria* to subgenus *Daphnia* and *D. rosea* and *D. hyalina* to subgenus *Hyalodaphnia*.

D. pulex/D. pulicaria and *D. hyalina/D. rosea* species pairs are relatively well resolved morphologically, but have low genetic divergence (Crease and Lynch, 1991; Lehman *et al.*, 1995; Schwenk *et al.*, 1998; Giessler *et al.*, 1999; Schwenk *et al.* 2000; Petrusek *et al.*, 2008). The question arises whether they represent recently differentiated sister species, or conspecific lineages that only differ in certain phenotypic characteristics. In either case, they are closely related species/lineages that have different habitat preferences (lakes or ponds), which is assumed to be the cause of divergent morphology (phenotypic adaptations) (Lehman *et al.*, 1995; Giessler *et al.*, 1999) and which we assume would also be the cause of different thermal sensitivity.

2.1.2 Temperature effects on *Daphnia* species

Temperature is the most important environmental factor that affects the distribution of organisms. It affects *Daphnia* at all levels of biological organisation: from the speed of enzymatic reactions (Simčič and Brancelj, 1997; Somero, 2004), membrane fluidity (Hazel and Williams, 1990; Schechtriem *et al.*, 2006), physiological rate processes (Simčič and Brancelj, 1997; Paul *et al.*, 1997; Clarke and Fraser, 2004), filtration rate (Kibby, 1971); swimming activity (Zeis *et al.* 2004b), behaviour (Gerritsen, 1982), life history parameters and fecundity (Stich and Lampert,

1984) and survival (MacArthur and Baillie, 1929). It affects population growth rates, phenology (Adrian *et al.,* 2006) and abundance (Pajk *et al.*, 2008). On ecosystem scale it can cause decoupling of trophic interactions (Winder and Schindler, 2004a) and changes in community structure. It also has indirect effects through the effect on oxygen solubility in water, and other abiotic factors, and on biotic factors such as food quality, quantity and timing, predation pressure, and occurrence of infections.

2.2 THERMAL REACTION NORMS FOR PERFORMANCE

2.2.1 Thermal performance curves

Thermal reaction norms for organismal performance are called thermal performance curves (Angilletta, 2009). Performance is any measure of an organism's capacity to function, usually expressed as a rate or probability. Common measures of performance include locomotion (swimming), assimilation (filtration rate), growth (juvenile growth rate), development (egg development rate), fecundity and survivorship. Performance curves for survivorship are sometimes referred to as tolerance curves (Gilchrist, 1995). General characteristics of a response can be captured with several parameters (Angilletta, 2009): (1) the thermal optimum (T_{opt}), (2) thermal breadth (or performance breadth), (3) the thermal limits, referred to as the critical thermal minimum (CT_{min}) and the critical thermal maximum (CT_{max}) and (4) the maximal performance (P_{max}). Organisms with broad thermal performance curves are called eurythermal organisms and organisms with narrow thermal performance curves are referred to as stenothermal organisms.

2.2.2 Estimation of fitness

The ultimate measure of organism's performance is fitness. Fitness, usually defined as the average number of offspring produced by individuals with a certain genotype, relative to the number produced by individuals with other genotypes, depends on two demographic variables: age-specific survivorship and age-specific fecundity (Stearns, 1992). In populations that remain stable for long periods of time, fitness can be measured as net reproductive rate (R_0), the total number of female offspring produced per individual in a single cohort (Kozlowski *et al.*, 2004; Sibly *et al.* 2007):

$$R_0 = \sum_{x=AFR}^{w} l_x m_x \qquad (1)$$

where l_x is the survival to age x, m_x is the fecundity at age x (female offspring per female), AFR is the age at first reproduction, and w is the maximum reproductive age. Most zooplankton populations, however, are not stable. In such cases, fitness is usually measured by the intrinsic rate of population increase, r (Odum, 1971; Angilletta, 2009). Most laboratory studies on cladocerans use the Euler-Lotka equation to estimate r iteratively (e.g. Lampert and Trubetskova, 1996; Boersma, 1997):

$$\sum_{x=AFR}^{w} l_x e^{-rx} m_x = 1. \quad (2)$$

Life table experiments used to calculate r are usually shortened to encompass the first two or three clutches (e.g. Boersma, 1997). Although the value of r, based on only the first few clutches, is an underestimation of life-time r, r-values from abbreviated life-table experiments have been shown to be highly correlated with lifetime values of r (Vanni, 1986); the contribution of the first two broods reflects around 80% of total r (Mooij and Boersma, 1996). In laboratory studies with abbreviated life tables, l_x is usually set to 1; that is, mortality observed during the experiment is assumed to be a result of handling rather than differences between individuals, as mortality in 'well-kept' cultures during experiments is normally very low (Vijverberg, 1989). Values of r obtained in the laboratory are useful as estimators of individual fitness but, due to the above mentioned shortcomings, cannot be used to calculate the rate of population increase in the field.

Computing values of the intrinsic rate of population increase using individual animals causes certain issues, as doing so makes it impossible to find negative values of r, except for the infinite negative, when an animal does not reproduce at all (Boersma, 1997). The production of one single egg in the animal's lifetime will lead to an r value equal or larger than zero. Moreover, even if r equals zero, this does not mean that fitness equals zero, as the individual is still capable of replacing itself, leading to a stable population density (Boersma, 1997). r does not scale linearly with the finite rate of population increase (λ; $r = \ln\lambda$), and hence taking r as a fitness measure could bias the results, but the relative differences between both estimates is small, because r is usually close to zero (Boersma, 1997).

Many studies on cladocerans use mass specific growth rate as an estimator of fitness (e.g. Mitchell and Lampert, 2000; Mitchell *et al.*, 2004). Mass specific growth rate is calculated from the equation:

$$g\ (day^{-1}) = \frac{\ln W_t - \ln W_0}{t}, \quad (3)$$

where W_0 is the dry mass at the beginning of the experiment, W_t is the individual dry mass at the end of the experiment and t is the duration of the experiment (in days). Especially the juvenile growth rate (g_j) – the growth rate from birth to maturity – is considered to be a good predictor of fitness in *Daphnia*, because of a strong linear corellation with r (Lampert and

Trubetskova, 1996). There is a theoretical basis for this correlation: *Daphnia* are supposed to allocate a fixed proportion of their total production into reproduction, regardless of the absolute growth varying in response to environmental conditions. Indeed, age specific proportion of total production invested into reproduction is similar among *Daphnia* species (Lynch *et al.*, 1986). A close correlation between g_j and r is expected when, across generations, the animals have the same average body mass (Lampert and Trubetskova, 1996). Values of g_j are higher than r because in the first few instars, all energy is invested into growth and none into reproduction (Lampert and Trubetskova, 1996).

2.2.3 Temperature and fitness

Three factors have the strongest effect on fitness: survival, age at first reproduction and fecundity, and all of them are affected by temperature. Temperature can have a strong impact on mortality. In a survey of 21 different clones from 6 temperate species of *Daphnia*, 15 minute-LT_{50} (the temperature at which 50 % of experimental animals die within 15 minutes) for animals acclimated to 25°C ranged from 34.4 to 40.1 °C (MacIsaac, 1985). The differences in thermal tolerance were correlated with the maximum temperatures of the environment from which the species originated (MacIsaac, 1985). A comparison of different heat tests by Kivivuori and Lahdes (1996) indicates that LT_{50} is higher if the temperature change is gradual. Shortening of life span in warmer environments appears to be a general trend in plants and animals (McCoy and Gillooly, 2008). This trend is attributed to intrinsic causes of mortality and the “rate of living”. Increased metabolic rates at higher temperatures incur higher rates of cell damage or decay, perhaps due to the accumulation of oxidative free radicals (Hulbert *et al.*, 2007), causing faster rates of senescence and shorter life span. Similar trends have been observed in *Daphnia magna* (MacArthur and Baillie, 1929), *Daphnia galeata* G. O. Sars, 1864 *mendotae* Birge, 1918 comb. Nov. Brooks, 1957 (Hall, 1964), and eight cladocerans from the river Thames (Bottrell, 1975). In tropical genera, life span shortens again at very low temperatures because the lower thermal limits of these species are approached (Lennon *et al.*, 2001; Benider *et al.*, 2002; Lemke and Benke, 2003). It has also been suggested that in tropics-adapted cladoceran species, the shortening of the lifespan with temperature reverses at higher temperatures (Han *et al.*, 2011).

However, extrinsic causes of mortality are thought to outweigh intrinsic causes in natural populations. Organisms in natural environments typically die as a result of disease, predation or accident, well before they reach their maximum possible life span (Ricklefs, 1998). Hall (1964) estimated that the physiological mortality rate of *Daphnia galeata* population is quite low, probably less than 3 % per day. Dodson (1972) found that above 90 % of total mortality in a population of *Daphnia rosea* can be attributed to predation (mostly by *Chaoborus* larvae). Wright (1965) correlated peak mortality in a population of *Daphnia schodleri* G. O. Sars, 1862, with

peak *Leptodora* density. The portion of mortality not attributed to predation by *Leptodora* was found to be constant during the season (Wright, 1965). However, temperature may affect mortality rates indirectly, through its influence on other abiotic and biotic factors of the environment, such as dissolved oxygen concentration, food quality and availability, virulence of diseases, abundance and activity of predators.

Many ectotherms respond similarly to variation in their developmental temperature; individuals at low temperatures grow relatively slowly, but delay maturation long enough to outgrow individuals at high temperatures (Atkinson, 1994). The general trend for cladoceran development rates to increase as temperature increases is well established (Brown, 1929; MacArthur and Baillie, 1929; Hall, 1964; Bottrell, 1975, Munro and White, 1975; Goss and Bunting, 1983). Age at maturity thus decreases as temperature increases. However, when development temperatures exceed the optimum, development time may increase again (Goss and Bunting, 1983).

Age specific fecundity (or clutch size) is highest at intermediate temperatures (15 or 20 °C for *D. pulex* and *D. magna*; Goss and Bunting, 1983). Clutch size usually decreases dramatically at higher temperatures (Green, 1956a).

Fitness, measured either as juvenile growth rate or *r*, increases slowly with temperature in the low temperature range up to an optimum temperature, after which it declines sharply. For example, g_j of various clones from two pond populations of *D. magna* increased slowly from a low level at 17 °C or 20 °C to a maximum at 26 °C or rarely 29 °C, followed by a sharp decline to low or zero growth at 32 °C, and clones from all periods showed relatively similar reaction norms (Mitchell *et al.*, 2004). Optimum temperatures can be species- and even clone-specific (Loaring and Hebert, 1981; Carvalho, 1987; Achenbach and Lampert, 1997). They depend on environmental conditions, especially food quantity and quality (Loaring and Hebert, 1981; Orcut and Porter, 1984).

2.2.4 Phenotypic plasticity of thermal performance curves and thermal adaptation

All organisms possess some capacity to modify their behavioural, physiological or morphological characteristics in response to environmental temperature (Angilletta, 2009). Brief exposure to extreme heat or cold often causes greater tolerance of thermal extremes within hours – a phenomenon called hardening. Prolonged exposures to moderate temperatures can trigger lasting changes in thermosensitivity. Generally, responses to environmental temperature are reversible, but some effects remain fixed throughout the life of the organism. Collectively, these examples of phenotypic plasticity are referred to as thermal acclimation. Acclimation to the complex of abiotic and biotic factors in the field is called acclimatization (Angilletta, 2009).

Acclimation is a phenotypic response to temperature that alters the performance curve. Developmental acclimation comprises irreversible responses to temperature experienced during onthogeny, while reversible acclimation comprises regulated responses to diel or seasonal changes in temperature (Angilletta, 2009). Temperature acclimation has been shown to affect the thermal preference (Lagerspetz, 2000) of *Daphnia*. Acute thermal tolerance increases with acclimation temperature (MacIsaac *et al.*, 1985) and respiration rate decreases with acclimation temperature (Armitage and Lei, 1979). Acclimation also affects the thermal performance curves for filtering rate (McKee, 1995) and swimming activity (Zeis *et al.*, 2004b) of *Daphnia*.

Maternal acclimation can influence thermal performance of offspring through what is called maternal effects. Maternal effects are intergenerational transmissions of individual quality affected by the environment in which the parents lived, and may act as a mechanism for adaptive phenotypic response to fluctuating environmental conditions (Mousseau and Fox, 1998). Maternal effects are mediated through provisioning the offspring (eggs) with energy reserves, nutrients, hormones, mRNA and other factors. They are ubiquitous in nature and have been demonstrated in a wide range of traits in plants and animals (Mousseau and Fox, 1998), including *Daphnia* (Lynch and Ennis, 1983; Agrawal *et al.*, 1999; Alekseev and Lampert, 2001). Maternal food quality has been shown to affect reproductive strategies (Abrusan *et al.*, 2007), the biochemical composition of the eggs (Wacker and Martin-Creuzburg, 2007) and the performance of offspring in *Daphnia* (Brett, 1993; Martin-Creuzburg *et al.*, 2005). Maternal food quantity affects offspring size, starvation resistance (Gliwicz and Guisande, 1992), growth rate and clutch size (Alekseev and Lampert, 2001), mode of reproduction (LaMontagne and McCauley, 2001) and longevity (Lynch and Ennis, 1983). Maternal photoperiod affected the age at first reproduction, survival to maturation and body mass of offspring (Alekseev and Lampert, 2001). Maternal temperature conditions were shown to affect the size of eggs and offspring in cladocerans (Perrin, 1988), which in turn affect important life history characteristics such as age and size at maturity (Ebert, 1991). The various maternal effects can also interact (Alekseev and Lampert, 2001). The complex set of environmental conditions of the previous generation may thus affect the performance of the next (Mousseau and Fox, 1998).

Thermal adaptation is an evolutionary change in genotype as a consequence of thermal selection that confers higher fitness under certain temperature regime (Mitchell and Lampert, 2000). There are three distinct approaches of investigation of thermal adaptation: (1) quantification of thermal selection in natural environments, (2) quantifying evolution during selection experiments (as in Van Doorslaer *et al.*, 2007), and (3) comparing organisms from different environments (as in Mitchell and Lampert, 2000). Although direct measures of selection are ideal, such observations are impractical or even impossible to accumulate for most species. Given this limitation, thermal adaptations are most often assessed through comparative

analyses (Angilletta, 2009). When thermal performances of different species are compared, their phylogenetic relationships must be taken into account (Angilletta, 2009).

A population may acclimatize to a change in environmental conditions with a shift in the clonal composition to clones more adapted to the new conditions. For example, Carvalho and Crisp (1987) found changes in the clonal composition of a *Daphnia* population during the season. Summer clones had greater fitness at higher temperatures while winter clones performed better at lower temperatures (Carvalho, 1987; Pinkhaus *et al.*, 2007).

2.3 MECHANISMS DETERMINING THE THERMAL PERFORMANCE CURVE

Which mechanisms and to what extent determine the shape of thermal performance curve depends on the performance being measured and the way it is measured (cronic/acute temperature exposure) (Angilletta, 2009). Recent work led to a model (e.g. Pörtner, 2001) that links thermal tolerance windows directly to oxygen supply in relation to energy demand on top of parallel adjustments at the molecular and membrane level (e.g. Somero, 2004).

2.3.1 Thermal effects on enzymes (and other proteins)

For any chemical reaction, temperature determines the proportion of reactants that possess the free energy required for reaction. Enzymes lower the energy of activation and therefore speed the reaction at any given temperature (Angilletta, 2009). The function of the enzyme requires both an initial conformation that can bind substrates and a change in this conformation that creates an ideal environment for the reaction (Angilletta, 2009). The ability of an enzyme to bind its substrate is indexed by the apparent Michaelis-Menten constant (K_m) (Somero, 2004). Conformation change is the speed limiting step of the reaction and determines the catalytic rate constant (k_{cat}) (Somero, 2004). Even a moderate drop in temperature alters the rate of conformation change, leading to inactivation at low temperatures (Angilletta, 2009). Either too high or too low a temperature might destroy the necessary conformation to bind substrate (Angilletta, 2009). Enzymes therefore have an optimum temperature range in which their activity is highest and this range affects the optimum temperature range of the organism.

Data gathered for a wide variety of ectothermic animals revealed a striking degree of independence of metabolic rate from influences of ambient temperature, following evolutionary adaptation, seasonal acclimatization or laboratory acclimation (Bullock, 1955) – a process known as temperature compensation of metabolism. Temperature compensatory shifts in enzymatic function can be a result of: (1) Changes in enzyme (protein) concentration through

increased transcription at certain temperatures (haemoglobin, heat shock proteins). (2) Genetically based differences in the kinetic properties of enzymes (allozymes – enzyme products of different alleles of the same gene; paralogous isozymes – different forms of the same enzyme resulting from gene duplication). The substitution of amino acids can alter the thermal properties of proteins by altering the conformational stability of enzymes. An enzyme with greater conformational stability functions better at higher temperatures and an enzyme with less conformational stability functions better at lower temperature (Somero, 2004). Lesser conformational stability means a greater k_{cat}, but it comes at the expense of greater instability at higher temperatures, which lowers K_m (Somero, 2004). (3) Modulation of the activity of pre-existing enzymes by low-molecular mass constituents of the cell or covalent modification of proteins (Somero, 2004).

A mutation in the structure of the enzyme can produce an allozyme that enhances performance at some temperatures but reduces performance at others. Duplication of genes can lead to the evolution of paralogous isozymes, which would enable an organism to operate over a wider range of temperatures (Angilletta, 2009). Differential expression of isozymes and allozymes (in heterozygotes), along with other above-mentioned mechanisms, enables temperature acclimation and acclimatization.

Protein expression changes with temperature. Twenty percent of all genes of *Campylobacter* were transiently significantly up- or downregulated over a 50 min period after a temperature increase (Stintzi, 2003). Approximately 25 % of the genes in the yeast genome were found to be involved in the response of yeast to low temperature (Sahara *et al.*, 2002). Seki *et al.* (2002) found 229 of about 7000 *Arabidopsis* genes with more than three times greater expression under cold stress (4 °C) than under normal conditions (22 °C). In goby fish, *Gillichthys mirabilis* Cooper, 1864, following 4 weeks of acclimation to 9 °C, 19 °C, or 28 °C, 150 of the 1,607 genes on the array had significantly different expression in the three acclimation groups (Logan and Somero, 2010). The protein expression of *Daphnia* is also affected by temperature. The concentration and oxygen affinity of *D. magna* haemoglobin decreased with decreasing acclimation temperature (30–10 °C) (Paul *et al.*, 2004b). The sub-unit composition of haemoglobin varied depending on acclimation temperature (Paul *et al.*, 2004b). Schwerin *et al*. (2009) found a marked change in protein expression of *D. pulex* with acclimation temperature (10 or 20 °C). They suggested that the increase of proteolytic enzyme concentration and the decrease of vitellogenin, actin and total protein concentration between 10 °C and 20 °C reflect the increased amino-acids demand and the reduced protein reserves in the animal's body, while the increase of actin concentration in cold-acclimated animals may contribute to a compensatory mechanism which ensures the relative constancy of muscular performance.

2.3.2 Membrane structure

In eukaryotic cells, phospholipids (PLs) are the predominant membrane lipids and consist of a hydrophilic head group to which are attached two hydrophobic acyl chains. These acyl chains are either saturated, monounsaturated or polyunsaturated fatty acid (SA- / MU- / PU- FA) residues that normally vary from 14 to 22 carbons in length (Hulbert, 2003). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the major PL classes and differ in the hydrophilic head group.

For normal function, membrane bilayers must be 'fluid', allowing lateral movement of membrane components (Hulbert, 2003). The fluidity of cellular membranes is determined by their lipid composition (fatty acids and cholesterol) and temperature (Hazel and Williams, 1990). High temperature increases the fluidity of membranes and can result in 'leaky' membranes, which jeopardises important proton and ion gradients. Cold environmental temperature decreases the fluidity of membranes resulting in gel phase which hinders lateral movement of membrane components, such as membrane bound enzymes (most of the electron transport system enzymes (ETS), ATP synthase and other important enzymes), reducing their function (Angilletta, 2009).

Although most organisms can maintain the full integrity of their cellular membranes over an interval of 10-15 °C, the optimal temperature depends on the FA composition of their phospholipids (Hazel and Williams, 1990). Saturated FAs (SAFA) provide less intrinsic fluidity than do unsaturated FAs. Consequently, membranes composed of primarily SAFA function better at high temperatures than membranes composed of primarily unsaturated FAs. The opposite is true at low temperatures; membranes with a higher proportion of unsaturated FAs function better than those composed mainly of SAFA. The proportion of different PL head groups also changes in response to temperature, particularly among winter-active crustaceans; PEs increase at the expense of PCs at low temperatures (Farkas *et al.*, 1984; Pruitt, 1990).

Many poikilothermic animals adapt to changing environmental temperatures by modifying the degree of unsaturation of their lipids (Chapelle, 1978; Hazel and Williams, 1990; Pruitt, 1990). Generally, the degree of unsaturation decreases with increasing temperature. Farkas and Herodek (1964) observed seasonal changes in the fatty acid composition of crustacean plankton. Schlechtriem *et al.* (2006) observed that cold acclimated *Daphnia* had an increased proportion of EPA, C20:5n3 polyunsaturated fatty acid (PUFA) in their membranes compared to warm acclimated animals. No such response was detected after short-term (48 h) exposure to cold temperatures (4–5 °C) (Farkas, 1979; Farkas *et al.*, 1981), indicating that *Daphnia* enzymes involved in the PUFA synthesis need longer adaptation periods to reach full activity. The molecular mechanism of temperature acclimation of membrane fluidity was studied in the ciliate

Tetrahymena pyriformis Ehr., 1830. The activity of fatty acid desaturase decreased during acclimation to high temperature and was regulated directly by membrane fluidity (Kasai *et al.*, 1976).

Sterols are found in the cell surface membranes of virtually all eukaryotic organisms. Cholesterol is a major constituent of the plasma membranes in animals. A widely accepted perspective is that cholesterol stabilizes membranes (Crockett, 1998). Cholesterol provides order to (rigidifies) membranes that are in the fluid phase. The ordering influence of cholesterol is particularly pronounced in membranes rich in saturated phospholipids compared with those containing unsaturated phospholipids (Kusumi *et al.,* 1986). Adjustments in cholesterol level could reverse, or at least ameliorate, temperature-induced changes of membrane fluidity. Hyperfluidization brought on by an increase in body temperature could be offset by increased levels of cholesterol. Similarly, hypofluidity triggered by a decline in body temperature could be countered by a decrease in membrane cholesterol. In addition, poikilothermic organisms whose plasma membranes are particularly enriched with cholesterol are less likely to experience a phase transition during a sudden change in body temperature than organisms whose membranes have little cholesterol (Crockett, 1998). Comparison of cholesterol levels in a stenothermal crab (*Cancer pagurus* L., 1758) with a eurythermal crab (*Carcinus maenas* (L., 1758)) supports this prediction (Cuculescu *et al.*, 1995).

Cholesterol levels have been shown to rise with temperature, stay the same, or decrease with an increase in acclimation temperature depending on the organism, tissue, or plasma membrane domain under investigation (Crockett, 1998). According to previous studies, bulk membrane fluidity in crustaceans at different temperatures does not appear to be regulated to any large degree by sterols (Pruitt, 1990). Gastaud (1977) found that sterol concentrations differed by less than 1% in the lipids of planktonic crustaceans from arctic and temperate seas and Cossins (1976) found no significant difference in the phospholipid to cholesterol ratio of sarcoplasmic reticulum membranes from crayfish acclimated to 4 and 25 °C. On the other hand Cuculescu *et al.* (1995) found that plasma membranes from cold-acclimated crabs had lower cholesterol to phospholipid ratios than those from warm-acclimated crabs, while the ratio of saturated to unsaturated fatty acids was little altered by temperature acclimation. Only one (*Calanus finmarchicus* (Gunner, 1765)) of the five copepod species studied by Hasset and Crockett (2008) altered its cholesterol content with acclimation temperature, but they did find a consistent positive relationship between cholesterol content and habitat temperature. Copepod species residing in warmer habitats had approximately twice the cholesterol of species living in colder waters. A similar pattern was observed for comparisons of species within genera (*Calanus*, *Acartia* and *Centropages*), with the species abundant at lower latitudes having more cholesterol than the northern congeners. There is little evidence about the influence of temperature on cholesterol content in *Daphnia*. Sperfeld and Wacker (2009) found that the

cholesterol content of *Daphnia magna* increased with increasing dietary cholesterol and this increase was enhanced at higher temperatures, indicating a higher demand for cholesterol for tissues and probably specifically for membranes at higher temperatures.

2.3.3 Oxygen limitation hypothesis

This model predicts that the optimum of performance should be near the temperature of maximum aerobic scope (= maximum metabolic rate - resting metabolic rate) (Angilletta, 2009). In the optimum range, the aerobic scope is maximum resulting in an optimal fitness. Thermal limits of performance are set by the temperatures at which aerobic respiration fails to meet energetic needs. Organisms respond to a temperature-dependent increase of oxygen demand with an increase of external and internal convective oxygen transport. Reaching the upper capacity limits of convective control, the upper limit of optimum range is reached. At even higher temperatures, the hemolymph Po_2 or the oxygenation of respiratory proteins start to fall due to a widening gap between further increasing energy demand and unchanged convection resulting in a decrease of aerobic scope. In the cold, the lower limit of optimum range is reached and blood Po_2 also falls (reducing the aerobic scope), when convections converge towards the lower control limits set by the quantities of ATP available (Paul *et al.*, 2004a).

Acclimation to seasonal cold causes an increase in mitochondrial aerobic capacity, which is reversed during seasonal warming (Guderley, 1998). The cold-induced increase of mitochondrial aerobic capacity, specifically in organs responsible for convection, is interpreted as to overcome energy limitations of convective control. The warm-induced decrease is interpreted as to reduce the temperature dependent increase of energy demand for mitochondria maintenance (i.e. proton leakage) leaving more energy for convective control (Paul *et al.*, 2004a). As one key mechanism, in combination with others on the membrane or protein level, it should be responsible for unidirectional shifts of the thermal tolerance range either towards lower or higher temperatures.

If this model correctly identifies the cause of critical thermal limits, adaptation to thermal extremes will require changes in mitochondrial, circulatory and respiratory capacities (Angilletta, 2009). As essential part of the oxygen transport system, haemoglobin (Hb) is very important also for thermal tolerance; Paul *et al.* (2004b) suggest that the regulation of haemoglobin expression is a central mechanism of adaptation to different temperature conditions in *Daphnia*.

Oxygen limitation might be especially important for thermal sensitivity in aquatic organisms because in aquatic ecosystems increased temperatures are often accompanied by decreased oxygen availability, due to not only decreased solubility of oxygen at higher temperatures but also stronger stratification and increased microbial activity.

2.4 METABOLIC ACTIVITY

Metabolic rate is a measure of the energy utilization of an organism, and is traditionally measured as the rate of oxygen consumption. This is a reasonable approximation, as in most organisms ATP is generated aerobically using oxygen as the final electron acceptor (Clarke and Fraser, 2004).

2.4.1 Respiration rate

Basal or maintenance metabolism is defined as the metabolic rate of an organism whose food intake is such that there is no net change in body mass (Clarke and Fraser, 2004). The major components of basal metabolic rate are non-mitochondrial oxidative processes, mitochondrial proton leak, Na^+ K^+-ATPase, protein synthesis and gluconeogenesis. Others include Ca^{2-}ATPase, nucleic acid turnover, signal transduction, urea synthesis, substrate cycling and protein degradation (Clarke and Fraser, 2004). For any given tissue, the absolute rate of basal metabolism, and the relative proportions of the various component processes, will differ depending on factors such as mitochondrial density, ribosomal concentration, requirement for ion gradients, protein synthesis and so on. Furthermore, the basal metabolic rate of a given organism will depend on the relative proportion of tissues with different inherent rates of basal metabolism and it will also vary during ontogenetic development. The proportion of body mass that is metabolically inert increases as the animal grows (Glazier, 1991; Simčič and Brancelj, 2003).

Often basal metabolic rate cannot be measured directly. Resting metabolism, the oxygen consumption of an inactive, postabsorbtive, non-growing and non-reproducing individual (Clarke and Fraser, 2004), is often used as an estimator. In situation when even resting metabolism cannot be measured, as is the case in *Daphnia*, where healthy animals are seldom inactive and healthy adult females are seldom non-reproducing, routine respiration rate is measured. That is the average respiration rate of organisms at their usual level of activity. The contribution of food digestion and assimilation on metabolic rate, termed “specific dynamic action” (Lampert, 1986), is usually avoided by starving the animals for some time before the onset of experiments. Metabolic scope is the difference between basal and maximum metabolic rate (Clarke and Fraser, 2004).

Respiration rate of *Daphnia* is well studied. Respiration rate increases with environmental temperature (Simčič and Brancelj, 1997; Paul *et al.*, 1997; Paul *et al.*, 2004a; Simčič and Brancelj, 2004), oxygen concentration (Paul *et al.*, 1997) and food quantity (Lampert, 1986).

Mass specific respiration rate decreases with organism's size and age (Glazier, 1991; Simčič and Brancelj, 1997; Paul *et al.*, 1997), and increases with activity and crowding. It differs between different species (Simčič and Brancelj, 1997) due to the difference in body size, lifestyle (more active species have a higher basal metabolic rate) and adaptation to prevailing environmental conditions. Within a species, respiration rate might differ due to acclimation (Simčič and Brancelj, 2004) or acclimatization to different environmental conditions.

2.4.2 Electron transport system (ETS) activity

Oxidative phosphorylation is the major source of ATP in aerobic organisms. The flow of electrons from NADH or $FADH_2$ (succinate) to O_2 through protein complexes located in the inner mitochondrial membrane – the electron transport system (ETS) – leads to the pumping of protons out of the mitochondrial matrix into the intermembrane space. A proton motive force is generated consisting of a pH gradient and a transmembrane electric potential. ATP is synthesized when protons flow back to the mitochondrial matrix through an enzyme complex (ATP-synthase). ETS activity is a biochemical measure of the potential metabolic activity (Lampert, 1984).

A specific and highly sensitive method for measurement of the rate of this process was developed. It is based on the reduction of a colourless tetrazolium salt to a coloured formazan (Curl and Sandberg, 1961; Gahan and Kalina, 1968; Packard, 1971; Kenner and Ahmed, 1975). The basic steps of the ETS method are the preparation of homogenate and its incubation in a solution of ETS substrates (NADH, NADPH) in excess and 2-(p-iodophenil)-3-(p-nitrophenil) tetrazolium chloride (INT) (G.-Tóth, 1993). INT is reduced by the oxidation of the coenzyme Q-cytochrome b complex in Complex III (Cytochrome reductase). Reaction product is an insoluble red formazan that can be measured spectrophotometrically at 490 nm (G.-Tóth, 1993). ETS activity is thus determined at the rate-limiting step of ETS; the oxidation of the coenzyme Q-citochrome b complex (Packard, 1971).

ETS activity can be expressed as electrochemical O_2 equivalent that is calculated from formazan production (Kenner and Ahmed, 1975). In the mitochondrion, 2 electrons (e^-) and 2 protons (H^+) are used to convert $½O_2$ to H_2O. 2 e^- are also used in the reduction of INT to formazan. Thus, 2 µmol of formazan are equivalent to 1 µmol O_2 which is equivalent to 22.4 µL O_2. The absorbance of 2 µmol formazan at 490 nm (path length 1 cm) is 31.8 A_{490nm}, so a factor of 1.42 A_{490nm} μl^{-1} O_2 is used to calculate ETS activity (Kenner and Ahmed, 1975).

ETS activity is affected by the concentration and activity of the enzymes of ETS, incubation temperature (Kenner and Ahmed, 1975; Borgmann, 1978), pH (Kenner and Ahmed,

1975), and substrate (Borgmann, 1978). It differs between and within species in a similar manner as respiration rate (Simčič and Brancelj, 1997).

2.4.3 The ETS/R ratio

ETS activity is a measure of the potential metabolic activity (Lampert, 1984). The ratio of ETS activity to respiration (ETS/R ratio) reflects the fraction of the maximum respiratory capacity that the organism is effectively using (Martinez, 1992). The ETS/R ratio is not constant. ETS/R of *Daphnia* ranges from 0.7 to 3.5 (Simčič and Brancelj, 1997). It varies with experimental temperature (Borgmann, 1978; Simčič and Brancelj, 1997). ETS/R ratio may differ between species from food-rich and food-poor habitats (ponds and oligotrophic lakes) (Simčič and Brancelj, 1997) since animals from habitats with food shortage are expected to evolve more efficient metabolisms.

2.4.4 Effect of body size on metabolic rate

Larger organisms have lower respiration rates and ETS activity per unit mass than smaller organisms (Hemmingsen, 1960; Borgmann, 1978; Simčič and Brancelj, 1997; Clarke and Fraser, 2004). Juvenile *Daphnia* have higher ETS activity and respiration rates per unit mass than adults (Simčič and Brancelj, 1997). The proportion of body mass that is metabolically inert increases as the animal grows, and this component contributes to the negative allometry of metabolic rate relative to body mass (Glazier, 1991). For example, the molt represents a progressively greater share of body mass of *D. pulex* as animals grow bigger (Lynch *et al.*, 1986).

The dependence of metabolic rate (Q) on body mass is usually expressed as:

$$Q = aM^{b}, \quad (4)$$

where *a* is a scaling constant, *M* is body mass and *b* is a mass scaling coefficient. For between-species comparisons *b* is close to 0.75 for most organisms (Hemmingsen, 1960; for the mass-specific rate Q/M, *b* is -0.25). For juveniles and adults of five *Daphnia* species, similar *b* was found for mass-specific respiration and ETS activity (-0.37) (Simčič, 1997). Literature reports for within species *b* for individual respiration rate of Cladocera range from 0.51 to 1.09, with most values around 0.80 (Lynch *et al.*, 1986). The high values of *b* were found for debrooded females (Lynch *et al.*, 1986; Glazier, 1991).

The exponent of mass dependence of oxygen uptake may depend on temperature. It increases from about 0.6 near 30 °C to about 0.8 at 0 °C among crustaceans (Ivleva, 1980). A

similar trend was found for an intertidal amphipod (Venables, 1981). For a predatory cladoceran *Bythotrephes cederstroemi*, however, the exponent increased with temperature (Yurista, 1999). Temperature dependence of the exponent was not observed among planktonic crustaceans by Vidal and Whitledge (1982).

2.4.5 Effect of temperature on metabolic rate

Variations in the metabolic rate of organisms with temperature have long been known, with the classic work in this field being that of Hemmingsen (1960). Respiration rate and ETS activity of *Daphnia* increase with increasing temperature (Paul *et al.*, 1997; Simčič and Brancelj, 1997; Paul *et al.*, 2004a; Simčič and Brancelj, 2004). The temperature sensitivity of metabolism is often described by Van't Hoff's Q_{10} – the magnitude of the acceleration of metabolic rate if the temperature changes for 10°C, or the Arrhenius activation energy (E_a) – which describes the accelerating influence of temperature on metabolic rate over the whole temperature range. E_a is obtained from the slope of linear regression of ln of metabolic rate against the inverse of absolute temperature. Low E_a values are characteristic of cold adapted animals and of animals that are adapted to wide temperature fluctuations (Simčič and Brancelj, 2004).

The combination of the mass scaling and temperature components led Gillooly *et al.* (2001) to propose a simple equation describing the variation of metabolic rate (*Q*) of all organisms:

$$Q = b_0 M^{3/4} e^{-E/kT}, \quad (5)$$

where b_0 is a scaling constant, *M* is body mass, *T* is absolute temperature, *k* is Boltzmann's constant and *E* is the activation energy of metabolism (defined as the average activation energy for the rate-limiting enzyme-catalysed biochemical reactions of metabolism).

This mechanistic approach makes no provision for acclimatization or evolutionary adaptation. The proposition of this equation is that the metabolic rate of organisms is driven directly by the kinetic energy of the cell, while in fact it is driven by energy demand. The enzymatic processes involved are under complex and subtle feedback control (Clarke and Fraser, 2004). Nevertheless, metabolism does scale with environmental temperature, due to its indirect effects on the resting metabolic rate through increased protein turnover and increased proton leakage across the mitochondrial membrane (Clarke and Fraser, 2004).

2.5 TEMPERATURE AND BIOCHEMICAL COMPOSITION

2.5.1 Daphnia haemoglobin (Hb) synthesis

O_2 concentration in aquatic environments is subject to spatial and temporal (seasonal, daily) fluctuations. The solubility of O_2 in water decreases with increasing water temperature. O_2 concentration is further influenced by biotic factors (respiration and photosynthesis) and by the stability of the water column. Diffusion and convection are the two mechanisms by which aerobic animals maintain a continuous flow of oxygen from the environment into their tissues (Pirow *et al.*, 2004). The surface to volume ratio in *Daphnia* is of a magnitude that allows integumentary respiration (Graham, 1988). Recent studies, however, indicate that O_2 flux into the animal occurs across the ventral body surface and is distributed to the tissues by the circulation of the hemolymph (Wiggins and Frappel, 2000). Mechanisms to improve oxygen supply to tissues when water O_2 concentration is low and/or the O_2 demand of the tissue is high (as at high temperatures) include increased heart rate (to increase hemolymph circulation) and changes in oxygen carrier capacity of the hemolymph – mostly through changes in the haemoglobin content of the hemolymph (Paul *et al.*, 1997). Apparently, the thoracic appendages are not involved in respiratory control (Paul *et al.*, 1997).

2.5.1.1 *Daphnia* Hb

Haemoglobin (Hb) is a respiratory protein that occurs in all kingdoms of living organisms (for an exhaustive review see Weber and Vinogradov (2001)), although not in all species. Extracellular Hbs composed of multiple two-domain subunits, playing important roles in oxygen transport, are found in the hemolymph of Cladocera (Weber and Vinogradov, 2001). *D. magna* Hb (molecular mass (*Mr)* of 494 kDa) is composed of 16 two-domain subunits (each with *Mr* = 31 kDa) (Ilan *et al.*, 1982) and *Daphnia pulex* Hb (*M*r of 420–460 kDa) is composed of 12 two-domain subunits (*M*r 32–35 kDa) (Dewilde *et al.*, 1999). The oxygen-binding properties of Hb depend on its specific assembly of subunits (Kobayashi *et al.*, 1988). Six different *D. magna* and four *D. pulex* Hb subunits were identified, presumably encoded by different genes. The O_2 binding properties of *Daphnia* Hbs suggest a maximum role for *D. magna* Hb in O_2 transport at low ambient O_2 tension (Weber and Vinogradov, 2001).

All Cladocera appear to synthesize Hb, in contrast to many other invertebrate lineages, in which only a few taxa share the trait (Hebert *et al.*, 1999). Hebert *et al.* (1999) have sequenced the Hb genes from *Ceriodaphnia, Daphnia, Daphniopsis, Scapholeberis,* and *Simocephalus.* Hb

was found also in *Bosmina* and *Chydorus* (Sell, 1998) and in *Moina* (Kato *et al.*, 2001). Within genus *Daphnia*, the presence of Hb has now been demonstrated in *D. magna*, *D. pulex*, *D. obtusa* Kurz, 1874, *D. hyalina*, *D. curvirostris* Eylmann, 1887, *D. atkinsoni* Baird, 1859, *D. thomsoni* Sars, 1884, *D. carinata* King, 1852, *D. hodgsoni* Sars, 1916, *D. longispina* (O. F. Müller, 1776) and *D. rosea* (Green, 1965b; Sell, 1998).

Interspecific and intraspecific differences in low oxygen tolerance and haemoglobin synthesis have been observed in a number of *Daphnia* species (Green, 1956b; Heisey and Porter, 1977; Weider and Lampert, 1985; Sell, 1998). When challenged by a reduction in ambient oxygen partial pressure, some *Daphnia* species (*D. magna, D. pulex, D.rosea)* show a striking increase in Hb concentration (Fox *et al.*, 1951; Fox and Phear, 1953; Kobayashi and Hoshi, 1982; Pirow *et al.*, 2001; Zeis *et al.*, 2003; Seidel *et al.*, 2005). Low oxygen conditions can induce a more than tenfold increase of Hb concentration within 10 days (Fox and Phear, 1953), which causes the animals to appear red-colored. Re-access to higher oxygen concentrations results in a decrease of Hb concentration and the animals become transparent again (Landon and Stasiak, 1983).

The rise in Hb concentration is accompanied by an increase in its oxygen affinity (Kobayashi *et al.*, 1988; Zeis *et al.*, 2003; Seidl *et al.*, 2005) resulting from an altered subunit composition of the multimeric protein (Zeis *et al.*, 2003). Exposure to hypoxic conditions increases the proportion of Hb components with high O_2 affinity. Such a mechanism minimizes the total Hb concentration required for transporting O_2 under a wide range of O_2 tensions (Weber and Vinogradov, 2001).

Extracellular Hbs are invariably synthesized intracellularly and then secreted. Fat cells and epithelial cells of the epipodites are the sites of Hb synthesis in *Daphnia* (Goldmann *et al.*, 1999). Tokishita *et al.* (1997) showed that the expression of Hb gene is regulated by mRNA level. Transcripts of hypoxia-inducible Hb genes occur within a few hours after exposure to hypoxia and 3 days are required to attain a stable high level (Seidel *et al.*, 2005). The critical oxygen concentration for the synthesis of large quantities of Hb in *Daphnia* seems to be about 3 mg L^{-1} (Fox *et al.*, 1951; Fox and Phear, 1953; Landon and Stasiak, 1983).

2.5.1.2 Advantages and disadvantages of Hb synthesis

The increase in Hb concentration leads to a higher oxygen transport capacity of the hemolymph, which improves the physiological performance of red animals exposed to hypoxia (Fox *et al.*, 1951; Weider and Lampert, 1985). The advantages of haemoglobin in *Daphnia* are well documented. Fox *et al.* (1951) found that *Daphnia* with considerable amounts of Hb have increases in lifespan, swimming and grazing speeds, and egg production as well as accelerated

embryonic development. Increase in Hb content leads to a reduction of the critical ambient oxygen tension (Weider and Lampert, 1985; Pirow *et al.*, 2001; Seidel *et al.*, 2005). During shorter periods of up to 3 h, Hb-rich *Daphnia* can survive even under anoxic conditions (Usuki and Yamaguchi, 1979). Hb-rich *Daphnia* produce more eggs, which contain threefold higher Hb concentration and higher Hb O_2 affinity than eggs of Hb-poor *Daphnia* (Kobayashi *et al.*, 1990), implying that the advantages of hypoxic acclimation are passed to offspring (Weber and Vinogradov, 2001).

Alternative strategies to cope with hypoxia are evading oxygen-poor microhabitats (Sell, 1998) and enduring short-term periods of oxygen deficiency by means of anaerobic metabolism (Usuki and Yamaguchi, 1979). However, predator avoidance or access to alternative food resources in hypoxic waters, combined with efficient food utilization largely independent from fluctuations in oxygen availability, conveyed by an increased Hb content, result in higher fitness (Pirow *et al.*, 2001). The benefits derived from Hb are diminished by two cost factors. The synthesis of Hb decreases the amount of matter and energy that can be invested into growth and reproduction (Fox *et al.*, 1951; Kobayashi and Hoshi, 1984). Moreover, an increased Hb content in the hemolymph results in a reddish coloration and can make these transparent animals more conspicuous to visually foraging predators (O'Brien, 1979). The synthesis of Hb seems to be useful only under conditions of reduced oxygen availability, because Hb then makes a substantial contribution to convective oxygen transport that outweighs any disadvantages (Pirow *et al.*, 2001).

2.5.1.3 Temperature and Hb content

Hb content affects the thermal preference of *D. carinata*. The preferred temperature of *D. carinata* is lowered under conditions of hypoxia, compared with normoxia. The benefits of this response depend largely on a Q_{10}-driven decrease in oxygen consumption (Wiggins and Frappel, 2000). Hb-rich specimens of *D. carinata* show a higher preferred temperature than control animals under hypoxic conditions (Wiggins and Frappel, 2000).

Environmental temperature affects Hb synthesis and loss. Wiggins and Frapell (2002) showed that in low temperatures where animals have low metabolic rates, increased Hb synthesis in hypoxia might not be necessary because the demand for O_2 is reduced. *D. magna* Hb concentration and Hb O_2 binding affinity increase along with increased acclimation temperature (Paul *et al.*, 2004b; Zeis *et al.*, 2004a). Since oxygen solubility is inversely correlated to temperature, these changes can be compared to the alterations as a function of oxygen concentration (Zeis *et al.*, 2004a). Hb-rich *Daphnia* lose Hb in aerated water. This loss is more rapid at higher temperature (Landon and Stasiak, 1983), probably due to the higher

metabolic rate that could speed up the breakdown of haemoglobin, as well as decrease the egg development time, resulting in a more frequent passage of Hb to the offspring.

2.5.1.4 Hb content and respiration rate

Low oxygen concentrations (<3 mg O_2 L^{-1}) in the surrounding water body usually cause a heavy decline of respiration and filtering rates in *Daphnia*. However, due to adaptation by the synthesis of Hb, several species are able to stabilize their metabolism at even lower oxygen concentrations (*D. magna* (Heisey and Porter, 1977), *D. pulex* (Weider and Lampert, 1985)). Hb-rich (hypoxia acclimated) animals display a lower metabolic rate than control animals under normoxic conditions at any given temperature (Wiggins and Frappel, 2000; Seidel *et al.*, 2005), but exposure to hypoxia fails to elicit a further reduction in respiration rate (Wiggins and Frappel, 2000). Changes in metabolic rate might arise from alterations in mitochondrial density and/or capacity, which are well known to occur in animals during thermal acclimation (Seidel *et al.*, 2005).

2.5.2 Interaction between temperature and food quality: the importance of sterols and long chain polyunsaturated fatty acids

Interactions of temperature effects with certain other environmental factors are well studied in *Daphnia*. Examples include interactive effects of temperature and dissolved oxygen content on *Daphnia* haemoglobin content (Wiggins and Frappell, 2002) and subcomponent composition (Zeis *et al.*, 2004a), studies regarding temperature interaction with *Chaoborus* (Hanazato, 1991) and fish (Bernot *et al.*, 2006) kairomones, and interaction between temperature and photoperiod (Armitage and Landau, 1982).

Interaction between food concentration and temperature was studied extensively (McKee and Ebert, 1996; Sushchenya and Trubetskova, 1981; Orcutt and Porter, 1984; Mourelatos and Lacroix, 1990; Gibelhausen and Lampert, 2001; Bunioto and Arcifa, 2007), especially with relation to temperature and food gradients in stratified lakes (Stich and Lampert, 1984; Kessler and Lampert, 2004). Generally, reduced temperature effects are found at reduced food conditions where growth rates are low. Animals are more sensitive to food shortage at higher temperatures, where energy demands are higher. Consequently, the temperature tolerance range is narrower when food quantity is lower.

Although researchers have long been aware that food quality also affects *Daphnia* ingestion, assimilation, survival, and reproduction (Arnold, 1971), fecundity and population

growth rate (Kilham *et al.*, 1997), lipid composition (Taub and Dollar, 1968; Weider *et al.*, 2005), and juvenile growth rate (von Elert *et al.*, 2003), its interaction with temperature has rarely been studied (D'Abramo, 1979; Masclaux *et al.*, 2009).

Phytoplankton communities undergo seasonal succession (Aleya, 1992; Berman *et al.*, 1992; Agbeti *et al.*, 1997). Different species of algae differ in food quality to zooplankton (Ahlgren *et al.,* 1990; Weers and Gulati, 1997a; von Elert and Stampfl, 2000). Even the nutrient and lipid composition of a single alga species changes in the season as a consequence of changes in nutrient availability and temperature (Holton *et al.*, 1964). Knowing how the effects of changing food quality interact with the effects of changing temperature is therefore very important for understanding *Daphnia* demography, physiology and biochemistry at different times in the season.

There are four mutually non-exclusive mechanisms by which the food affects its consumers (Gulati and Demott, 1997): (1) size and shape of the food particles, food selectivity, feeding inhibition and ingestion rates; (2) morphological defences against digestion; (3) nutritional inadequacy (amount of P, N, and fatty acids); and (4) presence of toxins. Ahlgren *et al.* (1990) pointed out that the poor food quality of cyanobacteria, and the high quality of flagellates and diatoms, could be accounted for by differences in fatty acid composition. Many studies point to the importance of certain long-chain polyunsaturated fatty acids (PUFAs) for determinantion of food quality for zooplankton (Weers and Gulati, 1997a; von Elert and Stampfl, 2000; Wacker and von Elert, 2001). Recent studies show that the presence of sterols is also important for food quality (von Elert *et al.*, 2003).

Sterols are an integral part of all eukaryote membranes. Cholesterol is the prevailing sterol in the membranes of animals. Sterols also serve as precursors of steroid hormones (Goad, 1981), such as ecdysteroids, which are involved in the process of molting (Harrison, 1990). Because arthropods are not capable of *de novo* synthesis of sterols, these compounds must be obtained from their food (Goad, 1981). Absence of dietary sterols constrains growth and reproduction of *Daphnia* (von Elert *et al.*, 2003; Martin-Creuzburg and von Elert, 2009). Somatic growth is affected by low sterol diet more than reproduction (Martin-Creuzburg and von Elert, 2009). The herbivorous cladoceran *Daphnia*, unlike carnivorous crustaceans, cannot rely on a dietary source of cholesterol because only trace amounts are found in many phytoplankton species (Patterson, 1991). Eukaryotic phytoplankton contains a great variety of plant sterols (Patterson, 1991), which can be distinguished from cholesterol by their chemical structure. These phytosterols are often characterized by additional substituents or by the position and/or number of double bonds in the side chain or in the sterol nucleus (Martin-Creuzburg and von Elert, 2004). The crustaceans examined to date are capable of converting dietary sterols to cholesterol, but not all sterols are suitable precursors for the synthesis of cholesterol (Martin-Creuzburg and von Elert, 2004). In many insects, 7-dehydrocholesterol is an intermediate in the

transformation of cholesterol to ecdysteroids. Supplementation of a sterol-free cyanobacterium with 7-dehydrocholesterol improved the growth of *D. galeata* more efficiently than cholesterol, indicating that the hormone production is an important sink of sterols (Martin-Creuzburg and von Elert, 2004).

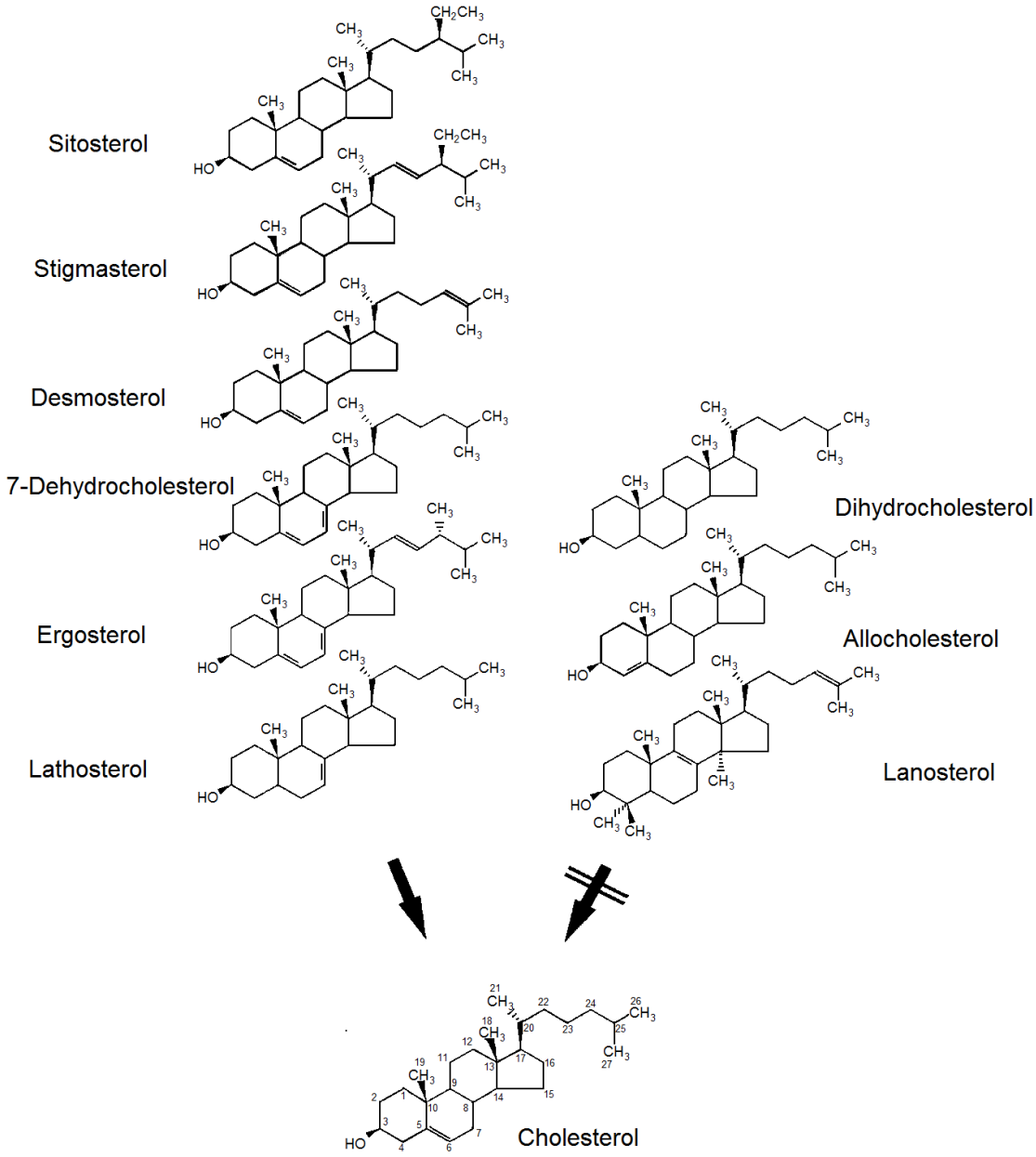

Figure 1: *Structural requirements for the conversion of dietary sterols to cholesterol in* Daphnia galeata*. Sterols on the left are suitable precursors for the synthesis of cholesterol, whereas sterols on the right are not. Reproduced from Fig. 1 in Martin-Creuzburg and von Elert (2004).*

Martin-Creuzburg *et al*. (2005) calculated that population growth rates of *D. magna* will decline at sterol contents of <3.4 µg mg^{-1} dietary C. Sperfeld and Wacker (2009) found that the growth rate of *D. magna* increased with increasing dietary cholesterol up to 7-9 µg cholesterol per mg^{-1} dietary C. The offset of growth limitation of *D. magna* by cholesterol increased with increasing temperature, indicating a higher demand for cholesterol at higher temperatures. Also, the cholesterol content was higher at higher temperatures. Cholesterol stabilizes lipid membranes; therefore increased cholesterol content is expected at high temperatures or in animals exposed to temperature fluctuation (Crockett, 1998). Furthermore, the frequency of molting and growth rate increases with temperature (e.g. Munroe *et al.*, 1975), also increasing the sterol requirements.

Fatty acids (FAs) can be used as an energy source and are primarily stored in the form of triacylglycerols (TAGs). TAGs serve as the major energy reserves in Cladocera (Goulden and Horning, 1980). They can represent up to 70 % of total lipids (Macedo and Pinto-Coelho, 2001; Mezek, 2009). FAs are also associated with membrane lipids, in particular phospholipids (PL) and sterol esters (Weers *et al.*, 1997). Polyunsaturated FAs (PUFAs) increase membrane fluidity and are therefore important for cold acclimation. They are also precursors for important animal hormones. PUFAs, in particular arachidonic acid (ARA; C20:4n6) and eicosapentaenoic acid (EPA; C20:5n3) serve as precursors to eicosanoids (Blomquist *et al.*, 1991). Eicosanoids, which include prostaglandins, thromboxanes, leukotrienes, hydroxy FAs and lipoxines, are critical in a very wide range of physiological processes in invertebrates. These include regulating egg-production, egg-laying, spawning and hatching, mediating immunological responses to infections, and regulating epithelial ion and water flux, temperature set points, and neurophysiology (Stanley-Samuelson, 1994).

PUFAs are almost exclusively synthesized by plants. Animals can convert from one form of PUFA to another through elongation and desaturation, but very few can synthesize PUFAs *de novo* (Brett and Müller-Navarra, 1997). Linoleic (LIN; 18:2n6) and α-linolenic acid (ALA; C18:3n3) are essential for almost all animals (with the exception of some terrestrial omnivorous insects; Blomquist *et al.*, 1991). Cladocerans have the capacity to convert LIN to ARA and ALA to EPA. However, the rates of conversion were shown to be low and probably do not meet metabolic demands, which explains the importance of C20 PUFAs as dietary compounds in the food of *Daphnia* (Weers *et al.*, 1997). A positive correlation has been established between the EPA content of phytoplankton and growth of *Daphnia* in the laboratory (Ahlgren *et al.*, 1990; von Elert, 2002) and in nature (Müller-Navarra, 1995; Wacker and von Elert, 2001). A dietary supply of long chain PUFAs is also important for many other aquatic animals (Brett and Müller-Navarra, 1997). Daphnids have an extremely low capacity to synthesize their lipids; more than 98 % of the accumulated lipid of *Daphnia* is derived from their diet (Goulden and Place, 1990). This stresses the importance of FAs in the food of *Daphnia*.

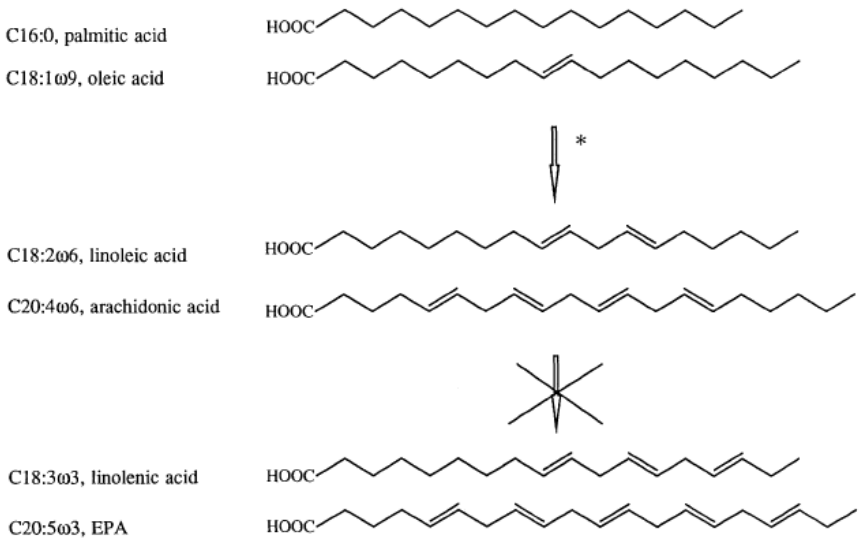

Figure 2: *Schematic presentation of fatty acid (FA) metabolism. Major non-essential FAs which are synthesized* de novo *are palmitic and oleic acid. Desaturation of oleic acid into linoleic acid (LIN) does not occur in most animals, with the exception of seventeen insect species (*). LIN can be further metabolized into arachidonic acid (ARA). Conversion of LIN or ARA (both n6 – referring to the position of the first double bond counting from the methyl end) into the n3-FAs (linolenic acid (ALA) and EPA) is not possible in animal tissues, but ALA can be transformed into EPA.*

Ectotherms have to adapt their biochemistry and physiology in order to function under the wide range of temperatures encountered in the field (Angilletta, 2009). Therefore an effect of temperature on the dietary requirements of organisms is expected. Recent studies on zooplankton indicate that the nature of the interaction between food quality and temperature may depend on the mechanism that determines the quality of the food (Masclaux *et al.*, 2009; Sperfeld and Wacker, 2009; Persson *et al.*, 2010). Masclaux *et al.* (2009) reported higher food quality constraints on zooplankton growth and reproduction at lower temperatures, which they attributed to temperature-dependent differences in the PUFA-requirements of the herbivores. On the other hand, cholesterol content and elemental nutrient content were found to impose higher constraints on *Daphnia* growth at high growth temperatures (Sperfeld and Wacker, 2009; Persson *et al.*, 2010).

3 MATERIALS AND METHODS

3.1 ORIGIN OF EXPERIMENTAL ANIMALS

Five species from the genus *Daphnia* were selected as model species: *Daphnia hyalina* Leydig, 1860; *D. magna* Straus, 1820; *D. pulex* Leydig, 1860; *D. pulicaria* Forbes, 1893; and *D. rosea* G. O. Sars, 1862. With the exception of *D. magna,* all species were collected from the field. *D. magna* individuals used in experiments are from a laboratory stock culture maintained for many years at 20°C. *D. hyalina* was collected from the largest natural Slovenian lake, Lake Bohinj (*Table 1*). Lake Bohinj is stratified in the summer. The highest epilimnion temperatures are around 20 °C. Hypolimnion temperatures during stratification are 4 °C and the oxygen concentration in the hypolimnion does not fall below 55 % (ARSO, 2007). The lake usually has some ice cover in winter, but the ice cover duration decreased and the incidence of ice-free winters increased in the last century (Frantar, 2004). It was never completely frozen during our study. Both fish and invertebrate predators are present in the lake. *D. hyalina* was present throughout the year, the density usually highest in the summer.

D. pulicaria was collected from two high-mountain lakes: Zgornje Kriško Jezero and Srednje Kriško Jezero (*Table 1*). These lakes are covered with ice from November to June, the upper lake a month longer (Brancelj, 2002). The growth season is thus very short. *D. pulicaria* forms resting eggs in autumn and spends the winter in inactive state. The lakes are stratified in the summer. At the end of summer epilimnion depth is 3-4 m. The highest epilimnion temperatures are around 15 °C. Hypolimnion temperatures during stratification are 4 °C. Oxygen concentrations are usually high but may fall down to 30 % in the bottom layer. There are no fish in these lakes.

D. pulex and *D. rosea* were collected from a lowland pond Hraški Bajer (*Table 1*). The Hraški Bajer pond is a permanent water body surrounded by a dense stand of *Typha latifolia* L., 1753. It is periodically covered with *Lemna minor* L., 1753, in the summer. Hraški Bajer is too shallow to develop proper stratification. Temperatures increased from 4 °C in winter, when the pond is usually periodically covered with ice, up to 29 °C in the summer. Temperature changes can be quite fast. Oxygen concentrations in the summer were usually below 50 % and probably decreased further at night. The rest of the year oxygen concentrations exceeded 70 %. No *Daphnia* were found in the pond from December to the middle of May. Both species coexisted but *D. rosea* was prevalent in the spring and summer, whereas *D. pulex* was prevalent in autumn. There are no fish in this pond, but a great density of *Chaoborus* larvae was observed in the summer.

Table 1: *Basic characteristics of water bodies that were sources of* Daphnia *species for our experiments.*

Lake or pond	*Height above sea level (m)*	*Geographical position*	*Median area (ha)*	*Maximum depth (m)*	*Maximum summer water temperature*	*Trophic state*
Zgornje Kriško Jezero[1]	*2150*	*N 46°24'32'' E 13°48'34''*	*0.662*	*9*	*16 °C*	*ultra-oligotrophic*
Srednje Kriško Jezero[1]	*1950*	*N 46°24'13'' E 13°48'26''*	*0.292*	*9*	*16 °C*	*ultra-oligotrophic*
Lake Bohinj[2]	*526*	*N 41°27' E 12°72'*	*328*	*45*	*21 °C*	*oligotrophic*
Hraški Bajer	*350*	*N 46°10'34'' E14°26'39''*	*0.6*	*1*	*29 °C*	*eutrophic*

Sources: [1]Brancelj (2002), [2] ARSO (2007)

Ideally, each sampling date would provide animals for physiological and biochemical analyses and growth experiments. Also ideally, sampling would be distributed throughout the season as to include the widest possible range of acclimatization temperatures. Possibly, these acclimatization temperatures should be the same for all four species. However, the reality of the seasonal dynamics of the abundance of the species in question and the rather large numbers of individual required for the analyses put certain limitations on our plans. For example 900 *D. hyalina* were required for the respiration experiments and measurement of the ETS activity, further 300 for lipid analyses (the bare minimum) and over 500 ovigerous adults to release approximately the required number of newborns for growth experiments. All these analyses are also time intensive and it proved impossible to run both growth and physiological experiments on the same sample. Therefore some compromises had to be made. The resulting pattern of sampling reflects the times of relatively high abundance of *Daphnia* in the field.

3.2 EXPERIMENTS WITH FIELD COLLECTED ANIMALS

3.2.1 Determination of respiration rate (R)

Individuals for analyses, experiments and laboratory cultures were collected (plankton net with 100 µm mesh size) from each population at different times during the season. Water temperature, dissolved oxygen content (MultiLineP4, WTW, Weilheim, Germany) and conductivity (Multi 340i, WTW, Weilheim, Germany) were recorded at each sampling (*Table 2*).

Table 2: *Sampling information for measurements of respiration rate and ETS activity.*

Group name	*Species*	*Lake/ Pond*	*Sampling date*	*T (°C)*	*Dissolved O_2 (%)*	*Conductivity ($\mu S\ cm^{-1}$)*
hyalina4	*D. hyalina*	*Lake Bohinj*	*18.2.08*	*4*	*100*	*160*
hyalina20	*D. hyalina*	*Lake Bohinj*	*22.7.08*	*20*	*116*	*178*
pulicaria15	*D. pulicaria*	*Zg. Kriško J.*	*12.9.07*	*15*	*90*	*104*
pulex12	*D. pulex*	*Hraški Bajer*	*8.10.07*	*12*	*75*	*266*
pulex4	*D. pulex*	*Hraški Bajer*	*16.11.07*	*4*	*87*	*285*
rosea21	*D. rosea*	*Hraški Bajer*	*19.5.08*	*21*	*70*	*295*
rosea28	*D. rosea*	*Hraški Bajer*	*1.7.08*	*28*	*35*	*320*

Respiration rate was measured at 6 different temperatures (5, 10, 15, 20, 25 and 30 °C) by the closed-bottle method using OXY-4 Oxygen Meter (PreSens, Regensburg, Germany). Special attention was taken to insure that the dissolved oxygen concentration did not fall below 75 % as in this range, respiration rate is unaffected by oxygen concentration (Paul *et al.*, 1997). The number of individuals per bottle was kept constant across all experimental temperatures (30 for *D. hyalina* and *D. rosea*, 15 for *D. pulex* and *D. pulicaria*) to avoid the effect of crowding on respiration rates, thus the duration of incubation was temperature dependent (15-6 hours at 5 °C – 30 °C respectively).

Only ovigerous females with early-stage eggs were used in experiments to avoid hatching during the experiment and to minimize the contribution of embryos to the respiration rate of brooding females. Due to their immobility, eggs and early stage embryos have lower respiration rates than late stage embryos and newborns (Glazier, 1991). Only ovigerous females were chosen to insure maturity and comparable physiological state (assuming that ill, starving or stressed individuals would not bear healthy parthenogenetic eggs). Animals were starved for 12 hours before measurement to exclude the effect of specific dynamic action. Thirty (*D. rosea* and *D. hyalina*) or 15 (*D. pulicaria* and *D. pulex*) ovigerous females were rinsed with synthetic water (294 mg L^{-1} $CaCl_2*2H_2O$, 123 mg L^{-1} $MgSO_4*7H_2O$, 65 mg L^{-1} $NaHCO_3$ and 6 mg L^{-1} KCl) and placed in 75 mL Erlenmeyer flasks filled with synthetic water. There were 5 replicates per treatment. Three 75 mL Erlenmeyer flasks filled with synthetic water only, served as control. Oxygen concentration was measured immediately after closing the bottles and at the end of incubation.

Oxygen consumption rate was calculated as:

$$R\ (\mu L\ O_2\ h^{-1}\ mg^{-1}\ dry\ mass) = \frac{(c_{s0} - c_{st})V_s - (c_{c0} - c_{ct})V_c}{Wt * 1.42}, \quad (6)$$

where:

R - respiration rate,

W - the calculated dry mass of *Daphnia* in the flask,

t - incubation time,

V_c and V_s - the volumes of control and sample closed flasks, respectively,

($c_{c0} – c_{ct}$) and ($c_{s0} – c_{st}$) - the difference in oxygen concentration between the beginning and the end of incubation for control and sample flasks, respectively. The control term was calculated as the average of three control flasks.

1.42 - the transformation factor from mg to µl of oxygen.

Dry mass of *Daphnia* in the flasks was calculated from wet mass measurements conducted before ETS activity analyses. The transformation factor was obtained by drying and weighing of 50-100 *Daphnia* of each species.

3.2.2 Determination of electron transport system activity (ETS) and the ETS/R ratio

Electron transport system (ETS) activity of *Daphnia* was measured using the method developed by Owens and King (1975) and improved by G.-Tóth (1993). Wet weighted *Daphnia* from respiration experiments, rinsed with distilled water, were homogenized in 4 mL of ice-cold homogenisation buffer for 1 minute with Eurostar homogeniser (IKA Labortechnik, Staufen, Germany) and 20 seconds with 4710 Ultrasonic Homogeniser (Cole-Parmer, Vernon Hills, Illinois, USA) and centrifuged at 0 °C and 10,000 rpm for 4 minutes (Sigma 2K15, Sigma Laborzentrifugen, Osterode am Harz, Germany). 0.5 mL of the homogenate supernatant (in triplicate) was transferred to glass vials where ETS assay was carried out. It was incubated in water baths of appropriate temperature (5, 10, 15, 20, 25 and 30 °C) within 10 minutes with 1.5 mL substrate solution and 0.5 mL dye. After 40 minutes of incubation, the reaction was stopped by adding 0.5 mL of stopper solution (concentrated orto-phosphoric acid : formaldehyde = 1 : 1, v : v). Blanks (1.5 mL substrate solution and 0.5 mL dye) were incubated and stopped as the samples, while 0.5 mL of homogenate was added after stopping. The formazan production was determined spectrophotometrically from the absorption of the sample against the blank at 490 nm within 10 min after stopping the reaction. The average absorption of three replicates of a sample was used in calculation.

ETS activity was calculated according to Kenner and Ahmed (1975) from the equation:

$$ETS\ activity\ (\mu L\ O_2\ mg^{-1}\ h^{-1}) = \frac{Abs_{490\,nm} * V_r * V_h * 60}{V_i * W * t * 1.42}, \quad (7)$$

where:

Abs_{490nm} – the average absorbance of sample replicates at 490 nm against the sample blank,

V_r – the end volume of reaction mixture (3 mL),

V_h – the volume of starting homogenate (4 mL),

V_i – the volume of incubated homogenate (0.5 mL),

W – the calculated dry mass of *Daphnia* homogenised,

t – the time of incubation (40 min) and

1.42 – the factor of transformation into the volume of oxygen (Kenner and Ahmed, 1975).

ETS/R ratio was calculated separately for each sample as the ratio of the ETS oxygen consumption measured *in vitro* to the respiration rate measured *in vivo*.

3.2.3 Mass correction of ETS activity and respiration rate

The scaling of metabolic activity (ETS and R) with body mass between species was determined by fitting our data to the logarithm transformed equation (Gillooly *et al.*, 2001):

$$\ln Q = \ln a + b \ln M - E / kT , \quad (8)$$

where:

Q – the respiration rate or ETS activity per individual,

a – a scaling constant

M – body mass,

b – the mass scaling coefficient

T – experimental temperature (K),

k – Boltzmann's constant (8.31 J mol^{-1} K^{-1})

E – the activation energy of metabolism.

E, *b* and *a* were fitted with linear regression. Measurements of ETS and R obtained at 25 °C and 30 °C were omitted from the fit to avoid distortion due to thermal stress. Each experimental group at each temperature represented one data point. We also tested whether *b* differs between temperatures by fitting our data to the shortened equation separately at each temperature:

$$\ln Q = \ln a + b \ln M \quad (9)$$

Since no trend of *b* with temperature was observed (see Results), one *b* was used for mass correction at all temperatures.

Mass scaling coefficients determined within each species would be preferable for mass correction, but they could not be established from our data. The size range of ovigerous females was quite narrow at each sampling date. There were usually not enough extremely big or small individuals to establish separate replicates at each of the six temperatures, therefore the mass range of the replicates was even narrower. However, our value of *b* calculated between species agrees well with the within species values reported in the literature (Lynch *et al.*, 1986) and we therefore deem it suitable for mass correction of the ETS activity and respiration rate.

To obtain mass corrected values of ETS activity and respiration rate suitable for between-group comparison, the value of ETS or R per individual was divided by M^b, where *M* is the individual dry mass and *b* is the mass scaling coefficient obtained from the fit (Gillooly *et al.*, 2001). These mass corrected values were then used for comparison of ETS and R between

groups that differed in body mass. This method was preferred to analysis of covariance (ANCOVA) because various ANCOVA assumptions were violated (Sokal and Rohlf, 1995); most notably the covariate “dry mass” depended on the independent variable “group”, slopes of ETS and R against dry mass differed between groups due to narrow within-group mass ranges, and variances were unequal. No transformation of the data could fix all these problems. Therefore for comparing groups that were found to differ in body mass, the above mass correction was used.

3.2.4 Calculation of Arrhenius activation energy (E_a) of ETS activity and respiration rate

The Arrhenius activation energy (E_a) describes the accelerating influence of temperature on metabolic rate over the whole temperature range. E_a is obtained from the slope of linear regression (*S*) of ln of metabolic rate against the inverse of absolute temperature (SPSS 13.0 statistical program). It was calculated from the equation (Simčič and Brancelj, 1997):

$$E_a\ (kJ\ mol^{-1}) = -RS\ , \qquad (10)$$

where:

R - the gas constant (8.31 J mol^{-1} degree K^{-1}) and

S - the slope of the Arrhenius plot.

3.2.5 Growth experiments

Mothers of experimental animals were collected from the field (*Table 3*), brought to the laboratory and acclimated to 20 °C. This way, the synchronisation of the release of offspring was better than at cooler temperatures. Only ovigerous females were chosen. When a sufficient number of offspring were released within 12 hours, a subsample (20 individuals of *D. pulex*, 30 *D. rosea* and *D. hyalina*) was used to determine the start mass of newborns. The remaining animals were distributed between the test temperatures. Ten individuals of *D. pulex* or 20 *D. hyalina* and *D. rosea* were placed into 1 L synthetic water and fed with 2 mg POC L^{-1} *Scenedesmus acutus* Meyen, 1829 (Chlorophyceae). The organic carbon content (mg POC mL^{-1}) of the algal suspension used for feeding was determined spectrophotometrically with the help of a pre-established calibration curve for organic carbon versus extinction at 470 nm. The jars were placed into thermostatic chambers at 10, 15, 20 or 25 (±0.5) °C with a 16 h light / 8 h dark photoperiod. There were 3 replicates at each temperature. Animals were fed daily. They were placed into fresh medium every second day at 10 and 15 °C and every day at 20 and 25 °C. When the animals in an experimental treatment had deposited their first clutch of eggs (after 5-

25 days, depending on experimental temperature), the animals were dried and weighted, after their clutch size (the number of eggs per ovigerous female) had been determined under a dissecting microscope.

Table 3: *Sampling information for growth experiments.*

Group name	*Species*	*Lake/ Pond*	*Sampling date*	*T (°C)*	*Dissolved O_2 (%)*	*Conductivity ($\mu S\ cm^{-1}$)*
hyalina4	*D. hyalina*	*Lake Bohinj*	*1.2.09*	*4*	*100*	*165*
hyalina20	*D. hyalina*	*Lake Bohinj*	*17.7.09*	*20*	*105*	*170*
pulex12	*D. pulex*	*Hraški Bajer*	*4.10.09*	*15*	*72*	*266*
pulex4	*D. pulex*	*Hraški Bajer*	*15.11.09*	*5*	*80*	*275*
rosea20	*D. rosea*	*Hraški Bajer*	*22.6.09*	*20*	*50*	*268*
rosea28	*D. rosea*	*Hraški Bajer*	*12.7.09*	*28*	*33*	*290*

Individual juvenile growth rate (g_j) was calculated from the formula:

$$g\ (day^{-1}) = \frac{\ln W_t - \ln W_0}{t}, \tag{11}$$

where

W_0 - the dry mass of a newborn individual,

W_t - the individual dry mass at the end of the experiment and

t - the duration of the experiment (days).

Growth rate calculated for the 'physiological' juvenile period from birth to the deposition of the first clutch was found to be a good predictor of population rate of increase r and therefore a good indicator of fitness (Lampert and Trubetskova, 1996).

Potential rate of increase (r_{pot} of Lampert and Trubetskova, 1996) was calculated from egg counts. Because only the first clutch was used in calculation and mortality in the experiments was negligible, the Lotka-Euler equation (van Doorslaer *et al.*, 2007) was shortened to:

$$r_{pot}\ (day^{-1}) = \frac{\ln m_x}{x}, \tag{12}$$

where

x - the age at first reproduction and

m_x - the age-specific fecundity.

As in van Doorslaer *et al.* (2007) r_{pot} here does not represent a value suitable for calculation of the real population growth rate, but is rather intended to quantify the reproductive performance of individuals, since age at first reproduction and fecundity are important components of fitness.

3.2.6 Determination of haemoglobin content

Haemoglobin content was measured by a modification of the method described by Pane *et al.* (2003). Thirty specimens of *D. rosea*, 120 of *D. hyalina,* 10 *D. pulicaria* or 10 of *D. pulex* were placed in plastic centrifuge tubes and homogenised (IKA Ultra-Turrax Homogenizer, IKA Labortechnik, Staufen, Germany) in 1.5 mL deionised water on ice. After addition of 1.5 mL of Drabkin's Reagent (Sigma, D 5941), the samples were incubated at room temperature for 30 minutes and centrifuged (5 min, 25 °C, 10,000 rpm). Absorption of the supernatant was measured at 420 nm (the Soret region) and haemoglobin content was determined from a calibration curve (*Figure 3*) constructed with standard solutions of bovine haemoglobin (Fluka, 51290). The estimated regression function (*Figure 3*, SPSS 13.0, n=92, t=271.3, p<0.001) through origin is:

$$Sample\ Hb\ concentration\ (mg\ L^{-1}) = 214.687 * Absorbtion_{420\,nm} \quad (13)$$

A 95 % confidence interval for the regression coefficient is (213.115, 216.260).

Haemoglobin content (*Hb*) per unit dry mass of *Daphnia* was calculated from the equation:

$$Hb = \frac{214.687 * Abs_{420\,nm} * V}{M}, \quad (14)$$

where:

Abs_{420nm} - absorbance of the sample at 420 nm,

V - the volume of the sample (3 mL) and

M - the calculated dry mass of *Daphnia*.

Table 4: *Sampling information for analysis of haemoglobin content in summer populations of* Daphnia*.*

Species	*Lake/ Pond*	*Sampling date*	*T (°C) min-max*	*O_2 (%) min-max*	*O_2 (mg L^{-1}) min-max*	*Conductivity (µS cm^{-1})*
D. pulex	*Hraški Bajer*	*8.8.2008*	*24*	*1-16*	*0.2-4*	*278*
D. rosea	*Hraški Bajer*	*17.7.2008*	*22*	*8-35*	*0.2-3*	*239*
D. pulicaria	*Zg. Kriško J.*	*10.9.2008*	*5-11*	*33-98*	*4-9*	*124*
D. hyalina	*Lake Bohinj*	*22.7.2008*	*6-17*	*93-116*	*9-12*	*178*

Haemoglobin content was determined in the summer field populations (for information on the oxygen and temperature conditions in their respective habitats see *Table 4*) and also in laboratory cultures of *D. pulex*, *D. rosea* and *D. hyalina*. For the latter, mothers of experimental animals were collected from the field (at the same time as for the growth experiments) and kept at 20 °C. Their offspring were transferred to either 15 or 25 °C upon hatching. Feeding and medium exchange proceeded as in the growth experiments. Oxygen concentration in the culture jars never fell below 80 %. There were four replicates at each temperature for *D. pulex* (20 animals per replicate) and *D. rosea* (30 animals per replicate). The *D. hyalina* experiment was

not replicated due to the large number of animals needed for the analysis. Hb content was determined when animals reached maturity.

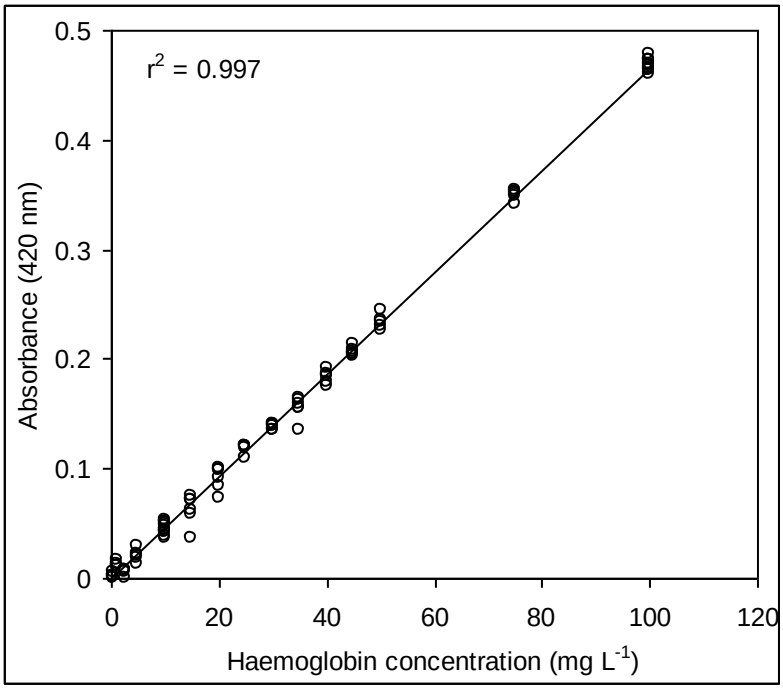

Figure 3: *Calibration curve for haemoglobin concentration determination.*

3.2.7 Statistical analysis

One-way ANOVA (SPSS 13.0) was used to test the differences in body mass between experimental groups. One-way ANOVAs were used to test the effect of experimental temperature on ETS, R, ETS/R, and fitness parameters of *Daphnia* (juvenile growth rate, clutch size and reproductive performance). ANOVA assumptions were checked with Levene's test of equality of variances and examination of residual plots. In cases of unequal variances the robust Welch statistic (Statistical program SPSS 13.0, SPSS Inc., Chicago, Illinois, USA) was calculated instead of the normal ANOVA. Pairwise group comparisons were conducted in case of significant omnibus test with Tukey's HSD post hoc tests in case of equal variances and Dunnett's T3 post hoc tests in case of unequal variances (SPSS 13.0).

The seasonal differences within a species and differences between closely related species (*rosea/hyalina* and *pulex/pulicaria*) in the response of ETS, R, ETS/R and fitness to temperature were tested with two-way ANOVAs (SPSS 13.0) with factors "experimental temperature" and "group". We were interested in the group and interaction effect. We conducted planned comparisons of the two groups at each experimental temperature, because we were interested to know in which temperature range the differences between groups were significant.

Since there were less than k-1 orthogonal comparisons (where k is the number of groups), correction for multiple comparisons was deemed unnecessary (Quinn and Keough, 2002). ANOVA assumptions were checked with Levene's test of equality of variances and examination of residual plots. In case of unequal variances an alternative ANOVA procedure without the assumption of equal variance (XPro*:* Ananda and Weerahandi, 1997) was used and the planned comparisons were made without the assumption of equal variances (SPSS 13.0). Mass corrected values of R or ETS were used for comparing groups that differed in body mass. Only groups collected at comparable water temperatures were used for between-species comparison.

Since multiple ANOVAs were conducted to test the effects of season, species and temperature, some authors recommend adjustment of p values for multiple comparisons. However, since our analyses were planned and are based on theory, such adjustment may not be necessary (Qiunn and Keough, 2002). As a compromise, we report unadjusted p values, but we also calculated stepwise Bonferroni adjusted (Holm, 1979) critical values of p and printed the p still significant after adjustment in bold.

The seasonal and species differences in E_a of ETS and respiration were tested with comparison of regression coefficients of the Arrhenius plots (GraphPad Prism 5) separately for each relevant group pair. Since there were less than k-1 orthogonal comparisons (where k is the number of groups), correction for multiple comparisons was deemed unnecessary (Quinn and Keough, 2002).

The relationship between metabolic variables and fitness variables was explored with Spearman's correlation. We correlated raw fitness variables with mass corrected ETS and R, the ETS/R ratio and experimental temperature. Then we normalized the variables by dividing them with the value at 20°C and made the same correlations. Finally we correlated the seasonal differences in fitness variables with the seasonal differences in mass corrected ETS and R, and the ETS/R ratio.

One-way ANOVA with post-hoc tests was used to test the differences in haemoglobin content between summer field populations. Planned contrast tests were used to test the difference in Hb content at 15 and 25 °C separately for each species. Since no temperature differences were detected, the values were averaged for between species comparison.

3.3 LIPID CONTENT OF DAPHNIA AND ITS EFFECT ON FITNESS AT DIFFERENT TEMPERATURES

3.3.1 Growth experiments with laboratory clones

These experiments were conducted at the Zoological Institute of the University of Cologne (Cologne, Germany).

Experiments were performed with one clone of *Daphnia magna* Straus (B) originally isolated from Großer Binnensee, Germany (Lampert, 1991) and one clone of *D. pulex* Leydig (FR) isolated from the eutrophic pond Hraški Bajer, Slovenia. All experimental and stock *Daphnia* cultures were run in aged and filtered tap water from the Zoological Institute. Food algae *Cryptomonas* sp. (Cryptophyceae, SAG 26.80) and *Chlamydomonas* sp. (Chlorophyceae, strain 56, culture collection of the Limnological Instiute at the University of Konstanz, Germany) were used to supply different quality foods to *Daphnia*. *Cryptomonas* is known to be rich in EPA and is considered to be high quality food for cladocerans, whereas *Chlamydomonas* lacks this long chain PUFA and is considered as lower quality food (Ahlgren *et al.*, 1990). Food algae were grown semicontinuously in Cyano medium (Jüttner *et al.*, 1983) with additional vitamins (Thiamine Hydrochloride 0.3 µM, Biotin 0.002 µM, and Cyanocobalamine (Vit. B12) 0.004 µM) at 20 °C and 100 µmol photons m^{-2} s^{-1}, at dilution rates of 0.25 d^{-1} (*Chlamydomonas* sp.) or 0.1 d^{-1} (*Cryptomonas* sp.). The organic carbon content (POC) of the algal suspension used for feeding was determined spectrophotometrically with the help of a previously established calibration curve for particulate organic carbon versus extinction. As the animals were fed *ad libitum* (2 mg POC L^{-1}) and both algal species are in the size range easily ingested by *Daphnia* (Ahlgren *et al.*, 1990) any differences between the effects of the two diets were attributed to food quality.

The *D. magna* and *D. pulex* clones were acclimated for at least three generations to four different combinations of constant temperature (15 or 20 °C) and food algae species (*Cryptomonas* sp. or *Chlamydomonas* sp.), namely Crypto15°C, Chlamy15°C, Crypto20°C and Chlamy20°C treatments in continuous dim light. During acclimation, animals were fed every day and the medium exchanged every two days. Throughout the dissertation, animals with a certain maternal diet are referred to as being “reared” on that diet. Synchronized third clutch newborns from the previous generation were used to start each new generation. The long acclimation time enabled complete acclimation of the maternal generation to the respective food and temperature conditions. Further, this long acclimation period confirmed that the chosen conditions were not deleterious, but supported good survival and reproduction of *Daphnia*. To avoid prohibitive complexity of the experimental design only two temperatures (15 and 20 °C) were chosen. They fall within the range commonly experienced by zooplankton of temperate lakes and ponds.

Furthermore, a 5 °C temperature difference between the environments of consecutive generations is not unrealistic.

In each growth experiment, newborn animals from one acclimation treatment were distributed randomly to all four experimental combinations. This resulted in 16 combinations of acclimation and experimental environments for each species (4 acclimation treatments x 4 experimental treatments). Synchronized third clutch newborns of acclimated animals were collected within 12 hours of birth. Two random sub-samples (10-15 *D. magna*, 24-29 *D. pulex*) of newborns served for determining the initial dry mass. 6 additional sub-samples of 30 newborns of *D. magna* were frozen for lipid analysis. The remaining animals were used in the growth experiments.

During the experiments, the animals were kept in glass jars (25 *D. magna* or 55 *D. pulex*, 3 replicates per treatment) with 1 L water and 2 mg POC L^{-1} of the assigned food alga (*Cryptomonas* sp. or *Chlamydomonas* sp.) and were transferred to fresh medium and fed daily. The jars were immersed in water baths of the appropriate constant temperature (15 $\pm$ 0.5 °C or 20 $\pm$ 0.5 °C). The position of the jars in the water bath was changed randomly on a daily basis. When the animals in an experimental treatment had deposited their first clutch of eggs into the brood pouch (after 5-10 days, depending on the treatment combination), a random sub-sample of animals from each jar (5 *D. magna* or 15 *D. pulex*) was used to determine their clutch size (the number of eggs per ovigerous female) under a dissecting microscope and subsequently the individual dry mass at the end of the growth experiment. The remaining daphnids were stored for fatty acid analyses. The juvenile growth rate and reproductive performance were calculated from equations (11) and (12).

3.3.2 Determination of lipid content in laboratory clones

At the end of the growth experiments, animals not used for end mass determination, collected from each jar, were distributed into two separate vials (10 *D. magna* or 20 *D. pulex*) with 5 mL of methylene chloride : methanol (2 : 1, v : v) and frozen at -20 °C, one for fatty acid and one for sterol analysis. Six sub-samples of 30 *D. magna* newborns from each acclimation treatment were prepared in the same way.

Before the beginning of fatty acid extraction 20 µg of C 17:0 and C 23:0 were added to the sample as internal standards. Fatty acids were extracted twice with 4 mL of methylene chloride : methanol (2 : 1, v : v). Particles were removed by centrifugation (4000 *g*, 5 min). The supernatant was evaporated to dryness under nitrogen atmosphere. The dried sample was resuspended in 3 mL of 3 M methanolic HCl and subsequently incubated 20 min at 70 °C in a sealed vial in order to trans-esterify fatty acids into methyl esters. After the sample had cooled

down fatty acid methyl esters (FAMEs) were extracted three times with 3 mL of *iso*-hexan. The fraction of *iso*-hexan was evaporated to dryness under nitrogen atmosphere and resuspended in a volume of 100 µL *iso*-hexan.

FAMEs were analysed by a gas chromatograph (GC, HP 6890) equipped with a flame-ionization detector. A total of 1 µL of the sample was injected onto a capillary GC-column (DB 225, J & W Fused Silica GC Columns, Agilent). FAMEs were identified by comparison of retention times with those of reference compounds. Quantification was performed by referring to the internal standards and to response factors determined for each FAME from mixtures of known composition.

FAs are reported in the form CX:YnZ, where X is the number of C atoms in the molecule, Y is the number of double bonds and Z is the position of the first double bond counting from the methyl end of the molecule. The more common and biologically relevant FAs are referred to by abbreviations of their common names (see Symbols and abbreviations). Results are reported as FA content (in µg mg^{-1} d.m. of sample) or relative FA composition (share of a FAME in total FA content). The FA composition is shown in graphs where the relative content of each FA that exceded 0.5 % average share in total FA content is shown and the remaining indentified FAs are designated as “Other”. These “Other” FAs were omitted from factor analyses and discriminant analyses.

We report the relative content of saturated FAs (SAFA – sum of FAs containing no double bonds), monounsaturated FAs (MUFA – sum of FAs containing one double bond) and polyunsaturated FAs (PUFA – sum of FAs containing more than one double bond). Fatty acid unsaturation of *Daphnia* lipids was assessed using the unsaturation index (*UI*), calculated as:

$$UI = (\sum P_j * d_j)/100, \quad (15)$$

where:

P_j - relative content of the fatty acid *j* (in % of total FA content) and

d_j - the number of double bonds in the fatty acid *j* (Lyons *et al.*, 1964).

Sterols were analysed after extraction and saponification as free sterols with GC (HP 6890) equipped with an HP-5 capillary column (Agilent) and a flame ionization detector. Cholesterol was quantified by comparison with an internal standard (5-o-cholestane) and with a response factor determined for cholesterol. The results are reported as cholesterol content per unit dry mass of sample.

3.3.3 Lipid content of field collected Daphnia

Ovigerous females of four *Daphnia* species were collected from the field (*Table 5*), rinsed with distilled water and stored in lipid free glass vials at -80 °C. 50-100 animals (depending on

body size) were joined in one sample. Before lipid extraction they were freeze-dried and the dry mass of the sample determined on a micro balance (to the nearest 1 µg; Sartorius ME 5). Synthetic lipid 5-α-cholestane (50 µg) was added to each sample prior to extraction as internal standard to provide indication of extraction efficiency. Subsequently lipids were extracted according to modified Folch procedure (Folch *et al.*, 1956; Mezek, 2009) in chloroform : methanol (2 : 1, v : v). Samples were homogenised (Fluka Ultra Turax T8) and particles removed by centrifugation (4000 rpm, 8 min, 4 °C). The joined supernatant was washed three times with chloroform : methanol : 0.9 % NaCl solution (2 : 1: 0.2, v : v : v). Sample was centrifuged each time (10 min, 4000 rpm, 4 °C) and the upper salt-water-methanol layer discarded. The lipid solution was evaporated to dryness under a nitrogen atmosphere.

The dried sample was resuspended in 1 mL of *iso*-hexan and two aliquots, 100 µl each, were transferred into pre-weighted aluminium vessels (Elemental Microanalysis Limited P/N 4057), and re-weighted after solvent has been evaporated. The average mass of the lipid residue was used for gravimetric determination of total lipids in the sample.

Table 5: *Sampling information for analysis of lipid content in field populations of* Daphnia*.*

Group	*Lake/pond*	*Date*	*T (°C)*	*n*
D. pulicaria SR	*Sr. Kriško J.*	*11.9.2007*	*15*	*2*
D. pulicaria ZG	*Zg. Kriško J.*	*12.9.2007*	*15*	*2*
D. hyalina	*Lake Bohinj*	*6.11.2007*	*4*	*2*
D. pulex Oct.	*Hraški Bajer*	*8.10.2007*	*12*	*1*
D. rosea Oct.	*Hraški Bajer*	*8.10.2007*	*12*	*1*
D. rosea May	*Hraški Bajer*	*5.5.2009*	*16*	*3*

The remaining resuspended sample (800 µl) was evaporated under nitrogen atmosphere and resuspended in a solution of methanol : toluene (2 : 1, v : v) in 1 % sulphuric acid (methylation reagent) and incubated for 24 h at 50 °C in sealed vials in order to trans-esterify fatty acids into methyl esters. After the sample had cooled down fatty acid methyl esters (FAMEs) were extracted from the methylation reagent three times with *iso*-hexan (2 : 1, v : v). The fraction of *iso*-hexan was evaporated to dryness under nitrogen atmosphere and resuspended in a volume of 100 µL *iso*-hexan. FAMEs were analysed by GC-MS (Agilent Technologies 6890N GC) equipped with a polar capillary column (Agilent Technologies; 60-m x 0,25 mm id x 0.15 µm DB-23 (P/N 122-2361)), an Agilent 7683B injector, and a mass selective quadrupole detector (Agilent 5973N). FAMEs were identified by comparison of retention times with those of reference compounds and from their mass spectra. Quantification was performed by referring to the internal standard and to response factors predetermined for each FAME from mixtures of known composition.

The results are reported as for the FA analysis of laboratory clones. The same species collected from different lakes or at different seasons were treated as different groups. Factor

analysis and cluster analysis of relative FA composition of FA representing at least 5 % of total FA in at least one group were used to analyse the difference in relative FA composition of different populations.

3.3.4 Statistical analysis

Four-way ANOVASs (SPSS 13.0) were used to test the effects of acclimation temperature (AT), acclimation food quality (AF), experimental temperature (ET) and experimental food quality (EF) and their interactions on the lipid content and fitness parameters separately for the two *Daphnia* clones in our experiment. ANOVA assumptions were checked by Levene's test of equality of variance and examination of residual plots. Tukey's HSD post hoc tests were used to determine which groups differed in fitness parameters. Some of the lipid variables had unequal group variances and in these cases we used an alternative procedure with contrasts without assumption of equal variances. Accordingly, planned contrasts comparing groups that differed in only one factor were performed without assumption of equal variances in these cases. Planned contrasts were preformed in SPSS 13.0 with the ONEWAY procedure.

The effect of maternal food quality and acclimation temperature and their interaction on the lipid content of newborn *D. magna* was tested with 2-way ANOVAs (SPSS 13.0). In case of unequal variances we ran an alternative ANOVA procedure without the assumption of equal variances with the program XPro. Differences in lipid content between adult and newborn *Daphnia* from the same acclimation treatment were tested with planned contrasts (SPSS 13.0).

Factor analyses (SPSS 13.0) of relative FA composition were used to analyse the differences in FA profiles between different experimental groups (in laboratory clones) or between field populations of *Daphnia*. Correlation matrix was analysed with the principal components method, factors with Eigenvalues over 1 were extracted and the unrotated factor solutions displayed. Additionally we performed cluster analysis (KyPlot) of the relative FA composition of field populations with standardized Euclidean distance measure and Ward's clustering method to verify the results of factor analysis with a different method. The results were presented in a dendrogram.

Discriminant analysis (SPSS 13.0) was used to determine the differences between the FA profiles of adult and newborn *D. magna* and differences between the FA profiles of *D. magna* and *D. pulex*. Dependents were "age" and "species", respectively. All independents (the relative FA content of FAs that represented more than 0.5 % average share in total FA content) were entered together. Prior probabilities of all groups were equal. We checked the model with leave-one-out classification, where each case is classified to a group according to a discriminant function derived from all other cases but that case.

Spearman's correlation (SPSS 13.0) was used to correlate the fitness of *Daphnia* (juvenile growth rate, clutch size, reproductive performance) with their lipid content (EPA, cholesterol, UI).

Linear regression (GraphPad Prism 5) was used to explore the relationship between juvenile growth rate and reproductive performance and to test the difference of slopes of the regression between different temperature groups. We also used this procedure to explore the relationship between fitness parameters (juvenile growth rate, clutch size, reproductive performance) and the EPA content of *Daphnia* (ln transformed) at different temperature combinations.

Finally we used model comparisons to determine whether the effect of food quality on the fitness of *Daphnia* can be explained by its effect on the lipid composition and whether the cholesterol content (Cho) or EPA content was more important in that respect. Different models were compared on the basis of Akaike's information criterion corrected for small sample sizes (AICc; SPSS 13.0, mixed-models procedure). Models with lower AICc are deemed better. We used different combinations of fixed effects predictors (AT, ET, AF, EF) and covariates (EPA, Cho) to predict the fitness parameters of *Daphnia*. We compared the best models with only temperature predictors (AT, ET), the best model with temperature and food quality predictors (AT, ET, AF, EF), the best model with temperature and EPA (AT, ET, EPA), the best model with temperature and cholesterol (AT, ET, Cho) and the best model with temperature and both lipids (AT, ET, EPA, Cho). If the best model with one or both lipids was as good as or better than the best food quality model we may assume that the effect of food quality was most likely mediated through its effect on that lipid. If the best lipid model was much worse than the best food quality model or even worse than the model with just temperature predictors, we may assume that the lipid in question is not related to the fitness parameter. The comparison of the best lipid models tells us whether EPA, cholesterol or both are the best predictors of fitness.

4 RESULTS

4.1 EXPERIMENTS WITH FIELD COLLECTED ANIMALS

4.1.1 Body mass

The species of *Daphnia* chosen for this study differed in body mass (Welch ANOVA, $F_{W(6, 45.3)}$=448.1, p<0.001). *D. pulex* and *D. pulicaria* had a much higher body mass than *D. rosea* and *D. hyalina* (*Figure 4*). Because body size importantly influences metabolic rates, only species pairs of similar body sizes were compared. In each species pair compared, pond dwelling species was larger than lake species; *D. pulex* was larger than *D. pulicaria* (*Figure 4*) and *D. rosea* was significantly larger than *D. hyalina* (*Figure 4*).

The average body mass of *D. rosea* from the two collection times did not differ significantly, but *D. pulex* and *D. hyalina* individuals collected at 4 °C were significantly bigger than those collected at warmer water temperatures (*Figure 4*).

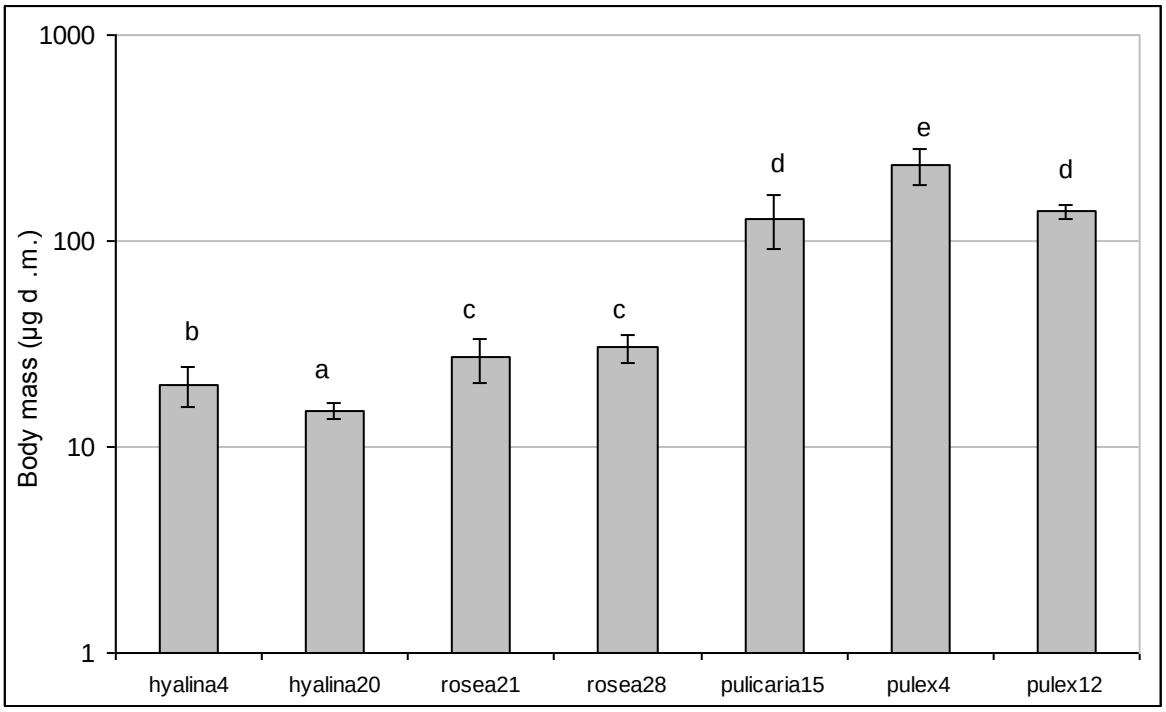

Figure 4: *Average dry mass of* Daphnia *used in experiments. The number next to the species name represents water temperature at the time of collection. Values are means±SD. Means marked with different letters differ significantly (post hoc Dunnett T3 test, p<0.05).*

4.1.2 Mass scaling of ETS activity and respiration

Individual ETS activity and respiration rate (ETS or R in mL O_2 h^{-1} $individual^{-1}$) scaled with body mass (M; in µg) and experimental temperature (T; in K) according to the following equations:

$$ln(ETS) = ln(a) + b \cdot ln(M) - (E_a/k) \cdot T^{-1} \text{ (n=34, } F_{(2,31)}=335.6, p<0.001, r^2=0.953), \quad (16)$$

$$ln(R) = ln(a) + b \cdot ln(M) - (E_a/k) \cdot T^{-1} \text{ (n=34, } F_{(2,31)}=148.9, p<0.001, r^2=0.900), \quad (17)$$

where k is the Boltzmann's constant (k = 8.31 J K^{-1} mol^{-1}), a is the scaling constant, b the mass scaling coefficient and E_a is the activation energy of metabolism (kJ mol^{-1}). Values of the coefficients are given in *Table 6*. Respiration rate per individual increased more with body mass than did ETS activity. In other words, respiration per unit mass decreased less with mass than ETS activity per unit mass (*b*, *Table 6*). On the other hand, ETS increased more with temperature than did respiration rate, as seen from the calculated activation energy (E_a, *Table 6*).

Table 6: *Coefficients from a multiple regression analysis of the relation of* Daphnia *ETS activity and respiration rate (R) to body mass (M) and experimental temperature (T). ETS and R were determined in four species of* Daphnia *(*D. pulicaria*,* D. pulex*,* D. hyalina *and* D. rosea*) at four different experimental temperatures (5, 10, 15, 20 °C).*

Variable	*Coefficient*	*mean*	*SE*	*t*	*p*	*95% CI*	
ETS	*ln(a)*	*13.917*	*1.707*	*8.2*	***<0.001***	*10.435*	*17.399*
	b	*0.656*	*0.031*	*21.4*	***<0.001***	*0.593*	*0.718*
	E_a	*58.738*	*4.045*	*14.5*	***<0.001***	*50.489*	*66.987*
R	*ln(a)*	*3.818*	*2.679*	*1.4*	*0.164*	*-1.646*	*9.281*
	b	*0.780*	*0.048*	*16.2*	***<0.001***	*0.682*	*0.878*
	E_a	36.742	6.346	*5.8*	***<0.001***	*23.799*	*49.684*

The *b* for ETS and R differed with temperature (*Table 7*), but there was no clear pattern. The values at 5 °C tended to be higher than at higher temperatures. The % of variance in respiration explained by the model was lower at higher temperatures; indicating differences due to factors other than body mass (acclimatization, adaptation) were stronger there. The highest temperatures (25 and 30 °C) were excluded from the analysis, because the individual temperature tolerance range was more important than body mass at these temperatures.

Table 7: *Mass scaling coefficients of* Daphnia *ETS activity and respiration rate (R) to body mass (M) in dependence of experimental temperature (T). Data were fit to the equation ln(Q) = ln(a) + b*ln(M), where Q is the metabolic rate per individual, M is the dry mass of Daphnia. ETS and R were determined in four species of* Daphnia *(*D. pulicaria*,* D. pulex*,* D. hyalina *and* D. rosea*).*

Variable	*T*	*N*	r^2	*p*	***b***	*95% CI*	
ETS	*5°C*	*7*	*0.951*	*<0.001*	***0.720***	*0.549*	*0.891*
	10°C	*9*	*0.953*	*<0.001*	***0.580***	*0.472*	*0.688*
	15°C	*9*	*0.962*	*<0.001*	***0.646***	*0.539*	*0.753*
	20°C	*9*	*0.905*	*<0.001*	***0.675***	*0.494*	*0.856*
R	*5°C*	*7*	*0.966*	*<0.001*	***0.891***	*0.716*	*1.066*
	10°C	*9*	*0.941*	*<0.001*	***0.885***	*0.701*	*1.069*
	15°C	*9*	*0.796*	*0.001*	***0.610***	*0.356*	*0.863*
	20°C	*9*	*0.873*	*<0.001*	***0.774***	*0.529*	*1.019*

4.1.3 Respiration rate (R), ETS activity and the ETS/R ratio

Respiration rate (*Figure 7*, *Figure 8*) and ETS activity (*Figure 5* and *Figure 6*) increased with increasing experimental temperature in all experimental groups (*Table 8*, results of post hoc tests in Annex B1 and Annex B2). A plateau or a decline in ETS activity was sometimes observed at high temperatures, indicating that the thermal optimum for ETS activity has been reached or exceeded, whereas respiration rate increased throughout the temperature range.

Two-way ANOVAs and contrast tests (Annex B3) were conducted to determine whether season (*Table 9*) and species (*Table 10*) affected ETS and R. There was a difference in metabolic rate between organisms of the same species collected at different times during the season (*Table 9*), even after the size difference has been accounted for.

Table 8: *Results of one-way ANOVAs for the effect of experimental temperature on the ETS activity (ETS) and respiration rate (R) of* Daphnia*. The number following the species name indicates the lake water temperature at the time of collection of organisms for experiments. Critical p values were step-wise Bonferroni corrected and significant p printed in bold.*

	ETS			*R*		
	df	*F**	*p**	*df*	*F**	*p**
D. pulex4	*3,6.3*	*726.3*	***<0.001***	*3,14*	*25.5*	***<0.001***
D. pulex12	*4,7.2*	*149.5*	***<0.001***	*4,7.2*	*83.2*	***<0.001***
D. pulicaria15	*2,6*	*7.7*	***0.022***	*2,6*	*10.9*	***0.010***
D. hyalina4	*5,12*	*43.2*	***<0.001***	*5,4.5*	*7.8*	***0.027***
D. hyalina20	*5,24*	*82.2*	***<0.001***	*5,10.2*	*22.0*	***<0.001***
D. rosea21	*5,10.7*	*109.6*	***<0.001***	*5,10.9*	*85.8*	***<0.001***
D. rosea28	*5,24*	*34.1*	***<0.001***	*5,10.8*	*19.0*	***<0.001***

** The Welch statistic and its P value are reported instead of normal ANOVA results in case of unequal variances. Note that in that case df are not integers.*

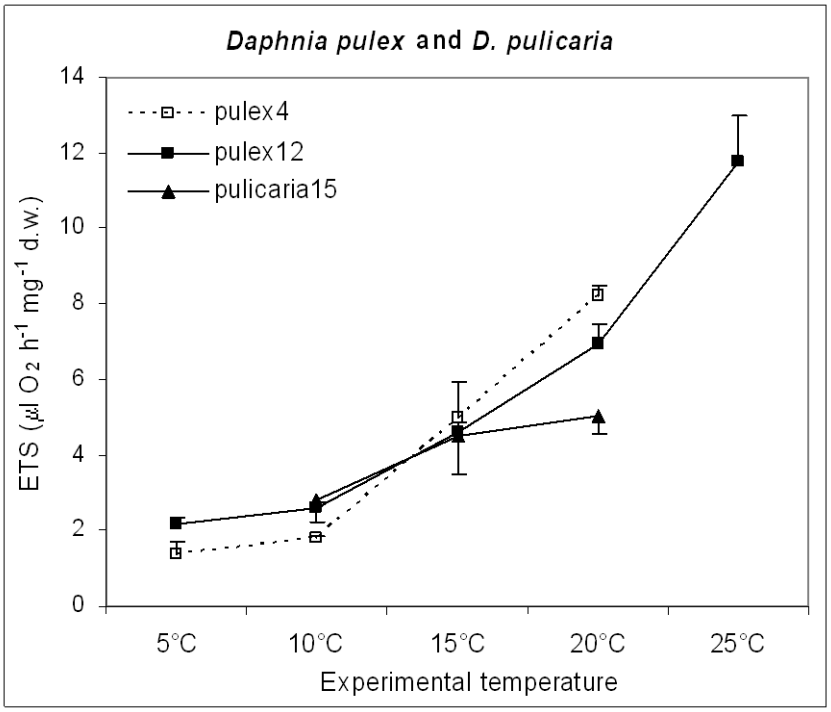

Figure 5: *ETS activity of* Daphnia pulex *and* D. pulicaria *as a function of experimental temperature. Two lines for* D. pulex *represent experiments on animals collected at two different dates in the season. Values are means of four replicates ± SD.*

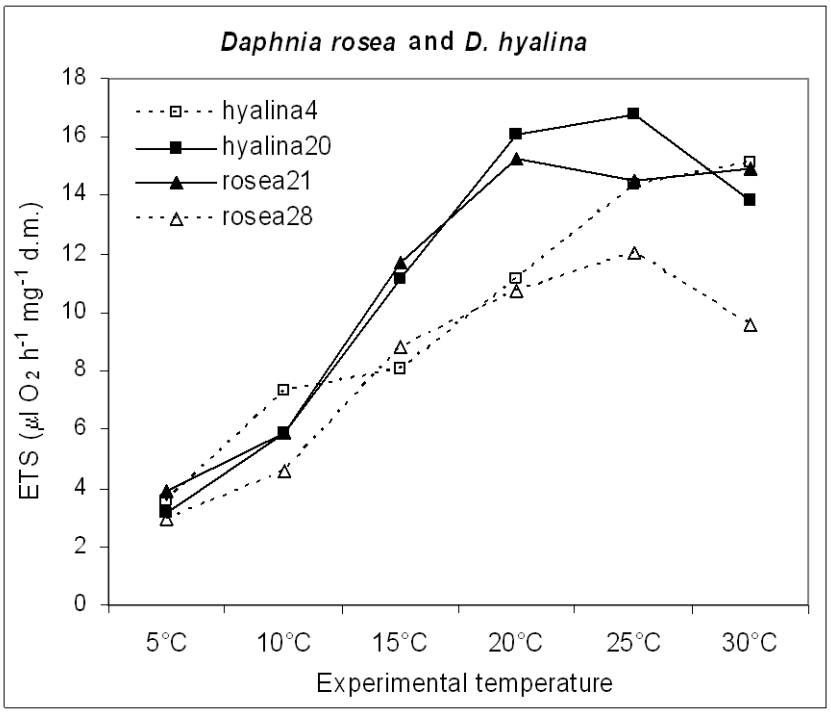

Figure 6: *ETS activity of* Daphnia rosea *and* D. hyalina *as a function of experimental temperature. Two lines for each species represent experiments on animals collected at two different dates in the season. Lake temperature at collection time is written next to the species name in the legend. Values are means of 5 replicates; SD is omitted for clarity (see Annex A).*

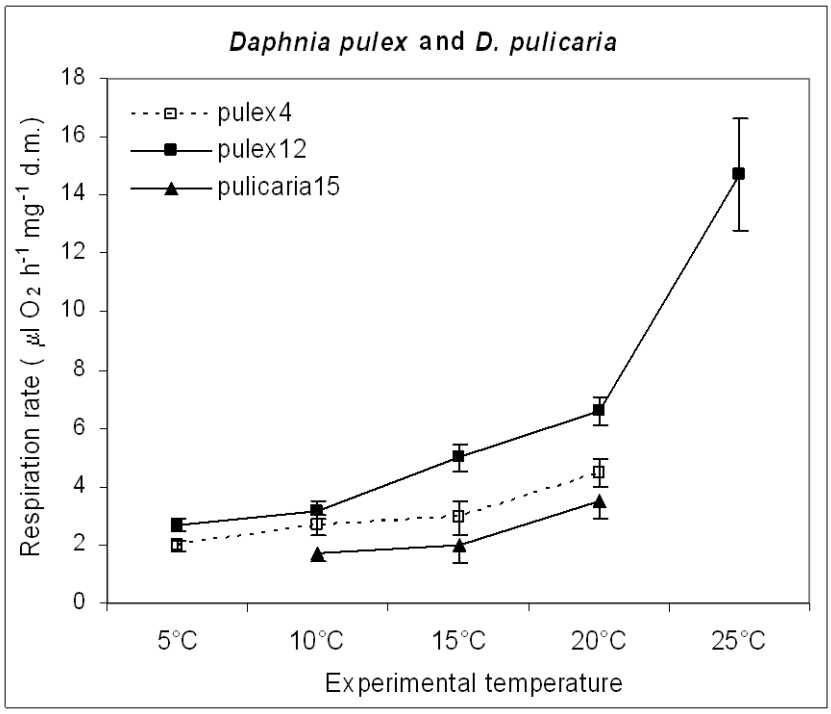

Figure 7: *Respiration rate of* Daphnia pulex *and* D. pulicaria *as a function of experimental temperature. Two lines for* D. pulex *represent experiments on animals collected at two different dates in the season. Values are means of four replicates ± SD.*

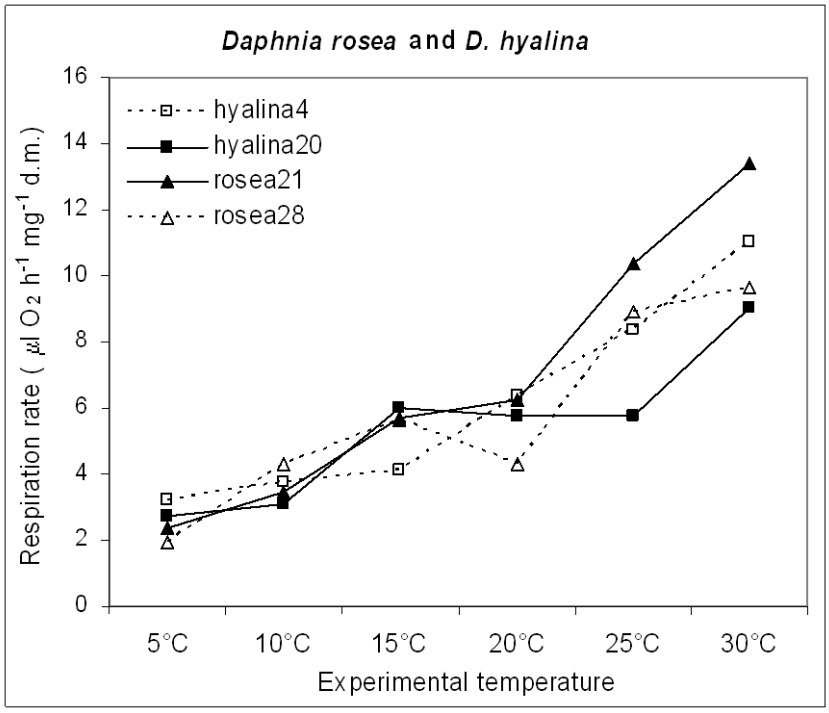

Figure 8: *Respiration rate of* Daphnia rosea *and* D. hyalina *as a function of experimental temperature. Two lines for each species represent experiments on animals collected at two different dates in the season. Lake temperature at collection date is written next to the species name in the legend. Values are means of 5 replicates; SD is omitted for clarity (see Annex A).*

Table 9: *Results of two-way ANOVAs testing the effect of season on the ETS activity (ETS), the respiration rate (R), and the ETS/R ratio of various* Daphnia *species at different experimental temperatures (T). The critical p value was adjusted for multiple comparisons with the stepwise Bonferroni correction. Significant p values are printed in bold.* [a]*Mass corrected values of ETS and R were used in the analysis for groups that differed in body mass.*

		season			*season x T*		
species	*variable*	*df*	*F/t*	*p*	*df*	*F/t*	*p*
D. pulex[a]	*ETS**	*1,13.0*	*6.7*	***<0.001***	*3,26*	*35.5*	***<0.001***
	R	*1,26*	*44.7*	***<0.001***	*3,26*	*9.2*	***<0.001***
	ETS/R	*1,26*	*14.6*	***0.001***	*3,26*	*9.1*	***<0.001***
D. rosea	*ETS**	*1,26.5*	*7.2*	***<0.001***	*5,48*	*3.0*	***<0.001***
	*R**	*1,15.9*	*2.6*	***0.019***	*5,48*	*2.4*	***<0.001***
	ETS/R	*1,48*	*6.5*	***0.014***	*5,48*	*1.7*	*0.164*
D. hyalina[a]	*ETS*	*1,36*	*0.7*	*0.399*	*5,36*	*4.5*	***0.003***
	*R**	*1,3.8*	*2.6*	*0.063*	*5,48*	*2.9*	***<0.001***
	ETS/R	*1,33*	*7.2*	***0.011***	*5,33*	*3.2*	***0.017***

**Variances not equal. Contrast test without the assumption of equal variance used to test the main effect, note that in this case df are not integers and t is reported instead of F. XPro program (Ananda and Weerahandi; 1997) used to test the interaction effect without the assumption of equal variances.*

Table 10: *Results of two-way ANOVAs testing the difference in the ETS activity (ETS), the respiration rate (R), and the ETS/R ratio between closely related* Daphnia *species from different habitats at different experimental temperatures (T). The critical p value was adjusted for multiple comparisons with the stepwise Bonferroni correction. Significant p values are printed in bold.* [a]*Mass corrected values of ETS and R were used in the analysis for groups that differed in body mass.*

groups compared	*variable*	*species*			*species x T*		
		df	*F/t*	*p*	*df*	*F*	*p*
pulex12/pulicaria15	*ETS**	*1,5.2*	*2.3*	*0.067*	*2,15*	*7.7*	*0.072*
	R	*1,15*	*162.5*	***<0.001***	*2,15*	*6.9*	***0.008***
	ETS/R	*2,15*	*165.6*	***<0.001***	*2,15*	*17.1*	***<0.001***
rosea21/hyalina20[a]	*ETS**	*1,20.4*	*5.5*	***<0.001***	*5,48*	*0.1*	***<0.001***
	*R**	*1,20.8*	*7.5*	***<0.001***	*5,46*	*12.5*	***<0.001***
	ETS/R	*1,46*	*7.0*	***0.011***	*5,46*	*6.3*	***<0.001***

**Variances not equal. Contrast test without the assumption of equal variance used to test the main effect, note that in this case df are not integers and t is reported instead of F. XPro program (Ananda and Weerahandi; 1997) used to test the interaction effect without the assumption of equal variances.*

The ETS of cold-water *D. pulex* was (after accounting for differences in body mass - *Figure 9*) higher than that of warm-water *D. pulex* at 15 and 20 °C (Annex B3), whereas the difference was smaller at lower temperatures. The opposite was true for respiration rate; cold-water *D. pulex* had lower respiration rate than warm-water *D. pulex* (*Figure 10*), especially at high temperatures. Consequently the cold-water *D. pulex* had higher ETS/R ratio, especially at high temperatures (*Figure 11*).

Summer *D. hyalina* had a significantly higher ETS activity than winter *D. hyalina* at 20 °C (Annex B3), but lower at low temperatures (*Figure 9*). The respiration rate of winter *D. hyalina* tended to be higher than that of the summer *D. hyalina* (*Figure 10*) but the effect depended on the test temperature (*Table 10*). These patterns resulted in a higher ETS/R ratio in summer *D. hyalina* at 20 and 25 °C (*Figure 12*).

D. rosea collected from the pond at 28 °C had on average a significantly lower respiration rate than *D. rosea* collected at 21 °C (*Figure 8*, *Table 10*), and also a significantly lower ETS activity at most temperatures (*Figure 6*, Annex B3). This resulted in very similar ETS/R-temperature curves, but the ETS/R was lower in *D. rosea* collected at 28 °C, especially at cold temperatures (*Figure 12*).

Because it was evident from our results that seasonal acclimatization had a significant influence on *Daphnia* metabolic rate (*Table 10*), only groups that were collected at comparable water temperatures were used for species comparison. We compared the metabolic rate of closely related pairs of species from different habitats (*Table 10*, see Annex B4 for contrast tests).

Mass corrected ETS activity was significantly higher in *D. rosea* than in *D. hyalina* (*Figure 9*) and so was the respiration rate, especially at high temperatures (25 and 30 °C; *Figure 10).* Therefore the ETS/R ratio did not differ much at lower temperatures, but was significantly higher in *D. hyalina* at 25 °C (*Figure 12*). *D. pulicaria* had a significantly higher respiration rate (*Figure 7*) and ETS activity (*Figure 5*) than *D. pulex* (*Table 10*).

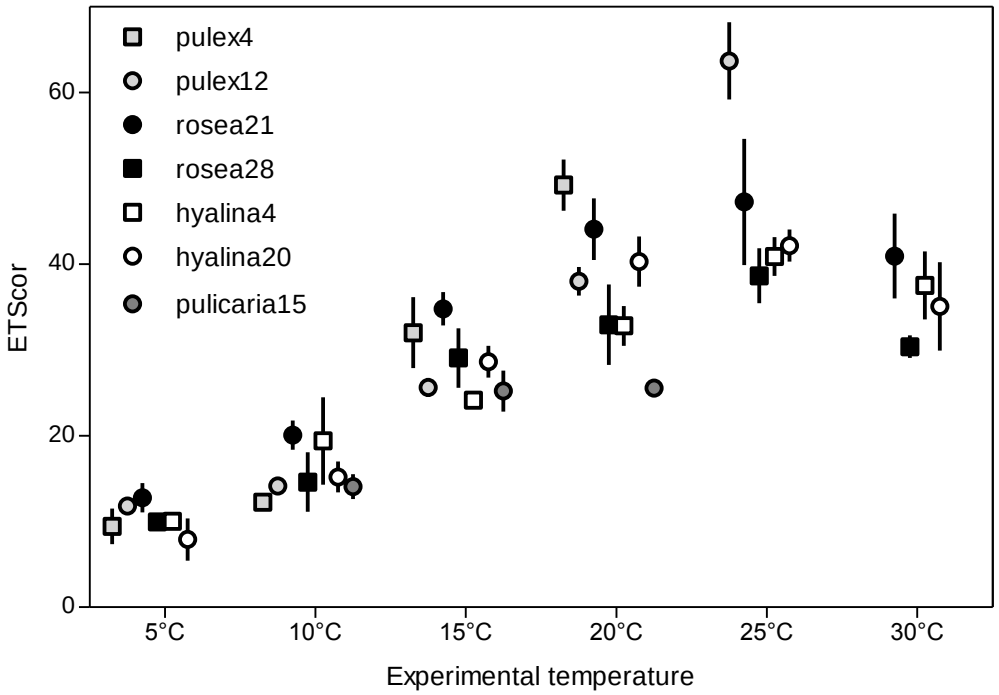

Figure 9: *Mass corrected rates of ETS activity (ETScor) of* Daphnia *(mean ± SD) as a function of experimental temperature. The number after the species name in the legend represents lake temperature at collection time.*

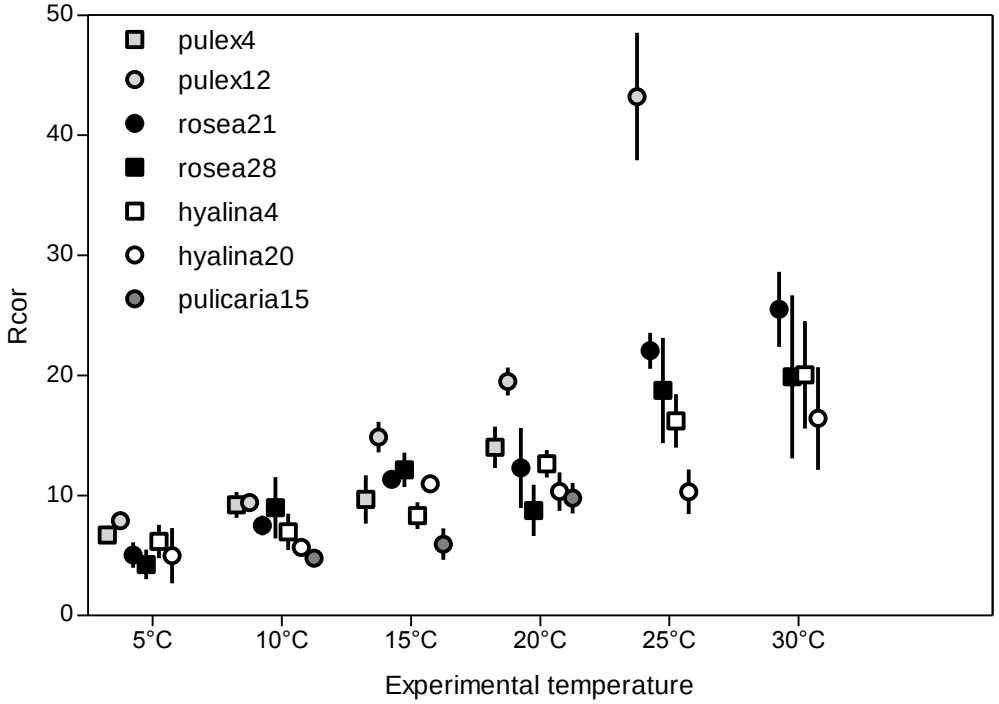

Figure 10: *Mass corrected rates of respiration (Rcor) of* Daphnia *(mean ± SD) as a function of experimental temperature. The number after the species name in the legend represents lake temperature at collection time.*

Mass corrected ETS activity did not differ much between *D. pulex* and *D. pulicaria,* except at 20 °C, where the ETS activity of *D. pulicaria* was lower (*Figure 9*, Annex B4). On the other hand there was a strong difference in respiration rate (*Table 10*); it was much higher in *D. pulex* at all temperatures (*Figure 10*). Consequently, *D. pulex* had a lower ETS/R ratio than *D. pulicaria* at all temperatures (*Figure 11*).

The ETS/R ratio also depended on experimental temperature, but not equally in all groups (*Table 11*; see Annex B1 and Annex B2 for post hoc tests). The ETS/R of 4 °C acclimatized *D. pulex* changed dramatically with temperature, values at 15 and 20 °C were twice as high as those at the lower temperatures (*Figure 11*). The ETS/R ratio of warm acclimatized *D. pulex* also changed significantly with experimental temperature (*Table 11*). A slight peak was at 20 °C, at which temperature the ETS/R was significantly higher than at other temperatures except 15 °C. The ETS/R ratio of *D. pulicaria* also changed notably with temperature (*Figure 11*). The peak ETS/R ratio in this species was at 15 °C, where it was significantly higher than at either 10 or 20 °C (Annex B1).

The ETS/R ratio temperature curves of both *D. rosea* groups were very similar in shape and had a significant peak at 20 °C (*Figure 12*, Annex B2). The ETS/R ratio of winter *D. hyalina* did not differ significantly with temperature (*Table 11*). The highest value was observed at 10 °C (*Figure 12*). On the other hand, the ETS/R of summer *D. hyalina* was affected by experimental temperature. There was a significant difference between ETS/R at 5 and 25 °C (Annex B2) – the lowest and the peak value of the ETS/R. *D. hyalina* was the only species in which the temperature optimum of ETS/R was changed by acclimatization.

Table 11: *Results of one-way ANOVA for the effect of experimental temperature on the ETS/R ratio of* Daphnia*. The number following the species name indicates the lake water temperature at the time of collection of organisms for experiments. Critical p values were step-wise Bonferroni corrected and significant p printed in bold. * The Welch statistic and its p value are reported instead of normal ANOVA results in case of unequal variances. Note that in that case df are not an integer.*

ETS/R	*df*	*F**	*p**
D. pulex4	*3,14*	*15.3*	***<0.001***
D. pulex12	*4,15*	*6.8*	***0.002***
D. pulicaria15	*2,6*	*12.1*	***0.008***
D. hyalina4	*5,4.4*	*3.1*	*0.133*
D. hyalina20	*5,22*	*9.1*	***<0.001***
D. rosea21	*5,24*	*8.4*	***<0.001***
D. rosea28	*5,24*	*16.3*	***<0.001***

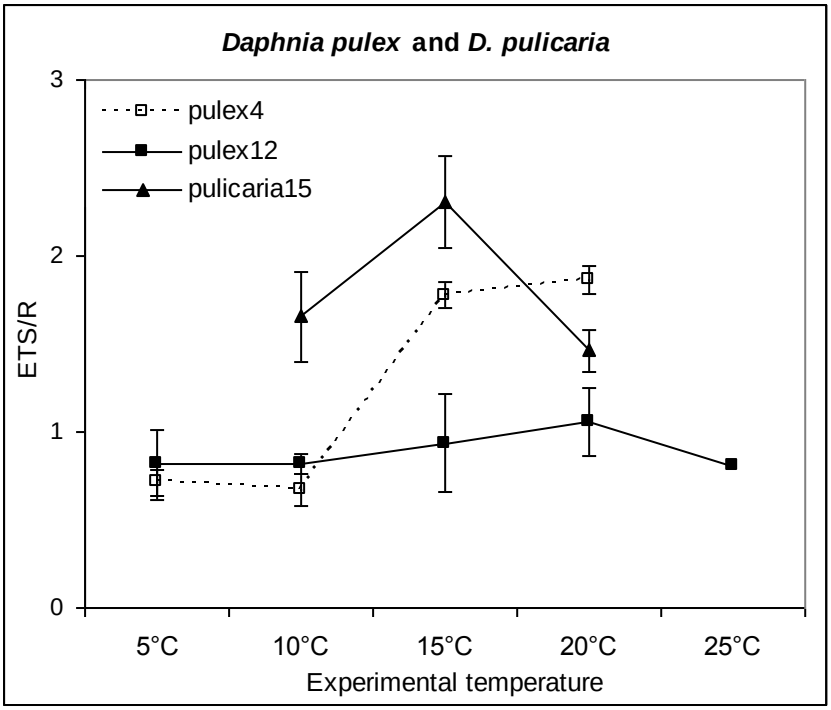

Figure 11: *ETS/R ratio of* Daphnia pulex *and* D. pulicaria *as a function of experimental temperature. Two lines for D. pulex represent experiments on animals collected at two different dates in the season. Values are means of four replicates ± SD.*

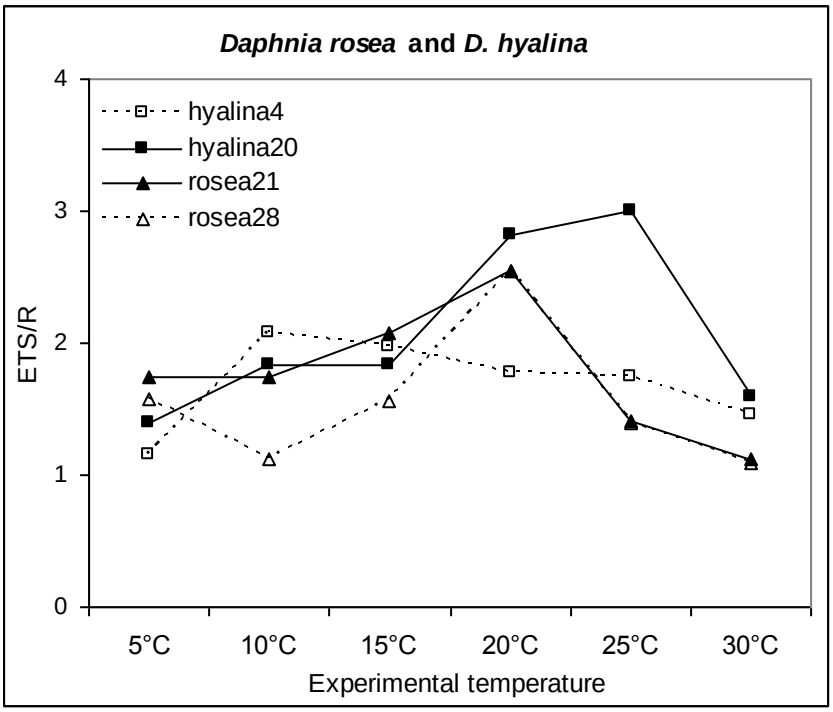

Figure 12: *ETS/R ratio of* Daphnia rosea *and* D. hyalina *as a function of experimental temperature. Two lines for each species represent experiments on animals collected at two different dates in the season. Lake temperature at collection time is written next to the species name in the legend. Values are means of 5 replicates; SD is omitted for clarity (see Annex A).*

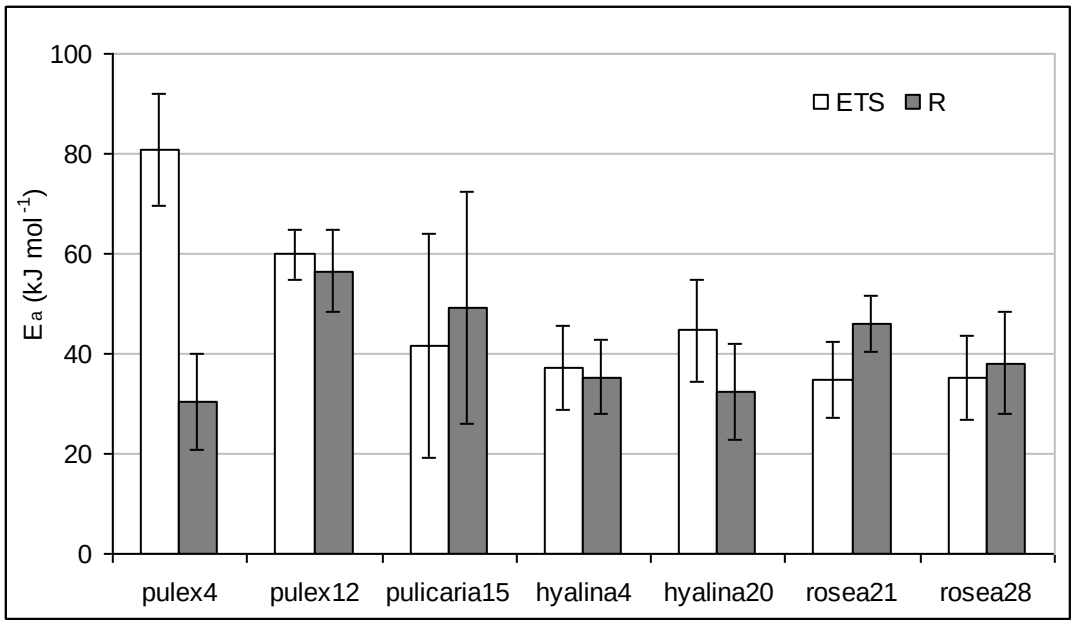

Figure 13: *Arrhenius activation energy (E_a) of mass corrected respiration rate (R) and ETS activity (ETS) of different experimental groups of field collected* Daphnia*. The number next to the species name represents water temperature at collection date. Values are means and 95 % confidence intervals.*

4.1.4 Arrhenius activation energy (E_a) of respiration rate and ETS activity

E_a of ETS activity (*Figure 13*) did not differ between the four *rosea/hyalina* groups, but E_a of respiration rate was significantly higher in *D. rosea* than in *D. hyalina* (*Table 12*). In the *pulex/pulicaria* groups *D. pulicaria* had lower E_a of respiration rate and ETS activity than *D. pulex* but the difference was only statistically significant for E_a of ETS due to the high variability of the *D. pulicaria* data.

The effect of season on E_a was detected only in *D. pulex* (*Table 12*). Winter *D. pulex* had more temperature sensitive ETS activity and less temperature sensitive respiration rate than warm acclimated *D. pulex* (*Figure 13*). Winter *D. pulex* was also the only group with a pronounced difference between the temperature sensitivity of ETS and respiration.

Table 12: *Results of t-tests for the effects of species and season on Arrhenius activation energy of ETS activity and respiration rate (R) of* Daphnia*. The number next to the species name represents water temperature at collection date. Mass corrected values of ETS and R were used in the analysis because groups differed in body mass.*

factor	group pair	ETS			R		
		df	t	p	df	t	p
season	pulex4/pulex12	1,34	3.9	**<0.001**	1,34	-4.3	**<0.001**
	hyalina4/hyalina20	1,44	-1.1	0.293	1,41	0.5	0.647
	rosea21/rosea28	1,56	0.1	0.951	1,56	-1.4	0.181
species	pulex12/pulicaria15	1,25	-2.4	**0.026**	1,25	-0.7	0.510
	rosea21/hyalina20	1,56	-1.6	0.119	1,54	2.6	**0.013**

4.1.5 Growth experiments

There was high mortality of experimental animals at 30 °C; they died before they reached maturity, usually after 2-3 days. Fitness parameters could not be calculated for this temperature. Less than 15 % of animals died before reaching maturity at the other experimental temperatures (10-25 °C). Percent of survival until maturity was higher at lower temperatures in all three species, but not significantly so (separate one-way ANOVAs for each group, $p>0.05$).

The juvenile growth rate was highest in *D. pulex* and lowest in *D. hyalina* at all experimental temperatures (*Figure 14*). It increased with temperature in all groups (*Table 13*, for post hoc tests see Annex C1), mostly due to faster maturation. Age at maturity increased from 5-6 days at 25 °C to 14-16 days at 10 °C. However, in both *D. hyalina* groups, the growth rates at 25 °C did not differ significantly from those at 20 °C.

The effect of seasonal acclimatization on the juvenile growth rate was small but significant in all three species (*Table 14*). The only significant difference in the growth rate of *D. pulex* from different seasons was at 15 °C (Annex C2), where the growth rate of cold-water *D. pulex* was lower. The juvenile growth rate was higher in *D. rosea* collected at 20 °C than in those collected at 28 °C, especially at lower temperatures. The juvenile growth rate of winter *D. hyalina* was higher than that of summer *D. hyalina* at low temperatures, but lower at high temperatures (Annex C2).

The size of first clutch increased with body size; it was highest in *D. pulex* and lowest in *D. hyalina* (*Figure 15*). It was affected by experimental temperature in all groups, except *D. rosea* collected at 20 °C (*Table 13*; for post hoc tests see Annex C1). In *D. rosea* collected at 28 °C and in cold-water *D. pulex*, clutch size at 25 °C was lower than at lower temperatures, whereas the clutch sizes at 10-20 °C did not differ significantly. In warm-water *D. pulex* the maximum clutch size was at 15 °C. The clutch size of both *D. hyalina* groups was the same at 10 and 15 °C but decreased at higher temperatures.

Table 13: *Results of one-way ANOVAs for the effect of experimental temperature on the juvenile growth rate (g_j), size of first clutch, and potential rate of population increase (r_{pot}) of* Daphnia*. The number following the species name indicates the lake water temperature at the time of collection of organisms for experiments. In case of unequal variances, the Welch statistic and p are reported. Note that in that case, df are not integers. Critical p values were step-wise Bonferroni corrected and significant p printed in bold.*

Group	g_j			Clutch size			r_{pot}		
	df	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*
pulex4	*3,11*	*554.6*	***<0.001***	*3,11*	*16.5*	***0.001***	*3,4.0*	*226.9*	***<0.001***
pulex15	*3,11*	*168.4*	***<0.001***	*3,11*	*29.2*	***<0.001***	*3,4.4*	*164.3*	***<0.001***
rosea20	*3,11*	*203.3*	***<0.001***	*3,11*	*1.1*	*0.410*	*3,11*	*151.8*	***<0.001***
rosea28	*3,11*	*360.7*	***<0.001***	*3,11*	*24.1*	***<0.001***	*3,11*	*225.7*	***<0.001***
hyalina4	*3,11*	*102.1*	***<0.001***	*3,11*	*251.7*	***<0.001***	*3,11*	*414.8*	***<0.001***
hyalina20	*3,4.1*	*240.1*	***<0.001***	*3,11*	*92.3*	***<0.001***	*3,11*	*201.2*	***<0.001***

Table 14: *Results of two-way ANOVAs testing the effect of season on the juvenile growth rate (g_j), size of first clutch, and potential rate of population increase (r_{pot}) of various* Daphnia *species at different experimental temperatures (T). Significant p values are printed in bold.*

		Season			season x T		
		df	*F*	*p*	*df*	*F*	*p*
D. pulex	g_j	*1,16*	*1.3*	*0.265*	*3,16*	*4.8*	***0.015***
	clutch	*1,16*	*20.6*	***<0.001***	*3,16*	*2.6*	*0.088*
	r_{pot}*	*1,6.7*	*2.1*	*0.078*	*3,16*	*1.5*	*0.101*
D. rosea	g_j	*1,16*	*16.7*	***0.001***	*3,16*	*1.7*	*0.197*
	*clutch**	*1,8.5*	*2.3*	*0.048*	*3,16*	*4.9*	*0.368*
	r_{pot}	*1,16*	*16.3*	***0.001***	*3,16*	*4.9*	***0.014***
D. hyalina	g_j*	*1,9.2*	*-1.9*	*0.084*	*3,16*	*19.8*	***0.012***
	clutch	*1,16*	*2.8*	*0.114*	*3,16*	*1.6*	*0.229*
	r_{pot}	*1,16*	*0.2*	*0.630*	*3,16*	*9.9*	***0.001***

** The Welch statistic and its P value are reported instead of normal ANOVA results in case of unequal variances. Note that in that case df are not integers.*

There was no effect of seasonal acclimatization on the clutch size of *D. hyalina* (*Table 14*). The clutch sizes of both *D. rosea* groups also differed little and only at 25 °C (Annex C2). The clutch sizes of warm and cold-water *D. pulex* were significantly different. They were higher in cold-water *D. pulex*, especially at 10 °C experimental temperature.

Since it is a combination of growth rate and clutch size, the reproductive performance was also highest in *D. pulex* and lowest in *D. hyalina* (*Figure 16*). It increased with experimental temperature in the range 10-20 °C in all experimental groups (*Table 13*; for post hoc tests see Annex C1). There were differences at the highest temperature. It continued to increase in *D. rosea* collected at 20 °C, was not significantly different from r_{pot} at 20 °C in *D. rosea* collected at 28 °C and both *D. pulex* groups and decreased dramatically in *D. hyalina*.

The effect of seasonal acclimatization on the r_{pot} of *D. pulex* was not significant (*Table 14*), even though r_{pot} of winter *D. pulex* was slightly higher at 10 °C. Time of collection had an effect on the r_{pot} of *D. rosea*. Those collected at 20 °C had generally higher r_{pot}, but the difference was only significant at 25°C (Annex C2). Winter *D. hyalina* had higher r_{pot} at lower temperatures, but lower r_{pot} than summer *D. hyalina* at 25 °C.

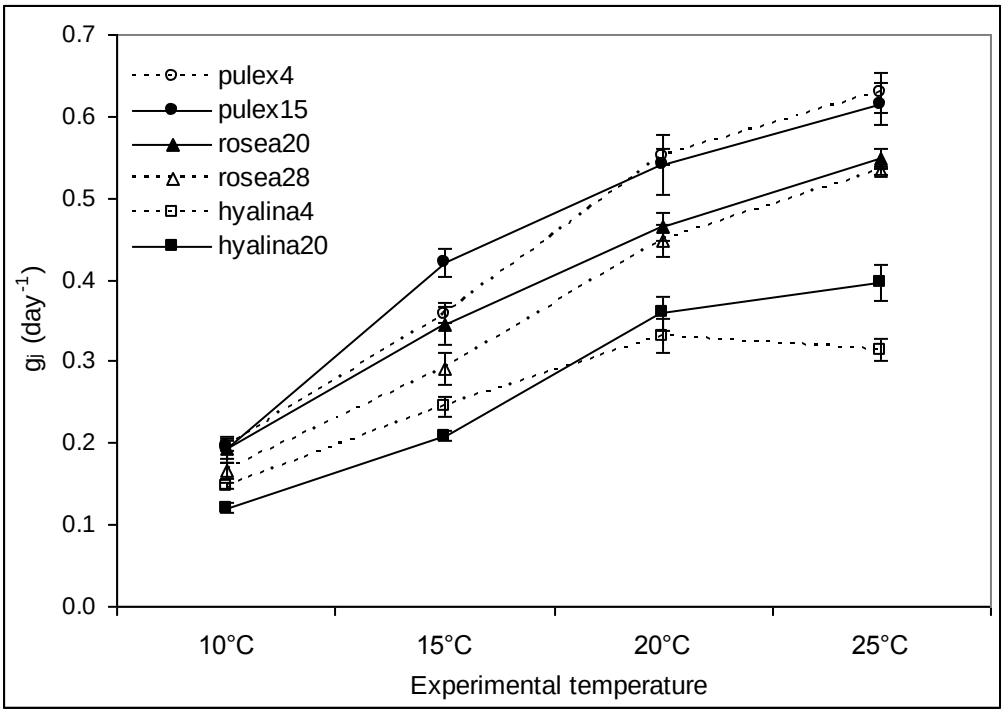

Figure 14: *Juvenile growth rate (g_j) of* Daphnia pulex*,* D. rosea *and* D. hyalina *as a function of experimental temperature. Two lines for each species represent experiments on animals collected at two different dates in the season. Lake temperature at collection time is written next to the species name in the legend. Values are means ± SD.*

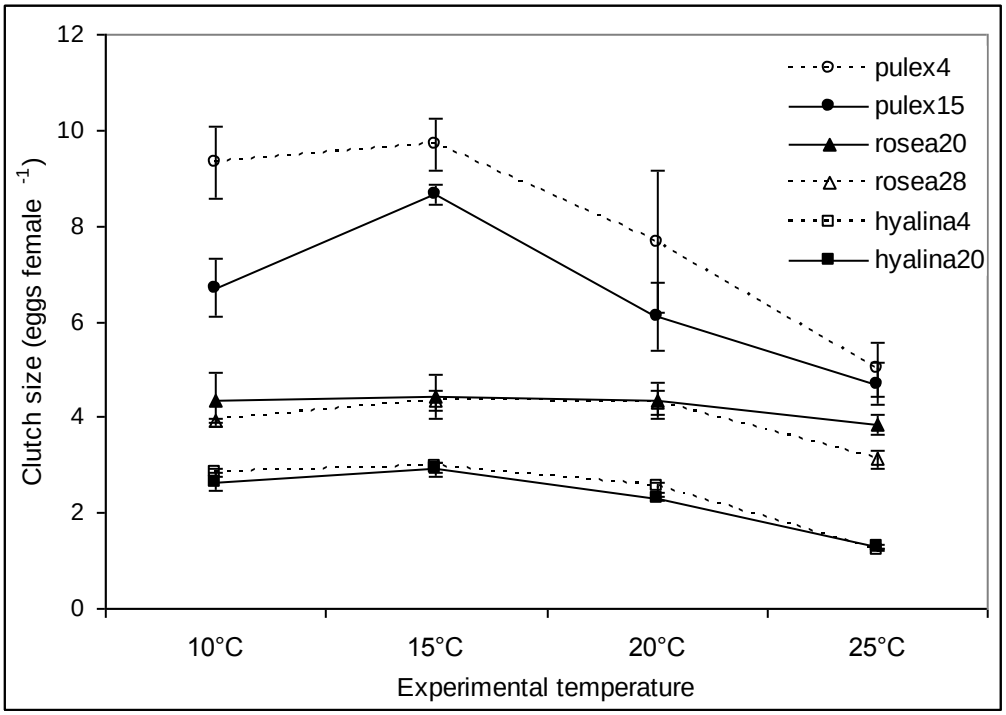

Figure 15: *Size of first clutch of* Daphnia pulex, D. rosea *and* D. hyalina *as a function of experimental temperature. Two lines for each species represent experiments on animals collected at two different dates in the season. Lake temperature at collection time is written next to the species name in the legend. Values are means ± SD.*

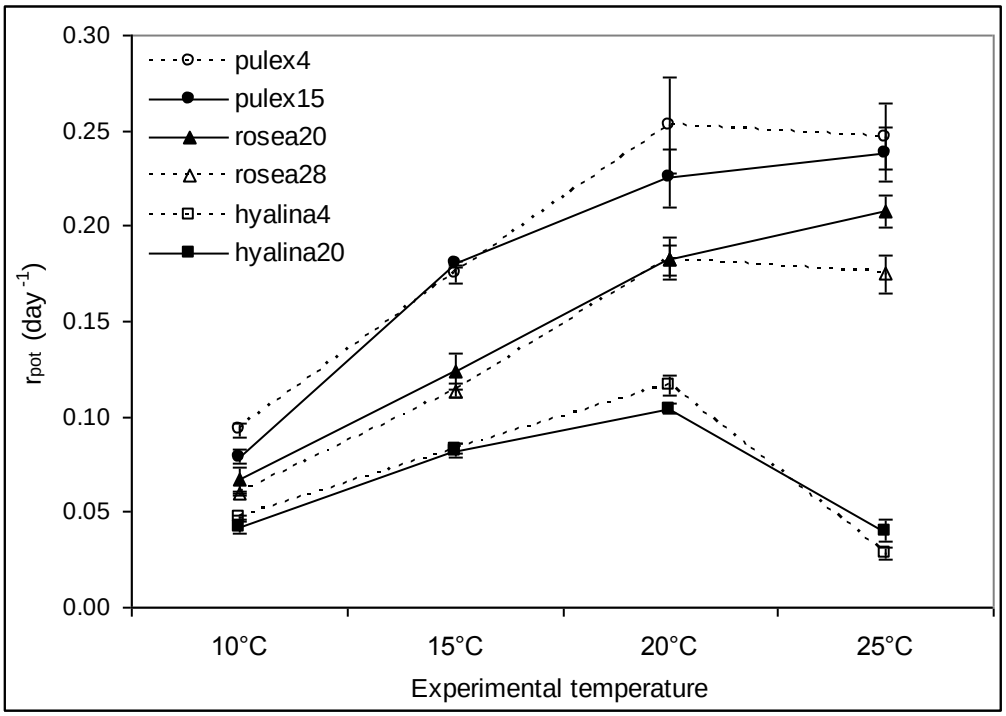

Figure 16: *Reproductive performance measured as the potential rate of population increase (r_{pot}) of* Daphnia pulex, D. rosea *and* D. hyalina *as a function of experimental temperature. Two lines for each species represent experiments on animals collected at two different dates in the season. Lake temperature at collection time is written next to the species name in the legend. Values are means ± SD.*

4.1.6 Correlations between fitness and metabolic rate at different temperatures

The relationship between metabolic rate (ETS activity, respiration rate and the ETS/R ratio) and the fitness parameters (g_j, clutch size, r_{pot}) was not straightforward. Clutch size depended on body size at maturity more than on any physiological or biochemical parameter. Both juvenile growth rate and reproductive performance were correlated to mass corrected ETS activity and respiration rate, but not the ETS/R ratio (*Table 15: Raw values*). The correlation of metabolic rate was stronger with g_j than with r_{pot}. These correlations took into account differences in ETS, R, ETS/R, g_j and r_{pot} between experimental temperatures as well as differences between groups. To test for the correlation of their change with temperature, we normalized the variables within each group by dividing them by their value at 20 °C. The general pattern of correlation remained the same: no correlation of fitness to ETS/R, stronger correlation with ETS than R, stronger correlations for g_j than r_{pot} (*Table 15: Normalized to 20 °C*).

Table 15: *Spearman's correlation between the fitness indicators of* Daphnia pulex*,* D. rosea *and* D. hyalina *(g_j – juvenile growth rate and r_{pot} – reproductive performance), physiological and biochemical variables (R – mass corrected respiration rate, mass corrected ETS activity, ETS/R ratio) and experimental temperature (T: 10-25°C). Correlations were first calculated for raw values testing the correlations at different temperatures and among different species and seasonal populations. Then the values of the variables were normalized by dividing all the values within a group with the value at 20°C. Finally the relative differences between seasonal populations were calculated for all variables.*

	g_j			r_{pot}		
	r_s	p	n	r_s	p	n
Raw values						
R	***0.788***	*<0.001*	*23*	***0.637***	*0.001*	*23*
ETS	***0.862***	*<0.001*	*23*	***0.549***	*0.007*	*23*
ETS/R	*0.000*	*0.998*	*23*	*-0.178*	*0.417*	*23*
T	***0.835***	*<0.001*	*24*	***0.481***	*0.017*	*24*
Normalized to 20°C						
R	***0.667***	*0.001*	*23*	***0.429***	*0.041*	*23*
ETS	***0.929***	*<0.001*	*23*	***0.581***	*0.004*	*23*
ETS/R	*0.280*	*0.196*	*23*	*0.218*	*0.318*	*23*
T	***0.945***	*<0.001*	*24*	***0.586***	*0.003*	*24*
Seasonal differences						
R	*-0.200*	*0.555*	*11*	*-0.158*	*0.644*	*11*
ETS	*0.264*	*0.432*	*11*	*0.374*	*0.257*	*11*
ETS/R	*0.560*	*0.073*	*11*	*0.406*	*0.215*	*11*
ETS/R without pulex	***0.898***	*0.002*	*8*	*0.539*	*0.168*	*8*

The correlation of fitness variables with ETS and R was stronger than with experimental temperature for raw values but slightly lower for normalized values, indicating that the change in

g_j and r_{pot} with temperature is predictable in this T range but the between group differences are related to differences in ETS and R. We further tested whether seasonal differences in fitness were correlated with seasonal differences in ETS, R and ETS/R. None of the correlations were significant, however ETS/R was weakly correlated with juvenile growth rate. Examination of the scatter plot showed a strong relationship between differences in g_j and differences in ETS/R in *D. rosea* and *D. hyalina*, but not in *D. pulex*. Correspondingly, the correlation between the two variables was significant if *D. pulex* was excluded from the analysis, despite the reduced df (*Table 15: Seasonal differences*).

4.1.7 Haemoglobin content

Hb content of summer field populations of *Daphnia pulex*, *D. rosea*, *D. pulicaria* and *D. hyalina* differed significantly (Welch ANOVA; $F_{W(2,6.1)}$=37.5, p<0.001). The pond species *D. pulex* and *D. rosea* had higher Hb content than lake species *D. pulicaria* and *D. hyalina* (*Figure 17*). For the oxygen conditions in their respective habitats at the time of collection see *Table 4*. Comparison of summer and spring *D. rosea* showed no statistically significant differences in the Hb content between the two (t-test: df=5, t=1.9, p=0.114) despite the somewhat higher oxygen concentration in the spring.

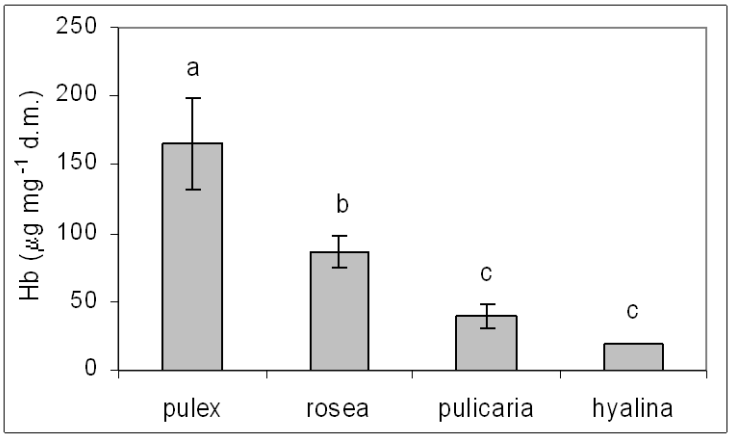

Figure 17: *Haemoglobin content (Hb) of summer field populations of four* Daphnia *species (mean ± SD). Bars marked with different letters represent significantly different means (Dunnett T3 post hoc test, p<0.05). One sample t-tests were used to compare the Hb content* of D. hyalina *(n=1) to the other three species.*

The offspring of field populations raised in the laboratory in normoxic conditions at 15 °C and 25 °C did not differ significantly in the Hb content either between species or between the two temperatures (2x2 ANOVA, p>0.05). The average Hb content of laboratory grown *D. pulex* and *D. rosea* was lower than in specimens taken directly from the summer field populations (37 ± 9

µg Hb mg^{-1} d.m.). Laboratory grown *D. hyalina* had on average 14 ± 3 µg Hb mg^{-1} d.m., which is significantly different lower than the other two species (t-Test, df=16, t=3.7, p=0.002).

4.2 LIPID CONTENT OF DAPHNIA AND ITS EFFECT ON FITNESS UNDER DIFFERENT TEMPERATURE CONDITIONS

4.2.1 Growth experiments

4.2.1.1 Body mass

Newborn mass of *D. pulex* from different acclimation treatments did not differ significantly (2x2 ANOVA, p>0.05). Offspring of *Cryptomonas* fed *D. magna* had significantly higher body mass than those of *Chlamydomonas* fed animals (2x2 ANOVA, p=0.023) while neither acclimation temperature nor its interaction with maternal food affected newborn size (2x2 ANOVA, p>0.05)

Body mass at maturity was higher in 15 °C acclimated animals than in 20 °C acclimated animals in both *D. magna* and *D. pulex*, while growth temperature had no significant effect (*Figure 18* and *Figure 19*, *Table 16* and *Table 17*). There was significant interaction between acclimation and experimental temperature in both species (*Table 16* and *Table 17*). The positive effect of cold acclimation on body mass at maturity was more pronounced at cold experimental temperatures (*Figure 18* and *Figure 19*, Annex D1).

Acclimation and experimental food as well as their interaction had a significant effect on body mass at maturity in both species (*Table 16* and *Table 17). Cryptomonas* fed animals achieved higher body mass at maturity than *Chlamydomonas* fed animals (*Figure 18* and *Figure 19*). Newborns of *Cryptomonas* fed mothers generally achieved higher body mass at maturity (*Figure 18* and *Figure 19*), but the effect was significant only when experimental food was *Chlamydomonas* (Annex D2). Significant interactions between food and temperature were found in both species (*Table 16* and *Table 17*).

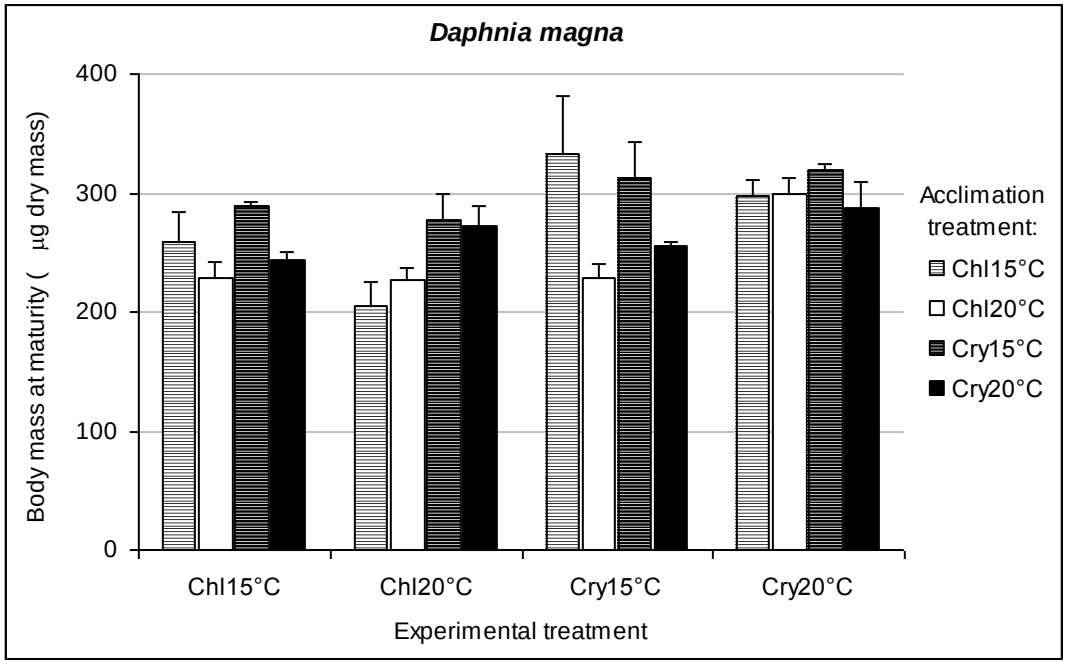

Figure 18: *Body mass at maturity (µg dry mass) of* Daphnia magna *as a function of acclimation and experimental food (Chl – Chlamydomonas sp.; Cry – Cryptomonas sp.) and temperature (15 °C; 20 °C).*

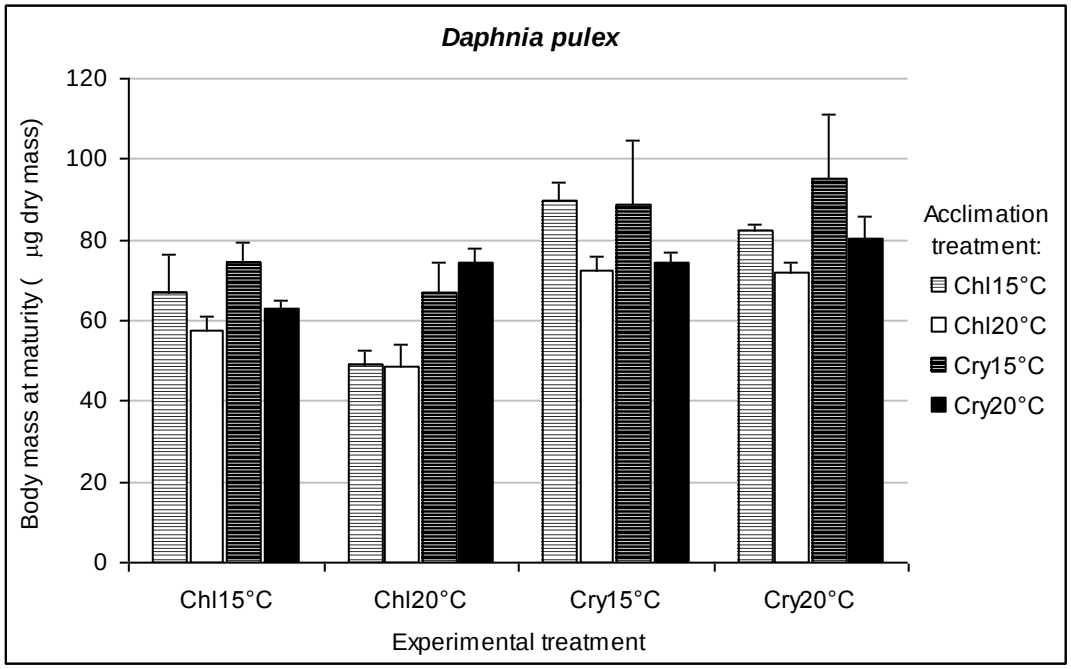

Figure 19: *Body mass at maturity (µg dry mass) of* Daphnia pulex *as a function of acclimation and experimental food (Chl – Chlamydomonas sp.; Cry – Cryptomonas sp.) and temperature (15 °C; 20 °C).*

Table 16: *Results of a four factor ANOVA test for the effects of acclimation food (AF), acclimation temperature (AT), experimental food (EF) and experimental temperature (ET) on juvenile growth rate (g_j), reproductive performance (measured as r_{pot}), size of first clutch and body mass at maturity of* Daphnia magna*.*

Daphnia magna	g_j		r_{pot}		*Clutch size*		*Body mass*	
Source of variation	$F_{(1,32)}$	*P*	$F_{(1,32)}$	*P*	$F_{(1,32)}$	*P*	$F_{(1,32)}$	*P*
AF	*36.6*	***<0.001***	*28.7*	***<0.001***	*2.3*	*0.141*	*14.4*	***0.001***
AT	*5.5*	***0.025***	*26.1*	***<0.001***	*85.8*	***<0.001***	*54.9*	***<0.001***
EF	*140.9*	***<0.001***	*106.7*	***<0.001***	*91.5*	***<0.001***	*89.2*	***<0.001***
ET	*4418.8*	***<0.001***	*2047.6*	***<0.001***	*21.0*	***<0.001***	*0.0*	*0.849*
AF x EF	*57.9*	***<0.001***	*17.0*	***<0.001***	*7.3*	***0.011***	*22.1*	***<0.001***
AT x ET	*15.1*	***<0.001***	*55.1*	***<0.001***	*4.3*	***0.047***	*43.8*	***<0.001***
AF x AT	*10.4*	***0.003***	*9.3*	***0.004***	*10.0*	***0.003***	*0.0*	*0.886*
EF x ET	*1.2*	*0.284*	*4.5*	***0.041***	*3.1*	*0.087*	*4.3*	***0.047***
AF x ET	*31.8*	***<0.001***	*3.6*	*0.067*	*0.2*	*0.662*	*7.8*	***0.009***
AT x EF	*0.7*	*0.406*	*0.8*	*0.378*	*12.9*	***0.001***	*18.5*	***<0.001***
AF x AT x EF	*6.1*	*0.019*	*0.7*	*0.418*	*1.3*	*0.269*	*5.2*	***0.030***
AF x AT x ET	*2.7*	*0.110*	*2.3*	*0.141*	*0.8*	*0.388*	*10.3*	***0.003***
AF x EF x ET	*0.3*	*0.598*	*0.0*	*0.856*	*0.7*	*0.406*	*1.0*	*0.329*
AT x EF x ET	*14.3*	***0.001***	*10.4*	***0.003***	*2.0*	*0.170*	*3.2*	*0.083*
AF x AT x EF x ET	*6.7*	***0.015***	*8.9*	***0.005***	*2.5*	*0.122*	*6.9*	***0.013***

Table 17: *Results of a four factor ANOVA test for the effects of acclimation food (AF), acclimation temperature (AT), experimental food (EF) and experimental temperature (ET) on juvenile growth rate (g_j), reproductive performance (measured as r_{pot}), size of first clutch and body mass at maturity of* Daphnia pulex**.**

Daphnia magna	g_j		r_{pot}		*Clutch size*		*Body mass*	
Source of variation	$F_{(1,32)}$	*P*	$F_{(1,32)}$	*P*	$F_{(1,32)}$	*P*	$F_{(1,32)}$	*P*
AF	*55.1*	***<0.001***	*59.0*	***<0.001***	*63.6*	***<0.001***	*24.6*	***<0.001***
AT	*8.9*	***0.005***	*84.2*	***<0.001***	*73.0*	***<0.001***	*20.2*	***<0.001***
EF	*202.1*	***<0.001***	*101.8*	***<0.001***	*91.5*	***<0.001***	*97.3*	***<0.001***
ET	*1685.7*	***<0.001***	*862.7*	***<0.001***	*62.9*	***<0.001***	*1.7*	*0.206*
AF x EF	*46.7*	***<0.001***	*14.9*	***0.001***	*2.6*	*0.117*	*5.8*	***0.022***
AT x ET	*8.5*	***0.006***	*40.2*	***<0.001***	*17.1*	***<0.001***	*5.4*	***0.027***
AF x AT	*2.0*	*0.172*	*0.2*	*0.647*	*0.2*	*0.701*	*0.2*	*0.673*
EF x ET	*1.3*	*0.260*	*0.1*	*0.789*	*1.7*	*0.208*	*2.9*	*0.097*
AF x ET	*1.9*	*0.174*	*15.7*	***<0.001***	*25.0*	***<0.001***	*10.1*	***0.003***
AT x EF	*6.0*	***0.020***	*1.1*	*0.310*	*2.2*	*0.146*	*7.1*	***0.012***
AF x AT x EF	*0.3*	*0.608*	*9.8*	***0.004***	*5.3*	***0.028***	*0.1*	*0.708*
AF x AT x ET	*0.4*	*0.552*	*0.6*	*0.458*	*0.3*	*0.614*	*0.1*	*0.786*
AF x EF x ET	*0.6*	*0.429*	*1.6*	*0.212*	*0.5*	*0.506*	*0.8*	*0.391*
AT x EF x ET	*3.3*	*0.078*	*2.5*	*0.124*	*4.2*	***0.048***	*1.4*	*0.245*
AF x AT x EF x ET	*1.0*	*0.318*	*10.1*	***0.003***	*6.1*	***0.019***	*0.9*	*0.344*

4.2.1.2 Juvenile growth rate

Experimental temperature had a strong effect on juvenile growth rate in both *D. magna* and *D. pulex* (*Table 16* and *Table 17)*. *Daphnia* growing at 15 °C had 28-40 % lower growth rates than animals of same origin and experimental food treatment growing at 20 °C. Growth rates were higher at 20 °C than at 15 °C experimental temperature, regardless of acclimation temperature and food quality (*Figure 20* and *Figure 21*, Annex D1). The effect of acclimation temperature on juvenile growth rate was more subtle and interacted with experimental temperature (*Table 16* and *Table 17)*. When an effect of acclimation temperature was detected (Annex D1), 20 °C acclimated animals had higher growth rates than 15 °C acclimated animals (*Figure 20* and *Figure 21*). In *D. magna* a negative effect of 15 °C acclimation on growth rate was only evident at 15 °C experimental treatments while in *D. pulex* the same effect was more pronounced at 20 °C experimental treatments.

Acclimation and experimental food quality and their interaction affected the juvenile growth rate of *D. magna* and *D. pulex* (*Table 16* and *Table 17)*. *Cryptomonas* fed animals had invariably higher growth rates than animals of same origin and experimental temperature treatment that were fed *Cryptomonas* (*Figure 20* and *Figure 21*, Annex D2), but the extent of the effect depended on acclimation food quality. Experimental food quality had no effect on juvenile growth rate of *D. magna* reared on *Cryptomonas* (*Figure 20*, Annex D2). In *D. pulex*, experimental food quality affected *Chlamydomonas* reared animals under all temperature combinations; whereas *Cryptomonas* reared animals were only affected when they experienced a temperature change between acclimation and experiment (*Figure 21*, Annex D2). *Cryptomonas* as maternal diet had a positive effect on juvenile growth rate of *D. magna* and *D. pulex* only in *Chlamydomonas* fed animals (*Figure 20* and *Figure 21*; Annex D2). *Cryptomonas* fed animals were not affected by maternal diet.

Results of four-way ANOVA (*Table 16* and *Table 17*) showed that there were significant interactions between temperature and food quality in juvenile growth rate of both species. However, no significant interactions between experimental food and experimental temperature were detected by the four-way ANOVAs. We additionally tested the interaction between experimental food and experimental temperature separately for each acclimation food by two-way ANOVAs on groups that were kept at one constant temperature throughout acclimation and experiment, and found no statistically significant interaction in either species (2x2 ANOVA, $p>0.05$).

An interaction between acclimation food and acclimation temperature on juvenile growth rate was detected in *D. magna* but not in *D. pulex* (*Table 16* and *Table 17*). *Chlamydomonas* as acclimation food had a more negative effect on juvenile growth rate of *D. magna* acclimated at 20 °C than at 15 °C (*Figure 20*, Annex D2). Experimental temperature affected the response to

acclimation food quality in *D. magna* (*Table 16*); *D. magna* juvenile growth rate were only affected by maternal diet at 15 °C experimental temperature (*Figure 20*, Annex D2).

The effect of experimental food quality on *D. pulex* juvenile growth rate interacted with acclimation temperature (*Table 17*); it was greater in 15 °C acclimated animals than in 20 °C acclimated animals (*Figure 21*). A complex interaction of temperature conditions and acclimation food quality determined the response of *D. magna* juvenile growth rate to experimental food quality (*Table 16*). *Chlamydomonas* as experimental food had a more negative effect on fitness in animals that experienced a temperature change between acclimation and experiment. A temperature shift from 20 °C to 15 °C resulted in a 20 % decrease in growth rate (relative to *Cryptomonas* fed animals under the same conditions) in *Chlamydomonas* reared and fed *D. magna*, while 15 °C acclimated *Chlamydomonas* reared and fed animals performed as well at 15 °C as the *Cryptomonas* fed animals (*Figure 20*, Annex D2).

4.2.1.1 Age at maturity

Age at maturity, defined as the age at the deposition of eggs into the brood pouch in days, was affected negatively by experimental temperature (*Table 18* and *Table 19*). At 20 °C experimental treatment *D. magna* required a day longer to reach maturity than *D. pulex*, and the age at maturity was independent of experimental food or acclimation treatments. At 15 °C the animals invariably needed longer to reach maturity than at 20 °C and the age at maturity depended also on experimental food and acclimation treatment.

Table 18: *Age at maturity (days) of* Daphnia magna *as a function of acclimation and experimental food (Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp.) and temperature (15 °C, 20 °C).*

D. magna		*Experimental treatment*			
		Chl15°C	*Chl20°C*	*Cry15°C*	*Cry20°C*
Acclimation treatment	*Chl15°C*	*11*	*7*	*11*	*7*
	Chl20°C	*11*	*7*	*9*	*7*
	Cry15°C	*10*	*7*	*10*	*7*
	Cry20°C	*9*	*7*	*9*	*7*

Table 19: *Age at maturity (days) of* Daphnia pulex *as a function of acclimation and experimental food (Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp.) and temperature (15 °C, 20 °C).*

D. pulex		*Experimental treatment*			
		Chl15°C	*Chl20°C*	*Cry15°C*	*Cry20°C*
Acclimation treatment	*Chl15°C*	*9*	*6*	*8*	*6*
	Chl20°C	*9*	*6*	*8*	*6*
	Cry15°C	*8*	*6*	*8*	*6*
	Cry20°C	*8*	*6*	*8*	*6*

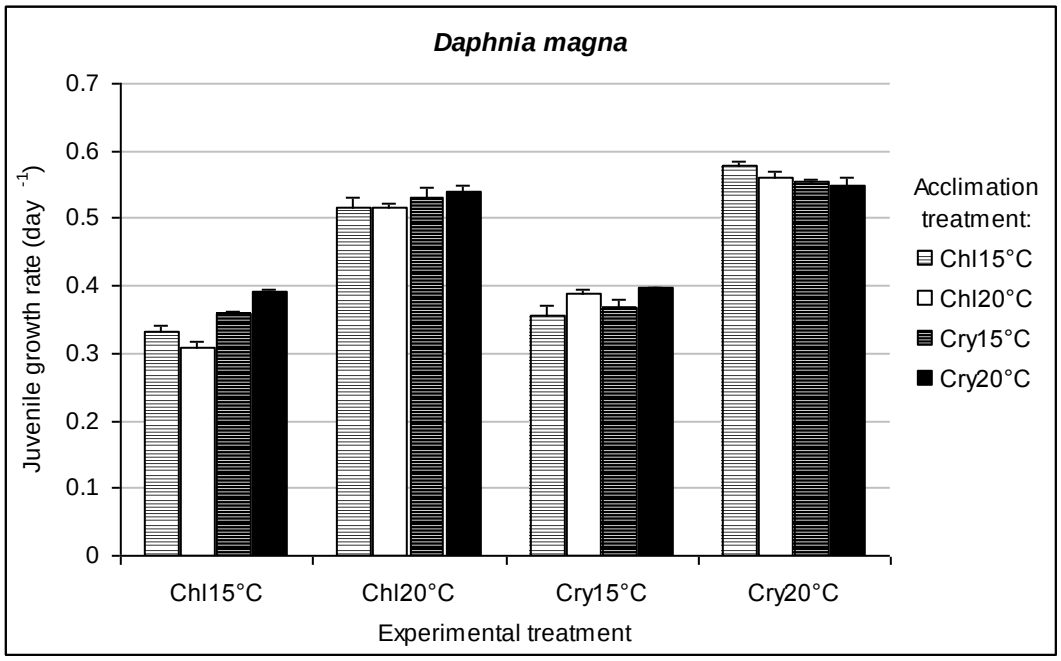

Figure 20: *Juvenile growth rate (day^{-1}) of* Daphnia magna *as a function of acclimation and experimental food (Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp.) and temperature (15 °C; 20 °C).*

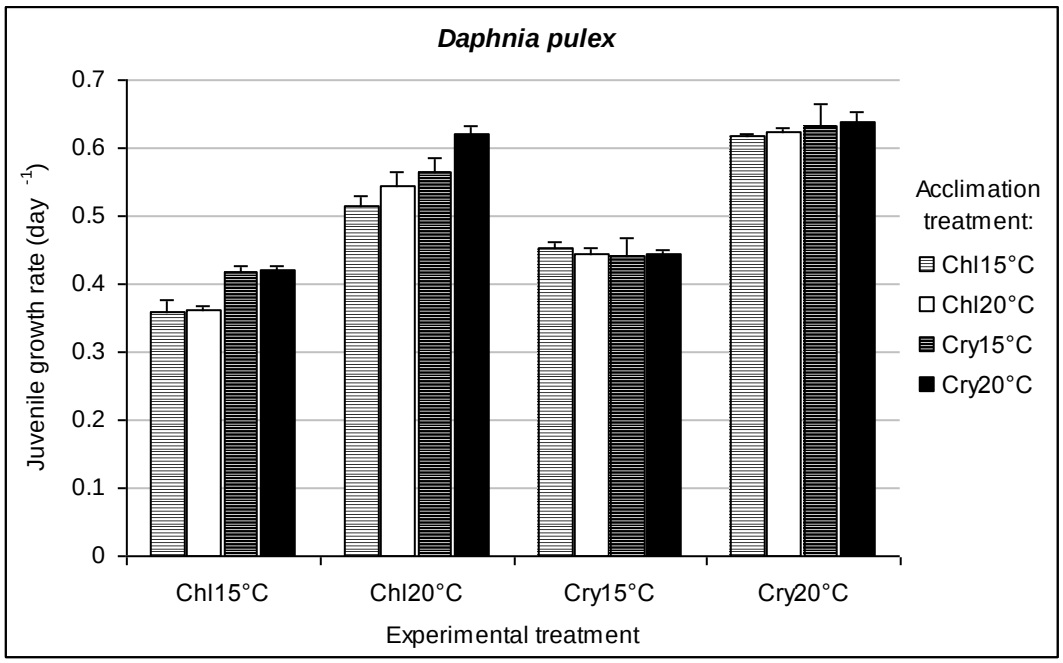

Figure 21: *Juvenile growth rate (day^{-1}) of* Daphnia pulex *as a function of acclimation and experimental food (Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp.) and temperature (15 °C; 20 °C).*

Chlamydomonas extended maturation time, especially when it was both acclimation and experimental food. In *D. magna* there was also an effect of acclimation temperature; 15 °C acclimated animals reached maturity later than those born at 20 °C. Acclimation temperature did not affect age at maturity in *D. pulex*.

4.2.1.2 Size of first clutch

Average clutch sizes of *D. pulex* and *D. magna* were affected by both acclimation temperature and experimental temperature and their interaction (*Table 16*, *Table 17*). The average size of first clutch was higher in *Daphnia* of both species acclimated to 15 °C than in 20 °C acclimated animals (*Figure 22* and *Figure 23*). Animals of both species had higher clutch sizes in cold experimental treatments. Acclimation temperature had a stronger effect at warm experimental temperatures.

D. pulex reared on *Cryptomonas* and/or fed *Cryptomonas* in the experiment had higher clutch sizes at 15 °C experimental temperature than at 20 °C (*Figure 23*, Annex D1). The effect of experimental temperature was stronger in 20 °C acclimated animals (Annex D1). 15 °C acclimated animals invariably had higher clutch size than 20 °C acclimated animals (*Figure 23*), but the difference was only significant at 20 °C experimental temperature (Annex D1).

Experimental temperature had only a slight effect on clutch size of *D. magna* (*Table 16*, Annex D1); *Chlamydomonas* fed animals had somewhat higher clutch size at 15 °C than at 20 °C (*Figure 22*). Acclimation temperature had a stronger effect (Annex D1); 15 °C acclimated animals had higher clutch sizes than 20 °C acclimated animals. This effect was stronger at 20 °C experimental temperature (*Figure 22*, Annex D1).

Effects of food quality on clutch size differed between the two species; acclimation food affected clutch size directly in *D. pulex* regardless of experimental food (*Table 17*), whereas in *D. magna* acclimation food did not have direct effects but interacted with the effects of experimental food (*Table 16*). Poor maternal diet had a stronger effect on clutch size in *D. pulex* (15 % reduction compared to that of animals reared on *Cryptomonas*) than in *D. magna* (4 % reduction). *Cryptomonas* fed animals had higher clutch size than *Chlamydomonas* fed animals in both species (*Table 16*, *Table 17*, *Figure 22* and *Figure 23*).

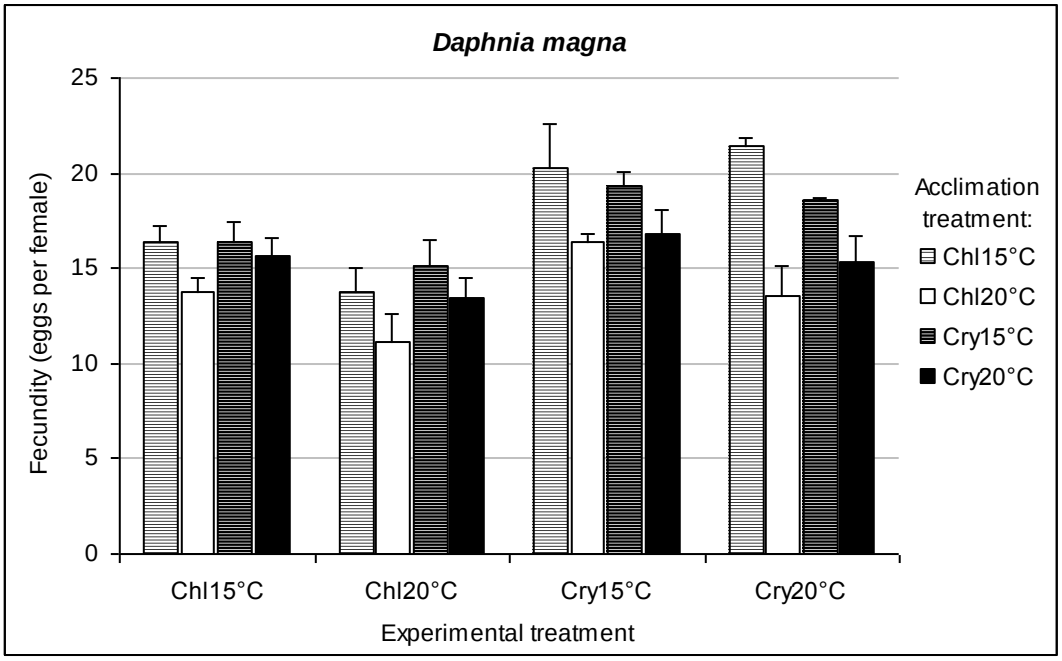

Figure 22: *Clutch size (eggs per female) of* Daphnia magna *as a function of acclimation and experimental food (Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp.) and temperature (15 °C; 20 °C).*

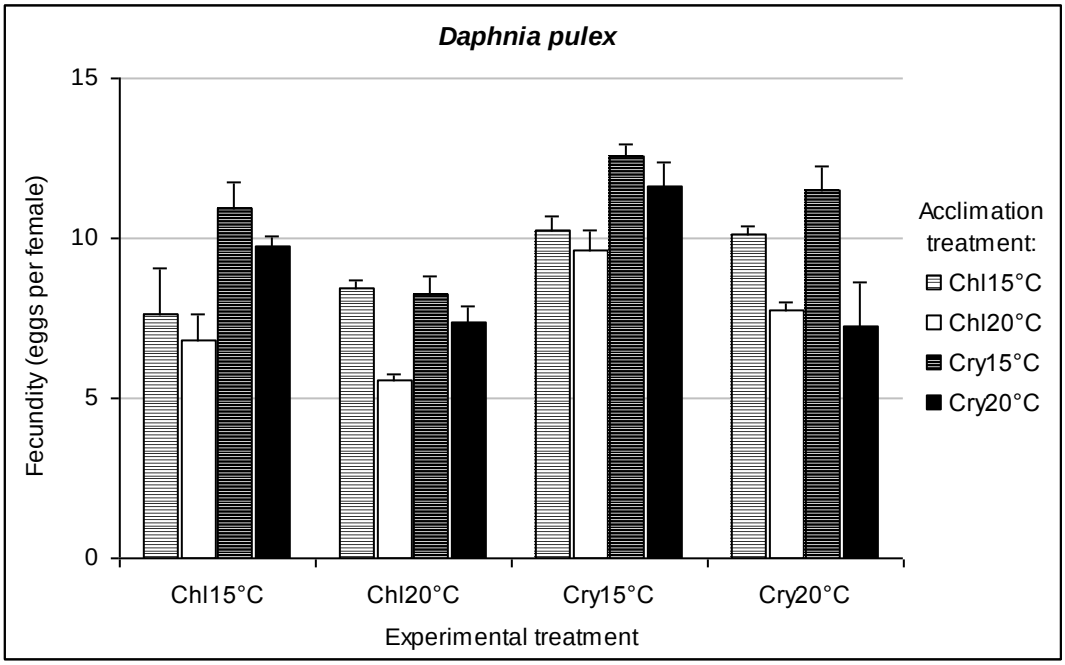

Figure 23: *Clutch size (eggs per female) of* Daphnia pulex *as a function of acclimation and experimental food (Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp.) and temperature (15 °C; 20 °C).*

Chlamydomonas reared *D. pulex* fed *Chlamydomonas* in the experiment had lower clutch size than *Cryptomonas* fed animals, under all temperature combinations (*Figure 23*, Annex D2). *Cryptomonas* reared animals were only affected by experimental food when they experienced a temperature change between acclimation and experiment. Acclimation food affected the clutch size of both *Chlamydomonas* and *Cryptomonas* fed animals but only at 15 °C experimental temperature (*Figure 23*, Annex D2), where clutch sizes were higher in animals reared on *Cryptomonas*.

D. magna fed *Chlamydomonas* had lower clutch size than animals fed *Cryptomonas* (*Figure 22*). This effect was greater in animals acclimated at 15 °C and reared on *Chlamydomonas* and was present in animals reared on *Cryptomonas* in only one temperature treatment (15°C→20°C, *Figure 22*, Annex D2). Acclimation food affected the clutch size of *D. magna* significantly only under optimal conditions: in *Cryptomonas* fed animals in the 15°C→20°C temperature combination (*Figure 22*, Annex D2), where *Chlamydomonas* reared animals actually had higher clutch size than the *Cryptomonas* reared animals. In all other cases animals reared on *Chlamydomonas* had the same or slightly lower clutch size compared with animals reared on *Cryptomonas*.

There were significant interactions between temperature and food quality in juvenile growth rate of both species (*Table 16* and *Table 17*). However, no significant interactions between experimental food and experimental temperature were detected either by the four-way ANOVAs or by the additional separate two-way ANOVAs for each acclimation food on groups that were kept at one constant temperature throughout acclimation and experiment (2x2 ANOVA, $p>0.05$).

An interaction between acclimation food and acclimation temperature on clutch size was detected in *D. magna* but not in *D. pulex* (*Table 16* and *Table 17*). *Chlamydomonas* as acclimation food had no effect on the clutch size of *D. magna* in 20 °C acclimated animals but a positive effect in 15 °C acclimated, *Cryptomonas* fed animals (*Figure 22*, Annex D2). Experimental temperature affected the response to acclimation food quality (*Table 17*); *D. pulex* clutch size was only affected by maternal diet at 15 °C experimental temperature (*Figure 23*).

The effect of experimental food quality on *D. magna* clutch size interacted with acclimation temperature (*Table 16*); it was greater in 15 °C acclimated animals than in 20 °C acclimated animals (*Figure 22*). A complex interaction of temperature conditions and acclimation food quality determined the response of *D. pulex* clutch size to experimental food quality (*Table 17*). *Chlamydomonas* as experimental food had a more negative effect on fitness in animals that experienced a temperature change between acclimation and experiment. *D. pulex* reared on *Cryptomonas* were only affected by experimental food quality when they experienced a temperature shift between acclimation and experiment (*Figure 23*).

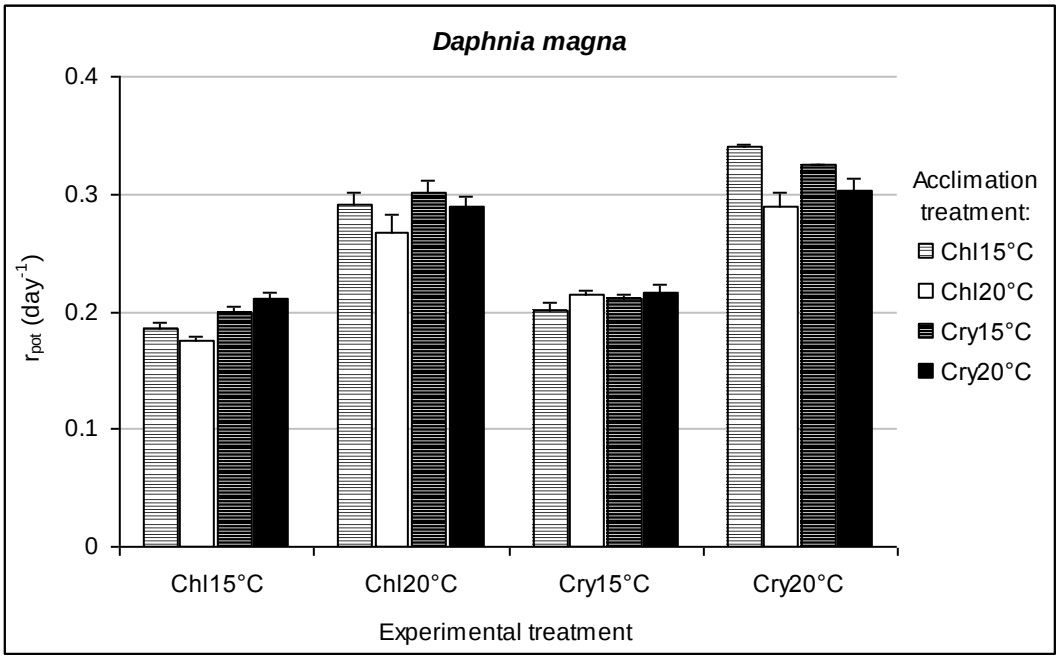

Figure 24: *Reproductive performance of* Daphnia magna*, measured as potential rate of population increase (day^{-1}), as a function of acclimation and experimental food (Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp.) and temperature (15 °C; 20 °C).*

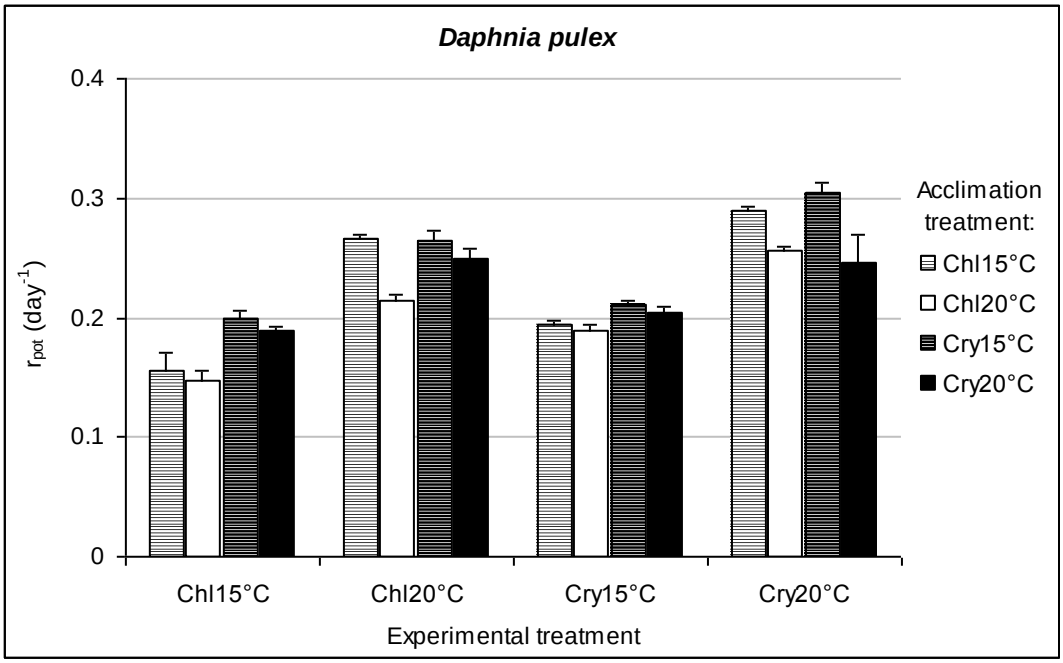

Figure 25: *Reproductive performance of* Daphnia pulex*, measured as potential rate of population increase (day^{-1}), as a function of acclimation and experimental food (Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp.) and temperature (15 °C; 20 °C).*

4.2.1.3 Reproductive performance

Reproductive performance (r_{pot}) as calculated in this study is meant as a joint estimator of fitness combining the results for growth rate and clutch size since it incorporates both clutch size and the time that was required to reach maturity.

Reproductive performance was invariably higher at higher experimental temperature in both species (*Figure 24* and *Figure 25*, *Table 16* and *Table 17*); regardless of acclimation temperature and food quality (Annex D1). 15 °C acclimated animals of both species had higher reproductive performance than 20 °C acclimated animals. This effect was only significant at 20 °C experimental temperature (*Figure 24* and *Figure 25*, *Table 16* and *Table 17*, Annex D1).

D. magna and *D. pulex* fed *Cryptomonas* as experimental food had higher reproductive performance than those fed *Chlamydomonas*, especially in groups with *Chlamydomonas* as maternal diet (*Figure 24* and *Figure 25*, *Table 16* and *Table 17*, Annex D2). Acclimation food had a significant effect on reproductive performance in both species (*Table 16* and *Table 17*); *Cryptomonas* as maternal diet generally increased reproductive performance (*Figure 24* and *Figure 25*). In *D. magna*, the effect was only significant in animals fed *Chlamydomonas* (Annex D2). In *D. pulex* it was also stronger in *Chlamydomonas* fed animals (Annex D2).

Significant interactions between temperature and food quality were detected in both species (*Table 16* and *Table 17*). In *D. magna* the effect of experimental food quality was slightly higher at 20 °C and the effect of the quality of the maternal diet was slightly stronger at 20 °C acclimation temperature (*Figure 24*). Acclimation temperature had a more negative effect on reproductive performance in animals from *Chlamydomonas* fed mothers (*Figure 24*). In *D. pulex* the effect of maternal food quality was more pronounced at higher experimental temperature (*Figure 25*).

Significant higher order interactions were detected in both species (*Table 16* and *Table 17*). In 20 °C acclimated *D. pulex* the interaction between maternal and experimental diet was strong; *Cryptomonas* reared animals were not affected by experimental food quality, while 15 °C acclimated animals were affected by experimental food regardless of maternal diet (*Figure 25*, Annex D2). Experimental food quality had a stronger effect on r_{pot} of *D. magna* when the animals experienced a temperature change between acclimation and experiment (*Figure 24*). The same was true for *D. pulex*, especially in *Cryptomonas* reared animals (*Figure 25*, Annex D2).

15 °C acclimated animals invested more into reproduction (as measured by r_{pot}) for the same juvenile growth rate (ANCOVA: $F_{(1, 91)}=47.0$, $p<0.001$) as evidenced by their steeper slope of linear regression of r_{pot} on g in *Figure 26* and *Figure 27*. This was observed in both species but was more pronounced in *D. pulex* (ANCOVA, species x acclimation T: $F_{(1, 91)}=5.7$, $p=0.019$). There was also a species difference; *D. pulex* invested less in reproduction for the same growth rate than *D. magna* (ANCOVA: $F_{(1, 91)}=261.3$, $p<0.001$).

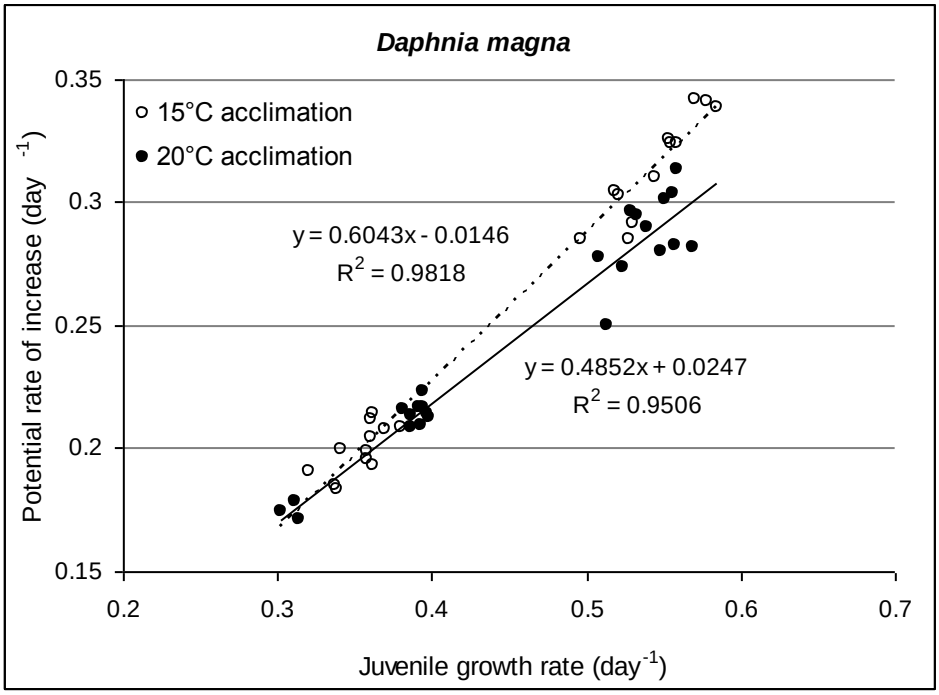

Figure 26: *Relationship between reproductive performance (measured as potential rate of population increase) and juvenile growth rate of* Daphnia magna *as a function of acclimation temperature (15 °C; 20 °C). Solid line – linear regression curve for 20 °C acclimation; dashed line – linear regression curve for 15 °C acclimation.*

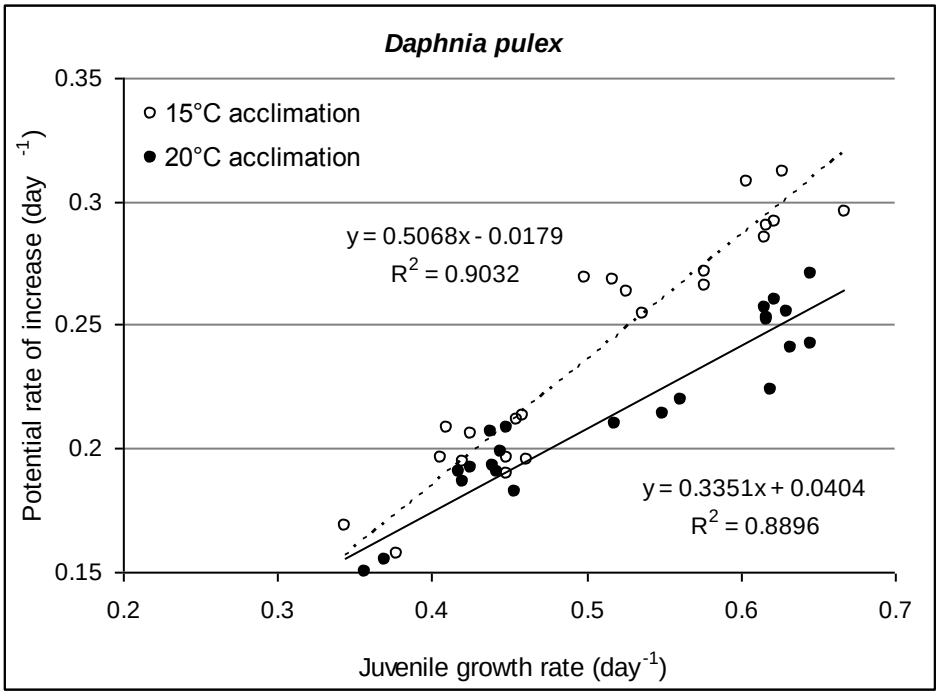

Figure 27: *Relationship between reproductive performance (measured as potential rate of population increase) and juvenile growth rate of* Daphnia pulex *as a function of acclimation temperature (15 °C; 20 °C). Solid line – linear regression curve for 20 °C acclimation; dashed line – linear regression curve for 15 °C acclimation.*

4.2.2 Lipid analysis of laboratory clones

4.2.2.1 Total fatty acid content

The total fatty acid (FA) content represented 2.7-10.8 % of dry mass in *Daphnia magna* and 3.7-9.4 % in *D. pulex* and was strongly influenced by both experimental and maternal conditions (*Figure 28* and *Figure 29*, *Table 20*; for between group comparisons see Annex H). The strongest effect was that of experimental diet; *Cryptomonas* fed *D. magna* and *D. pulex* had more FAs than *Chlamydomonas* fed animals. Maternal food quality did not have the same effect on the FA content in the two species (*Table 20*). In *D. magna*, offspring of *Chlamydomonas* fed mothers tended to have a higher FA content (*Figure 28*). In *D. pulex*, the effect of maternal diet depended on the other three factors (*Figure 29*).

Experimental temperature affected the FA content of both species. Animals growing at 20 °C generally had a higher FA content than those growing at 15 °C. However, in *D. pulex* the effect interacted with acclimation food; it was only significant in groups with *Chlamydomonas* as maternal diet. In *D. magna*, the effect was more pronounced in *Cryptomonas* reared animals.

Acclimation temperature affected the FA content of both species (*Table 20*). *D. magna* acclimated at 20 °C generally had more FA than those acclimated at 15 °C (*Figure 28*, Annex H). There was a similar trend in *D. pulex* with one exception (*Figure 29*, Annex H). The Chl15°C acclimation group had more FA than the Chl20°C acclimation group when both were fed *Cryptomonas* at 15 °C.

Table 20: *Results of a four factor ANOVA test for the effects of acclimation food, acclimation temperature, experimental food and experimental temperature on the total fatty acid content of Daphnia magna* and *D. pulex*.

Source	*Daphnia magna*		*Daphnia pulex*	
	$F_{(1,31)}$	*p*	$F_{(1,25)}$	*p*
Acclimation food (AF)	*18.6*	***<0.001***	*0.9*	*0.353*
Acclimation temperature (AT)	*53.1*	***<0.001***	*44.3*	***<0.001***
Experimental food (EF)	*125.6*	***<0.001***	*374.8*	***<0.001***
Experimental temperature (ET)	*25.4*	***<0.001***	*48.3*	***<0.001***
AF x EF	*10.7*	***0.003***	*18.2*	***<0.001***
AT x ET	*5.8*	***0.022***	*3.0*	*0.098*
AF x AT	*11.1*	***0.002***	*107.7*	***<0.001***
EF x ET	*8.4*	***0.007***	*7.7*	***0.010***
AF x ET	*0.9*	*0.341*	*50.9*	***<0.001***
AT x EF	*0.0*	*0.879*	*5.4*	***0.028***
AF x AT x EF	*5.9*	***0.022***	*24.4*	***<0.001***
AF x AT x ET	*1.4*	*0.240*	*3.8*	*0.062*
AF x EF x ET	*1.9*	*0.183*	*-*	*-*
AT x EF x ET	*2.7*	*0.112*	*0.0*	*0.875*
AF x AT x EF x ET	*0.4*	*0.545*	*-*	*-*

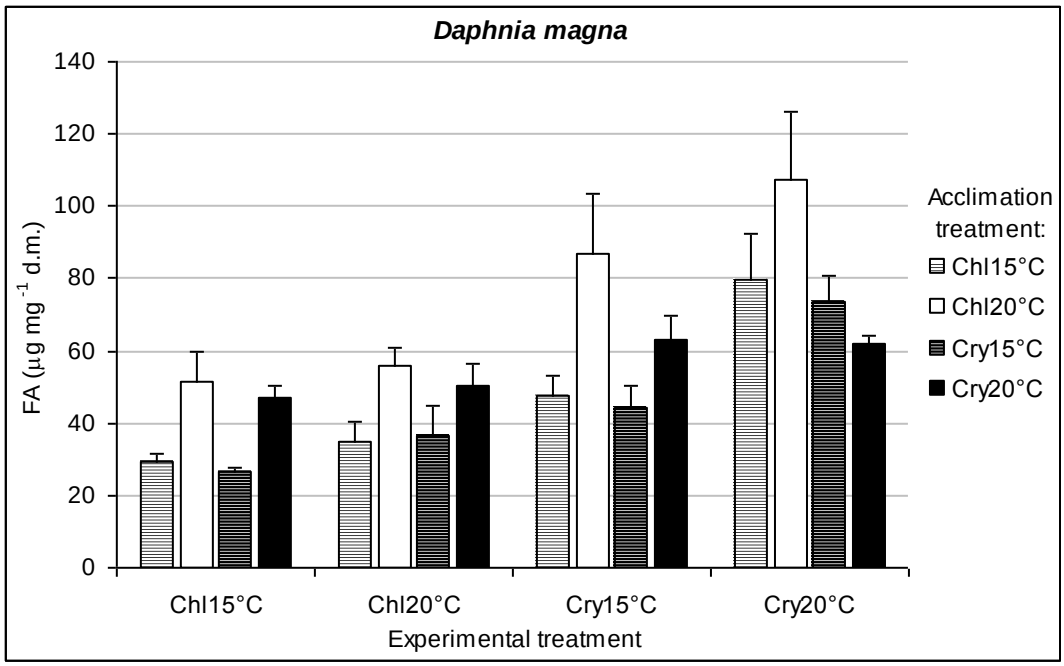

Figure 28: *Total fatty acid (FA) content of* Daphnia magna *under different acclimation and experimental treatments. Values are means of three replicates ± SD. Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp. as food. 15 °C; 20 °C – acclimation or experimental temperature.*

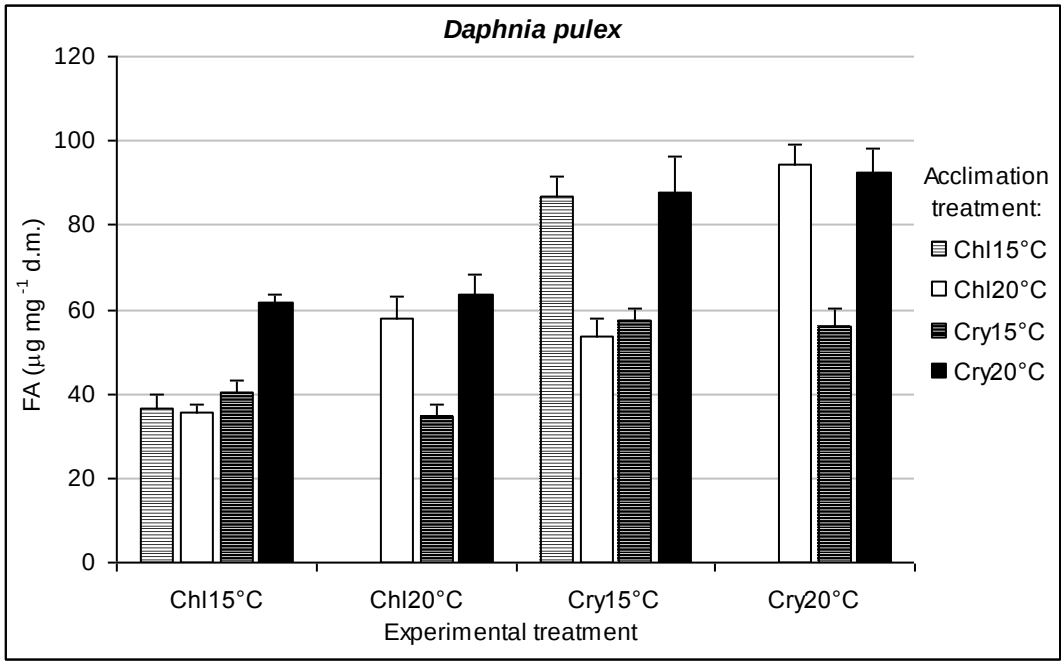

Figure 29: *Total fatty acid (FA) content of* Daphnia pulex *under different acclimation and experimental treatments. Values are means of three replicates ± SD. Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp. as food. 15 °C; 20 °C – acclimation or experimental temperature.*

4.2.2.2 Fatty acid composition

The same 34 fatty acids (FAs) were detected in both species (for complete list see Annex E). 15 of these accounted for the majority of FAs (*Figure 31* and *Figure 32*). Of the other 19, each had a less than 0.5 % average share in total FA composition. *Figure 31* and *Figure 32* reveal similarities between the FA composition of *D. magna* and *D. pulex*, and the profound effect of experimental diet on the total FAME composition of both species. *Daphnia magna* and *D. pulex* grown on *Cryptomonas* had a high content (>55 % share in the total FA composition) of long chain polyunsaturated fatty acids; notably SDA (C18:4n3), ARA (C20:4n6) and EPA (C20:5n3) and a relatively low (~10 %) content of ALA (C18:3n3). *Daphnia* grown on *Chlamydomonas* had a low (<5%) content of SDA, ARA and EPA whereas ALA represented over 50% of their total FA content (*Figure 31* and *Figure 32*).

The experimental food quality had a strong effect both on the EPA content of *D. magna* and *D. pulex* and the share of EPA in the total FA content (*Table 23*, *Table 24*). EPA represented over 38 % of all FAs in *Cryptomonas* fed *D. magna* and *D. pulex* and less than 3 % in *Chlamydomonas* fed animals (*Figure 31*, *Figure 32*). The 4-way ANOVAs showed other factors and interactions affected the EPA content as well, however, their effects were comparatively small. The effect of acclimation and experimental conditions on the content of EPA was a reflection of their effect on the total FA content (*Table 20*, *Figure 28*, *Figure 29*).The maternal diet affected the share of EPA in the total FA content of both species (*Table 23, Table 24*), especially in *Chlamydomonas* fed groups (Annex H). Those with *Cryptomonas* as maternal diet had a higher share of EPA than those reared on *Chlamydomonas* (*Figure 31*, *Figure 32*).

The share of EPA in the total FA content of *D. pulex* was not affected by experimental temperature (*Table 23*). The share of EPA in the total FA content of *D. magna* was on average slightly lower at 15 °C (*Table 24*, *Figure 31*), but the effect was only significant in Cry15°C acclimated animals fed *Cryptomonas* (Annex H). Acclimation temperature had no direct effect on the share of EPA in *D. magna*. It did have an effect in *D. pulex*: in the Chl15°C experimental group with *Chlamydomonas* as maternal food, 15 °C acclimated animals had a slightly higher share of EPA than 20 °C acclimated animals (*Figure 32*, Annex H). When only groups acclimated to the experimental conditions were compared (*Table 21*) there was also no evidence of an increase in EPA at 15 °C in either species. However, the ALA:EPA ratio tended to be lower at 15 °C than at 20 °C, indicating an increase in the conversion of ALA to EPA at 15 °C.

Table 21: *The effect of temperature (15 °C vs. 20 °C) on the content of the fatty acids (FAs) ALA (C18:3n3) and EPA (C20:5n3), and the ALA:EPA ratio of* Daphnia magna *and* D. pulex *fed either* Chlamydomonas *(Chl) or* Cryptomonas *(Cry). Only groups acclimated to the experimental conditions for three generations are compared (acclimation conditions/experimental conditions). T-Tests were conducted to compare groups with the same diet at different temperatures; means followed by different letters differ significantly (p<0.05).*

Experimental group	*ALA (% total FA)*			*EPA (% total FA)*			*ALA:EPA*		
	mean	*SD*	*t-test*	*mean*	*SD*	*t-test*	*mean*	*SD*	*t-test*
D. magna									
Chl15/Chl15	*59.45*	*1.10*	*a*	*0.24*	*0.11*	*a*	*286.07*	*105.84*	*a*
Chl20/Chl20	*62.13*	*0.55*	*b*	*0.10*	*0.00*	*a*	*627.34*	*11.14*	*b*
Cry15/Cry15	*9.66*	*0.37*	*a*	*42.60*	*0.33*	*a*	*0.23*	*0.01*	*a*
Cry20/Cry20	*10.61*	*0.18*	*b*	*44.46*	*0.83*	*b*	*0.24*	*0.01*	*a*
D. pulex									
Chl15/Chl15	*49.61*	*5.01*	*a*	*0.43*	*0.17*	*a*	*135.42*	*79.80*	*a*
Chl20/Chl20	*57.27*	*0.97*	*a*	*0.55*	*0.53*	*a*	*176.43*	*110.79*	*a*
Cry15/Cry15	*9.60*	*0.07*	*a*	*41.61*	*0.71*	*a*	*0.23*	*0.01*	*a*
Cry20/Cry20	*12.13*	*0.16*	*b*	*42.19*	*0.08*	*a*	*0.29*	*0.00*	*b*

Despite the apparent similarities between the FA compositions of *D. magna* and *D. pulex*, discriminant analysis showed that it is possible to distinguish the two species on the basis of their FA composition (*Figure 30*, for details of the analysis see Annex F). One discriminant function (D) was found with 0.978 canonical correlation between D and species. None of the individual predictor variables were strongly correlated with D, indicating that the separation cannot be made on the basis of any single FA. 100 % of all original cases were correctly classified using this discriminant function as well as a 100 % of cross-validated cases. (In cross validation, each case is classified by the discriminant function derived from all cases other than that case.) The standardized coefficients of D and the structure matrix showed that the major FAs (SDA, ARA, EPA, ALA and C18:1n9) contributed most to the separation: *D. magna* had a bigger share of these major FA while *D. pulex* had a greater share of the minor FA.

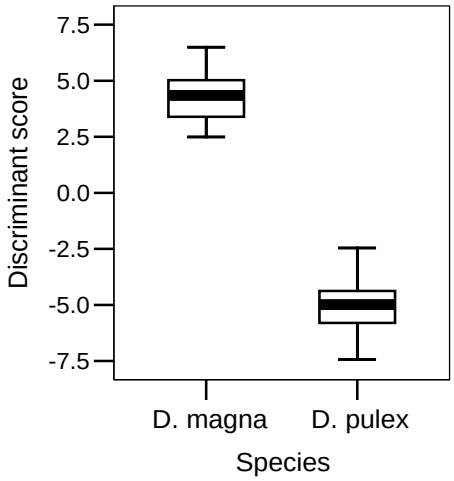

Figure 30: *Boxplot of the discriminant scores of* Daphnia magna *and* D. pulex *from the discriminant analysis of the fatty acid composition of the two species.*

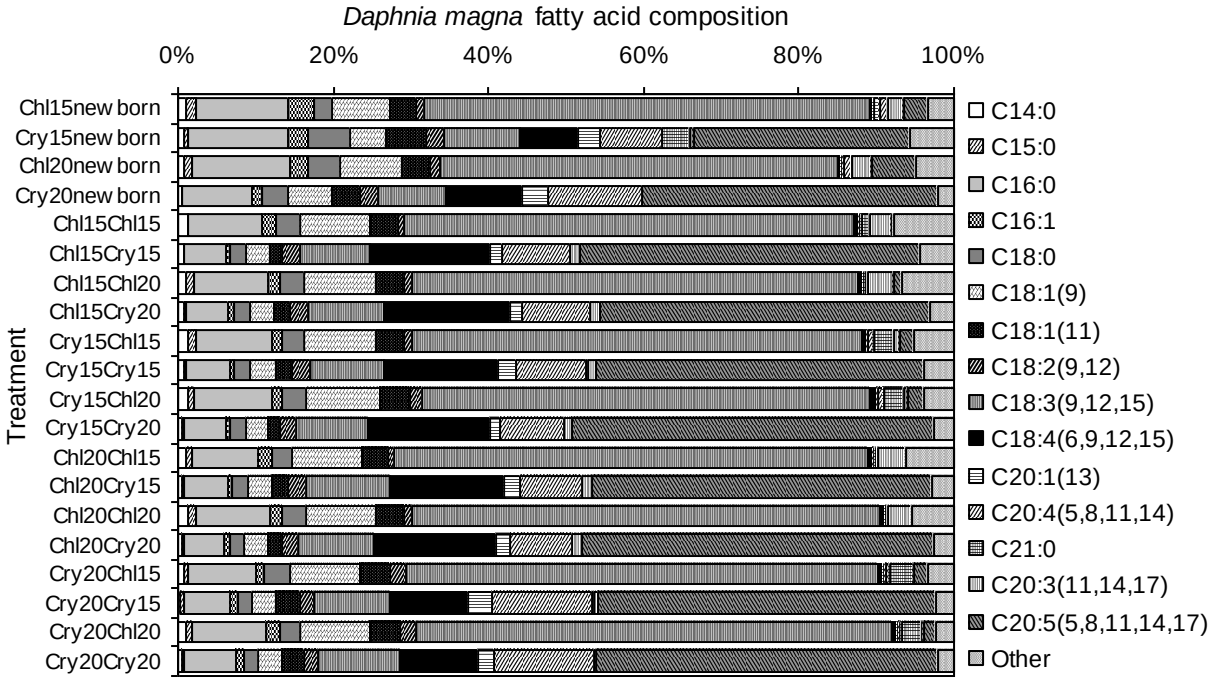

Figure 31: *Fatty acid (FA) composition of* Daphnia magna *in dependence on acclimation and growth conditions. Three replicates from each treatment were averaged. First three letters of the treatment name represent acclimation food (Cry-*Cryptomonas*, Chl-*Chlamydomonas*). The following number represents acclimation temperature (15 °C, 20 °C). The second half of the name indicates experimental conditions. Other-FAs with less than 0.5 % average share in total FA composition.*

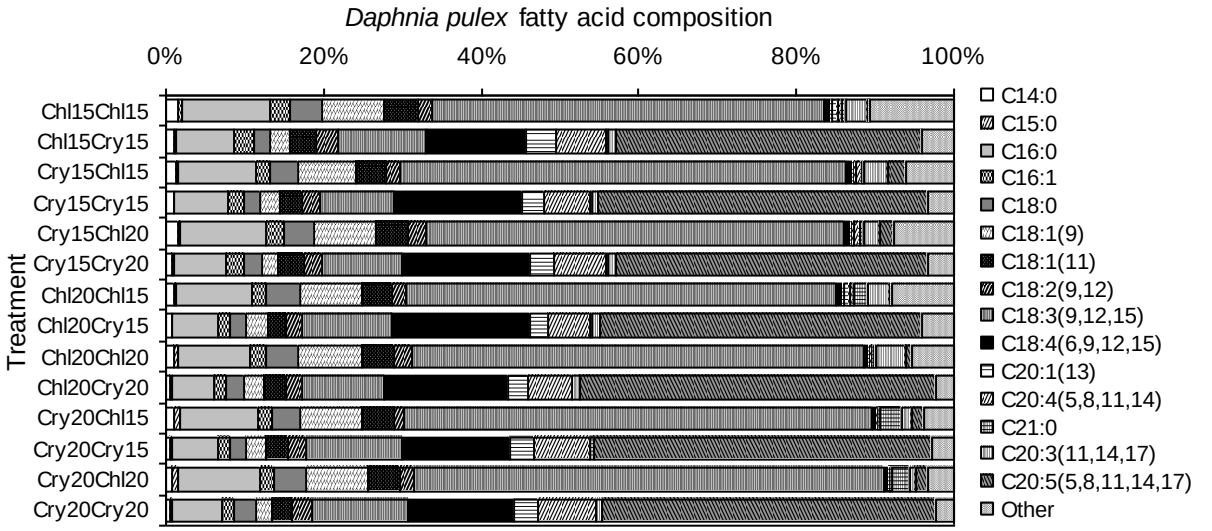

Figure 32: *Fatty acid (FA) composition of* Daphnia pulex *in dependence on acclimation and growth conditions. Three replicates from each treatment were averaged. First three letters of the treatment name represent acclimation food (Cry-*Cryptomonas*, Chl-*Chlamydomonas*). The following number represents acclimation temperature (15 °C, 20 °C). The second half of the name indicates experimental conditions. Other-FAs with less than 0.5% average share in total FA composition.*

Factor analysis of the fatty acid composition of *D. magna* resulted in two principal components (PC1 and PC2), which together accounted for 89.8 % percent of the total variation in the data. PC1 separated *Chlamydomonas* fed animals (*Figure 33*, clusters b and c) from *Cryptomonas* fed animals (*Figure 33*, cluster a) and explained 77.8 % of variation in FA composition. PC2 explained further 12.1 % of variation. It separated samples according to the maternal diet of the animals. This separation was more pronounced in *Chlamydomonas* fed animals, where *Chlamydomonas* reared animals (*Figure 33*, cluster c) were clearly separated from *Cryptomonas* reared animals (*Figure 33*, cluster b) while in the *Cryptomonas* fed animals this separation was less clear. Samples from Cry20°C acclimated animals (upper half of cluster a) differed somewhat from other *Cryptomonas* fed groups. A slight further separation according to temperature was observed along the PC2 in *Chlamydomonas* fed animals in clusters b and c (*Figure 33*). *Cryptomonas* reared animals (cluster b) were further separated according to acclimation temperature, while *Chlamydomonas* reared animals (cluster c) were further separated according to experimental temperature.

The first PC of *D. magna* FA composition correlated well with most FA but mainly it was a ratio of palmitic acid (C16:0), oleic acid (C18:1n9) and ALA to SDA, ARA and EPA (see Annex G for details of the analysis), which corresponds to the main difference between *Chlamydomonas* and *Cryptomonas* fed *D. magna* as seen in *Figure 31*. Contrast tests confirmed the strong connection between PC1 and experimental food quality. PC1 score was also slightly higher for animals at 20 °C experimental temperature (*Table 22*). The second PC mainly represented the ratio of C21:0 to C20:3n3, with more C20:3n3 in animals with *Chlamydomonas* as maternal diet. Contrast tests showed a strong effect of acclimation conditions on PC2 with a lot of interaction between the factors (*Table 22*). *Cryptomonas* reared animals had a higher PC2 score than *Chlamydomonas* reared animals and 20 °C acclimated animals had a higher PC2 score than 15 °C acclimated animals. The PC2 score of *Chlamydomonas* fed animals was either higher or lower than that of *Cryptomonas* fed animals, depending on maternal diet (*Figure 33*). The effect of experimental temperature on the PC2 score depended on the other three factors (*Table 22*).

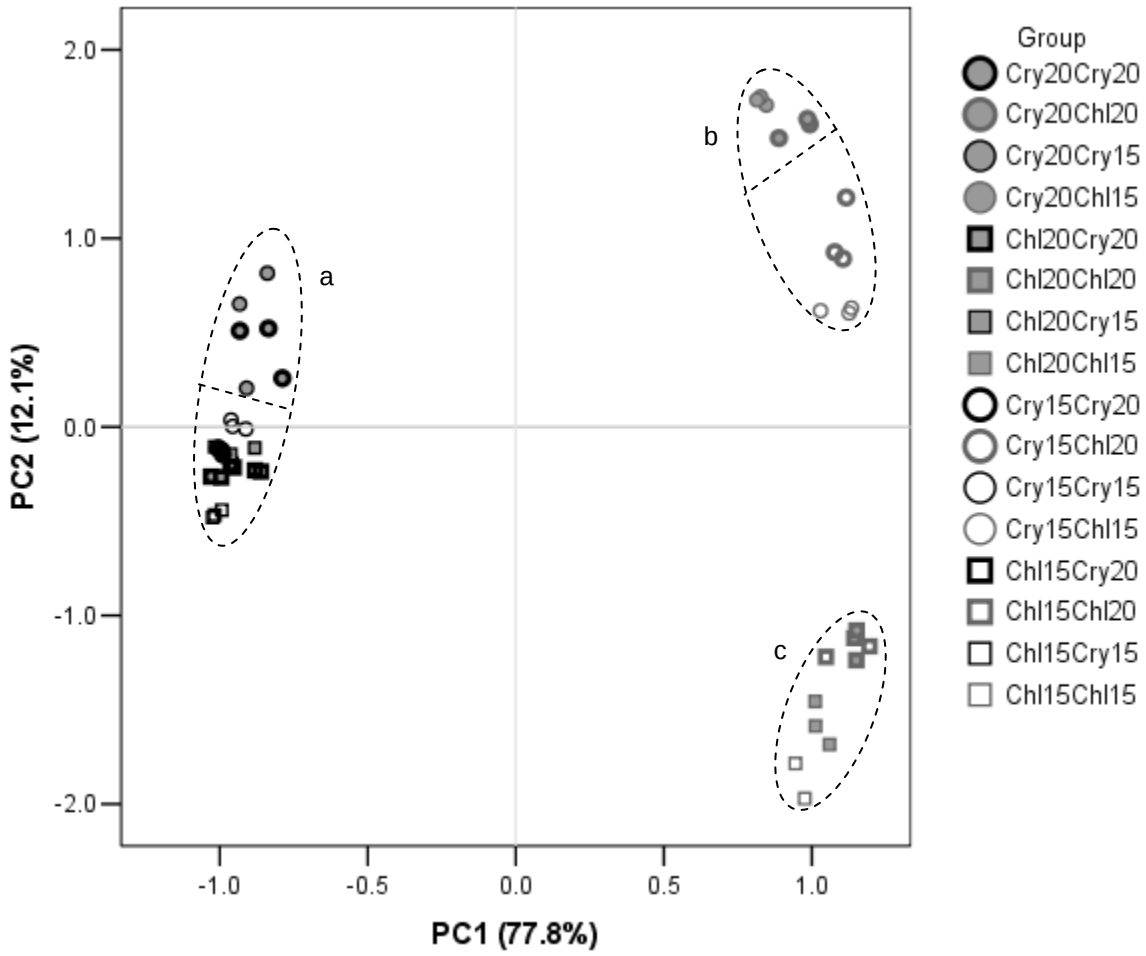

Figure 33: *A plot of the first two principal components (PC1, PC2 – number in the brackets indicates % of variance explained) from the factor analysis of fatty acid composition (percent share of individual fatty acids in the sum of all FA) of adult ovigerous* D. magna *from 16 experimental combinations of acclimation and experimental temperature (15 °C or 20 °C) and food algal species (Chl-*Chlamydomonas *sp., Cry-*Cryptomonas *sp.). The first part of the group name represents the acclimation conditions and the second part experimental conditions. Distinct clusters are outlined with dashed lines; a -* Cryptomonas *fed animals, b and c -* Chlamydomonas *fed animals; b – animals with* Cryptomonas *maternal food, c – animals with* Chlamydomonas *maternal food.*

Factor analysis of the fatty acid composition of *D. pulex* resulted in three principal components (PC1, PC2 and PC3), which together accounted for 90.2 % percent of the total variation in the data. PC1 separated *Cryptomonas* fed animals (*Figure 34*, clusters a and b) from *Chlamydomonas* fed animals (*Figure 34*, clusters c and d) and explained 68.5 % of variation in FA composition. PC2 explained further 13.9 % of variation. It separated animals acclimated to 15 °C (clusters a and c) from those acclimated to 20 °C (clusters b and d). PC3 explained 7.8 % of variation but was not clearly connected with a single factor.

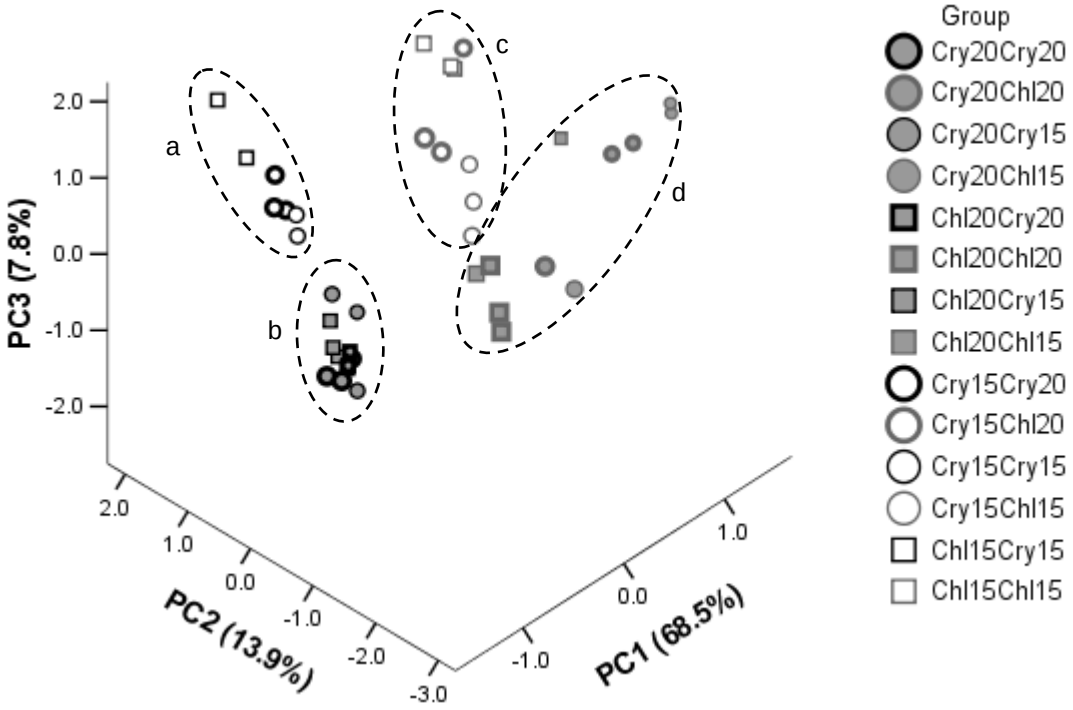

Figure 34: *A plot of the first three principal components (PC1, PC2, PC3 – number in the brackets indicates % of variance explained) from the factor analysis of fatty acid composition (percent share of individual fatty acids in the sum of all FA) of adult ovigerous* D. pulex *from 14 experimental combinations of acclimation and experimental temperature (15 °C or 20 °C) and food algal species (Chl-*Chlamydomonas *sp., Cry-*Cryptomonas *sp.). The first part of the group name represents the acclimation conditions and the second part the experimental conditions. Distinct clusters are outlined with dashed lines; a and b –* Cryptomonas *fed animals, c and d -* Chlamydomonas *fed animals; a and c – 15 °C acclimation, b and d –20 °C acclimation.*

The first PC of *D. pulex* was constructed similarly to the PC1 of *D. magna*. It correlated well with most FA but mainly it was a ratio of palmitic acid, oleic acid and ALA to SDA, ARA and EPA (see Annex G for details of the analysis), which corresponds to the main difference between *Chlamydomonas* and *Cryptomonas* fed animals as seen in *Figure 32*. Contrast tests confirmed the strong connection between PC1 and experimental food quality. PC1 score was also affected by the other three factors and their interactions (*Table 22*). The second PC mainly represented the ratio of C16:1n7 to C21:0, with more C16:1n7 in cold acclimated animals. Contrast tests showed a strong effect of acclimation conditions on PC2 (*Table 22*). 15 °C acclimated *D. pulex* had a higher PC2 score than 20 °C acclimated animals and *Chlamydomonas* reared animals had a higher PC2 score than *Cryptomonas* reared animals (*Figure 34*). The third PC had a weak correlation with individual FA. It was mostly a ratio of C16:1n7 and C21:0 to C20:3. The contrast analysis confirmed that there was no clear relationship between PC3 and the food and temperature conditions of *D. pulex*. Since PC3 had no interpretation it was omitted from further analysis.

Table 22: *Effects of acclimation and experimental food quality (*Cryptomonas *sp. vs.* Chlamydomonas *sp.) and temperature (15 °C vs. 20 °C) and their interactions on the principal components (PC) of the fatty acid composition of* Daphnia pulex *and* D. magna*. A 4-way ANOVA was used for PCs with equal group variances (Levene's test, p>0.05) and in these cases F is reported along with p. For PCs with unequal group variances (Levene's test, p<0.05) contrast tests without assuming equal variances were used and t is reported (note that in this case df are not integers). Statistically significant p (p<0.05) are printed in bold.*

	D. pulex			*D. magna*	
Source	PC1	PC2	PC3	PC1	PC2
Experimental food (EF)	$F_{1,25}$=10381 **p<0.001**	$t_{4.5}$=0.5 p=0.624	$t_{3.3}$=2.3 p=0.094	$t_{5.8}$=133.5 **p<0.001**	$t_{6.7}$=2.5 **p=0.043**
Experimental temperature (ET)	$F_{1,25}$=6.2 **p=0.020**	$t_{1.3}$=1.7 p=0.282	$t_{1.5}$=0.6 p=0.632	$t_{5.8}$=4.0 **p=0.008**	$t_{6.7}$=4.6 **p=0.003**
Acclimation food (AF)	$F_{1,25}$=10.8 **p=0.003**	$t_{4.5}$=6.1 **p=0.003**	$t_{3.3}$=0.2 p=0.876	$t_{5.8}$=0.9 p=0.416	$t_{6.7}$=48.0 **p<0.001**
Acclimation temperature (AT)	$F_{1,25}$=37.9 **p<0.001**	$t_{2.9}$=8.3 **p=0.004**	$t_{2.4}$=2.8 p=0.091	$t_{5.8}$=1.4 p=0.220	$t_{6.7}$=13.2 **p<0.001**
EF x ET	$F_{1,25}$=2.7 p=0.111	$t_{1.3}$=1.4 p=0.349	$t_{1.5}$=0.6 p=0.619	$t_{5.8}$=3.0 **p=0.026**	$t_{6.7}$=5.8 **p=0.001**
AF x AT	$F_{1,25}$=43.7 **p<0.001**	$t_{3.3}$=0.2 p=0.830	$t_{2.8}$=2.0 p=0.152	$t_{5.8}$=2.3 p=0.063	$t_{6.7}$=8.1 **p<0.001**
AF x EF	$F_{1,25}$=0.4 p=0.517	$t_{4.5}$=2.2 p=0.084	$t_{3.3}$=2.1 p=0.119	$t_{5.8}$=3.8 **p=0.010**	$t_{6.7}$=33.5 **p<0.001**
AT x ET	$F_{1,25}$=4.5 **p=0.044**	$t_{5.7}$=0.3 p=0.785	$t_{5.3}$=1.5 p=0.199	$t_{5.8}$=0.2 p=0.856	$t_{6.7}$=4.3 **p=0.004**
AT x EF	$F_{1,25}$=7.3 **p=0.012**	$t_{2.9}$=0.8 p=0.474	$t_{2.4}$=1.6 p=0.238	$t_{5.8}$=4.0 **p=0.008**	$t_{6.7}$=2.3 p=0.056
AF x ET	$F_{1,25}$=2.7 p=0.114	$t_{3.9}$=0.5 p=0.631	$t_{3.7}$=0.4 p=0.737	$t_{5.8}$=2.0 p=0.097	$t_{6.7}$=4.7 **p=0.002**
AF x EF x ET	$F_{1,25}$=7.5 **p=0.011**	$t_{3.9}$=0.4 p=0.701	$t_{3.7}$=0.9 p=0.405	$t_{5.8}$=0.6 p=0.580	$t_{6.7}$=2.1 p=0.072
AT x EF x ET	$F_{1,25}$=11.5 **p=0.002**	$t_{5.7}$=0.1 p=0.945	$t_{5.3}$=0.2 p=0.851	$t_{5.8}$=1.2 p=0.274	$t_{6.7}$=0.3 p=0.746
AF x AT x EF	$F_{1,25}$=43.8 **p<0.001**	$t_{3.3}$=1.5 p=0.214	$t_{2.8}$=0.7 p=0.526	$t_{5.8}$=6.3 **p=0.001**	$t_{6.7}$=1.8 p=0.111
AF x AT x ET	–	–	–	$t_{5.8}$=3.5 **p=0.014**	$t_{6.7}$=1.7 p=0.139
AF x AT x EF x ET	–	–	–	$t_{5.8}$=0.7 p=0.492	$t_{6.7}$=2.5 **p=0.045**

PCA analysis showed the existence of differences in the FA compositions between different experimental and acclimation groups of *Daphnia*. Next it was of interest of whether these differences affected the overall saturation of the FAs. The percent share of saturated FAs (SAFA), monounsaturated FAs (MUFA) and polyunsaturated FAs (PUFA) in the total FA content of *Daphnia* and the unsaturation index (UI) of different experimental and acclimation groups are presented in *Figure 35* through *Figure* 42 (see Annex H for the results of contrast tests).

The patterns of FA saturation were similar in both species, with *D. pulex* having a slightly greater share of SAFA than *D. magna* (*Figure 35*, *Figure 36*). Experimental food quality had the strongest effect on FA saturation in both species (*Table 23, Table 24*). Animals fed *Cryptomonas* had less SAFA (*Figure 35*, *Figure 36*), less MUFA (*Figure 37*, *Figure 38*), more PUFA (*Figure 39*, *Figure 40*) and a higher UI (*Figure 41*, *Figure 42*) than those fed *Chlamydomonas*.

The effect of maternal diet was less pronounced. *D. pulex* with *Cryptomonas* as maternal diet had on average more PUFA and a higher UI than *Chlamydomonas* reared animals, especially when acclimated at 15 °C (*Table 23, Figure 40, Figure 42*). However, no two groups of *D. pulex* that differed only in maternal diet had a significantly different share of SAFA, MUFA or PUFA, nor did their UI differ significantly (Annex H). In *D. magna* the effect of maternal food quality depended on acclimation temperature and experimental food quality with complex higher-order interactions (*Table 24*). *Cryptomonas* reared animals had on average less PUFA and a lower UI, especially at 20 °C acclimation temperature (*Figure 39*, *Figure 41*). All group pairs that differed only in maternal diet had significantly different PUFA content but no difference in the UI (Annex H). *Cryptomonas* reared animals also had a higher content of SAFA, which was especially evident in *Chlamydomonas* fed animals (*Figure 35*, Annex H).

Overall, the differences in FA saturation due to either acclimation or experimental temperature were not significant or opposite to our expectations. Exposure to the lower temperature never led to an increase in the unsaturation of FAs. Experimental temperature had no effect on the FA saturation of *D. pulex* (*Table 23*, Annex H). In *D. magna*, the effect of experimental temperature on the PUFA content and the UI depended on the experimental diet (*Table 24*). It had no effect in *Chlamydomonas* fed animals whereas in *Cryptomonas* fed animals those growing at 20 °C actually had slightly more PUFA and a higher UI (*Figure 39*, *Figure 41*).

Acclimation temperature affected the FA saturation in both species (*Table 23, Table 24*). *D. pulex* acclimated at 15 °C had on average less PUFA and a lower UI than 20 °C acclimated animals (*Figure 40, Figure 42*). However, the differences in SAFA, PUFA and UI between groups that differed only in acclimation temperature were not significant (Annex H). In *D. magna*, 20 °C acclimated animals had less SAFA, more PUFA and a higher UI, but only when fed *Chlamydomonas* in the experiment (*Figure 35*, *Figure 39*, *Figure 41*).

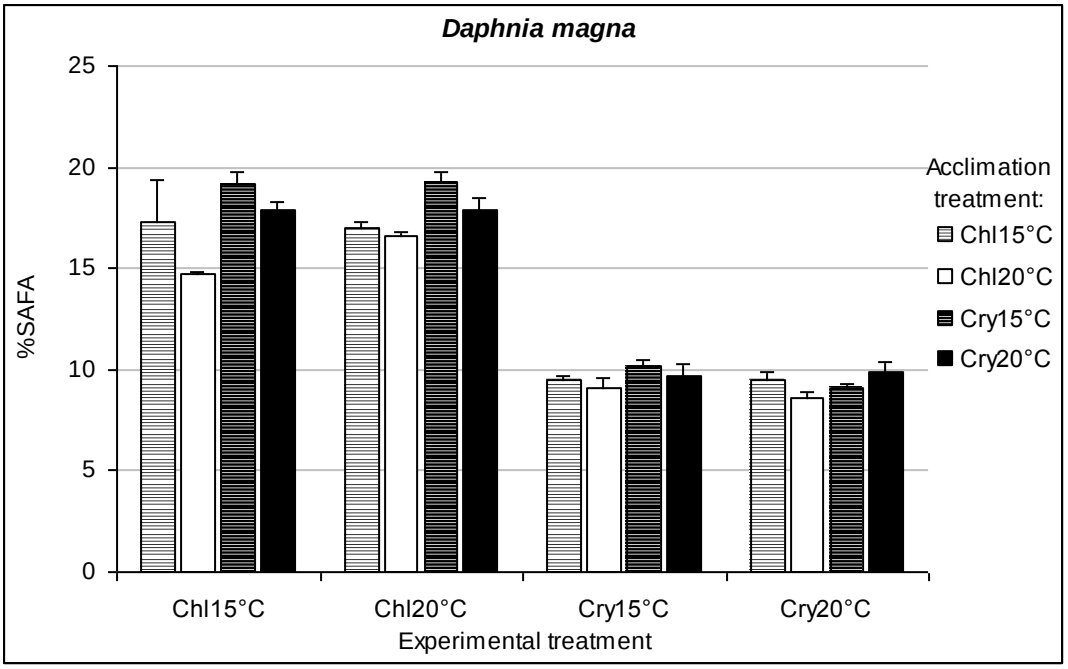

Figure 35: *Saturated fatty acid (SAFA) content (in % of total fatty acid content) of* Daphnia magna *under different acclimation and experimental treatments. Values are means of three replicates + SD. Chl* – Chlamydomonas *sp.; Cry* – Cryptomonas *sp. as food. 15 °C; 20 °C – acclimation or experimental temperature.*

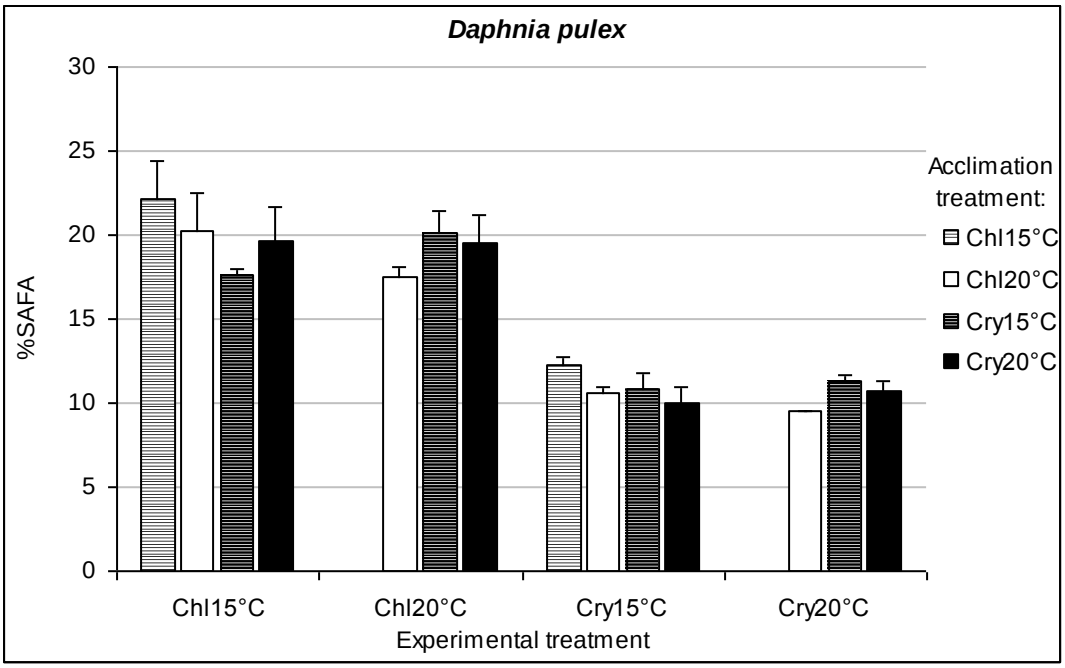

Figure 36: *Saturated fatty acid (SAFA) content (in % of total fatty acid content) of* Daphnia pulex *under different acclimation and experimental treatments. Values are means of three replicates + SD. Chl* – Chlamydomonas *sp.; Cry* – Cryptomonas *sp. as food. 15 °C; 20 °C – acclimation or experimental temperature.*

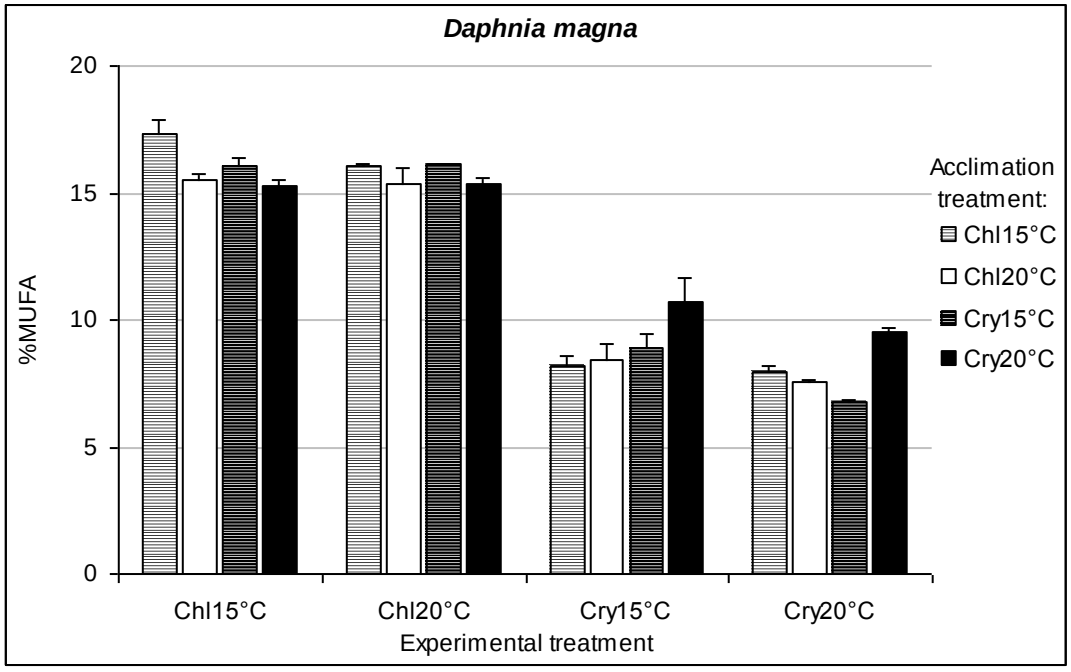

Figure 37: *Monounsaturated fatty acid (MUFA) content (in % of total fatty acid content) of* Daphnia magna *under different acclimation and experimental treatments. Values are means of three replicates + SD. Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp. as food. 15 °C; 20 °C – acclimation or experimental temperature.*

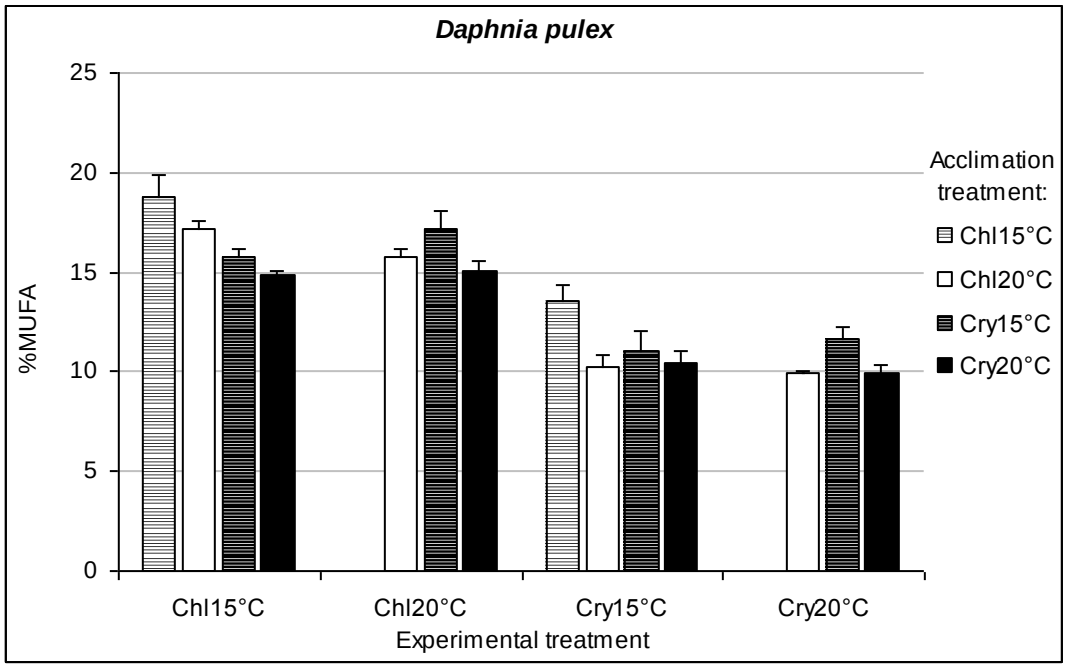

Figure 38: *Monounsaturated fatty acid (MUFA) content (in % of total fatty acid content) of* Daphnia pulex *under different acclimation and experimental treatments. Values are means of three replicates + SD. Chl –* Chlamydomonas *sp.; Cry – Cryptomonas sp. as food. 15 °C; 20 °C – acclimation or experimental temperature.*

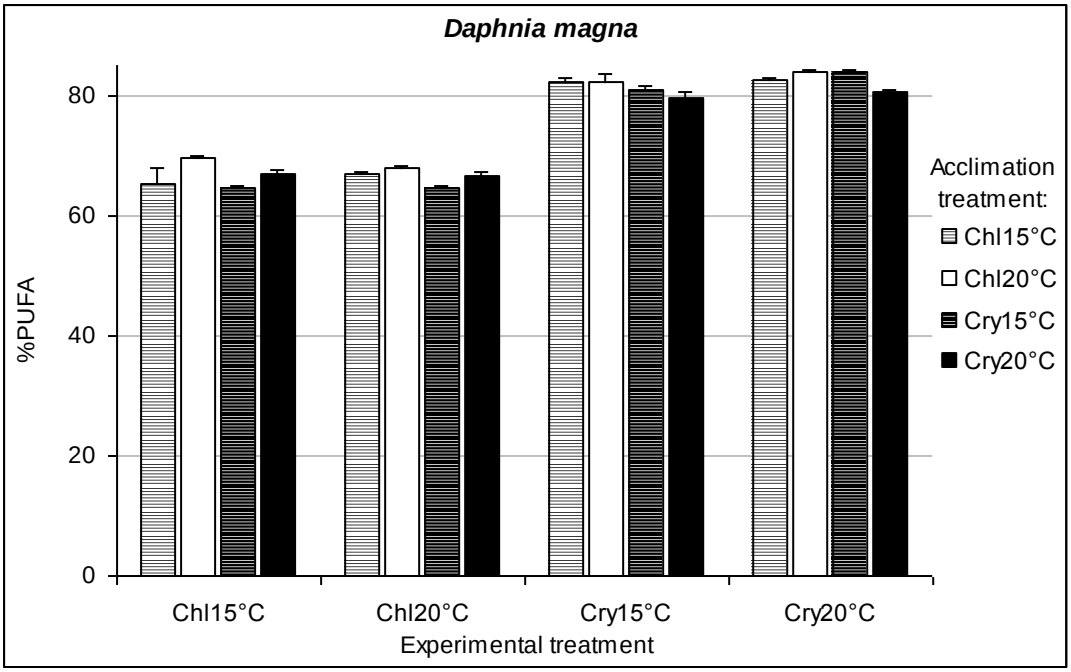

Figure 39: *Polyunsaturated fatty acid (PUFA) content (in % of total fatty acid content) of* Daphnia magna *under different acclimation and experimental treatments. Values are means of three replicates + SD. Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp. as food. 15 °C; 20 °C – acclimation or experimental temperature.*

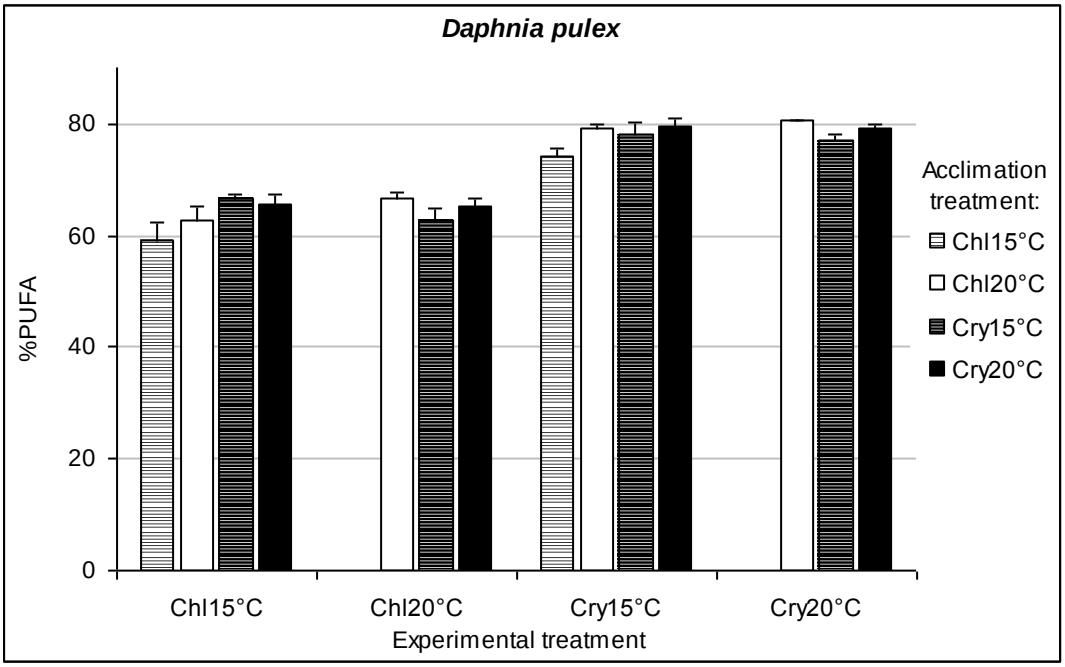

Figure 40: *Polyunsaturated fatty acid (PUFA) content (in % of total fatty acid content) of* Daphnia pulex *under different acclimation and experimental treatments. Values are means of three replicates + SD. Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp. as food. 15 °C; 20 °C – acclimation or experimental temperature.*

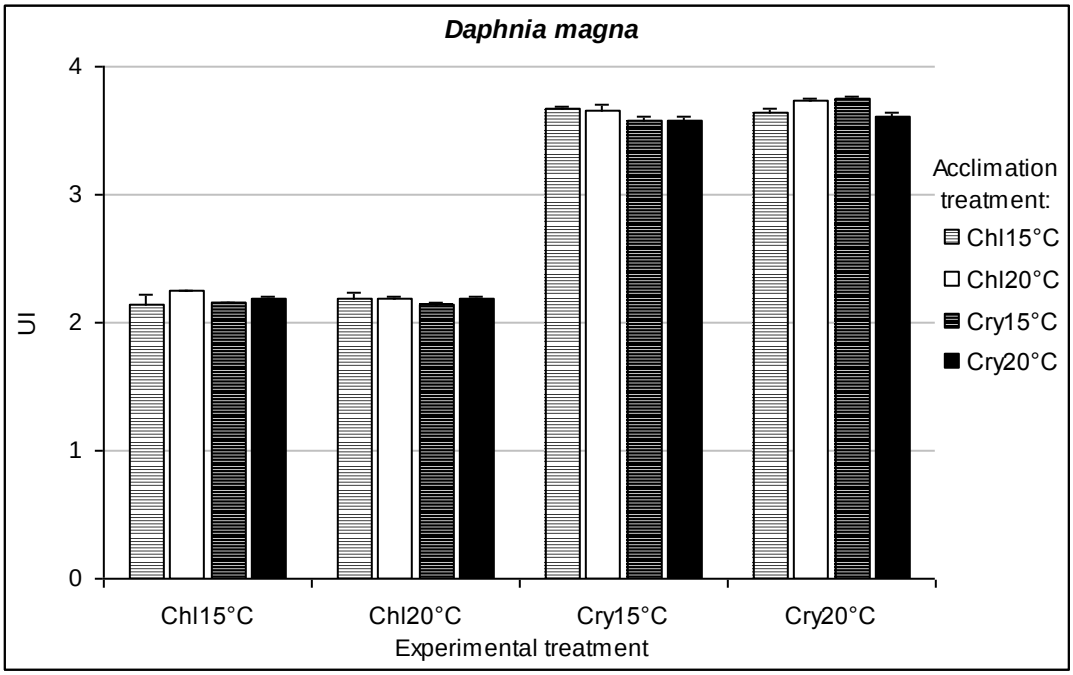

Figure 41: *Unsaturation index (UI) of* Daphnia magna *fatty acids under different acclimation and experimental treatments. Values are means of three replicates + SD. Chl* – Chlamydomonas *sp.; Cry* – Cryptomonas *sp. as food. 15 °C; 20 °C – acclimation or experimental temperature.*

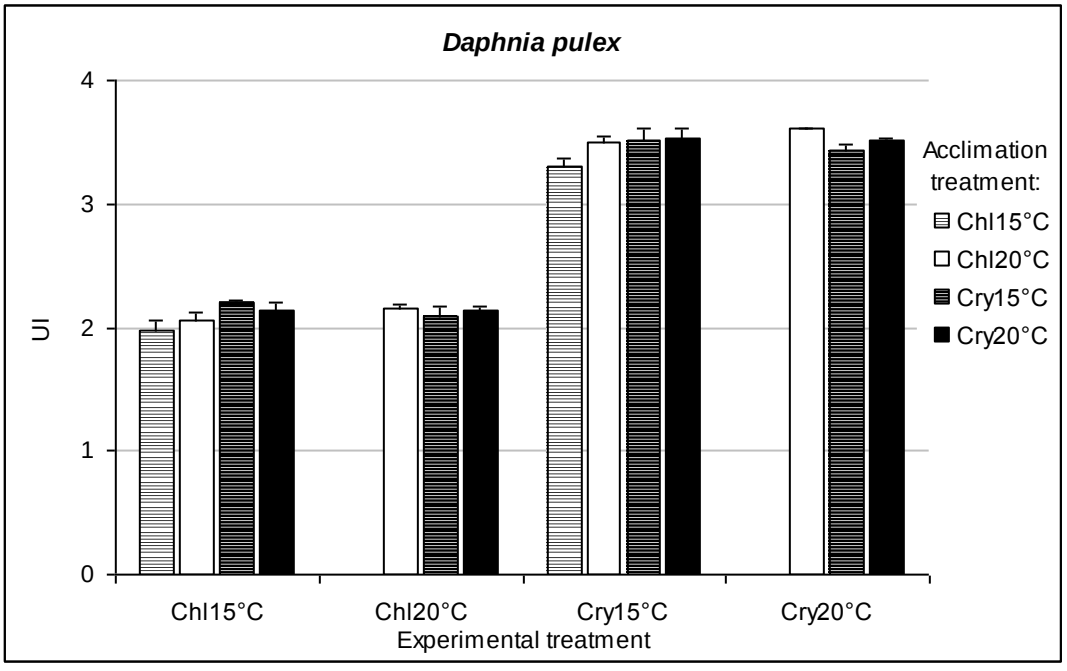

Figure 42: *Unsaturation index (UI) of* Daphnia pulex *fatty acids under different acclimation and experimental treatments. Values are means of three replicates + SD. Chl* – Chlamydomonas *sp.; Cry* – Cryptomonas *sp. as food. 15 °C; 20 °C – acclimation or experimental temperature.*

Table 23: *Effects of acclimation and experimental food quality (*Cryptomonas *sp. vs.* Chlamydomonas *sp.) and temperature (15 °C vs. 20 °C) and their interactions on the lipid composition of* Daphnia pulex*. A 4-way ANOVA was used for variables with equal group variances (Levene's test, p>0.05) and in these cases F is reported along with p. For variables with unequal group variances (Levene's test, p<0.05) contrast tests without assuming equal variances were used and t is reported (note that in this case df are not integers). Statistically significant p (p<0.05) are printed in bold. UI- fatty acid (FA) unsaturation index, % SAFA-saturated FAs, % PUFA-polyunsaturated FAs, C20:5n3 and % C20:5n3-the FA EPA content, per dry mass of* Daphnia *and in percent of total FAs respectively, Cho-Cholesterol.*

Source	UI	% SAFA	% PUFA	% C20:5n3	C20:5n3	Cho
Experimental food (EF)	$t_{7.2}$=64.4 **p<0.001**	$t_{5.1}$=16.7 **p<0.001**	$t_{5.6}$=19.5 **p<0.001**	$t_{2.8}$=243 **p<0.001**	$t_{3.5}$=32.9 **p<0.001**	$F_{1,30}$=20.8 **p<0.001**
Experimental temperature (ET)	$t_{5.7}$=0.2 p=0.856	$t_{2.8}$=0.8 p=0.507	$t_{5.3}$=0.3 p=0.754	$t_{7.9}$=1.5 p=0.168	$t_{6.8}$=8.0 **p<0.001**	$F_{1,30}$=7.1 **p=0.012**
Acclimation food (AF)	$t_{7.2}$=3.7 **p=0.007**	$t_{5.1}$=1.0 p=0.362	$t_{5.6}$=3.3 **p=0.018**	$t_{2.8}$=4.5 **p=0.024**	$t_{3.5}$=2.0 p=0.124	$F_{1,30}$=11.1 **p=0.002**
Acclimation temperature (AT)	$t_{7.1}$=3.9 **p=0.006**	$t_{5.2}$=1.8 p=0.138	$t_{7.0}$=4.2 **p=0.004**	$t_{5.6}$=5.1 **p=0.003**	$t_{3.8}$=2.2 p=0.092	$F_{1,30}$=12.0 **p=0.002**
EF x ET	$t_{5.7}$=0.5 p=0.636	$t_{2.8}$=0.3 p=0.818	$t_{5.3}$=0.2 p=0.850	$t_{7.9}$=2.0 p=0.077	$t_{6.8}$=8.1 **p<0.001**	$F_{1,30}$=3.9 p=0.059
AF x AT	$t_{8.6}$=2.7 **p=0.025**	$t_{5.7}$=1.5 p=0.178	$t_{7.8}$=2.4 **p=0.044**	$t_{4.6}$=1.2 p=0.276	$t_{3.6}$=5.4 **p=0.008**	$F_{1,30}$=24.5 **p<0.001**
AF x EF	$t_{7.2}$=1.3 p=0.223	$t_{5.1}$=1.0 p=0.362	$t_{5.6}$=1.2 p=0.283	$t_{2.8}$=3.1 p=0.057	$t_{3.5}$=2.6 p=0.065	$F_{1,30}$=4.7 **p=0.039**
AT x ET	$t_{9.5}$=1.7 p=0.117	$t_{8.1}$=1.1 p=0.294	$t_{10.6}$=1.9 p=0.080	$t_{5.3}$=1.3 p=0.237	$t_{6.9}$=1.3 p=0.222	$F_{1,30}$=21.4 **p<0.001**
AT x EF	$t_{7.1}$=1.2 p=0.255	$t_{5.2}$=0.1 p=0.951	$t_{7.0}$=0.5 p=0.664	$t_{5.6}$=5.7 **p=0.002**	$t_{3.8}$=2.1 p=0.106	$F_{1,30}$=2.8 p=0.104
AF x ET	$t_{4.7}$=2.9 **p=0.037**	$t_{4.4}$=2.2 p=0.087	$t_{5.2}$=2.4 p=0.060	$t_{4.5}$=4.6 **p=0.007**	$t_{7.9}$=7.5 **p<0.001**	$F_{1,30}$=1.1 p=0.312
AF x EF x ET	$t_{4.7}$=0.3 p=0.755	$t_{4.4}$=0.5 p=0.645	$t_{5.2}$=1.3 p=0.248	$t_{4.5}$=3.9 **p=0.014**	$t_{7.9}$=7.2 **p<0.001**	$F_{1,30}$=4.9 **p=0.035**
AT x EF x ET	$t_{9.5}$=0.6 p=0.533	$t_{8.1}$=1.4 p=0.187	$t_{10.6}$=0.9 p=0.386	$t_{5.3}$=0.8 p=0.453	$t_{6.9}$=1.2 p=0.277	$F_{1,30}$=8.0 **p=0.008**
AF x AT x EF	$t_{8.6}$=0.1 p=0.919	$t_{5.7}$=1.0 p=0.339	$t_{7.8}$=0.2 p=0.813	$t_{4.6}$=0.5 p=0.610	$t_{3.6}$=5.3 **p=0.008**	$F_{1,30}$=4.9 **p=0.035**
AF x AT x ET	–	–	–	–	–	$F_{1,30}$=11.5 **p=0.002**
AF x AT x EF x ET	–	–	–	–	–	$F_{1,30}$=10.3 **p=0.003**

Table 24: *Effects of acclimation and experimental food quality (*Cryptomonas *sp. vs.* Chlamydomonas *sp.) and temperature (15 °C vs. 20 °C) and their interactions on the lipid composition of* Daphnia magna*. A 4-way ANOVA was used for variables with equal group variances (Levene's test, p>0.05) and in these cases F is reported along with p. For variables with unequal group variances (Levene's test, p<0.05) contrast tests without assuming equal variances were used and t is reported (note that in this case df are not integers). Statistically significant p (p<0.05) are printed in bold. UI- fatty acid (FA) unsaturation index, % SAFA-saturated FAs, % PUFA-polyunsaturated FAs, C20:5n3 and % C20:5n3-the FA EPA content, per dry mass of* Daphnia *and in percent of total FAs respectively, Cho-Cholesterol.*

Source	UI	% SAFA	% PUFA	% C20:5n3	C20:5n3	Cho
Experimental food (EF)	$t_{10.5}$=156.7 **p<0.001**	$t_{4.8}$=42.4 **p<0.001**	$t_{6.7}$=61.7 **p<0.001**	$t_{4.4}$=216.9 **p<0.001**	$t_{7.8}$=32.1 **p<0.001**	$F_{1,30}$=57.0 **p<0.001**
Experimental temperature (ET)	$t_{10.5}$=3.0 **p=0.013**	$t_{4.8}$=0.3 p=0.755	$t_{6.7}$=2.6 **p=0.036**	$t_{4.4}$=4.2 **p=0.011**	$t_{7.8}$=5.3 **p=0.001**	$F_{1,30}$=0.3 p=0.595
Acclimation food (AF)	$t_{10.5}$=3.7 **p=0.004**	$t_{4.8}$=7.0 **p=0.001**	$t_{6.7}$=6.6 **p<0.001**	$t_{4.4}$=3.8 **p=0.016**	$t_{7.8}$=4.2 **p=0.003**	$F_{1,30}$=31.9 **p<0.001**
Acclimation temperature (AT)	$t_{10.5}$=1.6 p=0.140	$t_{4.8}$=4.4 **p=0.008**	$t_{6.7}$=3.1 **p=0.018**	$t_{4.4}$=0.05 p=0.962	$t_{7.8}$=4.4 **p=0.002**	$F_{1,30}$=22.3 **p<0.001**
EF x ET	$t_{10.5}$=3.6 **p=0.004**	$t_{4.8}$=2.0 p=0.103	$t_{6.7}$=3.2 **p=0.017**	$t_{4.4}$=2.9 **p=0.038**	$t_{7.8}$=5.1 **p=0.001**	$F_{1,30}$=1.6 p=0.221
AF x AT	$t_{10.5}$=3.3 **p=0.007**	$t_{4.8}$=1.3 p=0.258	$t_{6.7}$=3.8 **p=0.007**	$t_{4.4}$=2.5 p=0.058	$t_{7.8}$=3.9 **p=0.004**	$F_{1,30}$=1.8 p=0.189
AF x EF	$t_{10.5}$=0.7 p=0.476	$t_{4.8}$=4.2 **p=0.009**	$t_{6.7}$=0.5 p=0.608	$t_{4.4}$=3.6 **p=0.019**	$t_{7.8}$=4.8 **p=0.001**	$F_{1,30}$=2.1 p=0.161
AT x ET	$t_{10.5}$=2.1 p=0.065	$t_{4.8}$=1.9 p=0.121	$t_{6.7}$=2.1 p=0.071	$t_{4.4}$=1.2 p=0.281	$t_{7.8}$=2.4 **p=0.042**	$F_{1,30}$=0.4 p=0.543
AT x EF	$t_{10.5}$=3.4 **p=0.006**	$t_{4.8}$=3.2 **p=0.027**	$t_{6.7}$=6.7 **p<0.001**	$t_{4.4}$=2.1 p=0.096	$t_{7.8}$=4.4 **p=0.002**	$F_{1,30}$=4.1 p=0.051
AF x ET	$t_{10.5}$=1.8 p=0.098	$t_{4.8}$=1.3 p=0.253	$t_{6.7}$=1.4 p=0.219	$t_{4.4}$=2.5 p=0.060	$t_{7.8}$=1.1 p=0.296	$F_{1,30}$=17.7 **p<0.001**
AF x EF x ET	$t_{10.5}$=1.9 p=0.082	$t_{4.8}$=0.8 p=0.480	$t_{6.7}$=1.3 p=0.246	$t_{4.4}$=3.5 **p=0.020**	$t_{7.8}$=1.1 p=0.305	$F_{1,30}$=0.6 p=0.435
AT x EF x ET	$t_{10.5}$=1.1 p=0.308	$t_{4.8}$=0.8 p=0.480	$t_{6.7}$=1.1 p=0.324	$t_{4.4}$=0.5 p=0.638	$t_{7.8}$=2.3 p=0.055	$F_{1,30}$=0.2 p=0.674
AF x AT x EF	$t_{10.5}$=2.6 **p=0.025**	$t_{4.8}$=0.9 p=0.397	$t_{6.7}$=2.6 **p=0.039**	$t_{4.4}$=3.5 **p=0.021**	$t_{7.8}$=4.2 **p=0.003**	$F_{1,30}$=0.6 p=0.431
AF x AT x ET	$t_{10.5}$=1.8 p=0.106	$t_{4.8}$=0.4 p=0.702	$t_{6.7}$=0.002 p=0.998	$t_{4.4}$=3.8 **p=0.015**	$t_{7.8}$=1.8 p=0.111	$F_{1,30}$=3.8 p=0.060
AF x AT x EF x ET	$t_{10.5}$=4.6 **p=0.001**	$t_{4.8}$=2.7 **p=0.047**	$t_{6.7}$=3.4 **p=0.012**	$t_{4.4}$=4.5 **p=0.009**	$t_{7.8}$=1.8 p=0.110	$F_{1,30}$=0.03 p=0.870

4.2.2.3 Cholesterol content

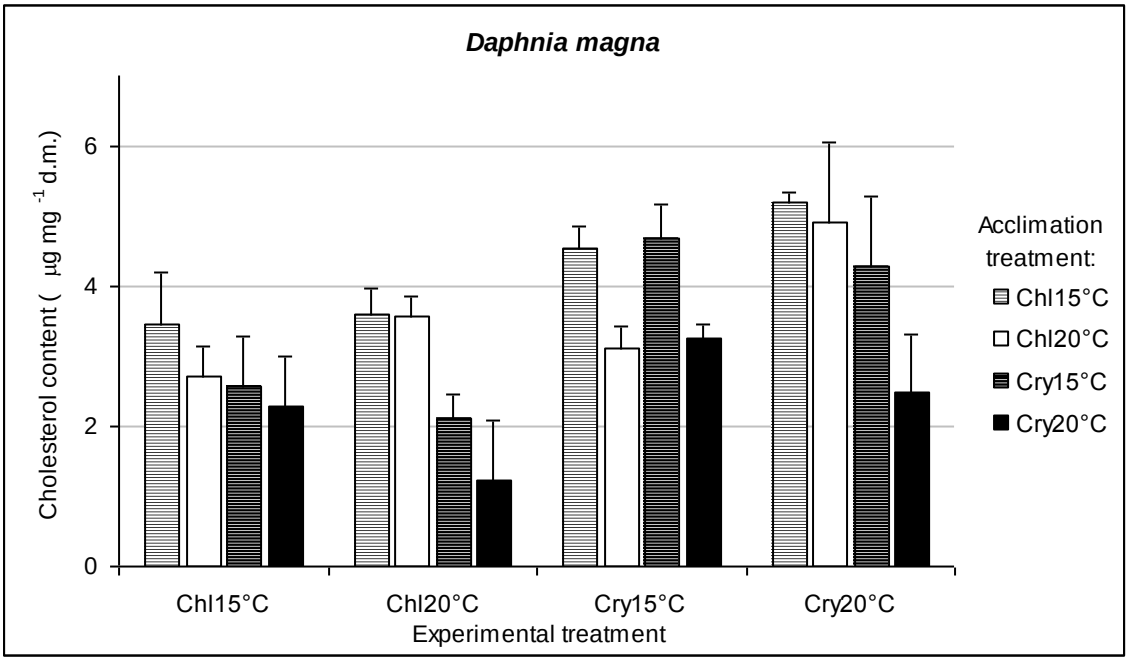

Figure 43: *Cholesterol content of* Daphnia magna *(mean of 3 samples + SD) as a function of acclimation food (Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp.) and temperature (15 °C; 20 °C) and experimental treatment.*

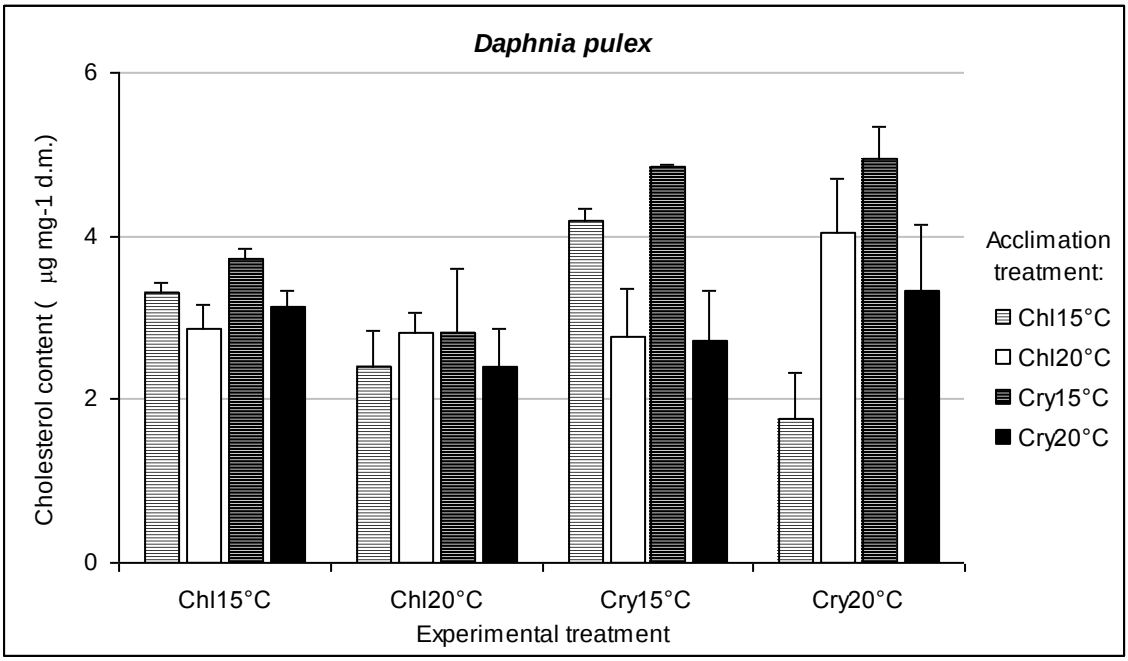

Figure 44: *Cholesterol content of* Daphnia pulex *(mean of 3 samples + SD) as a function of acclimation and experimental food (Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp.) and temperature (15 °C; 20 °C).*

Acclimation food, acclimation temperature and experimental food affected the cholesterol content of *D. magna* (*Table 24*). Experimental temperature did not have a direct effect on cholesterol content (*Table 24*). *Cryptomonas* fed animals had on average more cholesterol than *Chlamydomonas* fed animals (*Figure 43*). The effect of experimental food quality was significant only in animals from the Cry15°C acclimation treatment (Annex H). Contrary to expectations, warm acclimated animals had the same amount or less cholesterol than 15 °C acclimated animals (*Figure 43*, Annex H). Animals born to *Cryptomonas* fed mothers had less cholesterol at maturity than those from *Chlamydomonas* fed mothers, but only when growing at 20 °C (*Figure 43*, Annex H), resulting in a statistically significant interaction between acclimation food and experimental temperature (*Table 24*).

Cholesterol content of *D. pulex* was determined by a complex interaction of all four of the studied factors (*Table 23*). As in *D. magna*, *Cryptomonas* fed animals had more cholesterol than *Chlamydomonas* fed animals (*Figure 44*, Annex H). Acclimation food only affected cholesterol content in the Cry20°C experimental treatment where 15 °C acclimated animals had lower cholesterol content if their maternal diet was *Chlamydomonas* than if their maternal diet was *Cryptomonas* (*Figure 44*, Annex H). In all other cases there was no effect of maternal diet. In *D. pulex* fed *Cryptomonas* acclimation temperature significantly affected the cholesterol content (*Figure 44*, Annex H). Those also reared on *Cryptomonas* had lower cholesterol content when acclimated at 20 °C than when acclimated at 15 °C. In those reared on *Chlamydomonas* the effect of acclimation temperature depended on the experimental temperature; when growing at 20 °C, 20 °C acclimated animals had higher cholesterol content but when growing at 15 °C, 15 °C acclimated animals had higher cholesterol content (*Figure 44*). Animals at 20 °C experimental temperature generally had less cholesterol than animals growing at 15 °C, except in the Chlamy20°C acclimation group where the opposite was true (*Figure 44*, Annex H). The differences in cholesterol content among various acclimation treatments were larger in *Cryptomonas* fed animals (*Figure 44*), the cholesterol content of *Chlamydomonas* fed animals was not affected by acclimation conditions (Annex H).

4.2.2.4 The effect of age on the lipid composition of *Daphnia magna*

Discriminant analysis run on the FA composition of mature animals carrying their first clutch of eggs from all 16 treatment combinations (n=48) and newborns from the 4 acclimation treatments (n=9) showed that mature and newborn *Daphnia* can be distinguished on the basis of their FA composition (*Figure 45*, for details of the analysis see Annex J). One discriminant function (D) was found with 0.977 canonical correlation between D and age. None of the individual predictor variables were strongly correlated with D (Structure matrix, Annex J),

indicating that the separation cannot be made on the basis of any single FA. 100 % of all original cases were correctly classified using this discriminant function as well as a 100 % of cross-validated cases. The standardized coefficients of D showed that C16:0, C18:1n9, C18:1n7 and C20:1n7 contributed most to the separation: newborn animals had more C16:0, C18:1n9 and C20:1n7 while mature *Daphnia* had a bigger share of C18:1n7.

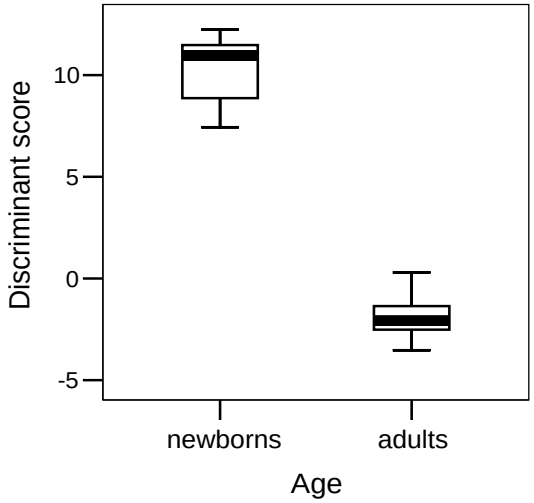

Figure 45: *Boxplot of the discriminant scores of newborn and mature* Daphnia mag*na from the discriminant analysis of the fatty acid composition of the two life stages.*

The total FA content of newborn *D. magna* was not affected by either acclimation temperature or its interaction with maternal diet (*Table 25*, Annex I). Maternal diet, however, did have a significant effect on the FA content of newborns (*Table 25*). Offspring of *Chlamydomonas* fed mothers had a higher FA content than offspring of *Cryptomonas* fed mothers (*Figure 46*). The cholesterol content of newborn *D. magna* was not affected by environmental conditions (*Table 25*, *Figure 47*).

There were differences between newborn and mature animals in the total FA content (*Figure 46*)). In the *Chlamydomonas* groups newborns had more FA than adults and the opposite was true in the *Cryptomonas* groups (*Figure 46*). Age also affected the cholesterol content of *D. magna* (*Figure 47*); the average cholesterol content of adult *D. magna* (3.4 ± 1.2 µg mg^{-1} d.m.) was higher than that of newborn *D. magna* (1.6 ± 0.8 µg mg^{-1} d.m.).

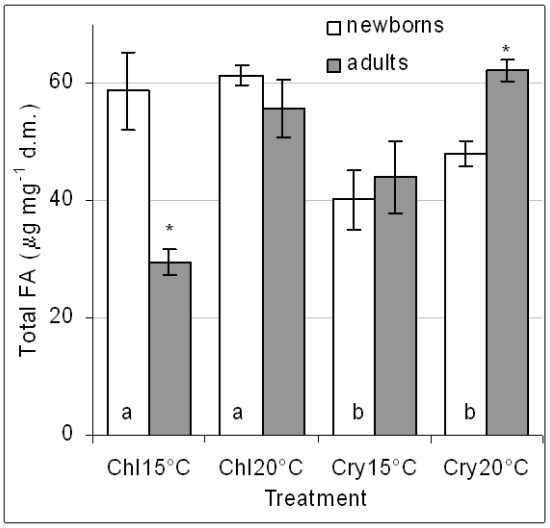

Figure 46: *Total fatty acid (FA) content of* Daphnia magna *newborns and adults under different acclimation treatments. Values are means of three replicates + SD. Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp. as food. 15 °C; 20 °C – acclimation temperature. * a significant difference between newborns and adults, bars marked by different letters differ significantly (contrast tests, Bonferroni adjusted, see Annex I).*

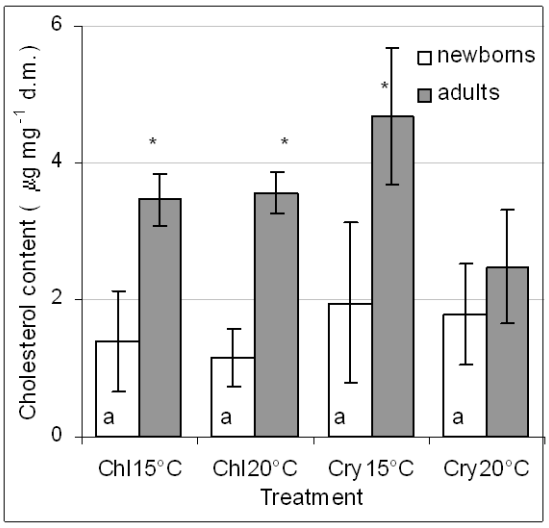

Figure 47: *Cholesterol content of* Daphnia magna *newborns and adults under different acclimation treatments. Values are means of three replicates + SD. Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp. as food. 15 °C; 20 °C – acclimation temperature. * a significant difference between newborns and adults, bars marked by different letters differ significantly (contrast tests, Bonferroni adjusted, see Annex I).*

The EPA content of newborn *D. magna* was affected by maternal food quality (*Table 25*); offspring of *Cryptomonas* fed animals had a higher EPA content than offspring of *Chlamydomonas* fed animals (*Figure 48*). Acclimation temperature and its interaction with food quality did not have a significant effect on the EPA content of newborns (*Table 25*).

The EPA content of newborns differed from that of mature animals (*Figure 48*): offspring of *Chlamydomonas* fed mothers tended to have more EPA than mature animals from the same treatment whereas offspring of *Cryptomonas* fed mothers tended to have less EPA than mature animals from the same treatment. However, the difference was statistically significant in only one group pair.

Acclimation temperature did not affect the FA saturation of newborn *D. magna* and did not interact with the effects of maternal diet (*Table 25*, *Figure 49*). The UI was affected by the quality of the maternal diet; offspring of *Cryptomonas* fed mothers had a higher UI (*Figure 49* d). The relative shares of SAFA, MUFA and PUFA were not affected significantly by maternal diet (*Table 25*).

Newborn *D. magna* reared on *Cryptomonas* had a lower UI and less PUFA than mature animals from the same treatment (*Figure 49* c and d). They also had more SAFA and MUFA, but the difference was not statistically significant (*Figure 49* a and b). Mature *Chlamydomonas* fed animals did not differ from the newborns in terms of the saturation of their FA (*Figure 49*).

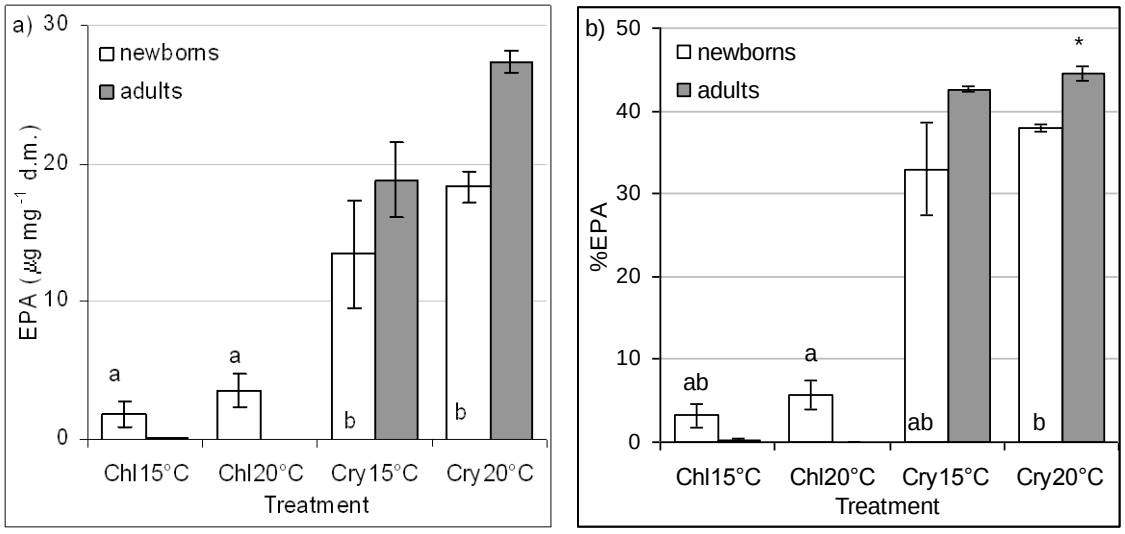

Figure 48: *a) The EPA (C20:5n3) content and b) the share of EPA in the total fatty acid content of* Daphnia magna *newborns and adults under different acclimation treatments. Values are means of three replicates + SD. Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp. as food. 15 °C; 20 °C – acclimation temperature. * a significant difference between newborns and adults, bars marked by different letters differ significantly (contrast tests, Bonferroni adjusted, see Annex I)*

Table 25: *Effects of maternal acclimation conditions; temperature (TEMP: 15 °C vs. 20 °C), food quality (FOOD:* Cryptomonas *sp. vs.* Chlamydomonas *sp.) and their interaction (TEMP X FOOD), on the lipid composition of newborn* Daphnia magna*. A two-way ANOVA was used for variables with equal group variances (Levene's test, p>0.05) and in these cases F is reported along with p. For variables with unequal group variances (Levene's test, p<0.05, marked with *) contrast tests without assuming equal variances were used and t is reported (note that in this case df are not integers). Statistically significant p (p<0.05) are printed in bold. FA-total fatty acid content, %SAFA-the share of saturated FA in the total FA content, %MUFA-the share of monounsaturated FA, %PUFA-the share of polyunsaturated FA, UI-unsaturation index, EPA-absolute amount of C20:5n3, %EPA-the share of EPA in the total FA content, Cho-total cholesterol content*

Source	*TEMP*			*FOOD*				*TEMP X FOOD*		
	df	*F/t*	*p*	*df*	*F/t*	*p*		*df*	*F/t*	*P*
FA	*1,5*	*2.5*	*0.177*	*1,5*	*22.3*	***0.005***		*1,5*	*0.6*	*0.468*
*%SAFA**	*1.3*	*-0.2*	*0.864*	*1.3*	*-2.2*	*0.218*		*1.3*	*-2.2*	*0.221*
*%MUFA**	*1.2*	*-1.5*	*0.347*	*1.2*	*-0.3*	*0.821*		*1.2*	*-0.8*	*0.572*
*%PUFA**	*1.0*	*0.8*	*0.579*	*1.0*	*1.5*	*0.364*		*1.0*	*1.7*	*0.331*
*UI**	*1.0*	*1.2*	*0.428*	*1.0*	*8.6*	*0.068*	[a]	*1.0*	*1.5*	*0.373*
EPA	*1,5*	*5.9*	*0.059*	*1,5*	*96.9*	***<0.001***		*1,5*	*1.5*	*0.278*
*%EPA**	*1.3*	*1.8*	*0.276*	*1.3*	*14.9*	***0.020***		*1.3*	*0.6*	*0.637*
Cholesterol	*1,7*	*0.1*	*0.803*	*1,7*	*0.6*	*0.480*		*1,7*	*0.7*	*0.423*

[a] *One-way Welch ANOVA for the effects of acclimation treatment showed that UI of newborn* D. magna *is affected by acclimation treatment (Welch statistic (3,3)=1205.6, P<0.001), which is also corroborated by between-group comparisons (see Figure 49 and Annex I1).*

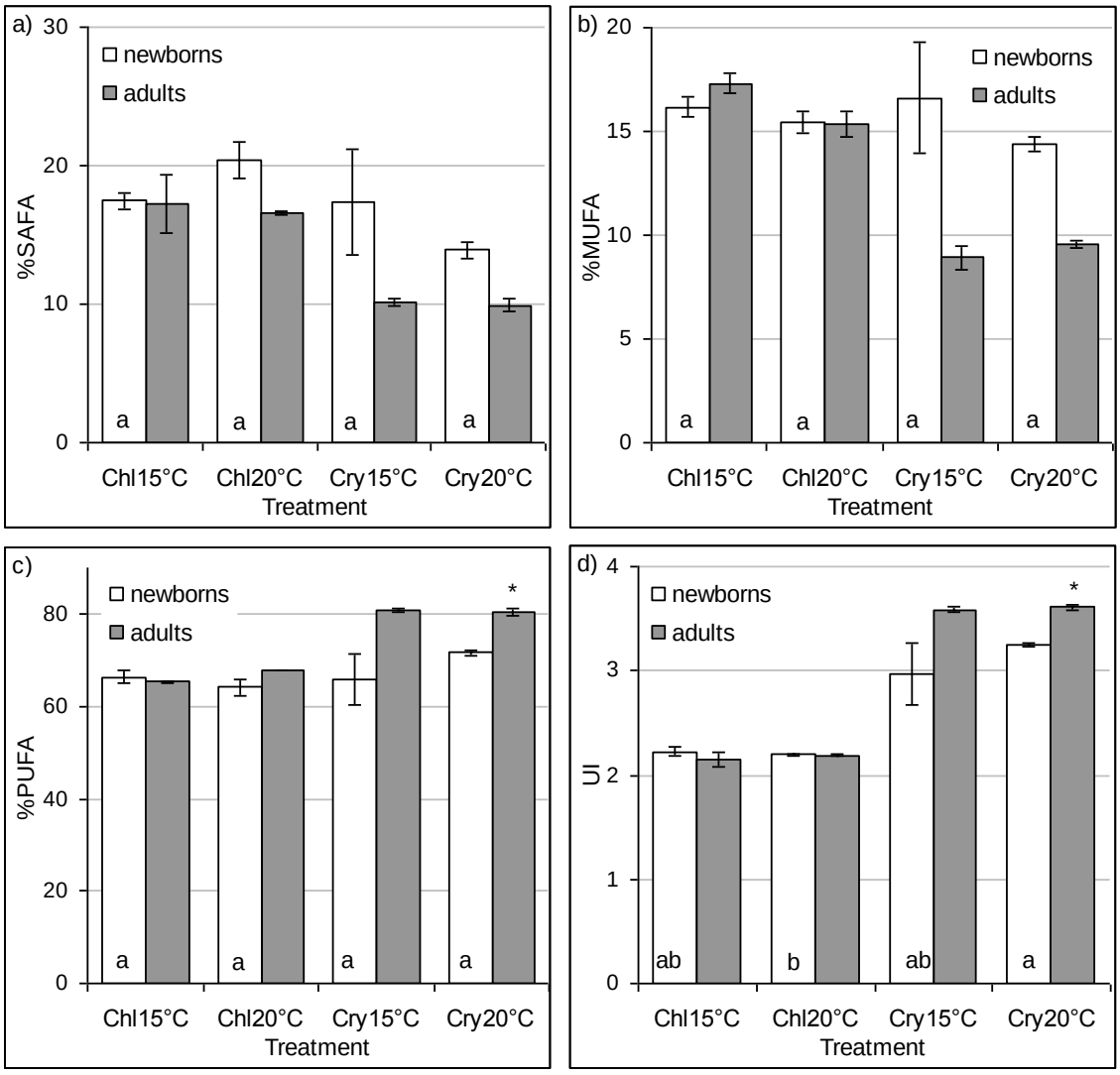

Figure 49: *a) Saturated fatty acid (SAFA), b) monounsaturated fatty acid (MUFA), c) polyunsaturated fatty acid (PUFA) content (in % of total fatty acid content) and d) the unsaturation index (UI) of* Daphnia magna *newborns and adults under different acclimation treatments. Values are means of three replicates + SD. Chl –* Chlamydomonas *sp.; Cry –* Cryptomonas *sp. as food. 15 °C; 20 °C – acclimation temperature. * a significant difference between newborns and adults, bars marked by different letters differ significantly (contrast tests, Bonferroni adjusted, see Annex I).*

4.2.3 The relationship between *Daphnia* fitness and their lipid content

Models that included food quality were better than models with only temperature combinations as predictors (*Table 26*). The best models of growth rate and reproductive performance including only temperature and EPA content were as good as or better than the models with temperature and food quality (they had similar or lower AICc, *Table 26*), indicating that the effect of acclimation and experimental diet quality on these parameters is mediated by EPA content. Adding Cho to these models did not improve them; therefore cholesterol content was probably not important determinant of juvenile growth rate and reproductive performance. The best EPA models for these parameters were additive, indicating no difference in the slopes of the regression at different temperatures, which was confirmed by regression analysis (*Figure 50*). This indicates a constant requirement for EPA across temperature regimes.

Models of clutch size showed a different pattern; the best lipids models were notably worse than the food quality models (*Table 26*) and far more interactions were included in the best models. This indicates that the effect of food quality on clutch size was not mediated entirely through its effect on EPA and cholesterol content.

Table 26: *Comparison of different models for fitness parameters of* Daphnia magna *and* D. pulex*. Predictors were acclimation temperature (AT), experimental temperature (ET), acclimation food (AF), experimental food (EF), ln of C20:5n3 content of* Daphnia *(EPA) and cholesterol content of* Daphnia *(Cho). AICc are reported for the best model (with the lowest AICc) with the designated combination of predictors and their formulations are reported under Model. T – models using only temperature variables as predictors, TxF – models using food and temperature conditions as predictors (and not the lipid content). The next three groups of models use the lipid content variables EPA and Cho as predictors instead of AF and EF. Within species and fitness parameter the best model is the one with the lowest value of AICc.*

Best model with the following predictors:	*Juvenile growth rate*		*Clutch size*		r_{pot}	
	AICc	*Model*	*AICc*	*Model*	*AICc*	*Model*
D. magna						
T	*-177*	*ET*	*190*	*ATxET*	*-201*	*ET*
TxF	***-202***	*ET+AFxEF+AFxET*	***107***	*ATxETxAFxEF*	***-214***	*ET+EF*
TxEPA	***-205***	*ET+EPA*	*157*	*ATxETxEPA*	***-220***	*ET+EPA*
TXCho	*-169*	*ET+Cho*	*169*	*ATxETxCho*	*-195*	*ET+Cho*
TxChoxEPA	*-197*	*ET+EPA+Cho*	***145***	*ATxETxEPAxCho*	*-210*	*ET+EPA+Cho*
D. pulex						
T	*-127*	*ET*	*149*	*ATxET*	*-163*	*ET*
TxF	***-162***	*ET+AFxEF*	***74***	*ATxETxAFxEF*	***-183***	*AT+ET+AF+EF*
TxEPA	***-162***	*ET+EPA*	*126*	*ATxETxEPA*	***-178***	*ATxET+EPA*
TxCho	*-122*	*ET+Cho*	*126*	*ATxETxCho*	*-164*	*ET+Cho*
TxChoxEPA	*-153*	*ET+EPA+Cho*	***102***	*ATxETxEPAxCho*	*-170*	*ATxET+EPA+Cho*

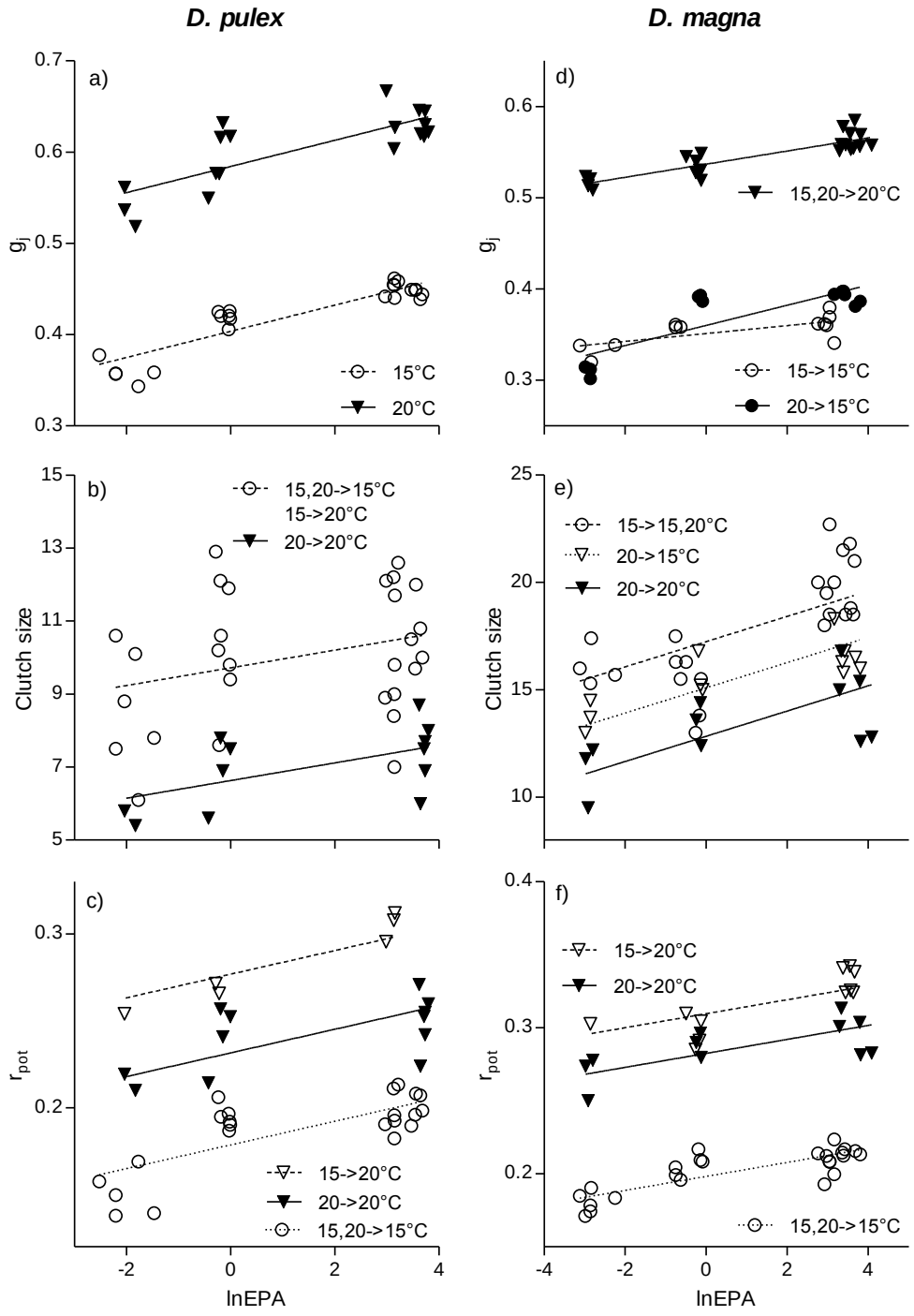

Figure 50: *Results of regression analyses of fitness parameters: juvenile growth rate (g_j; day^{-1}), size of first clutch (eggs female^{-1}) and reproductive performance (r_{pot}: day^{-1}) of* Daphnia pulex *(a, b, and c) and* D. magna *(d, e, and f) with EPA content (mg g^{-1} d.m., ln transformed) as a predictor. Temperature combinations with identical regression lines are represented by the same symbols.*

4.2.4 Fatty acid content of field collected *Daphnia*

The FAME profiles of different *Daphnia* species collected from, different water bodies at different seasons were similar (*Figure 52*). They had a more varied FA content than laboratory *Daphnia* clones fed mono-algal diets (*Figure 31* and *Figure 32*). All field-collected *Daphnia* had notable amounts of EPA, but not as much as laboratory clones fed *Cryptomonas*. *D. rosea* had more EPA in autumn than in spring (t-Test, df=2, t=17.6, p=0.003). *D. pulicaria* had the lowest EPA content.

The total FA content was lowest in *D. pulicaria* from lake Srednje Kriško Jezero and highest in *D. pulicaria* from lake Zgornje Kriško Jezero, but the differences were not statistically significant (Welch ANOVA, $p>0.05$). UI did not differ (Welch ANOVA, $p>0.05$) and was on average 1.5 ± 0.2.

All field-collected *Daphnia* contained similar amounts of PUFA, except for *D. pulicaria* from lake Srednje Kriško Jezero, which had a notably lower PUFA content (*Figure 51*). The ratio n3/n6 was higher in *D. pulex* and *D. rosea* collected in October from Hraški Bajer than in any other field population.

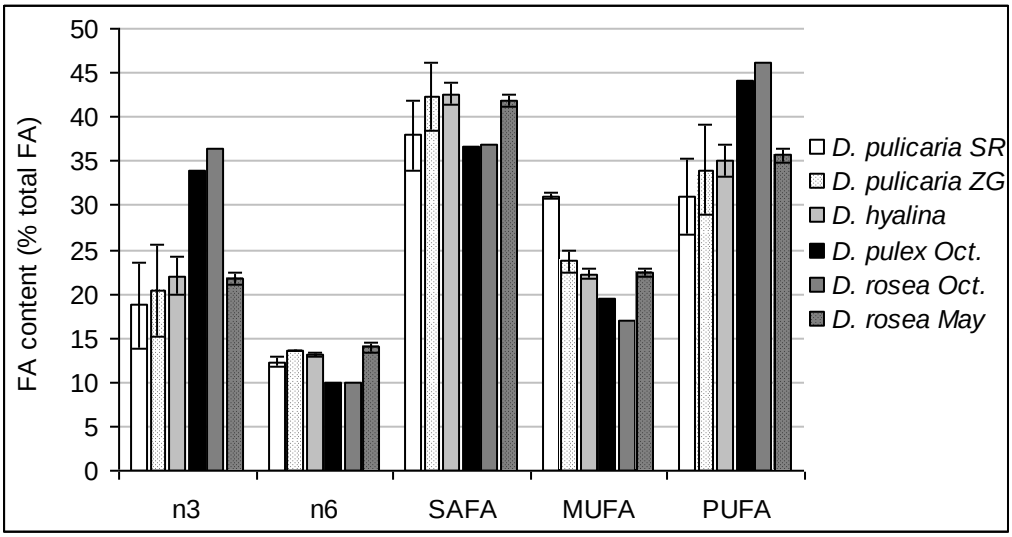

Figure 51: *Fatty acid (FA) content of field collected* Daphnia*. SAFA-sum of saturated FA, MUFA-sum of monounsaturated FA, PUFA-sum of polyunsaturated FA, n3 and n6- sum of n3 and n6 PUFA respectively. Oct. (October) and May designate the month of collection for* D. pulex *and* D. rosea *from Hraški Bajer pond.* D. pulicaria *was collected from two separate lakes: SR-Srednje Kriško Jezero, ZG-Zgornje Kriško Jezero in September.* D. hyalina *was collected from Lake Bohinj in November.*

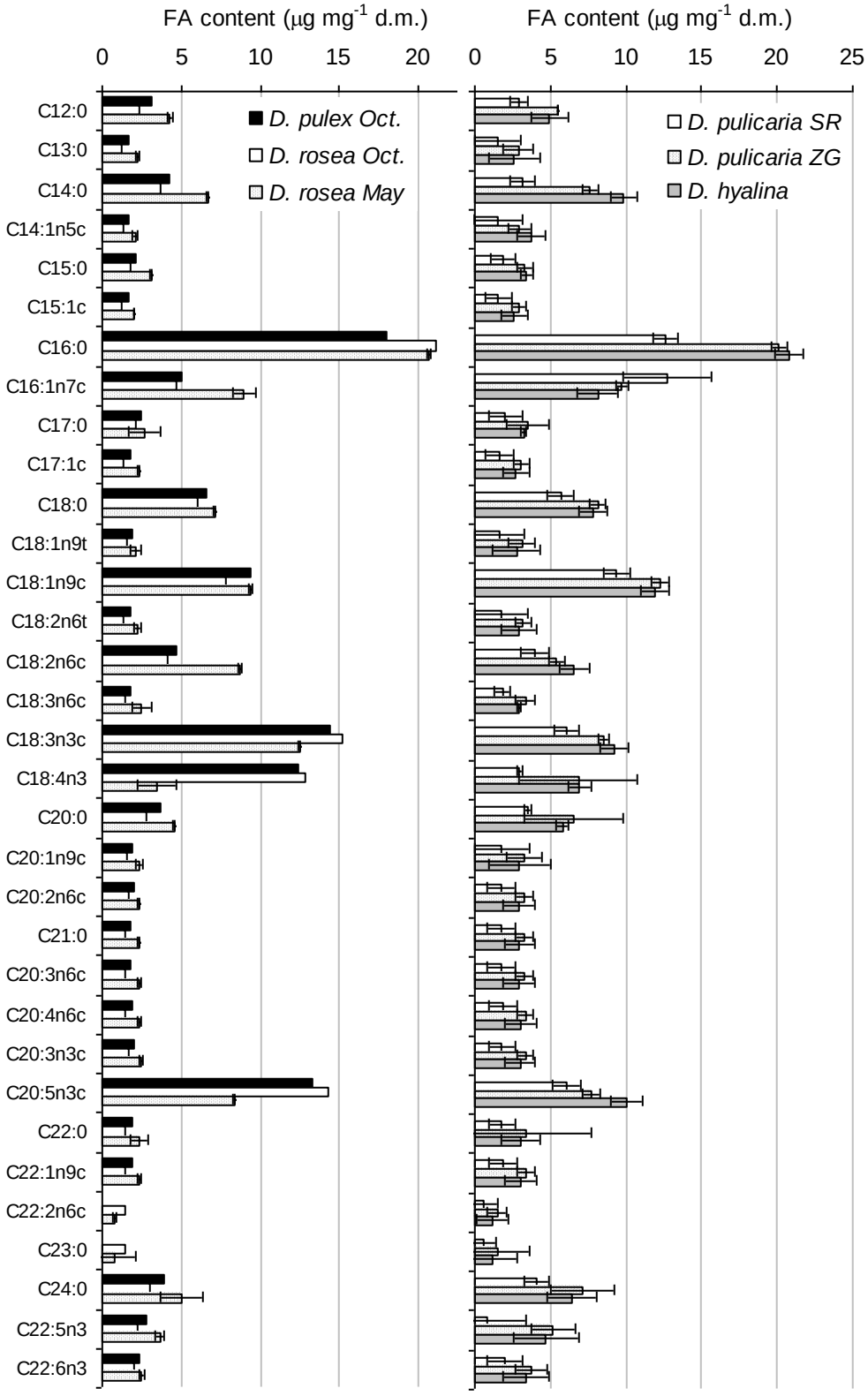

Figure 52: *Fatty acid (FA) content of field collected* Daphnia*.* D. pulex *and* D. rosea *were collected from the same pond.* D. pulicaria *was collected from two separate lakes: SR-Srednje Kriško Jezero, ZG-Zgornje Kriško Jezero.*

The differences in FA composition within a species due to differences in habitat (*D. pulicaria*) or time of collection (*D. rosea*) exceeded the differences between species (*Figure 53*). For example; the FA composition of *D. pulicaria* from Zgornje Kriško Jezero was more similar to the FA composition of *D. hyalina* from Lake Bohinj (*Figure 53*c) than to that of *D. pulicaria* from a nearby lake Srednje Kriško Jezero (*Figure 53*d). Furthermore, *D. pulex* and *D. rosea* collected from the same pond at the same time (Oct.) had similar PC scores (*Figure 53*a), whereas *D. rosea* collected from the same pond in spring (May) had a very different FA composition (b).

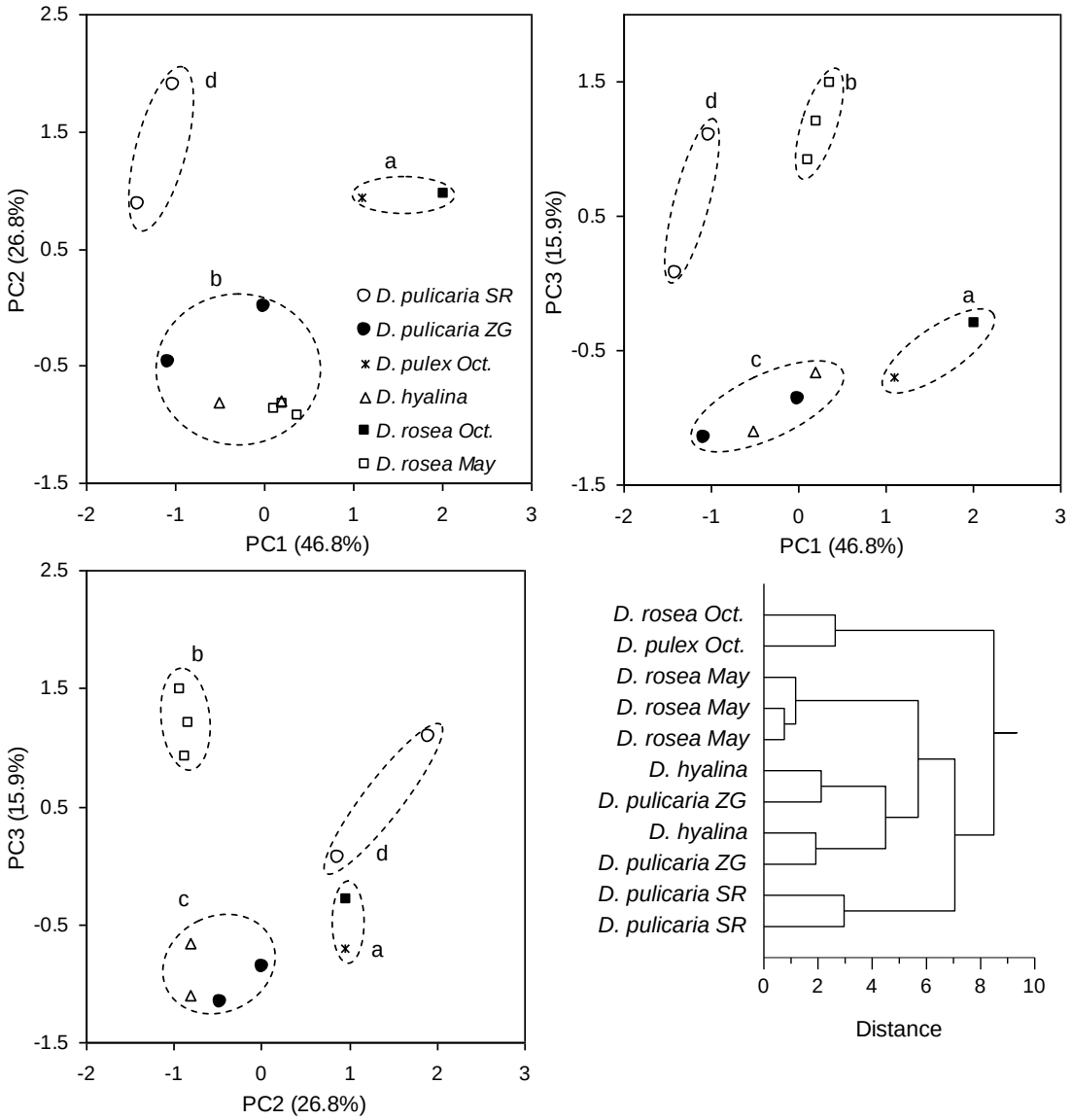

Figure 53: *Results of factor analysis of the fatty acid (FA) composition of field collected* Daphnia*. 3 principal components (PC1, PC2, PC3) were extracted, which together accounted for 89.5 % of variation in the FA composition. On the lower right there is a dendrogram of the same FA data showing the same pattern as the factor analysis.*

5 DISCUSSION

5.1 TEMPERATURE EFFECTS ON METABOLIC ACTIVITY AND FITNESS

5.1.1 Choice of measured variables

A change in temperature induces a complex response in the proteome of organisms (Stintzi, 2003; Schwerin *et al.*, 2009; Logan and Somero, 2010). The difference in gene expression between organisms at different temperatures is greatest immediately after transfer to a new temperature and much smaller after the initial acclimation is completed (Stintzi, 2003). The smaller transcriptional differences found among the acclimated groups may reflect an acclimation process that has largely remedied the effects of acute thermal stress and established a new steady-state condition involving changes in relative energy costs for different processes (Logan and Somero, 2010). Schwerin *et al*. (2009) found a marked change in protein expression of *D. pulex* with acclimation temperature (10 or 20 °C). They suggested that the increase of proteolytic enzyme concentration and the decrease of vitellogenin, actin and total protein concentration between 10 °C and 20 °C reflect the increased amino-acids demand and the reduced protein reserves in the animal's body, while the increase of actin concentration in cold-acclimated animals may contribute to a compensatory mechanism which ensures the relative constancy of muscular performance. Simple indicators for the complex process of thermal adjustment of the proteome would be difficult to find. Only the concerted action of all proteins allows for efficient acclimation of an organism and subsequent successful survival and reproduction under the new conditions. Correspondingly, only the success of the organism under the new conditions is a complete indicator of a successful adaptation of the proteome. A choice of proteins to monitor, in order to enable interspecific comparison, is therefore not an easy one. Nevertheless, certain proteins are of key importance. Haemoglobin has been shown to be important for temperature acclimation in *Daphnia* (Paul *et al.*, 2004b; Seidl *et al.*, 2005). The concentration and oxygen affinity of haemoglobin increases with increasing acclimation temperature (10–30 °C) in *D. magna* and the subunit composition varies with acclimation temperature (Paul *et al.*, 2004b). We therefore measured the Hb content of field collected *Daphnia* and in laboratory cultures acclimated to different temperatures. On the other hand, the enzymes of the ETS contribute >90% of energy available to an organism, and their activity is also temperature dependent (Simčič and Brancelj, 1997). We measured the ETS activity, which is a biochemical measure of the potential metabolic activity (Lampert, 1984). Sufficient adaptation of these proteins to temperature conditions could be of key importance for long-term fitness and serves in this study as a crude indicator of the proteome's flexibility under variable temperature conditions.

Adjustments of metabolic rate are also important for temperature acclimation and adaptation (Angilletta, 2009). In aerobic organisms, the rate of metabolism can be estimated by the rate of oxygen consumption. We measured the routine respiration rate (R) of *Daphnia.* That is the average respiration rate of an individual at its usual level of activity. The contribution of food digestion and assimilation on metabolic rate, termed specific dynamic action (Lampert, 1986), was avoided in this study by starving the animals for a period of time before the onset of experiments. The resting metabolism – the oxygen consumption of an inactive, postabsorptive (not digesting or absorbing food), non-growing and non-reproducing individual (Clarke and Fraser, 2004) – would be difficult to measure because healthy *Daphnia* are seldom inactive and healthy adult females are seldom non-reproducing. It is possible to restrain *Daphnia* during measurement of oxygen consumption by placing it in a very small container. However, with such treatment animals are exposed to high levels of stress; Zeiss (1963) found that the oxygen consumption of confined *Daphnia* is about 2.5 times that of unconfined animals. The measurement of routine metabolism is therefore the preferred alternative, especially since it is also a better approximation of the metabolic rate of the animals in the field. During our measurement of oxygen consumption, animals were free to move through the containers large enough (75 mL; 2.5-5 mL per individual *Daphnia*) to minimise the effects of crowding on respiration rate (Zeiss, 1963; Schindler, 1968). We calculated the ratio of ETS activity to respiration (the ETS/R ratio) which reflects the fraction of the maximum respiratory capacity that the organism is effectively using (Martinez, 1992).

The best estimator of success of an organism at different temperatures is naturally its fitness. In the current literature it is often represented by the intrinsic rate of population increase, *r.* Life table experiments used to calculate *r* are usually shortened to encompass the first two or three clutches. Although this value of *r*, based on only the first few clutches, is obviously an underestimation of life-time *r*, *r*-values from abbreviated life-table experiments have been shown to be highly correlated with lifetime values of *r* (Vanni, 1986); the contribution of the first two broods reflects around 80 % of total *r* (Mooij and Boersma, 1996).

In our study, we used three estimators of fitness: juvenile growth rate, clutch size, and the potential rate of population increase (r_{pot}). Juvenile growth rate (g_j), is considered to be a good predictor of fitness in *Daphnia* because it correlates well with *r* (Lampert and Trubetskova, 1996) and is often used as a measure of fitness (e.g. Mitchell and Lampert, 2000; Mitchell *et al.*, 2004). The theoretical basis for the correlation is that *Daphnia* are supposed to allocate a fixed proportion of its total production into reproduction, regardless of the absolute growth varying in response to environmental conditions. A close correlation between of the two parameters may be expected if, across generations, the animals have the same average body mass (Lampert and Trubetskova, 1996). However, the proportion of production allocated to reproduction may be affected by temperature, since a slight departure from linearity was observed in the relationship

between g_j and r if values obtained at different temperatures were included (Lampert and Trubetskova, 1996). The results of our study confirm that allocation into reproduction is affected by temperature. Therefore we also used a more direct estimate of r. We calculated the potential rate of population increase (r_{pot}) from egg counts of the first clutch (van Doorslaer *et al.*, 2007). As in our experiments there was no mortality and we only took the first clutch into account, this is not a true rate of population increase but rather a measure of individual reproductive performance (van Doorslaer *et al.*, 2007). Both age at maturity and fecundity factor in this calculation, allowing a comparison of reproductive performance between groups with differing life histories.

The effect of temperature on lifespan was not investigated in our study. Our experiments were too short to observe significant values of mortality. Lifespan of most organisms decreases with increasing temperature (McCoy and Gillooly, 2008). The same relationship was found in many cladocerans (MacArthur and Baillie, 1929; Hall, 1964; Botrell, 1975). However, exemptions may be found among the tropics-adapted species (Han *et al.*, 2011). In tropical genera life span shortens again at low temperatures because the lower thermal limits are approached (Lennon *et al.*, 2001; Benider *et al.*, 2002; Lemke and Benke, 2003). Intrinsic explanations of mortality relate life span to metabolic rate. High temperatures increase the metabolic rate and incur higher rates of cell damage or decay, perhaps due to the accumulation of oxidative free radicals (Hulbert *et al.*, 2007). However, extrinsic causes of mortality are thought to outweigh intrinsic causes in natural populations of zooplankton. Organisms in natural environments typically die as a result of disease, predation or accident, well before they reach their maximum possible life span (Ricklefs, 1998). Hall (1964) estimated that the physiological mortality rate of *Daphnia galeata* is quite low, probably less than 3 % per day. Dodson (1972) found that above 90 % of total mortality in a population of *Daphnia rosea* can be attributed to predation (mostly by *Chaoborus* larvae). Wright (1965) correlated peak mortality in a population of *Daphnia schodleri* with peak *Leptodora* density. The portion of mortality not attributed to predation by *Leptodora* was found to be constant during the season from April to late June and from late July to August (Wright, 1965). However, temperature may affect mortality rates indirectly, through its influence on other abiotic and biotic factors of the environment, such as dissolved oxygen concentration, food quality and availability, virulence of diseases, abundance and activity of predators.

5.1.2 Haemoglobin content

The summer populations of the two pond *Daphnia* species had high levels of Hb, whereas the two lake species had relatively low levels of Hb (*Figure 17*). *Daphnia* Hb synthesis is highly inducible and depends on ambiental O_2 concentration (Fox *et al.*, 1951; Fox and Phear, 1953;

Kobayashi and Hoshi, 1982; Pirow *et al.*, 2001; Zeis *et al.*, 2003; Seidel *et al.*, 2005). The critical O_2 value for induction of synthesis of large quantities of Hb appears to be 3 mg O_2 L^{-1} (Fox *et al.*, 1951; Fox and Phear, 1953; Landon and Stasiak, 1983). The Hb content of summer field populations of *D. pulex* and *D. rosea* from this study agrees well with the literature values for field populations of these species (Landon and Stasiak, 1983; Sell, 1998). It is also close to the values reported for red individuals of *D. magna* (Kobayashi and Hoshi, 1982) and *D. carinata* (Wiggins and Frappell, 2000). The Hb content of summer field populations of *D. pulicaria* and *D. hyalina* from our study is similar to the Hb content of pale individuals of *D. magna*, *D. pulex* and *D. carinata* (Kobayashi and Hoshi, 1982; Landon and Stasiak, 1983; Kobayashi and Hoshi, 1984; Wiggins and Frappell, 2000). In the pond Hraški Bajer, where *D. pulex* and *D. rosea* were collected, summer water temperatures reach up to 30 °C and O_2 levels routinely fall below 50%. On the other hand, Lake Bohinj and Zgornje Kriško Jezero, the origins of *D. hyalina* and *D. pulicaria*, respectively, are oligotrophic, their temperatures seldom exceed 20 and 15 °C, respectively, and in most of the water column O_2 saturation never falls below 80 %. The two pond species are therefore often exposed to oxygen levels below 3 mg L^{-1}, while the two lake species never experience low oxygen concentrations. Differences in Hb expression between species found in our study are therefore consistent with the likelihood that oxygen concentration will be low in their environment.

Furthermore, the synthesis of Hb potentially decreases the amount of matter and energy that can be invested into growth and reproduction (Fox *et al.*, 1951; Kobayashi and Hoshi, 1984). The synthesis of Hb seems to be useful only under conditions of restricted oxygen availability, because Hb then makes a substantial contribution to convective oxygen transport that outweighs any disadvantages (Pirow *et al.*, 2001). Selection pressures in oligotrophic lakes, where oxygen availability is usually high, and energy availability is low, would therefore favour *Daphnia* with low Hb content. Moreover, an increased Hb content in the hemolymph results in a reddish coloration and can make these transparent animals more conspicuous to visually foraging predators (O'Brien, 1979). *D. hyalina* which inhabits a lake with fish is therefore exposed to an additional selection pressure for low Hb content.

The results from the field populations are a reflection of the oxygen conditions in the habitats within a (short) period before the time of sampling and do not reflect the maximum capacity of a species for Hb synthesis. *D. pulex* and *D. rosea* were collected at the time of high oxygen stress in their environments, and so we can assume that they were displaying their maximum Hb content, but the same may not be true for *D. pulicaria* and *D. hyalina*. When the Hb content was compared in animals raised in the laboratory under normoxic conditions, the Hb content of *D. pulex* and *D. rosea* was lower than in field populations but still higher than in *D. hyalina*.

Acclimation of *D. magna* to high temperatures depends in part on the Hb expression (Seidl *et al.*, 2005). Seidl *et al.* (2005) found that *D. magna* acclimated to 30 °C under normoxic conditions had an increased Hb content (300 µmol L^{-1} hemolymph) compared to animals acclimated to 20 °C (120 µmol L^{-1}). In our study, we found no effect of growth temperature on the Hb content of *D. pulex*, *D. rosea* and *D. hyalina*. However, our growth temperatures were 15 °C and 25 °C. It is possible that growth at 25 °C under normoxic conditions does not cause an O_2 deficiency in the tissues of *D. pulex* and *D. rosea* that would signal the need for increased Hb synthesis. It was not possible to check the effects of growth at 30 °C because of high mortality of all three species at this temperature. However, Lamkemeyer *et al.* (2003) found a linear increase in Hb content with acclimation temperature in the range 10-30 °C from 75 to 205 µmol Hb L^{-1} hemolymph. It is possible that we failed to detect the temperature induced differences in Hb content due to overall low Hb content of our laboratory raised animals. Reports of Hb content from laboratory studies are often lower than from field studies, even with similar O_2 concentrations (Sell, 1997).

The seasonal differences in Hb content may help explain the seasonal differences in respiration rate. Hb-rich (hypoxia acclimated) animals display a lower metabolic rate than control animals under normoxic conditions at any given temperature (Wiggins and Frappel, 2000; Seidel *et al.*, 2005), but exposure to hypoxia fails to elicit a further reduction in respiration rate (Wiggins and Frappel, 2000). On the other hand, Weider and Lampert (1985) found no effect of acclimation to low O_2 conditions on the respiration rate of *D. pulex* over a broad range of O_2 concentrations (0.5-9 mg L^{-1}) at 20 °C.

5.1.3 Body mass and mass scaling of metabolic activity

The four species of *Daphnia,* used in our study, differed in body mass (*Figure 4*). *D. pulex* and *D. pulicaria* have a much higher body mass than *D. rosea* and *D. hyalina*. Because body size importantly influences metabolic rates, only pairs of species with similar body sizes were compared in further analyses. These correspond to closely related species pairs according to phylogenetic analyses (Lehman *et al.*, 1995; Giessler, 2001), namely *pulex/pulicaria* and *rosea/hyalina*. In each species pair compared, pond dwelling species (*D. pulex*, *D. rosea*) were larger than lake dwelling species (*D. pulicaria*, *D. hyalina*). This is considered to be an adaptation to different selective pressures in these habitats, especially the absence of visual predation in fishless ponds (Brooks and Dodson, 1965; Spitze, 1992). Giessler (2001) reports other convergent morphological characters of pond species. They have larger postabdomens and more anal spines, possibly associated with the extent of suspended particles in eutrophic

pond habitats. Furthermore, pond species tend to have bigger eyes, which may be adaptive in turbid habitats and not selected against because of the absence of visually hunting predators.

D. pulex and *D. hyalina,* collected at 4°C, had a significantly higher body mass than those collected at warmer water temperatures. A shift in body sizes seems to be a widespread seasonal pattern in cladocerans of temperate regions: adults as well as offspring are larger in winter than in summer (Stenson, 1976; Culver, 1980; Mangalo and Akbar, 1986; Pajk *et al.*, 2008). The size at maturity of cladocerans raised in the laboratory tends to decrease with temperature (Neill, 1981; Perrin, 1988; McKee and Ebert, 1996; Xie *et al.*, 2000; Giebelhausen and Lampert, 2001). The decrease of size at maturity with increased temperature is a general trend in ectotherms. A detailed discussion of plausible explanations for this phenomenon can be found in Atkinson (1994). Additionally, older (and larger) individuals are more common in winter samples because the life span of temperate cladocerans decreases with temperature (MacArthur and Baillie, 1929; Hall, 1964; Bottrell, 1975) and mortality due to predation is usually higher in the summer (Wright, 1965). The body mass of *D. rosea* from the two collection times did not differ significantly, probably because both were collected at warm temperatures (21 and 28 °C).

The respiration rate (R) and ETS activity of the four species of *Daphnia* fit well (r^2>0.9) to the combined mass scaling and temperature equation proposed by Gillooly *et al.* (2001). Smaller organisms have a higher metabolic rate per body mass than larger organisms (Hemmingsen, 1960; Borgmann, 1978; Simčič and Brancelj, 1997; Clarke and Fraser, 2004), because the proportion of body mass that is metabolically inert increases as the animal grows (Glazier, 1991; Simčič and Brancelj, 2003). The mass coefficient *b* for ETS activity obtained in our study (0.66) was similar to that obtained by Simčič (1997) for adults and juveniles of five *Daphnia* species at 20 °C (0.62). The *b* for respiration (0.78) was higher than for ETS in our study, whereas Simčič (1997) found similar *b* for ETS and R. Literature reports for *b* of individual *Daphnia* species (0.78-0.82) (Lynch *et al.*, 1986) agree well with our value. Higher values of *b* (0.9-1.1) were found only for debrooded females (Lynch *et al.*, 1986; Glazier, 1991).

5.1.4 The effect of experimental temperature on the metabolic activity and fitness of *Daphnia*

Metabolic rate generally increases with temperature (Hemmingsen, 1960; Gillooly *et al.*, 2001). Both the respiration rate and the ETS activity increased with increasing experimental temperature in all four *Daphnia* species and all acclimatization groups in our study. Other authors have found similar results for various species of *Daphnia* (Armitage and Lei, 1979; Paul *et al.*, 1997; Simčič and Brancelj, 1997; Paul *et al.*, 2004a; Simčič and Brancelj, 2004). Ivleva

(1980) demonstrated that the respiration rate of marine Crustacea increases with habitat temperature. There is a qualitative similarity between the within-species and the between-species relationships of metabolic rate with temperature (Clarke and Fraser, 2004).

As temperature increases, more ATP is required to fuel processes driven faster by higher cellular kinetic energy, higher rates of protein synthesis are required to replace degraded proteins and more ATP is needed to counteract increased proton leakage across the inner mitochondrial membrane resulting in a higher rate of basal metabolism (Clarke and Fraser, 2004). Temperature increases the rate of enzymatic reactions (Somero, 2004). However, extremely high as well as extremely low temperatures can lead to enzyme deactivation (Somero, 2004). The ETS activity of *D. rosea* and *D. hyalina* decreased above 25 °C. The ETS activity of *D. pulicaria* failed to increase from 15 to 20 °C. These results indicate that temperatures at and above the thermal optimum for the enzymes of the ETS have been reached. Further increases in temperature would destabilize the enzyme and ultimately render it unfunctional. Respiration rate continued to increase at these temperatures, lowering the ETS/R ratio.

The Arrhenius activation energy (E_a), which describes the accelerating influence of temperature on metabolic rate, ranged from 35 to 81 kJ mol^{-1} for ETS activity and from 30 to 57 kJ mol^{-1} for respiration rate. These values are close to those reported by Simčič and Brancelj (1997) for five species of *Daphnia* and near the range of values reported by Gillooly *et al.* (2001) for a wide range of organisms (unicells, plants, invertebrates, ectotherm vertebrates, birds and mammals). E_a of ETS activity and E_a for respiration rate reportedly differ (del Giorgio, 1992). This reflects the purely mechanistic nature of the *in vitro* enzymatic ETS reaction as opposed to the complex and tightly controlled process of respiration. As a consequence, the ETS/R ratio increases with temperature (Simčič and Brancelj, 1997). A linear increase in the ETS/R with temperature was reported for chironomid larvae (Simčič, 2005), an amphipod (Muskó *et al.*, 1995) and marine zooplankton (Bamstedt, 1980). The ETS/R ratio of *Daphnia* also changed with experimental temperature in this study. It fell within the range 0.7 to 3.5 previously reported for *Daphnia* (Simčič and Brancelj, 1997). However, in our study, the relationship of ETS/R with temperature was not linear. The ETS/R ratios decreased again at the highest temperatures, due to the decrease in the ETS activity and an increase in respiration. We detected this decrease because we used a wider temperature range (5-30 °C) than previous investigators (5-20 °C: Simčič and Brancelj, 1997; Simčič, 2005). The maximum ETS/R values were obtained at around 20 °C, with two exceptions. One was the winter population of *D. hyalina*, which had the maximum value of ETS/R at 10 °C, but it was the only group where the changes of ETS/R with temperature were not significant. The other exception was *D. pulicaria*. The highest ETS/R ratio in this species was at 15 °C. There was, therefore, a trend for peak ETS/R to be at lower temperatures in cold adapted/acclimated animals. ETS/R can be used as indication of metabolic

scope and the temperature of maximum of metabolic scope is related to optimum growth temperatures (Calow and Sibly, 1990).

The ETS/R ratio reflects the fraction of the maximum respiratory capacity that the organism is effectively using (Martinez, 1992). Values of the ETS/R are thus theoretically expected to be above 1. However, values below 1, as were found for *D. pulex* in our study, have also been reported before for *D. obtusa* (Simčič and Brancelj, 1997), *Acartia tonsa* Dana, 1849 (Bamstedt, 1980), chironomid larvae (*Zavrelimyia* sp. and *Paratanytarsus* sp.; Simčič, 2005) and marine zooplankton (King and Packard, 1975). This occurs because the ETS activity is measured *in vitro* while the respiration rate is measured *in vivo*. It means that respiration is also influenced by the intact intracellular environment, substrate concentrations, and structure and properties of intact lipid membranes (Simčič, 2005). Values of ETS/R at or below 1 indicate maximal exploitation of the metabolic potential.

Generally, organisms grow faster and mature earlier in warmer environments (Angilletta, 2009). The general trend for cladoceran development rates to increase as temperature increases is well established (Brown, 1929; MacArthur and Baillie, 1929; Hall, 1964; Bottrell, 1975, Munro and White, 1975; Goss and Bunting, 1983). Age at maturity thus decreases as temperature increases, as has been observed in our study. However, when development temperatures exceed the optimum, development time may increase again (Goss and Bunting, 1983). Growth rate usually increases with temperature up to an optimum and then decreases rapidly at higher temperatures (Mitchell *et al.*, 2004). In our study it increased in the range 10-25 °C. Most animals died before reaching maturity at 30 °C, therefore we assume the optimum is somewhere between 25 °C and 30 °C. It may be lower in *D. hyalina*, where g_j reached a plateau at 20 °C.

Age specific fecundity (or clutch size) of *Daphnia* is highest at intermediate temperatures (Green, 1956a; Goss and Bunting, 1983). As temperatures approach the thermal limits, clutch size decreases (Green, 1956a). Clutch size did not change with temperature in offspring of *D. rosea* acclimatized at 20 °C. In other groups it was higher at 15 °C than at warmer temperatures. It only decreased at 10 °C in offspring of *D. pulex* acclimatized at 15 °C. Due to temperature effects on clutch size, the optimum temperature for reproductive performance (r_{pot}) tended to be lower than for growth. There was a marked decrease in r_{pot} of *D. hyalina* between 20 and 25 °C. The relationship between r and g_j departs from linearity when temperatures exceed the optimum temperature range.

5.1.5 Seasonal acclimatization

5.1.5.1 Seasonal changes in metabolism

Body mass of *Daphnia* differed seasonally, also affecting the metabolic rates. However, even when differences in body mass were corrected for (*D. pulex, D. hyalina*), or there were none (*D. rosea*), significant seasonal differences in metabolic rate were still observed, indicating seasonal changes in biochemistry and physiology of *Daphnia*.

Seasonal acclimatization affected the ETS activity of all *Daphnia* species in this study (*Table 10*) but the responses were not uniform. According to Somero (2004) there are three basic strategies for adaptation of enzymes to temperature. (1) A shift in the concentration of enzymes; cold acclimated animals may have a higher concentration of enzymes to compensate for the lowered reaction rates at cold temperatures. This option would lead to higher rates of ETS activity in cold acclimated animals as compared to warm acclimated animals throughout the experimental temperature range. The seasonal acclimatization of ETS activity of *D. pulex* in our study is an example of this mechanism. After differences in body mass were accounted for, winter *D. pulex* had a higher ETS activity than warm water *D. pulex*, especially at high experimental temperatures. A fixed difference in the concentration of an enzyme makes for a larger difference in ETS activity at high temperatures where the activity per unit of enzyme is higher. Consequently, cold-water *D. pulex* also had a higher E_a of ETS – more temperature sensitive ETS activity – than warm-water animals. (2) Changes in amino acid sequence that cause adaptive variation in the kinetic properties and stabilities of proteins. Enzymes of cold acclimated animals tend to be more active but less stable than those of warm acclimated animals. Such changes would lead to a higher ETS activity of cold acclimated animals in the low temperature range but lower ETS activity in the high temperature range. The seasonal acclimatization of ETS activity of *D. hyalina* is an example of this mechanism. After the difference in body mass was accounted for, summer *D. hyalina* had higher ETS activity than winter *D. hyalina* at 20 °C but slightly lower ETS activity at 5 and 10 °C. (3) Changes in the milieu in which proteins function that may modulate protein activity in response to physiological needs. Since the ETS activity is measured *in vitro* these adjustments may be lost and no difference in the enzyme activity due to acclimatization would be observed. Of course a combination of these three strategies is usually at play and there is also the possibility that no compensation takes place for a specific enzyme. There may even be negative compensation. For example, *Acartia tonsa* acclimated to 17 °C had lower ETS activity than animals acclimated to 21 °C, measured at the same temperature (Bamstedt, 1980). ETS activity may increase with

acclimatization temperature to increase the metabolic potential, so that increased metabolic demands, induced by higher temperatures, can be met.

D. rosea collected from the pond at 28 °C had lower ETS activity throughout the experimental temperature range than *D. rosea* collected at 21 °C. In acclimation to high temperature and oxygen shortage animals can decrease their metabolic rates (Wiggins and Frappel, 2000; Seidel *et al.*, 2005) and thereby decrease the gap between oxygen supply and demand. This is accomplished in part through a reduction in mitochondria density, which reduces the energy losses through proton leakage (Paul *et al.*, 2004a). Reduction in mitochondria density also means a reduction in the quantity of enzymes of the ETS. Whole body homogenate ETS activity is therefore lowered. This effect was not observed in warm acclimated *D. pulex* and *D. hyalina*, probably because they were not exposed to such extremely high temperatures and low oxygen conditions before collection.

Acclimation to lower temperatures most often leads to a compensatory increase in respiration rate (Vollenweider and Ravera, 1958; Armitage and Lei, 1979). As a consequence, cold and warm acclimated animals can maintain similar metabolic rates at their respective habitat temperatures. However, Clarke (1993) warns that respiration is a cost factor in an organism's energy budget, and that respiration does not proceed regardless, but only if there is requirement for ATP. Therefore cold acclimated organisms would only compensate for temperature driven decrease of respiration rate, if energy requirements remain as high as in warm acclimated animals, which is usually not true. In many organisms, demands for energy for growth and reproduction, predator escape responses and basal metabolism decrease in winter (Clarke, 1993). Such organisms may lower their metabolic rate in response to cold, to conserve energy, since food is usually scarce in winter (Clarke, 1993). We have observed two different patterns of cold acclimatization of respiration rate. Winter *D. hyalina* had higher respiration rates than summer *D. hyalina*, whereas cold-water population of *D. pulex* had lower respiration rates than warm-water *D. pulex*. *D. hyalina* used in our study spends the winter in active state, while *D. pulex* does not. *D. hyalina* thus needs to maintain a relatively high level of activity at cold temperatures, and needs to compensate the temperature driven decrease in metabolic rate. On the other hand, *D. pulex* spends the winter in passive state (resting eggs). Prolonged activity in cold temperatures is thus not necessary. Higher basal metabolic rate is connected with higher potential for activity (Clarke, 1993). These differences may be caused for example, by variation in mitochondrial concentration or membrane proliferation, but also by higher costs of cardiovascular work or muscle tonus (Clarke and Fraser, 2004). If high activity is unnecessary or impractical (e.g. due to low food levels in the environment) basal metabolic rate may be lowered to conserve energy. However, this decreases the scope for activity; such cold acclimated organisms would be incapable (at least until they acclimatize to the new conditions) of a strong

increase in activity after exposure to warmer temperatures, resulting in the observed patterns of oxygen consumption.

D. rosea collected from the pond at 28 °C had on average a lower respiration rate than *D. rosea* collected at 21 °C. As we argued before for ETS activity, this can be attributed to a reduction in mitochondria density, reducing the energy loses through proton leakage and thus the energy requirements of the basal metabolism (Paul *et al.*, 2004a). Since 28 °C is a temperature at the upper limit of the tolerance range, it is also possible that *D. rosea* collected at this temperature was adversely affected, which would also contribute to the observed low respiration rates.

The differences in respiration rate due to seasonal acclimatization were not as pronounced as differences observed due to acclimation temperature in *Daphnia ambigua* Scourfield, 1947 (Armitage and Lei, 1979). Acclimatization is a complex process that involves simultaneous adjustment to many environmental factors, which may obscure temperature effects.

Zooplankton adapted to higher temperatures is supposed to have higher E_a of ETS activity (Packard *et al.*, 1975; Borgmann, 1978). Simčič and Brancelj (2004) observed an increase in E_a of ETS activity of two *Daphnia* hybrids at 25 °C compared to animals acclimated to room temperatures. However, E_a was even higher in animals acclimated to 7 °C. Similarly, E_a of ETS activity was higher in *D. pulex* acclimatized to 4 °C than in *D. pulex* acclimatized to 12 °C in our study. On the other hand, cold-water *D. pulex* had less temperature sensitive respiration rate than warm acclimated *D. pulex*, which is in line with the prediction. There was no significant seasonal difference in E_a in other *Daphnia* species.

The ETS/R ratio changed seasonally in all *Daphnia* species tested, due to different responses of ETS activity and respiration rate to seasonal acclimatization discussed above. Simčič and Brancelj (2001) also observed seasonal changes in the ETS/R ratio of *Daphnia* in Lake Bled. Body mass does not affect the ETS/R ratio (Bamstedt, 1988; Muskó *et al.*, 1995; Simčič and Brancelj, 2001) and can therefore not explain these results. A change in ETS/R may occur because organisms can react to changed environmental conditions without changing the enzyme concentration (Berges *et al.*, 1993). ETS/R may be lowered due to increased activity, stress, or decreased enzyme concentration. On the other hand, it may increase if the enzyme concentration increases, or the respiration rate decreases, for example due to some energy conservation strategy or a shift to anaerobic metabolism.

We have already mentioned different strategies of response of respiration rate to cold acclimatization in *D. pulex* and *D. hyalina*. These were reflected also in the seasonal changes of the ETS/R ratio. Cold-water *D. pulex* had a significantly higher ETS/R ratio than warm-water *D. pulex*, indicating energy conservation at low temperatures. On the other hand, the ETS/R ratio of winter *D. hyalina* was lower than that of summer *D. hyalina*, indicating a more active metabolism,

capable of supporting higher activity at low temperatures. The ETS/R ratio temperature curve of both *D. rosea* groups was very similar, but the extreme-temperature *D. rosea* had a lower ETS/R ratio at low temperatures, mostly due to decreased ETS activity.

5.1.5.2 Seasonal changes in fitness

The shape of temperature performance curves for fitness of *Daphnia* in our study did not differ much with seasonal acclimatization. Nevertheless, we observed some significant seasonal differences in fitness. These can be ascribed either to seasonal changes in the clonal composition of the populations, with different clones having different life history characteristics (e.g. Carvalho, 1987; Carvalho and Crisp, 1987; Pinkhaus *et al.*, 2007), or to maternal effects, since mothers of experimental animals were collected from the field, where they were exposed to different temperatures, but also to different food conditions and photoperiod, all of which have been shown to affect offspring fitness (e.g. the experiments with laboratory clones in this study, Alekseev and Lampert, 2001).

Considering all these possible sources of variation, the relative uniformity of the performance curves at different seasons requires some explanation. First of all, pronounced seasonal changes in clonal composition do not occur in all lakes and ponds (Mitchell *et al.*, 2004). For example, temperature reaction norms for juvenile growth rate did not differ in clones of *D. magna* collected at different seasons from Lebrader Teiche (Germany) (Giebelhausen and Lampert, 2001; Mitchell *et al.*, 2004). There was no evidence for genetically adapted seasonal groups. This was explained by the intermittent nature of the population, since the pond is dried during winter and is recolonized in spring from hatching ephippia, which probably reflect the clonal composition in autumn when they were produced (Giebelhausen and Lampert, 2001). Larger temperature fluctuations in shallow ponds may also lead to a more uniform clonal composition (Mitchell *et al.*, 2004). These considerations are also relevant to the pond dwelling species *D. pulex* and *D. rosea* in our study, which also spend the winter as resting eggs and are also exposed to high temperature fluctuations. On the other hand, cold-adapted clones of *D. hyalina* from Lake Bohinj always have a temperature refugium in the hypolimnion and may therefore be present throughout the year, thus decreasing the differences in the clonal composition of our samples. The average shape of temperature reaction norms for juvenile growth rate did not differ significantly between northern and southern populations of *D. magna* (Mitchell and Lampert, 2000). Within population variation was higher than variation between populations. The studies mentioned observed only a handful of clones per season or population. On the other hand, our replicates consisted of mixtures of randomly selected offspring of field collected animals, thus encompassing a wider variety of clones. With this method the observed

variability between replicates is lower, but the estimate of the mean value in the population is better. We expect that the clonal composition of our samples is a good reflection of the clonal composition of the field population.

In the studies mentioned above (Mitchell and Lampert, 2000; Giebelhausen and Lampert, 2001; Mitchell *et al.*, 2004) the main aim was estimation of genetic variation, therefore maternal effects were removed by acclimating clones to test temperatures for several generations. On the other hand, we wanted to estimate as closely as possible the fitness of field collected animals at different temperatures. Therefore we measured the growth rate and reproduction in offspring hatched from eggs that were produced in the field. The only common treatment was acclimation of mothers and developing eggs to 20 °C. This was done for two reasons: to synchronise hatching and to improve survival at the higher experimental temperatures. Reduction of temperature has little effect on survival but a rapid increase is often detrimental (Mitchell and Lampert, 2000). Maternal effects were thus probably the main cause of seasonal differences in fitness in our study.

Food conditions in the maternal environment affect egg size. Larger offspring are often born at lower food concentrations (Giusande and Gliwicz, 1992), and in our experiments with laboratory clones, offspring of *D. magna* were larger when maternal diet was of good quality (*Cryptomonas* sp.). The size of eggs and offspring decreases with temperature in *Simocephalus vetulus* (O. F. Müller, 1776) (Perrin, 1988). Size and age (clutch number) of the mothers shows a positive correlation with egg size and offspring size (Ebert, 1991; McKee and Ebert, 1996). As discussed before, animals collected at cooler temperatures tended to be bigger. Accordingly, newborn mass was lower at higher acclimatization temperatures in our study. Larger offspring of *D. pulicaria* and *D. parvula* Fordyce, 1901, grow to be larger and heavier at maturity and produce more eggs in approximately the same time as smaller offspring (Tessier and Consolatti, 1989), resulting in higher somatic growth rate and higher reproductive performance. Ebert (1991) found a significant positive relationship between neonate size and age and size at maturity and the size of first clutch in *D. magna*. Thus, offspring of cold-acclimatized animals are expected to have higher clutch sizes, which we observed in *D. pulex* and *D. rosea* in our study. Clutch size was not affected by acclimatization in the smallest species, *D. hyalina*.

The effect of acclimatization temperature on juvenile growth rate and reproductive performance is more difficult to predict, since both age and size at maturity correlate positively with body size (Ebert, 1991). The effect on growth therefore depends on which parameter is more affected. Furthermore, maternal food quality may affect offspring growth rate and reproductive performance without affecting newborn size, as we can see in *D. pulex* in our laboratory experiments. Accordingly, the effect of acclimatization on juvenile growth rate and reproductive performance was very variable. Offspring of *D. pulex* and *D. rosea*, collected at water temperatures at the edges of the thermal tolerance range (4 °C, 28 °C), had somewhat

lower juvenile growth rate than offspring of animals collected at water temperatures near the optimum (15 °C, 20 °C). This finding supports the optimum acclimation hypothesis of developmental acclimation (Angilletta, 2009). The detrimental effect of development at an extreme temperature probably overwhelms any effect of thermal acclimatization (Angilletta, 2009). Winter *D. hyalina* had higher juvenile growth rate than summer *D. hyalina* at 10 °C, and lower at 25 °C, supporting the beneficial acclimation hypothesis, which states that cold-acclimated organisms would outperform warm-acclimated organisms at low temperatures, while the reverse will be true at high temperatures (Angilletta, 2009). The r_{pot} of winter *D. hyalina* was higher than that of summer *D. hyalina* at 20 °C, but lower at 25 °C, lending further support to this idea. Temperatures around 4 °C are evidently not detrimental for this species, which is also supported by the fact that *D. hyalina* overwinters in active state.

5.1.6 Species differences in metabolic activity and fitness

Species differences in metabolic activity were estimated by comparing closely related pairs of species from different habitats. Between species differences in ETS activity and respiration rate were explained well (r^2>0.9) by the combined mass scaling and temperature model proposed by Gillooly *et al.* (2001), indicating a strong influence of body mass. However, significant differences between species remained even after mass correction of metabolic activity.

As we have discussed for seasonal differences, acclimation conditions can strongly affect the metabolic rate. Therefore we only compared groups collected at comparable temperatures. Nevertheless, since the two species in each pair originate from different habitats, there were still considerable differences in their environment, most notably a difference in food quantity. Food concentrations were high in the eutrophic pond, where we collected *D. rosea* and *D. pulex*, and low in both lakes that where the origins of *D. hyalina* and *D. pulicaria*. Differences in food quality and quantity may affect the respiration and ETS activity of zooplankton (Bamstedt, 1980). The conditions in the habitat have a twofold effect on *Daphnia* performance; through maternal effects and acclimatization and through selection. Since we did not acclimate the animals before measurements, we can only speculate on the mechanism behind the differences observed. Nevertheless, we can draw some conclusions by comparing our results to those of Simčič and Brancelj (1997), who measured the ETS activity and respiration rate in five species of *Daphnia* after one month of acclimation in the laboratory.

Our measurements of ETS activity fall within the range of values observed by Simčič and Brancelj (1997). Mass corrected values of ETS activity were higher in pond dwelling species than in lake dwelling species in our study. On the other hand, Simčič and Brancelj (1997)

observed similar ETS activity in *D. pulicaria* and *D. pulex*, even though *D. pulicaria* had much higher body mass. In our study, pond dwelling species also had higher respiration rates than lake dwelling species, especially at high experimental temperatures. No such trend was evident in Simčič and Brancelj (1997). Higher respiration rates of pond dwelling species in our study may be explained by the higher haemoglobin (Hb) content in pond dwelling *Daphnia*, which helps maintain a high oxygen supply at high temperatures and may therefore contribute to high temperature tolerance (Lamkemeyer *et al.,* 2003; Seidl *et al.*, 2005). *Daphnia* tend to lose Hb when transferred to normoxic laboratory conditions (Landon and Stasiak, 1983), which may explain the absence of this trend in laboratory acclimated cultures. Pond dwelling species had lower ETS/R ratios than lake dwelling species, both in our study and in Simčič and Brancelj (1997). The ETS/R ratio reflects the fraction of the maximum respiratory capacity that the organism is effectively using (Martinez, 1992). It has been proposed that pond species have higher energy demands for basal metabolism and locomotion than lake species (Simčič and Brancelj, 1997), due to differences in food quantity in these two environments. Higher values of basal metabolism enable greater activity and faster growth. *D. rosea* had higher growth rate than *D. hyalina* in our study, and *D. pulex* grows better than *D. pulicaria* at high food concentrations (Hrbáčková and Hrbáček, 1987). However, increased growth and activity come at a cost, which is reflected in higher minimum energy requirements (Achenbach and Lampert, 1997; Kreutzer and Lampert, 1999). This is not problematic in eutrophic ponds, where food is not limiting, but is a competitive disadvantage in oligotrophic lakes.

Food conditions and the strength of the selective pressure to conserve energy appear to be relatively more important for adaptation to differing habitats than prevailing temperatures. Elevated metabolic rate is expected with cold-stenotherm adaptation (Simčič and Brancelj, 1997; Paul *et al.*, 2004a). However, in our study, cold-stenotherm *D. pulicaria* had lower respiration rate than eurytherm *D. pulex*.

E_a of respiration was significantly higher in *D. rosea* than in *D. hyalina*. *D. pulicaria* had lower E_a of ETS activity than *D. pulex*. Simčič and Brancelj (1997) also found a lower value of E_a of respiration rate in *D. pulicaria* than in *D. pulex*. A *Daphnia* hybrid performing diurnal vertical migration in a stratified lake had lower E_a of ETS than the hybrid that stays in the warm water of the epilimnion (Simčič and Brancelj, 2004). E_a of respiration rate and ETS were significantly lower in cold-stenothermal chironomid larvae than in eurythermal chironomid larvae from a high mountain lake (Simčič, 2005). Warm-water freshwater zooplankton had a higher E_a of ETS than cold-water animals (Borgmann, 1987). It is suggested that low temperature sensitivity of organisms from variable temperature environments protects them against abrupt and frequent changes in activity (Simčič and Brancelj, 2004). Furthermore, organisms adapted to constantly cold environments have lower E_a due to adaptive mechanisms that increase metabolic efficiency at low temperatures and decrease it at high temperatures (Simčič and Brancelj, 2004). Warm

water organisms and organisms from environments with stable temperature are more metabolically sensitive to temperature changes (Simčič and Brancelj, 2004). Sarvala (1979) noted that for several species of copepods temperature effects on development were most marked for warm-water species.

In our study, clutch size, reproductive performance, and growth rate increased with species size, especially at higher temperatures. They were highest in *D. pulex*, intermediate in *D. rosea*, and lowest in *D. hyalina*. The same pattern was observed by Achenbach and Lampert (1997) in (in order of decreasing body size) *D. pulicaria*, *D. galeata*, and *D. ambigua*; the growth rate decreased with body size. This result is related to lower mass specific respiration rates and lower threshold food concentrations in larger animals. Size of first clutch and reproductive performance were higher in *D. magna* than *D. pulex* in our experiments with laboratory clones. However, the growth rate was higher in the smaller *D. pulex*. Because we only had one clone of each species, and considerable interclonal variation in growth rate is known to exist (Mitchell and Lampert, 2000), we cannot tell whether this is a genuine difference between the two species or just between the two clones.

The temperature performance norms for growth rate and reproductive performance were similar in *D. pulex* and *D. rosea*, showing a constant increase of performance in the temperature range 10-25 °C. Growth and reproduction were not evaluated at 30 °C due to high mortality – most animals died before maturity. Optimum temperatures for these two species appear to be between 25 °C and 30 °C, at least under laboratory conditions. Similar optimum temperatures were estimated for other temperate *Daphnia* (Hall, 1964; Mitchell *et al.*, 2004). An increase of *r* was observed between 25 °C and 30 °C in *D. parvula* (Orcutt and Porter, 1983) and between 20 °C and 30 °C in *D. pulex* (Loaring and Hebert, 1981). Optimum growth temperatures in the field may be lower, because of different food and oxygen concentrations. 25 °C was past the optimum temperature for growth of *D. hyalina*, with a strong decrease in r_{pot}. Optimum growth temperatures below 25 °C were observed in some clones of *D. magna* (Giebelhausen and Lampert, 2001; Masclaux *et al.*, 2009).

We expected an even lower optimum temperature in the cold-stenotherm *D. pulicaria*, but were unfortunately unable to perform growth experiments with this species. In samples collected from the high mountain lakes in 2009 and 2010 there were only a few adult animals, not enough to run growth experiments with their offspring. Mostly they carried resting eggs. We attempted to grow the collected sub-adults and offspring of field collected animals and use those as the parents of experimental generation, but they also produced resting eggs upon maturity. An early investment into resting eggs is considered to be an adaptation to short growth season in high alpine and arctic habitats, where lakes and ponds are covered with ice for most of the year. For example, the arctic cladoceran *Daphnia middendorffiana* Fischer, 1851, produces epphipia in the first clutch, before investing into parthenogenetic reproduction (Yurista, 1999).

The performance of all species at 10 °C was similarly low, but they all showed positive growth and reproduction. The minimum temperatures are similar in all temperate freshwater habitats (4 °C), and therefore this result could be expected. The clutch sizes at 10°C were close to maximum clutch sizes at 15 °C. On the other hand, the semi-tropical cladoceran *D. lumholtzi* G. O. Sars, 1885, produced no offspring at 5 and 10 °C (Lennon *et al.*, 2001).

5.1.6.1 The relationship between metabolic rate and fitness

Metabolism provides energy and building blocks for the production of tissue and gonads, and is therefore inextricably linked with growth and reproduction. Growth cannot occur at temperatures, at which metabolism fails to provide sufficient energy, or fails to function at all. Maximum growth and production is expected at the temperature of maximum metabolic scope – the maximum difference between basal metabolism (energy expended to counteract proton leakage, for protein turnover etc.) and potential metabolic rate (Angilletta, 2009). This difference was estimated in our study with the help of the ETS/R ratio. The temperature of maximum ETS/R ratio was lower in *D. pulicaria* than in *D. pulex*. There was a strong effect of acclimation in *D. hyalina*, winter animals had lower optimum temperature for ETS/R than summer animals. This difference was not reflected in optimum growth temperatures. Peak growth rate and reproductive performance occurred mostly at higher temperatures than the peak ETS/R ratio. Nevertheless, low values of ETS/R at 30 °C agree well with the failure to grow at that temperature.

One of the reasons, why we did not observe a better fit between optimum temperatures of ETS/R and optimum growth temperatures, may be the measurements of respiration. Since we measured routine metabolism, the energy required for swimming and filtration is included in our measurements of respiration rate. Filtration of *Daphnia* does note cease at low particle concentrations (McMahon and Rigler, 1963). The swimming rate of zooplankton is known to be affected by temperature (Zeis *et al.*, 2004b). The movement of thoracic limbs and the filtration rate are also temperature dependent (Kibby, 1971; Paul *et al.*, 2004). These processes consume energy above the resting metabolic rate and thus influence the response of respiration rate to temperature. Both swimming activity and filtration rate have bell shaped temperature curves (Kibby, 1971; Zeis *et al.*, 2004b) with maximum activity at intermediate temperatures. Personal observations during our experiments concur with these findings. Animals at 5 °C and 10 °C where lethargic, found at the bottom of their containers. The swimming activity was highest at intermediate temperatures and decreased again above 25 °C. Swimming activity therefore represented different portions of the total oxygen consumption at different temperatures. In nature such differences in activity would be reflected in the differences in growth, reproduction and survival – slow moving animals would have difficulties in escaping predation and reaching

fresh patches of food, whereas lowered filtration rate would result in lowered ingestion rate. Inactive animals may thus preserve energy at the cost of energy acquisition and low energy expenditure is therefore not necessarily reflected in high secondary production. However, these considerations could explain low growth with a high ETS/R ratio, but not the opposite.

As the energy demand continues to increase at high temperatures, the oxygen supply may become insufficient to support adequate respiration rates, causing a high ETS/R. This is especially true in aquatic ecosystems where the concentration of dissolved oxygen in the water decreases with increasing temperature. Under these conditions, animals may attempt to conserve energy (as by decreased swimming and filtration), increase oxygen supply (increased heart rate, and, after acclimation, increased Hb content) or else use anaerobic respiration (Paul *et al.*, 2004b). In the last case oxygen debt accumulates that is repaid when/if more favourable oxygen conditions are restored (Van den Thillart and Verbeek 1991; Zou *et al.* 1996; Lewis *et al.* 2007). However, this possibility could also only explain low growth with a high ETS/R ratio, but not the opposite.

In our experiments, animals were typically acclimated to test temperatures for three hours before measurements of respiration rate. This may not have been enough to avoid the measurement of a shock response at higher experimental temperatures, resulting in elevated respiration rates (e.g. *D. pulex* at 25 °C). This would result in a lowered ETS/R ratio. However, juvenile animals in growth experiments spent several days growing at 25 °C, and may have acclimated their metabolisms to the new conditions, thereby increasing growth. The ETS/R ratio may be a good predictor of the current thermal acclimation and preference – the peak was at lower temperatures in cold acclimated/adapted *D. hyalina* and *D. pulicaria*, but a less efficient predictor of long term effects of temperature on growth and reproduction, because, as we have seen, both ETS and R may change during acclimatization. On the other hand, had we incorporated mortality (which increases with temperature) into our experiments, we might have found a better agreement between optimum temperatures as predicted by ETS/R and by fitness.

Seasonal differences in metabolism were much more pronounced than seasonal differences in fitness. Chopelet *et al.* (2008) made a similar observation that important differences in metabolic rates among *Daphnia magna* from different thermal regimes were not always reflected in growth rate differences. Nevertheless, seasonal differences in fitness were related to seasonal differences in metabolic activity. There was a significant correlation between seasonal differences in ETS/R and seasonal differences in growth rate in *D. hyalina* and *D. rosea*. A seasonal increase in ETS/R was accompanied with an increase in growth rate in these two species. In *D. pulex* growth rate remained roughly the same despite large differences in ETS/R. This is perhaps a consequence of extremely low values of ETS/R observed in warm-acclimatized *D. pulex*.

The stress of *D. rosea* collected at 28 °C was evident in lowered metabolic rates and lowered ETS/R ratios, as well as lowered growth rates. Furthermore, the difference in efficiency of acclimatization to 4 °C between *D. hyalina* and *D. pulex* was detected by both metabolic measurements and growth experiments. Winter *D. hyalina* compensated for the temperature driven decrease in respiration, increased ETS activity at lower temperatures and had higher growth rate and r_{pot} at lower temperatures than summer *D. hyalina*. On the other hand, measurements in 4 °C acclimatized *D. pulex* indicated inverse compensation and energy conservation by reduced activity, which was reflected also in lower growth rate than that of 15 °C acclimatized *D. pulex*.

Interspecific differences in clutch size were related mostly to the body size of the species. However, higher growth rates were correlated with higher mass corrected rates of respiration. The pattern might have been different, if we had used lower food concentrations. E_a of metabolism was lower in *D. hyalina* and *D. pulicaria* than in *D. rosea* and *D. pulex*, indicating a lesser capacity to increase g_j and r_{pot} at higher temperatures, which was confirmed for *D. hyalina* with growth experiments.

5.2 INTERACTIVE EFFECTS OF TEMPERATURE, FOOD QUALITY, AND MATERNAL ENVIRONMENT ON *DAPHNIA* FITNESS PARAMETERS

5.2.1 Relevance to field conditions

Interactions between the effects of food quantity and food quality on zooplankton fitness indicate that food quality may be more important at high food quantities (Vijverberg, 1976). Apparently, cladocerans cannot overcome the effect of low nutrient content by simply eating more food. They shift from quantity limitation to quality limitation as food quantity increases (Sterner, 1997). However, nutrient limitation is still present even at very low food concentrations (Boersma and Kreutzer, 2002). The two daphnids used in this study are representatives of metazoan grazers of ponds, shallow lakes and lake littoral zones. High microbial production is observed in such habitats and since most daphnids can feed on bacteria and detritus that accumulate in the littoral (Burks *et al.*, 2002), there are relatively long periods in these habitats during which zooplankton are not limited by food quantity. This makes these species suitable for studying the interaction of food quality and temperature.

However, while the interaction between temperature and food quantity has been studied intensively (Sushchenya and Trubetskova, 1981; Orcutt and Porter, 1984; McKee and Ebert, 1996; Gibelhausen and Lampert, 2001) and it is well known that temperature has a stronger effect on fitness of *Daphnia* at higher food concentrations, whereas more food is needed at

higher temperatures, the research into the interaction between food quality and temperature has only just begun (Masclaux *et al.*, 2009; Sperfeld and Wacker, 2009; Persson *et al.*, 2010). The effect of food quality on zooplankton growth may either increase (Sperfeld and Wacker, 2009; Persson *et al.*, 2010) or decrease (Masclaux *et al.*, 2009) with temperature, depending on the mechanism that determines the quality of the food for the consumer. Dietary deficiency in EPA content has been shown to have greater impact on zooplankton growth and reproduction at lower temperatures (Masclaux *et al.*, 2009), whereas the C:P ratio (Persson *et al.*, 2010) and cholesterol content (Sperfeld and Wacker, 2009) are more important at higher temperatures.

Food quality changes during the season (Müller-Navarra and Lampert, 1996; Wacker and Von Elert, 2001) due to the shifts in the species composition of the phytoplankton community (Ahlgren *et al.*, 1990) as well as differences in the availability of light and nutrients (Hessen *et al.*, 2002). A notable change in food quality may be as rapid as a single *Daphnia* generation at spring water temperatures (Müller-Navarra and Lampert, 1996), so that food quality for mothers and offspring may differ considerably (Wacker and Von Elert, 2001). In shallow water habitats temperature variation may be so rapid that, while the phytoplankton community composition has not undergone changes, mothers and offspring face different environmental temperatures.

Daphnia in the field may thus experience a discrepancy between maternal and offspring environment in terms of either food quality, temperature, or both. Furthermore, the spatial variability in temperature and phytoplankton distribution, with patches that may be of different quality (Richerson *et al.*, 1970), in combination with unequal spatial distribution of adult and juvenile *Daphnia* (Dini and Carpenter, 1988; Brancelj and Blejec, 1994) may also add to changes of environment with respect to temperature and food conditions between consecutive generations. Therefore in our study, we have taken both offspring and maternal conditions into account when testing the interaction between food quality and temperature.

5.2.2 Effects of acclimation and experimental food quality

Experimental food quality affected fitness in both species in our study. *Cryptomonas* was found to be a better food for *Daphnia* than *Chlamydomonas*; *Cryptomonas* fed *Daphnia* having higher juvenile growth rates, clutch sizes, and reproductive performance. These results are in agreement with previous studies, which examined the nutritional quality of phytoplankton species as food source for *Daphnia* applying constant temperature and food regimes (Ahlgren *et al.*, 1990). The higher quality of *Cryptomonas* diet is ascribed to higher content of C20 PUFAs, especially EPA, while *Chlamydomonas* lacks this important PUFA (Ahlgren *et al.*, 1990). The decrease of growth and reproduction rate at low food quality varied between temperature and

maternal food quality treatments, indicating that temperature conditions and maternal effects influence the response of *Daphnia* to food quality.

An aspect of fitness not measured in our study is mortality. Mortality was negligible in our experiments; almost all animals reached maturity whereupon the experiment ended. Therefore we could not assess the effect of food quality on longevity or the contribution it would have to overall fitness. Food quality is known to affect survival of *Daphnia* (Infante and Litt, 1985). Generally survival is higher at good quality food. However, addition of EPA to P-limited algae decreased the survival of *Daphnia* feeding on this diet, but nevertheless increased the *r* (Becker and Boersma, 2003). But as discussed previously, it is the first few clutches that contribute most to the fitness of the animals. During acclimation, all *Cryptomonas* fed animals of both clones and at both temperatures survived until production of 3rd clutch (with sporadic losses due to handling). The survival was almost equally good in *Chlamydomonas* fed animals at 15 °C; however at 20 °C the mortality was higher but very variable. *Chlamydomonas* fed animals were more prone to infections.

Maternal food quality is known to mitigate the consequences of poor experimental food quality (Martin-Creuzburg *et al.*, 2005) and has been found to be less important under good environmental food quality conditions (Brett, 1993). The same pattern has been observed in our study. *Cryptomonas* as maternal food generally enhanced *Daphnia* growth and reproduction, especially so in offspring fed *Chlamydomonas*. Maternal diet was of less importance when offspring were fed *Cryptomonas*. Offspring of *Cryptomonas* fed mothers were also less sensitive to experimental food quality, which suggests that egg reserve quality is one of the factors determining zooplankton production during periods of poor food quality in the field.

Effects of maternal food quality treatment can be mediated through altered size and/or quality of eggs and offspring (Martin-Creuzburg *et al.*, 2005; Wacker and Martin-Creuzburg, 2007). In our experiment, newborns typically spent a few hours on maternal food treatment prior to collection for growth experiments; thus the quality of the food ingested by newborns immediately after hatching may also have affected their performance, but we would argue that this contribution is relatively small when compared to the contribution of egg reserves to the overall mass and fitness of newborns.

Maternal diet had a stronger effect on clutch size in *D. pulex* (15 % reduction at low quality maternal diet relative to that of animals reared on *Cryptomonas*) than in *D. magna* (4 % reduction). *D. pulex* was also more sensitive to low experimental food quality; its juvenile growth rate (on low *vs*. high experimental food quality) was reduced more than that of *D. magna*. Since only one clone of each species was used in our experiment we cannot test whether these differences were due to the difference between the two species or just between the two clones. Different susceptibilities of clones and taxa to the absence of EPA from the diet have been observed by Brzezinski and von Elert (2007), and von Elert (2002) suggested that they may

reflect different abilities to use other fatty acids as precursors for the synthesis of EPA. The same mechanism might also explain different sensitivities to the absence of EPA from maternal diet. Furthermore, differences in growth rate could result in differences in the discrepancy between metabolic demand and FA conversion capacities (Brett and Müller-Navarra, 1997). This would agree with the results of our study where *D. pulex*, which had higher juvenile growth rate at good food quality than *D. magna*, was more sensitive to poor experimental food quality. Furthermore, the shorter egg development time of *D. pulex*, during which more of the egg energy reserves are metabolized than in *D. magna* (Goulden *et al.*, 1987), together with its fast juvenile growth rate, might help explain the greater importance of maternal diet for clutch size in *D. pulex*. The accumulated nutrient shortage could manifest itself at the time of egg production, which is both energetically and nutritionally demanding (Wacker and Martin-Creuzburg, 2007). Different susceptibilities of clones and taxa to low food quality and the seasonal fluctuation of food quality of phytoplankton assemblages may contribute to seasonal changes in the composition of zooplankton assemblages, to the coexistence of clones and species, and the maintenance of genetic diversity in natural zooplankton communities.

5.2.3 Effects of acclimation and experimental temperature

Higher growth rate and lower clutch size at a higher experimental temperature found in this study are a well documented temperature effect (Orcutt and Porter, 1983; Giebelhausen and Lampert, 2001; Masclaux *et al.*, 2009). In the previous studies temperature effects on *Daphnia* have been investigated using a constant (Giebelhausen and Lampert, 2001; Masclaux *et al.*, 2009) or diurnally fluctuating (Orcutt and Porter, 1983) temperature regime, thus neglecting the effect of intergenerational temperature change, despite the fact that temperature varies in aquatic environments, especially in ponds and shallow parts of lakes that are the habitats of the two species used in this study. The importance of intergenerational temperature shifts for zooplankton abundance has been recognised (Verbitskii *et al.*, 2009), but the effect on offspring growth and reproduction of a temperature difference between the maternal and offspring environment has not previously been investigated. We have found that both acclimation temperature and intergenerational temperature change significantly affect *Daphnia* fitness.

There is assumed to be one linear relationship between juvenile growth rate and reproductive performance, regardless of experimental conditions (Lampert and Trubetskova, 1996) because *Daphnia* are assumed to allocate a fixed proportion of their total production to reproduction regardless of the absolute growth varying in response to environmental conditions. We found a slight discrepancy with this hypothesis; animals acclimated at 15 °C invested more into reproduction (as measured by r_{pot}) for the same juvenile growth rate as evidenced by their

steeper slope of linear regression of r_{pot} on *g* in *Figure 26* and *Figure 27*. This was evident in both species but more pronounced in *D. pulex*. There was also a species difference; *D. pulex* invests less in reproduction for the same growth rate.

Animals acclimated at 15 °C had higher clutch sizes than those acclimated at 20 °C, especially when both grew to maturity at 20°C experimental temperature. Newborn size of cladocerans tends to decrease with increasing temperature (Perrin, 1988; Gulbrandsen and Johnsen, 1990). Animals which are larger at birth often mature at a larger size and concomitantly produce larger clutches of eggs (Tessier and Consolatti, 1989; Ebert, 1991), which might help explain the observed effect of acclimation temperature.

The effect of acclimation temperature on *Daphnia* fitness in our experiment is a combination of maternal effects and developmental acclimation, since the experimental animals were not transferred to experimental temperature until after the release from the brood pouch of their mothers and so the eggs have developed at the acclimation temperature. Developmental acclimation comprises irreversible responses to temperature experienced during ontogeny (Angilletta, 2009), which might differ from acclimation responses at later life stages (Terblanche and Chown, 2006; Geister and Fischer, 2007). Different temperatures were optimal for different life stages of *Daphnia* in our study; 15 °C during acclimation (egg development) and 20 °C during experiment (juvenile growth). Animals acclimated at 15 °C had higher overall fitness (as estimated by r_{pot}) than those acclimated at 20°C (compared at the same experimental temperature), having higher clutch size and similar growth rates. On the other hand, animals at 20 °C experimental temperature had higher fitness than those at 15 °C experimental temperature due to their much higher somatic growth rates. Our results are contrary to the expectations of the hypothesis of beneficial acclimation (BAH), that animals acclimated to a certain temperature will have an advantage at that temperature over animals acclimated to different temperatures (Angilletta, 2009). Although intuitively appealing, only a few studies support the BAH, whereas the majority of experimental analyses to date have rejected its generality (reviewed in Woods and Harrison, 2002). In our study, fitness was greater in animals, acclimated at 15 °C and growing at 20°C, combining the advantage of faster growth rate at warm temperature and bigger clutch size of cold acclimated animals. This might be especially important in spring, when an increase in temperature often coincides with high food quality of natural seston (Müller-Navarra and Lampert, 1996; Wacker and Von Elert, 2001), so that this positive effect of a modest warming on *Daphnia* fitness might aid a rapid population increase.

5.2.4 Interactions between food quality and temperature

We found little direct interaction between experimental temperature and experimental food quality, even when we simulated the constant temperature conditions of previous studies (Masclaux *et al.*, 2009). Masclaux *et al.* (2009) also reported that the temperature effect on the response of *D. magna* growth rate to food quality was less pronounced at moderate temperatures (≤20 °C). Nevertheless, we found strong interactions between temperature and food quality, which would have been overlooked under constant environmental conditions, by observing the effects of the intergenerational temperature change on the dietary requirements of zooplankton.

Acclimation temperature affected the response of growth and reproduction to experimental food quality in both *Daphnia* species. The effect of experimental food quality on *D. magna* clutch size and *D. pulex* juvenile growth rate was greater in 15 °C acclimated animals than in 20 °C acclimated animals. On the other hand, at 15 °C experimental temperature, *D. magna* acclimated at 20 °C and *Chlamydomonas* reared and fed showed a 20 % decrease in growth rate (relative to *Cryptomonas* fed animals under the same conditions) while the corresponding group, acclimated at 15 °C, performed as well as the *Cryptomonas* fed animals, emphasizing the importance of acclimation temperature for food quality constraints. We suggest that the shift to lower temperature, coupled with the lack of EPA in the experimental as well as maternal diet, was especially detrimental to growth, since EPA and other long chain PUFAs play an important role in adaptation of lipid membranes to cold. This result also indicates that strong effects of food quality, that may be present in nature when temperatures vary, may be entirely overlooked in laboratory experiments run at constant temperature.

Temperature change also affected the dietary requirements of zooplankton. Food quality constraints were found to be more pronounced when animals experienced a temperature change between acclimation and experiment. The reduction of growth rate and reproductive performance at poor food quality in *D. magna* reared on *Chlamydomonas* was greater in groups that experienced a temperature change. Furthermore, *D. pulex* reared on *Cryptomonas* were only affected by experimental food quality when they experienced a temperature shift between acclimation and experiment. Organisms exposed to a change in temperature must acclimate to the new temperature. All forms of acclimation impose some energy costs that arise from the detection of and response to thermal change (Angilletta, 2009). Cellular responses include the expression of new proteins, the remodelling of cell membranes and the operation of molecular chaperones (Angilletta, 2009). These processes may increase not only the energy demand but also the nutrient requirement of organisms and may be responsible for the greater sensitivity to food quality in animals experiencing a temperature change.

It has been known for some time that the dietary requirements of zooplankton vary with experimental temperature (D'Abramo, 1979), but here we have demonstrated for the first time that the effects of maternal diet on offspring fitness are also temperature dependent. *Chlamydomonas* as maternal diet was more detrimental for fitness under suboptimal temperature conditions (20 °C acclimation and 15 °C during growth) in both species. Under optimal temperature conditions (15→20°C) and good experimental food quality, *D. magna* reared on *Chlamydomonas* had higher clutch size than those reared on *Cryptomonas*. We propose that under these favourable environmental conditions, where the quality of maternal diet is less crucial, a positive effect of a combination of *Chlamydomonas* egg reserves and *Cryptomonas* diet, similar to reported positive effects of dietary mixing of uni-algal diets (Boersma and Vijverberg, 1995), could manifest itself. Especially since *D. magna* clutch size was overall less sensitive to maternal diet than that of *D. pulex*. Under suboptimum environmental conditions, however, any such positive effects would be masked by the deficiency of *Chlamydomonas* as maternal diet. These results suggest that temperature conditions have a strong effect on the fitness consequences of maternal diet. A high quality maternal diet results in offspring more capable of coping, not only with conditions of poor food quality, but also with less favourable temperature conditions.

We have worked with a single clone of each species and they both showed the same general tendencies in their response to changes in food quality and temperature. Nevertheless, since interclonal differences in sensitivity to temperature (Carvalho, 1987) and food quality (Brzezinski and Von Elert, 2007) are known to exist, further research testing our results on a wider variety of clones of a single species to determine the extent of genetic variation that exists in natural populations would enable greater insight into the extent to which changes in temperature and food conditions affect zooplankton fitness.

We conclude that constraints exerted by food quality on somatic growth and reproduction of daphnids depend either on acclimation temperature or its combination with experimental temperature. These dietary constraints are higher when animals are exposed to temperature change, and increase with a lower quality of maternal diet, which is especially important under suboptimal environmental conditions. Zooplankton inhabiting shallow lakes or shallow parts of lakes is likely to experience intergenerational temperature change on a scale comparable to that simulated in our study. Furthermore, summer temperature fluctuations may become more common, extreme and less predictable due to climate change (Schar *et al.*, 2004; Kjellström *et al.*, 2007). Differences between maternal and offspring thermal environments may therefore become increasingly frequent and studies that examine the effects of food quality under constant temperature conditions may underestimate the dietary constraints in natural environments. Changing temperature conditions and the differences between maternal and offspring

environment should be taken into account when making predictions of matter transfer efficiency and production in natural populations.

5.3 LIPID CONTENT OF *DAPHNIA* AND ITS RELATION TO FITNESS UNDER DIFFERENT TEMPERATURE CONDITIONS

5.3.1 The effect of experimental food quality on Daphnia lipid content

Experimental diet had a strong effect on the lipid composition of *Daphnia* in our study. *Cryptomonas* fed animals had higher FA content and higher cholesterol content per unit of dry mass than *Chlamydomonas* fed animals, which is consistent with the higher FA and sterol content of their diet. *Cryptomonas* has a higher FA and sterol content per mg POC than *Chlamydomonas* (*Table 27*). The sterol composition of *Cryptomonas* and *Chlamydomonas* is different and different sterols are converted to cholesterol with different efficiencies (Martin-Creuzburg and von Elert, 2004). However, since *Chlamydomonas* contains sterols that are converted very efficiently (Martin-Creuzburg and von Elert, 2004), we assume that the phytosterol composition of the diet species was not responsible for this result.

Table 27: *The fatty acid (FA) profiles and sterol content of* Cryptomonas *sp. and* Chlamydomonas *sp. (data from [a]von Elert and Stampfl, 2000, and [b]Piepho et al., 2010). FA concentrations and total sterol content in µg mg^{-1} POC.*

	Cryptomonas	*Chlamydomonas*
C14:0	*4.3*	*1.3*
C15:0	*0.5*	*0.5*
C16:0	*14.4*	*32.7*
C16:1n7	*2.8*	*1.6*
C17:1n7	*0.7*	*--*
C18:0	*2.5*	*8.2*
C18:1n9/12	*1.1*	*4.6*
C18:1n7	*2.3*	*8.4*
C18:2n6	*4.9*	*12.7*
C18:3n6	*0.2*	*--*
C18:3n3	*37.1*	*58.1*
C18:4n3	*57.1*	*--*
C20:0	*--*	*1.7*
C20:1n9	*0.7*	*--*
C20:3n3	*--*	*--*
C20:4n6	*--*	*--*
C20:5n3	*40.0*	*--*
C22:6n3	*5.1*	*--*
Total FA[a]	*173.6*	*129.7*
Sterol[b]	*7-8*	*4*

-- not detected

Factor analysis showed that the majority of the variation in fatty acid (FA) profiles of different experimental groups can be explained by differences in experimental diet. *Daphnia magna* and *D. pulex* fed *Cryptomonas* had a high content (>55 % share in the total FA composition) of long chain polyunsaturated fatty acids; notably SDA (C18:4n3), ARA (C20:4n6) and EPA (C20:5n3) and a relatively low (~10 %) content of ALA (C18:3n3). *Daphnia* grown on *Chlamydomonas* had a low (<5 %) content of SDA, ARA and EPA whereas ALA represented over 50 % of their total FA content (*Figure 31* and *Figure 32*). This is a reflection of the FA composition of the food algal species (*Table 27*); *Chlamydomonas* lacks long chain polyunsaturated FA while they represent an important portion of *Cryptomonas* FA (von Elert and Stampfl, 2000). Other authors have also noted that the FAME profile of *Daphnia* reflects that of their diet (Weers *et al.*, 1997; Weiler, 2001; Brett *et al.*, 2006; Ravet *et al.*, 2010), probably due to low rates of *de novo* synthesis of FA in *Daphnia*. More than 98 % of the accumulated lipid of *Daphnia* is derived from their diet (Goulden and Place, 1990).

Nevertheless, there are certain systematic differences between the FA content of zooplankton and their diet; conversion and/or preferential accumulation of certain dietary FAs takes place. The changes depend on the taxonomy and trophic position (Persson and Vrede, 2006). The relative abundance of DHA (C22:6n3) is much lower in cladocerans than in their algal diet (Weers *et al.*, 1997; Brett *et al.*, 2009). In our study DHA levels were also low even in *Cryptomonas* fed *Daphnia*. On the other hand, copepods accumulate DHA (Brett *et al.*, 2009). Apparently DHA has no important function in Cladocera and the dietary DHA is at least partially converted to EPA (Weers *et al.*, 1997). The ability to convert DHA to EPA has already been demonstrated in various crustaceans (Merican & Shim 1996; Navarro *et al.*, 1999; von Elert, 2002).

The content of ARA and EPA is generally higher in *Daphnia* than in their diet (Weers *et al.*, 1997; Brett *et al.*, 2006; Persson and Vrede, 2006; Brett *et al.*, 2009; Mezek, 2009) indicating the biological importance of these C20 PUFAs. Zooplankton FA composition shows a “quasi-homeostatic” response – there is some, but much less, variation in the FA composition of consumers compared to their diet. For example, when feeding *Daphnia galeata* three algal cultures for which EPA concentrations varied by a factor of 20, EPA only varied by a factor of 2 in *D. galeata* (Müller-Navarra, 2006). In Schöhsee, *Daphnia* spp. had higher and much less variable PUFA composition than the seston, especially for LIN (C18:2n6) and EPA (Müller-Navarra, 2006).

Daphnia can use ALA and LIN as precursors for the synthesis of EPA and ARA, respectively (Farkas *et al.*, 1981; von Elert, 2002; Schlechtriem *et al.*, 2006). However, the rates of conversion are thought to be too low to meet metabolic demands (Weers *et al.*, 1997), which explains the importance of dietary EPA for *Daphnia* fitness. *Chlamydomonas* sp. contains a lot

of ALA, which could, theoretically, have been used by our experimental animals to synthesise EPA. However, we found no evidence of active EPA synthesis in *Chlamydomonas* fed animals, regardless of acclimation or growth temperature. Nevertheless, 3rd clutch newborns of *Chlamydomonas* fed animals did have small amounts of EPA, while mature animals from these treatments had almost none (*Figure 48*). Apparently whatever EPA is synthesised is invested in the offspring rather than somatic tissue. Wacker and Martin-Creuzburg (2007) report that eggs of *D. magna* contain over twice as much EPA as somatic tissue. This ratio was maintained even after 3 clutches produced on an EPA-free diet.

5.3.2 The effect of maternal diet on *Daphnia* lipid content

In our study, we tested the effect of maternal diet on the lipid composition of the offspring at hatching and after they reached maturity. The lipid content of newborns should closely reflect maternal investment, except for the part of lipid reserves which has been used by the developing egg. The proportion of egg energy reserves metabolized during embryonic development depends on neonate size and maternal food quality and quantity and ranges from 5-25 % of egg C (Goulden *et al.*, 1987; Boersma, 1995). In mature *Daphnia* we measured the lipid content of mothers along with their first clutch of eggs to evaluate the total lipid accumulated during growth. Only small differences in the overall FA composition were detected between newborns and adults from the same treatment.

The total FA content per g dry mass was higher in newborns of *Chlamydomonas* fed mothers than in newborns of *Cryptomonas* fed mothers, despite the fact that adult *Chlamydomonas* fed animals had less FA. This indicates that mothers on a poor quality diet invested more lipid reserves into each offspring. *Daphnia* are known to respond to low food quantity by increasing the allocation per offspring, i.e. by producing larger eggs with more protein, lipid and carbon, compared with eggs produced when high amounts of food are available (Guisande and Gliwicz, 1992), resulting in offspring more resistant to starvation (Tessier and Consolatti, 1989). Contrary to our results, Wacker and Martin-Creuzburg (2007) found a lower amount of total FAs in eggs of *D. magna* at poor food quality then at good food quality. However, in their experiment, mothers were switched to poor food quality after the release of their first clutch and they contained considerable PUFA reserves, whereas in our experiment they experienced poor food quality for three generations. A sudden deterioration of food quality may elicit a different response in investment to reproduction than continuous exposure to low food quality. For example, LaMontagne and McCauley (2001) found an increase in epphipia production in *D. pulex* when food quantity decreased between maternal and offspring

environment, but not when the food quantity was continuously low. Increasing lipid investment into reproduction in environments with constantly low food quality may be an adaptive response.

The FA content was higher in mature *D. magna* with *Chlamydomonas* maternal diet (the trend was similar but much less clear in *D. pulex*). The above mentioned differences in maternal investment cannot explain the differences between mature offspring with different maternal diets, because the difference in maternal investment is small (~10 ng FA) compared to the differences in individual FA content of mature *Daphnia* due to maternal diet (~2 µg FA). Therefore we can assume that the metabolism of the offspring was affected by maternal diet so that the rate of FA accumulation changed. This idea is also supported by the fact that the differences due to maternal diet were more pronounced when the total FA content was higher.

The cholesterol content of newborn *D. magna* was lower than that of adults (with eggs) and did not differ between acclimation treatments (*Figure 47*). Other studies found the cholesterol content of eggs to be equal or higher than in somatic tissues of *D. magna* (Wacker and Martin-Creuzburg, 2007; Sperfeld and Wacker, 2009). Wacker and Martin-Creuzburg (2007) report that cholesterol content of eggs is more homeostatic (with respect to changes in food quality) than that of somatic tissues. On the other hand, the cholesterol content of mature *Daphnia* was affected by maternal diet. *D. magna* with *Chlamydomonas* maternal diet had higher cholesterol content than those with *Cryptomonas* maternal diet (*Figure 43*). Since the cholesterol content of newborns did not differ between treatments, some aspect of maternal acclimation to algal diet must have been transmitted to the offspring, causing different rates of cholesterol accumulation as well as different rates of FA accumulation in animals with different maternal diets. Maternal diet influences gene expression in the intestine of offspring in chicken (Rebel *et al.*, 2006) and affects the cholesterol metabolism in adult offspring of rats through the effect on gene transcription in the hypothalamus (Orozco-Solís *et al.*, 2010).

Maternally induced differences in enzyme expression in offspring may also explain our results. The sterol content of *Cryptomonas* and *Chlamydomonas* differs; *Chlamydomonas* contains mainly ergosterol and 7-dehidrosterol while *Cryptomonas* contains mainly 24-methylcholesta-5,22-dienol (Patterson, 1991). *Daphnia* have the ability to convert all these sterol species to cholesterol (Martin-Creuzburg and von Elert, 2004). However, different enzymes are required. Furthermore, *Chlamydomonas* has a lesser sterol content than *Cryptomonas* (*Table 27*), and therefore *Chlamydomonas* reared animals might have more efficient mechanisms for assimilation and conversion of algal sterols to cholesterol (e.g. a higher concentration of the corresponding enzymes).

Maternal diet also affected the FA profile of *D. magna* and *D. pulex*. Significant effects of maternal diet on the PC2 score of the factor analysis of the relative FA composition were found in both species, however, the effect was much clearer in *D. magna*. When *Chlamydomonas* was the experimental food, the EPA content and the share of EPA in the total FA content was higher

in animals with *Cryptomonas* as maternal diet than in animals with *Chlamydomonas* as maternal diet. The difference in the EPA content per individual of *Chlamydomonas* fed *D. magna* with different maternal diets (~165 ng) roughly corresponded to the difference in EPA content per individual between newborns from different maternal diets (~136 ng). Therefore the differences in EPA content due to maternal diet can be ascribed to the differences in the EPA content of egg lipid reserves in different maternal treatments. This result is in line with Wacker and Martin-Creuzburg (2007), who found a strong effect of food quality on the maternal investment of EPA into parthenogenetic eggs of *D. magna*. There was no evidence in our study that EPA-poor maternal diet induces a higher activity of enzymes involved in the conversion of ALA to EPA. When *Cryptomonas* was the experimental diet, differences in the FA content due to the maternal diet were less pronounced. This trend also translated into the effects on fitness of *D. magna* and *D. pulex*. The negative effect of *Chlamydomonas* maternal diet on growth rate, clutch size and reproductive performance was less pronounced when *Cryptomonas* was the experimental diet, confirming a relationship between FA composition and fitness.

5.3.3 Temperature effects on the lipid composition

Proper membrane fluidity is critical for its function (Hulbert, 2003) and ectotherms can adjust the lipid composition of their membranes in order to counteract the effects of temperature on membrane fluidity (Chapelle, 1978; Hazel and Williams, 1990; Pruitt, 1990). We therefore expected a shift in fatty acid composition towards unsaturated fatty acids in response to cold acclimation and experimental temperature and an increase in cholesterol content in response to warm acclimation and experimental temperature. FA and cholesterol content of *Daphnia* in our study were influenced by temperature conditions, but not in the way we predicted. PUFA content, EPA content and UI were actually somewhat higher in 20 °C acclimated animals and animals growing at 20 °C, while SAFA and cholesterol content were higher in animals acclimated at 15 °C and animals growing at 15 °C. The temperature induced differences were small, especially when compared to those induced by experimental food quality.

Most laboratory acclimation studies on various species of crustaceans reported results consistent with the homeoviscous adaptation. Low temperature increased the degree of membrane unsaturation in crayfish (Cossins, 1976; Farkas and Nevenzel, 1981; Pruitt, 1988), shore crab (Chapelle, 1978), barnacles (Cook and Gabbott, 1972), amphipods (Dawson *et al.*, 1984), and copepods (Farkas *et al.*, 1984). Many crustacean species also accumulate higher amounts of log chain n3 PUFAs (EPA or DHA) at low temperatures (Chapelle, 1978; Farkas et al, 1984; Schlechtiem *et al.*, 2006). Experimental effects of acclimation temperature on the cholesterol content are more variable (Crockett, 1998). Copepod species from warmer habitats

have higher cholesterol content, but acclimation temperature affected the cholesterol content in only one of five tested copepod species (Hasset and Crockett, 2009). Cholesterol content increased with temperature and dietary cholesterol content in *D. magna* (Sperfeld and Wacker, 2009). However, the temperature effect was clear only at diet cholesterol concentrations exceeding those in our study.

We analysed the whole body lipid content of *Daphnia*. Therefore both the FAs from triacylglycerols (TAG), which function as the main energy reserves in freshwater crustaceans, and the FAs from phospholipids (PL), which are the predominant membrane lipids, were included in the analysis. The effect of environmental temperature on the saturation of FAs of different lipid classes may differ. Chapelle (1978) found a significant enhancement of degree of unsaturation of PLs of *Carcinus maenas* at lower temperatures, whereas less pronounced changes occurred in TAGs. Pruitt (1988) found a similar trend in crayfish. TAGs represent >50 % of total lipids in well fed laboratory raised cladocerans (Macedo and Pinto-Coelho, 2001) and in field collected *Daphnia hyalina* from Lake Bohinj, TAGs represented >60 % of total lipids while PLs represented only 5 % (Mezek, 2009). Thus in the analysis of total lipid FA composition the lack of temperature response in TAGs may obscure the changes in the saturation of the membrane PLs. Nevertheless, in *Carcinus maenas* the total lipid FA saturation showed a similar trend with temperature as the PL FA saturation (Chapelle, 1978). Furthermore, the FA composition of total lipids and PL FA composition of *D. magna* showed similar (lack of) responses to temperature (Farkas and Herodek, 1964; Farkas *et al.*, 1984). Also, the only study to date that has shown a response of *Daphnia* EPA content to temperature was conducted on whole body lipids, the same as our study (Schlechtriem *et al.*, 2006). Cholesterol does not act as an energy reserve in cladocerans and is mostly found in the membranes. Furthermore similar trends of cholesterol content were obtained for crude homogenates and plasma membranes in copepods (Crockett and Hassett, 2005). These results indicate that the analysis of whole body FA composition and sterol content would detect temperature related changes in FA saturation, had any occurred.

Most studies on crustaceans that detected a homeoviscous response of membrane lipids involved a temperature difference larger than that in our study. It is therefore possible, that no membrane adjustment was necessary in our temperature range (15-20 °C), but would be detected at more extreme temperature conditions. However, in a temperature range broader than that of our study (11 °C and 22 °C), Schlechtriem *et al.* (2006) actually observed a decrease in PUFA content in cold acclimated *D. pulex*. The UI of PLs did not differ between *D. magna* acclimated at 20 °C and 10 °C (Farkas *et al.*, 1984). It seems that bulk changes in the UI of membrane lipids are not an important mechanism of adjustment of membrane fluidity in *Daphnia*.

Only one study thus far examined cholesterol content of *Daphnia* in response to temperature (Sperfeld and Wacker, 2009). They found that cholesterol content increased with temperature (15-25 °C) and dietary cholesterol content in *D. magna*. However, the temperature effect was clear only at dietary cholesterol concentrations exceeding those in our study (and probably in nature, see Patterson, 1991). At sterol levels similar to those in our study, *Daphnia* cholesterol content did not differ between 15 °C and 20 °C, but it was higher at 25 °C, indicating that cholesterol content may be important for adaptation to high temperatures in *Daphnia*.

Furthermore, eurythermal animals, particularly those which undergo temperature change on a regular basis (e.g. diurnal fluctuations) may benefit from having membranes with higher cholesterol levels, because membranes enriched with cholesterol suffer less perturbation of their physical properties from a modest change in temperatures than membranes with lower cholesterol content (Crockett, 1998). Comparison of cholesterol levels in a stenothermal and a eurythermal crab species supports this prediction (Cuculescu *et al.*, 1995). Animals that underwent temperature change in our study did not have increased cholesterol content. However, a single temperature shift may not be enough to induce higher cholesterol content. *D. hyalina* collected from Lake Bohinj had higher cholesterol content than our laboratory clones (Mezek, 2009), especially in the summer (>6 µg Cho mg^{-1} d.m). Lake Bohinj is stratified in the summer and the temperatures in the water column range from 4 °C to 20 °C. *D. hyalina*, performing diel vertical migration, is thus exposed to great diurnal temperature fluctuations, whereas *D. magna* and *D. pulex* in our experiments were acclimated for generations to constant temperature conditions. However, as we have shown, the difference in cholesterol content may also be due to differences in dietary sterol content. Nevertheless, it seems unlikely that dietary supply of usable sterols would be higher in oligotrophic Lake Bohinj than with our two algal species supplied *ad libitum*. This indicates an enhanced accumulation of cholesterol in summer *D. hyalina*, possibly as a mechanism of acclimation to temperature fluctuation.

High dietary supply of EPA and high EPA content of lipid membranes is assumed to be relatively more important for zooplankton fitness at cooler temperatures (Masclaux *et al.*, 2009). Therefore a higher EPA content was expected at 15 °C than at 20 °C due to (1) higher accumulation of EPA from the diet in *Cryptomonas* fed animals or (2) higher rates of conversion of ALA into EPA in *Chlamydomonas* fed animals. This was not the case; at 15 °C (acclimation and/or experiment) animals tended to have lower EPA content. Short term (2-3 days) exposure to low temperatures (4-10 °C) did not cause appreciable changes in EPA levels of *D. pulex* and *D. magna* (Farkas, 1979; Farkas *et al.*, 1984). However, Schlechtriem *et al.* (2006) observed an increase of relative EPA content of *D. pulex* (on an EPA-free diet) after 1 month of cold acclimation from 3 (at 22 °C) to 13 % (at 11 °C) of total FA, along with a decrease of ALA – the precursor for its synthesis. Schechtriem *et al.* (2006) suggested that longer acclimation times may be necessary for enzymes involved in EPA synthesis to reach full activity. However,

comparison of groups acclimated for three generations at the experimental conditions showed no increase in EPA content at 15 °C in our study. Furthermore, cold acclimated animals did not invest their offspring with higher EPA levels. Only the ALA/EPA ratio tended to be lower at 15 °C, a possible indication of conversion of ALA to EPA at the lower temperature. These results indicate that an increase in EPA content is not required at 15 °C. However, the *D. pulex* used by Schechtriem *et al.* (2006) had a relatively high EPA content even at 22 °C compared to our *D. pulex* at 20 °C. This could indicate a lower capacity for synthesis of EPA in our clone. Von Elert (2002) has suggested that clones and taxa may have different capacities for conversion of C18 PUFAs to EPA. The observed differences in sensitivity of *Daphnia* clones and taxa to absence of EPA from their diet support this idea (von Elert, 2004; Brzeziński and von Elert, 2007). There was also no increase in EPA content in *Cryptomonas* fed animals at 15°C in our study, indicating that EPA accumulation was no higher at 15 °C than at 20 °C. Similar pattern emerged from FA analysis of field populations – *Daphnia* originating from cooler habitats did not have higher EPA content than those from warmer habitats. The relevance of the capacity to accumulate EPA for low temperature tolerance thus remains questionable.

The lack of homeoviscous changes in FA UI and cholesterol content in our study does not necessarily mean that membrane fluidity was not adjusted by some other means. For example, Cuculescu *et al.* (1995) observed changes in PUFA and cholesterol content that were opposite to homeoviscous theory in their spring experiment, but the membranes of cold acclimated crabs (5 °C and 8 °C) were nevertheless more fluid than those of warm acclimated crabs (22 °C) when compared at the same temperature. Membrane fluidity can be adjusted with a change in the proportion of different PL head groups. The content of PE increases at the expense of PC at low temperatures in various crustaceans, including *D. magna* (Farkas *et al.*, 1984; Pruitt, 1990). Direct measurements of membrane fluidity might therefore yield a clearer picture of *Daphnia* temperature adaptation at the membrane level. We therefore cannot completely exclude the possibility, that some aspect of membrane adjustment plays an important role in the determination of thermal sensitivity of *Daphnia* species, however it seems unlikely to be the major cause of different thermal preferences.

Acclimation temperature did not affect the FA or cholesterol content of newborns, or their UI, but nevertheless had an effect on the FA and cholesterol content and UI of mature animals. For the most part cholesterol content was lower in 20 °C acclimated animals, whereas total FA content and UI were higher in 20 °C acclimated animals. We can draw a similar conclusion as for the effects of maternal diet; acclimation temperature affected the lipid metabolism of the offspring.

The FA content was higher at 20 °C experimental temperature than at 15 °C in our study. This may be related to a well known phenomenon that the C:P ratio is lower in cold acclimated organisms. Cold-adapted ectotherms show higher cell-specific levels of P and rRNA than

individuals of the same species living under higher temperatures (Woods *et al.*, 2003). This has been interpreted as a compensatory response to a reduced efficiency of protein synthesis at low temperatures, i.e. more ribosomes are needed to maintain a given protein synthesis rate at low temperatures.

5.3.4 The effect of lipid composition on fitness under different temperature regimes

We examined the relationship between *Daphnia* lipid content and their fitness to answer two main questions: (1) Is the lipid content of *Daphnia* related to their fitness? and (2) Does the relationship between *Daphnia* lipid content and their fitness depend on the temperature conditions? Since experimental food quality had a strong effect both on fitness parameters and on the lipid content of *Daphnia*, a correlation between lipid content and fitness is not surprising. Indeed, in such circumstances, fitness would correlate with lipid content of *Daphnia* even if some other property of the food was actually responsible for the fitness effect. This is not likely the case in our study. The difference between the diets was caused by food quality, since ingestion rates are actually higher for *Chlamydomonas* (probably due to its smaller size) and therefore if the difference was solely in ingestion rates, *Chlamydomonas* fed animals would have performed better (De Lange and Lürling, 2003). Small differences in digestibility may exist, but both algae are among the easily digestible phytoplankton species (Ahlgren *et al.*, 1990). Obviously none of the diets is toxic since both supported excellent *Daphnia* growth both in our study and in previous studies (e.g. Ahlgren *et al.*, 1990). Both phytoplankton cultures were supplemented with vitamins and grown under non-limiting nutrient conditions, therefore it is unlikely that mineral ratios or vitamin content contributed to food quality. The threshold C:P ratio for elemental limitation of *Daphnia* growth is around 200 (Sterner and Hessen, 1994) and the C:P ratio of non-limited *Chlamydomonas* is below that (C:P=65, Weers and Gulati, 1997b). There is, however, a major difference in FA and sterol content of the two algal species (*Table 26*). Since EPA and sterols have been shown to affect *Daphnia* fitness (von Elert, 2002; von Elert *et al.*, 2003), we assume that these were, in fact, the main determinants of food quality.

The connection between UI, temperature and membrane fluidity lead to the assumption that animals with higher UI would perform better at 15 °C, while those with lower UI would perform better at 20 °C. However, no such pattern was found in our results. Animals with strikingly different UI often had similar fitness at both 15 °C and 20 °C.

Different levels of *Daphnia* cholesterol content did not affect the growth rate or reproductive performance regardless of temperature combination. Other authors found a correlation between *Daphnia* growth rate and dietary cholesterol content, but only up to a certain threshold value of dietary sterol content (von Elert *et al.*, 2003; Sperfeld and Wacker, 2009). The

slope of the sterol limited part increased with temperature as did the threshold value of dietary cholesterol content, indicating increased requirement for cholesterol at higher temperatures (Sperfeld and Wacker, 2009). *Daphnia* cholesterol content increases linearly with the log of dietary cholesterol content across a broad range of dietary cholesterol content (Sperfeld and Wacker, 2009). A threshold level for *Daphnia* cholesterol content is thought to be similar to that of the diet (Sperfeld and Wacker, 2009). The literature reports of the threshold level of dietary cholesterol content differ significantly; they range from 2 µg mg^{-1} POC for *D. galeata* (von Elert *et al.*, 2003) and 5.4 µg mg^{-1} POC for both *D. galeata* and *D. magna* (Martin-Creuzburg *et al.*, 2005), to 7-9 µg mg^{-1} POC for *D. magna* (Sperfeld and Wacker, 2009). The clones of *D. galeata* and *D. magna* were the same in all three studies (the same clone of *D. magna* was also used in our study), thus the differences probably arise from different methods used to produce the sterol gradient (causing e.g. different availability of sterols, different quality of food in terms of other nutrients). The estimate of Martin-Creuzburg *et al.* (2005) is probably the most relevant to our conditions (as well as natural conditions), since they produced a gradient of phytosterols with mixtures of intact algal and cyanobacterial cells. Judging from the reported sterol content of *Cryptomonas* and *Chlamydomonas* (Piepho *et al.*, 2010), the *Chlamydomonas* fed animals might have been sterol limited. However, *Chlamydomonas* phytosterols, ergosterol and 7-dehidrosterol, support higher growth rate and clutch size of *Daphnia* than cholesterol (Martin-Creuzburg and von Elert, 2004), so the incipient limiting level for these phytosterols may be lower. Our results support the lower estimates of the incipient limiting level of cholesterol content. The sterol levels in both our diets were probably above this value, therefore having little effect on fitness.

Müller-Navarra (1995) found EPA as a single FA, and not n3 FA as a family, was most strongly related to *Daphnia* growth. We also found a strong relationship between EPA content and the juvenile growth rate and reproductive performance of *Daphnia*. *Daphnia* performance increased linearly with a ln of EPA content. There was indication of a saturation threshold; the EPA content did not affect fitness in *Cryptomonas* fed animals, where it was probably above saturation point, whereas the EPA content of *Chlamydomonas* fed animals was below the threshold and therefore strongly affected fitness. The slopes of fitness with EPA did not differ significantly between temperature treatments, indicating that EPA requirements do not differ in our temperature range. Although Masclaux *et al.* (2009) found higher requirements of dietary EPA in colder temperatures (12-15 °C) as compared with higher temperatures (25 °C), the effect of dietary EPA content on the growth rate of *D. magna* did not differ between 15 °C and 20 °C (Masclaux *et al.*, 2009).

Size of first clutch was correlated with both EPA content and cholesterol content, but even the complex interaction model with both lipids was not as good as the model with temperature and food algae. This indicates that the clutch size was affected by the algal diet through more

than just its effect on the lipid content of mature *Daphnia*. EPA and cholesterol function not only as structural components but also as precursors of biologically active molecules (Goad, 1981; Blomquist *et al.*, 1991), therefore the dietary content of these lipids may affect fitness without affecting the lipid composition of *Daphnia*. For example, cholesterol-enriched diet enhanced egg production in a copepod *Acartia hudsonica* without altering cholesterol content (Crockett and Hassett, 2005). Furthermore, the effect of maternal diet on the early performance of the offspring may be much more pronounced than its effect on the adult lipid content. As we have shown, the metabolism and the rate of accumulation of different nutrients may be affected by maternal diet. Thus the rate of accumulation of the nutrients whose content we did not measure may have changed as well, contributing to this interaction.

5.3.5 Species differences in lipid content and temperature sensitivity

D. pulex and *D. magna* differed in their FA composition. Since their diet and environmental conditions were the same, this difference indicates differences in the FA metabolism of these two species. Von Elert (2002) has suggested that clones and taxa may have different capacities for conversion of C18 PUFAs to EPA. The observed differences in sensitivity of *Daphnia* clones and taxa to absence of EPA from their diet support this idea (von Elert, 2004; Brzeziński and von Elert, 2007). However, the effect of experimental diet on the FA profile was much more pronounced than that of species, indicating that food quality is more important for the FA content than metabolic differences. Nevertheless, the differences in metabolic capacity may have an important effect on fitness when food quality in the environment is very low (von Elert, 2004; Brzeziński and von Elert, 2007), for example during cyanobacterial blooms. Even under such extreme conditions the amount and quality of the previously accumulated lipid reserves may be more important than differences in FA metabolism. ARA and EPA are highly conserved during starvation in *D. pulex* (Schechtriem *et al.*, 2006). When switched to a cyanobacterium diet (no n3 PUFAs, low overall PUFA content) after a week of feeding on good quality food (high EPA content), *D. magna* continued to invest high amounts of EPA and other n3 PUFAs into eggs for the next three clutches (Wacker and Martin-Creuzburg, 2007).

The analyses of the FA composition of field collected *Daphnia hyalina*, *D. pulicaria*, *D. pulex* and *D. rosea* supported the notion that species specific differences in FA metabolism of *Daphnia* may not be as important for fitness as food quality. Within species differences in the FA composition due to different habitats or different seasons exceeded the between species differences. Other authors also found that intraspecific differences in FA profiles can exceed

interspecific differences in FA composition even when comparing different species from a single lake (Sekino *et al.*, 1997).

The dominant phytoplankton groups available as food to herbivorous zooplankton in freshwater ecosystems differ greatly in their FA composition (Ahlgren *et al.*, 1992). Since the phytoplankton composition varies both temporally (seasonal succession) and spatially (both between and within lakes: patches, vertical distribution), seston lipid composition also varies (Wacker and von Elert, 2001; Ravet *et al.*, 2010). Furthermore, temperature, nutrient limitation, light intensity, photoperiod and growth phase also affect the lipid content and FA composition of phytoplankton (Guschina and Harwood, 2009). Exposure to lower environmental temperatures generally causes algae to increase their relative amount of fatty acid unsaturation (Guschina and Harwood, 2009). Furthermore, differences in natural seston EPA content are much less pronounced than differences between uni-algal diets and as discussed *Daphnia* generally have higher and more homeostatic EPA content than the seston they feed on (Bret *et al.*, 2009). A combination of these facts and relatively uniform slopes of fitness against EPA content at different temperatures leads us to believe that interspecific differences in FA metabolism and membrane adjustment are probably not an important factor in determination of temperature sensitivity. For example, *D. hyalina*, the only actively overwintering species in our study, had lower EPA content and UI than pond species. However, we cannot exclude the possibility, that direct measurements of membrane fluidity would detect an adaptation on the membrane level by some mechanism other than FA unsaturation or cholesterol content. From our results it seems that the ability to adjust membrane lipid composition depends less on the species and more on the food quality in its habitat. This implies that changes in food quality (such as through increased eutrophication) may affect temperature sensitivity of *Daphnia*.

6 CONCLUSIONS

Temperature has a strong effect on the metabolic rate and fitness of *Daphnia*. Temperature optimum for ETS activity was above 25°C in *D. pulex*, close to 25 °C in *D. rosea* and *D. hyalina* and lowest in *D. pulicaria*. The optimum temperature was not affected much by seasonal changes. The temperature of maximum ETS/R ratio, indicating maximum metabolic scope was around 20 °C for most groups, but was lower in cold acclimatized (*D. hyalina*) and cold adapted (*D. pulicaria*) populations. Optimum temperatures for growth were similar in all tested species; 30 °C was outside their temperature tolerance range. Optimum temperature for reproductive performance was lower in *D. hyalina* than in *D. pulex* and *D. rosea*. *D. pulicaria* makes an early investment into resting eggs, a possible advantage in the short growth seasons of high alpine lakes.

We have detected an effect of body size on metabolism of *Daphnia* species, which were in line with results for many other species, including unicellular organisms and plants. Larger species also tended to have higher clutch sizes and higher growth rates. Even after metabolic rate was corrected for mass differences, there were still significant differences between animals of the same species, collected in different seasons, as well as between different species, collected at similar water temperatures.

There was an effect of habitat; a difference between lake and pond species in body mass, haemoglobin content, ETS/R ratio and thermal sensitivity of metabolism. Most of these differences were only indirectly linked with habitat temperature; larger body sizes of pond species are thought to be a result of size selective predation (absence of fish), higher haemoglobin content of pond species is an adaptation to more frequent hypoxic conditions, and the higher ETS/R ratio of lake species reflects higher food limitation and therefore stronger selection for energy conservation in their habitats. Productivity and oxygen concentration are affected by temperature. Thermal sensitivity of metabolism is higher in species from warm habitats, enabling an increase in activity at high temperatures that is absent in cold-water species, reflected also in lower optimum temperatures for growth.

Seasonal differences in thermal sensitivity were observed. Actively overwintering *D. hyalina* acclimatized successfully to winter water temperature (4 °C), as indicated by higher ETS activity at low temperatures, increase in respiration rate at low temperatures, a shift in the thermal optimum of ETS/R and better growth at lower temperatures than summer *D. hyalina*. On the other hand, development at extremely high or low temperatures (4 °C and 28 °C) caused slower growth of offspring in *D. rosea* and *D. pulex*. The metabolism of these two species acclimatized to these temperature extremes showed evidence of reduced basal metabolism and activity. *Daphnia* collected at cold water temperatures were bigger than those collected at warm temperatures, with bigger offspring, who grew to be bigger at maturity and have more eggs.

Cryptomonas sp. was better quality food for *Daphnia* than *Chlamydomonas* sp., supporting higher growth rates, higher clutch size and higher potential rate of population increase, connected with the higher EPA content of *Cryptomonas* sp. and *Cryptomonas* fed animals. The effect of experimental food quality on *Daphnia* fitness depends both on maternal food quality and on the temperature conditions. Both species showed similar responses, but the smaller and faster growing *D. pulex* was more sensitive to food quality than *D. magna*.

The positive effect of food quality on growth rate and clutch size was stronger if the maternal diet was of low quality. Good quality maternal diet generally increased growth rate and reproduction, especially if the experimental diet was of low quality.

Acclimation temperature – the temperature of egg development – affected the subsequent fitness of offspring. The maximum fitness was attained by animals acclimated at 15 °C and growing at 20 °C, combining the faster growth rate at warm experimental temperature and bigger clutch size of cold acclimated animals. Constant temperature throughout development and growth was not an advantage. Acclimation temperature affected the allocation of energy, animals acclimated at 15 °C invested more into reproduction for the same growth rate, than animals acclimated at 20 °C, indicating that the rate of somatic growth is not the best measure of fitness, when comparing animals at different temperatures. Clutch size should be included in the comparison.

Intergenerational temperature regime had a strong impact on the effects of maternal and experimental food quality. Good maternal food quality was more important for fitness under less favourable temperature conditions. Low experimental food quality was more detrimental to fitness in animals experiencing temperature change between acclimation and experiment and less so under constant temperature conditions.

Our results suggest a strong interaction between intergenerational changes in temperature and dietary requirements of organisms. Extrapolation from studies conducted under constant temperature conditions may seriously underestimate dietary constraints in natural environments.

Fatty acid composition and cholesterol content of *D. magna* and *D. pulex* were mostly a reflection of experimental diet. Other factors had a lesser effect. Acclimation and experimental temperature affected the lipid composition of *Daphnia*, but the changes were contrary to homeoviscous adaptation. Apparently no membrane adjustment is required in the optimum range 15 °C – 20 °C.

There was no increase of synthesis or accumulation of EPA at 15 °C compared to 20 °C. Also, there was little evidence of active EPA synthesis in *Chlamydomonas* fed animals. Whatever EPA was synthesised was invested into offspring and this investment was independent of temperature.

Maternal diet and acclimation temperature affected the metabolism of offspring, changing the rate of accumulation of lipids.

Intra-specific differences in fatty acid composition due to differences in food quality in different habitats and different seasons exceeded the inter-specific differences. *Daphnia* species adapted to lower temperatures (*D. pulicaria*) or spending the winter in active state (*D. hyalina*) did not have higher levels of fatty acid unsaturation or EPA content than warm water pond species (*D. pulex*, *D. rosea*). Changes in fatty acid composition do not seem to be an important mechanism of temperature adaptation and acclimation of *Daphnia*.

Acclimation of metabolism is important for thermal tolerance. Sustained performance at lower temperatures was related to the ability to compensate the temperature driven decrease in metabolic rate. Performance at higher temperatures was related to haemoglobin content and the ability to increase respiration rates at higher temperatures.

Despite the lower thermal optima of cold water species *D. pulicaria* and *D. hyalina*, we estimate that the pond species *D. rosea* and *D. pulex* are more directly threatened by climate change. Temperatures in their habitat are already stressfully high in the summer, and may increasingly often reach temperatures outside their thermal tolerance (30 °C or more), with strong consequences for the phenology of these two species.

On the other hand warmer temperatures and longer seasons may increase the productivity in the lakes that are the home of *D. pulicaria* and *D. hyalina*. Consequently, such lakes could become voulnerable to invasion by cladocerans better capable of increased metabolic activity and increased growth at higher temperatures and they could outcompete *D. pulicaria* and *D. hyalina* under such changed circumstances. A change in the species composition of *Daphnia* in alpine lakes or a change in phenology in lowland ponds can therefore serve as signals of climate change in freshwater ecosystems.

Most working hypotheses have been confirmed except for hypothesis (2), which was confirmed only partially:

(1) *Daphnia pulex*, *D. pulicaria*, *D. rosea*, and *D. hyalina* did have different thermal sensitivity, which depended on their evolutionary adaptations (biochemical – e.g., haemoglobin content, physiological – e.g., respiration rate) to the prevailing environmental conditions in their habitats.

(2) Only in *D. hyalina* seasonal acclimatization resulted in cold-acclimatized individuals that outperformed warm-acclimatized individuals at low temperatures, and the opposite at high temperatures. In the other two species tested acclimatization to extreme temperatures impaired fitness. Laboratory acclimation gave results contrary to our hypothesis; the same acclimation temperature appeared optimum, regardless of subsequent test temperatures. Acclimatization/acclimation was achived mostly through adjustments on the enzyme level and

adjustments in the metabolic rate, whereas adjustments of membrane fluidity were not as important.

(3) Species differed in their ability to acclimate/acclimatize to different temperatures. The differences were most pronounced at high temperatures. The capacity for acclimatization to certain environmental conditions was reflected also in growth, reproduction and survival.

(4) Food quality interacted with temperature conditions to determine the fitness of *Daphnia*.

(5) Maternal conditions in terms of food quality and temperature interacted with the offspring environment to affect offspring fitness.

7 SUMMARY

We estimated the thermal sensitivity of four *Daphnia* species, representing pairs of closely related species; the relatively large bodied *D. pulex* and *D. pulicaria* and smaller *D. rosea* and *D. hyalina*. Species from each pair have different habitat preferences; *D. pulicaria* and *D. hyalina* originate from cool water oligotrophic lakes: Zgornje Kriško Jezero and Lake Bohinj, respectively. *D. pulex* and *D. rosea* originate from a lowland pond Hraški Bajer. We expected different thermal sensitivity as a consequence of adaptation to different thermal regimes in these habitats.

We measured the ETS activity (respiratory potential), respiration rate (R), the ETS/R ratio, juvenile growth rate, clutch size and reproductive performance at different temperatures in field collected *Daphnia* at two different times during the season. In addition, we measured the haemoglobin content in summer populations and the effect of growth temperature on haemoglobin content under normoxic laboratory conditions.

We found a strong effect of experimental temperature and body size on the ETS activity and respiration rate (R) of the four *Daphnia* species, which can be described well (r^2>0.9) with common temperature (T in K) and body mass (M in µg d.m.) equations:

$\ln(ETS) = 13.917 + 0.656M – (58.738/k)T^{-1}$

$\ln(R) = 3.818 + 0.780M – (36.742/k)T^{-1}$

R increased with temperature in the measured range, but the thermal optimum for ETS activity was exceeded in some species. The thermal optimum for ETS was highest in *D. pulex*, close to 25 °C in *D. rosea* and *D. hyalina* and lowest in *D. pulicaria*. It was not affected much by seasonal changes. The temperature of maximum ETS/R ratio, indicating maximum metabolic scope, was around 20 °C for most groups, but was lower in cold acclimatized (winter *D. hyalina*) and cold adapted (*D. pulicaria*) *Daphnia*. Optimum temperatures for growth were similar in all tested species; at or above 25 °C. Clutch sizes were higher at lower temperatures. 30 °C was outside the temperature tolerance range of the tested *Daphnia*. Optimum temperature for reproductive performance was lower in *D. hyalina* than in *D. pulex* and *D. rosea*. *D. pulicaria* makes an early investment into resting eggs, a possible advantage in the short growth seasons of high alpine lakes.

Significant differences remained between seasons and between species even after the metabolic activity was corrected for body mass. Respiration rate was higher in pond species, especially at high experimental temperatures. This was connected with the high haemoglobin content of pond species, compared with lake species. The ETS/R ratio was higher in species from oligotrophic lakes, probably as an energy saving adaptation to low food conditions in these habitats.

Seasonal differences in thermal sensitivity were observed. Actively overwintering *D. hyalina* acclimatized successfully to winter water temperature (4 °C), as indicated by higher ETS activity at low temperatures, increase in respiration rate at low temperatures, a shift in the thermal optimum of ETS/R and better growth at lower temperatures than summer *D. hyalina*. On the other hand, development at extremely high or low temperatures (4 °C and 28 °C) caused slower growth of offspring in *D. rosea* and *D. pulex*. The metabolism of these two species acclimatized to these temperature extremes showed evidence of reduced basal metabolism and activity. *Daphnia* collected at cold water temperatures were bigger than those collected at warm temperatures, with bigger offspring, who grew to be bigger at maturity and have more eggs.

We also investigated the interaction of intergenerational temperature and food quality change on the fitness and lipid content of two species of *Daphnia*. The effect of a change in temperature (15 °C vs. 20 °C) and food quality (*Cryptomonas* sp. as high quality food, containing a high level of an n3 fatty acid EPA, important for *Daphnia* growth *vs*. *Chlamydomonas* sp. as relatively low quality food, containing no EPA) on juvenile growth rate, clutch size, reproductive performance, fatty acid and cholesterol content of a clone of *Daphnia magna* and *D. pulex* was measured in 16 combinations of maternal and experimental environments in standardized growth experiments. Both species showed similar responses, but *D. pulex* was more sensitive to food quality.

The positive effect of food quality on growth rate and clutch size was stronger if the maternal diet was of low quality. Good quality maternal diet generally increased growth rate and reproduction, especially if the experimental diet was of low quality. The maximum fitness was attained by animals acclimated at 15 °C and growing at 20 °C, combining the faster growth rate at warm experimental temperature and bigger clutch size of cold acclimated animals. Intergenerational temperature regime had a strong impact on the effects of maternal and experimental food quality. Good maternal food quality was more important for fitness under less favourable temperature conditions (20°C→15°C). Low experimental food quality was more detrimental to fitness in animals experiencing temperature change between acclimation and experiment and less so under constant temperature conditions. These results suggest that intergenerational changes in temperature strongly affect the dietary requirements of organisms. Extrapolation from studies conducted under constant temperature conditions may seriously underestimate dietary constraints in natural environments.

The lipid content of *Daphnia* was affected most strongly by experimental diet; other factors had only subtle effects. At 20 °C *Daphnia* had a higher UI, more EPA and less cholesterol than at 15 °C, contrary to expectation of homeoviscous theory. Apparently membrane fluidity did not need to be adjusted in this temperature range, or was adjusted by some other mechanism. Maternal diet and acclimation temperature affected the metabolism of offspring, changing the rate of accumulation of lipids. The lipid content of newborns differed from that of adults. They

had less cholesterol and, in case of *Chlamydomonas* fed animals, more EPA. Apparently whatever EPA was produced was invested into offspring.

We found no evidence of active EPA synthesis in either species at either temperature. There was no evidence for higher rates of EPA accumulation at cooler temperature in *Cryptomonas* fed animals. Intra-specific differences in fatty acid composition due to differences in food quality in different habitats and different seasons exceeded the inter-specific differences in field collected *Daphnia*. *Daphnia* species adapted to lower temperatures (*D. pulicaria*) or spending the winter in active state (*D. hyalina*) did not have higher levels of fatty acid unsaturation or EPA content than warm water pond species (*D. pulex*, *D. rosea*). Changes in fatty acid composition do not seem to be an important mechanism of temperature adaptation and acclimation in *Daphnia*.

Acclimation of metabolism is important for thermal tolerance. Sustained performance at lower temperatures was related to the ability to compensate the temperature driven decrease in metabolic rate. Performance at higher temperatures was related to haemoglobin content and the ability to sustain high respiration rates at higher temperatures.

Despite the lower thermal optima of cold water species *D. pulicaria* and *D. hyalina*, we estimate that the pond species *D. rosea* and *D. pulex* are more directly threatened by climate change. Temperatures in their habitat are already stressfully high in the summer, and may increasingly often reach temperatures outside their thermal tolerance (30 °C or more), inducing summer production of resting eggs, with strong consequences for the phenology of these two species.

On the other hand, warmer temperatures and longer seasons may increase the productivity in the lakes, which are the home of *D. pulicaria* and *D. hyalina*, opening them to competitors better capable of increased metabolic activity and increased growth at higher temperatures. A change in the species composition of *Daphnia* in alpine lakes or a change in phenology in lowland ponds can therefore serve as signals of climate change in freshwater ecosystems.

8 POVZETEK

Ocenili smo temperaturno občutljivost štirih vrst vodnih bolh iz rodu *Daphnia*, ki predstavljajo dva para ozko sorodnih vrst; relativno veliki *D. pulex* in *D. pulicaria* ter manjši *D. rosea* in *D. hyalina*. Vrsti iz vsakega para naseljujeta različne habitate. *D. pulicaria* in *D. hyalina* izvirata iz hladnih oligotrofnih jezer; Zgornjega Kriškega jezera in Bohinjskega jezera. *D. pulex* in *D. rosea* pa izvirata iz nižinske mlake Hraški bajer. Pričakovali smo različno temperaturno občutljivost kot posledico prilagoditev na različne temperaturne režime v teh habitatih.

Merili smo aktivnost ETS (dihalni potencial), hitrost dihanja (R), razmerje ETS/R, hitrost juvenilne rasti (hitrost rasti od izkotitve do spolne zrelosti), število jajc in reproduktivni uspeh pri različnih temperaturah pri dafnijah nabranih na terenu v dveh časih med sezono. Poleg tega smo merili tudi vsebnost hemoglobina pri poletnih populacijah in vpliv rastne temperature na vsebnost hemoglobina pri normoksičnih laboratorijskih pogojih.

Odkrili smo močan vpliv inkubacijske temperature in telesne mase na aktivnost ETS in hitrost dihanja pri vseh štirih vrstah dafnij, ki ga lahko dobro opišemo (r^2>0.9) s kombinirano temperaturno (T v K) in masno (M v µg d.m.) enačbo:

$\ln(ETS) = 13.917 + 0.656M – (58.738/k)T^{-1}$

$\ln(R) = 3.818 + 0.780M – (36.742/k)T^{-1}$

R je naraščala s temperaturo v območju meritve, temperaturni optimum za aktivnost ETS pa je bil pri nekaterih vrstah presežen. Temperaturni optimum za ETS je bil najvišji pri *D. pulex* (nad 25 °C), blizu 25 °C pri *D. rosea* in *D. hyalina* in najnižji pri *D. pulicaria*. Nanj ni veliko vplivala sezonska aklimatizacija. Temperatura najvišjega razmerja ETS/R, ki nakazuje maksimalni metabolni razpon, je bila okoli 20 °C pri večini eksperimentalnih skupin, a nižja pri hladno aklimatiziranih (zimska *D. hyalina*) in hladno adaptiranih (*D. pulicaria*) dafnijah. Optimalne temperature za rast so bile podobne za vse vrste; pri ali nad 25 °C. Število jajc na samico je bilo višje pri nižjih temperaturah. 30 °C je zunaj tolerančnega območja testiranih vrst. Optimalna temperatura za reproduktivni uspeh je bila nižja za *D. hyalina* kot za *D. pulex* in *D. rosea*. *D. pulicaria* je zgodaj investirala v trpežna jajca, kar je morda prednost v kratkih rastnih sezonah visokogorskih jezer.

Značilne razlike med sezonami in vrstami so ostale tudi po tem, ko smo metabolno aktivnost popravili za razlike v telesni masi. Hitrost dihanja je bila višja pri vrstah iz mlak, še posebej pri višjih inkubacijskih temperaturah. To je bilo povezano z višjo vsebnostjo hemoglobina pri vrstah iz mlake kot pri vrstah iz jezera. Razmerje ETS/R je bilo višje pri vrstah iz oligotrofnih jezer, verjetno kot adaptacija za varčevanje z energijo v teh s hrano revnih habitatih.

Opazili smo tudi sezonske razlike v temperaturni občutljivosti. Aktivno prezimujoči osebki vrste *D. hyalina* so se uspešno aklimatizirali na zimsko temperaturo vode (4 °C), kot kaže povišana aktivnost ETS pri nizkih temperaturah, povišanje hitrost dihanja pri nizkih

temperaturah, premik temperaturnega optimuma za ETS/R in boljša rast pri nizkih temperaturah v primerjavi s poletnimi osebki vrste *D. hyalina*. Nasprotno pa je razvoj pri ekstremno nizkih ali visokih temperaturah (4 °C in 28 °C) povzročil počasnejšo rast mladičev pri vrstah *D. rosea* in *D. pulex*. Metabolizem teh dveh vrst, aklimatiziranih na ti ekstremni temperaturi, je kazal znake redukcije bazalnega metabolizma in aktivnosti. Osebki določene vrste, nabrani pri nižjih temperaturah vode, so bili večji kot tisti nabrani pri višjih temperaturah, imeli so večje mladiče, ki so bili ob zrelosti večji in so imeli več jajc.

Raziskovali smo tudi vpliv interakcije med medgeneracijskimi spremembami hrane in temperature na fitnes in lipidno sestavo dveh vrst iz rodu *Daphnia*. Vpliv spremembe temperature (15 °C ali 20 °C) in kvalitete hrane (*Cryptomonas* sp. kot visoko-kvalitetna hrana, ki vsebuje veliko n3 maščobne kisline EPA, ki je pomembna za rast dafnij, ali *Chlamydomonas* sp. kot relativno manj kakovostna hrana, ki ne vsebuje maščobne kisline EPA) na juvenilno rast, število jajc, reproduktivni uspeh ter vsebnost maščobnih kislin in holesterola pri enem klonu vrste *Daphnia magna* in *D. pulex* smo merili v šestnajstih kombinacijah maternalnega in eksperimentalnega okolja s standardiziranimi rastnimi poskusi. Obe vrsti sta imeli podobne odzive, le da je bil *D. pulex* bolj občutljiv na kvaliteto hrane.

Pozitivni vpliv kvalitetne hrane na hitrost rasti in število jajc je bil večji, če je bila maternalna hrana slabše kakovosti. Kvalitetna maternalna hrana je na splošno izboljšala rast in reprodukcijo potomcev, še posebej, če je bila eksperimentalna hrana slabše kakovosti. Največji fitnes so dosegle živali, aklimirane na 15 °C, ki so rasle pri 20 °C, zaradi kombinacije hitrejše rasti pri višji eksperimentalni temperaturi in večjega števila jajc pri živalih, aklimiranih na nižjo temperaturo. Medgeneracijski temperaturni režim je močno vplival na učinke kvalitete maternalne in eksperimentalne hrane. Kvalitetna maternalna hrana je bila bolj pomembna za fitnes v manj ugodnih temperaturnih pogojih (20°C→15°C). Slaba kvaliteta eksperimentalne hrane je imela večji vpliv na fitnes pri živalih, ki so doživele temperaturno spremembo med aklimacijo in poskusom, in manjši vpliv pri živalih na konstantni temperaturi. Ti rezultati nakazujejo, da medgeneracijske spremembe temperature močno vplivajo na prehranske potrebe organizmov. Z ekstrapolacijo iz študij, opravljenih pri konstantnih temperaturah, lahko resno podcenimo vpliv kvalitete prehrane na sekundarno produkcijo v naravnih okoljih.

Na vsebnost lipidov je najbolj vplivala eksperimentalna hrana, drugi faktorji so imeli le majhen vpliv. Pri 20 °C sta imela *D. magna* in *D. pulex* višji UI, več maščobne kisline EPA in manj holesterola kot pri 15 °C, kar je v nasprotju s pričakovanji homeoviskozne teorije. Kaže, da v tem temperaturnem območju ni bilo potrebe za regulacijo fluidnosti membrane, ali pa je bila ta regulirana s kakšnim drugim mehanizmom. Maternalna hrana in aklimacijska temperatura sta vplivali na metabolizem potomcev, tako da se je spremenila hitrost akumulacije lipidov. Vsebnost lipidov pri novorojenih osebkih se je razlikovala od tiste pri odraslih. Imeli so manj holesterola in,

v primeru živali, hranjenih z algo *Chlamydomonas*, več maščobne kisline EPA. Kaže, da živali, hranjene z algo *Chlamydomonas*, vso EPA, ki jo producirajo, investirajo v potomce.

Pri nobeni vrsti in na nobeni temperaturi nismo našli nobenega dokaza aktivne sinteze maščobne kisline EPA. Prav tako ni bilo dokazov za hitrejšo akumulacijo EPA pri nižji temperaturi pri živalih, hranjenih z algo *Cryptomonas*. Znotraj-vrstne razlike v maščobno-kislinski sestavi, zaradi razlik v kvaliteti hrane med sezonami in habitati, so bile večje kot med-vrstne razlike pri osebkih iz rodu *Daphnia*, nabranih na terenu. Vrste iz rodu *Daphnia*, ki so evolucijsko prilagojene na nižje temperature (*D. pulicaria*) ali preživljajo zimo v aktivnem stanju (*D. hyalina*), niso imele večje stopnje nesaturiranosti maščobnih kislin ali vsebnosti EPA kot vrsti iz tople mlake (*D. pulex*, *D. rosea*). Kaže, da spremembe v maščobno-kislinski sestavi niso pomemben mehanizem za temperaturno adaptacijo in aklimacijo pri rodu *Daphnia*.

Aklimacija metabolizma je pomembna za temperaturno toleranco. Uspešna rast in razmnoževanje pri nizkih temperaturah sta bili povezani s sposobnostjo kompenzacije upada hitrosti metabolizma, ki ga povzroča nižanje temperature. Uspeh pri višjih temperaturah pa je bil povezan z vsebnostjo hemoglobina in sposobnostjo za visoko hitrost dihanja pri visokih temperaturah.

Kljub nižjima temperaturnima optimumoma pri vrstah iz hladnih jezer (*D. pulicaria* in *D. hyalina*) ocenjujemo, da sta vrsti iz mlak, *D. rosea* in *D. pulex*, bolj neposredno ogroženi s klimatskimi spremembami. Temperature v njunem habitatu so že sedaj poleti stresno visoke in utegnejo vedno pogosteje dosegati vrednosti zunaj njunega tolerančnega območja (30 °C ali več), kar bi povzročilo poletno produkcijo trpežnih jajc, s posledicami za fenologijo teh dveh vrst.

Po drugi strani pa lahko višje temperature in daljše sezone povečajo produktivnost jezer, ki so dom vrst *D. pulicaria* in *D. hyalina*, ter jih tako odprejo za kompetitorje, ki so bolj sposobni povečanja metabolne aktivnosti in rasti pri visokih temperaturah. Sprememba v vrstni sestavi vodnih bolh iz rodu *Daphnia* v alpskih jezerih ali sprememba fenologije v nižinskih mlakah lahko torej služita kot znaka klimatskih sprememb v sladkovodnih ekosistemih.

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ANNEXES

ANNEX A

MEANS AND STANDARD DEVIATIONS OF THE RESPIRATION RATE (R), ETS ACTIVITY (ETS) AND THE ETS/R RATIO OF *DAPHNIA ROSEA* AND *D. HYALINA* AT DIFFERENT TEMEPRATURES (T)

The number following the species name represents lake water temperature at the time of collection of organisms for experiments.

	T	R (µL mg^{-1} h^{-1})		ETS (µL mg^{-1} h^{-1})		ETS/R	
	(°C)	Mean	SD	Mean	SD	Mean	SD
D. hyalina4	5	3.19	0.73	3.55	0.16	1.15	0.23
	10	3.75	0.90	7.30	2.08	2.07	0.86
	15	4.15	0.65	8.08	0.31	1.97	0.22
	20	6.34	0.75	11.13	1.03	1.77	0.21
	25	8.35	1.45	14.40	1.53	1.74	0.11
	30	11.01	2.76	15.15	0.66	1.45	0.33
D. hyalina20	5	2.75	1.29	3.15	1.02	1.39	0.86
	10	3.11	0.40	5.91	0.76	1.83	0.27
	15	6.02	0.44	11.14	0.67	1.83	0.18
	20	5.76	0.88	16.10	1.60	2.83	0.31
	25	5.73	1.06	16.77	0.59	3.00	0.52
	30	9.04	2.35	13.83	2.51	1.59	0.38
D. rosea21	5	2.38	0.50	3.91	0.43	1.74	0.61
	10	3.43	0.38	5.92	0.63	1.75	0.30
	15	5.67	0.35	11.73	0.66	2.08	0.22
	20	6.26	1.98	15.27	2.49	2.55	0.54
	25	10.39	0.82	14.54	2.46	1.40	0.24
	30	13.38	2.01	14.89	2.24	1.12	0.17
D. rosea28	5	1.96	0.61	2.94	0.38	1.57	0.35
	10	4.31	1.30	4.62	1.21	1.12	0.31
	15	5.66	0.69	8.82	1.26	1.56	0.15
	20	4.31	1.28	10.74	2.07	2.55	0.31
	25	8.92	2.31	12.04	1.53	1.40	0.24
	30	9.61	3.82	9.56	1.19	1.09	0.35

ANNEX B

RESULTS OF MUTIPLE COMPARRISON TEST FOR RESPIRATION RATE, ETS ACTIVITY AND THE ETS/R RATIO

Annex B1: Results (p values) of post hoc tests for the effects of experimental temperature (T) on respiration rate (R), ETS activity (ETS) and the ETS/R ratio of *Daphnia pulicaria* and *D. pulex*

The number following the species name represents lake water temperature at the time of collection of organisms for experiments. Post hoc tests were conducted after one-way ANOVAs for the effect of experimental temperature within each group were found to be statistically significant. Tukey HSD test was used in case of equal variances and Dunnett T3 test was used in case the Levene's test of equality of variances indicated the variances were not equal. Significant p values are printed in bold.

Group	T_1 (°C)	T_2 (°C)	ETS	R	ETS/R
			Tukey HSD	Tukey HSD	Tukey HSD
pulicaria15	10	15	0.064	0.752	**0.026**
		20	**0.022**	**0.011**	0.567
	15	20	0.676	**0.026**	**0.008**
			Dunnett T3	Tukey HSD	Tukey HSD
pulex4	5	10	0.261	0.139	0.998
		15	**0.002**	**0.027**	**0.002**
		20	**<0.001**	**<0.001**	**0.001**
	10	15	**0.007**	0.862	**0.002**
		20	**<0.001**	**<0.001**	**0.001**
	15	20	**0.005**	**<0.001**	0.982
			Dunnett T3	Dunnett T3	Tukey HSD
pulex12	5	10	0.056	0.206	1.000
		15	**<0.001**	**0.004**	0.306
		20	**0.001**	**<0.001**	**0.007**
		25	**0.002**	**0.005**	1.000
	10	15	**<0.001**	**0.009**	0.345
		20	**0.001**	**<0.001**	**0.008**
		25	**0.003**	**0.006**	0.999
	15	20	**0.006**	**0.019**	0.267
		25	**0.005**	**0.008**	0.250
	20	25	**0.010**	**0.014**	**0.005**

	T_1 (°C)	T_2 (°C)	rosea21	rosea28	hayalina4	hyalina20
			Dunnett T3	Dunnett T3	Dunnett T3	Dunnett T3
R	5	10	0.066	0.102	0.993	1.000
		15	**<0.001**	**<0.001**	0.732	**0.027**
		20	0.080	0.098	**0.049**	**0.036**
		25	**<0.001**	**0.015**	0.073	**0.046**
		30	**0.001**	0.077	0.377	**0.018**
	10	15	**<0.001**	0.543	0.999	**0.001**
		20	0.220	1.000	0.135	**0.011**
		25	**<0.001**	0.070	0.093	**0.028**
		30	**0.002**	0.241	0.399	**0.033**
	15	20	0.999	0.528	0.134	1.000
		25	**<0.001**	0.228	0.126	1.000
		30	**0.007**	0.467	0.429	0.290
	20	25	0.059	0.070	0.554	1.000
		30	**0.006**	0.240	0.599	0.239
	25	30	0.200	1.000	0.881	0.240
			Dunnett T3	Tukey HSD	Tukey HSD	Tukey HSD
ETS	5	10	**0.006**	0.405	**0.020**	**0.041**
		15	**<0.001**	**<0.001**	**0.005**	**<0.001**
		20	**0.003**	**<0.001**	**<0.001**	**<0.001**
		25	**0.004**	**<0.001**	**<0.001**	**<0.001**
		30	**0.002**	**<0.001**	**<0.001**	**<0.001**
	10	15	**<0.001**	**0.001**	0.960	**<0.001**
		20	**0.006**	**<0.001**	**0.018**	**<0.001**
		25	**0.008**	**<0.001**	**<0.001**	**<0.001**
		30	**0.004**	**<0.001**	**<0.001**	**<0.001**
	15	20	0.222	0.264	0.068	**<0.001**
		25	0.391	**0.012**	**<0.001**	**<0.001**
		30	0.227	0.953	**<0.001**	**0.049**
	20	25	1.000	0.671	**0.046**	0.971
		30	1.000	0.746	**0.012**	0.132
	25	30	1.000	0.081	0.966	**0.026**
			Tukey HSD	Tukey HSD		Tukey HSD
ETS/R	5	10	1.000	0.184	*	0.769
		15	0.740	1.000		0.767
		20	**0.032**	**<0.001**		**0.002**
		25	0.727	0.932		**<0.001**
		30	0.151	0.143		0.987
	10	15	0.746	0.206		1.000
		20	**0.033**	**<0.001**		0.062
		25	0.720	0.670		**0.020**
		30	0.147	1.000		0.977
	15	20	0.417	**<0.001**		0.063
		25	0.096	0.949		**0.020**
		30	**0.007**	0.160		0.976
	20	25	**0.001**	**<0.001**		0.992
		30	**<0.001**	**<0.001**		**0.008**
	25	30	0.857	0.584		**0.002**

Annex B2: Results (p values) of post hoc tests for the effects of experimental temperature (T) on respiration rate (R), ETS activity (ETS) and the ETS/R ratio of *Daphnia rosea* and *D. hyalina*

The number following the species name represents lake water temperature at the time of collection of organisms for experiments. Post hoc tests were conducted after one-way ANOVAs for the effect of experimental temperature within each group were found to be statistically significant. Tukey HSD test was used in case of equal variances and Dunnett T3 test was used in case the Levene's test of equality of variances indicated the variances were not equal. Significant p values are printed in bold.

*Post hoc tests not performed because ANOVA was not significant.

Annex B3: Contrast tests for the effect of season of collection on the ETS activity, respiration rate (R) and the ETS/R ratio of *Daphnia* at specified experimental temperatures

The critical p value was step-wise Bonferroni corrected and significant p values printed in bold. In case of unequal variances contrast tests without the assumption of equal variances were used. Note that in that case df are not integers. *Analyses run on mass corrected rates of ETS and R because body masses differed significantly between collection times.

Species	T	ETS			R			ETS/R		
	(°C)	t	df	p	t	df	p	t	df	p
*D. pulex**	5	2.2	3.4	0.102	1.3	26	0.215	0.6	26	0.583
	10	5.4	3.4	**0.009**	0.2	26	0.847	0.8	26	0.419
	15	-3.3	4.7	**0.023**	5.9	26	**<0.001**	-4.8	26	**<0.000**
	20	-7.1	6.4	**<0.001**	6.3	26	**<0.001**	-4.5	26	**<0.000**
D. rosea	5	3.8	7.9	**0.005**	1.2	7.7	0.274	0.8	48	0.431
	10	2.1	6.0	0.076	-1.5	4.7	0.210	2.9	48	**0.006**
	15	4.6	6.1	**0.004**	0.0	5.9	0.988	2.4	48	0.021
	20	3.1	7.7	**0.015**	1.8	6.8	0.108	0.0	48	0.993
	25	1.9	6.7	0.097	1.3	5.0	0.240	0.0	48	0.977
	30	4.7	6.1	**0.003**	2.0	6.1	0.098	0.1	48	0.883
*D. hyalina**	5	1.0	36	0.327	0.9	5.9	0.395	-0.7	33	0.473
	10	2.0	36	0.057	1.4	2.7	0.278	0.7	33	0.505
	15	-2.1	36	0.043	-3.6	3.2	0.032	0.4	33	0.698
	20	-3.5	36	**0.001**	2.4	5.6	0.059	-3.1	33	**0.004**
	25	-0.6	36	0.555	3.9	3.7	0.021	-3.7	33	**0.001**
	30	1.1	36	0.265	1.0	1.8	0.438	-0.4	33	0.714

Annex B4: Contrast tests for the effect of species on the ETS activity, respiration rate (R) and the ETS/R ratio of *Daphnia* at specified experimental temperatures

Only groups collected at comparable field water temperatures were compared. The critical p value was step-wise Bonferroni corrected and significant p values printed in bold. In case of unequal variances contrast tests without the assumption of equal variances were used. Note that in that case df are not integers. *Analysis run on mass corrected rates of ETS and R because body masses differed significantly between species.

Species	T	ETS			R			ETS/R		
	(°C)	t	df	p	t	df	p	t	df	p
D. pulex/	10	-0.6	2.2	0.601	4.3	15	**0.001**	-5.4	2	**0.021**
D. pulicaria	15	0.2	2.2	0.874	8.7	15	**<0.001**	-8.8	2	**0.009**
	20	5.0	4.6	**0.005**	9.1	15	**<0.001**	-5.1	3	**0.011**
D. rosea/	5	3.6	7.1	**0.008**	0.1	5.6	0.958	1.3	46	0.215
*D. hyalina**	10	4.4	8.0	**0.002**	3.5	6.8	**0.010**	-0.3	46	0.775
	15	5.2	8.0	**0.001**	0.8	6.3	0.456	0.8	46	0.407
	20	1.8	7.7	0.106	1.2	5.8	0.284	-1.0	46	0.320
	25	1.5	4.5	0.200	11.0	7.7	**<0.001**	-5.8	46	**<0.001**
	30	1.8	8.0	0.104	3.8	7.3	**0.006**	-1.7	46	0.099
D. pulex/	5	-1.2	4.9	0.290	4.9	6.7	**0.002**	-3.4	4.1	**0.026**
*D. rosea**	10	-7.0	5.5	**0.001**	3.8	6.9	**0.007**	-6.5	4.9	**0.001**
	15	-8.9	6.5	**<0.001**	5.0	4.3	**0.006**	-10.9	5.1	**<0.001**
	20	-3.3	5.9	**0.016**	4.5	5.2	**0.006**	-6.0	4.2	**0.003**
	25	4.1	6.7	**0.005**	7.8	3.4	**0.003**	-5.0	5.4	**0.003**

ANNEX C

RESULTS OF MUTIPLE COMPARRISON TEST FOR GROWTH EXPERIMENTS ON DAPHNIA FROM NATURAL POPULATIONS

Group	T1 (°C)	T2 (°C)	g_i Tukey HSD	clutch size Tukey HSD	r_{pot} Dunnett T3
pulex4	10	15	**<0.001**	0.959	**<0.001**
		20	**<0.001**	0.193	**0.023**
		25	**<0.001**	**0.002**	**0.010**
	15	20	**<0.001**	0.098	0.094
		25	**<0.001**	**0.001**	**0.047**
	20	25	**0.001**	**0.030**	0.999
			Tukey HSD	Tukey HSD	Dunnett T3
pulex15	10	15	**<0.001**	**0.008**	**<0.001**
		20	**<0.001**	0.537	**0.007**
		25	**<0.001**	**0.007**	**0.005**
	15	20	**0.001**	**0.002**	0.100
		25	**<0.001**	**<0.001**	**0.053**
	20	25	**0.026**	**0.047**	0.861
			Tukey HSD	Tukey HSD	Tukey HSD
rosea20	10	15	**<0.001**	0.997	**<0.001**
		20	**<0.001**	1.000	**<0.001**
		25	**<0.001**	0.522	**<0.001**
	15	20	**<0.001**	0.991	**<0.001**
		25	**<0.001**	0.425	**<0.001**
	20	25	**0.003**	0.573	**0.036**
			Tukey HSD	Tukey HSD	Tukey HSD
rosea26	10	15	**<0.001**	0.108	**<0.001**
		20	**<0.001**	0.191	**<0.001**
		25	**<0.001**	**0.005**	**<0.001**
	15	20	**<0.001**	0.976	**<0.001**
		25	**<0.001**	**<0.001**	**<0.001**
	20	25	**0.001**	**<0.001**	0.541
			Tukey HSD	Tukey HSD	Tukey HSD
hyalina4	10	15	**<0.001**	0.305	**<0.001**
		20	**<0.001**	**0.012**	**<0.001**
		25	**<0.001**	**<0.001**	**<0.001**
	15	20	**<0.001**	**0.001**	**<0.001**
		25	**0.002**	**<0.001**	**<0.001**
	20	25	0.477	**<0.001**	**<0.001**
			Dunnett T3	Tukey HSD	Tukey HSD
hyalina20	10	15	**<0.001**	0.129	**<0.001**
		20	**0.005**	0.083	**<0.001**
		25	**0.004**	**<0.001**	0.923
	15	20	**0.014**	**0.003**	**<0.001**
		25	**0.010**	**<0.001**	**<0.001**
	20	25	0.347	**<0.001**	**<0.001**

p values are printed in bold.

Annex C1: Results (*p* values) of post hoc tests for the effects of experimental temperature (T) on juvenile growth rate (g_j), size of first clutch, and reproductive performance (r_{pot}) of *Daphnia pulex, D. rosea* and *D. hyalina*

The number following the species name represents lake water temperature at the time of collection of mothers of experimental animals. Post hoc tests were conducted after one-way ANOVAs for the effect of experimental temperature within each group were found to be statistically significant. Tukey HSD test was used in case of equal variances and Dunnett T3 test was used in case the Levene's test of equality of variances indicated the variances were not equal. Significant

Annex C2: Contrast tests for the effect of season of collection on the juvenile growth rate (g_j), size of first clutch, and reproductive performance (r_{pot}) of *Daphnia* at specified experimental temperatures (T)

Species	T (°C)	g_j			Size of first clutch			r_{pot}		
		t	df	p	t	df	p	t	df	p
D. pulex	10	0.1	16	0.910	4.3	16	**0.001**	4.8	3.9	**0.009**
	15	-3.8	16	**0.002**	1.7	16	0.109	-1.9	2.8	0.154
	20	0.5	16	0.592	2.6	16	0.020	1.6	3.3	0.199
	25	0.8	16	0.410	0.5	16	0.629	0.7	3.8	0.513
D. rosea	10	1.9	16	0.072	1.3	2.0	0.323	1.1	16	0.279
	15	4.0	16	**0.001**	0.2	2.7	0.839	1.6	16	0.122
	20	1.2	16	0.235	0.1	3.6	0.907	0.1	16	0.902
	25	1.1	16	0.299	4.3	4.0	**0.013**	5.2	16	**<0.001**
D. hyalina	10	7.2	3.4	**0.003**	1.9	16	0.082	1.7	16	0.110
	15	4.4	2.6	0.029	0.4	16	0.715	0.3	16	0.762
	20	-1.6	4.0	0.194	1.9	16	0.082	3.1	16	**0.006**
	25	-5.5	3.2	**0.010**	-0.7	16	0.468	-4.1	16	**0.001**

ANNEX D

RESULTS OF MUTIPLE COMPARRISON TEST FOR GROWTH EXPERIMENTS ON LABORATORY CLONES

Annex D1: Tukey's HSD post hoc pair-wise comparisons for the effects of acclimation (acc.) or experimental (exp.) temperature (T) within the designated temperature and food (Crypto – *Cryptomonas* sp., Chlamy – *Chlamydomonas* sp.) treatment combinations on the juvenile growth rate (g_j), size of first clutch, reproductive performance (r_{pot}) and body mass at maturity of *Daphnia magna* and *D. pulex*.

Species	*Daphnia magna*				*Daphnia pulex*			
Effect of	Acclimation T		Experimental T		Acclimation T		Experimental T	
Within	exp. 15 °C	exp. 20 °C	acc. 15 °C	acc. 20 °C	exp. 15 °C	exp. 20 °C	acc. 15 °C	acc. 20 °C
g_i								
CryptoCrypto	**0.015**	0.718	**<0.001**	**<0.001**	0.998	0.995	**<0.001**	**<0.001**
ChlamyCrypto	**0.020**	0.216	**<0.001**	**<0.001**	0.460	0.766	**<0.001**	**<0.001**
CryptoChlamy	**0.008**	0.669	**<0.001**	**<0.001**	0.979	**0.003**	**<0.001**	**<0.001**
ChlamyChlamy	0.125	1.000	**<0.001**	**<0.001**	0.999	0.214	**<0.001**	**<0.001**
Clutch size								
CryptoCrypto	0.062	**0.018**	0.814	0.354	0.593	**0.002**	0.470	**0.001**
ChlamyCrypto	**0.048**	**0.001**	0.744	0.153	0.337	**0.001**	0.980	**0.003**
CryptoChlamy	0.819	0.273	0.531	0.134	0.115	0.326	**0.002**	**0.005**
ChlamyChlamy	0.087	0.092	0.092	0.096	0.660	**0.013**	0.632	0.337
r_{pot}								
CryptoCrypto	0.700	**0.008**	**<0.001**	**<0.001**	0.925	**0.002**	**<0.001**	**0.019**
ChlamyCrypto	0.140	**<0.001**	**<0.001**	**<0.001**	0.454	**<0.001**	**<0.001**	**<0.001**
CryptoChlamy	0.261	0.188	**<0.001**	**<0.001**	0.387	0.145	**<0.001**	**<0.001**
ChlamyChlamy	0.473	0.061	**<0.001**	**<0.001**	0.729	**0.001**	**<0.001**	**<0.001**
Body mass								
CryptoCrypto	**0.030**	0.253	0.968	0.265	0.464	0.423	0.884	0.915
ChlamyCrypto	**<0.001**	1.000	**0.001**	**0.001**	**0.001**	**0.020**	0.107	1.000
CryptoChlamy	**0.017**	0.975	0.764	0.119	0.095	0.351	0.356	0.093
ChlamyChlamy	0.260	0.536	**0.034**	1.000	0.276	0.999	**0.024**	0.293

Annex D2: Tukey's HSD post hoc pair-wise comparisons for the effects of acclimation (acc.) or experimental (exp.) food quality within the designated temperature and food (Crypto – *Cryptomonas* sp., Chlamy – *Chlamydomonas* sp.) treatment combinations on the juvenile growth rate (g_j), size of first clutch, reproductive performance (r_{pot}) and body mass at maturity of *Daphnia magna* and *D. pulex*.

Species	*Daphnia magna*				*Daphnia pulex*			
Effect of	Acclimation food		Experimental food		Acclimation food		Experimental food	
Within	Exp. Crypto	Exp. Chlamy	Acc. Crypto	Acc. Chlamy	Exp. Crypto	Exp. Chlamy	Acc. Crypto	Acc. Chlamy
g_j								
20°C→20°C	0.385	0.061	0.733	**0.002**	0.652	**0.001**	0.593	**0.001**
15°C→15°C	0.659	0.057	0.773	0.077	0.815	**0.015**	0.386	**0.001**
15°C→20°C	0.132	0.335	0.113	**0.001**	0.830	0.085	**0.016**	**0.001**
20°C→15°C	0.193	**<0.001**	0.496	**<0.001**	0.994	**<0.001**	**0.009**	**<0.001**
Clutch size								
20°C→20°C	0.447	0.238	0.389	0.203	0.815	0.069	0.987	**0.032**
15°C→15°C	0.861	1.000	0.132	**0.041**	**0.048**	**0.007**	0.198	**0.029**
15°C→20°C	**0.026**	0.318	**0.010**	**<0.001**	0.051	0.964	**<0.001**	**0.021**
20°C→15°C	0.961	0.125	0.483	**0.030**	**0.018**	**0.002**	**0.025**	**0.003**
r_{pot}								
20°C→20°C	0.512	0.189	0.475	0.172	0.772	**0.039**	0.974	**0.018**
15°C→15°C	0.091	**0.042**	0.079	**0.037**	0.128	**0.001**	0.366	**0.002**
15°C→20°C	0.098	0.304	**0.018**	**<0.001**	0.070	0.936	**<0.001**	**0.014**
20°C→15°C	0.971	**<0.001**	0.473	**<0.001**	**0.045**	**<0.001**	0.062	**<0.001**
Body mass								
20°C→20°C	0.792	**0.033**	0.712	**0.003**	0.189	**<0.001**	0.404	**0.001**
15°C→15°C	0.074	0.396	0.589	**0.002**	0.999	0.808	0.341	0.087
15°C→20°C	0.441	**0.003**	0.052	**<0.001**	0.337	0.136	**0.018**	**0.008**
20°C→15°C	0.050	0.330	0.516	1.000	0.877	0.204	**0.010**	**0.002**

ANNEX E

A LIST OF FATTY ACIDS DETECTED IN LABORATORY CLONES OF *DAPHNIA MAGNA* AND *D. PULEX*

FA
C8:0
C10:0
C11:0
C12:0
C13:0
C14:0
C14:1n5
C15:0
C15:1n5
C16:0
C16:1n7
C17:1n7
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n6
C18:3n3
C18:4n3
C20:0
C20:1n9
C20:1n7
C20:2n6
C20:3n6
C20:4n6
C21:0
C20:3n3
C20:5n3
C22:0
C22:1n9
C22:2n6
C22:6n3
C24:0
C24:1n9

ANNEX F

RESULTS OF THE DISCRIMINANT ANALYSIS OF THE FAME COMPOSITION OF *DAPHNIA MAGNA* AND *D. PULEX*

Discriminant analysis for "species" was run on the percent FA composition (% share of FA in total FA content) of the FAs with more than 0.5% average share in total FA content of *Daphnia magna* and *D. pulex.* The results of the SPSS 13.0 analysis are presented below.

Eigenvalues				
Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	22.18	100	100	**0.978**

First 1 canonical discriminant function was used in the analysis.

Wilks' Lambda				
Test of Function(s)	Wilks' Lambda	Chi-square	df	Sig.
1	0.043	240.5	15	**<0.001**

Standardized Canonical Discriminant Function Coefficients		Structure Matrix	
C14:0	1.74	C14:0	-0.03
C15:0	-0.23	C15:0	0.07
C16:0	-1.28	C16:0	-0.03
C16:1n-7	0.04	C16:1n-7	-0.19
C18:0	-0.12	C18:0	-0.08
C18:1n-9	**15.32**	C18:1n-9	0.04
C18:1n-7	-0.25	C18:1n-7	-0.08
C18:2n-6	0.84	C18:2n-6	-0.06
C18:3n-3	**14.42**	C18:3n-3	0.01
C18:4n-3	**5.74**	C18:4n-3	0.00
C20:1n-7	0.58	C20:1n-7	-0.05
C20:3n-3	0.28	C20:3n-3	-0.02
C20:4n-6	**7.09**	C20:4n-6	0.04
C20:5n-3	**16.14**	C20:5n-3	0.01
C21:0	0.47	C21:0	0.02

Standardized canonical discriminant function coefficients show which of the predictor variables contribute most to the separation between groups. The structure matrix shows pooled within-group correlations between discriminating variables and the standardized canonical discriminant function.

Unstandardized canonical discriminant functions evaluated at group means:

Functions at Group Centroids						
Species	Mean	SD	95% CI		Minimum	Maximum
			Lower Bound	Upper Bound		
D. magna	**4.2**	0.96	4.0	4.5	2.5	6.5
D. pulex	**-5.1**	1.05	-5.4	-4.8	-7.4	-2.5

ANNEX G

FACTOR ANALYSES OF THE FAME COMPOSITION OF *DAPHNIA MAGNA* AND *D. PULEX*

Annex G1: Factor analysis of the FAME (fatty acid methyl ester) composition of mature *Daphnia magna* with their first clutch of eggs from different combinations of acclimation and experimental treatments.

Factor analysis was run on the percent FA composition (% share of FA in total FA content) of the FAs with more than 0.5 % average share in total FA content of *Daphnia magna.* The results of the SPSS 13.0 analysis are presented below.

Communalities		
	Initial	Extraction
C14:0	1	**0.78**
C15:0	1	0.81
C16:0	1	0.96
C16:1n-7	1	0.85
C18:0	1	0.81
C18:1n-9	1	0.99
C18:1n-7	1	0.91
C18:2n-6	1	**0.79**
C18:3n-3	1	0.99
C18:4n-3	1	0.95
C20:1n-7	1	0.85
C20:3n-3	1	0.97
C20:4n-6	1	0.90
C20:5n-3	1	0.99
C21:0	1	0.94

Total Variance Explained			
PC	Eigenvalue	% of Variance	Cumulative %
1	**11.66**	**77.76**	**77.76**
2	**1.81**	**12.08**	**89.83**
3	0.65	4.33	94.16
4	0.29	1.95	96.11
5	0.21	1.42	97.54
6	0.16	1.07	98.61
7	0.10	0.69	99.30
8	0.04	0.27	99.56
9	0.03	0.21	99.78
10	0.02	0.11	99.88
11	0.01	0.07	99.95
12	0.00	0.02	99.98
13	0.00	0.02	99.99
14	0.00	0.01	100.00
15	0.00	0.00	100.00

Two factors were retained, explaining 90 % of the total variance.

Component Matrix		
	Component	
	PC1	PC2
C14:0	0.83	-0.31
C15:0	0.85	0.30
C16:0	**0.98**	0.02
C16:1n-7	0.91	-0.12
C18:0	0.90	0.00
C18:1n-9	**1.00**	0.00
C18:1n-7	0.92	0.25
C18:2n-6	-0.83	0.32
C18:3n-3	**0.99**	0.01
C18:4n-3	**-0.97**	-0.07
C20:1n-7	-0.92	0.04
C20:3n-3	0.35	**-0.92**
C20:4n-6	**-0.94**	0.06
C20:5n-3	**-1.00**	0.00
C21:0	0.60	**0.76**

Annex G2: Factor analysis of the FAME (fatty acid methyl ester) composition of mature *Daphnia pulex* with their first clutch of eggs from different combinations of acclimation and experimental treatments.

Factor analysis was run on the percent FA composition (% share of FA in total FA content) of the FAs with more than 0.5 % average share in total FA content of *Daphnia pulex.* The results of the SPSS 13.0 analysis are presented below.

Communalities		
	Initial	Extraction
C14:0	1	0.90
C15:0	1	**0.69**
C16:0	1	0.98
C16:1n-7	1	0.90
C18:0	1	0.90
C18:1n-9	1	0.99
C18:1n-7	1	0.93
C18:2n-6	1	**0.58**
C18:3n-3	1	0.99
C18:4n-3	1	0.96
C20:1n-7	1	0.94
C20:3n-3	1	0.91
C20:4n-6	1	0.97
C20:5n-3	1	0.99
C21:0	1	0.91

Total Variance Explained			
PC	Eigenvalue	% of Variance	Cumulative %
1	**10.28**	**68.54**	**68.54**
2	**2.08**	**13.86**	**82.40**
3	**1.17**	**7.79**	**90.19**
4	0.69	4.57	94.76
5	0.33	2.18	96.94
6	0.15	1.01	97.95
7	0.13	0.87	98.82
8	0.08	0.52	99.34
9	0.04	0.29	99.63
10	0.02	0.13	99.77
11	0.02	0.11	99.87
12	0.01	0.07	99.94
13	0.01	0.03	99.98
14	0.00	0.02	100.00
15	0.00	0.00	100.00

Three factors were retained, explaining 90 % of the total variance.

Component Matrix			
	Component		
	PC1	PC2	PC3
C14:0	0.71	0.56	0.30
C15:0	0.60	-0.55	-0.16
C16:0	0.96	0.18	0.15
C16:1n-7	0.23	**0.79**	0.47
C18:0	0.94	-0.04	-0.12
C18:1n-9	**0.99**	-0.02	-0.06
C18:1n-7	0.94	0.15	0.16
C18:2n-6	-0.61	0.39	-0.22
C18:3n-3	**0.99**	-0.09	-0.09
C18:4n-3	**-0.98**	0.01	0.06
C20:1n-7	-0.96	0.11	0.13
C20:3n-3	0.55	0.46	**-0.63**
C20:4n-6	**-0.98**	0.05	0.07
C20:5n-3	**-1.00**	-0.03	0.04
C21:0	0.47	**-0.63**	**0.53**

ANNEX H

MULTIPLE COMPARISSON TESTS OF THE LIPID COMPOSITION OF *DAPHNIA MAGNA* AND *D. PULEX*

Species	*Daphnia magna*				*Daphnia pulex*			
Effect of	Acclimation food		Experimental food		Acclimation food		Experimental food	
Within	Exp. Crypto	Exp. Chlamy	Acc. Crypto	Acc. Chlamy	Exp. Crypto	Exp. Chlamy	Acc. Crypto	Acc. Chlamy
FA								
20°C→20°C	0.051	0.306	0.072	0.034	0.661	0.142	**<0.001**	**<0.001**
15°C→15°C	0.515	0.186	0.034	0.024	**<0.001**	0.365	**<0.001**	**<0.001**
15°C→20°C	0.506	0.876	**0.004**	0.018			**<0.001**	
20°C→15°C	0.123	0.446	0.042	0.052	**<0.001**	**<0.001**	**<0.001**	**<0.001**
%SAFA								
20°C→20°C	**0.001**	**0.001**	**<0.001**	**<0.001**	0.047	0.104	**0.005**	**<0.001**
15°C→15°C	0.063	**<0.001**	**<0.001**	**<0.001**	0.134	0.099	**0.002**	**0.020**
15°C→20°C	0.225	**<0.001**	**<0.001**	**<0.001**			**0.005**	
20°C→15°C	0.069	**<0.001**	**<0.001**	**<0.001**	0.387	0.712	**0.009**	0.084
%MUFA								
20°C→20°C	**<0.001**	0.994	**<0.001**	**0.001**	0.897	0.112	**<0.001**	**0.001**
15°C→15°C	0.139	0.179	**<0.001**	**0.003**	0.031	0.028	**0.009**	**0.004**
15°C→20°C	**0.006**	0.684	**<0.001**	**<0.001**			**0.001**	
20°C→15°C	0.033	0.392	**0.010**	**<0.001**	0.686	0.059	**0.002**	**0.001**
%PUFA								
20°C→20°C	**<0.001**	**0.024**	**<0.001**	**<0.001**	0.102	0.237	**<0.001**	**0.001**
15°C→15°C	**0.010**	**0.001**	**<0.001**	**<0.001**	0.059	0.058	**0.007**	**0.009**
15°C→20°C	**0.004**	**<0.001**	**<0.001**	**<0.001**			**0.002**	
20°C→15°C	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.717	0.334	**0.001**	0.052
UI								
20°C→20°C	0.008	0.567	**<0.001**	**<0.001**	0.009	0.541	**<0.001**	**<0.001**
15°C→15°C	0.019	0.225	**<0.001**	**<0.001**	0.044	0.036	**0.001**	**<0.001**
15°C→20°C	0.018	0.372	**<0.001**	**0.001**			**<0.001**	
20°C→15°C	0.130	0.027	**<0.001**	**0.001**	0.444	0.319	**<0.001**	**0.007**
EPA								
20°C→20°C	0.042	**0.001**	**0.013**	**0.009**	0.134	0.068	**0.001**	**0.001**
15°C→15°C	0.352	**0.003**	**0.007**	**0.005**	0.218	**0.001**	**0.030**	**0.013**
15°C→20°C	0.902	0.487	**0.003**	**0.006**			**0.002**	
20°C→15°C	0.101	**0.001**	**0.005**	**0.011**	**0.001**	**<0.001**	**0.001**	**0.003**
%EPA								
20°C→20°C	0.058	**0.002**	**<0.001**	**<0.001**	**0.008**	0.110	**<0.001**	**<0.001**
15°C→15°C	**0.004**	**0.004**	**<0.001**	**<0.001**	0.086	**0.001**	**0.008**	**<0.001**
15°C→20°C	**0.008**	0.523	**<0.001**	**0.004**			**<0.001**	
20°C→15°C	0.505	**<0.001**	**<0.001**	**<0.001**	0.126	**0.001**	**<0.001**	**<0.001**
Cho								
20°C→20°C	**<0.001**	**<0.001**	0.021	0.024	0.076	0.291	0.022	**0.003**
15°C→15°C	0.775	0.085	**0.001**	0.144	0.128	0.299	0.014	0.032
15°C→20°C	0.088	0.013	**<0.001**	0.010	**<0.001**	0.295	**<0.001**	0.162
20°C→15°C	0.780	0.397	0.062	0.430	0.912	0.519	0.291	0.759

Species	*Daphnia magna*				*Daphnia pulex*			
Effect of	Acclimation T		Experimental T		Acclimation T		Experimental T	
Within	exp. 15 °C	exp. 20 °C	acc. 15 °C	acc. 20 °C	exp. 15 °C	exp. 20 °C	acc. 15 °C	acc. 20 °C
FA								
CryptoCrypto	0.025	0.089	**0.005**	0.891	**<0.001**	**<0.001**	0.748	0.224
ChlamyCrypto	0.046	0.111	0.030	0.228	**<0.001**			**<0.001**
CryptoChlamy	**0.003**	0.089	0.156	0.516	**<0.001**	**<0.001**	0.164	0.620
ChlamyChlamy	0.037	0.024	0.184	0.510	0.798			**<0.001**
%SAFA								
CryptoCrypto	0.186	0.019	**0.003**	0.515	0.288	0.230	0.649	0.387
ChlamyCrypto	0.201	0.014	0.995	0.202	0.024			0.047
CryptoChlamy	**0.001**	**<0.001**	0.691	0.935	0.270	0.621	0.080	1.000
ChlamyChlamy	**0.001**	0.280	0.026	**<0.001**	0.539			0.269
%MUFA								
CryptoCrypto	0.061	**<0.001**	0.019	0.158	0.447	0.021	0.450	0.246
ChlamyCrypto	0.528	0.061	0.538	0.100	**0.005**			0.486
CryptoChlamy	0.029	0.032	0.916	0.670	0.047	0.030	0.078	0.560
ChlamyChlamy	0.108	0.179	0.214	0.785	0.112			0.063
%PUFA								
CryptoCrypto	0.013	**<0.001**	**<0.001**	0.066	0.388	0.032	0.467	0.892
ChlamyCrypto	0.791	0.018	0.687	0.013	0.009			0.131
CryptoChlamy	**<0.001**	**<0.001**	0.765	0.897	0.401	0.139	0.072	0.980
ChlamyChlamy	**<0.001**	0.064	0.886	**0.001**	0.296			0.254
UI								
CryptoCrypto	0.829	0.008	**0.004**	0.367	0.706	0.067	0.285	0.566
ChlamyCrypto	0.725	0.024	0.360	0.120	0.014			0.050
CryptoChlamy	0.037	0.042	0.770	0.644	0.210	0.386	0.113	0.879
ChlamyChlamy	0.144	1.000	0.899	0.009	0.345			0.293
EPA								
CryptoCrypto	0.027	0.049	**0.003**	0.968	**0.004**	**0.001**	0.343	0.518
ChlamyCrypto	0.040	0.066	0.024	0.154	0.136			**<0.001**
CryptoChlamy	**<0.001**	0.547	0.070	0.479	0.132	0.268	0.302	0.199
ChlamyChlamy	0.458	0.498	0.523	0.941	0.356			0.363
%EPA								
CryptoCrypto	0.091	0.024	**<0.001**	0.249	0.280	0.019	0.068	0.470
ChlamyCrypto	0.904	0.010	0.106	0.177	0.053			0.010
CryptoChlamy	0.990	0.010	0.046	0.132	**0.001**	0.769	0.481	0.026
ChlamyChlamy	0.319	0.487	0.543	0.309	0.346			0.525
Cho								
CryptoCrypto	0.009	**0.001**	0.453	0.136	**<0.001**	**<0.001**	0.822	0.120
ChlamyCrypto	0.010	0.644	0.207	**0.004**	**0.001**	**<0.001**	**<0.001**	**0.002**
CryptoChlamy	0.610	0.094	0.435	0.051	0.141	0.295	0.026	0.068
ChlamyChlamy	0.100	0.919	0.930	0.104	0.276	0.291	0.026	0.871

FA-total fatty acid content, %SAFA-the share of saturated FA in the total FA content, %MUFA-the share of monounsaturated FA, %PUFA-the share of polyunsaturated FA, UI-unsaturation index, EPA-absolute amount of C20:5n3, %EPA-the share of EPA in the total FA content, Cho-total cholesterol content, Crypto-*Cryptomonas* sp., Chlamy-*Chlamydomonas* sp., acc.-acclimation, exp.-experiment, T-temperature. P of contrast tests is reported, the critical p value is step-wise Bonferroni adjusted and the significant values are printed in bold.

ANNEX I

MULTIPLE COMPARISSON TESTS OF THE LIPID COMPOSITION OF NEWBORN *DAPHNIA MAGNA*

The results of contrast tests for the effects of maternal food and temperature (T) on the lipid content of newborn *Daphnia magna* under the environmental conditions indicated in the parentheses. Animals were fed either *Chlamydomonas* sp. (Chl) or *Cryptomonas* sp. (Chl) and kept at 15 °C or 20 °C for at least three generations. The lipid composition of newborns from different acclimation treatments was compared with contrast tests with or without the assumption of equal variances according to the results of the Levene's test of equality of variances. Note that in the case of unequal variances df are not integers. The critical p was step-wise Bonferroni adjusted and significant results are printed in bold.

	Food (15 °C)			Food (20 °C)			T (Cry)			T (Chl)		
	t	df	p	t	df	p	t	df	p	t	df	p
FA	-4.1	5	**0.010**	-2.7	5	**0.045**	-1.6	5	0.172	-0.6	5	0.585
%SAFA	0.0	1.0	0.991	-6.3	1.3	0.058	1.3	1.0	0.416	-2.9	1.2	0.168
%MUFA	0.2	1.0	0.845	-2.3	1.8	0.164	1.2	1.0	0.445	1.6	2.2	0.240
%PUFA	-0.1	1.0	0.941	12.6	1.1	0.037	-1.2	1.0	0.430	3.2	1.8	0.096
UI	3.6	1.0	0.167	94.4	1.5	**0.001**	-1.4	1.0	0.402	0.9	2.1	0.446
EPA	4.3	6	**0.005**	5.5	6	**0.002**	-2.8	6	0.031	-0.7	6	0.534
%EPA	7.5	1.1	0.073	24.9	1.1	**0.017**	-1.3	1.0	0.420	-1.7	1.8	0.248
Cho	-0.1	7	0.946	1.2	7	0.270	-0.7	7	0.481	0.4	7	0.671

FA-total fatty acid content, %SAFA-the share of saturated FA in the total FA content, %MUFA-the share of monounsaturated FA, %PUFA-the share of polyunsaturated FA, UI-unsaturation index, EPA-absolute amount of C20:5n3, %EPA-the share of EPA in the total FA content, Cho-total cholesterol content

ANNEX J

DISCRIMINANT ANALYSIS OF THE FATTY ACID COMPOSITION OF NEWBORN AND ADULT *DAPHNIA MAGNA*

Discriminant analysis for "age" was run on the percent FA composition (% share of FA in total FA content) of the FAs with more than 0.5 % average share in total FA content of *Daphnia magna.* The results of the SPSS 13.0 analysis are presented below.

Eigenvalues				
Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	20.83	100	100	**0.977**

First 1 canonical discriminant function was used in the analysis.

Wilks' Lambda				
Test of Function(s)	Wilks' Lambda	Chi-square	df	Sig.
1	0.046	148.0	14	**<0.001**

Standardized Canonical Discriminant Function Coefficients	
C14:0	-2.16
C15:0	0.49
C16:0	**4.07**
C16:1n-7	2.06
C18:0	0.87
C18:1n-9	**4.64**
C18:1n-7	**-4.48**
C18:2n-6	0.76
C18:3n-3	-1.39
C18:4n-3	1.49
C20:1n-7	**3.28**
C20:3n-3	-0.35
C20:4n-6	-0.88
C21:0	-0.02

Structure Matrix	
C14:0	-0.02
C15:0	0.07
C16:0	0.16
C16:1n-7	0.22
C18:0	0.09
C18:1n-9	0.02
C18:1n-7	0.09
C18:2n-6	-0.01
C18:3n-3	0.00
C18:4n-3	-0.04
C20:1n-7	0.05
C20:3n-3	0.00
C20:4n-6	0.00
C20:5n-3	-0.02
C21:0	-0.06

Standardized canonical discriminant function coefficients show which of the predictor variables contribute most to the separation between groups. The structure matrix shows pooled within-group correlations between discriminating variables and the standardized canonical discriminant function.

Unstandardized canonical discriminant functions evaluated at group means:

Functions at Group Centroids						
Species	Mean	SD	95% CI		Minimum	Maximum
			Lower Bound	Upper Bound		
newborns	10.4	1.65	9.1	11.6	7.4	12.3
adults	-1.9	0.84	-2.2	-1.7	-3.5	0.3