# The role of food quality for local adaptation in *Daphnia*

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*„Du bist was Du isst.*

*Jeder Stoff, den Du isst, wird im Blut zu Gesinnungsstoff.“*

**Ludwig Feuerbach (1804-72), dt. Philosoph**

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# INTRODUCTION

# INTRODUCTION

## Resources and growth – general limitations

Organisms are made of more than one substance, and all living organisms require resources in order to maintain their metabolism, growth and reproduction. The most prominent factor that leads to a divergent response of species is the available food which can vary both in quantity and quality, influencing species metabolism directly. Contrasting to food quantity, food quality effects have surprisingly been neglected in many ecological and evolutionary studies (Sterner and Elser 2002).

All organisms transform energy, convert elements into organic forms and thereby create a distinct biological, chemical and physical internal environment. The first constraint in biological systems is the abundance and availability of chemical elements, which in many cases both are limited (Williams 1997). As for all living things, the elements that constitute the majority of organic biomolecules show unique chemical properties, and their abundances in biological tissues do not reflect their relative abundance on earth, often referred to as "the evolution of chemical elements by biological systems" (Frausto da Silva and Williams 2001). For example, carbon is present in all known organisms and constitutes the second most abundant element by mass in biological tissues (about 18.5%), but its percent by weight is below one percent in the lithosphere and only 0.04% is bounded as atmospheric $CO_2$ (Frausto da Silva and Williams 2001).

Primary producers such as algae or plants show considerable variation in their elemental composition (Sterner and Elser 2002), contrasting to most consumers with a rather stable elemental body tissue ratio. Thus, the elemental composition of the food often does not match the demands of consumers, so they have to cope with food below their nutritional or energetic requirements. As a result, primary consumers have to adjust their pathways or rates of metabolism in order to balance the variations in resource supply.

The focus of this study lies on the effects of nutrient limitation in freshwater filter-feeders of the genus *Daphnia*, i.e. on the effects of resource shortage. Phosphorus (P) concentration of seston is regarded as the key factor of eutrophication (Schindler 1978) and consequently I tested if *Daphnia* species supplied with food algae limited in phosphorus show a differential response in their life-history traits compared to non-limiting conditions.

Moreover, I studied the evolutionary consequences of food quality limitations to assess the potential for local adaptation in *Daphnia* for this essential resource.

**Evolutionary ecology and natural selection**

In the past, evolutionary studies used phylogenetic and molecular methods to focus on historical processes, such as natural selection, however, these studies often neglected ecological aspects shaping evolutionary processes. On the other hand, ecological studies often explained variation between populations and species solely in terms of contemporary biotic and abiotic environmental effects. In order to bridge these gaps, the field of evolutionary ecology emerged, integrating both, the historical and contemporary mechanisms explaining the origin and maintenance of genetic variation and diversity (e.g. see MacArthur 1964, Pianka 1976, Rosenzweig 1991, Urban et al. 2008). Thus evolutionary ecology explores the functional biological basis at the interface between the fields of ecology and evolution (Hairston et al. 2005, Carroll et al. 2007).

One of the main aims in evolutionary ecology is to reveal the patterns that led to the observed geographical distribution of species; because not all species are distributed all over, but can be found in restricted areas of defined ecological parameters. This pattern has frequently been attributed to local adaptation of species, but the degree to which local adaptation occurs depends on the potential for natural selection to occur (Darwin 1859) as well as the potential for populations to evolve differences from each other. Local adaptation is the result of directional or disruptive selection, and it is one of the central themes in the field of evolutionary ecology because it is a direct consequence of natural selection. Thus it has been accepted as the main mechanism leading to adaptation in biology (Futuyma 1999). Natural selection favours certain genotypes or genetic lineages and directly influences the genotypic composition of a resident population (Endler 1986).

If selection favours different phenotypes in different environments, also the corresponding genotypes become more frequent. However, this is only true when the phenotypic response to selection can be classified as genotypic response, i.e. if it is based on heritable traits. In addition, local adaptation may lead to reproductive isolation if the character states under divergent selection are heritable and associated with mate choice, so that migrating individuals have a reduced mating success compared to the resident, adapted phenotypes (Fox et al. 2001).

In order to test if natural selection occurred, one might compare neutral genetic markers and quantitative traits of species. This is conducted by a comparison against the null hypothesis that variation is selectively neutral. Spitze (1993) developed the idea of testing selective divergence by a comparison of population differentiation in quantitative traits ($Q_{ST}$) with the differentiation obtained from neutral molecular markers ($F_{ST}$). Phenotypic variation for quantitative traits results from the simultaneous segregation of alleles at multiple quantitative trait loci (QTL), i.e. the phenotypes are influenced in degree by the interaction of two or more genes and their interaction with the environment. Three outcomes, each having a unique interpretation, are possible (Merila and Crnokrak 2001): (i) $Q_{ST}$ values exceeds $F_{ST}$ values: this is commonly interpreted as evidence of divergent selection and adaptation to local environments (e.g. see Podolsky and Holtsford 1995, Bonnin et al. 1996, Luttikhuizen et al. 2003, Fox 2004); (ii) $Q_{ST}$ values do not differ from $F_{ST}$ values: here genetic drift alone is sufficient to explain the pattern of detected variation (Yang et al. 1996, Fox et al. 2001), although effects of natural selection and drift may not be indistinguishable in certain cases (Sokal and Wartenburg 1983); and (iii) $Q_{ST}$ values are smaller than the $F_{ST}$ values: here the explanation is the existence of convergent selection favouring the same phenotype in different environments (Kuittinen et al. 1997, Fox et al. 2001, Edmands and Harrison 2003).

All these concepts for the comparison of quantitative traits with molecular markers help to distinguish between the alternative scenarios shaping populations subdivision and to test for directional selection, a pre-requisite for local adaptation. Here I present studies on the evolutionary ecology and local adaptation of the freshwater zooplankter *Daphnia.* I will study the impact of food quality differences to assess the potential for adaptation within this genus. Based on a comparison of quantitative and qualitative traits this will help to understand the different life-history responses of species and to assess the impact of food quality on local adaptation in *Daphnia.*

**The study organism *Daphnia***

Species of the genus *Daphnia* play an important role in freshwater food webs as they link primary production with higher trophic levels. As it was found by genetic methods, the genus *Daphnia* can be subdivided in the subgenera *Daphnia*, *Ctenodaphnia* and the *D. longispina group* (Colbourne and Hebert 1996). Because of the high phenotypic plasticity and the common interspecific hybridization (Flößner and Kraus 1986, Schwenk et al. 2000, Schwenk et al. 2001), the genus *Daphnia* is still not taxonomically resolved, but recent studies

recognize 32 species in Europe (Schwenk et al. 2000, Benzie 2005). However, the genus was subjected to a major revision recently (Petrusek et al. 2008).

*Daphnia* species show a wide distribution and are found in nearly every freshwater system around the globe. Most *Daphnia* species reproduce by cyclical parthenogenesis, i.e. via obligate parthenogenesis. For cyclical parthenogens, parthenogenetic females produce sexual females as well as sexual males when conditions become harsh, e.g. as a response to daylength, temperature, predation, crowding, food quantity or oxygenic stress (Banta and Brown 1929, Carvalho and Hughes 1983, Hobæk and Larsson 1990, Kleiven et al. 1992). The eggs are encapsulated in a carapace structure (ephippium) and are shed off during moulting or decomposition of the carapax. Within the asexual reproduction cycle, *Daphnia* reproduces by amictic parthenogenesis. The females produce subitaneous eggs (clonal lineage), and this form of reproduction is much more frequent in *Daphnia* than the sexual mode of reproduction, resulting in natural populations consisting of clonal lineages (e.g. see Hebert 1978, Hebert and Crease 1983, Hebert 1984, Schwenk et al. 2004, Thielsch et al. 2009). This mode of reproduction also allows the establishment of clonal lineages in the laboratory and testing ecological parameters on animals with identical genetic composition. Combined with its short generation time it represents an ideal candidate for studies on evolutionary ecology. In addition, well established techniques, both for genetic and life-history studies, exist, allowing a deep insight into the evolutionary ecology of *Daphnia*.

For *Daphnia*, several studies exist that integrated genetic and ecological aspects (for a review see Mort 1991). In addition, Spitze (1993) and Lynch et al. (1999) experimentally combined ecological and molecular genetic approaches, and many other studies exist that compared the ambient environmental conditions with species genetics (e.g. see De Meester 1996, Hebert and Taylor 1997, Elser et al. 2000b, Palsson 2000, Pfrender et al. 2000, Schwenk et al. 2001, Brendonck and De Meester 2003, Caceres and Tessier 2003, DeMott et al. 2004).

In this thesis, I will study the evolutionary ecology and local adaptation in the genus *Daphnia* with special attention on food quality differences between habitats as selective factors, focussing on the effect of phosphorus limitation. Up until now several studies on local adaptation were published (e.g. see Carvalho 1984, Bachiorri et al. 1991, Leibold and Tessier 1991, Parejko and Dodson 1991, Pijanowska et al. 1993, Spitze 1993, Teschner 1995,

Mitchell and Lampert 2000, Cousyn et al. 2001, DeClerck et al. 2001), none dealing with the effects of food quality as a selective factor in this genus. This is remarkable as recent evidence has shown that nutrient stoichiometry of food plays a vital role in the success of *Daphnia*.

**Food quality and *Daphnia***

As for all consumers, also for the filter-feeder *Daphnia* the ideal food source would be easily ingestible, digestible and contains all essential compounds matching the nutritional demands of the organism at all developmental stages. Under natural conditions, these requirements are rarely met, and *Daphnia* often encounters food sources of sub-optimal quality causing limited growth.

Several factors that determine food quality in algae are identified which can be classified in size and morphology (DeMott 1995), toxicity (e.g. see Lampert 1982, Nizan et al. 1986, Reinikainen et al. 1994, Jang et al. 2003), nutrient content (e.g. (C)arbon:(P)hosphorus molar ratio (C:P), C:(N)itrogen, Sterner and Elser 2002) and biochemical content (Müller-Navarra 1995a, Müller-Navarra 1995b, Sundbom and Vrede 1997). For all the different variables that determine the quality of a food resource, phosphorus has been suggested to be the main limiting nutrient in most freshwater systems, lakes and rivers (Schindler 1978). It is essential for growth and maintenance, for the metabolism of energy rich compounds (e.g. ATP) and as structural component of phospholipids and DNA (Frausto da Silva and Williams 2001). Studies on phosphorus limitation often use the (C)arbon: (P)hosphorus molar ratio (C:P) to describe the degree of phosphorus limitation compared to carbon content. An increased C:P ratio represents a low P-content of the food item and thus a lower food quality for the consumer.

Compared to other zooplankters, *Daphnia* shows a high requirement for phosphorus because of their high body P-content (C:P ratio= 30, Hessen and Lyche 1991). This makes them more likely to be affected when P becomes scarce and they are assumed to be limited by a C:P ratio above 80-300 (DeMott 1998, Brett et al. 2000, Vrede et al. 2002). At the same time, phosphorus concentrations, or more specifically C:P ratios of the seston, can show large variation between different lakes both in space and time (Elser and Hassett 1994, Kreeger et al. 1997). Numerous studies on food quality effects, especially on P-limited food, were conducted on *Daphnia* (e.g. see Sterner 1993, Müller-Navarra and Lampert 1996, Van Donk et al. 1997, DeMott and Gulati 1999, Boersma 2000, Brett et al. 2000, Elser et al. 2001, Urabe

and Sterner 2001, Becker and Boersma 2003, Weider et al. 2005), and it is know that *Daphnia* can counter-act phosphorus limitation by several life-history adjustments, e.g. by filter screen morphology (Repka et al. 1999a, Repka et al. 1999b) or beat rate adjustment of the filter screens appendices (Plath and Boersma 2001).

Although much is known on the contemporary effects of nutrient limitation and the consequences of food quality limitation in *Daphnia*, only little is known on its potential for adaptation to food with changing qualities. In this thesis, I studied several life-history traits on various organisation levels in *Daphnia,* i.e. between subgenera, species and hybrids as well as for clonal lineages, to reveal the potential for local adaptation to food quality differences in this genus.

**Questions on the role of food quality for local adaptation in *Daphnia***

During the last centuries, many European lakes have undergo severe changes in their ecology because of man-made eutrophication, i.e. an overenrichment with nutrients, but many recovered to their original trophic state due to pollution control (Correll 1998). Only little is known on the response of the zooplankton communities to these fast changing environmental conditions, and we do not know if established populations can keep pace with these rapid changes. Thus we lack information if populations do show local adaptation on food limited in quality and how they cope with changes in nutritional availability. In northern temperate lakes, total phosphorus (P) concentration of seston is regarded as the key factor of eutrophication (Schindler 1978), and I will use this elemental factor as a surrogate for nutritional load in algae applied to *Daphnia* species. This study provides a basis for future hypothesis testing on the ecological mechanisms that influence local adaptation in this genus concerning food quality differences.

Thus I will focus on the influence of food quality both on quantitative and qualitative traits; this will allow the assessment of the impact of phosphorus limitation across several hierarchical levels in *Daphnia*: I will study the response to variation in food quality among (i) subgenera, species and interspecific hybrids, (ii) clones, (iii) and at the molecular level.

More specifically, I want to answer the following questions:

(i) Subgenera, species and interspecific hybrids

- Do *Daphnia* species and interspecific hybrids vary in their response (in life-history traits) to different phosphorus levels of their food?
- Are interspecific hybrids superior at certain environmental conditions?
- Are species different in their susceptibilities to variation in food quality?
- Is there a trade-off between susceptibility to variation in food quality and growth rate at optimal conditions?
- Is the response of species to variation in food quality explained by habitat preferences or phylogenetic history?
- What is the time frame of adaptation to food quality?

(ii) Clonal level

- Do *Daphnia* clones vary in their response (in life-history traits) to variation in food quality?
- Is the response of daphniids to food quality explained by habitat differences?
- Is variation in life-history traits between populations explained by directional selection?

(iii) Molecular level

- What is the molecular basis for a differential response according to food quality differences in *Daphnia*?
- Which genes show a differential expression pattern under various food quality conditions?

**Thesis outline**

My thesis examines the role of food quality for local adaptation in *Daphnia* covering several hierarchical levels: subgenera (chapter one), species (chapter two) and clones (chapter three), as well as the molecular level (chapter four).

In chapter one I report about a life-history experiment conducted for twelve *Daphnia* species which belong to three different subgenera. I studied somatic growth rate differences on P-sufficient and P-limited algae to investigate differences between subgenera and species. In addition, I revealed a trade-off in susceptibility to food quality changes and growth at optimal conditions. A phylogenetic contrast analysis (PIC) showed potential associations between species-specific habitat preferences and their response to variation in food quality.

To understand the impact of different food qualities for a hybrid complex in *Daphnia*, I conducted life-history experiments with clones of *Daphnia galeata*, *Daphnia cucullata*, and their interspecific hybrids and measured fitness-related life-history traits at two food quality conditions (chapter two). The results of the single-clone life-history studies were confirmed by a multi-clone experiment. All clones were inoculated in an experimental tank supplied with a diet of either P-limited or P-sufficient algae. After several generations, the frequency of taxa was determined by molecular methods. These experiments allowed the assessment of the impact of food quality differences on hybrid maintenance in *Daphnia*.

In chapter three, I applied a combined approach of ecological and genetic analyses to reveal the potential for local adaptation to food quality differences in *Daphnia*. I describe the variation for quantitative traits and molecular markers estimated within and among four lake populations of *Daphnia galeata* representing two different types of habitat. I studied the reaction norm for susceptibility to variation in food quality and compared it to the genetic differentiation based on microsatellite analysis using six polymorphic loci. A comparison of both measurements, i.e. $Q_{ST}$ and $F_{ST}$, allowed the differentiation between genetic drift and natural selection, and shows whether the precondition for local adaptation (i.e. directional selection) occurs in *Daphnia*.

To understand the impact of food quality differences at the molecular level (chapter four), I addressed the molecular basis for a differential response to variation in food quality. A clone of the microcrustacean *Daphnia magna* was subjected to phosphorus-rich and P-limited

algae and somatic growth rate was measured. In addition, cDNA of experimental animals were subjected to DD-PCR and fragments of up- or down-regulated loci were sequenced after DNA cloning. With these methods I found and analysed candidate genes associated with a change in somatic growth rate due to food quality differences in *Daphnia*.

The general discussion highlights the results in the light of microevolutionary change in *Daphnia* and provides suggestions for further research directions.

# CHAPTER 1: EVOLUTIONARY CONSTRAINTS AND TRADE-OFF: SUSCEPTIBILITY OF *DAPHNIA* SPECIES TO PHOSPHORUS-LIMITED

# Chapter 1: Evolutionary constraints and trade-off: susceptibility of *Daphnia* species to phosphorus-limited diets

## 1.1 Introduction

The field of ecological stoichiometry deals with the balance of energy and multiple chemical elements in ecological interactions (Sterner and Elser 2002), and stoichiometrical considerations have lead to several interesting and testable hypotheses. The most prominent question in this context is the limitation of essential elements, that is the elemental composition of the food is below the nutritional demands of the consumer. Stoichiometric theory makes predictions about the relationship between a consumer's element contents and two key traits. It is assumed that maximal growth rate and the sensitivity to element deficiency are interrelated (Sterner and Hessen 1994). The first relationship, the phosphorus content of the food and the growth rate of the consumer, is summarized in the *growth rate hypothesis* (GRH). Assuming that P-rich ribosomal RNA is the largest repository of phosphorus in an organism, somatic growth rate will be directly affected by P-limitation (see also Vrede et al. 2004). Thus, when P-levels are below the nutritional demands of a particular species, reduced growth rates and enzyme activities will follow. As these considerations imply that the P-content of organisms is correlated with their growth rate, recent studies have broadened the stoichiometric concept for use in evolutionary studies (reviewed in Elser et al. 2000b).

The GRH predicts that organisms with high body P-content also show a higher RNA content and higher growth rates when supplied with P-sufficient food than organisms with lower P-contents (Elser et al. 1996, Acharya et al. 2004). Secondly, those organisms which show high P-demands at maximal growth rates should do poorly when resources are P-deficient, i.e., they should face a trade-off between maximum performance and sensitivity to changes in phosphorus supply (Sterner and Hessen 1994).

Many studies on various aquatic and terrestrial species indicate that animal growth rate, rRNA allocation, the transcription of several genes as well as P requirements are inherently associated (Schulz and Sterner 1999, DeMott et al. 2001, Kay et al. 2005, Elser 2006), but a growing number of studies failed to verify these associations (DeMott et al. 2004, DeMott and Pape 2005). For example, DeMott and Pape (2005) studied the interactions

between body P-content, growth rate and habitat preference for several species of *Daphnia* and observed no relationship of body P-content in the *Daphnia* and phosphorus content of their food. Moreover, they did not observe higher sensitivities of taxa with higher body P to food with low phosphorus content. In consequence, they proposed that other factors than body P-content are responsible for variation in somatic growth rate and sensitivity to variation in P-content of food items. The inconsistent results for *Daphnia* might be explained by the gap between ecologically and evolutionary motivated approaches in stoichiometry (Sterner and Elser 2002, Elser 2006). However, recent phylogenetic studies, for example on insects (Fagan et al. 2002) or fishes (Hendrixson et al. 2007), have shown that organismal stoichiometry shows a strong phylogenetic signal (Kay et al. 2005), i.e., the body C:N:P ratio of species is explained by their evolutionary history. Physiological studies have shown that *Daphnia* species differ in their elemental composition, i.e. P-content (Hessen and Lyche 1991, Weider et al. 2004). Although these differences have been associated with the length of ribosomal rDNA spacer (Crease and Lynch 1991, Gorokhova et al. 2002, Weider et al. 2004), however, the underlying evolutionary processes are controversially discussed (Sterner and Hessen 1994, DeMott and Pape 2005).

In contrast to the studies on organismal stoichiometry which already have implemented evolutionary approaches, we lack this level of integration in studies on the response of species to variation in food quality in *Daphnia,* but see the noticeable exceptions of Weider et al. (2005), Seidendorf et al. (2007) and Tessier and Woodruff (2002b). Several field and laboratory studies have shown that *Daphnia* species vary in their life-history traits if they are subjected to food sources of different quality, in particular if the key element phosphorus is limiting (e.g. see Urabe et al. 1997, DeMott 1998). As a consequence, it is to be expected that species from different habitats, also differ in their phosphorus demands and thus exhibit different maximum growth rates (Tessier and Leibold 1997, Tessier et al. 2000). For example, the advantage of high growth rates and the sensitivity to changes in food quality can differ between lakes of different size, depth and food structure (Tessier and Woodruff 2002a, 2002b). Thus, interspecific variation in P-content of *Daphnia* species might represent different P requirements and might explain ecological differentiation. On the other hand, physiological demands might reflect primarily the phylogenetic history of species, rather than a response to selection. It still remains unclear whether species responses to variation in food quality (in terms of phosphorus content) are explained by their evolutionary history and possible phylogenetic constraints, or whether these responses are associated more with their

ecological niche, and local adaptation. Here we present a phylogenetically comparative approach testing 12 *Daphnia* species from deep to shallow to temporary lakes of three different subgenera for variation in key life-history parameter i.e. somatic growth rate (SGR) and susceptibility to phosphorus changes in food quality. Hairston et al. (2001) suggested that resistance to harsh conditions might have evolved as a decrease in phenotypic plasticity and an increase in growth rate at limiting conditions, in comparison to other species which show a broader range of phenotypic plasticity but a higher sensitivity to changing conditions. Although there is strong evidence on specialization to resource environments in *Daphnia* (Tessier and Woodruff 2002a, 2002b), we still lack a comparison of different species on phosphorus limitation. We test the hypothesis of Hairston et al. (2001) by comparing the susceptibility to food quality changes with the somatic growth rate at high-P and P-limited food conditions among 12 *Daphnia* species of 3 different subgenera. Specifically, we focus on the following questions: 1) is the response to varying food quality conditions among *Daphnia* subgenera and species associated with their phylogenetic history or linked to habitat preferences? and 2) Is somatic growth rate under optimal food conditions associated with a different susceptibility to variation in food quality?

## 1.2 Material and Methods

### *Taxon selection*

Most species of the genus *Daphnia*, apart from a monotypic subgenus *Australodaphnia* with a restricted distribution, belong to three major monophyletic and widely distributed taxonomic groups (Adamowicz et al.), a highly diversified subgenus *Ctenodaphnia*, and two large species groups within the subgenus *Daphnia* – the *D. pulex* group and the *D. longispina* group (often denoted as a separate subgenera, *Hyalodaphnia* and *Daphnia*). Four lineages of each of these three groups (some of them representing yet undescribed cryptic lineages), all occurring in the Western Palaearctic region, were subjected to a food quality experiment (table 1).

Table 1: *Daphnia* species and taxonomic affiliations (subgenera in bold) and environmental parameters used for phylogenetic independent contrast analysis (for more information see material and methods). All = *Daphnia* found in all types of habitats, Habitat type: Pu = puddle, Po = pond, La = lake, Re = reservoir, Habitat permanency: P = permanent, T = temporary, Habitat size: S = small, M = medium, L = large, Water transparency: C = clear, T = turbid, Fish: A = absent and P = present. Names of undescribed or nomenclaturally problematic cryptic species are enclosed in quotation marks. European *D.* "*pulicaria*" corresponds to *D.* gr. *pulicaria* sp2, and *D.* "*obtusa*" to *D.* gr. *obtusa* sp3 in Adamowicz et al.; *D.* "*atkinsoni*" is a distinct lineage different from *D. atkinsoni* sensu stricto. Nomenclature of *D. longispina* follows Petrusek et al. (2008).

| | Habitat | Permanency | Size | Water colour | Fish |
|---|---|---|---|---|---|
| ***Ctenodaphnia*** | | | | | |
| *D. magna* | All | P, T | S, M, L | C, T | A, P |
| *D. similis* | Pu, Po | T | S | C | A |
| *D. lumholtzi* | Po, La, Re | P, T | S, M, L | C | A, P |
| *D. "atkinsoni"* | Pu, Po | T | S, M | C, T | A, P |
| ***D. pulex* group** | | | | | |
| *D. pulex* | Po | P, T | S, M, L | C | A, P |
| *D. "pulicaria"* | La, Re | P | M. L | C, T | A, P |
| *D. obtusa* | Pu, Po | T | S | C, T | A |
| *D. "obtusa"* | Pu, Po | T | S | C, T | A |
| ***D. longispina* group** | | | | | |
| *D. galeata* | La, Re | P | M. L | C, T | A, P |
| *D. cucullata* | La, Re | P | M. L | T | P |
| *D. curvirostris* | Po | P, T | S, M, L | C | A, P |
| *D. longispina (hyalina morph)* | Po, La, Re | P, T | S, M, L | C, T | A, P |

We used one clone per species, although we were aware of the potential clonal variation affecting the measured traits. However, in a previous study focusing on closely related sister species (Seidendorf et al. 2007), we observed that interspecific differences in *Daphnia* measured on life-history traits affected by food quality differences exceeded the clonal variation. Thus, if strong phylogenetic or habitat-dependent signal exists within the

variation of the measured traits, we should be able to find this even with one clone per species. All clones had been kept for at least 5 generations under identical conditions in the experimental medium (see below) in order to minimize maternal effects.

**Life-History Experiments**

For the food quality experiment, we used semi-continuous cultures of *Scenedesmus obliquus* which were established in Z/4 medium (Zehnder and Gorham 1960) with sufficient phosphorus or with limited phosphorus content generated in a similar way as in Becker and Boersma (2003). These cultures resulted in algal cells with a molar carbon to phosphorus (C:P) ratio of 70 - 80 for P-rich cells ($P^+$) and about 1000 for P-limited algae ($P^-$). Every day, 700 mL (total volume: 1.5 L) of culture medium was replaced with fresh medium: algae were centrifuged at 5000 rpm for 10 min and diluted in phosphorus-free medium (AdaM, Kluttgen et al. 1994). C-content of the cultures was measured photometrically using a calibration curve for both culture conditions. The calibration curve was established by measuring the extinction of different algae suspensions at 800 nm using a spectrophotometer (Hitachi, U-2000). For each dilution, C-content was measured subsequently by filtration of algae onto precombusted 24 mm diameter glass-fiber filters (Whatman GF/C) and C-content was quantified by a CHN-analyzer (Perkin Elmer). P-content of algae was measured spectrophotometrically after digestion with potassium persulfate (Langner and Hendrix 1982).

All experimental *Daphnia* cultures were kept at 18°C with a light:dark cycle of 16:8 h. Before starting the experiments, each species was adjusted to ADaM medium for at least five generations. Juvenile animals were collected from stock cultures and placed into 250 mL jars filled with ADaM medium and fed 1 mg C $L^{-1}$ of $P^+$-algae to guarantee a food supply above the incipient limiting level (Lampert 1987). We inoculated 10-15 neonates (≤ 24 h old) in 250 mL experimental vessels which were placed in a flow-through system. Both algal suspensions (P-sufficient and P-limited algae) were set to 1 mg C $L^{-1}$ and the flow rate through the chambers was set to 55 mL $h^{-1}$, resulting in a replacement rate of total chamber volume of about 5 $d^{-1}$. Each culture condition was represented by 5 replicates each.

**Data analysis**

To obtain the somatic growth rate (SGR) of all experimental animals, the initial weight was measured on a cohort of newly born animals (≤ 24 h), as well as the weight after 4 days of incubation under experimental conditions. Animals were dried at 65°C and weighed on a

microbalance (Sartorius 4503 micro). SGR was calculated following the formula SGR = $\ln(M_t/M_0)/t$, where $M_0$ is the average initial mass per individual and $M_t$ is the mass of the animals at time t (Sterner and Elser 2002).

We used the approach described in Hairston (2001), estimating two measures of susceptibility: first, we calculated a simple difference between SGR at optimal and limiting conditions (dSGR = SGR[$P^+$] - SGR[$P^-$]). SGR[P+] is the growth rate at high P conditions, and SGR[P-] represents the somatic growth rate at P-limited conditions. Secondly, we measured the differences on log-transformed values of SGR ($dSGR_{log}$ = logSGR[$P^+$] - logSGR[$P^-$]). Hairston (2001) showed that scaling of reaction norms is critical for interpreting data on the evolution of phenotypic plasticity. Ideally, a reaction norm should reflect the change in fitness associated with the alternate phenotypes induced by a change in environment, here food quality. If the fitness of species is a linear function of dSGR, it is appropriate to investigate these differences by carrying out graphical or statistical analysis using $dSGR_{log}$, which is the same as analyzing the ratio of dSGR. However, if the fitness of a species is an exponential function of dSGR, it is appropriate to use dSGR directly without any transformation. Since we have no a priori information on the function relating the growth rate and fitness, we estimated and compared both dSGR and $dSGR_{log}$.

All experimental data were analyzed by ANOVA. Species were nested within subgenus and were implemented as random factor within the model, all other effects were treated as fixed factors (Statistical model: *subgenus + food quality + species (subgenus) + subgenus*food quality + species (subgenus)*condition*). The *F*-values and the accompanying degrees of freedom (df) were calculated according to the methods described in Satterthwaite (1946). Differences among subgenera were tested *a posteriori* conducting a Fisher-LSD test using type III GLM.

**Phylogenetic independent contrast analysis (PIC)**

Phylogenetic independent contrast analysis (PIC) allows the comparison of characters of species, such as morphology or life-history traits that are phylogenetically dependent (Harvey and Pagel 1991). One of the most common applications is to determine if two traits are associated after correcting for their evolutionary history. Here we test for an association of life-history traits (SGR) and habitat structure of closely related *Daphnia* species. In order to conduct a PIC, DNA sequences of the 12S ribosomal subunit (12S rDNA) of *Daphnia* species

were either obtained from current sequencing efforts (Moritz Salinger, personal communication), or were downloaded from GenBank. Phylogenetic reconstruction of *Daphnia* species was based on the Bayesian inference of phylogeny performed by MrBayes v. 3.2.2 (Ronquist and Huelsenbeck 2003) based on combined information from sequences of fragments of mitochondrial genes for 12s rRNA, cytochrome c oxidase subunit 1 (COI) and, for some taxa, also the mitochondrially encoded NADH dehydrogenase (ND2). We used the general time reversible model of sequence evolution with among-site rate variation following a gamma distribution, enforced the molecular clock, and evaluated support at nodes with posterior probabilities generated in MrBayes. Sequences of two other related cladoceran genera, *Ceriodaphnia* and *Moina*, were used as outgroups.

To evaluate the potential effects of environmental conditions on the susceptibility of *Daphnia* to food quality variation, we selected various key habitat characteristics (locality type and size, permanency, water turbidity, fish presence) and split each into two to several categories (Table 1). We then assigned these categories for each species used in the experiment, based on literature records (which could be reliably linked to the taxon in question) and field observations. These environmental data were then correlated with direct (dSGR) and indirect measurements ($dSGR_{log}$) for the susceptibility to phosphorus-limited food after correcting for phylogenetic history. In addition, the same calculation was done without phylogenetic correction. Comparative analysis was achieved by the PDTREE module of Mesquite (Midford et al. 2005, Maddison and Maddison 2006). All assumptions for phylogenetic contrast analysis were tested according to the PDTREE protocol.

### 1.3 Results

All *Daphnia* species responded with a significant reduction in SGR at limited phosphorus conditions (table 2, figure 1) compared with SGR at P-sufficient supply. Overall, we observed neither a significant subgenus nor a significant species effect on somatic growth rates (table 2). In addition, we did not detect a differential response (no interaction of and the subgenera with the food quality) of the three subgenera. However, species responded differentially under both food conditions (table 2; *"species"*"food quality"* interaction).

A post-hoc comparison of the three different subgenera derived from the ANOVA analysis on SGR showed a more differential pattern. All subgenera were significantly different from each other at P-sufficient, but not at P-limited conditions (table 3). Interestingly, they responded with large differences in their variation to food quality (figure 1).

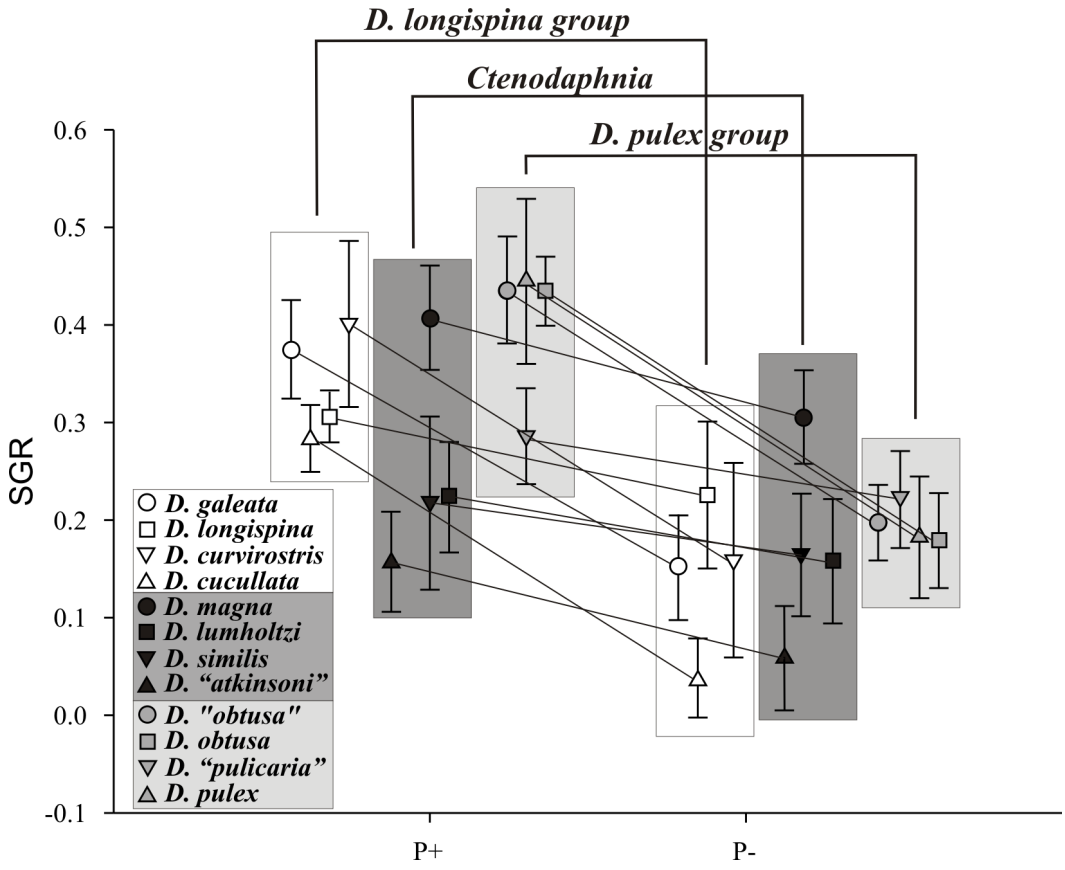


Figure 1: Reaction norms for 12 *Daphnia* species, raised under P-limiting (P-) and P-sufficient (P+) conditions. Species are grouped according to their phylogenetic affiliations to the three subgenera *D. longispina* group (white), Ctenodaphnia (dark grey) and *D. pulex* group (light grey). Error bars indicate 95% confidence limits.

The *D. longispina* group showed higher variation between species when fed P-limited algae, whereas for the other two groups variation between species was higher on P-sufficient food levels.

Table 2a.) Results of an ANOVA analysis of somatic growth rate (SGR) among 12 *Daphnia* species, species were nested within the subgenera. Species(subgenera and *Species(subgenera)*food quality* have been treated as random factors, all others were set as fixed, 2b.) ANOVA analysis of susceptibility to variation in food quality. Both types of calculations, dSGR and dSGRlog (for details see Material and Methods) are shown, species were nested within subgenera and provide a fully nested statistical design. Significant results are emphasized in bold letters ($P < 0.05$). SS = sum of squares, *df* = degrees of freedom, MQ = mean squares

a.)

| | SS | df | MQ | *F* | *p* |
|---|---|---|---|---|---|
| subgenus | 0.158 | 2 | 0.079 | 1.81 | 1.809 |
| food quality (P+/P-) | 0.768 | 1 | 0.768 | 40.31 | **<0.001** |
| Species (Subgenus) | 0.393 | 9 | 0.044 | 2.29 | 0.116 |
| Subgenus *food quality | 0.088 | 2 | 0.044 | 2.31 | 0.155 |
| Species (Subgenus)*food quality | 0.171 | 9 | 0.019 | 3.00 | **0.003** |
| Error | 0.610 | 96 | 0.006 | | |

b.)

| | SS | df | MQ | F | p |
|---|---|---|---|---|---|
| ***dSGR*** | | | | | |
| species(subgenus) | 0.362 | 9 | 0.040 | 5.52 | **<0.001** |
| subgenus | 0.090 | 2 | 0.045 | 1.14 | 0.360 |
| ***dSGRlog*** | | | | | |
| species(subgenus) | 1.823 | 9 | 0.203 | 2.31 | **0.035** |
| subgenus | 0.137 | 2 | 0.218 | 1.09 | 0.375 |

When species were nested within subgenera and treated as random factors, only the species were significantly different (using both measures of dSGR; table 2b). However, ignoring the hierarchical structure, we found a significant subgenus effect (df=2, MQ= 0.048, F=3.49, p=0.038).

Table 3: Results of the *post hoc* comparison tests of the somatic growth rate among three subgenera of *Daphnia*, derived from an ANOVA analysis on their somatic growth rate. Significant results are emphasized in bold letters ($p < 0.05$). Lower matrix shows p-values derived under P-sufficient conditions, upper matrix represent p-values derived under P-limited conditions.

| | *Ctenodaphnia* | *D. pulex group* | *D. longispina group* |
|---|---|---|---|
| *Ctenodaphnia* | | 0.448 | 0.266 |
| *D. pulex group* | **0.001** | | 0.063 |
| *D. longispina group* | **<0.001** | **0.022** | |

Furthermore, we detected a relationship between the susceptibility of the 12 *Daphnia* species to variation in food quality (dSGR) and somatic growth rate under optimal food quality conditions (SGR P+; figure 2). Species which showed a rather small SGR at optimal food quality conditions showed low susceptibilities at high food quality changes, whereas species with a high somatic growth rate at P-sufficient conditions exhibited a higher susceptibility to food quality changes. Species were grouped (figure 2, circles) based on their susceptibility to changes in food quality (high or low susceptibility). Both groups differed significantly ($p > 0.05$) in their SGR at P-sufficient conditions.

In a next step, we correlated the habitat characteristics given in table 1 with the measurements of susceptibility (dSGR and SGR) without a phylogenetic correction. We observed no significant correlations here, i.e., none of the given habitat parameters explained the differences in dSGR (all p-values <0.05). In order to conduct an analysis of a

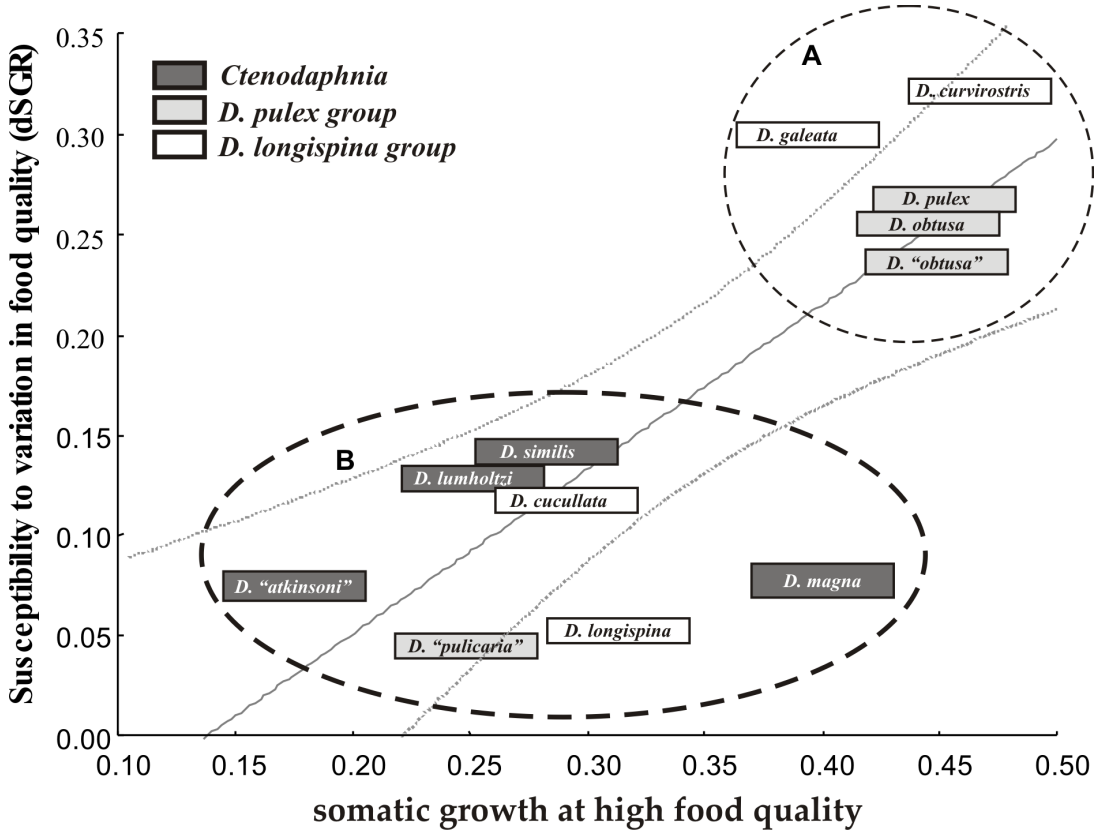


Figure 2: Relationship between the susceptibility of 12 *Daphnia* species to variation in food quality (dSGR) and somatic growth rate under optimal food quality conditions (SGR P+). Average values per species (represented by species names) are based on two to five clonal replicates ($R^2 = 0.578$; $P = 0.002$). Species were grouped (circles) based on their susceptibility on changes in food quality (high or low susceptibility). Both groups differed significantly in their SGR at sufficient P-conditions ($t = 6.9$, $P < 0.001$). Subgenera classification of species is depicted by boxes (white = *D. longispina group*, dark grey = *Ctenodaphnia*, light grey = *D. pulex group*).

phylogenetically corrected data set with the measurements of susceptibility (phylogenetic contrast analysis), we reconstructed the relationships among the 12 *Daphnia* species used in the experiment based on a phylogenetic analysis using mitochondrial DNA (figure 3) resulting in a branching pattern that was in concordance with previously published analyses (Colbourne and Hebert 1996, Schwenk et al. 2000, Adamowicz et al., Petrusek et al. 2008).

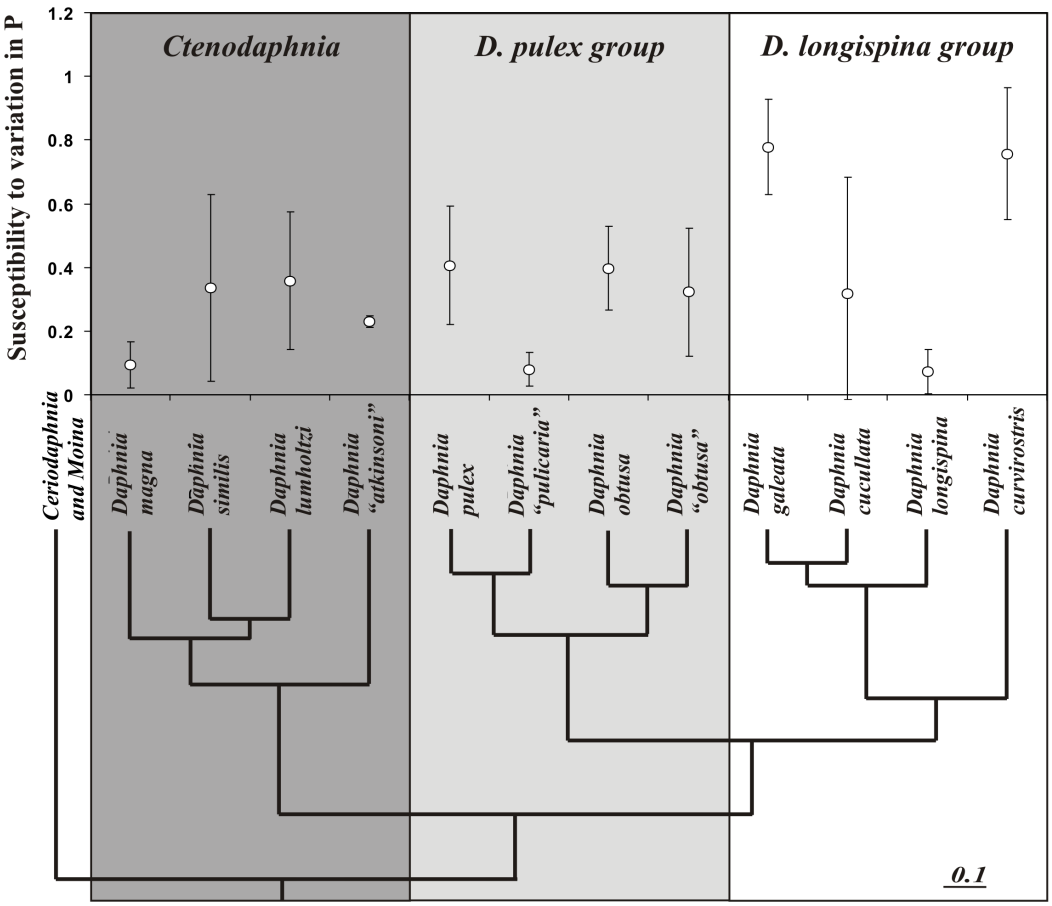


Figure 3: Combined plot of species susceptibility to changes in P with their phylogenetic history. Species from each taxonomic group are grouped by boxes (white = *D. longispina* group, dark grey = *Ctenodaphnia*, light grey = *D. pulex* group). Phylogenetic reconstruction is based on a Bayesian approach using 12S rDNA, COI and ND2 sequences.

Prior to the phylogenetic contrast analysis, we tested all inherent assumptions according to the manual instructions of PDAP (Midford et al. 2005, Maddison and Maddison 2006), a software module that analyses data by the method of phylogenetically independent contrasts (PIC), as described by Felsenstein (1985). PDAP includes a series of diagnostics to check the inherent assumptions of PIC, thus all assumptions were tested within the software module. When analyzing data by the method of see phylogenetically independent contrasts

branch lengths need to be statistically adequate (e.g., see the discussion in Garland et al. 1991, Garland et al. 1992, Blomberg et al. 2003). Analyzing branch length, we found no significant correlation of absolute values of the standardized PIC versus their standard deviations and branch lengths needed to be transformed. The software module can be used to transform branch lengths to the arbitrary branch lengths of S. Nee (Purvis 1995), and transformation was achieved according to the published protocols (Midford et al. 2005, Maddison and Maddison 2006). Branch length transformation was successful only for dSGR but not for $dSGR_{log}$ thus $dSGR_{log}$ was not considered for further analysis. Only one significant regression, between dSGR and water color ($P = 0.017$) was found.

## 1.4 Discussion

The response of *Daphnia* species to food quality differences does not reflect their phylogenetic history, since species differences are significantly larger than differences among subgenera (table 2b). These results are in concordance with previously published data (Tessier and Woodruff 2002b), however their study was based on a limited number of species and food of unknown P-content. We tested for an association between environmental parameters (table1) and susceptibility to food quality changes using phylogenetically corrected and un-corrected data sets. Susceptibility to variation in food quality showed only a significant relationship with water colour ($p = 0.017$), however, because of only two character states, this might be of stochastic nature. The failure to detect any explanatory variables among the habitat characteristics might indicate that the ecological parameters were not sufficient to characterize the species specific habitats, or that ecological differentiation in life-history traits represents a random process. Furthermore, we did not include a parameter that is directly linked to phosphorus content of seston. This lack of any significant phylogenetic signal at the subgenus level may indicate that, in contrast to the traditional view on *Daphnia* evolution, the main ecological differentiation among species was acquired much later (2 - 30 Myr, see also Colbourne et al. 1997) than the split into three subgenera (100 - 900 Myr, Schwenk et al. 2000).

In concordance with previous studies, we showed that twelve different *Daphnia* species collected across a broad habitat range differed significantly in SGR due to limited phosphorus supply (table 2a, e.g. see Gulati and DeMott 1997, Boersma 2000, Plath and Boersma 2001, Becker and Boersma 2003, Seidendorf et al. 2007). Although our experimental design based on two different food qualities reflects only a fraction of the natural variation in food quality, our results indicate that species respond with up to a 3-fold difference in somatic growth rates (figure 1). The three major species groups represented in our experiment did not differ in their response to food quality, i.e., we observed no *subgenus* nor a *subgenus*food quality* interaction (table 2a). However, although they differed from each other at P-sufficient conditions (table 3), no significant differentiation was found at P-limited conditions. In addition, differences between both measurements, dSGR and dSGRlog were only marginal in our study. Since SGR as any fitness surrogate is much more likely to be represented by a saturation function, we tend to support of a non-linear relationship between fitness and SGR (Hairston et al. 2001).

It is know that freshwater habitats represent not only distinct regimes according to their predator selective forces (Zaret 1980), but also differ substantially with respect to food quality (Tessier and Woodruff 2002a, 2002b). Shallow and temporary habitats are much richer in food quality than deep and permanent lakes, but food qualities vary seasonally, and zooplankton is likely to be physiologically challenged by these distinct seasonal shifts (Kreeger et al. 1997). The high resource quality in shallow lakes is an indirect consequence of greater mortality on grazers in such lakes (Tessier and Woodruff 2002a), due to high nutrient recycling (Jeppesen et al. 1997). Taxa from deep lakes, which are adapted to a reduced resource availability, do poor in rich resource environments compared to those which originate from non-limited habitats (Tessier and Woodruff 2002b). We showed that species which are typical grazers in resource limited habitats (table 1) like *D. pulicaria, D. longispina, D. cucullata, D. lumholtzi* show all the same reduced susceptibility to changes in food quality. In addition, these species are characterized by only moderate changes to their low somatic growth rates at high food qualities (figure 2). For *D. lumholtzi* and *D. cucullata* we know that they commonly occur in relatively eutrophic habitats with high fish predation, and it is known that these two taxa cope with predation pressure differently (Pijanowska 1991, DeClerck and De Meester 2003, Dzialowski et al. 2003), although they apparently share their way of response on food quality differences. In contrast, species of shallow or temporary ponds like *D. pulex* or *D. obtusa*, showed a high somatic growth rate at optimal food conditions, facing a trade-off in higher susceptibility to variation in food quality (figure 2, group A). Interestingly, *D. magna* showed a high somatic growth rate, but also a rather low susceptibility to changes in food quality. This advantage, combined with a larger clutch size than most of the other species tested here (Ebert 1993, Boersma 1997), might contribute to its success as a very versatile (and extremely widespread) species. However, we know that *D. magna*can hardly be found in freshwater system with size-selective predation pressures, that is although they can be found in Holartic, Oriental, and Ethiopian biogeographic regions, *D. magna* will not be the dominating species in these types of habitat.

On a species level, susceptibility to variation in food quality among the different *Daphnia* species was found to be associated with somatic growth rate at optimal food quality conditions, and it is known that variation in sensitivity is largely independent of body size in daphniids (Tessier and Woodruff 2002b). If species show a relatively high somatic growth rate, they also show a higher susceptibility to changes in food quality (figure 2). Conversely, those which show a rather low somatic growth rate have a reduced susceptibility on food

quality changes as it was proposed by Sterner and Hessen (1994). This is in concordance with previously published data (Tessier and Woodruff 2002b), and trade-offs in sensitivity or efficiency of resource use may be central to understanding diversity in consumer–resource interactions (Tessier et al. 2000), especially when food is phosphorus limited.

Our study uncovered the trade-off between the species susceptibility to food quality changes and growth rate at optimal conditions. Fast growing species are faced with a higher susceptibility on food quality changes as proposed by Sterner and Hessen (1994), which was uncoupled of species phylogeny. Differences between species were not explained by ecological parameters on phylogenetically corrected data sets. Hairston et al. (2001) proposed that a resistance to limiting conditions might have evolved as a decrease in phenotypic plasticity and an increase in growth rate at limiting conditions in *Daphnia*. We hypothesise that a low susceptibility on food quality changes will explain a resistance to harsh conditions and helps to explain species distribution in the wild, which is consistent with an adaptive match of exploitation ability to specific phosphorus levels in *Daphnia*.

In summary, we found no phylogenetic association or phylogenetic constraints for *Daphnia* according to food quality differences on somatic growth rate. In addition, we found no association of somatic growth rates with environmental parameters, but a trade-off between the susceptibility to food quality changes and growth at optimal conditions. We conclude that the ecological differentiation of species is consistent with an adaptive match of exploitation ability to the specific resource conditions. We encourage further studies using controlled food quality conditions with additional ecological parameters, i.e. predation, temperature and parasite load. These experiments will help to elucidate the impact of food quality differences for the ecological differentiation in *Daphnia*.

# CHAPTER 2: EVOLUTIONARY STOICHIOMETRY: THE ROLE OF FOOD QUALITY FOR CLONAL DIFFERENTIATION AND HYBRID MAINTENANCE IN A *DAPHNIA*

# Chapter 2: Evolutionary stoichiometry: The role of food quality for clonal differentiation and hybrid maintenance in a *Daphnia* species complex

## 2.1 Introduction

It has long been thought that interspecific hybridization among animal species represents a rare phenomenon (Mayr 1963). Recent studies, however, have shown that interspecific hybridization is in fact fairly common and contributes significantly to evolutionary changes (Harrison 1990, Grant and Grant 1992, Bullini 1994, Dowling and Secor 1997, Seehausen 2004). The origin and establishment of hybrid lineages can occur rapidly within a few generations, which allows evolutionary biologists to study a number of basic ecological and genetic processes under natural conditions (i.e., reproductive isolation, ecological differentiation, and speciation). Consequently, interspecific hybridization has become a major research field in evolutionary biology and molecular ecology (Hewitt 1988, Harrison 1990, Seehausen 2004). Well isolated populations on islands or in lakes and ponds offer a unique opportunity to study the consequences of interspecific hybridization in syntopic populations which lack any geographic isolation or gradients. In particular, the analysis of ecological differentiation among these hybridizing taxa allows the analysis of exogenous factors responsible for the origin and maintenance of hybrid lineages (e.g. Grant and Grant 1994, Bert and Arnold 1995, Turgeon et al. 1999). Cyclic parthenogenetic organisms are especially suited to study hybridization processes, as their reproductive mode allows the experimental differentiation between exogenous and endogenous selection. Studies on interspecific hybridization among several cladoceran taxa, mainly *Daphnia* species, showed that parental taxa co-occur over large areas, populations are well isolated and interspecific hybrids are found frequently in syntopy with at least one parental species (Wolf 1987, Hebert et al. 1989, Schwenk and Spaak 1995). However, species are genetically well differentiated despite frequent interspecific hybridization and backcrossing (Schwenk et al. 2000).

Most attempts to explain the origin and maintenance of interspecific hybrids can be classified as derivates of two different types of models: First, *tension zone models* explain the formation of hybrids by a balance between dispersal of parental species into a hybrid zone and the subsequent selection against interspecific hybrids. This selection against hybrids is mainly attributed to endogenous factors, such as genetic incompatibilities between parental genomes. In contrast, *cline or ecotone models* are based on exogenous factors, i.e., environmental

gradients, which determine the origin and fate of interspecific hybrids (e.g. Endler 1977, Arnold 1997). Both types of models have been used to explain hybrid maintenance in many plants and animal taxa (e.g. see Arnold 1997, Rieseberg 1997, Avise 2000, Scribner et al. 2000, Seehausen 2004). Among species of the microcrustacean genus *Daphnia*, a number of studies described that under certain environmental conditions interspecific hybrids exhibited a higher relative fitness (e.g., intrinsic rate of increase) than parental species (Spaak and Hoekstra 1995, Repka et al. 1999b, DeClerck and De Meester 2003). This phenomenon motivated Spaak and Hoekstra (1995) to propose the *temporal hybrid superiority* (THS) model. This model assumes higher fitness for interspecific hybrids only during certain periods of the year when several environmental conditions are met. This model is a derivative of a cline model (i.e., bounded hybrid superiority model) which defines fitness among parental species and interspecific hybrids based on exogenous factors (Moore 1977). The THS model is supported by recent field data, which demonstrated temporal dominance of interspecific hybrids during a season (DeClerck and De Meester 2003). In addition, a number of life-history studies provided evidence for different environmental factors that are responsible for hybrid maintenance, such as variation in fish predation (DeClerck and De Meester 2003), temperature (Weider and Wolf 1991) and food quantity (Boersma and Vijverberg 1994b, 1994c). Here we tested the question if food quality differences, which often occur during a season in lakes (Kreeger et al. 1997), help to explain the maintenance of hybrid lineages.

Although low food quality is known to affect fitness in *Daphnia* adversely (Boersma 2000) and food quality plays an important role explaining community structure and population dynamics of zooplankton (Elser et al. 2000a, Sterner and Elser 2002), the effect of food quality has so far not been tested in the framework of studies on interspecific hybridization. One of the best studied determinants of food quality in freshwater environments is phosphorus (P) content of food particles, since it is essential as a component of proteins, nucleic acids, lipids, and energetic nucleotides (Sterner and Elser 2002). It represents one of the limiting factors for growth in freshwater zooplankton species (Scheffer 2001), and several studies indicated that *Daphnia* species responded to a decrease in P-content of algae with a reduction in fitness (Vanni and Lampert 1992, Sterner et al. 1993, Weers and Gulati 1997, Boersma 2000, Becker and Boersma 2003). It is known that above a critical carbon to phosphorus ratio (C:P ratio) of about 225-375, growth is limited in *Daphnia*, a value which can be found in a substantial subset of lakes (Brett et al. 2000), although recent

evidence suggests that the use of these thresholds in field situations should be done with care (DeMott and Tessier 2002).

The general aim of our study was to investigate life-history variation of the two *Daphnia* species *Daphnia galeata*, *D. cucullata* and their interspecific hybrids at two food quality treatments (P-rich and P-limited algae) in order to assess the potential contribution of food quality variation to hybrid maintenance. Specifically we addressed the following question: Do *Daphnia* species and their interspecific hybrids differ in their response to variation in food quality?

## 2.2 Material and methods

### Life-history experiments

Three clones of *D. galeata,* two clones of *D. cucullata* and three clones of their interspecific hybrid *D. cucullata x galeata* were subjected to three different experiments. All interspecific hybrids and one *D. galeata* clone originate from laboratory crosses (Schwenk et al. 2001). We used the *D. galeata* clone G100 (isolated from lake Tjeukemeer, The Netherlands) and G44 (Grote Brekken, The Netherlands) and one clone, GL5, which resulted from an intraspecific cross between G100 and G44. *D. cucullata* was represented by the two clones, C33 (Lake Tjeukemeer) and V50 (Lake Vechten, The Netherlands). Interspecific hybrids originate from laboratory crosses of G100 and C33, resulting in clone X1 and X3, and a cross between V50 and G100 resulted in clone GCL1. The offspring of all crosses was verified using several genetic markers (Schwenk et al. 2001).

Semi-continuous cultures of *Scenedesmus obliquus* were established in Z/4 medium (Zehnder and Gorham 1960) with full phosphorus or with reduced phosphorus content in a way similar to Becker and Boersma (2003), resulting in algal cells with a molar carbon to phosphorus (C:P) ratio of 70 – 80 for P-rich cells ($P^+$) and about1000 for P-limited algae ($P^-$). Every day, 700 mL (total volume: 1.5 L) of culture medium was replaced with fresh medium: Algae were centrifuged at 5000 rpm for 10 min and diluted in phosphorus-free medium ("Aachener Daphnien Medium", AdaM, Kluttgen et al. 1994) to remove traces of dissolved P of algal culture media. C-content of the cultures was established photometrically using a calibration curve for both culture conditions. The calibration curve was established by measuring the extinction of different diluted algae suspensions at 800 nm using a spectrophotometer (Hitachi, U-2000). For each dilution, C-content was measured subsequently by filtration of algae onto precumbusted 24 mm diameter glass-fiber filters (Whatman GF/C) and C-content was quantified by a CHN-analyzer (Perkin Elmer). P-content of algae was measured spectrophotometrically after digestion with potassium persulfate (Langner and Hendrix 1982).

All experiments and cultures were kept at 18°C with a light:dark cycle of 16:8 h. Before starting the experiments, daphniids of each clonal lineage were adjusted to ADaM medium for at least five generations. Juvenile animals were collected from stock cultures and placed into 250 mL jars filled with ADaM medium and fed 1 mg C $L^{-1}$ of $P^+$-algae to

guarantee a food supply above the incipient limiting level (Lampert 1987). Neonates, born within 24 h, were subjected to three different experiments. First, newborns were kept individually in 250 mL beakers at $P^+$- and $P^-$-conditions at 1mg C $L^{-1}$ until they reached maturity and reproduced. Medium and algae were replaced every day. Size of the newborns (JUS), as well as size of the females at reproduction (SAR), was measured under a microscope to the nearest 0.02 mm. The number of newborns (NJU) and days to reproduction (DTR) under both conditions ($P^+$ and $P^-$) were counted. In a second experiment, 10 newborns of each clone were kept at $P^+$ and $P^-$-conditions (in 250 mL beakers) for four days to measure their somatic growth rate (SGR). The initial size of juveniles was measured using ten newborns per clone, and the size after 4 days of the experimental animals was measured to the nearest 0.02 mm under a microscope. The size of the animals was obtained by measuring the maximum length excluding the caudal spine. Somatic growth rate (SGR) was calculated following the formula $SGR = \ln(L_t/L_0)/t$, where $L_0$ is the average initial size of a clone and $L_t$ is the size of the animals at time t (Sterner and Elser 2002). To test for the susceptibility of a taxon to variation in food quality, we estimated the relative growth rate (RGR) using the formula: $RGR = \mu/\mu_m$ as described in Sterner and Elser (2002), where $\mu_m$ represents the somatic growth rate under P-rich and $\mu$ the somatic growth rate at P-limited conditions.

In order to investigate if results from life-history experiments of individuals can be extrapolated to population experiments, we carried out a third experiment. We determined the effect of different food sources on population growth rates of different clones of *Daphnia* species and hybrids in a multi-clone experiment. Hence, we set up a flow-through system with six 15-L containers. Three containers were supplied with $P^+$ algae medium and three containers with $P^-$ medium. Media and algae were replaced once a day using a flow-through system based on a peristaltic pump which continuously replaced the old vs. new media. Each container was initially inoculated with 10 juveniles of each clone. Once a week each container was cleaned from algae and bacteria growing at the glass walls. After 6 weeks, the experiment was terminated, and all animals (per replicate) were counted after they had been preserved in 70% ethanol. In addition, we subjected a random sample of 35 animals per replicate to genetic analyses to determine the abundance of clones and taxa in each container.

**Genetic analysis**

DNA preparation of individuals of experiment III was conducted following a standard protocol (Schwenk et al. 1998). For each individual, a Polymerase Chain Reaction (PCR) was

accomplished using the primers ITS2-5.8S and ITS-18S to amplify a ~1500 base pair (bp) segment consisting of ITS2, 5.8S, ITS1 and a part of 18S rDNA (Billiones et al. 2004). A subsequent Restriction-Fragment-Length-Polymorphism (RFLP) analysis was conducted to determine taxon affiliation following the protocol of Billiones et al. (2004).

In order to analyze the clonal affiliation of each individual (eight different clonal lineages) we conducted microsatellite analyses in combination with mitochondrial DNA PCR-RFLP analyses. Since two hybrid clones (generated by different maternal species) showed the same genotype at all microsatellite loci we applied PCR-RFLP of maternally inherited mitochondrial DNA. We amplified mitochondrial DNA using the primer S1 and S2 as described in Schwenk (1998) and conducted a RFLP-analysis with the restriction enzyme *Rsa* I. Mitochondrial DNA analysis was used to identify the two hybrid clones X3 and GCL1 since they originate from different maternal lineages (G100 and C33). Thus it was possible to assign each individual to one of the 8 clonal lineages and to determine the relative frequencies of taxa and clones used in the multi-species/-clones experiment.

A subsample of each replicate ($n$ = 35) of Experiment III was subjected to microsatellite analysis using six microsatellite loci (Dove et al., unpub.). Fragments were separated on Polyacrylamid (PAA) gels with the help of an automatic DNA sequencer (Automatic Length Fragment Analyser, ALF; Amersham Pharmacia). Allelic variation at each microsatellite locus was combined to diagnostic multi locus genotypes (MLG).

**Numerical analyses**

*Experiment I and II*

Rates of population increase ($r$) for each taxon and treatment were calculated using the Euler-Lotka-equation (Stearns 1992). Since our cultures consisted of individual animals, no age specific-survivorship ($l_x$) could be determined, therefore $l_x$ was set to 1. To estimate the variance for $r$, two random combinations between the three replicates per clone and treatment were drawn to calculate an average and standard-deviation to obtain a rough estimate of variation.

Because of an unbalanced experimental design (three clones for *D. galeata* and the interspecific hybrids and two clones of *D. cucullata*) we used a General Linear Model (GLM). Clones were nested within taxon and were implemented as random factor within the model,

all other effects were treated as fixed factors (Statistical model: *taxon* + *condition* + *clone (taxon)*+ *taxon*condition* + *clone(taxon)*condition*). The *F*-values and the accompanying degrees of freedom (df) were calculated according to the methods described in Satterthwaite (1946). Differences between the taxa were tested *a posteriori* conducting a Fisher-LSD test using type III GLM. In addition, we conducted a nested GLM analysis for each life-history trait using taxon and clones (nested within taxon) as two levels to assess the proportion of total variance for this life-history trait.

For intraspecific comparisons (among clones), 2-way ANOVA analyses were performed, and one-by-one comparisons between taxa and clones were analyzed using Mann-Whitney-*U* tests. Total variance of a trait and taxon ($\sigma^2_T$) was decomposed by a one-way ANOVA into clonal variances ($\sigma^2_G$). Broad-sense heritabilities ($H^2$) for each trait and taxon were calculated following the approach described in Lynch and Walsh (1998) for clonal organisms ($H^2 = \sigma^2_G / \sigma^2_T$, $\sigma^2_G$ = genetic variance, $\sigma^2_T$ = total variance).

*Experiment III*

A comparison of taxon and clonal composition among replicates and treatments was conducted using a multiple comparison procedure (Holm-Sidak method, Sidak 1967, Holm 1979). For the analysis of the clonal composition we excluded the taxon *D. cucullata*, since only a relatively small number of *D. cucullata* individuals were detected (0 – 11 % of total).

## 2.3. Results

*Daphnia* clones of each taxon reared at high concentrations (1 mg C $L^{-1}$) of P-rich and P-limited green algae (*S. obliquus*) differed significantly in their life-history traits and showed a reduced fitness at low P-conditions. Somatic growth rate of all taxa was on average reduced by 45% (from 0.11 ± 0.03 to 0.06 ± 0.03; mean ± SE, based on the average across all clones *n*= 8), the number of juveniles declined by 29% (from 3.42 ± 1.47 to 2.44 ± 1.16 and individuals required 20% more time to reproduce (from 10.56 d ± 2.46 to 13.28 d ± 3.74).

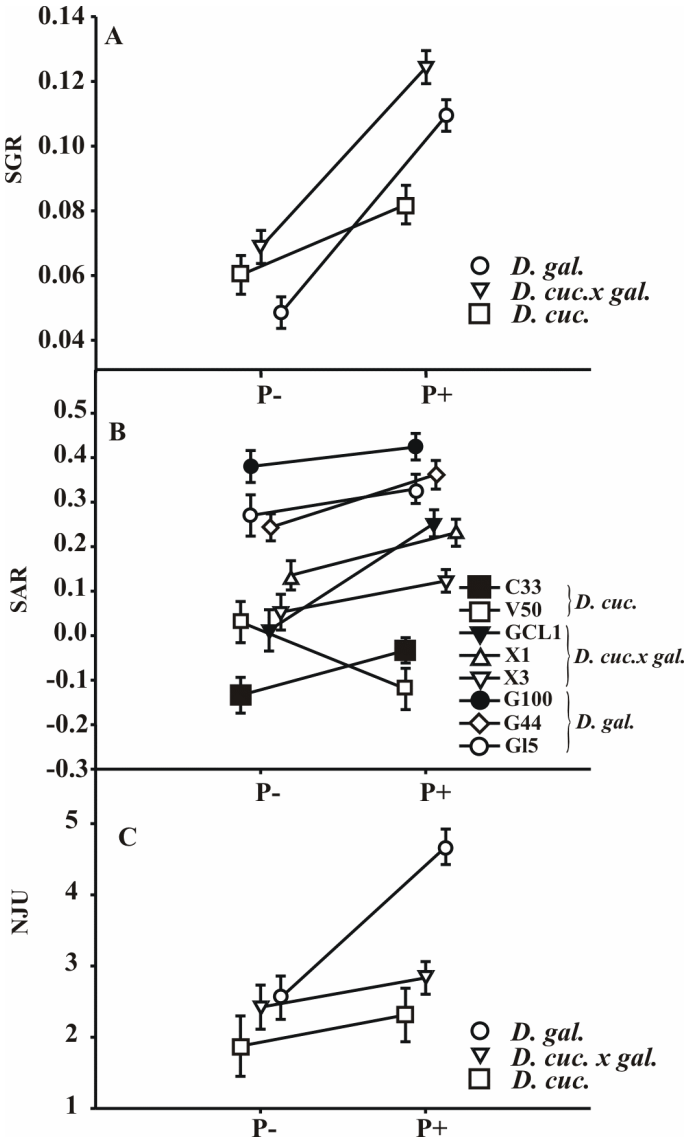


Figure 1: Reaction norms of *D. galeata, D. cucullata* and their interspecific hybrids *D. cucullata x galeata* for three life-history traits. (A) Somatic growth rates (SGR) under two food quality conditions ($P^+/P^-$), (B) size at first reproduction (SAR) presented on log-scale, and (C) number of juveniles of first clutch (NJU), error bars represent standard errors.

Using a nested statistical analysis of variances, no significant interaction for SGR of taxa with experimental conditions were detected (Table 1), but interspecific hybrids were significant different to both parental taxa at $P^+$-conditions, and to clones of *D. cucullata* at $P^-$-conditions (Table 2). For SAR, no effect of food quality was detected, but clones differed in their reaction to the different qualities (Table 1 and 2, Fig. 1). In addition, JUS was not altered by P-limited algae. Number of juveniles (NJU) was different for all three taxa but not among clones within taxa. For the NJU, a significant food quality effect was detected, and clones of *D. galeata* differed significant in their number of juveniles at P-sufficient conditions. Furthermore, NJU varied much more at $P^+$ than at $P^-$-conditions (Fig. 2). In addition, the time it took the taxa to

reproduce was significantly longer when fed P-limited algae, and also an interaction of clonal lineages with food quality was detected (Table 1).

Table 1: Results of the General Linear Model (GLM) analysis of variation in six life-history traits among eight clones of *D. galeata, D. cucullata,* and *D. cucullata x galeata*. Taxon written in brackets indicates that clones were nested within taxa. Clone(Taxon) and *Clone(Taxon)*Condition* have been treated as random factors, all others were set as fixed factors. SGR = somatic growth rate, NJU = number of juveniles, DTR = days to reproduction, SAR = size at reproduction, JUS= size of juveniles, *r* = rate of population increase. Significant results are emphasized in bold letters ($p < 0.05$). Degrees of freedom (df) are non-integers due to the special type of calculation (see Material and Methods section).

| | | sum of squares | df | mean square | *F* | *p* |
|---|---|---|---|---|---|---|
| SGR | Taxon | 0.015 | 4.994 | 0.004 | 1.735 | 0.268 |
| | food quality (P+/P-) | 0.076 | 4.978 | 0.001 | 83.928 | **<0.001** |
| | Clone(Taxon) | 0.022 | 5.000 | 0.001 | 4.815 | 0.055 |
| | Taxon*Conditon | 0.009 | 4.971 | 0.001 | 5.060 | 0.063 |
| | Clone(Taxon)*Condition | 0.005 | 124.000 | 0.001 | 1.407 | 0.226 |
| | Error | 0.080 | | | | |
| SAR | Taxon | 1.729 | 5.189 | 0.026 | 33.492 | **0.001** |
| | food quality (P+/P-) | 0.077 | 5.282 | 0.021 | 3.729 | 0.108 |
| | Clone(Taxon) | 0.137 | 5.000 | 0.022 | 1.250 | 0.406 |
| | Taxon*Conditon | 0.067 | 5.237 | 0.021 | 1.607 | 0.286 |
| | Clone(Taxon)*Condition | 0.109 | 73.000 | 0.006 | 3.390 | **0.008** |
| | Error | 0.471 | | | | |
| JUS | Taxon | 0.773 | 6.143 | 0.055 | 7.053 | **0.026** |
| | food quality (P+/P-) | 0.024 | 16.666 | 0.013 | 1.861 | 0.191 |
| | Clone(Taxon) | 0.337 | 5.000 | 0.010 | 6.693 | **0.029** |
| | Taxon*Conditon | 0.019 | 14.749 | 0.013 | 0.760 | 0.485 |
| | Clone(Taxon)*Condition | 0.050 | 247.000 | 0.020 | 0.501 | 0.775 |
| | Error | 4.966 | | | | |
| NJU | Taxon | 28.728 | 5.793 | 1.401 | 10.256 | **0.012** |
| | food quality (P+/P-) | 18.860 | 5.918 | 1.466 | 12.869 | **0.012** |
| | Clone(Taxon) | 7.077 | 5.000 | 1.490 | 0.950 | 0.522 |
| | Taxon*Conditon | 13.900 | 5.751 | 1.470 | 4.729 | 0.061 |
| | Clone(Taxon)*Condition | 7.452 | 75.000 | 1.232 | 1.210 | 0.313 |
| | Error | 92.396 | | | | |
| DTR | Taxon | 6.277 | 5.282 | 23.346 | 0.134 | 0.877 |
| | food quality (P+/P-) | 160.816 | 5.426 | 18.838 | 8.537 | **0.030** |
| | Clone(Taxon) | 123.552 | 5.000 | 20.012 | 1.235 | 0.411 |
| | Taxon*Conditon | 23.864 | 5.350 | 19.027 | 0.627 | 0.569 |
| | Clone(Taxon)*Condition | 100.058 | 75.000 | 7.832 | 2.555 | **0.034** |
| | Error | 587.436 | | | | |
| *r* | Taxon | 0.025 | 5.000 | 0.001 | 12.219 | **0.012** |
| | food quality (P+/P-) | 0.040 | 5.000 | 0.002 | 18.645 | **0.008** |
| | Clone(Taxon) | 0.005 | 5.000 | 0.002 | 0.477 | 0.782 |
| | Taxon*Conditon | 0.008 | 5.000 | 0.002 | 1.808 | 0.257 |
| | Clone(Taxon)*Condition | 0.011 | 16.000 | 0.001 | 1.577 | 0.223 |
| | Error | 0.022 | | | | |

Table 2: Results of the *post hoc* comparison tests, derived from a General Linear Model (GLM) analysis of three life-history traits among eight clones of *D. galeata* (gal), *D. cucullata* (cuc), and *D. cucullata x galeata* (cg). SGR= somatic growth rate, SAR= size at reproduction, NJU= number of juveniles. Significant results are emphasized in bold letters ($p < 0.05$).

| SGR | cg P- | cg P+ | cuc P- | cuc P+ | gal P- | gal P+ |
|---|---|---|---|---|---|---|
| cg P- | | | | | | |
| cg P+ | **<0.001** | | | | | |
| cuc P- | 0.222 | **<0.001** | | | | |
| cuc P+ | 0.125 | **<0.001** | **0.011** | | | |
| gal P- | **0.003** | **<0.001** | 0.133 | **<0.001** | | |
| gal P+ | **<0.001** | **0.025** | **<0.001** | **0.001** | **<0.001** | |

| SAR | cg P- | cg P+ | cuc P- | cuc P+ | gal P- | gal P+ |
|---|---|---|---|---|---|---|
| cg P- | | | | | | |
| cg P+ | **<0.001** | | | | | |
| cuc P- | **<0.001** | **<0.001** | | | | |
| cuc P+ | **<0.001** | **<0.001** | 0.860 | | | |
| gal P- | **<0.001** | **<0.001** | **<0.001** | **<0.001** | | |
| gal P+ | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **0.005** | |

| NJU | cg P- | cg P+ | cuc P- | cuc P+ | gal P- | gal P+ |
|---|---|---|---|---|---|---|
| cg P- | | | | | | |
| cg P+ | 0.925 | | | | | |
| cuc P- | 0.875 | 0.375 | | | | |
| cuc P+ | 1.000 | 0.960 | 0.874 | | | |
| gal P- | 0.976 | 1.000 | 0.520 | 0.988 | | |
| gal P+ | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | |

The taxa differed in their rate of population increase, which also was significant negatively affected by P-limited algae (Fig. 2). In addition, the variation that emerges from the different analyses is mostly attributable to interspecific variation (more than 90 %, Table 3), intraspecific (clonal) variation was low except for SGR (32% intraspecific variation of total).

In order to reveal the sensitivity of clones and taxa to the different experimental treatments we calculated the relative growth rate, the growth rate at low-P relative to high-P food (RGR). The clones of *D. galeata* showed the lowest values for RGR (0.44 ± 0.05), the interspecific hybrid were intermediate (0.55 ± 0.07) and clones of *D. cucullata* showed highest values for RGR (0.77 ± 0.25). The parental species differed significantly in their RGR

(Mann-Whitney-*U* test, $p = 0.0133$), indicating that parentals exhibit different levels of susceptibility to variation in food quality.

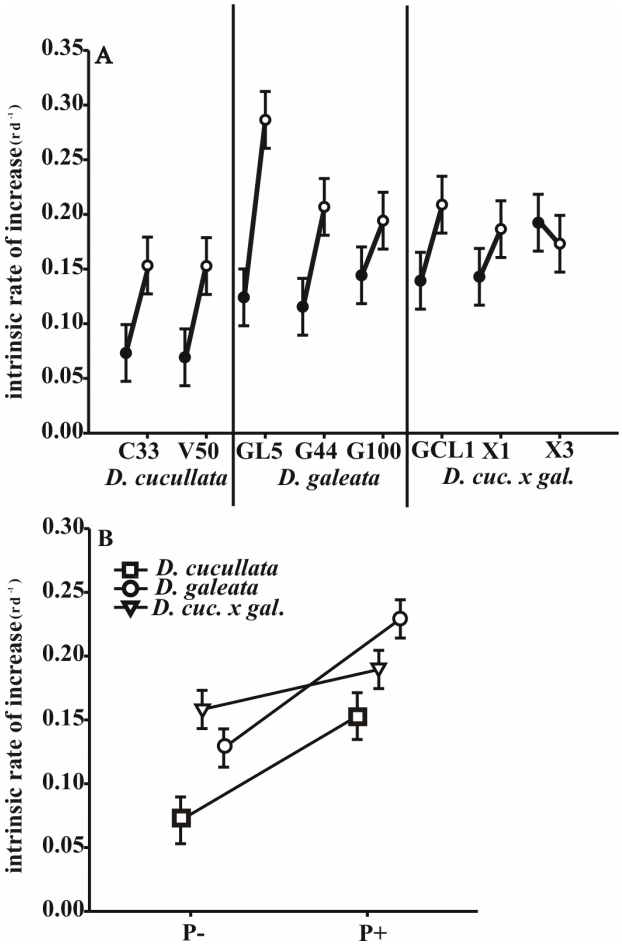


Figure 2: Rates of population increase (r) for (A) each clonal lineage of D. galeata, D. cucullata and their interspecific hybrids. Open circles represent P-rich, closed circles P limited conditions. Black lines are connecting the means of each clonal lineage under two food quality conditions (P+ and P-). (B) Reaction norms of the rates of population increase per taxon at both conditions. *D. cuc. * gal.* = interspecific hybrids. Error bars represent standard errors.

The rate of population increase (*r*) differed among taxa and among different treatments (Table 1), and all taxa showed a reduced level of population increase at $P^-$-conditions (Fig. 2). *D. galeata* showed the highest *r* at P-rich conditions, but at P-limited conditions, clones of the hybrid *D. cucullata x galeata* exhibited the highest values (Fig. 2), but differences were not significant (results not shown). Interestingly, one hybrid clone (X3) revealed a very shallow reaction norm compared to the other clones, i.e. no significant difference between rates of population increase at P-limited and P-rich conditions (Fig. 2A).

Table 3: Partitioning of variation in life-history traits between intraspecific (clone within taxon) and interspecific (taxon) components. Significant results are emphasized in bold letters ($p < 0.05$), SGR = somatic growth rate, NJU = number of juveniles, DTR = days to reproduction, JUS = size of juveniles, SAR = size at first reproduction, *r* = rate of population increase, MS= mean square

| | | DF | MS | proportion of variance | F | P |
|---|---|---|---|---|---|---|
| **SGR** | taxon | 2 | 0.008 | 0.635 | 5.646 | **0.004** |
| | Clone (taxon) | 5 | 0.004 | 0.365 | 3.243 | **0.009** |
| **SAR** | taxon | 2 | 0.866 | 0.968 | 92.782 | **<0.000** |
| | Clone (taxon) | 5 | 0.028 | 0.032 | 3.045 | **0.014** |
| **JUS** | taxa | 2 | 0.697 | 0.909 | 35.039 | **<0.000** |
| | Clone (taxon) | 5 | 0.070 | 0.091 | 3.518 | **0.004** |
| **NJU** | taxon | 2 | 19.690 | 0.917 | 12.099 | **<0.000** |
| | Clone (taxon) | 5 | 1.775 | 0.083 | 1.091 | 0.372 |
| **DTR** | taxon | 2 | 2.759 | 0.147 | 0.259 | 0.773 |
| | Clone (taxon) | 5 | 15.994 | 0.853 | 1.500 | 0.199 |
| ***r*** | Taxon | 2 | 0.012 | 0.924 | 3.752 | **0.038** |
| | Clone (taxon) | 5 | 0.001 | 0.076 | 0.307 | 0.904 |

*Daphnia* taxa showed marked differences in broad-sense heritabilities of various life-history traits (Table 4). For example, *D. galeata* showed the highest heritability values in SGR, whereas interspecific hybrids showed the highest heritability in NJU, DTR, JUS, and SAR. In general, *D. cucullata* showed for all traits the lowest broad-sense heritabilities compared to the other two taxa (Table 4).

Table 4: Broad-sense heritabilites ($H^2$) for the three different taxa *D. galeata*, *D. cucullata x galeata* and *D. cucullata*, for five life-history traits. SGR = somatic growth rate, NJU = number of juveniles, DTR = days to reproduction, JUS = size of juveniles, SAR = size at first reproduction.

| **Trait** | *D. galeata* | *D. galeata x D. cucullata* | *D. cucullata* |
|---|---|---|---|
| SGR | 0.163 | 0.078 | 0.028 |
| SAR | 0.128 | 0.264 | 0.011 |
| JUS | 0.190 | 0.201 | 0.007 |
| NJU | 0.057 | 0.101 | 0.034 |
| DTR | 0.083 | 0.174 | 0.040 |

Densities of individuals in multi-clonal vessels of experiment III varied strongly after six weeks between both conditions; we found on average 1429 ± 77.8 individuals at $P^+$-conditions and 245 ± 96.1 individuals at $P^-$-conditions. Differences between both treatments were significant ($F$ = 15.073, $p$ = 0.018). Discrepancies between the individuals subjected to DNA-analysis and the number of individuals given in Table 5 can be explained by a non-sufficient yield of DNA for genetic analysis.

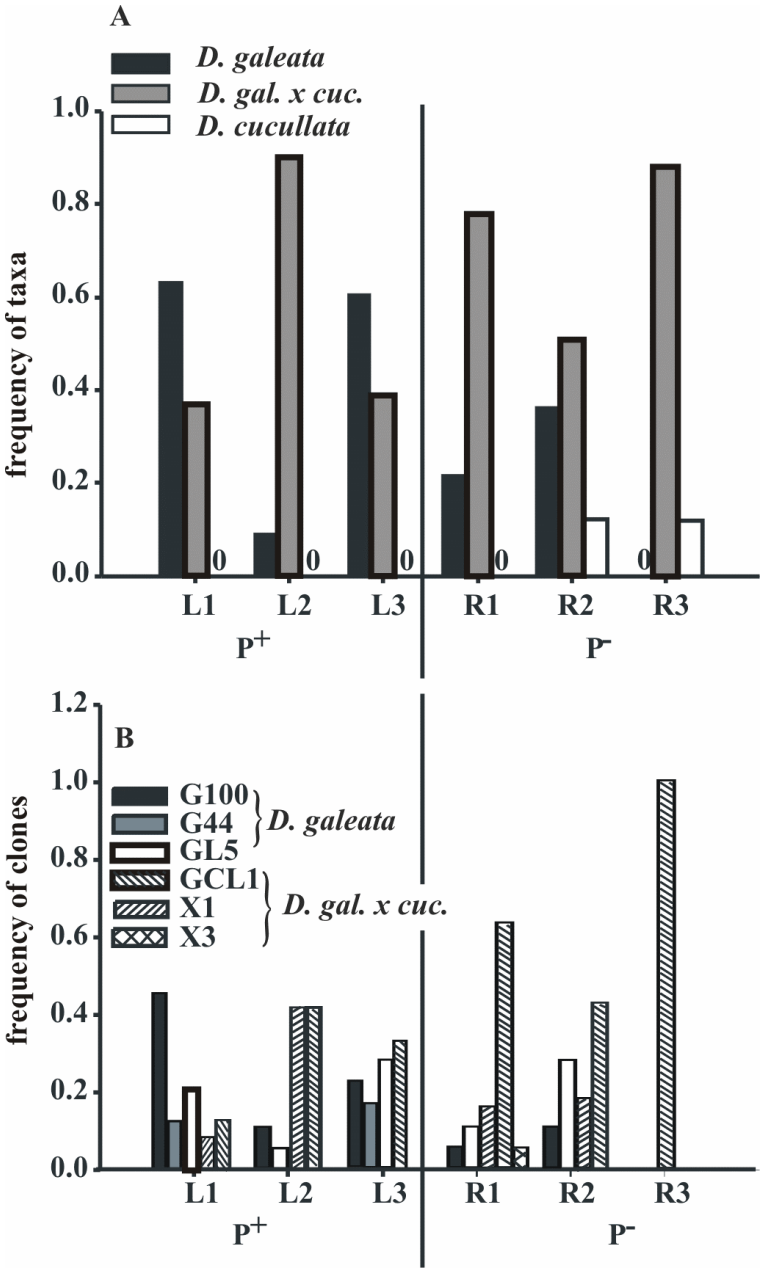


Figure 3: A. Relative abundance of *Daphnia* taxa per replicate (L1, L2, and L2 for $P^+$, R1, R2, and R3 for $P^-$-conditions) of the multi-clone experiment (experiment III). Zero numbers indicate frequencies of corresponding taxa not identified within the different replicates. (B) Relative abundances of multi locus genotypes per replicate (see table 5). *D.cuc x D.gal.* = interspecific hybrids. Clones of *D. cucullata* were excluded from microsatellite analysis because they occurred only in very low frequencies.

Relative abundances of taxa did not differ significantly among treatments (ANOVA, Holm-Sidak method, $F = 1.617$, $p = 0.239$). However, one replicate (L2) of the high quality treatment contained much lower densities of animals (L1 = 1484 individuals, L3 = 1374 individuals, compared to L2 = 722 individuals). Based on this large deviation we considered this value as an outlier and calculated a comparison between the relative abundances without replicate L2. The reduced data set revealed a significant difference in taxon composition between treatments (ANOVA, Holm-Sidak method, $F = 4.394$, $p = 0.037$). In general, frequencies of *D. galeata* were higher at P-rich conditions than those of interspecific hybrids (Fig. 3A). In addition, no individual of *D. cucullata* was detected at $P^+$-conditions (Table 4). In contrast, interspecific hybrids dominated at $P^-$-conditions (Table 5, Fig. 3A). *D. galeata* and interspecific hybrid clones occurred in similar proportions at $P^+$-conditions (Fig. 3B), except for clone X3, which was not found in any replicate. In contrast, the interspecific hybrid clone X1 was clearly dominant in all three replicates at $P^-$-conditions, including the extreme case of replicate R3, in which only clone X1 was found. Clonal frequencies differed significantly among treatments and an interaction of clonal composition with treatment was detected (Holm-Sidak method, $F = 3.654$, $p = 0.016$)

Table 5: Results of RFLP and microsatellite analysis of the multi-clone experiment (experiment III). ΣT = number of individuals assigned to taxon level, ΣC = number of individuals assigned to each clone, ΣΣC = total number of assigned individuals per replicate (L1, L2, L3 for P-rich conditions and R1, R2, R3 for P-limited conditons), ΣΣT = total number of investigated individuals per replicate.

| | Replicate | *D. cucullata* | *D. galeata* | | | | | *D. cucullata x galeata* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **ΣT** | 100 | G44 | GL5 | **ΣC** | **ΣT** | GCL1 | X1 | X3 | **ΣC** | **ΣT** | **ΣΣC** | **ΣΣT** |
| | L1 | 0 | 11 | 3 | 5 | 19 | 20 | 2 | 3 | 0 | 5 | 13 | 24 | 3 |
| **P+** | L2 | 0 | 2 | 0 | 1 | 3 | 3 | 8 | 8 | 0 | 16 | 27 | 19 | 3 |
| | L3 | 0 | 4 | 3 | 5 | 12 | 14 | 0 | 6 | 0 | 6 | 10 | 18 | 2 |
| | | | | | | | | | | | | | **Σ** | 8 |
| | | **ΣT** | 100 | G44 | GL5 | **ΣC** | **ΣT** | GCL1 | X1 | X3 | **ΣC** | **ΣT** | **ΣΣC** | **ΣΣT** |
| | R1 | 0 | 1 | 0 | 2 | 3 | 7 | 3 | 12 | 1 | 16 | 23 | 19 | 3 |
| **P-** | R2 | 4 | 3 | 0 | 8 | 11 | 12 | 5 | 12 | 0 | 17 | 18 | 32 | 3 |
| | R3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 21 | 0 | 21 | 23 | 24 | 2 |
| | | | | | | | | | | | | | **Σ** | 9 |

## 2.4 Discussion

Our study shows that variation in food quality causes inter- and intraspecific variation in fitness related life-history traits of *Daphnia* species and their interspecific hybrids. Clones and taxa showed a differential response to an exogenous factor which is bound to determine the fitness of evolutionary lineages. All life-history traits (except juvenile size and size at reproduction) of *D. galeata*, *D. cucullata* and their interspecific hybrids were negatively affected by P-limitation (Table 1, Fig. 1). Several studies had already demonstrated that a chemical unbalanced food source (such as P-limited algae) reduces fitness in *Daphnia* (e.g., Sommer 1992, Boersma and Vijverberg 1995, Repka 1996, Weers and Gulati 1997, Repka et al. 1999a, Repka 1999, Sterner and Schwalbach 2001, DeMott 2003). At P-sufficient conditions, clones of *D. galaeata* showed the highest values at several life-history traits, e.g. number of juveniles, which was significantly higher than in the other taxa. However, interspecific hybrids responded differentially, showing highest values in somatic growth rate at both food quality conditions (Fig. 1, Table 2) and they also dominated in our multi-clone experiment at $P^-$-conditions (Fig. 3). Like in some other studies (Grant and Grant 1996, Schluter 1996), we found that hybrids are superior to their parental species in several life-history traits under certain environmental conditions.

In general, we observed a considerable amount of clonal variation for *D. galeata* and *D. cucullata x galeata*, but interactions between clones and experimental conditions were hardly found. *D. cucullata* clones differed in none of the life-history traits, whereas *D. galeata* and hybrid clones varied in 60% of their traits, but only two interactions with food quality were detected (results not shown). This pattern is in agreement with previous studies which showed that variation between *D. galeata* and interspecific hybrid clones is smaller than the variation between taxa (e.g. Weider 1993). Our results suggest that life-history variation under various food quality conditions is mainly based on the differentiation between taxa rather than on differentiation between clones, although the selection of clones was not representative for natural variation of field populations. In this study, we investigated only one family (all interspecific hybrids which originate from the same paternal and maternal clone), and hence we potentially underestimated clonal variation among hybrids and thus overestimated between species effects. Thus, field hybrids might exhibit a much larger variance in response to food quality variation than observed in our experiments. However, the clonal variation of one parental species (*D. galeata*) was comparable to the variation among interspecific hybrids (Table 3), thus hybrid variation is at least in the range of clonal variation within species.

Hence, we conclude that the consistent differential response of *Daphnia* species and hybrids to variation in food quality may facilitate co-existence and niche differentiation.

Our results from single-clone life-history experiments were supported by a multi-clone experiment: *D. galeata* showed the highest rate of population increase and the highest relative frequencies during the multi-species experiment under P-sufficient conditions. In contrast, *D. cucullata x galeata* dominated in all three replicates at P-limited conditions. *D. cucullata* was only detected at P-limited conditions, indicating a higher ability to compete with *D. galeata* and the interspecific hybrids at those conditions. At the clonal level we found a more complex pattern: the single-clone experiments did not exactly predict the most successful clone of our multi-clone experiments, however, those clones which had a relatively high population increase were among the most frequent clones in the multi-clone experiment. Based on the values for somatic growth rate and *r*, we had expected clone X3 to represent the most frequent clone at $P^-$-conditions, however clone X1 dominated in all three replicates. The differences between our expectations and what we observed in the population experiment might be the result of several processes: clonal interactions, stochastic effects or a differential mortality rate during experimental conditions. The inoculation or the build-up of the X3 populations might have been different among replicates which caused large variation of clonal abundances among vessels at $P^+$-conditions (outlier L2; Fig. 2). We excluded a feedback between grazing and nutrient recycling from the grazer onto its resource to be able to compare the single and multi-clonal experiments. These feedback mechanisms are probably fairly important in nature as they may alter the stoichiometry of the grazer and the resources (Andersen 1997), and hence should be included in future experiments.

Phenotypic plasticity has often been assumed to limit natural selection by buffering the effects of selection, but recent studies showed that phenotypic plasticity represents a fundamental component of evolutionary change (Behera and Nanjundiah 2004). Based on the considerable phenotypic plasticity of *D. galeata* and the *D. cucullata x galeata* hybrids and the environmental variability of food quality, we suspect a large potential for the establishment of new evolutionary trajectories. To understand how different phenotypes perform under different environmental conditions, we need to evaluate whether the phenotypic differences among populations and species represent the outcome of evolution by natural selection. Our results indicate that interspecific hybrids showed higher heritability estimates for most of their life-history traits than parental taxa (except somatic growth rate,

see Table 2), which may allow them to adapt faster to changes in food quality. Increased levels of heritability provides a greater scope for directional natural selection and thus adaptation (Grant and Grant 1994), resulting in fast adaptation of novel genotypes and phenotypes that may serve as the starting point of a new evolutionary trajectory. Increased heritability in interspecific hybrids is caused by higher genetic variation among hybrids due to additive effects (Graham 1992), new associations between nuclear and mitochondrial genomes (Ferris et al. 1983) and mutation (Woodruff 1989). However, since our experimental design did not allow the comparison of heritability among hybrids of different families, heritability levels might be elevated due to within-family comparisons. Further studies on the quantitative variation in life-history traits are required to determine the phenotypic consequences of interspecific hybridization and the resulting potential for evolutionary change.

Several theories have been developed to explain hybrid maintenance based on tension zone or cline and ecotone models; a derivative, the temporal hybrid superiority model (THS) by Spaak and Hoekstra (1995), assumes temporally fluctuating levels of exogenous selection among taxa. Since the THS represents a hypothesis, rather than an explicit mathematical model, we used our data to qualitatively test assumptions of the temporal hybrid superiority model. First, we found that fitness in parental species and hybrids varies with environmental conditions, i.e., food quality and secondly, interspecific hybrids show higher somatic growth rate at low food quality conditions. Therefore, we add food quality to the list of parameters, such as predation levels and food quantity which contribute to the temporary superiority of interspecific *Daphnia* hybrids. Although the THS model seems closely related to models on species coexistence and maintenance of diversity (e.g. see Chesson 2000), hybrid superiority, as described by the THS model, is solely based on fitness differences among taxa during the asexual phase of reproduction. This is largely caused by the focus of most studies on this phase: nearly all empirical data on fitness variation in *Daphnia* rely on measurements of the rate of population increase during the asexual phase. Further empirical studies of hybrid complexes should include the sexual phase of reproduction, as well as diapause and the 'storage effect' thus providing data to rigorously test hypotheses on species coexistence.

Which exogenous factor, fish, invertebrate predators, food quality or food quantity, represent the most important factor responsible for hybrid maintenance in *Daphnia* remains an open question for further research. Most likely, however, food quality has an important

impact on population dynamics of *Daphnia* species and their intraspecific hybrids, in particular in syntopic populations. Our data suggest that food quality differences during a season (Kreeger et al. 1997) or between lakes might facilitate species and hybrid differentiation and that food quality certainly could play a role determining the fate of hybrid lineages. Hybrids occur in high frequencies during certain periods of the growing season (Wolf 1987, Weider and Stich 1992), showing that hybrids show different fitness optima than their parental taxa. Wolf (1987) showed that the relative abundance of hybrids increased after the mid-summer decline, that is when food quality (measured as C:P ratio) is usually low (Kreeger et al. 1997). In addition, several studies document large variation in food quality within and among seasons (Lampert et al. 1986, Müller-Navarra and Lampert 1996). Thus, the functional link between differential ecological demands of taxa and large temporal variation in food quality might explain the co-occurence of hybrids with their parental taxa. This might explain the observation made by Schwenk (1997) and also Hessen et al. (1995) who observed associations of different *Daphnia* taxa with different environmental factors in lakes. However, further field studies which monitor food quality, food quantity, and taxon frequencies are necessary to test hypotheses derived from our experimental study. In addition, this approach should be supplemented by multi-clone life- history studies considering more than one potential factor explaining hybrid maintenance.

In conclusion, we have shown that different taxa from the *Daphnia galeata hyalina* complex react differently to changes in food quality (measured as P-content) conditions. This implies that elemental stoichiometry of the food is not only expected to influence the performance of individual species or hybrid lineages, but also the composition of zooplankton communities in aquatic environments.

# CHAPTER 3: LOCAL ADAPTATION TO FOOD QUALITY IN A FRESHWATER CRUSTACEAN

# Chapter 3: Local adaptation to food quality in a freshwater crustacean

## 3.1 Introduction

The microcrustacean *Daphnia* is a key species in many pelagic food webs, and as a result one of the best studied organisms in freshwater systems. A broad phenotypic plasticity coupled with the available genomic information makes it a versatile model system to investigate fundamental mechanisms in various fields, such as ecology, evolution and ecotoxicology (Mort 1991, Weltje 2003). Phylogenetic relationships, phylogeography and morphological variation are well described (Schwenk 1993, Taylor and Hebert 1994, Schwenk and Spaak 1995, Spaak and Hoekstra 1995, Colbourne et al. 1997, Flößner 2000). Moreover, a wealth of information exists on the population structure of different *Daphnia* species (Korpelainen 1984, Dufresne and Hebert 1995, Hebert and Finston 1996, Crease et al. 1997, Vanoverbeke and De Meester 1997, Weider and Hobaek 1997) demonstrating that even populations in proximity may show considerable genetic differentiation.

Despite its predominantly parthenogenetic mode of reproduction, and the resulting slower rates of evolution, *Daphnia* species seem to adapt quite rapidly to their local environment. Several authors have shown, for example, local adaptation with respect to the presence of predators (e.g. Leibold and Tessier 1991, Parejko and Dodson 1991, Pijanowska et al. 1993, Spitze 1993, Cousyn et al. 2001, DeClerck et al. 2001). Others focused on environmental factors, such as salinity (Teschner 1995), temperature (Mitchell and Lampert 2000), oxygen stress (Carvalho 1984), pollution (Bachiorri et al. 1991), or other unspecified factors (DeClerck et al. 2001) to study local adaptation.

Interestingly, even though food quality and quantity belong to the most important factors influencing population dynamics in *Daphnia*, relating food quality to local adaptation has proven to be difficult. Numerous studies have shown food quality effects for herbivorous zooplankton (e.g. Sterner et al. 1993, Müller-Navarra 1995b, DeMott et al. 1998, Boersma 2000, Elser et al. 2001, Urabe and Sterner 2001, Hessen et al. 2002, Becker and Boersma 2003, Seidendorf et al. 2007). Several factors determine the quality of algae as food for zooplankters, such as size and morphology, toxicity/palatability, nutrient and biochemical content. Here we will focus on the nutrient content, especially the carbon (C) to phosphorus (P) ratio of sestonic algae. In freshwater environments, phosphorus is the element that has

attracted most attention, as in most systems it is the limiting element for phytoplankton growth (Sommer 1992, Elser and Hassett 1994). Members of the genus *Daphnia* show low body tissue C:P ratios compared to other cladocerans (Sterner and Elser 2002), illustrating their high phosphorus demand (Sterner and Schulz 1998, Plath and Boersma 2001). Indeed, a low P diet may have a considerable impact on the life-history of *Daphnia* species (e.g. Sterner 1993, Müller-Navarra 1995b, Müller-Navarra and Lampert 1996, Plath and Boersma 2001, Boersma and Kreutzer 2002, Becker and Boersma 2003). The results of these studies suggest that variation in phosphorus content of primary producers should represent a key factor for local adaptation. It is therefore surprising that given the enormous interest of food quality effects in *Daphnia,* only a few studies addressed the ability for these species to show local adaptation with regard to food quality. Repka (1997, 1998) studied food quality effects using four different food types of algae and cyanobacteria which occur in lakes of different trophic status. Several *Daphnia* clones isolated from different environments were subjected to different algal species, however the life-history response of clones could not be explained by the type of food sources. She concluded that other factors than food quality determined ecological differentiation. In contrast, some field studies showed that local adaptation in response to variation to food quality might represent a possible explanation for population differentiation (Elser et al. 2000b) since growth rate and phosphorus content of individuals was correlated with the trophic status of the lake the animals were sampled from.

Adaptive variation and local adaptation is most convincingly demonstrated by a comparison of variation in a trait against the null hypothesis that variation is selectively neutral (Lande 1992, Whitlock 1999). To test if populations adapted to their particular environmental conditions, we need to combine results of life-history studies with genetic information. Only a few studies successfully integrated genetic and ecological differentiation among *Daphnia* populations. Lynch (1999) and Spitze (1993) were the first who experimentally combined ecology and molecular genetics. Others tried to link their findings to the ambient environmental conditions in lakes (Hairston et al. 1999, Mitchell and Lampert 2000, Cousyn et al. 2001, DeClerck et al. 2001). However, we are aware of only one study which linked clonal variation with a differential response to variation in food quality (Weider et al. 2005). Although this study indicated that variation in food quality may play a role in microevolution, it did not provide information on the potential for local adaptation to food quality differences in *Daphnia*.

Many studies on local adaptation focused on a comparison of population differentiation at quantitative traits ($Q_{ST}$) and neutral molecular markers ($F_{ST}$), which provide a powerful test for the role of selection in phenotypic divergence (Lande and Arnold 1983, Spitze 1993, Merila and Crnokrak 2001, McKay and Latta 2002). If the quantitative differentiation ($Q_{ST}$) equals the genetic differentiation ($F_{ST}$) among populations, than quantitative traits, such as e.g. somatic growth rate, should not have been subjected to directional selection (Spitze 1993). Departures from this neutral expectation are considered as evidence for selection, i.e. $Q_{ST} < F_{ST}$ indicates homogeneous selection, while for $Q_{ST} > F_{ST}$ indicates diversifying selection (Merila and Crnokrak 2001). For a wide range of taxa, these expectations were tested to detect different modes of selection or levels of local adaptation. This type of comparison was conducted for *Daphnia* before (Morgan et al. 2001), but food quality differences were never tested explicitly in this context. Here we will use the combined analysis of between population genetic differentiation using neutral genetic markers (microsatellites) and variation in susceptibility to food quality in the freshwater species *Daphnia galeata.* This species inhabits permanent lakes and therefore experiences a relatively stable environment in which prolonged periods of clonal selection may be frequent. We tested for the prerequisites of local adaptation ($Q_{ST}>F_{ST}$) by comparing the genetic differentiation at neutral loci (microsatellites) and in a fitness related trait, i.e. the susceptibility to food quality changes.

### 3.2 Material and Methods

Zooplankton and water samples of lakes across Germany were collected during spring (Mai to July) of 2004 (see table 1). The geographical distances between populations ranged between 17 and 572 kilometers. All lakes were sampled by a Ruttner-sampler and key lake parameters were recorded (table 1). Water samples were stored at 8°C prior to laboratory treatment. Carbon content of the seston was measured by filtration of algae onto pre-combusted 24 mm diameter glass-fiber filters (Whatman GF/C) and later analysis using a CHN-analyzer (Perkin Elmer). Phosphorus-content of algae was measured spectrophotometrically after digestion with potassium persulfate (Langner and Hendrix 1982). Zooplankton samples of each lake site, collected by a plankton net, were preserved in 70% ethanol and stored at 12°C for further genetic analysis. Live animals were inspected directly at the sampling site and individuals of D. *galeata* were identified using morphological criteria (Flößner 2000). About 20 randomly selected individuals per lake were transferred alive to the laboratory and set-up as clonal stock cultures, and clones were maintained individually in 200 ml beakers filled with phosphorus-free artificial (ADaM) medium (Kluttgen et al. 1994). Animals were fed 1 mg C $L^{-1}$ of *Scenedesmus obliquus* which were cultured in Z/4 medium (Zehnder and Gorham 1960) with sufficient phosphorus supply (resulting in algae cells with a molar carbon to phosphorus (C:P) ratio of 70 – 80). All individuals in stock cultures were double-checked by genetic methods in order to verify their taxonomic status (see below for details on genetic methods). Individuals identified as *D. galeata* were used in the life-history experiments and microsatellite analysis (see below for details on genetic methods and Brede et al. 2006).

Table 1: *Daphnia* populations subjected to molecular and experimental analysis. Clones were sampled during spring 2004 and verified using microsatellite analysis. Carbon to phosphorus (C:P) ratio of seston are provided, and all lakes are grouped to different categories according to their C:P ratio ( C:P ratio >300 = high, C:P ratio <200 = low). Average distance between *Daphnia* populations was 304 km. abbr. = abbreviation

| lake | abbr. | C:P ratio | position (lattitude/longitude) | category |
|---|---|---|---|---|
| Meerfelder Maar | MMM | 359 | 50°06'02.09"N/ 6°45'23.84"E | high |
| Teterow | GTTS | 470 | 53°47'28.73"N/ 12°36'27.18"E | high |
| Ober Moos | GOMC | 70 | 50°27'52.19"N/ 9°22'17.53"E | low |
| Lake Gedern | GGG | 155 | 50°25'58.41"N/ 9°10'58.71"E | low |

**Measurement of quantitative traits- life-history experiments**

Experiments were set-up as a common garden experiment measuring the variation in somatic growth rate (SGR) which is strongly correlated to the intrinsic rate of population increase *r* (Lampert and Trubetskova 1996). For this we directly manipulated the nutrient (C:P) supply ratio of the algal food supplied to experimental animals. All experiments were conducted at 18°C with a light:dark cycle of 16:8 h. Before starting the experiments, individuals were adjusted to ADaM medium for at least five generations. Juvenile animals were collected from stock cultures and placed into 250 mL jars filled with ADaM medium and fed 1 mg C $L^{-1}$ of phosphorus sufficient-algae to guarantee a food supply above the incipient limiting level (Lampert 1987).

All clones were subjected to two different food quality conditions. Semi-continuous cultures of *S. obliquus* were established in Z/4 medium (Zehnder & Gorham 1960) with sufficient phosphorus or with limited phosphorus content in a way similar to Becker & Boersma (2003) resulting in algal cells with a molar C:P ratio of 70-80 for P-rich cells (P+) and about 1000 for P-limited algae (P-). Every day, 700 mL (total volume: 1.5 L) of culture medium was replaced with fresh medium: Algae were centrifuged at 2700 rpm for 10 min and resuspended in phosphorus-free medium (AdaM) to remove traces of dissolved P of the algal culture media. Carbon-content of algae was established photometrically using a calibration curve for both culture conditions. The calibration curve was established by measuring the extinction of differently diluted algae suspensions at 800 nm using a spectrophotometer (Hitachi, U-2000). For each dilution, the C-content was measured.

For each experiment, 20 neonates, born within 24 h, were placed into 250 mL experimental vessels of a flow-through system. Both algal suspensions (P-sufficient and P-limited algae) were set to 1 mg C $L^{-1}$ in order to be well above the minimum C-content level of algae for *Daphnia* (Lampert 1987), the flow-through rate of the chambers was set to 55 mL $h^{-1}$, resulting in a replacement of culture media of about 5 $d^{-1}$. The animals were kept at $P^{+}$- and $P^{-}$- conditions for four days at 1 mg C $L^{-1}$ to define their somatic growth rate (SGR) at both food quality conditions. We used clones of *D. galeata* sampled in lakes which showed a broad differentiation in their ecological parameters (see table 1), especially C:P ratio. In total, we used four different lakes, 2 lakes per category (high/low C:P ratio). In total, 15 lakes were sampled during spring 2004, but only two lakes with a high C:P ratio showed clones of *D. galeata* which could be used for our experiments. All clones were tested on P-sufficient and

P-limited algae. Each lake was represented by three clones of *D. galeata* (6 replicates each). In addition to the results of the life-history experiment, we obtained microsatellite data for a total of 40 animals per lake used in this experiment or from preserved ethanol samples (for details on genetic methods see below).

To determine SGR the initial and final mass for each clone was determined by transferring 10 juveniles, reared within 24 h, or the experimental animals after 4 days, to pre-weighted aluminum boats. Samples were dried at 60° C and weighted on a Sartorius microbalance (Sartorius 450J micro). Somatic growth rate (SGR) was calculated following the formula $dM/Mdt = \ln(M_0/M_t)/t$, where $M_0$ is the average initial mass of a clone, $M_t$ is the mass of the animals at time t (Sterner and Elser 2002). To test for the susceptibility of a taxon to variation in food quality, we determined the realized growth rate (RGR) by $RGR = SGR_{P-}/SGR_{P+}$ as described in Sterner & Elser (2002), where $SGR_{P-}$ represents the somatic growth rate under P deficient, and $SGR_{P+}$ the somatic growth rate at P-sufficient conditions.

**Genetic analysis**

*Taxonomic evaluation of the sampled species/ clones*

In order to determine species affiliation of our stock cultures sample animals were used directly following the protocol of Billiones et al. (2004) and Brede et al. (2006).In addition, DNA of preserved plankton was screened for species affiliation by genetic analysis, too. For this, animals were transferred to 1 ml TE buffer (10 mM TrisHCl, 1mM EDTA, adjusted to pH 8.0) for a minimum of 4h. Animals were directly transferred to 30-50 µl H3 buffer (10 mM Tris–HCl; pH 8.3 at 25°C, 0.05 M potassium chloride, 0.005% Tween 20 and 0.005% NP-40) and 2 µl proteinase K (Sigma 10mg/ml) was added. After 12 h incubation at 42°C proteinase K was deactivated by heating the sample at 95°C for 10 min. About 2 µl of template was used directly for the amplification of the ITS fragment by Polymerase Chain Reaction (PCR) (3 mM $MgCl_2$, 1x PCR buffer, 0.2 mM dNTP, 0.3 mM of each primer (ITS2-5.8S: 5´-GGA AGT AAA AGT CGT AAC AAG G-3, ITS1-18S: 5´-CGG TGG TCG ACG ACA CTT CGA CAC GC-3´, and 1 U TAQ DNA Polyermase, all chemicals and primers by Invitrogen). PCR was set to 94°C for 3 min, five cycles at 94°C for 1 min; 52°C for 1 min; 72°C for 1.5 min; 35 cycles: 94°C for 1 min; 50°C for 30 s; 72°C for 1 min; final synthesis step at 72°C for 5 min. A restriction fragment length polymorphism analysis (RFLP) was conducted on PCR-products. Amplification products of the ITS region were enzymatically digested by MwoI (5´-GVNNNNNN↓NNGC-3´; NEB) for 2.5 h at 60°C, total volume of 9.6

µl, 8 µl were PCR product and 10x NEBuffer for MwoI, 5 U of the restriction enzyme and autoclaved $dH_2O$. Digestion products were transferred to a 2% agarose gel and RFLP products were separated by applying 115 volts. Specific banding patterns allowed the identification of the taxa. All individuals in stock cultures were double-checked by genetic methods to verify their taxonomic status.

*Microsatellite analysis*

We conducted a microsatellite analysis using six different loci (DaB 10/15, DaB 17/17, DaB 17/16, DaB 10/14, Dp512 andDp519) which were shown to be highly variable for *Daphnia* (Brede et al. 2006). PCR reactions were performed in 0.2 mL tubes using either a Biometra T3 or a DYAD thermal cycler. All reactions were first performed in a 10 µL reaction volume containing 2.4 mM $MgCl_2$, 1× PCR buffer (see above), 0.25 mM dNTPs, 0.2 µM of each primer and 0.5 U Taq DNA polymerase (chemicals and primers by Invitrogen). Cycling conditions: 3 min 95°C, 35 cycles of 1 min steps at 95°C, 55°C and 72°C with a final of 7 min synthesis step at 72°C. Depending on the specificity of each primer set, PCR conditions varied mainly in annealing temperature, for details see see Brede et al. (2006). When pure PCR products were obtained, the PCR was repeated with labelled forward primers (Invitrogen, MWG). Amplification products were diluted and electrophoresed on an ALF sequencer (Amersham) with self-designed size standards based on Lambda virus DNA (Symonds and Lloyd 2004). We summed up in total for 40 individuals screening on variation for the six microsatellites per lake (total of 160 individiduals).

**Data analysis**

We used a General Linear Model (GLM) for the analyses of SGR and RGR. Clones were nested within lake and were implemented as random factor within the model, lake and quality were treated as fixed factors. The F-values and the accompanying degrees of freedom (df) were calculated according to Satterthwaite (1946). Differences between the lakes were tested *a posteriori* conducting a Fisher-LSD test using type III GLM. For clonal comparisons (within lakes), 2-way ANOVA analyses were performed, and one-by-one comparisons between lakes and clones were analyzed using Mann-Whitney-U tests. All analysis were conducted in STATISTCA (StatSoft 2005).

*$F_{ST}$ vs. $Q_{ST}$ comparison and genetic analysis*

The genetic structuring of populations was examined by a hierarchical, nested analysis of molecular variance in ARLEQUIN v. 3.11 (Excoffier et al. 2005). Different data file formats of multilocus genotypes were transformed using the software CONVERT (Glaubitz 2004). F-statistics were computed from the descriptive components of variance as implemented within the software of Arlequin v. 3.11 by permutation analysis (Excoffier et al. 1992). Variance was partitioned between both categories of natural C:P (high and low, table 2), between populations nested within these two groups, and among individuals within populations. Pairwise population differentiation was calculated based on $F_{ST}$ (Weir and Cockerham 1984). Global and population pairwise estimates of $F_{ST}$ were also calculated using AMOVA in ARLEQUIN v. 3.11. Significance was assessed after 16 000 permutations for global estimates and 3000 permutations for pairwise estimates, using the default settings in ARLEQUIN. We also estimated $R_{ST}$ (Slatkin 1995), and further analyses were done using both, $F_{ST}$ and $R_{ST}$, to ensure that our conclusions were free on the choice of statistics. $R_{ST}$ is a measure of genetic differentiation based on the stepwise mutation model (SMM), and is often more appropriate for microsatellites. This is because differentiation might be underestimated by $F_{ST}$ if mutations create allelic homoplasy and mutation rate is high relative to the migration rate. However, if mutation rates are low relative to migration rates, $F_{ST}$ can be expected to provide more accurate estimates of genetic differentiation than $R_{ST}$ (Slatkin 1995). As an estimator of $R_{ST}$ we used Goodman's unbiased q, calculated using RstCalc 2.2 (Goodman 1997). P-values of global $R_{ST}$ estimates over all populations were obtained by permutation tests, and approximate 95% confidence intervals by the range of the central 95% of 10000 bootstrap estimates.

For the quantitative trait RGR we estimated $Q_{ST}$ as a measurement of susceptibility to food quality changes. To obtain $Q_{ST}$ for this polygenic trait we followed the protocol of Koch et al. (2004). The following variance components were calculated: Lake ($V_p$), individuals ($V_i$), and the residual error ($V_r$). From these, the $Q_{ST}$ values were calculated according to the formula $Q_{ST} = V_p / ( V_p + V_i + 2 V_r)$. The variance components for RGR were obtained by a ANOVA analysis in STATISTICA (StatSoft 2005) and acquired variance were portioned accordingly. This was conducted for the different groups (high/low, table 1) and all pairwise population comparisons.

### 3.3 Results

*Life-history*

In the life-history experiment with two different food qualities (P+/P-) we observed a significant food quality effect, with SGR lower under P- conditions (table 2, figure 1). Also the clones of the different populations were significantly different from each other, but interactions between clones and experimental conditions were not found. In addition, we observed a lake x food quality interaction: that is a differential response of the populations originating from different lakes in SGR on food of different qualities (table 2, figure 1). No significantly different response was observed for the two categories (high/low C:P ratio, table 1, Wilks lambda=0.996, F(2,139) = 0.235, $p$ = 0.790).

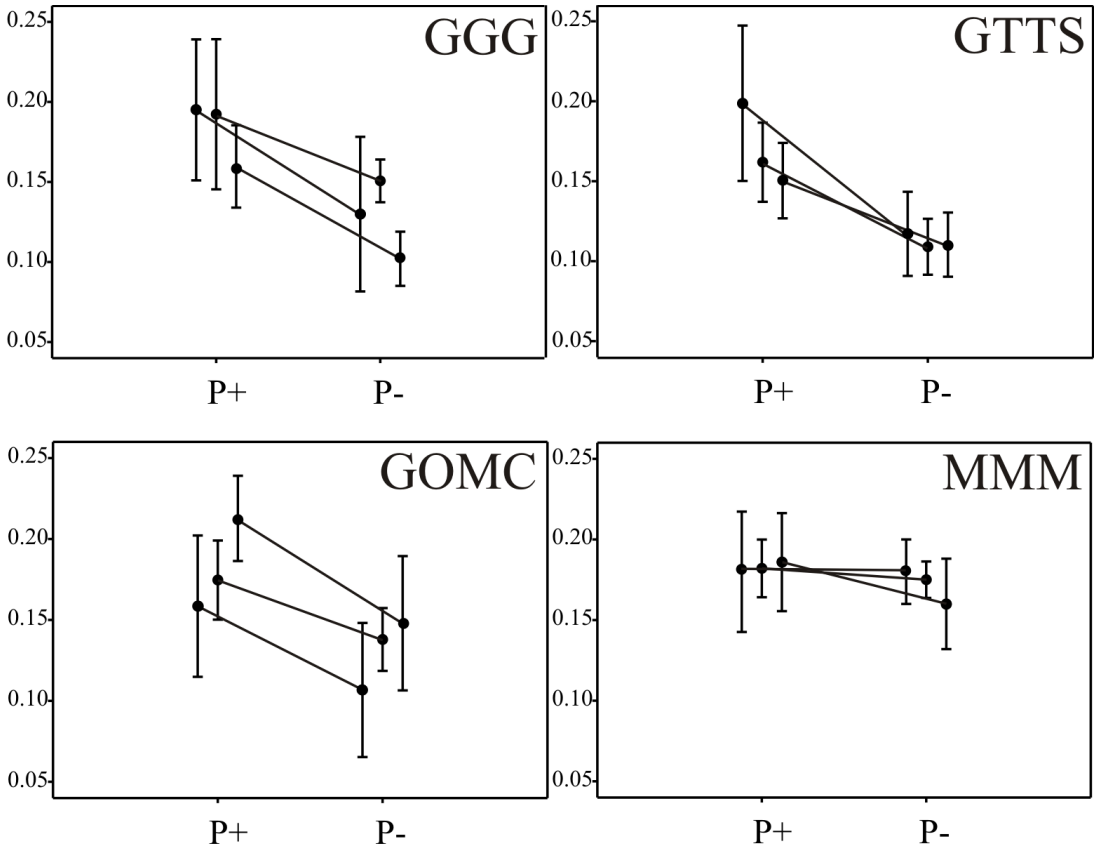


Figure 1: Reaction norms of *Daphnia* clones for variation in somatic growth rate (SGR) under two food quality conditions. Three clonal lineages per population were subjected to two different food qualities (P-sufficient food = P+, P-deficient food = P-). Error bars indicate standard deviations. Depending on the food quality and clonal composition, SGR showed a significant different reaction norm (table 2). For lake abbreviations see table 1.

Table 2: Comparison of the somatic growth rate (SGR) for different populations of *D. galeata* to variation in food. Populations were sampled in lakes across Germany from locations of different trophic categories. Results were obtained by a GLM, clones were nested to the different populations and treated as random factor, df = degrees of freedom, significant results are emphasized in bold letters ($p < 0.05$).

| | Sum of squares | df | mean square | F | *p* |
|---|---|---|---|---|---|
| Population | 0.108 | 3 | 0.036 | 1.209 | 0.367 |
| Clone(Population) | 0.239 | 8 | 0.030 | 4.899 | **0.019** |
| Food quality (P+/P-) | 0.198 | 1 | 0.198 | 32.505 | **<0.001** |
| Population*quality | 0.109 | 3 | 0.036 | 5.974 | **0.019** |
| Clone(Population)*quality | 0.049 | 8 | 0.006 | 0.293 | 0.967 |
| Error | 2.502 | 120 | 0.021 | | |

In addition, the populations showed a significant difference for their RGR (F=6.26, $p$=0.001, figure 2), thus populations differed in their susceptibility to food quality changes. However, this significant difference in susceptibility between populations could not be linked to the initial grouping of the lakes (figure 2).For both categories, we found some variation in susceptibilities between the lakes within a group.

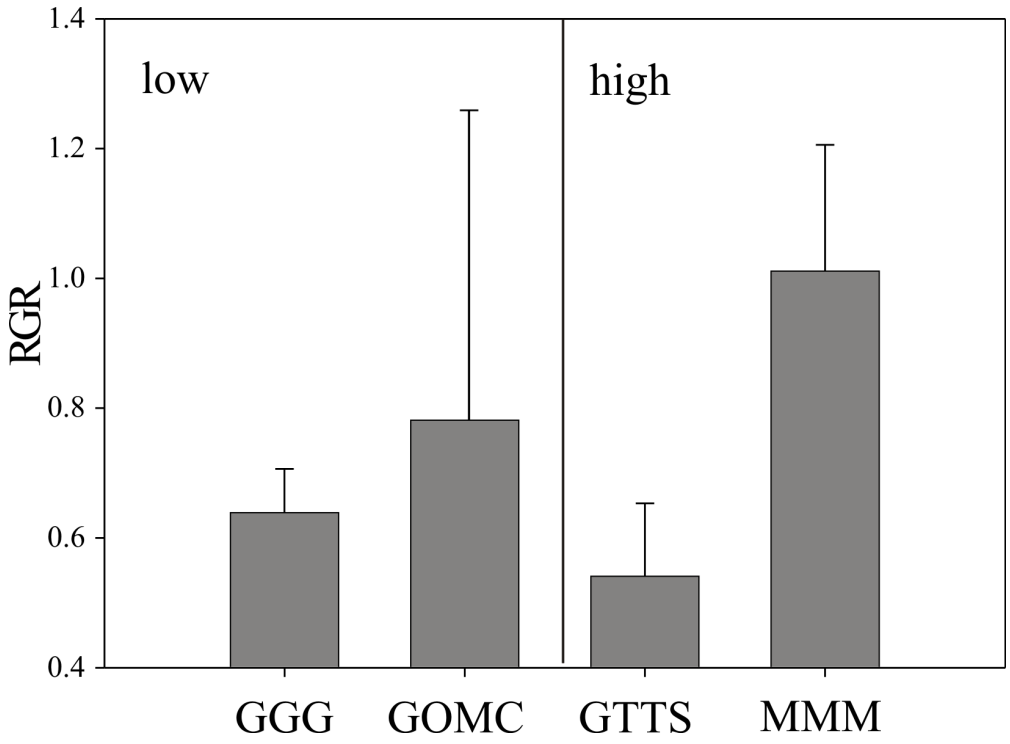


Figure 2: Comparison of the realized growth rate (RGR) of the different populations of *D. galeata*. Populations differed significantly in their susceptibility to changes in food quality of experimental algae (P-sufficient, P-limited). For the different categories depicted in table 1, no significant differences was found. Error bars indicate standard deviations.

*Genetic structure*

An analysis of molecular variance showed that the *Daphnia* populations were weakly structured; only about four percent of the variation was attributable to the two categories (table 3). Populations within the groups explained about eight percent of the total variation,

Table 3: Hierarchical analysis of molecular variance (AMOVA) for *D. galeata* populations, grouped in two categories (see table 1). Molecular variation was determined by six variable microsatellite loci.

| Source of variation | d.f. | Sum of squares | Variance components | Percentage of variation | *p* -value | $F_{ST}$ |
|---|---|---|---|---|---|---|
| Among categories | 1 | 7.812 | 0.02335 | 3.741 | 0.3412 | 0.0374 |
| Among populations within categories | 2 | 8.526 | 0.04951 | 7.933 | **<0.0001** | 0.0824 |
| Among individuals within populations | 148 | 74.089 | -0.05069 | -8.120 | | |
| Among individuals | 152 | 91.500 | 0.60197 | 96.446 | | |

but most of the variation was found within populations (table 3).The global estimates of divergence between the two different categories was $F_{ST}$ = 0.037 (± 0.01480, $p$ = 0.34115), the divergence between the populations within the categories was slightly higher ($F_{ST}$= 0.082 ± 0.0013, $p < 0.001$). For $R_{ST}$ variables, we obtained values not correlated to those determined by $F_{ST}$ analysis (global $R_{ST}$ = 0.05 ± 0.0013, p < 0.001, 10000 bootstrap estimations, table 4), but both values indicate only a moderate population differentiation.

Table 4: Population pairwise $R_{ST}$ estimates of genetic variation (above diagonal) and pairwise estimates of Weir-Cockerham $F_{ST}$ (below diagonal). Population abbreviations as depicted in table 1. Significant values are highlighted in bold.

| | MMM | GGG | GOMC | GTTS |
|---|---|---|---|---|
| MMM | | **0.078** | **0.059** | **0.092** |
| GGG | **0.150** | | 0.016 | **0.053** |
| GOMC | **0.048** | **0.112** | | 0.016 |
| GTTS | **0.040** | **0.149** | **0.082** | |

*Genetic and ecological differentiation*

$Q_{ST}$ comparisons for populations were determined for values of RGR, which represents a measurement of susceptibility to food quality changes. In all pairwise comparisons, values for $Q_{ST}$ exceeded the amount of $F_{ST}$, showing a larger diversification at ecological than at neutral loci, indicating directional selection (figure 3).

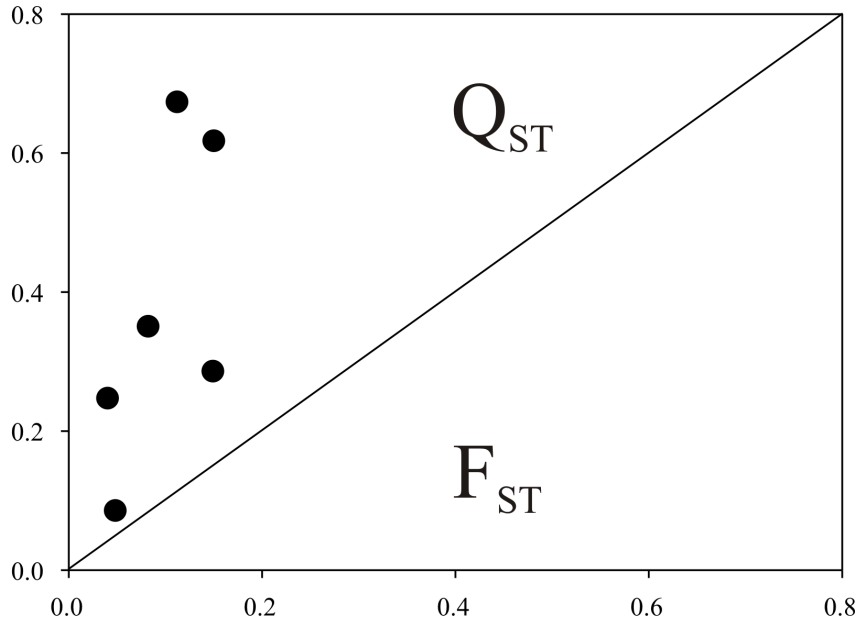


Figure 3: Population differentiation in quantitative traits ($Q_{ST}$) in relation to neutral genetic differentiation ($F_{ST}$) in *D. galeata* for variation in food quality. Differences between realized growth rate on food with different qualities are larger than expected from neutral differentiation. $F_{ST}$ was based on six polymorphic microsatellite markers, whereas the quantitative trait (RGR = realized growth rate) was used as a measurement of populations susceptibility to changes in food quality ($Q_{ST}$).

### 3.4 Discussion

We found a significant differentiation in susceptibility to variation in food quality between different populations (RGR), but no significant differentiation among the two categories (high/low C:P ratio, table 1). Due to the low number of populations within each category and the artificial differentiation into high and low phosphorus lakes, we might have failed to detect a signal of ecological differentiation. Since lakes vary seasonally and annually in their phosphorus availability (Kreeger et al. 1997) and we reduced the natural variation into one value per lake, our categories might not represent the true natural phosphorus conditions which determined the life-history of local clones. Nevertheless, we did observe the significant between-population variation with respect to the susceptibility to changes in food quality. As was shown in several life-history studies, *Daphnia* clones respond with a decline in somatic growth rate (and other life-history traits), if exposed to food of low quality (figure 1, table 2, and see e.g. Sterner et al. 1993, Boersma 2000, Elser et al. 2001, Becker and Boersma 2003, Seidendorf et al. 2007). Moreover, populations showed a significant differential response to either P-sufficient or P-limited algae (*population*quality* effect, table 2, figure 1), suggesting local adaptation in *Daphnia*.

During the last century most European lakes went through a phase of eutrophication, with subsequent recovery to their original trophic state due to effluent control (Correll 1998). Thus, an adaptation to locally different C:P ratios occurred most likely during a relatively short time period. Although we are not able to determine the time frame of adaptation, we know that local adaptation to different environments can be a rather fast process in *Daphnia* (De Meester et al. 1994, Hairston et al. 1999, Cousyn et al. 2001, Morgan et al. 2001, De Meester et al. 2002, but see Spaak and Keller 2004). Since eutrophication represents a multi-factorial process, we also have to consider indirect effects that accompany changes in phosphorus load. It is known that under phosphorus limitation species composition of sestonic algae changes (Kreeger et al. 1997), and different algal species can have differential effects on life-histories in *Daphnia* (Boersma and Vijverberg 1994a, 1995). Since *Daphnia* species are non-selective filter feeders that are not able to discriminate between different food particles (DeMott 1986), they are highly susceptible to changes in species composition of the seston. Thus *Daphnia* clones might be adapted not only to the different C:P ratios of algae, but also to morphological characteristics of the food that come along with an adaptation to variation in P (e.g. see Brendelberger 1991, Lampert and Brendelberger 1996).

Based on the presented evidence for local adaptation in *Daphnia* to variation in food quality, we tested the assumption for this process, i.e. directional selection. We used an approach based on the comparison of neutral genetic markers (microsatellites) and a quantitative trait (RGR). As a first step we determined the population structure, which revealed a level of differentiation that was comparable to other studies in *Daphnia* which is moderate (e.g. Michels et al. 2003). Since most studies did not find a correlation of genetic differentiation and geographical distances among populations, other factors than geographic distances explain local differentiation, e.g. the size of the dormant egg bank, the length of the growing season and the strength of clonal selection (De Meester et al. 2006).

In a second step we compared the neutral genetic markers with the response to variation in food quality (RGR) and as reported for many species across a broad range of taxa (Merila and Crnokrak 2001, McKay and Latta 2002), $Q_{ST}$ values greatly exceeded $F_{ST}$ values for populations of *D. galeata* (figure 3). This pattern indicates directional selection, a pre-requisite for local adaptation. Differences among traits of $Q_{ST}$ may reflect differences in both inheritance and selection. For example, a trait may show $Q_{ST} > F_{ST}$ values not because it has been a target of selection but because of co-variation of selection on genetically correlated traits. For *Daphnia* it was shown that the trait we used here, somatic growth rate and its resulting measurement of susceptibility (RGR), is fitness related when food quality changes (Seidendorf et al. 2007). Thus local adaptation to variation in food quality is most likely in *Daphnia galeata* because of directional selection. To differentiate between the different parameters discussed for local adaptation, it is necessary to assess the role of food quality vs. other parameters such as salinity (Teschner 1995), temperature (Mitchell and Lampert 2000), oxygen concentration (Carvalho 1984), levels of pollution (Bachiorri et al. 1991), or other unspecified factors (Declerck et al. 2001).

In summary, our data support local adaptation in *D. galeata* based on a test on directional selection and a *population*food quality* interaction, even though we could not link it with the current C:P conditions in the lakes directly. These findings highlight the role of food quality for population differentiation in *Daphnia* besides the other factors discussed for local adaptation in *Daphnia*. We showed that patterning of pairwise $Q_{ST}$ between populations of *D. galeata* is most likely attributable to spatially varying selection to food quality changes and/or the direct influence of habitat specific environmental effects that come along with changes in phosphorus availability.

# CHAPTER 4: RAPID IDENTIFICATION OF ECOLOGICALLY RELEVANT GENES IN *DAPHNIA*: DIFFERENTIAL GENE EXPRESSION PATTERNS AS A RESPONSE TO VARIATION IN FOOD QUALITY

# Chapter 4: Rapid identification of ecologically relevant genes in *Daphnia*: differential gene expression patterns as a response to variation in food quality

## 4.1. Introduction

Adaptation requires first that organisms differ in fitness and second that those fitness differences are heritable. Understanding how adaptive traits evolve requires that we integrate genetic analyses of phenotypic differences with analyses of phenotypic variation in an ecological context. Several classical studies of adaptation, e.g. the evolution of beak morphology in Darwin´s finches, have recently been extended using molecular techniques which allow the identification of the genetic background for variation in life-history traits or behaviour (Luikart et al. 2003, Stearns and Magwene 2003, Abzahnov et al. 2004, Fitzpatrick et al. 2005). Expression patterns of these functional genes, so-called candidate genes, were described for a number of ecologically relevant traits (Fitzpatrick et al. 2005). Candidate genes are defined as genes that contribute to the development of a particular life-history trait, phenotype or behaviour. Changes in the expression patterns of these candidate genes provide a description of the molecular basis of phenotypic variation (Fitzpatrick et al. 2005). Expression of candidate genes which are associated with ecological relevant traits (mediated by natural selection) provide information on the genetic basis of evolutionary changes (Abzahnov et al. 2004).

The aim of our study was to provide a method that enables the identification of the genetic basis of phenotypic plasticity for ecologically relevant traits in the freshwater crustacean *Daphnia*. Although several candidate genes were described for a number of invertebrate species (Liao and Freedman 2002), and the freshwater cladoceran *Daphnia pulex* is one of the species with an almost completely described genome (for more information on the *Daphnia* genome project visit https://dgc.cgb.indiana.edu/display/daphnia/Introduction or http://wfleabase.org/), we are not aware of any study where candidate genes related to ecologically relevant environmental conditions were identified in *Daphnia*. Here we used an interdisciplinary approach combining life-history experiments with differential display-PCR (DD-PCR) analysis (Liang and A.B. 1992, Xiong et al. 1998). This method is very well suited to identify genes which show any qualitative difference among different treatments. The main advantage of the method is that, in contrast to other techniques developed to characterize candidate genes such as quantitative realtime PCR (qPCR) and microarray technology, DD-PCR is a less laborious and time consuming approach. DD-PCR captures approximately 90%

of the transcriptome (Liang 2002) and provides sequence information of differentially expressed genes. In addition, it is rather cost un-expensive and can be applied in almost every laboratory.

Here we tested this approach to one of the main issues in current zooplankton ecology, that is the importance of food quality as a factor determining growth and reproductive success. *Daphnia* species are in general highly variable in its phenotype, e.g. on fish predation (e.g. formation of helmets, Tollrian 1990, 1993), UV radiation (melanization of the carapax, Rautio and Korhola 2002) or food limitation (adaptation of filtering screen, Repka 1999). In addition, life-history studies demonstrated that chemically unbalanced food resources, such as algae grown under phosphate-limited conditions ("P-limited algae"), reduce fitness in *Daphnia* (e.g. Sterner et al. 1993, Elser et al. 2001, Boersma and Kreutzer 2002). Only a limited number of *Daphnia* studies (e.g. Gorokhova et al. 2002, Pijanowska and Kloc 2004) combined ecological data with molecular analyses in an attempt to provide the genetic background for differential responses to experimental conditions. For example, growth rate in *Daphnia* is positively correlated with the length and content of the intergenic spacer (IGS) region of the rDNA tandem repeat unit (Elser et al. 2000b, Gorokhova et al. 2002, Weider et al. 2004). In addition, exposure of *Daphnia* to invertebrate and vertebrate kairomones showed that life-history changes are associated with changes in heat-shock proteins (HSP´s) level and the actin and tubulin cytoskeleton (Pijanowska and Kloc 2004, Pauwels et al. 2005). Moreover, Diener et al. (2004) observed that there are changes in genes expression under starvation versus feeding conditions. But they did not identified the genes or proteins correlated with these changes in expression levels. Thus we are not aware of any other studies which focused on the isolation of candidate genes (in particular ecological relevant genes) associated with life-history responses caused by environmental stress in *Daphnia* by differential display.

Here we extended the approach of Diener et al. (2004) to identify and address ecologically relevant genes with regard to variation in food quality. The specific aim of our study was to show that is possible to identify differentially expressed candidate genes in *Daphnia magna* as a result of differences in food quality using the combination of ecological experiments and an fast and effective PCR based technique.

## 4.2 Material and Procedures

All molecular techniques were based on the protocols by Diener et al. (2004) and Sambrook et al. (1989), any modifications are mentioned below. Pilot experiments showed that 20 juveniles (born within 24h) of *D. magna* resulted in a sufficient amount of total RNA (> 1 µg, determined spectrophotometrically at 260/280 nm wavelength) for reverse transcription and PCR.

### Total RNA extraction

Total RNA was extracted using the Trizol method, a procedure adapted from the guanidine thiocyanate-phenol-chloroform method for extraction of total RNA based on US-Patent 5.346.994 (Chomczynski and Sacchi 1987, Chomczynski 1993). The frozen animals were immediately transferred using the end of an RNase-free pipette tip into a pre-cooled 1.5 mL microcentrifuge tube containing 500 µl TriReagent as described by Chomczynski and Sacchi (1987, 1993). Animals were homogenized using RNAse free pistils within 30s. Subsequent steps were accomplished following the manufacturers' protocol. Total RNA was re-suspended in 20 µl of RNase-free water. RNA concentrations and purity was determined spectrophotometrically at 260 and 280 nm. DNA:RNA-ratios varied between 1.5 and 1.9 and in addition the yield of RNA isolation was determined using 1.2% agarose gels. Samples of extracted RNA were stored at -20°C. In order to test for potential DNA contamination of RNA, we subjected isolated RNA to two different treatments: 1.) total RNA (~1 µg) was mixed with 1 U of RQ1 RNase-free DNase I (Promega Corp., Madison, WI, USA) and 1 µl of 10X DNase buffer (400 mM Tris-HCl pH 8.0, 100 mM $MgSO_4$, 10 mM $CaCl_2$, total volume 10µl) and incubated at 37°C for 30 min. The reaction was terminated by adding 1 µl of stop-solution (20 mM EDTA, pH 8.0) and heating at 65°C for 10 min. 2.) In addition, in a second step total RNA (1 µg) was mixed with 1 µl of RNase (Promega Corp., Madison, WI, USA) and 1 µl of 10X buffer (400 mM Tris-HCl pH 8.0, 100 mM $MgSO_4$, 10 mM $CaCl_2$) for 30 min at 37°C in a final volume of 10 µl. The so treated RNA samples were used for reverse transcription and PCR amplification. Both samples (total RNA treated with RNAse and total RNA treated with DNAse) were subjected to reverse transcription and PCR.

### Reverse Transcription and PCR

Reverse transcription was generated with RevertAid$^{TM}$ Moloney murine leukemia virus (M-MuLV, MBI-Fermentas) according to the supplier´s protocol. In contrast to Diener et al. (2004), we used an oligo$(dT)_{18}$-Primer for cDNA synthesis. Total RNA (1 µg in 10 µl of

RNase-free water) and 0.5 µg oligo$(dT)_{18}$ was added up to a final volume of 11 µl with nuclease-free deionized water. The mix was incubated at 70°C for 5 min and stored on ice. Subsequently, 4 µl 5x reverse transcription buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 2 µl of 10 mM deoxynucleotide triphosphate (dNTP) and 20 U of ribonuclease inhibitor (MBI-Fermentas) were added and filled up with nuclease-free deionized water to a final volume of 19 µl. This solution was incubated at 25°C for 5 min and 200 U of RevertAid$^{TM}$ M-MuLV Reverse Transcriptase was added. The reaction mixture was incubated at 42°C for 60 min. The reaction was terminated by heating at 70°C for 10 min and cDNA was chilled on ice before DNA amplification.

cDNA of experimental animals were subjected to polymerase chain reaction with all possible primer combinations (see Table 1). Although this is not the typical way a DD-PCR is performed because mostly the single stranded cDNA is amplified with the oligo dT primer used to create the cDNA initially, and subsequently using an arbitrary primer, we followed the approach of Diener *et al.* (2004) because they showed with their approach that they can produce a reasonable amount of fragments. PCR reactions (total volume of 25 µl) consisted of 1 µl of 1:10 diluted cDNA, 25 mM of each arbitrary primer, 3 mM $MgCl_2$, 0.2 mM dNTP, 1x amplification buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), and 1 U *Taq*-Polymerase (Invitrogen Corp., USA) per reaction (Diener et al. 2004). DNA was amplified using the following temperature profiles: 1 cycle at 1 min at 94°C, 5 min at 35°C and 5 min at 72°C and 39 cycles at 94°C for 1 min, 2 min at 50°C and 2 min at 72°C.

Table 1: DNA sequences of primes used for DD-PCR analyses of *D. magna* as described in Diener et al. (2004).

| Primer for DD-PCR analysis | Sequence |
|---|---|
| A2 | 5´- AACTAGAGCTCCTCCTC-3´ |
| A3 | 5´- AACTAGAGCTCCAGCAG-3´ |
| A4 | 5´- AACTAGAGCTCTCCTGG-3´ |
| A5 | 5´- AACTAGAGCTCTCCAGC-3´ |
| A6 | 5´- AACTAGAGCTCCCTCCA-3´ |

**Visualization and preparation of PCR products**

PCR products were separated on 1.2% agarose 1x TBE gels (LE Agarose, Biozym). Electrophoresis was carried out at 120 V for 4-6 h. PCR products were visualized by subsequent staining with ethidium bromide. Bands varying in intensity levels among

treatments were isolated and transferred to a sterile 1.5 mL microcentrifuge tube. Subsequently, DNA was extracted from agarose gels using the NucleoSpin Extract II Kit (Machery-Nagel Inc., Easton) following the manufactures instructions; DNA was eluted into 50 µl TE-Buffer. All eluted fragments were re-amplified by the corresponding primer-sets in order to maximize the yield of DNA for the subsequent cloning procedure. Total PCR products were separated on a 1.2% agarose gel and DNA was purified using the NucleoSpin Extract II Kit. Although we know that in most cases DD-PCR products are run on polyacrylamide gels because of higher resolution power of fragments, we decided to use agarose instead because it allowed us to easy extract the DNA from the gel in order to clone these differentially expressed fragments.

**Cloning of differentially expressed cDNA and identification of candidate genes**

Purified cDNA was cloned using the pGEM-T Easy Vector System (Promega, Promega Corp., Madison, WI, USA) following the manufactures protocol. We added cDNA (3 µl) to 1 µl of pGEM-T Easy Vector (50 ng) and 2x Rapid T4 Ligation Buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% PEG). Reactions were started by subjecting 3U of T4 DNA-ligase to the mix for 1 h at RT. Subsequently, the vector was cloned into highly competent *Escherichia coli* (strain Xl1-Blue MRF; Stratagene Inc., USA) by a standard heat-shock protocol. After incubation for 1 h with SOC medium 100 µl each transformation culture was plated on LB/ampicillin/IPTG/X-Gal plates for blue-white screening. Plates were incubated overnight at 37°C. Two white colonies of each transformation were isolated and grown in 50 mL LB-Medium (IDG, corp., USA) containing 100 µg/mL ampicillin. Plasmids were isolated using the GFX Micro Plasmid Prep Kit (Amersham Bioscience) according to manufactures protocol. Purified plasmids were re-suspended in 100 µl TE-buffer. Sequences of the cDNA inserts were obtained by SRD GmbH, Frankfurt am Main, a sequencing laboratory, using the primer UCP 5´-GTTTTCCCAGTCACGTTGTA-3´ or RCP 5´-GGAAACAGCTATGACCATGATTAC-3´. All sequences were visually verified and edited using BIOEDIT (Hall 1999) and compared with previously published sequences using the NCBI database (*http://www.ncbi.nlm.nih.gov*). In addition, we also compared our sequence on the genome of *D. pulex*, a rather close related species to *D. magna* (*http://wfleabase.org/blast/*).

### 4.3. Assessment

In order to test our method we conducted a life-history experiment with one clone of *D. magna*, which originated from a stock culture at the MPI, Plön, (M24, originally collected at Großer Plöner See, Germany), and which was subjected to two different food quality conditions. Semi-continuous cultures of *Scenedesmus obliquus* were established in Z/4 medium (Zehnder and Gorham 1960) with sufficient phosphorus or with limited phosphorus content in a way similar to Becker and Boersma (2003), resulting in algal cells with a molar carbon to phosphorus (C:P) ratio of 70 - 80 for P-rich cells ($P^+$) and about 1000 for P-limited algae ($P^-$). Every day, 700 mL (total volume: 1.5 L) of culture medium was replaced with fresh medium: Algae were centrifuged at 2700 x g at 10 min and diluted in phosphorus-free medium ("Aachener Daphnien Medium", AdaM, Kluttgen et al. 1994) to remove traces of dissolved P of the algal culture media. Carbon (C) content of algae was established photometrically using a calibration curve for both culture conditions. The calibration curve was established by measuring the extinction of different diluted algae suspensions at 800 nm using a spectrophotometer (Hitachi, U-2000). For each dilution, C-content was measured subsequently by filtration of algae onto precombusted 24 mm diameter glass-fiber filters (Whatman GF/C) and C-content was quantified by a CHN-analyzer (Perkin Elmer). P-content of algae was measured spectrophotometrically after digestion with potassium persulfate (Langner and Hendrix 1982).

All experiments were performed at 18°C with a light:dark cycle of 16:8 h. Before starting the experiments, individuals of *D. magna* were adjusted to ADaM medium for at least five generations. Juvenile animals were collected from stock cultures and placed into 250 mL jars filled with ADaM medium and fed 1 mg C $L^{-1}$ of $P^+$-algae to guarantee a food supply above the incipient limiting level (Lampert 1987). 20 Neonates, born within 24 h, were placed into 250 mL experimental vessels of a flow-through system. Both algal suspensions (P-sufficient and P-limited algae) were set to 1 mg C $L^{-1}$ and the flow-through rate through the chambers was set to 55 mL $h^{-1}$, resulting in a replacement rate of total chamber volume of about 5 $d^{-1}$. Each culture condition was represented by 5 replicates: three replicates were used to determine the somatic growth rate (SGR), two replicates were subjected to molecular analyses. Samples for molecular analyses were immediately frozen into liquid nitrogen at the end of the experiment and kept at -80°C until RNA extraction. For SGR, the initial size of juveniles was measured using 20 newborns, and the size after 4 days of the experimental animals was measured to the nearest 0.02 mm under a microscope. Somatic growth rate

(SGR) was calculated following the formula $dM/Mdt = \ln(M_0/M_t)/t$, where $M_0$ is the average initial size of a clone and $M_t$ is the size of the animals at time t (Sterner and Elser 2002). Differences in SGR between treatments were analyzed calculating a t-test.

*D. magna* showed a significant (P = 0.013) decrease in somatic growth rate (SGR) of about 33% in P-limited conditions compared with animals raised on a P-sufficient diet (SGR $P^+$ = 0.407 $d^{-1}$+/- 0.053 standard deviation (std. dev.), SGR $P^-$ = 0.306 $d^{-1}$+/- 0.047 std. dev., Figure 1). Several life-history studies have shown that *Daphnia* responds with a decline in

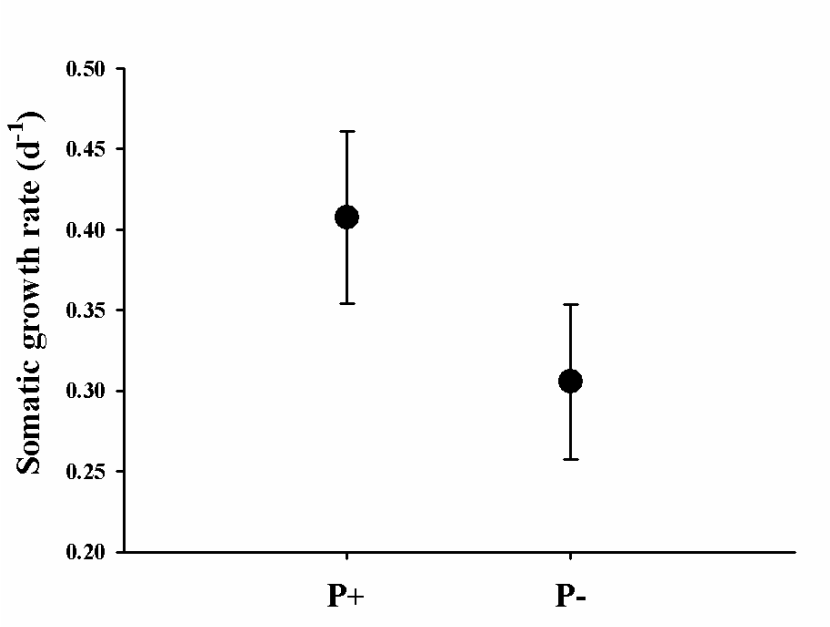


Figure 1: Results of a life-history study of *D. magna* individuals cultured in a flow-through system at two food quality conditions. SGR = average somatic growth rate, $P^+$ = individuals cultured at P-sufficient conditions, $P^-$ = individuals cultured at P-limited conditions, error bars indicate standard deviations.

somatic growth rate and other life-history traits due to food of lower quality, especially P-limited food (e.g. Boersma 2000, Becker and Boersma 2003). A similar pattern was observed in our study rearing a clone of *D. magna* under P-limited and P-sufficient conditions. The significantly reduced SGR of *D. magna* under poor food conditions supports the observations of previous studies indicating the important role of phosphorus for fitness related traits in *Daphnia*. DD-PCR amplification of RNA treated with RNAse before cDNA synthesis failed to generate any banding profile. The DD-PCR profiles for the DNAse treatment and for amplification with untreated extractions showed that the isolated RNA was not contaminated with genomic DNA because both amplification patterns were identical. The same was shown when cDNA of 24 hours old juveniles was used for RNAse and DNAse treatment. Thus,

observed banding patterns represented patterns of differential gene expressions since no false-products were generated.

Animals cultured at limiting P-conditions showed a differential response in RNA expression compared to RNA expression of P-sufficient organisms. For six primer combinations we obtained a differential banding profile (A2/A4, A2/A6, A3/A4, A3/A5, A3/A6 and A4/A5), the other combinations revealed no differential DD-PCR products (Table 2). For each of these combinations at least one fragment was different to the controls, that is it was possible to obtain more than one differential fragments per primer combination. Comparing the different banding profiles of all combinations, we found that some of the bands were either absent at P-limited conditions (Figure 2) or were down- or up-regulated (Figure 2; Table 2). In total, we were able to identify 9 bands that were up- or down-regulated which were subsequently used for DNA cloning and sequencing. For 5 out of 9 of these cloned sequences we found homologous sequences by database searches (Table 2). As a

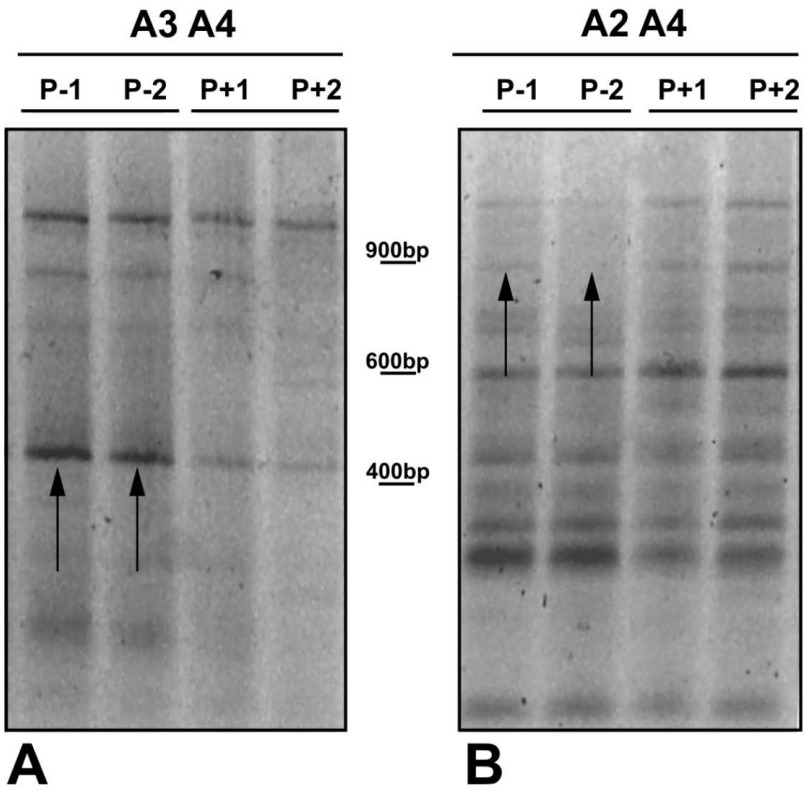


Figure 2: Example for a differential display PCR profile of *D. magna* using the primer combinations (A) A3/A4, and (B) A2/A4. P-1 and P-2: Two cDNA-ddPCR profiles each based on 20 pooled individuals of *D. magna* cultured at P-limited conditions, $P^{+}1$ and $P^{+}2$: Two cDNA DD-PCR profiles each based on 20 pooled individuals of *D. magna* cultured at P-sufficient conditions, Arrows indicate up-regulated genes at P-limited conditions at (A) and down-regulated genes at P-limited conditions (B). Sizes for the internal 100-bp-ladder are given in basepairs (bp).

threshold we defined any sequence consilience that was 20 bp or more. Four loci which showed no similarity with previous published sequences and one DNA sequence which showed highest similarity with human mRNA and were not considered in further analyses.

Table 2: Primer combinations which resulted in differential DD-PCR profiles of *Daphnia magna* subjected to different food qualities. The primer combinations A2/A3, A4/A6 and A5/A6 showed no differential profile between $P^+$ and $P^-$ conditions. Amplified product length (basepair; bp) and loci (gene) with the highest similarity to the *Daphnia* DNA sequences are provided. Arrows indicate if genes have been up- (↑) or down-regulated (↓) at P-limited conditions compared to P-sufficient conditions. Function classifies the function of these genes. Accession numbers (Acc. No.) for the NCBI databank, and similarity among target and reference sequences are presented in the two last columns. Identity reflects the similarity of *D. magna* sequences with sequences of the NCBI database per sequence given in numbers and percentages. A BLAST of the fragments for the sequences given for the genome of *D. pulex* revealed no additional information (http://wfleabase.org/)

| Primers | size (bp) | | Gene | Function | Acc. No. | Identity |
|---|---|---|---|---|---|---|
| **A2/A4** | **900** | ↓ | A2-Topoisomerase I binding RS Protein, mRNA, *Aspergillus fumigatus* | *Cell cycle* | XM 749616.1 | 23/24 (95%) |
| | **1100** | ↓ | *no analogy with previously published sequences* | | | |
| **A2/A6** | **1200** | ↑ | mitochondrial glycerol-3-phosphate dehydrogenase *(Aspergillus* ), mRNA (G3P-DH) | *Enery* | BX016916.1 | 41/46 (89%) |
| **A3/A4** | **450** | ↑ | myosin heavy chain genes (*D. melanogaster)* , alternatively spliced products and isoforms of myosin heavy chain genes *(D. melanogaster* ), 12 Isoforms | *Movement* | X53155.1, and NM 165181.1 to NM 165192.1 | 214/267 (80%) 214/267 (80%) 214/267 (80%) |
| | **850** | ↑ | nicotinamid adenine dinucleotide trans-dehydrogenase *(similar to C. familiaris* ) (NADH-DH) | *Energy* | XM 536481.1 | 55/65 (84%) |
| | **1050** | ↑ | ATPase ( *Caenorhabditis elegans* ), mRNA | *Energy* | NM 058764.2 | 180/224 (80%) |
| **A3/A5** | **950** | ↓ | *no analogy with previously published sequences* | | | |
| **A3/A6** | **800** | ↑ | *no analogy with previously published sequences* | | | |
| **A4/A5** | **900** | ↓ | *no analogy with previously published sequences* | | | |

Only for the comparisons at NCBI we were able to obtain results on sequence alignment with previously published genes but not for the search on the genome of *D. pulex.* All sequences obtained by the DD-PCR approach resembled other mRNA genes (obtained by NCBI database searches), which confirmed a) that no DNA contamination occurred and b) that the DD-PCR indeed revealed differential gene expression patterns of *D. magna*. More specifically, using primer combinations A2/A6 we found mRNA of the glycerol-3-phosphate dehydrogenase gene which was upregulated at P-limited conditions (G3P-DH). This enzyme plays an important role in the glycolysis as it is involved in the generation of high-energy molecules (ATP and NADH). In addition, we identified mRNA of the ATPase gene, which was up-regulated at P-limited conditions (primer combination A3/A4). This type of enzyme is necessary to provide cell energy in form of ATP which is needed for nearly any energy consuming biological pathways in cells. A third enzyme which is relevant for respiration and thus is involved to provide cell energy as well (nicotinamid adenine dinucleotide trans-dehydrogenase, NADH-TDH) was up-regulated at P-limited conditions. The enzyme NADH-TDH catalyses the reduction of nicotinamide adenine dinucleotide ($NAD^+$/NADH).

In addition to proteins relevant for respiration, also structural genes coding for muscle proteins (myosin heavy chain) were up-regulated at P-limited conditions (primer combination A3/A4). Together with the protein actin, myosin generates the contractile force responsible for many aspects of cell locomotion and muscle contraction. Also a small protein related to the A2-Topoisomerase I (RS Protein) has been found to be down-regulated at P-limited conditions. This type of enzyme affects the topology of DNA, i.e. topoisomerases change the supercoiling of DNA.

In general, we showed that it is possible with this approach to gain information on the molecular background for a specific trait (here SGR) for *D. magna* on food with different quality. It allowed us to reveal information on genes involved in a differential response when food quality differs (qualitative information), but not for any quantitatively interpretation. Based on the fact that the raw material for this study is cDNA, which can easily be obtained in almost every lab as was shown here, we believe that our method is applicable for a huge number of closely related species of *Daphnia magna* and for an innumerable number of questions.

### 4.4. Discussion

Up until now, the molecular mechanisms responsible for changes in life-histories are largely unknown, and we have very little knowledge about the response of daphniids to counteract the effects of low-quality food. Some reports exist describing an increased uptake of food particles in order to compensate the lack for phosphorus. Plath and Boersma (2001) found that P-limitation of food increased the beat rate of the filter screens in *Daphnia* (located on appendages). They concluded that daphniids counterbalance a lower P-content of their food by spending more energy on acquiring the limiting resource. Other studies indeed described increased respiration rates of animals at P-limited conditions (Jensen et al. 2001, Darchambeau et al. 2003). The reported increase of clearance rates with the rise of seston P-deficiency seems to be an appropriate adaptive behavioural response to nutrient deficiency. A prerequisite for this phenotypic response is an increase energy metabolism, especially the generation of ATP, and will most likely cause a higher turn-over rate for proteins related to the movement of filter-screen appendages, such as myosin. Our data do support the observation of Plath and Boersma (2001), because we found a gene up-regulated at P-limited conditions necessary for muscle contraction and thus feeding activity (table 2).

The genes related to a differential response on food quality differences can be categorized to different aspects of either a) energy metabolism or b) the regulation of (appendage) movement. For example, myosin, the energy-consuming part of the actin/myosin muscle complex, was up-regulated at P-limited conditions, as well as genes necessary for cell respiration and thus for providing more energy due to higher demands by the behavioural response due to P-deficiency (Table 2). Furthermore we found a gene which could not be directly related to a response to P limitation: the A2-Topoisomerase binding RS protein, which is necessary for the replication of the DNA during a cell cycle, was down-regulated at P-limited conditions. One explanation might be that at P-limited conditions replication and cell divisions is reduced to allocate energy and other recourses for maintenance and food uptake. Since individuals at both phosphorus treatments differed in somatic growth rate, they most likely will have differed also in other characteristics such as for example their developmental stage, number of cells in particular cell cycles, etc. Thus, the differential response in transcription factors might not be directly caused by different P levels but represent the indirect response resulting in a number of molecular changes. Even though we are not able to differentiate among these direct or indirect effects of P limitation, our results

show that variation in P can be the cause for the differential expression of genes producing proteins found in P dependent biochemical processes.

Some information exists that the correlation between the expression of a gene and the amount of translated protein might not be as tight as we would like (Caderas et al. 2000, Rockman and Kruglyak 2006). Therefore, differentially expressed genes may not convert into different protein levels with functional or ecological implications. Other methods and tools such as 2D-Page or Western blotting techniques will complete the picture of understanding the complex interactions between genes and proteins.

**Comments and recommendations**

We showed that we developed a fast, cheap and easy applicable method which is not restricted to studies based on food quality differences, but for a huge field of ecological motivated studies interested in the molecular background of a differential response in *Daphnia* or closely related species. Although DD-PCR does only detect a specific sub-sample of all expressed genes (mainly house keeping genes) in a cell we have identified potential candidate genes for further studies, using e.g. quantitative PCR or studies on between species differences.In addition, it would be very interesting to test if the same patterns of differential gene expression occur if the growth rate of *Daphnia* varied for other reasons, such as lower temperature, nitrogen limitation or lower food density that is to test for a general stress reaction That is we highly motivate studies applying other factors known to alter life-history strategies in *Daphnia* in combination with the method presented here. Furthermore, with the use of these candidate genes, further research on natural populations and among different taxonomic units (clades, ecotypes and species) are bound to unravel the molecular genetic architecture of ecological differentiation and allows the test of hypotheses on local adaptation and natural selection (Agrawal 2001).

# GENERAL DISCUSSION

# GENERAL DISCUSSION

In northern temperate lakes, total phosphorus (P) concentration is regarded as the key factor of eutrophication (Schindler 1978), and this element is limiting phytoplankton growth and production in most systems (e.g. see Sommer 1992, Elser and Hassett 1994). Many lakes in Europe are known to have gone through severe ecological changes in the last decades (eutrophication), mostly induced by anthropogenic impact, but many recovered to their original trophic state due to pollution control (Correll 1998). Despite the large fluctuations in available phosphorus, body C:P ratios of zooplankters are relatively constant. Different genera of zooplankton show different phosphorus contents (e.g. Hessen and Lyche 1991) which leads to differences in their requirement for phosphorus (Schulz and Sterner 1999) and consequently their susceptibility to changes in phosphorus availability. This seems to be most prominent in the genus *Daphnia*, because *Daphnia* shows a higher requirement for phosphorus relative to other zooplankters explained by their high body P-content (Hessen and Lyche 1991).

Most studies on food quality differences that determined the response in life-history traits were conducted with a limited number of taxa or clones only. These studies did not provide a sufficient amount of data to infer the process of local adaptation in *Daphnia* with respect to food quality as a selective factor. Consequently, I have studied the response to food quality in twelve different *Daphnia* species and several clones of different populations. This approached allowed me to study local adaptation across all hierarchical levels from the molecular to the subgenus level.

In this last part of my thesis I develop a more general view and investigate the response (measured in life-history traits) to food quality differences across several organization levels, i.e. between subgenera, species, interspecific hybrids and different clones. Based on the results of my thesis and recent studies in the field of evolutionary ecology I will give future perspectives with regard to local adaptation in *Daphnia*.

**The role of food quality for differentiation in *Daphnia* subgenera, species and interspecific hybrids**

The differential response of species to food with different qualities is of great importance in *Daphnia* not only because it directly affects biological production, and ultimately the production of commercially important species such as fish, but also may influence the species composition and thus the aquatic environment as a whole. My findings on the life-history responses of *Daphnia* species are to a great extent in concordance with those presented in previously published studies (e.g. see Urabe et al. 1997, DeMott 1998) because *Daphnia* individuals were negatively affected in their life-history traits if subjected to food with limited quality. However, nothing was known on the response of different subgenera of *Daphnia* to food with different quality, and here I was able to show that the three different subgenera (*D. longispina*, *Ctenodaphnia* and the *D. pulex* group) all responded with a decline in their somatic growth rate at P-limited conditions (chapter one). However, no differential interaction with food quality for the different subgenera was found, but the twelve *Daphnia* species responded differentially to the different food qualities. Moreover, the subgenera showed a significant differentiation at P-sufficient conditions, but no significant differentiation at food limited in quality (chapter one). This implies that differentiation in *Daphnia* is more prominent when food qualities are non-limited. Comparing the susceptibilities of all twelve *Daphnia* species, I revealed a trade-off between the somatic growth rate at optimal conditions and the susceptibility to food quality changes. Although for several cases an association of species distribution and habitat preferences was detected, no explanatory environmental variable for the species and the subgenera was found (chapter one). In addition, phylogenetic analysis revealed no phylogenetic constraint for the parameters tested here, thus I conclude that *Daphnia* species acquired their ecological differentiation after their separation into the different subgenera.

For *Daphnia* it is know that species often co-occur, and that these sympatric species form interspecific hybrids, a common phenomena in this genus (Schwenk 1993). Recent studies have shown that interspecific hybridization is in fact fairly common and contributes significantly to evolutionary changes (Harrison 1990, Grant and Grant 1992, Bullini 1994, Dowling and Secor 1997, Seehausen 2004). Moreover, for *Daphnia* it is discussed that hybrids often are superior to their parental species when certain environmental conditions are met (temporal hybrid superiority Model, Spaak and Hoekstra 1995). This model is supported by recent field data (DeClerck and De Meester 2003), and a number of life-history studies

(Weider and Wolf 1991, Boersma and Vijverberg 1994c, 1994b). Although a number of factors were described for hybrid maintenance, one of the main factor which determines fitness in *Daphnia*, i.e. food quality (Vanni and Lampert 1992, Sterner et al. 1993, Weers and Gulati 1997, Boersma 2000, Becker and Boersma 2003), was not studied so far. Here I present data on the differences between two closely related *Daphnia* species, *D. galeata* and *D. cucullata* and their interspecific hybrid, *D. cucullata* x *D. galeata* on P-sufficient and P-limited algae (chapter two). Measurements on several life-history traits revealed that the interspecific hybrids showed highest fitness at low food quality conditions, relative to the parental species, whereas *D. galeata* was superior at P-rich conditions. In addition, all traits measured showed broad-sense heritabilities that allow evolutionary changes to happen. These results, based on single-clone life-history studies, were confirmed by a multi-clone experiment and indicate that variation in food quality represents an additional factor explaining hybrid maintenance in *Daphnia*.

In my thesis I present evidences for local adaptation to food qualities (chapter three) and heritability of somatic growth rate in *Daphnia* (chapter two), as well as species and hybrid specific susceptibilities to variation in food quality (chapter one and two), which represent the prerequisites for evolutionary changes. As shown in chapters one, two and three, natural selection on food quality differences causes differential responses of species, interspecific hybrids and clones, thus I expect that major evolutionary changes will happen on periods of changes in food quality. It was also shown that hybrids are superior to their parental species (chapter two) when food quality conditions are low. Hybrids often occur in high frequencies during certain periods of the growing season (Wolf 1987, Weider and Stich 1992), and recent findings support my prediction since interspecific hybrids occurred in higher frequencies during periods of massive changes in phosphorus availability (Brede et al. 2009). Because hybridization may lead to gene flow between species, hybridizing populations are capable of a rapid evolutionary response to perturbed habitats. Variation in food quality, mediated by phosphorus content, seems to represent a major factor causing and maintaining interspecific hybridization and the potential generation of new evolutionary trajectories.

**Fitness consequences of variation in food quality at the clonal level**

Evolutionary change requires two prerequisites: first, individuals should vary in their phenotype, and second, these phenotypic differences should at least in part be heritable. When genetic structure is estimated using neutral markers, valuable insights can be obtained into the level to which genetic drift, gene flow, population subdivision and inbreeding influence patterns of variability within and among populations. Natural selection, however, results in changes of fitness related (quantitative) traits such as life-history parameters. A commonly used method to estimate the relative impacts of drift and selection on polygenic traits and consequently to study local adaptation and adaptive variation is the comparison of genetic differentiation at neutral markers and quantitative traits.

Here I used this comparative method for *Daphnia galeata* to study local adaptation with respect to variation in food quality. For *Daphnia*, a suite of environmental factors have been described for local adaptation e.g. salinity (Teschner 1995), temperature (Mitchell and Lampert 2000), oxygen concentration (Carvalho 1984), levels of pollution (Bachiorri et al. 1991), or other unspecified factors (Declerck et al. 2001), but a discussion on local adaptation with respect to the feeding environment has yet not been described in detail. To address this issue, I studied the reaction-norm for a life-history trait on food with different quality and determined the genetic variation by microsatellite analysis using six polymorphic loci. Estimates of population subdivision for molecular ($F_{ST}$) and quantitative traits ($Q_{ST}$) were concordant, with $Q_{ST}$ generally exceeding the values of $F_{ST}$, indicating directional selection. As shown in several chapters of my thesis, *Daphnia* species show a strong response to variation in food quality, and the lack of any significant phylogenetic signal indicates that the main ecological differentiation among *Daphnia* species represents a rather young evolutionary process (chapter one).

Recent studies have documented rates of evolution of ecologically important phenotypes adapting sufficiently fast that they have the potential to impact the outcome of ecological interactions, i.e. for evolution in ecological time-scales (Hairston et al. 2005, Carroll et al. 2007). It is known that *Daphnia* species are able to respond microevolutionary to rapid ecological changes (Hairston et al. 2005, Decaestecker et al. 2007), however, the response on variation in food quality remained unclear. Here I showed that variation in food quality has a major impact at all hierarchical levels in *Daphnia* (all chapters). Moreover, I showed that *Daphnia* hybrids are superior to their parental species when certain

environmental conditions are met, and recent empirical data on the evolutionary changes in a hybrid complex in lake Constance showed that in less than a century significantly microevolutionary changes related to phosphorus occurred (Brede et al. 2009). Thus *Daphnia* species represent not only an ideal model organisms to study the impact of variation in food quality, but is also a suitable system to reveal the general interaction of ecological and evolutionary processes.

**Candidate genes responsible for adaptation to variation in food quality**

The project leading to chapter four was motivated by the idea to reveal the molecular basis of adaptation to variation in food quality. My aim was to detect and characterize the regulation of candidate genes in *Daphnia,* combining ecological experiments and differential display polymerase chain reaction (DD-PCR). I exposed a clone of *Daphnia magna* to phosphate-rich and P-limited conditions and measured its somatic growth rate. In addition, cDNA of experimental animals were subjected to DD-PCR and fragments of up- or down-regulated loci were sequenced. I detected an up-regulation of genes coding for three different enzymes relevant to cell respiration and also genes coding for muscle proteins which were up-regulated at P-limited conditions. Thus I provide a technique that enables the identification of the molecular basis of phenotypic plasticity for ecologically relevant traits. This was revealed by the application of life-history experiments in combination with genetic analyses.

Although the method provides a fast, cost-efficient and easily applied approach that is relevant to the whole field of evolutionary ecology, experimental results need to be verified by further genetic techniques like RealTime PCR or Western-Blot analysis. However, the up- or down-regulated genes were related to the physiological response of individuals to counter-balance nutrient deficiencies. This is the first step to reveal genes underlying local adaptation in *Daphnia.* Further studies using more advanced techniques such as microarrays are bound to reveal how genomes respond to environmental stress and cause ecological differentiation.

**Future perspectives**

During the recent past, many European lakes were subjected to severe changes in their trophic status by the overenrichment of nutrients (mainly phosphorus). Consequently, this affected the food quality of herbivorous zooplankton such as *Daphnia*. Despite many ecological studies on the key parameter phosphorus, there is only little information on the evolutionary consequences of these environmental changes. My studies show that *Daphnia* species, hybrids and clones are subjected to natural selection causing local adaptation by variation in food quality. The impact of food quality differences is demonstrated at the molecular level (expression of candidate genes), among different *Daphnia* populations (directional selection) and between hybrids and parental species, indicating hybrid superiority at limited food quality conditions. Thus I expect that interspecific hybridization is more common on periods with changes in food quality.

Recent studies showed that adaptation to fast changing conditions can happen on ecological time-scales, and I showed that the response to food quality changes is not constrained by the phylogenetic history of *Daphnia* species. Thus I expect *Daphnia* to adapt to fast changes in their habitat characteristics, and it will be interesting to reveal the impact of food quality differences in this multi-factorial process of adaptation. My first step to identify and describe genes responsible for adaptation shows that it is promising to have a deeper insight into the molecular basis for microevolutionary changes. By now, the genome of *D. pulex* is sequenced (http://wfleabase.org/), and the development of modern applications like SNP analyses, microarrays and the application of different gene libraries will allow to study specific questions according to local adaptation not only in *Daphnia*.

The need to predict nature's reaction to global changes becomes more urgent during recent years. Man-made changes of global ecology strongly influences almost all habitats around the globe. Observing the changes as they happen in natural populations when habitats alter in their characteristics are necessary to make reliable predictions about future populations.

## SUMMARY

**SUMMARY**

Man-made eutrophication, the ecosystemic response to the fertilization of water bodies with nitrogen (N) and phosphorus (P), degrades water and habitat quality. Intense studies on the effects of eutrophication culminated in the scientific basis for banning phosphate detergents (a major source of P, the most frequent culprit in eutrophication of lakes), and the regulation of nutrient entry by pollution control. However, the evolutionary and ecological consequences of these fast changes in trophic state (eutrophication/ re-oligotrophication) remain unclear for most freshwater species. Within this thesis, I studied the evolutionary ecology for a prominent genus in freshwater systems, i.e. for species of the genus *Daphnia,* to changes in food quality by directly controlling the elemental composition of algae. Various life history traits in *Daphnia* are affected by the elemental composition of seston, measured as the carbon (C) to phosphorus (P) ratio (C:P). Here I studied the impact of food quality differences (measured as C:P of algae) for this genus and its ability for local adaptation on changes in food quality.

In the first chapter of my thesis I present a life-history survey of three different subgenera and 12 *Daphnia* species originating from a broad range of habitats that differ in environmental parameters. Species varied in fitness on food with different quality, but no associations between fitness of species and environmental parameters were detected. In addition, a trade-off between susceptibility to food quality changes and somatic growth rate at optimal conditions was revealed. In several cases, this trade-off explained species distribution with their preferred habitat types. However, I found neither explanatory environmental variables nor phylogenetic constraints for differences in somatic growth rate, indicating that *Daphnia* species acquired ecological differentiation after their separation into subgenera.

In chapter two I study the effect of food quality differences on a *Daphnia* hybrid complex, because *Daphnia* species and interspecific hybrids have been shown to be ecologically differentiated and often co-occur in the same lake. Thus I conducted life-history experiments with clones of *Daphnia galeata*, *Daphnia cucullata*, and their interspecific hybrids and measured fitness-related traits at two different food quality conditions. Hybrids showed highest fitness values in some traits at low food quality conditions, relative to their parental species, whereas *D. galeata* was superior at P-rich conditions. These results, based on single-clone life-history studies, were confirmed by a multiclone experiment.

To investigate the pre-requisite for local adaptation, i.e. directional selection, I compared the genetic differentiation at neutral genetic markers ($F_{ST}$) and at quantitative traits ($Q_{ST}$) for populations of *D. galeata* sampled at different trophic levels (chapter three). This comparative method is a commonly used approach to estimate the relative impacts of drift and selection on polygenic traits. This was done within and between four lake populations of *Daphnia galeata* representing two different types of habitat. Estimates of population subdivision for molecular and quantitative traits were concordant, with $Q_{ST}$ generally exceeding $F_{ST}$, indicating directional selection. However, no significant differences in quantitative traits were found between lakes of different trophic category.

Finally, to address the impact of food quality differences at the molecular level, I present an approach based on the combination of ecological experiments and differential display polymerase chain reaction (DD-PCR) to identify genes potentially responsible for adaptation (chapter four). To address this issue, cDNA of experimental animals was subjected to DD-PCR and fragments of up- or down-regulated loci were sequenced after vectorial cloning technique. Here I detected an up-regulation of genes coding for three different enzymes relevant to cell respiration and also genes coding for muscle proteins which were up-regulated at P-limited conditions. In conclusion, I showed the impact of food quality differences on various levels in *Daphnia,* i.e. between subgenera, species, interspecific hybrids and the molecular level and its consequences for the evolutionary ecology within this genus.

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## ZUSAMMENFASSUNG

Während der letzten Jahrzehnte waren viele Seen in Europa starken anthropogenen und ökologischen Veränderungen ausgesetzt, wobei die Einbringung von Medikamenten-Rückständen und Chemikalien, die Einschleppung von Neobiota und die Eutrophierung dieser Habitate einen besonders gravierenden Einfluss auf deren natürliches Gleichgewicht hatte. Durch große Anstrengungen im Umweltschutz, wie z.B. die Errichtung von Kläranlagen, wurde in den letzen Jahren versucht, vor allem die Eutrophierung der Gewässer rückgängig zu machen, um diese Habitate wieder in einen naturnahen und ursprünglichen ökologischen Status zurückzuführen. Diese schwankenden Umweltbedingungen stellen eine Herausforderungen für die meisten Süßwasserorganismen dar. In dieser Dissertation wurden die Auswirkungen, die sich durch eine Veränderung der Futterqualität (Phosphorlimitierung) auf verschiedene Organisationsebenen der Gattung Daphnia ergeben, untersucht.

Die meisten Daphnien besitzen einen zyklisch parthenogenetischen Reproduktionszyklus, d.h. asexuelle und sexuelle Reproduktion wechseln sich ab. Die sexuelle Reproduktion, aus der Dauereier (Ephippien) hervorgehen, wird durch eine Verschlechterung der Lebensbedingungen induziert. So kommt es meist im Anschluss an die Klarwasserstadien der Seen zur sexuellen Reproduktion. Es wurde aber auch eine Induktion durch andere Stimuli, wie hohen Prädatorendruck oder drastische Veränderungen in der Habitatstruktur (z.B. Trockenheit o.ä.) beobachtet. Während der sexuellen Reproduktion ist außerdem die Bildung von interspezifischen Hybriden möglich, ein häufig beobachtetes Phänomen dieser Gattung. Unter optimalen Lebensbedingungen, wie sie meist im Frühjahr und Sommer herrschen, vermehren sich Daphnien jedoch hauptsächlich asexuell, d.h. über die größte Zeit des Jahres werden genetisch identische Individuen gebildet. Dies erlaubt in Verbindung mit einer kurzen Reproduktionszeit die Etablierungen klonaler Linien im Labor, welche verschiedensten experimentellen Bedingungen ausgesetzt werden können.

Im Gegensatz zu Copepoden und manchen Rotatorien sind Daphnien nicht in der Lage, Futterpartikel wie Algen aufgrund der Futterqualität zu diskriminieren. Für sie als unselektive Filtrierer ist deshalb die Qualität des verfügbaren und des aufgenommenen Sestons identisch. Die Futterqualität hängt dabei nicht nur von der Partikelform und -größe, sondern auch von der mineralischen und biochemischen Zusammensetzung des filtrierten Sestons ab. Die Nährstofflimitierung von Algen ist streng an deren Umweltbedingungen und

die verfügbaren Ressourcen gekoppelt, d.h. Algen können, je nach Habitat, große Schwankungen in ihrem Kohlenstoff(C):Phosphor(P)-Verhältnis (C:P) aufweisen. Im Gegensatz dazu sind die einzelnen Arten des Zooplanktons in Bezug auf ihre Kohlenstoff-, Stickstoff- und Phosphorverhältnisse homöostatisch, d.h. sie zeigen nur sehr geringe Schwankungen und Toleranzen in ihren stöichiometrischen Verhältnissen. Daher ist die Futterqualität von Algen für Daphnien von großer Bedeutung.

Obwohl bereits Arbeiten über die Auswirkungen von P-limitierten Futterquellen auf *Daphnia* existieren, ist wenig über das Potential zur lokalen Anpassung an schnelle Veränderung im Phosphathaushalt vieler Seen und die Auswirkung auf die verschiedenen hierarchischen Stufen (d.h. zwischen und innerhalb verschiedener Subgenera, zwischen Arten, zwischen Klonen und interspezifischen Hybriden) bekannt. Um die ökologischen und evolutionären Prozesse, die sich durch die schnelle Veränderung des Trophiegrades der letzen Jahrzehnte ereigneten, besser verstehen zu können, wurden für diese Arbeit verschiedene Arten und interspezifische Hybride aus drei Subgenera der Gattung Daphnia als Untersuchungsobjekt ausgewählt. An diesen wurde der Einfluss schwankender Futterqualität (C:P ratio von Futteralgen) auf die somatische Wachstumsrate, verschiedene *life-history*-Parameter und die Genexpression gezeigt.

Kapitel eins dieser Arbeit beschäftigt sich mit verschiedenen *life-history*-Experimenten, in denen die Auswirkungen unterschiedlicher Futterqualitäten auf die somatische Wachstumsrate untersucht wurden. Die somatische Wachstumsrate kann als Indikator für die allgemeine Fitness der Individuen angesehen werden. Daher lassen sich durch gemessene Unterschiede Rückschlüsse auf die Fitness der Arten und Klone bei verschiedenen Futterqualitätsbedingungen ziehen. In den Experimenten wurde eine Durchflussapparatur verwendet, in der Individuen zwölf verschiedener Daphnien Arten aus drei unterschiedlichen Subgenera P-limitierten oder nicht-P-limitierten Futterqualitäten ausgesetzt waren. Für die verschiedenen Arten konnte ein *trade-off* nachgewiesen werden, d.h. die Wachstumsrate unter optimalen Futterbedingungen war durch die Empfindlichkeit gegenüber Schwankungen in der Futterqualität bedingt. In einigen Fällen erlaubte dies, die Verbreitung der Arten auf ihre spezifischen Habitate und deren Umweltparameter zu erklären. Die Ergebnisse der *life-history*-Experimente wurden außerdem mit phylogenetischen Methoden untersucht, um eine mögliche Assoziation zwischen der Reaktion auf Futterqualitätsunterschiede und artspezifischen Habitatsmerkmalen nachzuweisen. Es konnte

jedoch kein Zusammenhang zwischen der Wachstumsrate und der Empfindlichkeit gegenüber Qualitätsschwankungen des Futters mit den überprüften Merkmalen abgeleitet werden. Da weder eine erklärende Variable, noch eine phylogenetische Einschränkung nachgewiesen werden konnte, sondern nur eine artbedingte Empfindlichkeit gegenüber Schwankungen in der Futterqualität, scheint eine ökologische Differenzierung der Arten nach der Aufteilung in die Subgenera stattgefunden zu haben.

Um den Einfluss unterschiedlicher Futterqualitäten auf die *life-history*-Parameter der in der Natur häufig auftretenden interspezifischen Hybride in *Daphnia* zu untersuchen, wurden weitere Experimente mit den hybridisierenden Arten, *D. galeata*, *D. cucullata*, und deren interspezifischen Hybrid *D. galeata x D. cucullata* durchgeführt (Kapitel zwei). Eine ökologische Differenzierung zwischen Elternarten und interspezifischen Hybriden wurde für *Daphnia* bereits beschrieben, jedoch war der Einfluss von Futterqualitätsschwankungen für diesen Hybridkomplex, wie er sympatrisch in vielen Seen vorkommt, unbekannt. Es zeigte sich, dass Klone von *D. galeata* in den meisten Parametern unter optimalen Futterbedingungen überlegen waren, unter Mangelbedingungen jedoch Klone des interspezifischen Hybrids *D. galeata x D. cucullata* eine höhere Fitness aufwiesen. Dieses Ergebnis, das auf Einzelversuchen basierte, wurde durch ein Multiklon-Experiment erhärtet. Hierzu wurden alle Klone der Einzelexperimente zusammen in einer Durchflussapparatur gehältert und mittels genetische Methoden die relative Häufigkeit der Hybride nach mehreren Generationen bei unterschiedlichen Futterqualitäten bestimmt. Hier zeigte sich, dass unter limitierten Nährstoffbedingungen eine erhöhte Frequenz der interspezifischen Hybride auftrat, was als Indiz für eine höhere Fitness von Hybriden gegenüber ihren Elternarten gewertet werden kann.

Um zu überprüfen ob es, wie in Kapitel eins und zwei angedeutet, zu einer lokalen Anpassung an unterschiedliche Futterqualitäten kommen kann, war es notwendig, die Vorraussetzungen für einen solchen Prozess zu überprüfen. Für die Untersuchungen in Kapitel drei wurden daher vier *D. galeata* Populationen in unterschiedlich eutrophierten Seen gesammelt (nähstoffreich/ nährstoffarm). Für verschiedene Klone aus den unterschiedlichen Seen wurde sowohl die somatische Wachstumsrate unter zwei Futterqualitätsbedingungen bestimmt, als auch deren Empfindlichkeit gegenüber Schwankungen in der Futterqualität ermittelt. Diese Ergebnisse wurden mit der genetischen Differenzierung, basierend auf sechs unterschiedlichen Mikrosatteliten, verglichen. Beide Parameter, die quantitative und die

molekulare Differenzierung waren miteinander vergleichbar. Jedoch war die Differenzierung basierend auf quantitativen Merkmalen in jedem Vergleich größer als die Differenzierung basierend auf neutralen genetischen Markern. Da die Differenzierung in den Selektion unterliegenden Merkmalen größer war als die Differenzierung gemessen mit neutralen genetischen Markern, kann dies als Nachweis für gerichtete Selektion und somit als Vorraussetzung zur lokalen Anpassung an unterschiedliche Futterqualitäten gedeutet werden. Jedoch konnte keine Differenzierung basierend auf quantitativen Merkmalen alleine, d.h. der Empfindlichkeit gegenüber Schwankungen im C:P-Verhältnis der Futteralgen, zwischen den Populationen aus unterschiedlichen Kategorien gezeigt werden. Daher scheint eine Anpassung an unterschiedliche Futterqualitäten nicht allein durch Unterschiede im C:P-Verhältnis der Algen erklärt werden zu können. Vielmehr müssten auch die komplexen Veränderungen im Seston wie Artenzusammensetzung und morphologische Anpassungen berücksichtigt werden, die mit einem veränderten Nährstoffeintrag einhergehen.

Obwohl die phänotypische Reaktionen auf verschiedene Futterqualitäten gut untersucht sind, wissen wir noch wenig über die molekularen Grundlagen, die diese Unterschiede hervorrufen. In Kapitel 4 dieser Arbeit wurde durch eine Kombination von ökologischen (*life-history*) und molekularbiologischen Experimenten (DD-PCR) eine einfache Methode entwickelt, um Gene aus Daphnien zu isolieren, die bei Gabe unterschiedlicher Futterqualitäten einer differentiellen Expression unterliegen. Diese Gene bilden möglicherweise die molekulare Grundlage für eine Anpassung an unterschiedliche Futterqualitäten. Unter P-limitierten Bedingungen zeigte sich eine verstärkte Expression von Genen, die für die Zellatmung zuständig sind, wie auch von Genen, die im Metabolismus von Muskelproteinen eine Rolle spielen. Eine erhöhte Schlagfrequenz der Filterbeine wurde bei Daphnien unter limitierten Futterbedingungen bereits in anderen Arbeiten beobachtet, und auch eine erhöhte Zellatmung ist unter limitierten Bedingungen plausibel, um den Mangelerscheinungen entgegenzuwirken. Die Ergebnisse ermöglichen damit einen Einblick in die molekulare Prozesse, die bei der Anpassung an unterschiedliche Futterqualitäten ablaufen.

Zusammenfassend lässt sich sagen, dass sich Unterschiede in der Futterqualität (gemessen als C:P) signifikant auf die verschiedenen Subgenera, auf die verschiedenen Arten, Klone und Hybriden, als auch auf molekularer Ebene auswirken. Trotz der teils unterschiedlichen Reaktionen in fitnessrelevanten Parametern, zeigten alle untersuchten

Individuen unter limitierten Bedingungen eine verminderte Wachstumsrate und somit eine geringere Fitness unter Mangelbedingungen. Hybride waren in ihrer Fitness ihren Elternarten bei limitierten Futterqualitäten überlegen. Es konnte außerdem gerichtete Selektion durch Schwankungen in der Futterqualität nachgewiesen werden. Weiterhin zeigten sich keine phylogenetischen Einschränkungen der Arten in deren Reaktion auf unterschiedliche Futterqualitäten. Damit scheint eine Adaptation nicht nur in evolutionären, sondern auch in ökologisch kurzen Zeiträumen möglich zu sein. Welchen Einfluss die Futterqualität im Vergleich zu anderen Parametern hat, die ebenfalls lokale Anpassung von Daphnien beeinflussen (z.B. Temperatur, Fraßdruck), bleibt in weiteren Studien zu überprüfen.

# CURRICULUM VITAE

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**Erklärung**

Ich erkläre hiermit, dass ich mich bisher keiner Doktorprüfung unterzogen habe.
Aschaffenburg, den

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**Eidesstattliche Versicherung**

Ich erkläre hiermit an Eides Statt, dass ich die vorgelegte Dissertation selbständig angefertigt habe und mich keine anderen, als die angegeben Hilfsmittel verwendet habe. Entlehnungen aus anderen Schriften, soweit sie in der Dissertation nicht ausdrücklich als solche mit Angabe der betreffenden Quelle gekennzeichnet sind, haben nicht stattgefunden.

Einzelne Kapitel wurden in internationalen Fachjournalen eingereicht bzw. veröffentlicht/ Separate chapters are publicated in international scientific journals or are submitted:

*Chapter 1: Seidendorf B., Meier, N., Petrusek A., Boersma, M, Streit, B. and Schwenk, K., submitted, Susceptibility of Daphnia species to phosphorus-limited diets: the role of phylogenetic constraints and trade-offs, Oecologia, submitted*

*Chapter 2: Seidendorf B., Boersma, M, and Schwenk, K, 2007, Evolutionary stoichiometry: The role of food quality for clonal differentiation and hybrid maintenance in a Daphnia species complex, Limnology and Oceanography, 52(1), 385-394*

Aschaffenburg, den

..... (Unterschrift)

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