Ecological genetics of the benthic feeding habits of *Daphnia*

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Abstract

The environment of most organisms vary over different scales of space and time. Examples of evolutionary responses to environmental heterogeneity are well investigated in cladoceran crustaceans of the genus *Daphnia*. While, traditionally, studies of *Daphnia* have largely focused on its planktonic lifestyle, few authors have highlighted an important role of the benthic environment for the ecology of some species and for the evolutionary history of the genus. In this perspective, my thesis investigated the behavioural and morphological feeding adaptation of *Daphnia* to benthic environments mostly using the traditional model *Daphnia magna* which, despite being primarily pelagic, often dwells in benthic habitats.

In the first part of my thesis, I presented my work on a poorly studied feeding behaviour of *D. magna*, termed sediment browsing. I demonstrated genetic variation and high heritability for the behaviour among *D. magna* genotypes and began to investigate the ecological determinants of such variation. While local pelagic predation in the original habitats of the clones did not appear to influence browsing behaviour, clones from big lakes and ponds were shown to differ in browsing levels, suggesting how the proximity of the benthic environment might influence the evolution of benthic feeding preferences. Next, I described the genetic architecture of the behaviour by QTL analyses and identified three genomic regions associated with its variation. In another study, I analysed how genetic variation in browsing behaviour influences the establishment of microbial associations in *D. magna*. This study showed how genetic variation in behaviour might play a role in determining the genotypespecific microbiota observed in a particular environment.

In the second part of my thesis, I focused on morphological variation in a previously poorly studied limb of *Daphnia*, trunk limb II, which has been proposed to serve to collect food by scraping. This study was conducted at different levels: plastic responses to food treatments within *D. magna* genotypes, genetic variation between *D. magna* clones spanning the geographical and habitat range of the species and morphological comparisons between species of the genus. The analyses did not detect a plastic response in setal morphology to the feeding treatments applied. However, I found high heritability for trunk limb II setal morphology and that variation is partially explained by geographic genetic lineage differences between clones. Finally, a preliminary comparison of trunk limb II among eleven *Daphnia* species found a phylogenetic distribution suggestive of convergent evolution of setal morphology in some species with similar ecologies.

Together, my work on *Daphnia* benthic feeding functional morphology and on the ecological genetics and functional aspects of sediment browsing behaviour highlighted the interactions with the benthic environment as an important, yet often overlooked, aspect of the ecology of *Daphnia*. Recently, this line of research has gained momentum in the light of a novel focus of ecological studies considering the coupling of benthic and pelagic lentic habitats. In this perspective, the work presented in my thesis might contribute to a better integration of the benthic habitats into *Daphnia* ecoevolutionary models.

Introduction

Environmental heterogeneity as a driver of evolutionary change

A long-standing goal of evolutionary biology is understanding the processes that underlie the origin of the diversity that we observe in biological systems (Lewontin 1974). Most organisms live in heterogeneous environments that vary over various scales of space and time and this has a great impact on population, species and ecological dynamics (Pigliucci 2001). Environmental change has long been recognized as a major determinant of evolutionary processes since the rate of environmental change determines the intensity of selection (Barton & Partridge 2000). Moreover, environmental heterogeneity can maintain genetic variation in traits of adaptive significance, thereby influencing rates of phenotypic evolution (Byers 2005). Organisms' adaptations to heterogeneous environments also have the potential to greatly modulate the intensity of selection and maintaining genetic variation. Responses to environmental heterogeneity include local adaptation, phenotypic plasticity, and behavioural adaptations. In the case of patchy environments, local selective conditions and some degree of isolation set the conditions for population genetic differentiation possibly leading to local adaptation (De Meester 1996). This process is of great importance in maintaining genetic variation within a species and can result in macro-evolutionary processes of speciation and adaptive radiation. Plasticity is defined as the ability of a single genotype to exhibit a range of different phenotypes in response to variation in the environment (Forsman 2015). Phenotypic plasticity can promote population divergence by facilitating phenotypic diversification and genetic divergence (Schneider & Meyer 2017). Behaviour has long been recognized as a driver of evolutionary change as it influences the interactions of an organism with the environment, determining type and magnitude of selection. For example, the exposure to new selective pressures as a result of behavioural changes can result in the rapid evolution of morphological, life history and physiological traits and might initiate adaptive shifts (Duckworth 2009). Habitat selection (i.e. the choice of habitat across numerous scales of space and time) can influence the intensity of selection and population structure and can drive both intra- and inter- population differentiation (Pigliucci 2001). Finally, environmental heterogeneity can also influence the dynamics of gene flow (e.g. migration and dispersal) with a great impact on evolutionary processes.

Adaptation and behaviour in freshwater zooplankton

The above-mentioned examples of evolutionary responses to environmental heterogeneity are well exemplified and investigated in zooplankton species inhabiting lentic freshwater environments (e.g. pools, ponds and lakes). Lakes and ponds are characterised by high heterogeneity and spatial structure. Different zones are defined within water bodies, each associated with a more or less specific set of biotic and abiotic conditions: the pelagic (open-water), the benthic (bottom sediment) and the littoral (submerged shoreline) zones. Therefore, within a water body, organisms might encounter distinct but interconnected micro-habitats. While specialization to a restricted niche is a common strategy in freshwater organisms, many have evolved as generalist species, able to dwell in different microhabitats where they perform specific activities

(e.g. foraging, resting and reproduction). Intra-population genetic differentiation associated to spatial habitat structure has been found for many freshwater species and is regarded as an important mechanism maintaining genetic variation in ecologically relevant traits (De Meester 1996). The patchy distribution of lentic habitats and their often well-defined boundaries generate specific local environmental conditions and limits gene flow between populations, creating opportunities for local genetic differentiation and local adaptation (Slarkin 1985). In the context of the adaptations to environmental heterogeneity, habitat selection behaviours in zooplankton have been extensively investigated. Many zooplankton species, including cladocerans and copepods, migrate between different zones within water bodies in response to predation, exposure to physical damage (e.g. UV light) and food availability (De Meester 1993; Cousyn *et al.* 2001). Habitat selection has been found to be heritable and to evolve in cases of changes in selective regimes. For example, in *Daphnia,* Cousyn *et al.* (2001) found that genetic changes in phototactic behaviour, a predatory avoidance strategy, correlate to variable levels of fish predation over a period of 30 years. Predation by fish and invertebrates is generally regarded as the main selective pressure acting on the evolution of habitat selection behaviours in *Daphnia.* However, food conditions have been shown to influence habitat selection in freshwater zooplankton, but studies on this regards are surprisingly rare. Some zooplankton species, including members of the cladocerans, copepods, and fairy shrimps, actively feed on benthic substrates such as microbial mats when trophic and grazing conditions limit phytoplankton abundance (Rautio & Vincent 2006). *D. magna* and *D. pulex* switch from suspension filter feeding to feeding on substrates such as periphyton when the concentration of suspended food drops below a critical threshold (Horton *et al.* 1979; Siehoff *et al.* 2009). This hitherto poorly investigated aspect of the feeding biology of freshwater zooplankton species might have relevant implications for their population dynamics. Due to the great impact of zooplankton populations on freshwater environments, the integration of behavioural responses to feeding conditions into habitat selection studies might improve our understanding of the eco-evolutionary dynamics of these habitats.

Alternative feeding strategies and habitat selection in *Daphnia*

Planktonic cladoceran crustaceans of the genus *Daphnia* are key species in worldwide freshwater ecosystems. Being both primary consumers of phytoplankton (primary producers) and the preferred prey of many predators, species of the genus occupy a central position in freshwater food-webs (Lampert 2011). Top-down and bottom-up effects of *Daphnia* on freshwater community dynamics have been reported, thereby pinpointing these species as "strong ecological interactors" (Miner *et al.* 2012). *Daphnia* can reproduce both asexually (resulting in the production of clonal offspring) or sexually. Clonal reproduction permits to replicate *Daphnia* genotypes in laboratory experiments, offering exceptional resolution in genetic analysis where, for example, the aim is to disentangle genetic and environmental determinants of a given phenotype (Simon *et al.* 2011). This genetic tractability, combined with extensive knowledge of its ecology, makes *Daphnia* an ideal eco-genomic model organism (Miner *et al.* 2012). A genome is available for two species of the genus, and gene expression analysis and manipulation techniques are rapidly being established (Colbourne *et al.* 2011; Miner *et al.* 2012). Behavioural interactions between *Daphnia* and the environment have been extensively studied (De Meester 1993; Burks *et al.* 2001; Decaestecker *et al.* 2002). Nevertheless, *Daphnia* feeding habits other than filter feeding have been surprisingly neglected, despite their number and importance (Fryer 1991). *Daphnia* are primarily filter feeders in the water column. However, when feeding conditions deteriorate, some species adopt an alternative feeding strategy, termed sediment browsing behaviour (Hor-

ton *et al*. 1979). The animals swim along a sediment surface, stirring up particles with movements of the second antennae; the sediment particles are then ingested by filter feeding. Some species might also be able to feed on periphyton, the complex mixture of algae, cyanobacteria, heterotrophic microbes, and detritus that is attached to submerged surfaces, by scraping by means of a robust seta on their second trunk limbs. As highlighted by Fryer (1991) in his monography about *Daphnia* functional morphology, the heavily build species *Daphnia magna* displays a series of morphological and behavioural adaptations for inhabiting the bottom environments of lakes and ponds. This species is often found dwelling in the proximity of sediments and within submerged plant beds where it might find refuge from vertebrate and invertebrate predators. *D. magna* is able to perform sediment browsing and surface scraping behaviours as alternative feeding strategies to suspension filter feeding. Due to these features, *D. magna* represents an ideal model for integrating the study of behavioural responses to feeding conditions into habitat selection models of freshwater zooplankton.

Thesis outline

Throughout my PhD project, I studied the adaptations of the ecological and evolutionary model organism *Daphnia magna* to the benthic environments. This species, although primarily feeding in the water column, can feed by browsing on sediments. However, this strategy may increase the exposure to benthic predation and infection from parasite transmission stages. Therefore, the evolution of feeding behaviour in this species is expected to be influenced by multiple and possibly contrasting selective forces. In **Chapter I** of this thesis (published as Arbore *et al.* 2016), I first demonstrated genetic variation for the behaviour among 15 *D. magna* genotypes (clones) from natural populations. Next, I used an F2 recombinant population and QTL analyses to describe the genetic architecture of the behaviour and identified three regions in the *D. magna* genome associated to its variation. This work provided the genetic background to the study of the different selective pressures that might act on the evolution of browsing behaviour. In a following study (**Chapter IV**), I analysed the behaviour of 40 clones sampled throughout the known geographical range of the species and found that browsing behaviour can differ between habitat types (ponds, small lakes and big lakes), highlighting how local environment can affect browsing across a broad geographical range. In another study (**Chapter II**, published as a shared first author in Mushegian *et al.* 2019), I analysed how genetic variation in browsing behaviour influences the establishment of microbial associations (e.g. microbiota) in *D. magna*. In this study, 12 clones from natural populations were either exposed to sediments with different levels of bacterial diversity or blocked from browsing on sediments with a permeable barrier. Then, their microbiota was characterized using a next generation DNA sequencing approach. I found host genotype effects on microbiota composition and that the bacterial diversity of the environment had multiple, sometimes opposing effects on microbiota diversity. This study showed how genetic variation in behaviour might play a role in determining the genotype-specific microbiota observed in a particular environment. This work highlighted behavioural genetic variation as a significant, yet often overlooked, factor potentially influencing microbiota composition and, in turn, suggested how microbiota acquisition might be important for behavioural evolution. Besides being able to feed on particulate sediment, *D. magna* is also able to feed by scraping on submerged surfaces. Fryer (1991) hypothesized that scraping might be accomplished by means of a robust seta located on trunk limb II but no other authors have provided evidence in this regard. In **Chapter III**, I present the results of two experiments where replicate individuals of six clones of *D. magna* were raised in two feeding treatments, namely in

the presence of algae in suspension or in the presence of a layer of algae on the bottom of glass jars. The morphology of the "scraping" seta on trunk limb II was documented from dissected exuviae on multiple subsequent instars of each individual. The induction of a plastic response in the morphology of the seta might have provided an indirect evidence of its function in scraping. However, no such change was observed in the experiments. Nevertheless, genetic variation in setal morphology was found between clones. In continuation to this study, I performed an analysis of setal morphology and browsing behaviour using 40 *D. magna* clones from water bodies of different sizes distributed across the wide geographical range of the species (**Chapter IV**). This work identified lineage and region-specific genetic variation for setal morphology and differences between habitats in the propensity of the clones to browse on bottom sediments. Finally, in the concluding chapter of my thesis (**Chapter V**), I present a preliminary comparative analysis of seta morphology between several species of the genus *Daphnia* and discuss the results in the context of the phylogenetic relationships between the species.

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Chapter I

Ecological genetics of sediment browsing behaviour in a planktonic crustacean

Abstract

Zooplankton can display complex habitat selection behaviours that influence the way they interact with their environments. Some species, although primarily pelagic, can exploit sediment borne particles as a food source or use sediments as a refuge from pelagic predation. However, this strategy may increase the exposure to other risks such as benthic predation and infection from sediment-borne parasite transmission stages. The evolution of habitat selection behaviour in these species is thus expected to be influenced by multiple and possibly contrasting selective forces. Here we study the browsing behaviour of the water flea *Daphnia magna* on bottom sediments. First, we demonstrated genetic variation for sediment browsing among *D. magna* genotypes from natural populations sampled across a broad geographic range. Next, we used an F2 recombinant panel to perform a QTL analysis and identified three regions in the *D. magna* genome contributing to variation in browsing behaviour. We also analysed the correlation between our data and previously published data on the phototactic behaviour of genotypes from the same F2 panel. Clonal means of the two behavioural traits were not correlated, suggesting that they may evolve independently. Browsing behaviour is likely to be a relevant component of habitat selection in *D. magna*, and its study may help to incorporate the interactions with the sediment into eco evolutionary models of this key freshwater species.

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Introduction

The structural complexity of ecosystems has a profound impact on their ecological and evolutionary dynamics. In heterogeneous environments, different habitats are linked by the movement of energy, material and organisms across habitat boundaries (Polis *et al.* 1997). Such habitat coupling has fundamental consequences for ecosystems as it can influence, for example, nutrient recycling as well as community and food-web structures (Schindler & Scheuerell 2002). Lakes and ponds are characterised by distinct but interconnected habitats: the pelagic (open-water), the benthic (bottom sediment) and the littoral (submerged shoreline) zones. In shallow waters, as in small ponds or in the littoral zone of large lakes, benthic-pelagic interactions can play a crucial role in determining ecosystem organization (Threlkeld 1994). Organisms that migrate between the benthic and pelagic zones are important vectors mediating habitat coupling (Polis *et al.* 1997). For example, opportunistic cross-habitat foraging by some fish can generate trophic pairing between the benthic and the pelagic zones (Schindler & Scheuerell 2002; Vander Zanden & Vadeboncoeur 2002). Diapausing organisms that hatch from sediment eggbanks (e.g. cladocerans, rotifers and copepods) heavily influence zooplankton population dynamics with whole-ecosystem effects (e.g. Brendonck & De Meester 2003; Gyllstrom & Hansson 2004). Benthic algae and detritus represent alternative food sources for some species that otherwise feed primarily in the water column. When trophic and grazing conditions limit phytoplankton abundance, as for example in high-latitude lakes and ponds (Rautio & Vincent 2006), some zooplankton species, including members of the cladocerans, copepods, and fairy shrimps, can exploit benthic food sources (Horton *et al.* 1979; Fryer 1991; Rautio & Vincent 2006; Siehoff *et al.* 2009). The ability to consume these alternative resources may confer competitive advantages over strictly pelagic feeders, especially in shallow water bodies (Horton *et al.* 1979; Siehoff *et al.* 2009).

Beside the role of sediments in the feeding ecology of many aquatic animals, the watersediment interface also plays an important role in the interaction with natural antagonists, such as predators and parasites. Bottom sediments may offer visual protection from pelagic predators (De Meester 1993; Destasio *et al.* 1993) or from predators that are attracted by plant beds (Tavsanoglu *et al.* 2012). Conversely, benthic predators, such as larval odonates, can pose a threat for zooplankton populations (Burks *et al.* 2001). Pond sediments can also harbour the transmission stages of microparasites and epibionts of planktonic organisms (Green 1974; Ebert 1995; Decaestecker *et al.* 2002; Decaestecker *et al.* 2004; Lawrence *et al.* 2002), and infections from the sediment can have important effects on parasite epidemiology and host population dynamics (Ebert 1995; Ebert *et al.* 1997). Spores can remain infectious in sediments of freshwater environments for many years and reinitiate epidemics after periods of absence of the host (Decaestecker *et al.* 2004; Andras & Ebert 2013). The interaction with benthic microbial communities may also influence how zooplankton species acquire and maintain their microbiomes (Qi *et al.* 2009; Sison-Mangus *et al.* 2015).

Variation in habitat selection behaviour has been well studied in aquatic crustaceans of the genus *Daphnia*. These studies reveal that variation in this behaviour within and between populations may be maintained by the dynamic balance between positive and negative fitness effects. In the water flea *Daphnia magna*, there is a behaviourally mediated trade-off between the risk of predation by planktivorous fish and the risk of infection by parasite spores taken up from the sediment (Decaestecker *et al.* 2002). The degree to which *Daphnia* stay higher or lower in the water column, and thus farther from or closer to the sediment, is largely influenced by phototactic behaviour. As a consequence, more negatively phototactic genotypes have a higher

infection risk compared to more positively phototactic genotypes. The cost associated with the avoidance of pelagic predators has been proposed to maintain genetic polymorphism in habitat selection in *D. magna* (Decaestecker *et al.* 2002). When feeding conditions in the water column deteriorate, this species displays a sediment browsing behaviour whereby the animals swim along the sediment surface, stirring up particles with movements of the second antennae (Movie S1). The sediment particles are then ingested by filter feeding (Horton *et al.* 1979). This behaviour brings *Daphnia* into direct physical contact with the sediments and is likely an important component of habitat selection in *D. magna*.

Due to the central role of *Daphnia* in fresh water ecosystems, habitat selection can have ecologically relevant effects, for example by influencing predators' population dynamics or by triggering parasite epidemics. The study of habitat selection in *Daphnia* can therefore shed light on how behavioural variation can affect whole-ecosystem processes. Here we performed a genetic analysis of sediment browsing behaviour in *D. magna* with the aim of expanding our understanding of the genetic basis of habitat selection. Our aims were i) to quantify the magnitude of genetic and phenotypic variation for the browsing behaviour in *Daphnia* from diverse natural habitats, ii) to gain insights into the genetic architecture of this behaviour and iii) to analyse the genetic correlation between browsing and phototactic behaviours. For these purposes we measured the browsing behaviour of 15 clones (i.e. genotypes), one from each of 15 *D. magna* populations sampled across a wide geographical range. Browsing behaviour was measured by analysing the traces left by individual animals on the surface of fine sediments on the bottom of glass jars (Fig. 1). The same assay was used for 185 *D. magna* genotypes from an F2 QTL panel (Routtu *et al.* 2010; Roulin *et al.* 2013; Routtu *et al.* 2014), with the aim to describe the genetic architecture of the behaviour. Finally, we assessed the genetic correlation between sediment browsing and phototactic behaviours using previously published data on the phototactic behaviour of a subset of clones from the same mapping panel (Routtu *et al.* 2014).

Materials and Methods

Study organism

D. magna can reproduce both asexually and sexually by cyclical parthenogenesis. Asexual reproduction makes it possible to produce unlimited cultures of genetically identical individuals and to replicate genotypes in laboratory experiments. Hereafter, we refer to such genetic lines as "clones" and we refer to individuals from a given clone simply as "animals" or "replicates". Asexual females can also produce male offspring and sexual reproduction makes it possible to cross different clones or the same clone (i.e. self-fertilization). Repeated rounds of self-fertilization are used to generate inbred clones which can subsequently be propagated asexually.

Clones from natural populations

In one experiment, we used *D. magna* clones sampled from different locations distributed throughout the northern hemisphere and clonally propagated in the laboratory (belonging to the *Daphnia magna* Diversity Panel) (Table S1). We selected 15 unique clones from as many sampling locations spanning from Canada to Europe to western Russia. The ecological information available for the sampling locations of the panel was sparse and the ecosystem types varied considerably between locations. Therefore, we chose locations with a known record of presence or absence of fish (8 and 7 respectively), as predation by fish is known to be an important factor influencing habitat selection in *D. magna* (e.g. De Meester 1993; Boersma *et al.* 1998; Decaestecker *et al.* 2002). However, no data are available for type of fish and their density.

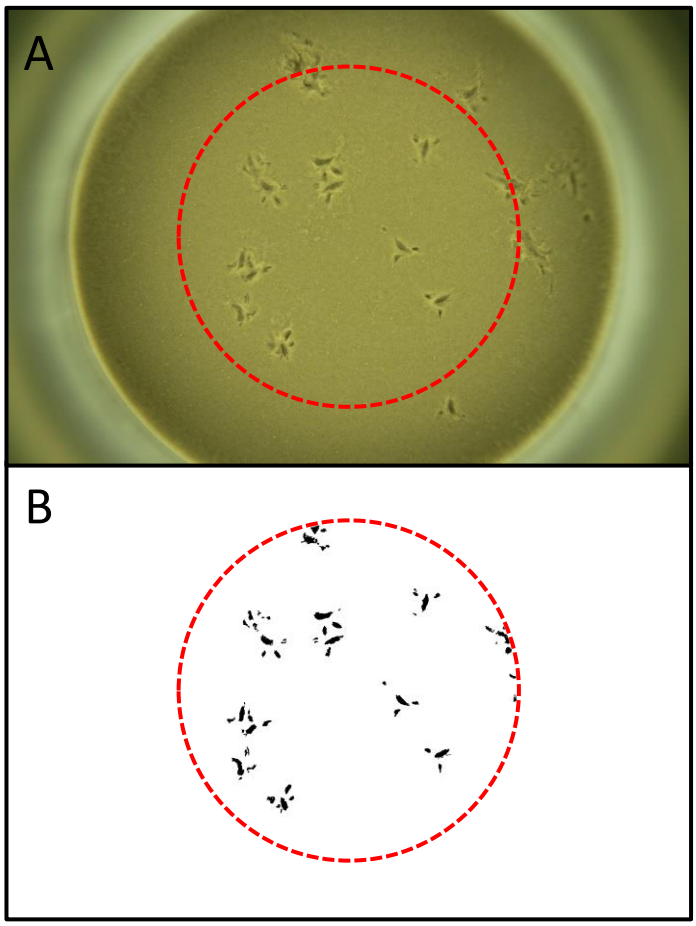

Figure 1: Browsing behaviour assay. A: photograph of the traces left by one *Daphnia* browsing for 30 minutes on the layer of sediment at the bottom of the experimental glass jar; B: the same picture after processing to analyse the area of the browsing traces (black zones). The red dotted line defines the area included in the analysis. See also Movie S1.

Clones from the QTL panel

In order to analyse the genetic architecture of browsing behaviour, we used 185 genotyped clones from an F2 QTL panel (F2 panel hereafter) maintained in the laboratory. This F2 panel was previously used to create the first (Routtu *et al.* 2010), second (Routtu *et al.* 2014), and third (**1**: unpublished data) *D. magna* genetic maps. Briefly, two inbred parental clones were crossed, and one F1 offspring clone was then self-fertilized several times to generate the F2 clones. This F2 panel has been previously used to map several reproductive, life history, parasite resistance, and behavioural traits (Routtu *et al.* 2010; Roulin *et al.* 2013; Routtu *et al.* 2014; Routtu & Ebert 2015). While there is no evidence for differences in browsing behaviour between the parental clones, variation was observed within the F2 panel.

Experimental conditions

All animals used in this study were females kept individually in 100-ml jars filled with 80 ml of *Daphnia* medium (ADaM) (Kluttgen *et al.* 1994) at 20 °C with a 16:8 light/dark cycle, fed daily with chemostat grown green algae *Scenedesmus sp.* and propagated clonally. Positions in the incubator were randomized to minimize microenvironmental effects. The animals were kept in standardized conditions for three generations before each experiment in order to minimize variation in maternal effects. To establish each generation, 4-day-old juvenile females were

isolated from their mothers' third or fourth clutches and fed daily with 1 x 10^6 algal cells. The amount of algae fed to the animals was increased to 2 x 10^6 on day 6, 2.5 x 10^6 on day 9, 3 x 10^6 on day 11, and 5 x 10^6 on day 11. The animals were transferred to fresh medium when they were 12 days old and thereafter every 4th day or when a clutch was released.

In the first experiment, we measured the browsing behaviour of genetically identical replicate animals for each of the 15 clones from natural populations. For each clone, we randomly chose seven female offspring from three animals (3x7=21 replicate animals per clone) and raised them as described above. After laying their first clutches, these animals were analysed in the browsing behaviour assay (see below). Animals that were accidentally damaged or lost were excluded from the analysis.

In the second experiment, we measured the behaviour of replicate animals of 185 clones from the F2 QTL panel. For every clone, two animals born four days apart from each other were used to establish the maternal generation. Three juvenile offspring from the third or fourth clutch of these animals were randomly chosen (2x3=6 replicate animals per clone) and raised as described above. When 12 days old, these animals were analysed in the browsing behaviour assay (see below). With this procedure, we were able to distribute the behavioural assays of 12 days old animals over a period of 10 days, despite their unsynchronized ages, and measure half of the replicates for each clone on two different days. Every day, we assayed three replicate animals of 40 clones (n=120, see below). Animals that were damaged or lost were excluded from the analysis. In total, we assayed the behaviour of 941 animals with an average of five animals analysed from each of the 185 F2 clones.

Quantification of browsing behaviour

Browsing behaviour was quantified by analysing the traces left by single animals on a layer of loess (fine silt) covering the bottom of glass jars (Fig. 1). The jars (height = 20 cm; diameter = 6.5 cm) were filled with 400 ml of medium and 20 ml of a suspension of loess. The loess was previously passed through a 200 µm filter, washed several times to remove very fine particles and autoclaved. Jars with the suspension were left for three days to settle until the loess formed a smooth 1 cm-layer on the bottom of the jars. The bottom loess layer of a jar was photographed (time 0) with a digital camera using a ring light to ensure uniform illumination of the loess surface. The jar was carefully transferred into a darkened cardboard tube and illuminated with a neon light (lm=1600, W965) positioned 10 cm above the tube. Then one animal was carefully introduced. This procedure was repeated at one minute intervals for 12 animals, using a different jar for each animal. After exactly 30 minutes in the experimental jars the animals were removed, and the jars were again photographed (time 1) under the same position and light conditions. A maximum of 120 animals were assayed every day. Replicates where the loess surface at time 1 was disturbed or the animals damaged during the handling were not considered for the further analysis. The photographs were processed with ImageJ (http://rsb.info.nih. gov/ij/). The raw pictures were converted to grey scale, and a central circular area was cropped to exclude shadows from the edge of the jar. Further edge shadows in the selected area were distinguished by visual inspection and were deleted manually. After picture processing, the browsing traces of the animals on the loess surface resulted in shadows that appeared as black areas against a white background and were quantified by the number of black pixels (Fig.1B). Pictures taken at time 0 were processed in the same way and used to correct the values calculated for the browsing traces in those cases when irregularities on the sediment surface were detected (i.e. pixel count > 0 at time 0). The values were then log-transformed [$\log_{10}(X+1000)$] to ensure normal distribution. We added 1000, because 1000 pixels correspond approximately to the minimum area of one browsing trace.

Phototactic behaviour data

The values of the phototactic index (De Meester 1991, 1993) of some of the F2 clones from the same F2 panel used in the present study were retrieved from the dataset published by Routtu *et al.* (2014). Briefly, the authors quantified the phototactic behaviour of the clones by counting the proportion of animals occupying the upper (U; 12 cm), middle (M; 10 cm) or lower (L; 3 cm) compartments of a 25 cm-high glass column illuminated from above. The phototactic index for each clone was then calculated as [(U-L)/(U+M+L)] averaged over 5 observations, each with 10 animals per trial. For this analysis, the phototactic indices, measured in the absence of fish kairomones, for 113 of the 185 clones included in our experiment were available. The Pearson's correlation between the browsing behaviour and the phototactic index was conducted in JMP (v. 11.0: SAS Institute Inc, NC, USA).

Statistical analyses

The intra-class correlation coefficient (ICC, equivalent to the calculation of broad-sense heritability) for the browsing behaviour of the clones from natural populations was calculated by fitting a linear mixed effect (LMM) model (allowing for the slightly unbalanced number of animals per clone), with clone as a random effect, in the R package rptR (Nakagawa & Schielzeth 2010). Confidence intervals and statistical significance were calculated with a restricted maximum likelihood (REML) estimation method (using parametric bootstrapping with 5,000 iterations and a randomization procedure with 5,000 permutations). The effect of the presence or absence of fish in the site of origin of the clones on browsing behaviour was tested by fitting a mixed model with clone as a random effect and fish presence/absence as a fixed effect using JMP (v. 11.0: SAS Institute Inc, NC, USA).

QTL analysis

Linkage mapping was performed by Haley-Knott regression with the R/qtl package (version 1.27-10, Broman *et al.* 2003; R version 3.0.0). For each F2 clone, the mapped phenotype corresponded to the average value of the browsing behaviour of the replicate animals. Following Churchill & Doerge (1994), we calculated significant ($\alpha = 0.05$) and suggestive ($\alpha = 0.10$) genome-wide LOD thresholds of 3.78 and 3.45 respectively (10,000 permutation tests). A two-QTL scan was performed to identify interactions among QTLs. This analysis permits to assess epistatic interactions and to identify additional QTLs of modest effect (Broman *et al.* 2003). The LOD-1.5 support intervals for the QTLs (the interval in which the LOD score is within 1.5 units of its maximum) were calculated using the lodint() function in R/qtl. The phenotypic variances explained by the single QTLs and by multiple-QTL models were estimated using the fitqtl() function in R/qtl. A post-hoc Tukey's analysis was performed in R to test for the difference in browsing behaviour between genotypes at the QTL locations.

Results

The total phenotypic variance for browsing behaviour explained by the clones from 15 natural populations corresponded to 21.3% (ICC = 0.213, 95% CI = 0.059-0.372, P = 0.0002) (Table S2). We found no significant effect of the presence or absence of fish in the clones' site of origin (Fig. 2) ($F_{1, 13}$ = 0.37, P = 0.55).

Across the 185 F2 clones analysed, browsing behaviour displays a normal distribution ranging from high levels of browsing, with most of the sediment surface disturbed during the 30 minute assay, to no browsing activity (Fig. 3, Table S3). The single-QTL genome scan (Fig. 4A) identified one QTL (Q1) located on linkage group (LG) 4 surpassing the significant genome-wide LOD threshold (LOD_{Q1} = 3.94; $LOD_{\alpha=0.05}$ = 3.78). The proportion of the phenotypic variance explained by this QTL corresponded to 9.34% with the genotype at the marker associated with the highest LOD score showing a significant effect on the browsing behaviour ($F_{2, 182}$ = 6.39, P = 0.0022). Significant differences in the browsing behaviour were found between the AA and the AB genotypes (Tukey's P-value = 0.022) and between the AA and BB genotypes (Tukey's P-value = 0.0041), with the AA genotype showing the lowest browsing levels (Fig. 4B). No significant differences were detected between the BB and the AB genotypes (Tukey's P-value = 0.34). Additive and dominance effect sizes for Q1 were 0.17 (SE = 0.03) and 0.06 (SE = 0.04) respectively. The LOD-1.5 support interval for Q1 spanned about 14 cM on LG4 (corresponding to about 2.5 Mb) and included several scaffolds and contigs of the current genome assembly (version 2.4).

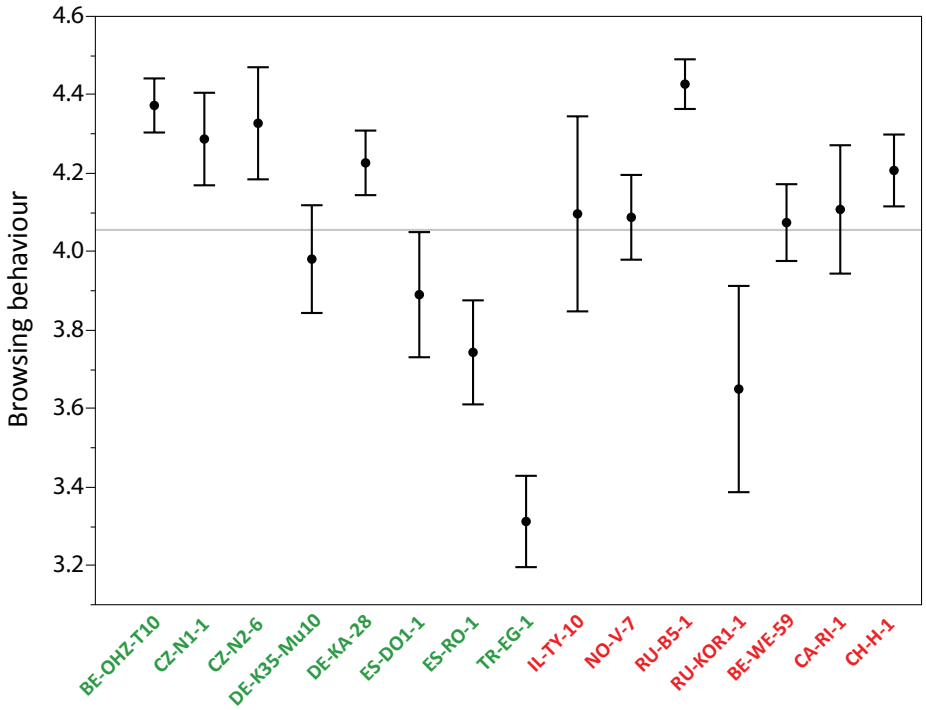

Figure 2: Browsing behaviour of 15 *Daphnia magna* clones from natural populations. Mean and standard error of the browsing behaviour of clones sampled from ponds or lakes with fish (green) and without fish (red). The clone ID includes the country of the sample location and population and clone name: (BE) Belgium, (CZ) Czech Republic, (DE) Germany, (ES) Spain, (TR) Turkey, (IL) Israel, (NO) Norway, (RU) Russia, (CA) Canada, (CH) Switzerland (Table S1). The behaviour was defined as the $\log_{10}$ of the area of the browsing traces left by replicate animals after browsing for 30 minutes on a sediment layer (Fig. 1). The grey solid line corresponds to the mean browsing behaviour (mean = 4.05).

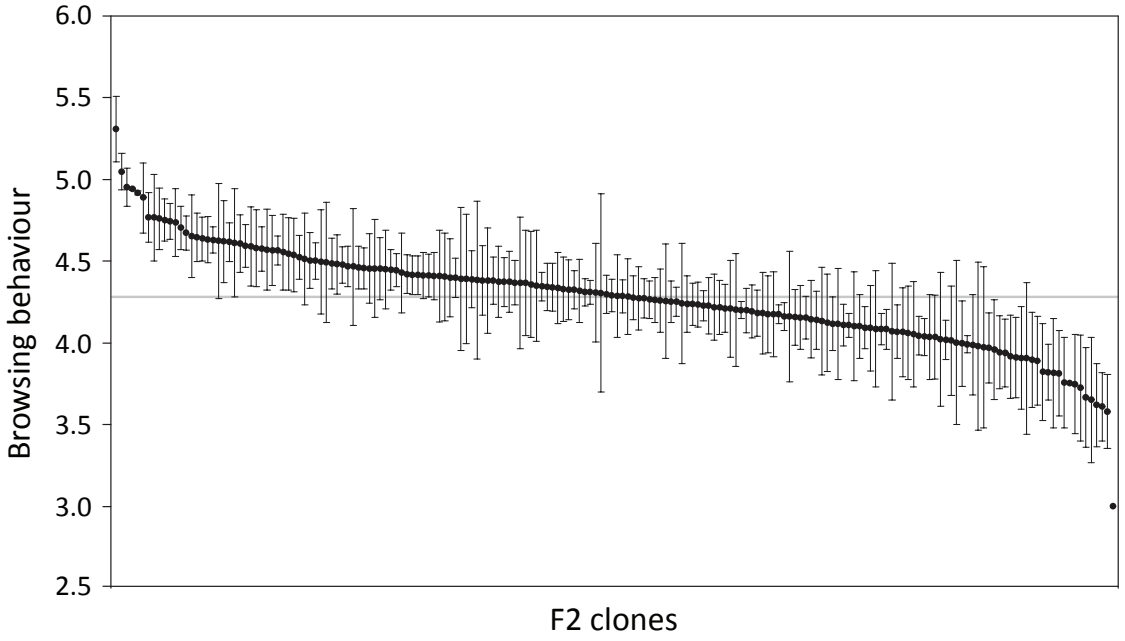

Figure 3: Distribution of the browsing behaviour of 185 *Daphnia magna* F2 clones. Mean and standard error of the browsing behaviour of replicate F2 clones. The behaviour was defined as the $\log_{10}$ of the area of the browsing traces left by replicate animals after browsing for 30 minutes on a sediment layer (Fig. 1). The grey solid line corresponds to the mean browsing behaviour of the panel (mean = 4.28).

An additional suggestive QTL (Q2) was identified on LG 1 ($LOD_{Q2} = 3.56$; $LOD_{\alpha=0.10} = 3.45$), explaining 8.54% of the phenotypic variance (Fig. 4A). The genotype at the marker associated with the highest LOD score had a significant effect on the browsing behaviour ($F_{2, 182} = 7.66$, $P = 0.0007$). At this locus, the BB genotype was associated with the highest browsing levelsand showed significant differences with the AA (Tukey's P-value = 0.0012) and AB genotypes (Tukey's P-value = 0.0019) (Fig. 4C). No significant difference between the AA and the AB genotypes was detected (Tukey's P-value = 0.84). Additive and dominance effect sizes for Q2 were 0.10 (SE = 0.03) and -0.07 (SE = 0.04) respectively. The LOD-1.5 support interval for Q2 spanned about 20 cM (corresponding to about 3.5 Mb).

The two-QTL genome scan revealed no significant interactions among QTLs. Nevertheless, the analysis identified a third QTL (Q3) whose interaction LOD score with Q1 approached the suggestive threshold ($LOD\ fv1_{Q1\text{-}Q3} = 6.18$; $LOD\ fv1_{\alpha=0.10} = 6.97$). Figure 4D shows the browsing behaviour values of all the genotype combinations between Q1 and Q3 with the lowest and the highest values being associated with the AA-BB and the BB-AB genotypes respectively. A genetic model including these three QTLs (Q1, Q2 and Q3) and the interaction between Q1 and Q3 explained 27.52 % of the total variance in browsing behaviour within the F2-panel ($F_{10, 174} = 6.57$, $P < 0.0001$).

No significant correlation was found between browsing behaviour and the phototactic index (data from Routtu *et al.* 2014) of the analysed clones ($r_{112} = 0.0058$, $P = 0.42$)(Fig. 5; Table S4).

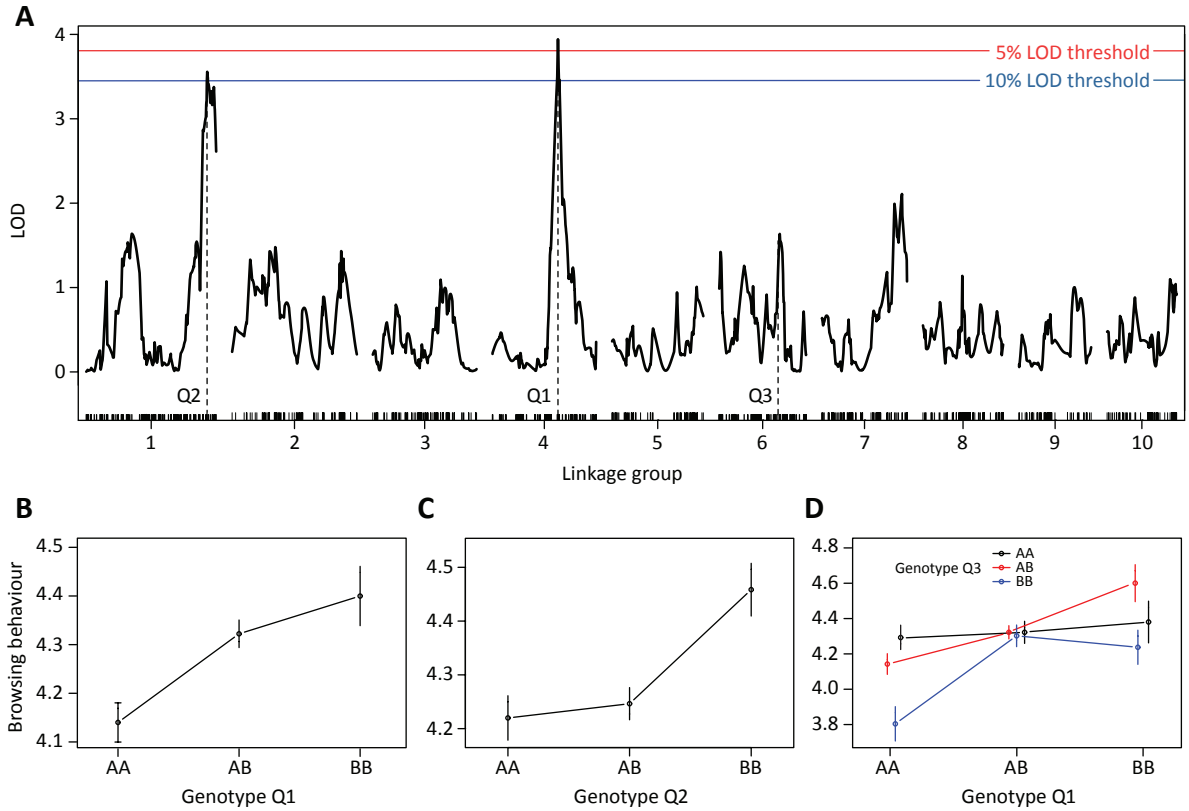

Figure 4: Quantitative trait loci (QTL) mapping analysis of browsing behaviour in *Daphnia magna.* A: single-QTL LOD scores for browsing behaviour. The solid red line represents the significance LOD threshold ($LOD_{\alpha=0.05}$ = 3.78). The blue solid line represents the suggestive LOD threshold ($LOD_{\alpha=0.10}$ = 3.45). The black dashed lines indicate the position of the markers associated with the highest LOD scores (Q1 and Q2) and of the marker at the locus interacting with Q1 (Q3); B: effect of the QTL on linkage group 4 (Q1); C: effect of the QTL on linkage group 1 (Q2); D: effect of the interaction between the QTLs on linkage group 4 (Q1) and on linkage group 6 (Q3).

Discussion

The aim of our analysis of *Daphnia* clones from natural populations was to investigate the extent of the genetic contribution to variation in browsing behaviour. Therefore, we chose clones originating from locations distributed throughout a wide geographical range and with very different ecological and climate conditions in an attempt to maximise variation. In our analysis, we estimated that about 21% of the observed variation could be attributed to genetic differences among the clones. Measures of the proportion of phenotypic variance attributable to genetic differences between clones are common in *Daphnia* literature (e.g. De Meester 1989; De Meester 1991; Ebert *et al.* 1993; Cousyn *et al.* 2001). Notably, estimates for another behavioural trait, phototactic behaviour, vary considerably between studies, populations and environmental conditions (from 20% up to 80%) (De Meester 1989; Cousyn *et al.* 2001). While our estimate for browsing behaviour might be to some degree dependent on the choice of clones it nevertheless provides evidence of a substantial genetic component underlying browsing behaviour.

Daphnia populations are often behaviourally adapted to their environment (De Meester 1996; Cousyn *et al.* 2001). For example, *Daphnia* clones from populations with a history of fish predation are more negatively phototactic and show an increased plastic response (inducing a more negatively phototactic behaviour) to fish kairomones than clones from populations that do not co-occur with fish (De Meester 1993; Boersma *et al.* 1998, Cousyn *et al.* 2001). As predator avoidance is regarded as an important determinant of habitat selection in *Daphnia,* we

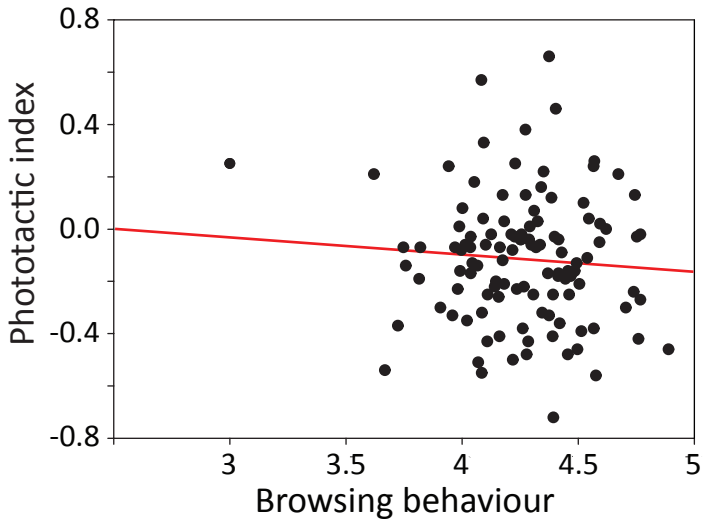

Figure 5: Correlation between the browsing and the phototactic behaviours of *Daphnia magna* F2 clones. Linear regression between the browsing behaviour (analysed in this study) and the phototactic index (from Routtu *at al.* 2014) of 113 F2 clones (Table S4).

tested whether, by inducing a closer contact with the bottom environments, pelagic predation would also favour higher levels sediment browsing. We found no effect of the presence or absence of fish in the pond or lake of origin of the analysed clones, suggesting that selection by fish predation might not be of overall importance for browsing behaviour. Nevertheless, a role of predation in the evolution of browsing behaviour cannot be ruled out, given that the environmental differences between the ponds of origin of the analysed clones and our sampling design might have hindered the detection of such an effect. Furthermore, without detailed knowledge of the strength of predation by fish or by other invertebrate predators, strong conclusions are not possible.

The QTL panel employed in our analysis was designed to encompass variation in many traits that show variation in the environment of origin of the two parental lines, namely a fish breeding pond in Germany (parental line: Iinb1) and a very shallow, fishless, intermittent rock pool population in South-western Finland (parental line: Xinb3) (Routtu *et al.* 2010; Roulin *et al.* 2013; Routtu *et al.* 2014; Routtu & Ebert 2015). At the markers associated with the browsing QTLs on linkage group 1 and 4, the alleles from the Xinb3 clone (BB genotype) show a higher propensity for browsing than the alleles from the Iinb1 clone (AA genotype). Heterozygote clones at these markers tended to show intermediate values. This suggests that the Finnish genotypes might be associated with a higher browsing activity. The small depth of the rock pools in the Finish habitat might favour a close link with the sediment layer. At the marker associated with the QTL on LG 6, which interacts with the locus on LG 4, the finish B-allele shows the opposite effect of reducing the browsing behaviour. However, the combination of alleles from different populations might obscure the effects of individual alleles on the behaviour due to the interactions between new alleles. Notably, the clone showing the lowest levels of browsing among the clones natural populations (TR-EG-1) was sampled from a lake population. It is therefore possible that ecosystem type (e.g. lake, pond or rock pool) or depth of the water body might be important determinants of browsing behaviour.

The three QTLs we identified together explain about 28% of the total phenotypic variance across the QTL panel. The effects of the main QTL on LG 4 and of the QTL on LG 1 were mainly additive. The QTL on LG 4 and another QTL on LG 6 showed weak evidence of epistatic interaction. Polygenic determination, with multiple loci of small effect and epistasis, has been

found to be common for behavioural traits (Bleakley & Danielson-François 2014) suggesting that the evolution of behaviour might more often be driven by the inheritance patterns of complex genetic architectures, as seems to be the case for the browsing behaviour of *D. magna*. The genetic basis of behavioural traits is generally still poorly understood, and few causal genes have been identified (van Oers & Mueller 2010; Bleakley & Danielson-François 2014). Notable exceptions are foraging behaviours, which in many species are influenced by genes homologous to the *foraging* gene (*for*) first identified in *Drosophila melanogaster* (Osborne *et al.* 1997, Ben-Shahar *et al.* 2002). In *Caenorhabditis elegans* another gene, neuropeptide Y receptor homolog (*npr-1*), was shown to influence foraging activity probably by acting on the same signalling pathway of *for* (Fujiwara *et al.* 2002). For these genes, multiple BLAST hits were found in the D. magna genome (version 2.4). However, hits aligning to scaffolds or contigs represented in our genetic map do not colocalize with our QTLs.

The LOD-1.5 support intervals for the QTLs identified in our study on LG 4 and LG 1 span about 14 and 20 cM respectively (corresponding to about 2.5 and 3.5 Mb). These large regions include several scaffolds and contigs of the current *D. magna* genome assembly (version 2.4) and the small effect sizes of our QTLs limit the possibility of identifying the genes responsible for the observed variation in browsing behaviour. However, improvements in the genome assembly might allow a more targeted candidate gene approach in these genomic regions. This task might be assisted by the QTL mapping of the behaviour in a larger number of F2-clones and by current advancements in genotyping of *D. magna* clones from a variety of natural environments (the *Daphnia magna* Diversity Panel). The latter analysis might also reveal signs of selection in these regions and, possibly, the nature of the selective pressures acting on browsing behaviour. No co-localization with loci identified in other genetic mapping analyses for *D. magna* (Routtu *et al.* 2010; Roulin *et al.* 2013; Routtu *et al.* 2014; Routtu & Ebert 2015) was found for the browsing behaviour QTLs.

Our analysis found no correlation between browsing and phototactic behaviours within the F2 panel. This result suggests that these behavioural traits may evolve independently. The absence of a correlation was surprising for us, as it has been suggested that negative phototactic behaviour is key in determining browsing behaviour (Decaestecker *et al.* 2002). It seems reasonable to assume that in natural settings, positive phototactic clones browse less because the distance to the benthos precludes contact with the sediment. However, negative phototaxis does not necessitate sediment browsing, and under the given experimental conditions these two traits may have been decoupled. Nevertheless, most of the analysed F2 clones had a phototactic index below 0. The division of the containers for the phototactic behaviour assay was asymmetrical, therefore, most of the analysed clones tended to occupy the lower portion of the containers and this skew might have hindered the detection of a correlation between the behaviours. Our analysis was performed with an F2 panel from one single biparental crossing scheme and thus is limited with regard to the genetic diversity included in the study. Correlations between behavioural traits have been shown to vary in magnitude and direction between populations exposed to different selective environments. For example, different behavioural traits (aggression, general activity, and exploration-avoidance) are correlated in three-spined stickleback populations from lakes with piscivorous predators, but such correlations are absent or are very weak in populations from ponds without predators (Bell 2005). Although our results might rule out the existence of a tight link between browsing and phototactic behaviours (e.g. a shared physiological or genetic regulation), it is nevertheless possible that favourable combinations of these behaviours might be brought together under specific selection regimes. Habitat selection in *D. magna* can be regarded as a composite trait including phototactic and browsing behaviours, but also

other predator avoidance strategies such as macrophyte avoidance in the presence of predatory fish that are attracted by plant beds (Tavsanoglu *et al.* 2012). From this perspective, a larger survey of behavioural correlations among populations from different environments and with an appropriately large sample size would be required to shed light on the existence and structure of behavioural syndromes (suites of correlated behavioural traits) in *D. magna*. Accordingly, we interpret our finding of the absence of a correlation between the browsing and the phototactic behaviours in *D. magna* cautiously.

Although the role of behaviour in influencing exposure risk to parasites is generally acknowledged, its study from a genetic and evolutionary point of view has received little attention compared to, for example, the study of variability in host susceptibility after exposure (Parker *et al.* 2011). Many microparasites of *Daphnia* are transmitted horizontally from dead hosts decaying on bottom substrates, and browsing behaviour is likely an important determinant of infection risk for *Daphnia* (Ebert 2005). Browsing behaviour almost certainly did not evolve in direct response to infection risk but rather in relation to feeding (Fryer 1991). Nevertheless, it is possible that, as for phototactic behaviour (Decaestecker *et al*. 2002), infection risk would contribute to the maintenance of genetic variation in browsing behaviour, an intriguing hypothesis whose formal testing was beyond the scope of our analysis. Although infection avoidance behaviours have been described in several animals (e.g. Meisel & Kim 2014; Curtis 2014), a broader characterization in multiple species has been invoked in order to expand the understanding of non-immunological defences and their influence on hostparasite dynamics (Parker *et al.* 2011; Curtis 2014). An analysis of plastic responses in browsing behaviour to the presence of parasites in the sediments might contribute to the understanding of the epidemiology of *Daphnia* infection risk.

In *D. magna*, microbiota plays a major role in host fitness (Sison-Mangus *et al.* 2015) and both host genetic and environmental factors are determinants of microbiota community structure (**2**, unpublished data). Although little is known of how this species acquires its microbiota from the environment, sediments might represent important sources of bacteria. The effect of genetic variation in browsing behaviour on microbiota acquisition from sediments is supported by preliminary results (**3**, unpublished data) and is currently under investigation.

Conclusions

Given its well-studied and central role in fresh water ecosystems and the availability of genomic tools, the genus *Daphnia* serves as an ideal model for eco-genomic studies aimed at linking genome and ecosystem structure, function, and evolution (Miner *et al.* 2012). Despite growing interest in this model for ecological genomics, studies of ecologically relevant traits, including behavioural traits, with the goal of identifying causal genes are still largely missing. Such studies can broaden our perspective on the evolution of habitat selection behaviours that mediate the interactions between *Daphnia* and its environment.

Our study highlights sediment browsing behaviour, a trait relatively uncommon among *Daphnia* species other than *D. magna*. A benthic life style is ancestral in the Cladocera, with a planktonic life style being derived (Fryer 1991). In this regard, *D. magna* combines an ancestral with a derived life style. This aspect is still poorly integrated in ecoevolutionary studies of the species. A multidimensional study is still required in order to identify and disentangle the different environmental factors related to genetic variation in browsing behaviour. Here we provide a preliminary genetic characterization of sediment browsing behaviour which, as a component of habitat selection, can have important implications for the ecology and evolution of *Daphnia.*

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Data Accessibility

The data used in this work are provided as supporting information for online publication (https://onlinelibrary.wiley.com/doi/full/10.1111/jeb.12923) and include: 1) coordinates and ecological information on the sampling locations; 2) browsing behaviour values of individual replicates of the clones from natural populations; 3) browsing behaviour values of individual replicates of the clones from the QTL panel; 4) browsing and phototactic clonal means of the clones from the QTL panel.

Chapter II

Environmental sources of bacteria and genetic variation in behavior influence host-associated microbiota

Abstract

In many organisms, host-associated microbial communities are acquired horizontally after birth. This process is believed to be shaped by a combination of environmental and host genetic factors. We examined whether genetic variation in animal behavior could affect the composition of the animal's microbiota in different environments. The freshwater crustacean *Daphnia magna* is primarily planktonic, but exhibits variation in the degree to which it browses in benthic sediments. We performed an experiment with clonal lines of *D. magna* showing different levels of sediment-browsing intensity exposed to either bacteria-rich or bacteria-poor sediment or whose access to sediments was prevented. We find that the bacterial composition of the environment and genotype-specific browsing intensity together influence the composition of the *Daphnia*-associated bacterial community. Exposure to more diverse bacteria did not lead to a more diverse microbiome, but greater abundances of environment-specific bacteria were found associated with host genotypes that exhibited greater browsing behavior. Our results indicate that, although there is a great deal of variation between individuals, behavior can mediate genotype-by-environment interaction effects on microbiome composition.

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Introduction

Every multicellular organism is colonized by a community of microorganisms: its microbiota (1). The host provides a habitat for a complex and dynamic consortium of microorganisms, many of which have fundamental influences on the host's well-being . A central concern in both infectious disease epidemiology and in studies of host-associated microbial community ecology is the transmission of microbes between host individuals and between hosts and the environment. Many bacterial assemblages are transmitted from host mother to offspring (2) or within social groups (3), but the diversity of microbiota typically changes over time depending on the microbes available in the environment (4, 5). In some cases, environmentally acquired microbes are even essential for the completion of postembryonic development (6, 7). Thus microbes from the environment can be co-opted as part of the microbiota, or can affect host health during a transient occupation (8).

Environmental effects on microbiota community structure have been extensively documented (9, 10) and studies on model organisms have started to shed light on the relative importance of environmental and host genetic factors in determining microbiota composition (11, 12). Recently, the focus has been moving towards a better understanding of the mechanisms of bacterial aqusition from the environment. Host genetics have been shown to play a role in the establishment of microbial associations through microbial recognition, immune selection, and determination of the biochemical niche (12). Importantly, these processes select microbes after the host has come in contact with bacterial communities in the environment. The initial encounter may be a key phase of the host's colonization by microbes. If host genetics influence interaction with the environment, for example through the expression of behavioral variation, it may influence the initial encounters with environmental bacteria and thus affect the composition of the host microbiota.

Many animals utilize different habitats according to behavioral strategies collectively termed habitat selection. If habitats differ in their microbial communities, host behavior influencing habitat choice and the microbiome may influence each other. Hosts may have evolved strategies to ensure or avoid encounter with beneficial and pathogenic microorganisms. Avoidance behaviors of harmful bacteria are well documented, and behavior is considered one of the first lines of defense against infectious disease. For example, the nematode *Caenorhabditis elegans* actively avoids pathogenic bacteria and the genetic determinants of this behavior have been worked out (13). The opposite case, where a host's behavior is involved in the acquisition of beneficial bacteria from the environment, has received less attention, despite speculation about the role of human behaviors such as outdoor play in preventing autoimmune diseases (14). The overall effects of host habitat choice behavior on microbiome composition have not, to our knowledge, been explored in any system. An analysis of natural genetic variation in behavioral traits potentially influencing microbiota acquisition is therefore timely (15). If variation in behavior affects the composition of the host's microbial community, then behavior could underlie some genotype-environment interaction effects on microbiota. The goal of this study was to examine the effect of genetic variation in host behavior on microbiota composition in different environments using the freshwater planktonic crustacean *Daphnia magna*.

Recently, it has been shown that *D. magna* microbiota play a major role in host fitness (16), that both host clonal line and environmental factors are determinants of microbiota community structure (17) and that genotype-specific microbiomes can mediate daphnids' adaptive traits (18). However, little is known about the mechanisms by which the host acquires microbiota from the environment. A specific behavior, termed sediment browsing, mediates the interaction between *D. magna* and bottom sediments of ponds and lakes (19, 20). During browsing, the

animals swim along the sediment surface, stirring up particles, and then ingest the particles by filter feeding. Besides representing valuable food reservoirs, sediments are likely important environmental sources of bacteria. Therefore, the physical contact with the sediments resulting from browsing might present both disease risks and benefits from increased contact with bacteria. Previous work found evidence of genetic variation for the levels of browsing activity in *D. magna* (21).

We performed a laboratory experiment in which we analyzed the browsing behavior and microbiota of 12 genetically distinct *D. magna* clones allowed to browse in sediment. The animals were exposed to three different treatment conditions, where they had access to either previously autoclaved (i) or untreated ("natural" and therefore microbe-rich) sediments (ii), or where their access to natural sediment was prevented (iii) (Figure 1A). This setup amounts to an external manipulation of the behavior that mediates the acquisition of environmental microbiota, meaning it allows us, to some extent, to isolate the effect of genotype-specific behavior from other traits that vary between genotypes. We hypothesized that *D. magna* clones exhibiting more intense browsing behavior would have more diverse microbiota in conditions where they had access to bacteria-rich sediment, whereas the microbiome would be less affected by the bacterial environment in genotypes that browsed less. In this experiment, we made no assumptions about whether bacteria found in the sediments were beneficial, harmful, or neutral for the host, nor whether they colonized *Daphnia* stably or transiently; therefore, the patterns observed here could be applicable to studies of disease, microbiota, or general environmental microbial community dynamics. Our analysis illustrates how a behavioral trait can mediate the interplay between genetic and environmental variation in the establishment of host-microbe associations.

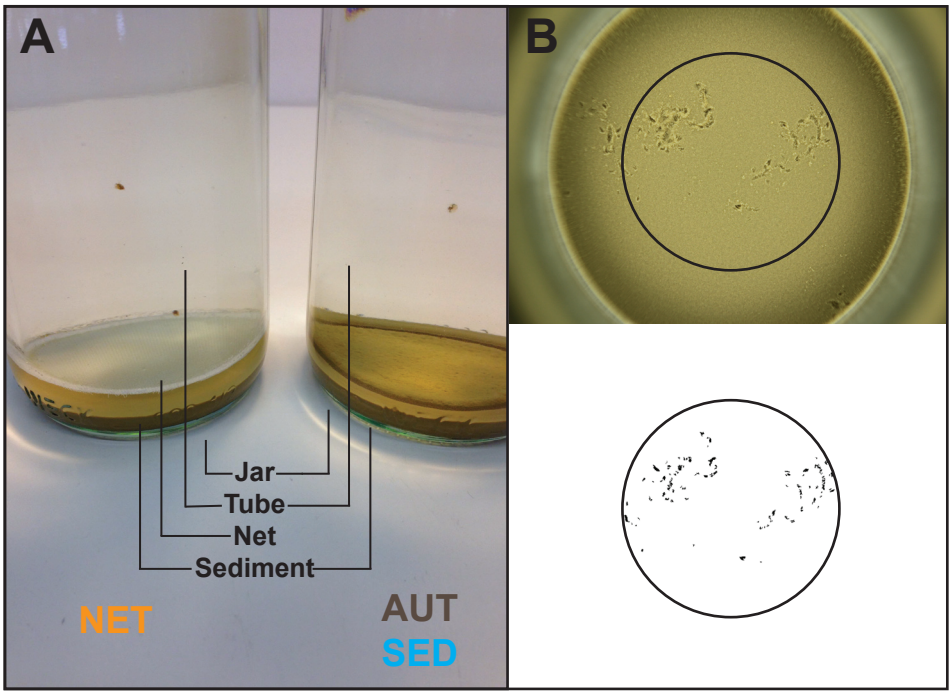

Figure 1: Experimental set-up and browsing behavior assay. A: the jars used in the experiment had a bottom layer of fine loess and contained two animals each; the animals were prevented from browsing on untreated sediments by a net placed 5 mm above the sediment surface (NET, right) or were allowed to browse on autoclaved sediments (AUT) or untreated sediments (SED) (left). B: traces left by one animal browsing on a sediment surface for 30 minutes and the same picture after processing for quantification of the browsing behavior.

Methods

Overview of the experiment

In this study, we combined the analysis of animals with constitutive (genetic) differences in browsing behavior with manipulations of the environment that affected animals' access to the sediments. Animals were either exposed to natural sediment, to autoclaved sediment, or to natural sediment blocked by a permeable net barrier (Figure 1A). In order to analyze both the browsing behavior of the animals and their microbiota, we placed two animals in each jar; of these pairs of animals, after 6 days of exposure to the different treatments, one animal was used to assay browsing behavior while the other was used for microbiota analyses.

Experimental animals

D. magna reproduces by cyclical parthenogenesis. Clonal populations can be generated and propagated in the laboratory through asexual reproduction. Here, we refer to such genetically identical individuals as "replicates" or "animals" while we refer to different genetic lines as "clones." In this study we used 12 *D. magna* clones from our stock collection, originating from different populations (Table 1). The animals were propagated from stock cultures maintained in the laboratory in standardized conditions and without any effort to modify their microbiota. The browsing behavior of these clones has been assessed before (21) and was shown to differ among genotypes.

D. magna reproduces mostly asexually, with males being rare. Therfore, all animals used in this study were females. Prior to the experiment, every clone was propagated in individual replicates for three generations in order to minimize variation due to maternal effects. These animals were kept individually in 100-ml glass jars filled with 80 ml of ADaM (*Daphnia* medium (22)) randomly distributed within trays in incubators with a 16:8 light/dark cycle and constant temperature of 20 °C. To establish every generation, the animals were isolated at 4 days old and fed daily with chemostat-grown green algae *Scenedesmus* sp: 1 x 10^6 algae cells/animal until day 5, 2 x 10^6 until day 8, 2.5 x 10^6 until day 10, 3 x 10^6 until day 12, and 5 x 10^6 onwards. The animals were transferred to fresh medium when they were 12 days old and thereafter every day.

Table 1. Names, number of individual replicates included in the microbiota analyses in the three treatments (AUT, NET and SED) and origin information of the 12 *Daphnia magna* clones used in this study. AUT: Exposure to autoclaved sediment; NET: prevented exposure to untreated sediment; SED: exposure to untreated sediments.

Clone ID	N (AUT)	N (NET)	N (SED)	Country	Latitude, N	Longitude E/W	Source	Description
BE-OHZ-T10	**4**	**5**	**3**	Belgium	50º50'00"N	4º39'00"E		
CZ-N1-1	**8**	**8**	**8**	Czech Rep.	48°46'31.14"N	16°43'24.70"E		
CZ-N2-6	**6**	**7**	**6**	Czech Rep.	48°46'31.14"N	16°43'24.70"E		
DE-K35-Mu10	**4**	**3**	**4**	Germany	48°12'23.93"N	11°42'34.98"E	*D. magna* Diversity panel	A geographically diverse collection of clones maintained asexually in the laboratory since 2012
DE-KA-F28	**8**	**7**	**7**	Germany	50°56'02"N	6°55'41"E		
ES-DO1-1	**7**	**6**	**7**	Spain	36°58'42.1"N	6°28'39.5"W		
TR-EG-1	**5**	**7**	**8**	Turkey	39°49' 25"N	32°49' 50"E		
BE-WE-G59	**7**	**8**	**7**	Belgium	51°04'04"N	3°46'25"E		
No-V-7	**4**	**3**	**2**	Norway	67°41'13.06"N	12°40'19.09E		
Clone ID				**Description**			**Source**	**Description**
IXF1	**5**	**8**	**7**	F1 clone				
F2-82	**7**	**7**	**4**	F2 clone			*D. magna* QTL panel	An intercross F2 recombinant panel maintained asexually in the laboratory since 2006/2007
F2-918	**5**	**6**	**6**	F2 clone				

For the experiment, we used animals from the 4th generation of each of the 12 clones. These animals were kept in groups of 8 siblings belonging to one clutch of one mother. At 4 days old (± 1 day), 6 animals from every clutch were randomly assigned in pairs to individual jars divided into the three different treatments (split brood design); each such jar containing a pair of animals was an experimental replicate. In total, we included in the experiment 540 animals (270 pairs) corresponding to 15 pairs of clone BE-OHZ-T10, 18 pairs of clones DE-K35-Mu10 and NO-V-7, 21 pairs of clone F2-918, and 24 pairs of each of the remaining clones. Variation in replicate numbers resulted from differences in availability of female offspring at the time that the treatments were established.

Experimental design

The experiment was conducted in cylindrical glass jars (height = 20 cm; diameter = 6.5 cm) (Fig. 1A) kept in cardboard boxes on shelves in a climate room (16:8 light/dark cycle at 20 °C), loosely covered with transparent plastic film and top-illuminated with neon lights. In this way, light only entered the jars from the top. All the experimental jars were first filled with 400 ml of medium. 15 ml of a suspension of loess (fine silt) was then carefully deposited on the bottom using a serological pipette. The loess was previously collected from a soil stock from a pit near Biel-Benken, Switzerland. To prepare it for the experiment, the loess was suspended in water, passed through a 200 µm filter and washed to remove very fine particles. After two days of sedimentation in the experimental jars, the loess formed a 1 cm layer at the bottom of the jar. Then, an acrylic tube (height = 21 cm; diameter = 5 cm) was inserted into the jars and kept in position with a plastic ring fixed to the opening of the jar, so that its lower end was positioned close to the sediment surface. In one treatment (NET), the acrylic tube was closed with a 500 µm net at the lower end (suspended 5 mm above the sediment surface) preventing animals from direct contact with the sediment (Fig.1A left). In the other two treatments (AUT and SED), the acrylic tubes had no net so that the animals had free access to the sediment (Fig.1A right). In the AUT treatment, the loess was previously autoclaved while in the SED and the NET treatment the loess was left untreated ("natural"). After autoclaving, AUT sediment was handled in the same way as natural sediment, i.e. exposed to nonsterile media and laboratory environment. After inserting the tubes, the jars were left undisturbed for two days before the animals were introduced in order to allow the sediment to settle. Immediately before the experiment, the sediments of three jars of each of the SED and the AUT treatment were sampled and frozen at -20 °C; these sampled jars were not used further. Two animals from the same clutch were carefully introduced into the inner tube of each jar. The 264 jars, each containing one pair of animals, were evenly distributed among the treatments and their positions in the incubator room were randomized. The animals remained in the experimental jars for 6 days. During this time, the animals were carefully fed twice daily with 2.5 x 10^6 algal cells. At day 6, all animals were collected and one member of every pair was assigned to the behavioral assay (see below) and the other was frozen for later DNA extraction. 32 pairs of animals were lost or damaged during the experiment and were excluded from further analyses. At the end of the experiment, 3 sediment samples from the NET treatment and 3 sediment samples from the AUT treatment were collected and frozen at -20 °C.

Behavioral analysis

The animals for the behavioral assay were transferred individually from the sediment jars to 100-ml glass jars filled with medium and kept in an incubator and fed daily with 5 x 10^6 algal cells. The behavior assay was conducted over two days when the animals were 12 to 14 days old with all replicates for the different clone by treatment combinations evenly distributed across

time. The behavior assay was performed as described previously (21). Briefly, we measured the traces left by individual replicate animals on a smooth surface layer of sediment (loess) at the bottom of tall cylindrical glass jars (20 cm tall, 6.5 cm diameter; Fig. 1B) during 30 minutes. The sediment surface was photographed before animals were released (time 0), using a ring light to ensure uniform illumination. The jar was then transferred into a cardboard tube and illuminated from the top with a neon light and one animal was introduced in each jar. After exactly 30 minutes, the animal was removed and the sediment surface was again photographed (time 1), in the same position and under the same light conditions. Using the software ImageJ (http://rsb.info.nih. gov/ij/), the pictures were converted to grey scale and a central circular area was cropped to exclude shadows from the edge of the jar (Fig. 1B). Pictures were processed such that the browsing traces of the animals on the sediment surface resulted black areas against a white background. Then the number of black pixels was quantified. Pictures taken at time 0 were used to correct the values calculated for the browsing traces when irregularities on the sediment surface were detected (i.e. in cases the picture of time 0 was not entirely white). The pixel values were then log-transformed ($[\log_{10}(X+1000)]$; 1000 corresponds approximately to the number of pixels of one individual browsing trace. During the assay, four animals were accidentally damaged while handling and were excluded from the analyses. The jar-mate counterparts of these individuals were still sequenced, but were excluded from sequencing analyses in which individual jar-mate behavior was used as the behavior proxy. The body lengths of the animals used for behavior analysis were measured after the behavioral assay.

The adjusted intra-class correlation coefficient for the browsing behavior (equivalent to broad sense heritability) was calculated with a linear mixed effect (LMM) model, with treatment as a fixed effect and clone as a random effect (R software package rptR developmental version; (23)). Confidence intervals and statistical significance were calculated using parametric bootstrapping with 5000 iterations and a randomization procedure with 5000 permutations.

DNA extraction, library preparation and sequencing

The animals assigned to the microbiota analysis were transferred individually from the sediment treatment jars to 40 mL of autoclaved ADaM for about 2 hours to dilute carryover of unattached bacteria. Then, the animals were transferred into 1.5 ml Eppendorf tubes, the ADaM was removed and the tubes were stored at -20 °C until DNA extraction.

DNA was extracted from single animals using a cetyltrimethylammonium bromide (CTAB)-based protocol. The animals were ground with a sterile pestle in 1.5 ml Eppendorf tubes in a 10 mg/ml lysozyme solution and mixed at 850 rpm and 37 °C for 45 minutes. Then, a 20 mg/ml solution of proteinase K was added and the tubes mixed at 850 rpm and 55 °C for 1 hour. After an RNase treatment (20 mg/ml) at room temperature for 10 minutes, a preheated 2X solution of CTAB was added and the tubes mixed at 300 rpm and 65 °C for 1 hour. After two rounds of chloroform isoamyl alcohol (CIA) purification (1 volume CIA; 8 minutes centrifugation at 12,0000 rpm and 15 °C), a solution of sodium acetate 3M pH 5.2 and isopropanol were added to the DNA solution and the tubes were stored overnight at -20 °C. The following day, DNA was purified by two rounds of 70 % ethanol precipitation and suspended in water. The extractions were then incubated at 4 °C overnight and then stored at -20 °C.

All DNA extractions were conducted over a period of 6 days with samples from the different clone by treatment combinations randomly distributed between the days and one reagent-only negative control extraction included every day. DNA from the sediment samples and from one negative control was extracted on a different day using a commercial kit (PowerSoil® DNA Extraction kit; MO BIO Laboratories, cat. 12888-100).

We sequenced amplicons of the V3-V4 variable region of the bacterial 16S rRNA gene using the Illumina MiSeq platform. Amplicons were generated using NEBNext High Fidelity PCR Master Mix (New England Biolabs catalog #M0541L) for 27 cycles in 25 µl reactions containing 3% DMSO. The primers used were 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGA-3') and 785R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGA-3') with Illumina adapter sequences and 0-3 bp random frameshifts. PCR product was purified with Ampure beads at a 0.6x ratio of beads to PCR product, amplified for 9 cycles with Nextera XT v2 indexing primers, and purified again. Libraries were quantified with Qubit and quantitative PCR, normalized, and pooled, followed by additional bead purification to remove remaining short fragments before sequencing on the Illumina MiSeq (reagent kit v3, 300 bp paired-end reads). The same library pool was used for two MiSeq runs; after checking that there was no statistical difference in community composition between the runs (PERMANOVA (Adonis) analysis of Bray-Curtis dissimilarity between samples, p=0.394), the data from the two runs were merged using the default settings in phyloseq.

Sequence quality control

Raw reads were quality controlled with FastQC (Babraham Institute, UK). Paired reads were merged (FLASH v1.2.9), primers trimmed (Cutadapt v1.5), and quality filtered (PRINSEQ-lite v0.20.4). OTU clustering including abundance sorting and chimera removal was performed using the UPARSE workflow (24). Only those OTUs represented by 5 or more reads in the run were included. Taxonomic assignment was performed using UTAX against the GreenGenes v13/5 database. We analyzed samples with more than 5000 total reads. This left 214 samples; numbers of replicates for each combination of variables are reported in Table 1.

Since individual *Daphnia* contain low bacterial biomass, we considered the issue of reagent contamination with bacterial DNA (25). Samples were processed in haphazard order, so erroneous sequences originating from reagent contamination were expected to be distributed randomly and not confounded with any treatment or genotype. For our research question, we were interested in patterns of diversity and changes in composition in response to experimental factors rather than in the presence or absence of any particular strain. For all analyses, we first tested for processing batch effects and stratified the main analysis by batch if they were significant.

For statistical analyses in which host clone was a fixed effect, we excluded clone NO-V-7, since it did not have at least 3 replicates in each treatment; we included this clone in analyses where clone was treated as a random effect. We examined the effects of experimental factors on both overall diversity and the community composition of each animal's microbiota using standard ecological diversity indices and ordination methods. To evaluate the effect of animal behavior on microbiota, we used as proxies for individual behavior either the mean browsing intensity index of the clone or the browsing intensity of the individual co-housed with the sequenced individual in the same jar ("jar-mate").

Analyses were carried out in R (3.4.3), using the packages phyloseq (1.22.3), vegan (2.4.6), plyr (1.8.4), dplyr (0.7.4), DESeq2 (1.10.1), nlme (3.1.131), lme4 (1.1.15), metacoder (0.3.0.1), and ggplot2 (2.2.1).

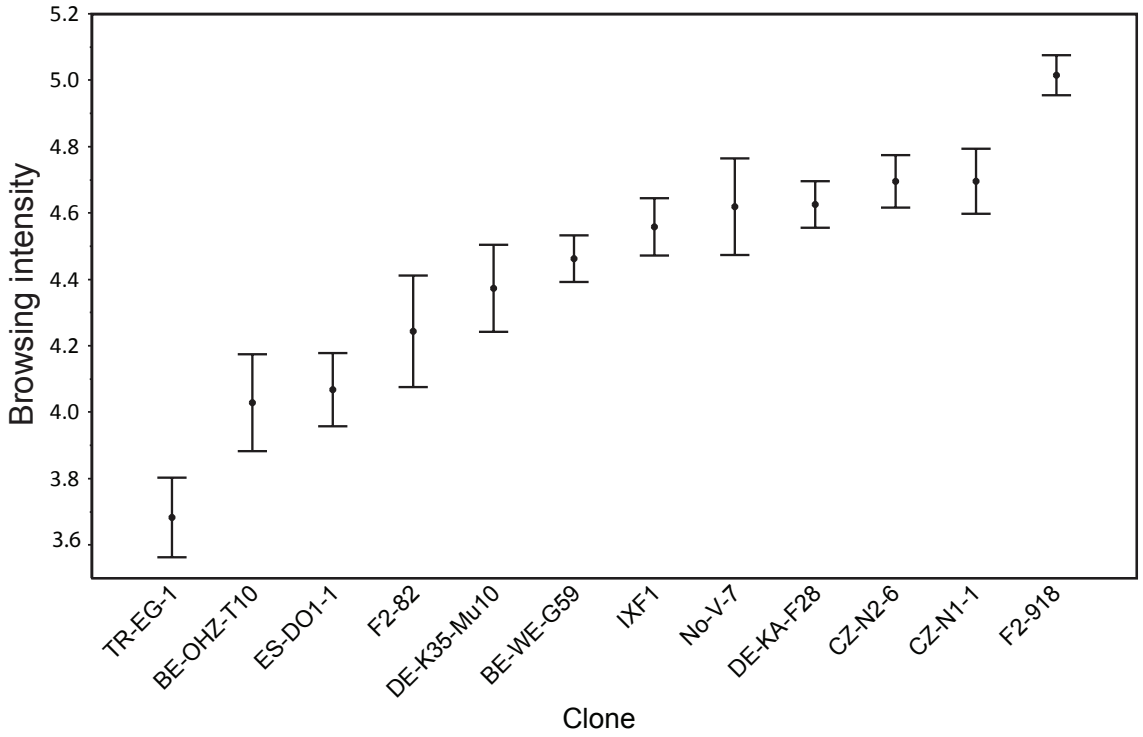

Figure 2: Browsing intensity of 12 *D. magna* clones (mean and SE). Browsing intensity was defined as the Log_{10} of the area of the browsing traces left by individual replicate animals browsing on a sediment surface for 30 minutes (see Fig. 1).

Results

Browsing intensity, animal microbiota, and sediment bacteria

Consistently with previous studies (21), browsing behavior intensity varied among *Daphnia* clones (Fig. 2). Clone and treatment, but not their interaction, had a significant effect on browsing behavior (analysis of variance: clone F=12.717, df=11, p<0.0001; treatment F=4.100, df=2, p=0.018; clone*treatment F=1.274, df=22, p=0.193). The average browsing intensity of animals from the NET treatment was lower than that of animals in the SED and AUT treatments (Fig. S1). The total phenotypic variance for browsing behavior explained by clone, after controlling for the treatment effect, corresponded to 36.5% (95% CI = [13.2, 55.3%], p = 0.0002). Clone but not treatment had a significant effect on body size, so we assume that access to (and type of) sediment did not substantially affect nutrition and growth over the timeframe of the experiment (analysis of variance: clone F=8.08, df=11, p < 0.001; treatment F=2.01, df=2, p=0.137; clone*treatment F=1.43, df=22, p=0.103). Individual body size was uncorrelated with behavior (analysis of variance: F=0.346, df=1, p=0.56; Fig. S2).

A total of 370 OTUs were found among the animal samples; of these, 318 were found in less than 10% of samples. (See Fig. S3A-C for taxonomic heat trees of OTUs with presence/absence information) (26). Consistently with multiple previous studies of *Daphnia* microbiota (27–30), the most abundant bacterial species was a single OTU (OTU_1) of *Limnohabitans* sp (Betaproteobacteria, Comamonadaceae), with a mean relative abundance across all clones of 0.39 (s.e.m. 0.02). Interestingly, a second type of *Limnohabitans* (OTU_2) was a dominant OTU only in the three clones originating from clones bred in the laboratory as part of a genetic breeding design (QTL panel; 0.32 mean relative abundance among individuals of clones IXF1, F2-82, F2-918; 0.0016 mean relative abundance in remaining clones). As expected, the sediment used in the SED treatment had much higher bacterial species richness than that used in the AUT treatment (Fig. S4).

Table 2. Results of analyses of variance of different alpha diversity indices. All treatments included. clone NO-V-7 is excluded.

	Richness	Shannon	Inverse Simpson
Clone	F=0.40, df=10, **p=.944**	F=2.43, df=10, **P=.00997 ***	F=1.98, df=10, **p=.0383 ***
Treatment	F=4.91, df=2, **p=.00842 ***	F=12.21, df=2, **p<.0001 ***	F=13.25, df=2, **p<.0001 ***
Clone:Treatment	F=0.75, df=20, **p=.770**	F=0.78, df=20, **P=.734**	F=0.65, df=20, **P=.8124**

Effects of treatment and clone on alpha diversity

Both *Daphnia* clone and treatment, but not their interaction, had significant effect on the Shannon and inverse Simpson alpha diversity indices (Table 2). For further analyses, we focused on the Shannon index, because it takes into account not only species richness but also evenness (with additional species given more weight as they become more abundant). The Shannon index displayed no significant effect of processing batch (DNA extraction and amplification) (df=5, F=1.42, p=0.22). Shannon diversity estimates for the 12 clones arranged in order of increasing average browsing intensity and the three groups (AUT, NET and SED) are shown in Fig. 3 (species richness and inverse Simpson index are shown in Fig. S5A-B). Unexpectedly, the highest average alpha diversity in most clones (9/12) was observed in the AUT treatment group, despite their exposure to less-diverse sediment than the SED group. Therefore, diversity of animal microbiota does not directly reflect diversity of bacteria in the environment.

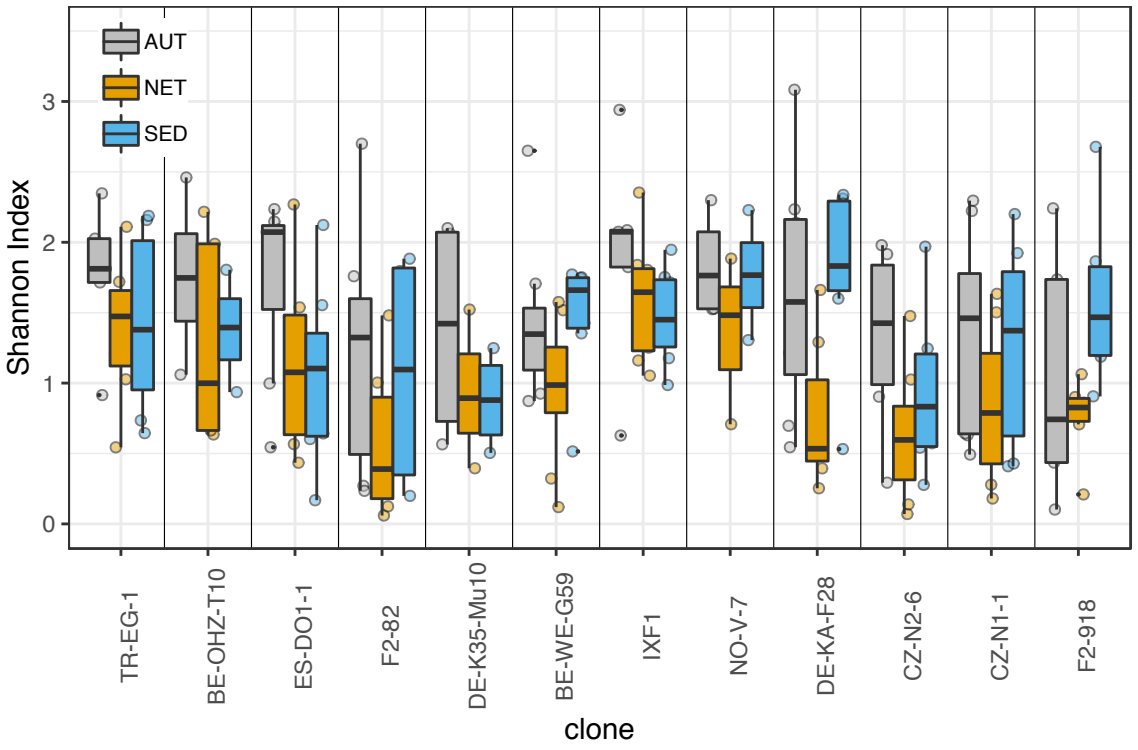

Figure 3: Microbiota diversity (Shannon index) of *Daphnia* clones under three different treatment conditions (AUT, NET and SED). AUT: Exposure to autoclaved sediment; NET: prevented exposure to untreated sediment; SED: exposure to untreated sediments. Clones are arranged left-right by increasing average clone browsing intensity.

To specifically investigate the effect of direct access to the same bacteria-rich sediment, we compared the NET and SED treatment groups' diversity as a function of clonal average behavior in each group (Fig. 4A). The difference in mean Shannon diversity between SED and NET animals was greatest at the highest average clonal level of browsing intensity (Fig. 4B; linear regression p=0.055). A similar tendency could be seen when the browsing intensity of each individual's jar-mate was used as the proxy for individual behavior (Fig. S6). Shannon diversity significantly depended on the interaction between treatment and clonal average browsing intensity in a linear mixed-effects model with clone included as a random effect (Table 3); the same was true when treatment-specific clonal average behavior was used as the behavior proxy, but not when individual jar-mate behavior was used (Table S1).

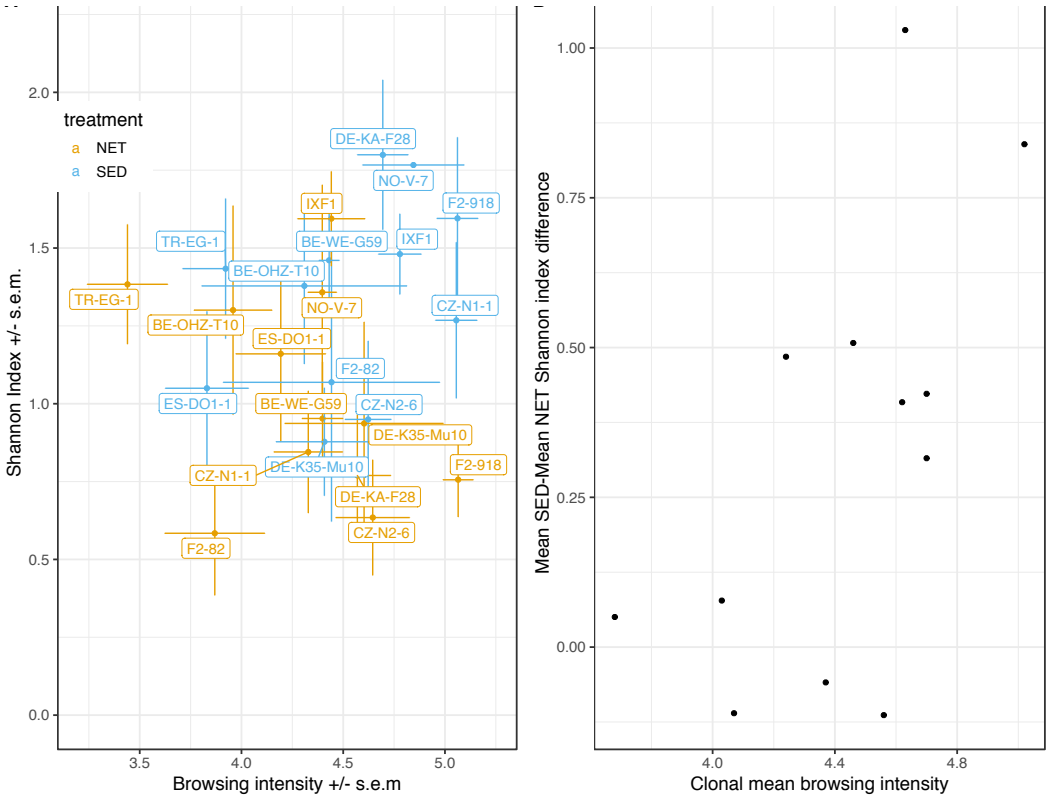

Figure 4: Average browsing intensity and average microbiota diversity in the NET and SED treatments. A: average clone browsing intensity and average clone microbiota diversity in the NET and SED treatments. Average browsing intensities were calculated based on samples whose jar-mates passed the sequence quality control (N=214, Table 1). B: average clone browsing intensity and the difference between average Shannon diversity in the SED treatment and average diversity in the NET treatment. Here, average browsing intensities were calculated based on the complete set of samples (N=228, i.e. all assayed jar-mates). Error bars represent standard error of the mean.

Table 3. Effect on Shannon index. NET and SED treatments only, all clones included. Linear mixed-effects model with treatment, clonal average browsing intensity and clonal average size as fixed effects and clone as random effect.

	numDF	denDf	F-value	p-value
(Intercept)	1	129	270.12	<.0001
Treatment	**1**	**129**	**12.26**	**0.0006 ***
Clone average behavior	1	9	0.275	0.613
Clone average size	1	9	2.568	0.144

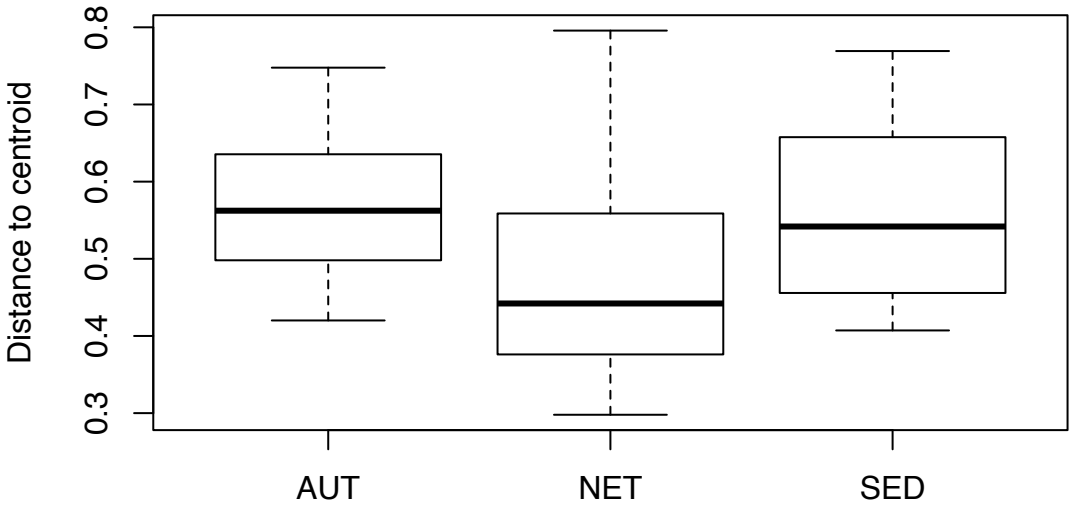

Figure 5: Within-group dispersion of community similarity. The median distance to the centroid is lower in the NET treatment group than in the others (Permutation test of multivariate dispersion p<0.0001, 999 permutations), meaning that NET communities are less variable than AUT or SED microbiotas.

Community composition and acquisition of bacteria from sediment

To examine shifts in bacterial community composition in response to environmental treatments, we Hellinger-transformed the bacterial abundances by taking the square root of the relative abundance of each taxon in each sample to reduce the influence of rare taxa, and then calculated pairwise Bray-Curtis distances between samples. The average distance to the centroid (dispersion) was lower in the NET group than in the AUT and the SED groups (Fig. 5), suggesting that access to sediment increases variability of microbiota regardless of the composition of the sediment. To see whether the different sediments resulted in systematically different microbiota composition, we excluded the NET group and performed principal coordinates analysis (PCoA) (Fig.6). PERMANOVA (adonis) analysis stratified by processing batch showed that both treatment and clone had a significant effect (treatment: R^2=0.05, p=0.001; clone: R^2=0.16, p=0.001), but not their interactions (treatment:clone R^2=0.07, p=0.63). However, clones also showed significant differences in dispersion (p<0.001). The R^2 values suggest that most variance in the dataset is not explained by these two factors, meaning that clonal and environmental factors have small – albeit detectable – effects on composition.

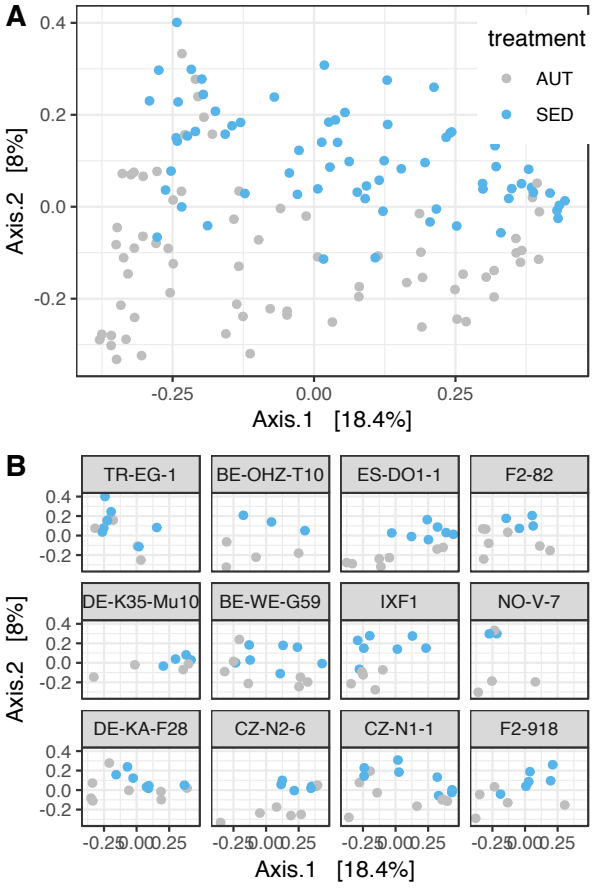

Figure 6: Similarity of bacterial community composition in the AUT and SED treatments. A: first and second axis of a principal coordinates analysis (PCoA) of bacterial community composition based on Hellinger-transformed Bray-Curtis dissimilarities. B: first and second axis of a principal coordinates analysis (PCoA) of bacterial community composition by *Daphnia* clone.

Having confirmed that differences in the sediment environment resulted in differences in animal microbiota composition, we next explored the extent to which environment-specific bacteria contributed to these differences. We used the implementation of DESeq2 in phyloseq to determine which bacteria were significantly more present in natural sediment than autoclaved sediment (n=3 each). 115 OTUs were calculated to be significantly differentially present between the two sediment types (Supplementary Table 2); of these, 48 had at a log2-fold increase of at least 8 in natural sediment compared to autoclaved sediment. We refer to these as natural-sediment-derived taxa. The 8-fold threshold was chosen based on inspecting the data; similar results were seen when sediment-derived bacteria were defined by a log2-fold change of 5 or 10; see Fig. S7. Only one of these OTUs was found in a majority of animals, and the median number of animals in which a given OTU was found was 6.5. We therefore concluded that animals likely acquired environmental bacteria randomly rather than selectively from the environment. Accordingly, we looked at the total relative abundance of reads from all natural sediment–derived bacteria in each individual.

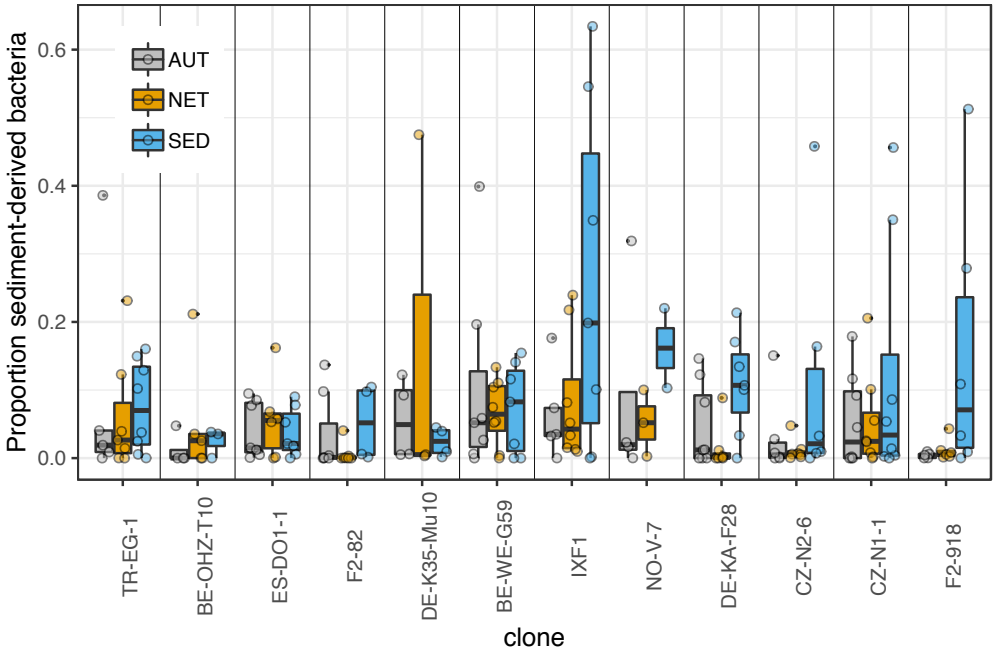

Figure 7: Analysis of sediment-derived bacteria. Proportion of sediment-derived bacteria in the microbiota of animals from AUT, NET and SED treatments. Sediment-derived bacteria were identified by comparing autoclaved and untreated sediment samples (log2-fold increase of at least 8 in natural sediment compared to autoclaved sediment). Clones are arranged leftright by increasing average clone browsing intensity.

The relative abundance of natural-sediment-derived bacteria was generally low in both the AUT and NET treatment groups, and increased with browsing intensity in the SED treatment group (Fig. 7), with a significant interaction effect between treatment and clonal average browsing intensity (Table 4). Treatment-specific clonal average behavior showed the same significant interaction effect with treatment, but the interaction effect was not significant when jar-mate behavior was used as the behavior proxy. Among the set of clones examined here, an appreciably high relative abundance of sediment-derived bacteria was detectable mainly in clones with a browsing intensity index higher than mean 4.4 (clones IXF1, NO-V-7, DE-KA-F28, CZ-N2-6, CZ-N1-1, F2-918). The mean relative abundance of sediment-derived bacteria in the SED treatment in the pooled animals from these clones was 0.14 (s.e.m. 0.026), whereas it was 0.048 (s.e.m. 0.0097) in the lower-browsing clones. Across all clones in the AUT and NET treatment groups, the average relative abundance of sediment-derived bacteria was 0.051, nearly identical to that of the low-browsing clones in the SED conditions.

Table 4. Effect on relative abundance of sediment-derived bacteria. All treatments and all animals included. Linear mixed-effects model with treatment and clonal average browsing intensity as fixed effects and clone as random effect.

	numDF	denDf	F-value	p-value
(Intercept)	1	198	53.999	<.0001
Treatment	**2**	**198**	**6.500**	**0.0018***
Clone average behavior	1	10	0.490	0.4998
Treatment:Clone average behavior	**2**	**198**	**4.185**	**0.0166***

Discussion

Our results have several implications for studies of animal-associated microbiota in diverse environmental settings. First, we confirm the intuition that environmental sources of bacteria affect the diversity of animal microbiota, but not because more diverse environments always create more diverse microbiota; rather, the animals we exposed to the less species-rich autoclaved sediments had higher overall diversity in their microbiota than those exposed to untreated, bacterial-species-rich sediment. We hypothesize that this might be due to competitive interactions between *Daphnia* microbiota and the particular microbes found in these sediments. The untreated sediments may contain bacteria that can outcompete multiple OTUs of "native," preexisting *Daphnia* microbiota. If this were the case, then browsing in sediment could have multiple opposing effects on overall microbiota diversity: on the one hand, it would bring daphnids into contact with more diverse bacteria, but on the other hand those bacteria could reduce existing microbiota diversity. In the NET treatment, animals might be exposed to some sediment-derived bacteria in the water column but lack access to the full diversity of bacteria in the sediment. An experiment designed to explicitly test this hypothesis would be required to determine whether there are competitive interactions between exogenous sediment-derived bacteria and those typically carried by *Daphnia* in the laboratory; it would also be interesting to see how these competitive effects interact with early colonization events in young *Daphnia.*

We also saw that having access to either sediment increased the variability of community composition as measured by multivariate dispersion. These results suggest that having access to multiple habitats with different bacterial communities can affect the diversity and composition of an animal's microbiota. Therefore, fine-scale heterogeneity in a host's habitat might be a relevant aspect to take into account when examining effects of environment on animal microbiota. This is especially important when considering ecological immunology, because disease-causing bacteria in the environment may cause short-term risk but also long-term fitness benefit via processes like immune priming (31, 32).

Our data further suggest that the diversity of *Daphnia*-associated microbiota in a particular environment may to some extent be mediated by genotype-specific sediment browsing intensity. This was apparent as the net barrier made the greatest difference in microbial alpha diversity in high-browsing host clones. However, this effect may be partially obscured by several factors: the hypothesized competitive exclusion effects we allude to above, and also non-behavior-related host genotype effects on microbiota diversity. While host genotype had an effect on microbial diversity, the highest- and lowest-browsing clones in our study had similar microbial alpha diversity overall. The only way to conclusively determine that differences between the microbiotas of different genotypes are mediated by host behavior independently of other host traits would be to genetically manipulate behavior on an otherwise identical genetic background; we approximate this in our experiment with the treatment where *Daphnia* are blocked from sediment browsing, contrasted with the treatment where they are allowed to

browse freely. Our cautious conclusions about the effect of behavior on microbiome are based on examining the contrast between these treatments within each genotype, not based on the observation of genotype-dependent differences alone. It was only in evaluating the difference between presence and absence of the barrier that an effect of browsing on diversity could be seen. We conclude that the effect of environmental bacteria on host-associated microbiota is not additive. The clearest effect of environmental bacteria on host-associated microbiota was not on alpha diversity, but relative abundances of certain taxa.

Clones with low average browsing intensity had no greater amount of sediment-specific bacteria than animals exposed to autoclaved sediment or prevented from browsing, whereas those with high browsing intensity could reach over 60% of reads from environment-derived bacteria in some individuals. While many studies of animal microbiota rightly concern themselves with distinguishing between truly "host-associated" microbiota versus "transient environmental" microbiota, these results raise the possibility that the amount of environmental microbes found in association with an animal could itself be a host-genotype-specific feature of the microbiome. Another key question is whether browsing behavior affects community composition by simple exposure to more colonizing bacteria, or by more frequent replenishment of bacterial taxa that would not otherwise persist in association with the host. For example, browsing frequently enough may replenish bacteria that would otherwise be lost when the animal molts. In *Drosophila*, some functionally important bacteria do not persist at replacement rate within the host, and must be continuously replenished from the environment (33). It is not known how widespread such situations are in nature, or whether a fraction of the microbiome that requires continual environmental replenishment is missing in microbiome surveys carried out in "cleaner" laboratory conditions. Conversely, hosts' recent behavior should be considered as a potential source of variability when sampling animal microbiomes in nature, and behavior as an interface between animals and environments should be considered when examining host traits that affect host-microbe interactions. In this study, we made no assumptions about the types of interactions between the sediment-associated bacteria and the *Daphnia*, but still were able to demonstrate a link between the environmental and host-associated microbiome.

It would also be interesting to investigate whether carriage of bacteria on *Daphnia* from the sediment into the water column affects bacterial dynamics in the larger environment; previous studies have shown that movement of *Daphnia* between benthic and limnetic environments represents a mechanism of bacterial dispersal in the environment (34). Studies using classification methods more sensitive than 16S-based taxonomy may be necessary to unambiguously distinguish and assign sources to different bacteria.

Conclusion

We show that at least some characteristics of host-associated microbial community composition result from genotype-by-microhabitat interactions, specifically ones resulting from genotype-specific variation in behavior. We show this using an experimental treatment that externally manipulated behavior, but genetically manipulating behavior to confirm these results would be a natural next step when the molecular tools to do so become available. Behavior could thus be considered a genetic factor that shapes microbial exposure in a given environment. Overall, these results provide further evidence that environment, behavior, genetics, disease risk, and microbial community composition are interrelated in potentially complex ways. Our observations indicate a need for more integrative eco-immunology studies, in which the interfaces between behavioral ecology, microbial community ecology and evolution of immune function are explored. Studies can take advantage of the experimental tractability of the *Daphnia*-microbiota system to further investigate these relationships in mechanistic detail.

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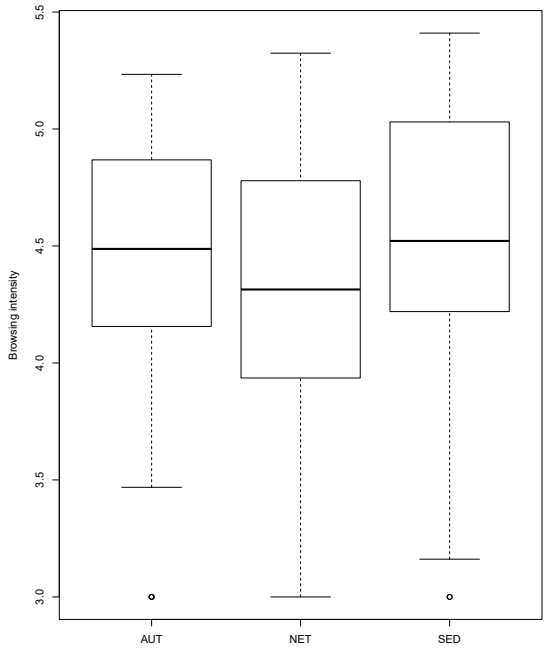
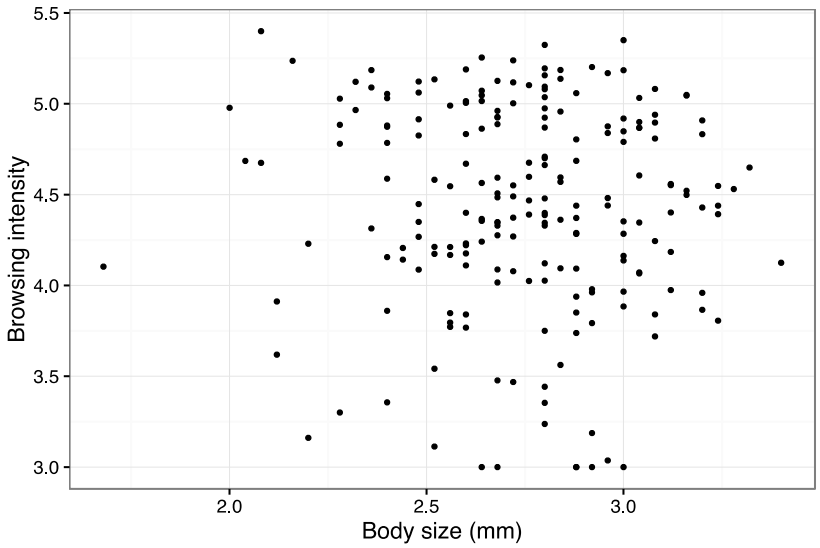
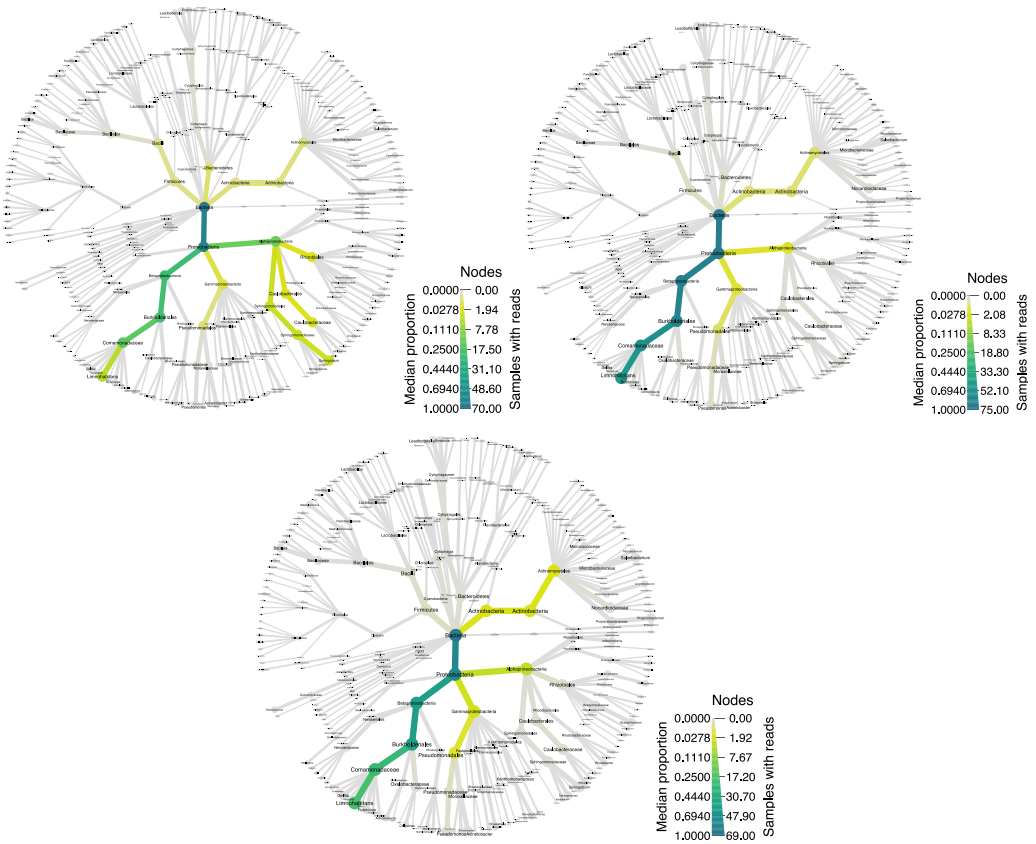
Supplementary Material – Chapter II



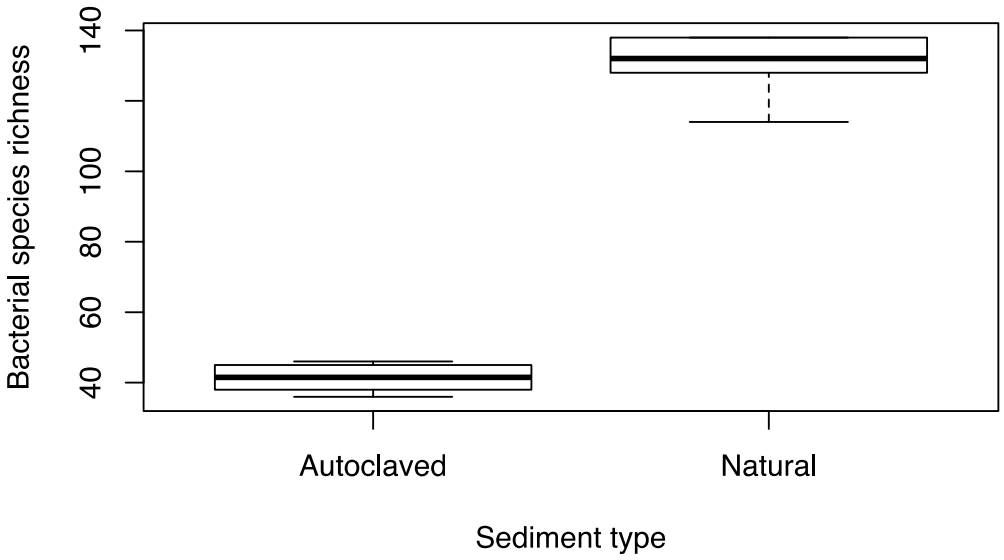
Supplementary Figure 1. Browsing intensity index by environmental treatment is shown.



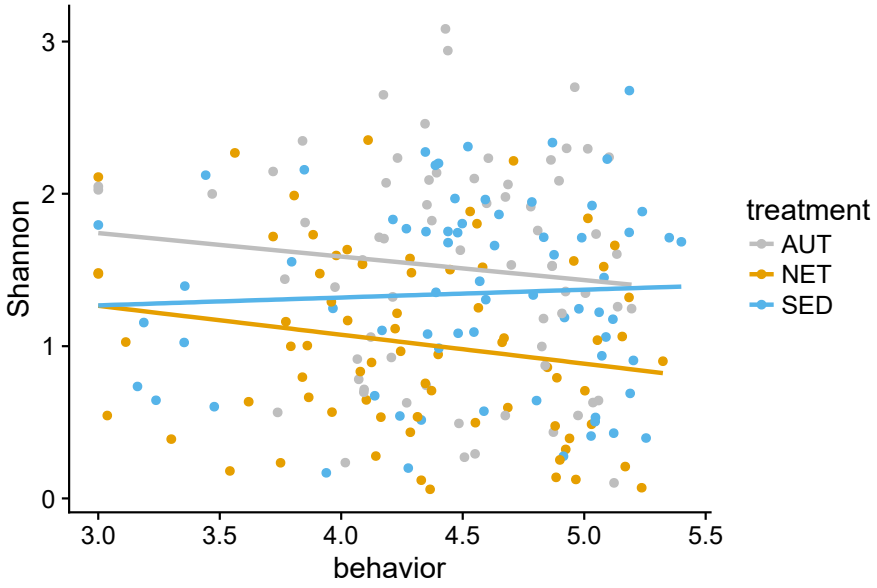
Supplementary Figure 2. Clonal average size and clonal average browsing intensity are uncorrelated.



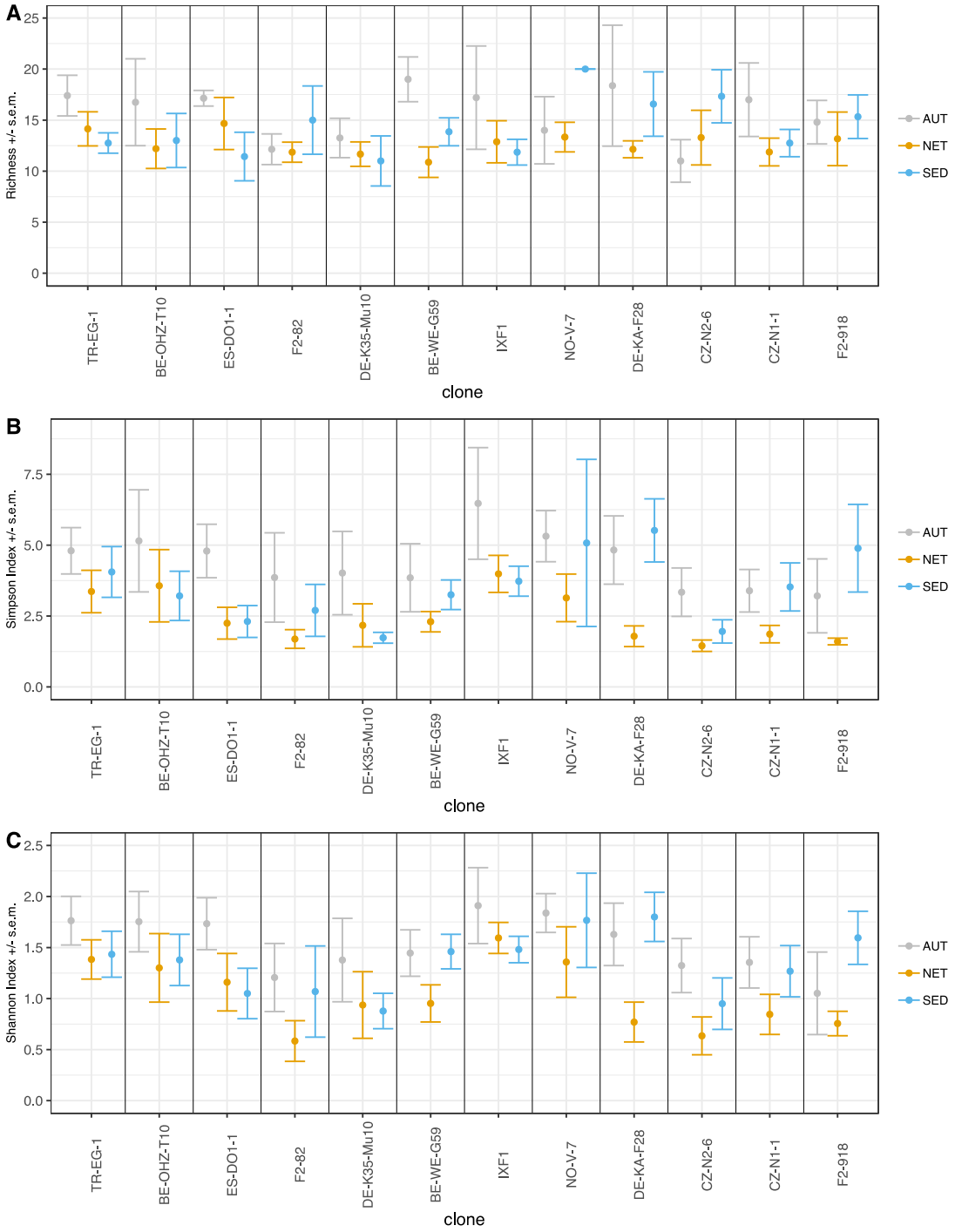
Supplementary Figure 3: Taxonomic trees of OTUs found in sequenced animals, highlighting presence/absence and relative abundance in AUT (A), NET (B) and SED (C) treatment groups. Node size represents the number of samples in the treatment group in which a given taxon is found, whereas node color represents the median relative abundance of the taxon among samples in the treatment group.



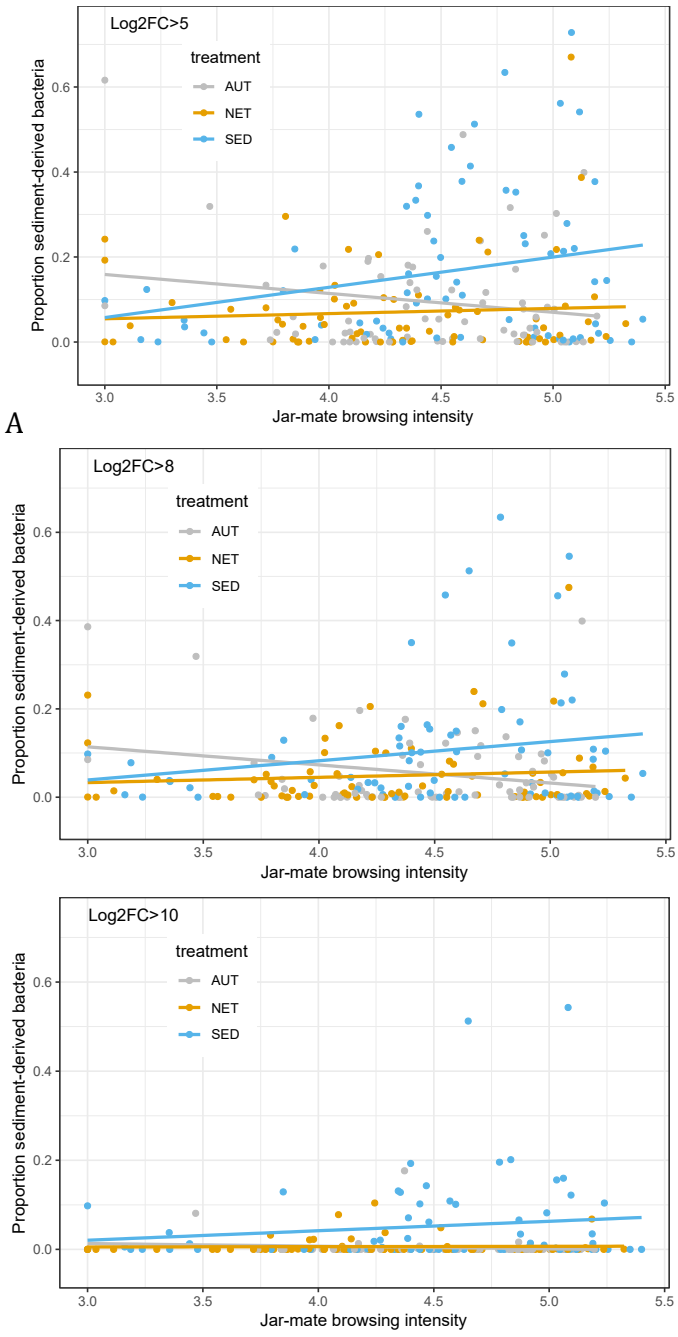
Supplementary Figure 4: Bacterial species richness of autoclaved and untreated sediment. N=6 samples per treatment, 3 each collected at beginning and end of experiment.



Supplementary Figure 6. Shannon diversity index as a function of the browsing intensity calculated for each individual's jar-mate. Lines represent linear regression; shaded area represents 95% confidence interval.



Supplementary Figure 5. Average microbiota diversity by clone and treatment using species richness after rarefying to an even sampling depth (A), inverse Simpson index (B), or Shannon index (C).



Supplementary Figure 7. Relationship between browsing intensity and proportion of sediment-derived bacteria in the microbiota using jar-mate's browsing intensity as behavior proxy and different thresholds for defining sediment-derived bacteria. (A) Sediment-derived bacteria defined by log2-fold change of 5 or more between autoclaved and natural sediment samples (B) Sediment-derived bacteria defined by log2-fold change of 8 or more between autoclaved and natural sediment samples (C) Sediment-derived bacteria defined by log2-fold change of 10 or more between autoclaved and natural sediment samples.

Supplementary Table 1. Effect of treatment (NET or SED) and behavior on Shannon diversity index, using treatment-specific clonal average behavior (A) or jar-mate behavior (B) as behavior proxy.

A. Linear mixed-effects model with clone as random effect

	numDF	denDF	F-value	p-value
(Intercept)	1	124	262.61367	<.0001
treatment	1	124	11.76313	0.0008
trtbehavior	1	124	0.05191	0.8201
cloneavgsize	1	9	2.07427	0.1837
treatment:trtbeh	1	124	4.62296	0.0335
treatment:cloneavgsize	1	124	0.01471	0.9036

trtbehavior=treatment-specific clonal average behavior; cloneavgsize=clonal average body size

B. Analysis of variance

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
treatment	1	3.85	3.845	10.490	0.0015 **
jar-mate behavior	1	0.27	0.267	0.727	0.3953
treatment:behavior	1	0.72	0.722	1.969	0.1628
Residuals	138	50.58	0.367		

Supplementary Table 2. (https://aem.asm.org/content/early/2019/02/06/AEM.01547-18.long). List of bacterial OTUs significantly differentially present between natural and autoclaved sediment samples, as determined by adjusted p-value <0.05 in DESeq analysis implemented in phyloseq. OTUs with a log2foldchange of at least 8 between autoclaved and natural sediment were considered to be "sediment-derived OTUs" in further analyses.

Data availability

Sequence data are deposited in the European Nucleotide Archive of the EBI under accession number PRJEB30308 (http://www.ebi.ac.uk/ena/data/view/PRJEB30308). Data tables, OTU sequences and code used for analysis can be found on Github at https://github.com/amusheg/Daphnia-microbiota-behavior and will be deposited in Dryad upon publication. The data used in this work are provided as supporting information for online publication (https://aem.asm.org/content/early/2019/02/06/AEM.01547-18.long)

Chapter III

An assessment of morphological and functional plasticity in *Daphnia magna* setae in relation to surface feeding

Abstract

The ancestors of *Daphnia* were benthic species that collected food by scraping over substrates using specialized structures on their trunk limbs II. During the invasion of free water habitats, the limb lost its primary function in feeding. However, one seta on the trunk limb II of some *Daphnia* species has been suggested to having retained or secondarily acquired a function in food scraping. Here, we performed experiments where replicate individuals of six clones of *D. magna* were raised in two feeding treatments, namely in the presence of algae in suspension or in the presence of a layer of algae on the bottom of glass jars. The morphology of the seta on trunk limb II was documented from dissected exuviae on multiple subsequent instars of each individual replicate. We did not find a plastic response in setal morphology as induced by the feeding treatments. Therefore, we could not support the hypothesis of the role of the seta in food collection by scraping. However, the seta differed between clones in both environments suggesting a strong genetic component underlying setal morphological variation in *D. magna* and suggesting that selection could act on these traits.

Manuscript in preparation. Arbore R., Vellnow N. and D. Ebert. An assessment of morphological and functional plasticity in *Daphnia magna* setae in relation to surface feeding.

Introduction

At the heart of the arthropod radiation lies an astonishing diversification in limb morphology and function. Limb evolution, from a simple, unjointed ancestral hypothetical lobopoan-like appendix, has resulted in the high variety of complex and greatly specialized structures of extant arthropods (Fryer 1996). Paradigmatic examples are the wings of insects, the legs of spiders and the elaborate mouthparts of crustaceans. Controversies on the monophyletic origin of arthropods are glowing but there is consensus about the series of innovations that gave origin to the general features of the arthropod limb. Cuticular sclerotization has allowed for the evolution of strong, polyramous joined limbs followed by their sub-functionalization to a variety of novel utilities such as locomotion, feeding, sensing and defence. This extreme diversification in function has been achieved not only by means of the possibilities offered by the mechanics of a robust exoskeleton but also, to a large extent, by modifications in the ornamentation of the exoskeleton itself (Ruppert *et al.* 2004).

In all arthropods, the exoskeleton is adorned by a variety of articulated and unarticulated outgrows such as setae, spines and scales. Among them, setae are found in all subphyla and have the greatest functional diversity and importance (Keil 1997). They are elongated, flexible structures mostly found on the appendages and in the head. Often in conjunction, and together with their surface outgrows termed setulae, setae form functional units that serve a variety of purposes. In insects, where they are studied the most, setae are almost exclusively sensory organs (e.g. mechano-, chemo- and thermo-receptors) (Caldwell & Eberl 2002). In other groups, such as spiders and crustacean, setae also have vital mechanical functions in, for example, feeding, locomotion and copulation (Garm & Watling 2013). Comparative analyses in many groups have shown how the morphology of the setae and their mechanical function are highly correlated. Garm and Watling (2013) classify crustacean limb setae into seven morphological types each associated with a particular set of functions, from filtering to prey handling. The functional morphology of setae is, therefore, of great help to understand the natural history of non-insect arthropods.

Cladocerans are small branchipod crustaceans ubiquitous in fresh water habitats from artic to tropical latitudes (Smirnov 2014). Cladocerans have successfully colonized a great variety of habitats from large lakes to smaller water bodies such as ponds and ephemeral pools and even accumulations of water in soil and epiphytic plants. A morphological radiation in the setal apparatus throughout the evolutionary history of the order is tightly linked to this high niche specialization and species differentiation (Smirnov & Kotov 2010). In cladocerans, setae can be broadly classified into *stiff setae* and *soft setae*. Stiff setae are armed by rows of denticles or setulae and are found in different types and modifications on the anterior margin of the limbs. Soft setae bear flexible and elongated spinules and are locate more medially on the limb surface; they also vary in morphology even on the same limb and often form feather-like structures (*plumose setae*). Emancipation of some families from the ancestral benthic habitat, with the acquisition of a planktonic life style and filter feeding, has been accompanied by great modifications in the types and arrangements of these setae on the thoracic limbs (Fryer 1995). The ancestors of the cladocerans were benthic species that collected food by scraping (Fryer 1995, Smirnov & Kotov 2010). Many extant cladocerans (belonging to the families *Chydoridae* and *Macrothricidae*) maintain a close association with the substrata and still collect food by scraping using rows of stiff setae on their trunk limbs II (Smirnov & Kotov 2010). The other limbs are armed with complex assemblages of stiff and soft setae which are not directly involved in food collection and serve other purposes, such as removal of non-edible material from the feeding chamber. This setal apparatus on the trunk limbs functions as a unit and the detailed

morphology and arrangements of the setae are associated to the circumstances in which food is collected in the different species. In contrast, in planktonic filter feeders, such as members of the genera *Daphnia* and *Moina*, food collection is achieved by the next pairs of limbs (trunk limb III and IV) where soft setae are predominant and form a highly sophisticated filtering apparatus (Fryer 1991). The proximal endites of these limbs (gnatobases) are greatly enlarged and bear many soft setae which form a mesh by means of interconnected rows of flexible setulae. Water is pumped by movements of the limbs within a chamber formed by the limbs and the valves of the carapace, while food particles are retained by the filter. Again, the setal apparatus of all trunk limbs forms a highly integrated functional unit enabling efficient feeding in the water column. In pelagic species, stiff setae are heavily reduced in number on the trunk limbs and do not contribute directly to filter feeding. With their high diversity in feeding specializations, including the major ecological transition to pelagic filter feeding, Cladocerans exemplify the potential of setal morphogenesis as a driver to niche specialization and adaptive radiation in arthropods (Smirnov & Kotov 2010).

Pelagic filter feeding has evolved to a high level of sophistication in the genus *Daphnia* (Fryer 1991). This genus diverged from its anomopod ancestor some 120 million years ago and all extant species have acquired suspension filter feeding as a primary mode of food acquisition. Some species are truly planktonic (e.g. *D. cucullata* and *D. galeata*) while others, despite being primarily suspension filter feeders, can exploit benthic food sources (e.g. *D. magna*, *D. obtusa*, *D. pulex*) (Fryer 1991). Some species can feed on sediment particles that are stirred up by movements of the second antennae and then filtered (Horton *et al.* 1979, Chapters I, II and IV of this thesis). In his monograph about functional morphology of the genus, Fryer (1991) highlights how some species might be able to collect material from surfaces by scraping in a way similar to that of many benthic cladocerans. *D. magna* is equipped with a single stiff seta on its trunk limb II (Figure 1). The seta is located on a part of the limb (the endopodite) that is projected frontally in correspondence of the carapace mid-line towards which it can swing. On its distal portion, the seta is armed by a single row of strong spinules which become smaller and more tightly spaced towards the middle of the seta. *D. magna* sometimes glide on its carapace margins parallel to surfaces and rapidly moves the limbs, thus generating a propelling current;

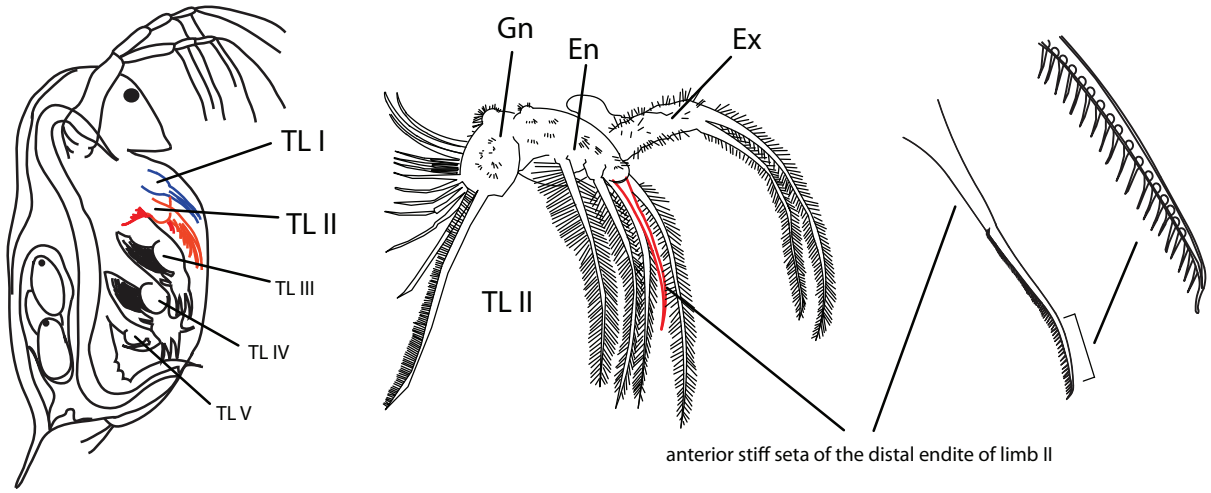


Figure 1: Size and position of the stiff seta on trunk limb II in *Daphnia magna*. Left: relative position of trunk limbs I-V in an adult female; modified from (Ebert 2005). Center: Trunk limb II; Gn: gnatobase, En: endopodite, Ex: exopodite. Right: the stiff seta on the distal endite of the endopodite and details of its distal portion armed with strong spinulae.

particles are filtered from the water current probably with the aid of the scraping action of the seta. Indeed, in laboratory conditions, *D. magna* is able to grow and reproduce when feeding in this way on a layer of green algae deposited on the bottom of glass jars (Siehoff *et al.* 2009). In nature, surface feeding might be induced by limiting food conditions in the water column (Rautio & Vincent 2006). Moreover, this might expose the animals to different food items then generally found in solution. Phenotypic plasticity in the setal apparatus of *D. magna* enables morphological responses to the quantity and quality of food available in suspension by altering the shape and mesh size of the gnatobasic filters (Lampert 1994). However, plastic responses to food conditions in the setal equipment in relation to surface feeding have never been investigated.

Here, we performed laboratory experiments where individual replicates of *D. magna* clones were raised either in standard feeding conditions with algae in solution or in the presence of only algae that formed a layer on the bottom of glass jars. In the experiments, we analysed growth of body size and morphology of the stiff seta of trunk limbs II (and I) along subsequent instars of the individual replicates from their exuviae (i.e. the released exoskeleton after moulting). We did not observe significant differential growth or changes in morphology of the setae between treatments. Not surprisingly, the biggest differences between treatments were found in body size of the animals. Size differences mainly resulted from qualitative differences in food between treatments but the magnitude of this difference was influenced by the way the food was presented to the animals. Overall, our study did not support the feeding role and plasticity of the stiff seta on trunk limb II. However, we found differences in setal morphology between clones upon which we elaborated in a subsequent analysis (Chapter IV of this thesis).

Materials and methods

Experimental animals

In this study, we used six *D. magna* clones (e.g. clonal lines) from stock cultures belonging to a large collection sampled throughout the geographic range of the species and propagated asexually in the laboratory (the *Daphnia magna* Diversity Panel). The clones were originally sampled in ponds in Belgium (2 clones), Czeck Republic (1), Russia (1), and Switzerland (1) and from a lake in Turkey (1) (Table S1). We chose clones from distant sides to maximize genetic variation for trunk limb morphology, growth and plasticity. Prior to the experiments, individual replicate female lines of all six clones were propagated asexually in standardized conditions for three generations. The animals were kept in 100-ml glass jars filled with 80 ml of *Daphnia* medium (Klüttgen *et al.* 1994) in randomized positions in an incubator at 20 °C with a 16:8 light/dark cycle. The animals were isolated from their clutches when four-day old, transferred to fresh medium when twelve-day old and then transferred every three to four days when they released a new clutch. The animals were fed daily with increasing amounts of *Scenedesmum sp*. algal cells: 1 x 10^6 until day five, 2 x 10^6 until day eight, 2.5 x 10^6 until day ten, 3 x 10^6 until day twelve and 5 x 10^6 afterwards. Offspring from the third or fourth clutches were used to establish each new generation and for experiment 1.

Experiment 1

At the beginning of experiment 1, four-day old animals were fed daily with increasing amount of suspended algae until they reached sexual maturity (1st adult instar). From then on, ten siblings

for each of two females per clone were randomly allocated in each of two treatments (split brood design; 6 clones x 2 mothers x 5 siblings x 2 treatments = 120 animals). In the control treatment, the animals were transferred every day to new jars and 5 x 10^6 of fresh algae were added in suspension to the medium. In the other treatment, the animals were transferred every day in jars previously prepared with 5 x 10^6 algae settled on the bottom. The settled algae were prepared by adding fresh algae to jars filled with medium and leaving the jars untouched for four days in the same incubator where the experiment took place. The position of the animals in the incubators was randomized in order to minimize possibly confounding microenvironmental effects. Exuviae of the first eight adult instars were collected and dissected daily for a period of 30 days as described below. During the experiment, some animals died or the dissections failed and, at the end, we documented 591 instars with a mean of 6 replicates per clone per treatment for each instar. Offspring from late clutches of the animals used here (7^{th}, 8^{th} or 9^{th} clutches) were isolated after birth and used in experiment 2.

Experiment 2

In experiment 2, we allocated replicate individuals from five clones to the same treatments as their mothers in experiment 1 (one clone was excluded because not enough replicates were available). We included one offspring per mother and on average 6 replicates per treatment per clone. Here, the treatments were applied within 24 hours from when the animals were released from the brood pouch. From then on, the animals were transferred every day to new jars with either suspended or settled algae prepared as before. Algae amounts in each treatment were increased regularly to accommodate the growing food requirements of the animals: 1 x 10^6 until day five, 2 x 10^6 until day eight, 2.5 x 10^6 until day ten, 3 x 10^6 until day twelve and 5 x 10^6 afterwards. Of these animals, we dissected the exuviae of the second and the fourth pre-reproductive instars (hereafter 2^{nd} and 4^{th} instar) and of the first three adult instars (hereafter 6^{th}, 7^{th} or 8^{th} instars). Overall, we documented 239 exuviae collected over 28 days with a mean of 4.8 replicates per clone per treatment for each instar stage.

Experiment 3

In this experiment, we only measured body size of one clone (BE-WE-59) grown in four different treatments. In the control treatment (C), we fed the animals daily with fresh *Scenedesmus* algae added in suspension to the medium. In the other treatments, we fed the animals every day with 3-days aged food that was either resuspended right before the animals were introduced into the jars (R) or settled on the bottom of the jars as in experiment 1 and 2 (S). In addition, another group of animals received resuspended aged algae until they laid their 1^{st} clutch and then received settled aged algae (RS). At the beginning, eight individual females were isolated from stock cultures and raised until they laid at least two clutches. Then, on average, 10 female offspring of one clutch for each female (mother) were distributed across the treatments when 2-day old. Afterwards, the animals were transferred daily to new jars within their treatments with the same increased feeding schedule as before. Of these animals, we measured the body size when four-days old and after they laid their 4^{th} clutches, using a dissecting microscope (body size was measured from the base of the spine and the top of the head). In total we measured 11 control animals (C), 25 animals that received resuspended algae (R), 24 animals that received settled algae during the entire experiment (S) and 23 animals that received settled algae after maturation (RS).

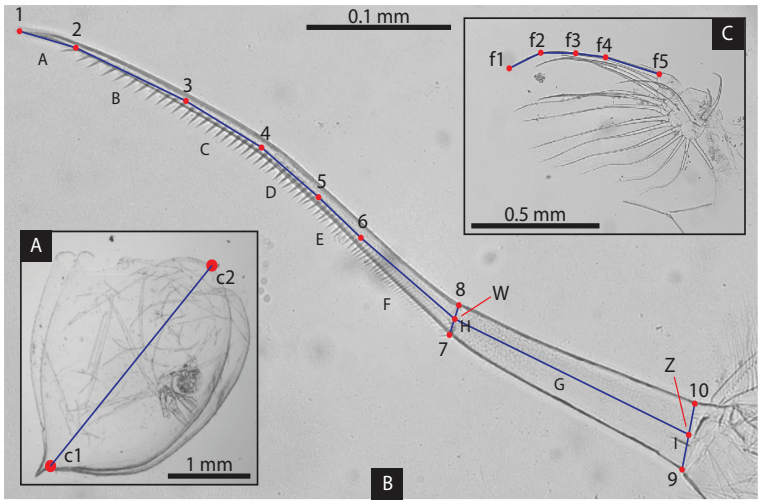


Figure 2: Photographs of the exuvia of an adult *D. magna* female and landmarks for the morphometric analysis in experiments 1 and 2. A: Body size was measured as the distance from the base of the carapax spine (c1) and the top of the head of the animals (c2). B: Armature of the stiff seta of trunk limb II: ten landmarks were placed on the seta (1-10) and two semi-landmarks were defined as the midpoint between landmarks 7 and 8 (W) and between landmarks 9 and 10 (Z). Length of the seta was measured as the sum of segments A-G. Thickness of the seta was measured as the mean length of segments H and I. C: Armature of trunk limb I and landmarks on the long stiff seta of the exopodite (f1-f5).

Morphological analyses

For dissection, the exuviae of recently moulted animals (<24 h) collected in experiment 1 and 2 were rinsed briefly in ADaM and transferred individually to a microscope slide with a glass pipette. We performed the dissections using two thin metal needles under a dissecting microscope with dark field illumination. First, by pulling the second antennae apart from the carapax, we separated the two carapax valves from the exuvia. The residual exoskeleton, including the armature of the trunk limbs and the post abdomen, remained attached to the second antennae. The setae of limb II were spread out with attention on the slide in order to avoid the overlapping with the stiff seta (anterior seta of the distal endite of limb II) and the gnatobase. A glass cover slip, covering the carapax valves and the trunk limbs I and II, was then gently placed on the specimen, liquid in excess was removed with filter paper to make it adhere, and its sides were sealed with nail polish to avoid dehydration. The specimens were kept covered in plastic boxes with water-soaked paper towels before being photographed. For each specimen, we documented the carapax, the stiff seta of the distal endite of trunk limb II and the stiff seta on the exopodite of trunk limb I. For the morphometric analyses, we defined fixed landmarks on the carapax and the setae and measured their coordinates for each specimen using the software ImageJ (Figure 2). The size of the animals was estimated as the Cartesian distance between the base of the carapax spine and the distal margin of one of the carapax valves (Figure 2A). Ten landmarks were defined on the stiff seta of trunk limb II (Figure 2B): one (1) at the extremity of the tip, one (2) at the emergence of the first spinulae, four (3-6) at the base each 10th subsequent spinules, two (7, 8) at both sides of the end of the portion of the armature bearing spinules and two (9, 10) at both sides of the site of emergence of the seta from the endite (the exact position of emergence on the side opposite from the raw of spinules was often uncertain; landmark 10 was therefore instead placed in its proximity where the armature consistently displays a minute crease). Two semi-landmarks (W and Z) were then defined as the midpoints between landmarks 7 and 8 and between landmarks 9 and 10 respectively. Cartesian distances of the segments between adjacent landmarks and semi-landmarks were then calculated. The total length of the seta was measured as the sum of segments A to G and its width as the mean of segments H and I. Five landmarks were placed on the stiff seta of trunk limb I, from its tip to the emergence of the base from the exopodite (Figure 2C). Specimen where the carapax or the setae revealed to be damaged or folded or where landmarks could not be placed were removed from the analysis. In the first experiment, we estimated body size based on 507 exuviae, trunk limb II stiff seta length and thickness on 512 exuviae and trunk limb I stiff seta on 400 exuviae. In the second experiment we measured 276 exuviae for body size, 248 exuviae for trunk limb II seta length and thickness and 217 exuviae for trunk limb I seta length.

Statistical analyses

Statistical analyses were conducted in R (ver. 3.2.4; R Development Core Team, 2008). For experiments 1 and 2, the effects of treatment and clone on the different measurements were first analysed with a linear model within single instar stages (TableS2). These were: 1 to 8 adult instars in experiment 1 and 2 to 8 pre- or post-maturation instars in experiment 2. In early and late instars, we sometimes did not have animals for each clone by treatment combination and these clones were therefore excluded from the within instar analyses. Due to the difficulties of working with exuviae, our dataset was relatively unbalanced, with a minority of animals being documented at each instar stage. Linear mixed models are robust in this regard and easily deal with longitudinal studies where multiple scattered measurements are taken on the same individuals. Growth curves were analysed within a linear mixed model framework to estimate the effect of treatment over consecutive instars. In general, growth was best modelled by a cubic polynomial function of size over subsequent instars (considering lower and higher degree polynomials and logistic functions did not improve curve fitting). For every measurement in every experiment (e.g. body size), we constructed a linear mixed model with linear, quadratic and cubic terms of instar stage number as fixed effects (e.g. 1-8 in experiment 1). We used the poly function in R to compute orthogonal polynomials for the series of instars and to avoid correlation between polynomial terms. Orthogonal polynomials are centred at zero and, consequently, the treatment effects (shown in Table S3) were estimated at the middle time point of the experiment, between the 4^{th} and 5^{th} instars in experiment 1 and at the 5^{th} (unmeasured) instar in experiment 2. For every polynomial term, we included its interaction with treatment as a fixed effect. A significant interaction would indicate differential growth speed (linear term) or differences in shape (quadratic and cubic terms) of the growth curves of the two treatments. In all models, clone was considered a random effect and each individual slope was allowed to vary (i.e. random intercept and slope for individual female). In experiment 2, some polynomial terms were not significant, nor were their interactions with treatment, and we removed them from the models (Table S3B). The linear mixed models were performed using the R package Lme4 and the statistical significance of the fixed effect was estimated with Type III F tests with the function anova in the R package lmerTest (Kenward-Roger's approximation for denominator degrees of freedom). In the same way, we evaluated clonal effects on size measurements along the experiments with linear mixed models with treatment, clone and instar (as a categorical variable) as fixed effects and individual as random effect. A linear model with treatment and mother ID as fixed effects was used to analyse the results of experiment 3 (on average 10 female offspring of one clutch for each female, the "mother", were distributed across the treatments: split brood design). Tukey's HSD test p values for the contrast between treatments were calculated with the lsmeans package in R.

Results

Animals' growth

Among all measurements, body size of the animals was the most affected by feeding treatment in experiment 1 and 2. In the first experiment, the animals were allocated to the feeding treatments after they laid their first clutch, when the clones did not differ in average body size (Table S2). At the end of the experiment, when we measured the 8th adult instar exuviae, we found significant differences between clones and between treatments (Table S2). For every clone, the animals that were fed settled algae were bigger than animals fed with food in suspension (maximum difference 0.273 mm). Growth curves of the animals for the two treatments are shown in Figure 3A. Growth of the animals was modelled by a cubic polynomial function of size over subsequent instars (R^2 = 0.77; Table S3). We found a significant difference between treatments only in the linear term of the model ($F_{1, 95.441}$= 15.7, p = 0.0001). The speed of growth was therefore higher when the animals had to collect algae from the bottom but the overall shape of the curves did not differ between treatments. The treatment effect was estimated at the middle of the experiment where the size of the animals already diverged (treatment: $F_{1, 93.253}$ = 40.0, p < 0.0001). Clonal effects on size along the entire experiment remained highly significant (clone: $F_{5, 90.83}$ =37.26, p < 0.0001; Table 1).

Table 1: Results of the mixed models for body size and setal morphology in experiment 1 (A) and 2 (B).

A	Experiment 1 (maternal generation)		
	Fixed effects	F_{df}	p
Body Size	Treat.	$40_{1,91.43}$	< 0.0001 ***
	Clone	$8.89_{5,91.33}$	< 0.0001 ***
	Instar	$491_{7,421.39}$	< 0.0001 ***
Seta length (TL II)	Treat.	$18.1_{1,93.48}$	< 0.0001 ***
	Clone	$24.1_{5,93.79}$	< 0.0001 ***
	Instar	$998.8_{7,419.46}$	< 0.0001 ***
Seta thickness (TL II)	Treat.	$12.7_{1, 90.27}$	0.0005 ***
	Clone	$29.4_{5,29.4}$	< 0.0001 ***
	Instar	$129.3_{7,439.3}$	< 0.0001 ***
Seta length (LT I)	Treat.	$16.19_{1,90.66}$	0.0001 ***
	Clone	$37.26_{5,90.8}$	< 0.0001 ***
	Instar	$553.58_{7,318.8}$	< 0.0001 ***

B	Experiment 2 (offspring generation)		
	Fixed effects	$F_{(df)}$	p
Body Size	Treat.	$3.79_{1, 57.8}$	0.0056 **
	Clone	$8.51_{4, 58.3}$	< 0.0001 ***
	Instar	$799.27_{4, 175.7}$	< 0.0001 ***
Seta length (TL II)	Treat.	$0_{1,58.2}$	0.9487
	Clone	$14.35_{4,58.7}$	< 0.0001 ***
	Instar	$771.23_{4,158.94}$	< 0.0001 ***
Seta thickness (TL II)	Treat.	$3.01_{1,57.5}$	0.08783
	Clone	$3.01_{4,53.28}$	< 0.0001 ***
	Instar	$131.4_{3,181.9}$	< 0.0001 ***
Seta length (TL I)	Treat.	$0.48_{1,57.6}$	0.48958
	Clone	$6.1_{4,54.9}$	0.0003 ***
	Instar	$170.5_{3,153.1}$	< 0.0001 ***

In the second experiment, the animals were allocated from birth to the same food treatments of their mothers. When the animals moulted for the second time, no differences in size between treatments and clones were found (Table S2). As before, the animals that had to collect algae from the bottom grew slightly bigger (Figure 3B). These animals grew linearly from birth to the third adult instar stage, while animals fed with suspended food grew slower at the beginning of the experiment. However, when the last instars were measured (3rd adult instar) differences in size were found between clones but not between treatments (Table S2). The growth curves of the animals showed a significant difference in the cubic term of the polynomial model ($F_{1, 126.470}$= 5.52, p = 0.02, Table S3), and differences between treatments were found in the middle of the experiment where the effect was estimated (Table S3). As before, clones differed in size throughout the experiment (Table 1B).

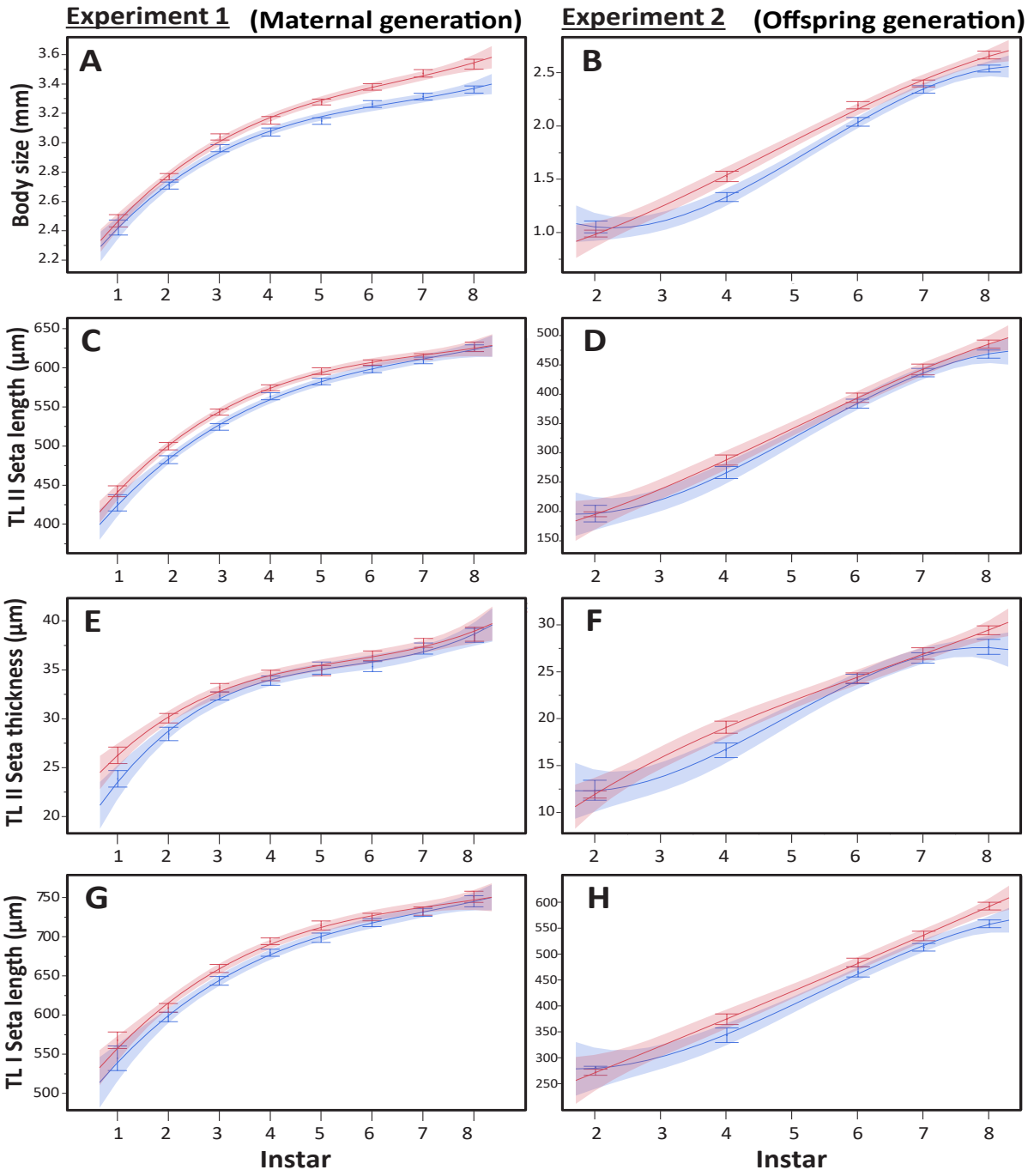


Figure 3: Growth of the animals and of the setae of trunk limbs II and I along subsequent instars in two feeding treatments (experiments 1 and 2). Red: settled algae treatment. Blue: control animals fed with algae in suspension. In experiment 1, we measured replicate individuals of six clones at eight subsequent adult instar stages. In experiment 2, we measured the 2nd and the 4th pre-reproductive instars and the first three adult instars (6-8) of replicate individuals of five clones. Body size (A-B); length (C-D) and thickness (E-F) of the stiff seta on the distal endite of trunk limb II; G-H: length of the stiff seta of the exopodite of trunk limb I.

In the third experiment, control animals (C) were considerably smaller than animals that received aged food (R, RS and S) (Figure 4). However, differences were also found between the aged-food treatments, indicating how the way the animals collect food might affect their size (the results are reported in Table 2). The animals fed with resuspended algae during the whole experiment (R) were bigger than in the other aged food treatments. The animals that had to collect food from the bottom (R and RS) were significantly bigger than the control animals.

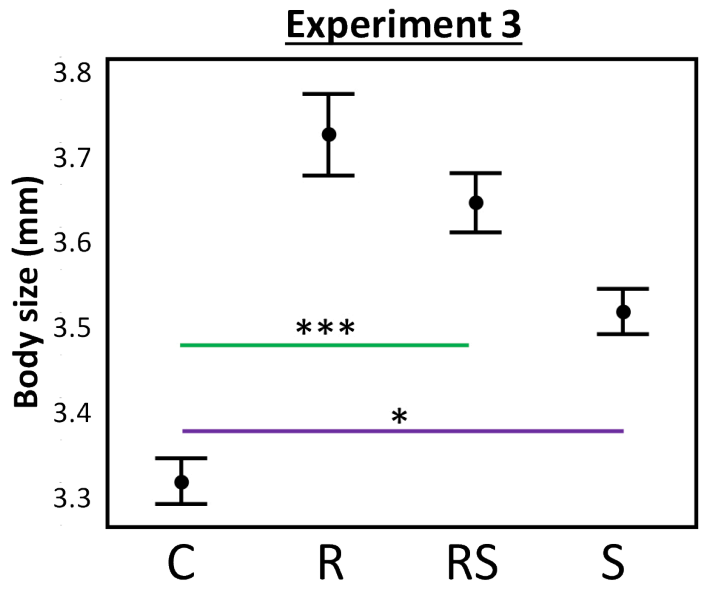


Figure 4: Body size of adult *D. magna* females (4th adult instars) raised in four feeding treatments from birth. In the control treatment (C), the animals were fed fresh algae in suspension. In the resuspended algae treatment (R), the animals received 3-day aged algae in suspension. In another treatment (RS), the animals received 3-day aged algae in suspension until they reached sexual maturity and then aged algae settled on the bottom of glass jars. In the settled algae treatment (S), the animals received 3-day aged algae settled on the bottom of glass jars for the entire experiment.

These differences correspond to those found in the previous experiments, i.e. control animals vs. animals that received settled algae after reaching adulthood (RS) for the first experiment and control animals vs. animals that always received settled food for the second experiment (S). Overall, these results suggest that while food quality affected body size in our previous experiments, this seems probably also modulated by the way the animals had to collect food. In experiment 1 and 2, the bigger size of the animals fed with settled algae certainly resulted from differences in food quality between treatments but the magnitude (and direction) of the size differences we observed was also affected by the way food was presented to the animals (Figure 4).

Trunk limb II analysis

At the end of two experiments 1 and 2, we did not find differences in stiff seta length between treatments (Figure 3C-D, Table S2). However, we found significant differences between clones along the entire experiments (Table 1). In the first experiment, the growth rate of the seta slowed down over subsequent moults but did not differ statistically between treatments (Table S3). In the second experiment, we found no difference in the trajectories of the growth curves of the seta for the two treatments (Table S3). Similar results were found for seta thickness: at the end of the experiments, we found differences in thickness between clones but not between treatments and the growth trajectories along the experiments did not differ statistically between treatments (Table S2 and S3).

Trunk limb I analysis

In the two experiments, we also measured the length of second stiff seta of the exopodite of trunk limb I (Figure 2C) to test if the response to the treatments was the same for trunk limb I and II. However, no substantial differences were found between the growth of the stiff setae on trunk limb I and II. As before, the seta was overall longer in the settled food treatment, but no differences between the growth curves were supported statistically (Table S2 and S3). As for trunk limb II, we found clonal effects over the entire experiments (Table 1 and Table S2).

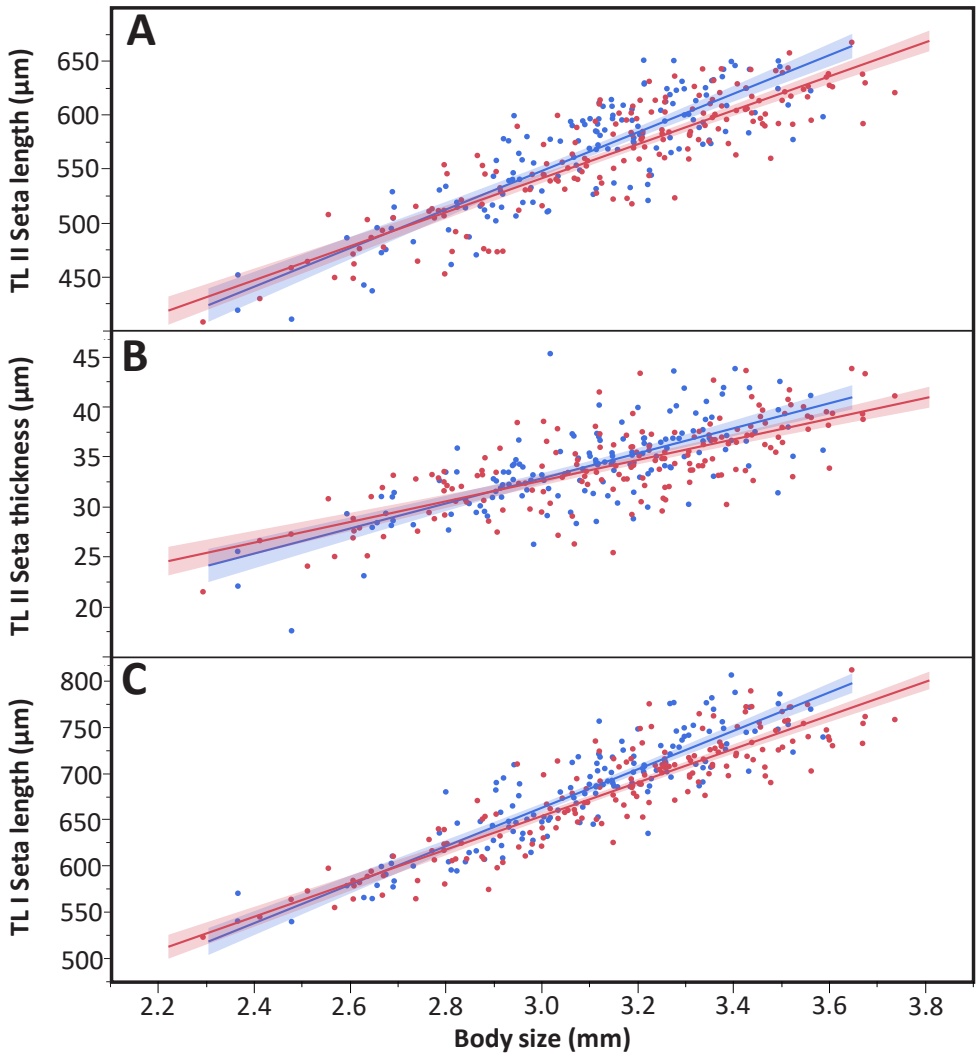


Figure 5: Allometric relationships between body size and size of the setae of trunk limbs II and I of animals raised in two feeding treatments (experiment 1). Red: settled algae treatment. Blue: control animals fed with algae in suspension. A-B: Length and thickness of the stiff seta on the distal endite of trunk limb II. C: length of the stiff seta of the exopodite of trunk limb I.

Table 2: Results of Experiment 3. A: Linear model with treatment and mother ID as fixed effects. On average 10 female offspring of one clutch for each female (Mother ID) were distributed across the treatments (split brood design). **B: Results of the Tukey's HSD test for the contrast between treatments.** C: control treatment (the animals were fresh algae in suspension). R: resuspended algae treatment (the animals received 3-day aged algae in solution). RS: resuspended/settled algae treatment (the animals received 3-day aged algae in suspension until they reached sexual maturity and then settled 3-day aged algae. S: settled algae treatment (the animals received 3-day aged algae settled on the bottom of glass jars for the entire experiment.

A)	Fixed effects	F	Den d.f.	p
Linear model	Treat.	15.2	3.72	<0.0001 ***
	Mother ID	2.9	7.72	<0.009 **
B)	**Contrast**	**T**	**d.f.**	**p**
Tukey's HSD test	C - R	-6.096	72	<0.0001 ***
	C - RS	-4.955	72	<0.0001 ***
	C - S	-2.779	72	0.034 *
	R - RS	1.428	72	0.486
	R - S	4.299	72	0.0003 ***
	RS - S	2.735	72	0.038 *

Allometry

The relationship between length of the setae (TL II and I) and body size for experiment 1 are shown in Figure 5. Overall, animals in the settled algae treatment had slightly shorter setae, compared to body size, than control animals. This difference increased with body size due to differential growth of the animals in the two treatments. No differences were found between treatments for seta thickness on TL II. We also analysed if the setae on the two trunk limbs grew differently along subsequent instars. In experiment 1, the relative length of each seta (on TL II and TL I) remained similar between treatments until the 4th adult instar. For the last four instars, relative seta length of both limbs remained constant in the control animals and decreased continuously in the settled algae treatment (Figure 6). However, this difference is clearly driven by differential growth in body size of the animals in the two treatments while setae length remained constant.

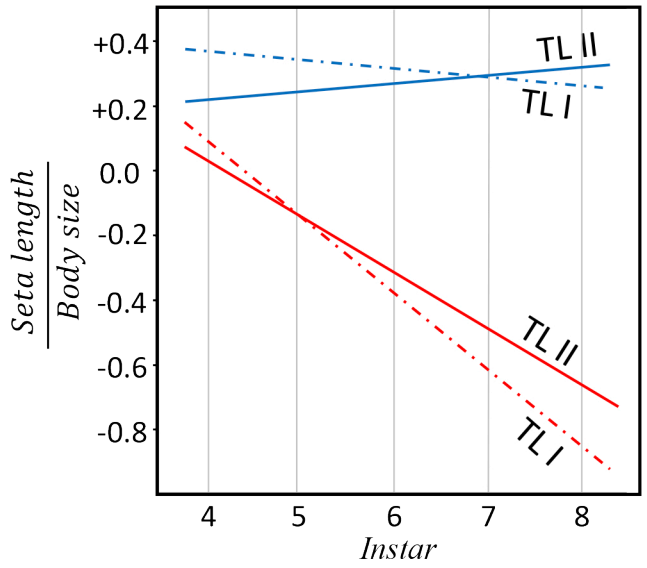


Figure 6: Differential growth between treatments of the stiff setae on trunk limb II and I relative to body size. Red: settled algae treatment. Blue: control animals fed with algae in suspension. In experiment 1, the relative lengths of both setae diverged between treatments after the animals reached the 4th adult instar stage. Length of the setae and body size were previously scaled (Z transformed) in order to compare their rate of growth.

Discussion

Here, we studied a hitherto poorly investigated aspect of the feeding biology of *D. magna*, that is its retention (or secondary acquisition) of scraping habits in a species that otherwise fully evolved planktonic filter feeding. Little is known about the impact of surface feeding on the physiology, life history, and ecology of this species, nor of any other species of the genus. A handful of publications have touched this subject and can be summarised into the conclusions that some species of *Daphnia* (i.e. *D. magna*, *D. pulex* and *D. middendorffiana*) can engage in surface feeding, normally switch to such a strategy when suspended food is scarce and that surface feeding can ensure individual and population growth and reproduction (Horton *et al.* 1979, Fryer 1991, Rautio & Vincent 2006, Siehoff *et al.* 2009, Cazzanelli *et al.* 2012). Overall, our results confirmed these findings for *D. magna* as, in our experiments, the absence of suspended food could be fully compensated by surface feeding and the animals fed with deposited algae were able to grow and reproduce.

Interestingly, in Experiment 1 and 2, the animals fed with deposited algae grew faster and bigger than the animals fed with suspended algae. However, experiment 3 revealed that such an effect in the previous experiments was likely due to a qualitative difference between the two food treatments. The deposited food in experiment 1 and 2 has been aged during the time necessary for its settling on the bottom of the experimental jars (3 days). It appears that the changes in the food occurring during this period resulted in food of better quality enabling faster growth and bigger size of the animals at the end of the experiment. While, most likely, the algae could reproduce during this time, the animals were fed with an excess of food in both treatments; therefore, it is unlikely that differences in food quantity between the treatments can explain our results. Qualitative changes between the treatments could be related, for example, to bacteria growing in the food or partial degradation of the algae facilitating processing and digestion by the animals. *D. magna* can feed on bacteria and animals fed on bacteria-enriched algal diets (e.g. 80% *Scenedesmus obliquus* and 20% *Escherichia coli*) have been shown to perform better in terms of growth and reproduction than animals fed with algae alone (Freese & Martin-Creuzburg 2012). These findings are in accordance with our results and might explain the unexpected differences we found between our treatments. However, the results of experiment 3 suggest that while food quality affected body size in our previous experiments, this effect seems also modulated by the way the animals had to collect food. In experiment 3, the animals fed with aged resuspended algae grew bigger than the animals for all other treatments; the animals fed with aged algae in suspension until they reached sexual maturity and then aged settled algae and animals receiving settled aged algae for the entire experiment grew at intermediate values. Finally, controls animals that only received fresh algae in suspension were the smallest at the end of the experiment. Therefore, in experiment 1 and 2, the bigger size of the animals fed with settled algae certainly resulted from differences in food quality between treatments, but the magnitude of the size differences was also affected by the way food was presented to the animals. With our data, we are not able to elucidate whether the smaller size of the animals that had to scrape, compared to the size of the animals that could filter food of the same quality from the water, resulted from a less efficient food ingestion or from the higher energetic expenditure possibly associated with scraping. The clear cost of scraping that we observed most likely explains the strategy adopted by some *Daphnia* species to only engage in surface feeding when the food conditions in the water column deteriorate (Horton *et al.* 1979). However, certain benefits resulting from bottom feeding as, for example, the access to benthic complements of the diet might influence the feeding biology of *Daphnia* and deserve further attention (Siehoff *et al.* 2009, Cazzanelli *et al.* 2012).

Several life history, behavioural and morphological plastic responses to environmental clues have been documented in *Daphnia* species (e.g. Boersma *et al.* 1998, Riessen 1999). Morphological alterations can represent adaptations to vertebrate (e.g. Tollrian 1994) and invertebrate (e.g. Rabus & Laforsch 2011) predation and to variations in feeding conditions. A few studies have investigated the changes in the morphology of the trunk limbs of *Daphnia* in relation to food quality and quantity (e.g. Pop 1991, Lampert 1994, Lampert, & Brendelberger, 1996, Macháček & Seda 2013, Wejnerowski *et al.* 2017). These changes include the alteration of the area of the filtering fan, the number and morphology of filtering setae and the mesh size of the filter. For example, daphniids can adapt to low food conditions by increasing their filtering screen area (Lampert 1994) and can adapt to high levels of toxic of inedible food (e.g. filamentous cyanobacteria) by reducing the mesh size of the filters and by increasing the thickness of the filtering setae (Wejnerowski *et al.* 2017). While these studies have highlighted the high plastic potential of the setal apparatus *Daphnia* thoracic limbs and its role for the species' feeding biology, they have focused exclusively on the gnatobasic filter system of limbs III and IV. This

is not surprising as planktonic filtering is undoubtedly the main feeding mechanism in *Daphnia*. Nevertheless, as surface feeding might represent a relevant, albeit complementary, strategy for species as *D. magna* (Siehoff *et al.* 2009), a lack of attention in plasticity studies to other trunk limbs possibly involved in food acquisition is unfortunate. Here, building on the known plastic potential of *D. magna* limbs (e.g. Lampert 1994, Lampert and Brendelberger 1996, Wejnerowski et al. 2017), the considerations of Fryer about the species' benthic habits (Fryer 1991) and to broaden our understanding *Daphnia* feeding biology, we aimed at experimentally inducing a plastic response in the stiff seta of trunk limb II of *D. magna*. This structure has been proposed to serve as a scraper for surface feeding (Fryer 1991) and a plastic response to our treatments would have provided indirect evidence of the involvement of the seta in collecting food by scraping from submerged surfaces. To our knowledge, our study is the first explicitly focusing on its role in food collection, its plastic potential and its morphological variation among clones.

In our experiments, we could not observe a plastic response of the stiff seta on trunk limb II to our feeding treatments. Our allometric analysis highlighted how body size was the most relevant measure affected by our feeding treatments. However, we relied on a simple system to provide the animals with deposited food which might have not be enough or suitable to induce a response. The algae were presented to the animals as a thin layer resulting from the settling of the algae for three days. Although the layer was compact and seemed resistant to perturbations, it is possible that it was soft enough to be handled without requiring any specific alteration of the seta as we hypothesised. In a study specifically focusing on the effects of surface feeding on *D. magna* populations (Siehoff *et al.* 2009), the authors fed daphniids in the laboratory with a layer of periphyton (e.g. complex community of diatoms, green algae, cyanobacteria and filamentous bacteria) previously reared for two months on artificial substrates in an aquatic outdoor mesocosm. It is possible that, by using a more natural benthic food source as in Siehoff *et al.* 2009, a plastic response in the stiff seta of trunk limb II could be induced, thereby supporting the hypothesis of its role in scraping food from submerged surfaces.

Our study spanned two generations and the experimental animals were repeatedly exposed to changes in the food treatments so that the animals were to feed on deposited algae in different ontogenetic stages. Previous works on the plastic responses of the setae of trunk limb III and IV of *Daphnia* to feeding conditions have highlighted how, for some morphological parameters, as filtering setae length, such responses can be rapid and can occur within the lifespan of one individual (e.g. Pop 1991). In experiment 1, the animals were raised in normal conditions (i.e. food in suspension) until they reached adulthood and then were allocated to the treatments. This experiment was designed to observe a possible rapid response in adults and thereby testing the hypothesis that individuals might adapt morphologically to current shortage of suspended food. A rapid behavioural response to this feeding regime has been reported in *Daphnia*, although in this study the animals where given the choice to feed on sediments (Horton *et al.* 1979). The animals of the second experiment were exposed during their entire ontogeny to the different food treatments. Having measured the animals from their first instars, we cannot support the hypothesis that feeding conditions early in life alter the development of the seta on trunk limb II. A recent study (Macháček & Seda 2013) found that filtering setae number in *D. galeata* is fixed for an individual, is established during embryonic development and that temporal variations in a clonal population can only be found as a transgenerational response. Interestingly, the authors also found that temperature is the main determinant of filtering setae number in this species. Recent work on morphological plasticity of the filtering apparatus (Macháček & Seda 2013, Macháček & Seda 2016) has highlighted the importance of experiments that follow the growth of individual animals and include more than one generation, in order to understand the

mechanisms behind trunk limb plasticity in *Daphnia*. The method we adopted, the analysis of morphological parameters from the exuviae of animals that can remain in the experiment, had been introduced by Pop (Pop 1991). However, in this study, the author could follow only few individual replicates. After, the method has been reported only in two recent studies (Macháček & Seda 2013, Macháček & Seda 2016). Future studies on morphological variation in cladocerans should take advantage of this approach. Moreover, images of the setal apparatus of trunk limb I and II can be taken easily as the stiff setae are more resistant then the gnatobasic setae of the other limbs. This method might therefore be a useful tool for further studies expanding the focus to the entire thoracic limb apparatus of *Daphnia*.

Conclusions

Our experiments, albeit explorative, represent the first attempt to investigate plasticity in the second trunk limb of a *Daphnia* species; we deliberately focused on the dynamic measure of morphological change, by following individual animals over their lives, in order to increase our chances to detect an effect of our feeding treatments and to possibly understand its underlying mechanisms. Due to experimental constrains we could not include more than six clones in the present study. However, the seta differed between clones in both treatments suggesting a genetic component underlying setal morphological variation in *D. magna* and providing the bases for further studies (Chapter IV and V of this thesis).

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Table S1: Sampling locations and original habitat type of the six *D. magna* clones used in this study.

Clone ID	Country	Latitude	Longitude	Habitat
BE-OHZ-T10	Belgium	50°50'00"N	4°39'00"E	Pond
CZ-N1-1	Czech Rep.	48°46'31"N	16°43'24"E	Pond
TR-EG-1	Turkey	39°49'25"N	32°49'50"E	Lake
BE-WE-G59	Belgium	51°04'04"N	3°46'25"E	Pond
CH-H-1	Switzerland	43°38'19"N	8°51'46"E	Pond
RU-B-51	Russia	49°59'11"N	46°41'34"E	Pond

Table S3: Results of the mixed models for growth of body size and seta on trunk limb I and II in experiment 1 (A) and 2 (B). In the models, treatment, instar (linear, quadratic and cubic orthogonal polynomial terms) and interaction between instar polynomial terms and treatment were fixed effects (random effect: clone).

A — **Experiment 1 (maternal generation)**

	Fixed effects	F	Den d.f.	p
Body Size	Treat	37.6	93.3	<0.0001 ***
	Instar	1946.1	95.7	<0.0001 ***
	Instar^2	175.3	94.6	<0.0001 ***
	Instar^3	26.8	93.2	<0.0001 ***
	Treat:Instar	15.7	95.4	0.0001 ***
	Treat:Instar^2	0.1	94.5	0.7381
	Treat:Instar^3	0.8	92.9	0.3765
Seta length (TL II)	Treat	18.8	102.3	<0.0001 ***
	Instar	2784.8	116.6	<0.0001 ***
	Instar^2	556.2	99.6	<0.0001 ***
	Instar^3	23.0	98.2	<0.0001 ***
	Treat:Instar	2.6	116.9	0.109
	Treat:Instar^2	2.3	99.5	0.130
	Treat:Instar^3	1.4	97.6	0.245
Seta thickness (TL II)	Treat	11.7	94.9	0.0009 ***
	Instar	630.6	92.2	<0.0001 ***
	Instar^2	49.0	94.9	<0.0001 ***
	Instar^3	12.7	91.4	0.0006 ***
	Treat:Instar	0.4	92.4	0.512
	Treat:Instar^2	0.3	94.5	0.604
	Treat:Instar^3	0.1	90.6	0.733
Seta length (LT I)	Treat	16.1	99.4	0.0001 ***
	Instar	1840.2	109.8	<0.0001 ***
	Instar^2	312.2	102.7	<0.0001 ***
	Instar^3	15.3	101.8	0.0002 ***
	Treat:Instar	0.0	109.6	0.951
	Treat:Instar^2	4.5	103.4	0.037 *
	Treat:Instar^3	0.5	100.9	0.475

B — **Experiment 2 (offspring generation)**

	Fixed effects	$F_{(df)}$	Den d.f.	p
Body Size	Treat	4.5	63.3	0.037 *
	Instar	63.1	2279.7	<0.0001 ***
	Instar^2	---	---	---
	Instar^3	66.8	17.2	0.0001 ***
	Treat:Instar	---	---	---
	Treat:Instar^2	---	---	---
	Treat:Instar^3	61.3	4.5	0.037 *
Seta length (TL II)	Treat	0.0	62.1	0.874
	Instar	1583.7	62.0	<0.0001 ***
	Instar^2	---	---	---
	Instar^3	69.7	12.6	0.0007 ***
	Treat:Instar	61.9	0.1	0.744
	Treat:Instar^2	---	---	---
	Treat:Instar^3	69.6	2.6	0.109
Seta thickness (TL II)	Treat	59.0	0.9	0.359
	Instar	923.3	41.1	<0.0001 ***
	Instar^2	46.1	6.1	0.017 *
	Instar^3	---	---	---
	Treat:Instar	41.1	0.0	0.908
	Treat:Instar^2	46.4	0.2	0.653
	Treat:Instar^3	---	---	---
Seta length (TL I)	Treat	4.2	63.0	0.045 *
	Instar	60.7	894.7	<0.0001 ***
	Instar^2	---	---	---
	Instar^3	---	---	---
	Treat:Instar	0.1	60.6	0.810
	Treat:Instar^2	---	---	---
	Treat:Instar^3	---	---	---

Table S2: Linear models for body and limb sizes for each time point in the two experiments (treatment and clone, fixed effects). Measurements differences between the treatments for each data point are reported in micrometers. P-values < 0.05 are highlighted.

A) Experiment 1 (mat. gen.)		Body size			Seta length (TL II)			Seta thickness (TL II)			Seta length (TL I)		
Adult instar	Fixed effects	F_{df}	p	Treat. diff. µm	F_{df}	p	Treat. diff. µm	F_{df}	p	Treat. diff. µm	F_{df}	p	Treat. diff. µm
1		$0.089_{1,27}$	0.770	45.9	$0.13_{1,21}$	0.7157	14.8	$1.11_{1,21}$	0.30	2.4	N/A	N/A	22.9
2		$4.52_{1,64}$	0.0373	60.0	$9.60_{1,67}$	**0.0028**	17.2	$5.08_{1,67}$	**0.0274**	1.6	$0.65_{1,43}$	**0.0422**	11.6
3		$9.95_{1,72}$	**0.0023**	76.5	$20.3_{1,79}$	**<0.0001**	19.0	$6.19_{1,79}$	**0.0149**	0.8	$10.03_{1,57}$	**0.002**	15.8
4		$6.67_{1,60}$	0.01179	79.5	$17.3_{1,66}$	**<0.0001**	10.8	$2.46_{1,66}$	0.121	0.5	$16.72_{1,52}$	**0.0001**	14.6
5	Treat	$33.25_{1,62}$	**<0.0001**	129.6	$16.3_{1,60}$	**0.0001**	13.3	$0.1_{1,60}$	0.75	-0.2	$10.85_{1,52}$	**0.0025**	15.6
6		$23.45_{1,61}$	**<0.0001**	116.5	$3.81_{1,62}$	**0.0554**	8.4	$2.73_{1,62}$	0.10	1.1	$4.91_{1,55}$	**0.030**	7.1
7		$25.31_{1,59}$	**<0.0001**	158.4	$4.94_{1,66}$	**0.0298**	4.9	$4.59_{1,63}$	**0.0036**	0.5	$6.97_{1,46}$	**0.01125**	1.6
8	1->	$22.33_{1,46}$	**<0.0001**	172.4	1-> $0.02_{1,36}$	0.89	1.8	$0.15_{1,36}$	0.69	0.1	$0.67_{1,32}$	0.41	5.6
1		$2.28_{5,27}$	0.0746		$0.99_{5,21}$	0.4131		2-> $1.36_{3,21}$	0.2823 [2]		3-> N/A	N/A	
2		$1.94_{5,64}$	0.0990		$7.40_{5,67}$	**<0.0001**		$4.49_{5,67}$	**0.013**		4-> $4.56_{4,43}$	**0.0037**	
3		$4.56_{5,72}$	**0.0011**		$9.5_{5,79}$	**<0.0001**		$13.17_{5,79}$	**<0.0001**		$0.86_{5,57}$	**<0.0001**	
4		$2.50_{5,60}$	**0.0399**		$22.6_{5,66}$	**<0.0001**		$11.417_{5,66}$	**<0.0001**		$19.10_{5,52}$	**<0.0001**	
5	Clone	$4.89_{5,62}$	**0.0007**		$15.9_{5,60}$	**<0.0001**		$2.50_{5,60}$	**0.03995**		$15.56_{5,52}$	**<0.0001**	
6		$5.28_{5,61}$	**0.0004**		$12.7_{5,62}$	**<0.0001**		$6.1_{5,62}$	**0.0001**		$27.73_{5,55}$	**<0.0001**	
7		$2.98_{5,59}$	0.018		$25.0_{5,63}$	**<0.0001**		$9.395_{,63}$	**<0.0001**		$16.87_{5,46}$	**<0.0001**	
8	1->	$7.30_{4,46}$	**0.0001**		1-> $7.87_{4,36}$	**0.0001**		1-> $8.48_{4,36}$	**<0.0001**		1-> $24.21_{4.32}$	**<0.0001**	

1: clone CH-H1 was not measured in both treatments and was excluded.
2. clones RU-B-5 and TR-EG-1 were not measured in both treatments and were excluded
3: not enough 1st adult instars were successfully measured
4: clone BE-OHZ-t10 was not measured in both treatments and was excluded

B) Experiment 2 (off. gen.)		Body size			Seta length (TL II)			Seta thickness (TL II)			Seta length (TL II)		
Instar	Fixed effects	F_{df}	p	Treat. diff. µm	F_{df}	p	Treat. diff. µm	F_{df}	p	Treat. diff. µm	F_{df}	p	Treat. diff. µm
2	1->	$0.76_{1,12}$	0.39	-62.5	$0.004_{1,11}$	0.098	-1.1	$0.07_{1,11}$	0.79	-0.4	$0.59_{1,4}$	0.4857	-9.0
4		$7.90_{1,37}$	**0.0078**	193.2	$1.85_{1,33}$	0.1126	21.2	$5.49_{1,33}$	**0.025**	2.4	$3.21_{1,27}$	0.0839	30.7
6	Treat	$4.06_{1,52}$	**0.049**	156.0	$0.01_{1,51}$	0.90	10.0	$0.32_{1,51}$	0.5719	0.1	$0.81_{1,41}$	0.3721	18.3
7		$0.01_{1,51}$	0.991	55.8	$2.50_{1,47}$	0.12	5.3	$0.1_{1,47}$	0.996	0.4	$0.16_{1,46}$	0.69	21.7
8		$2.52_{1,50}$	0.11	126.8	2-> $0.10_{1,44}$	0.299	c	2-> $3.44_{1,44}$	0.070	c	2-> $8.34_{1,42}$	**0.006**	c
2	1->	$5.7_{3,12}$	0.99		$0.90_{4,11}$	0.49		$0.93_{4,11}$	0.48		$50.9_{4,4}$	0.72	
4		$6.59_{4,37}$	**0.0004**		$5.1_{4,33}$	**0.0025**		$5.41_{4,33}$	**0.001**		$2.91_{4,27}$	0.039	
6	Clone	$6.90_{4,52}$	**0.0001**		$9.2_{4,51}$	**<0.0001**		$6.63_{4,51}$	**0.0002**		$3.35_{4,41}$	**0.0182**	
7		$5.67_{4,51}$	**0.0007**		$18.05_{4,47}$	**<0.0001**		$14.85_{4,47}$	**<0.0001**		$9.42_{4,46}$	**<0.0001**	
8		$4.45_{4,50}$	**0.0037**		2-> $14.58_{3,44}$	**<0.0001**		2-> $6.48_{3,44}$	**0.0009**		2-> $6.63_{3,42}$	**0.0008**	

1: clone RU-B-5 was not measured in both treatments and was excluded
2: clone CH-H-1 was not measured in both treatments and was exclude

Chapter IV

Genetic variation in the benthic feeding habits of *Daphnia magna* across its geographical and habitat range

Abstract

Species with broad geographical ranges that encompass a variety of habitats are suitable for the analysis of the interplay between local selective conditions on adaptive traits and the historical and demographic influences on genetic variation. The distribution of freshwater lentic habitats is often patchy and habitat boundaries are often well defined in these environments. These conditions favour isolation of planktonic species populations, a situation that, coupled with often large population sizes and strong founder effects, might facilitate local differentiation and adaptation. Habitat selection behaviours influence the selective regime on loci that affect adaptation to the environment and might set the early condition for adaptive shifts. In the freshwater zooplankton crustacean *D. magna*, habitat selection occurs in response to differences in food availability in suspension in the water. In these circumstances, *D. magna* is able to exploit benthic food sources such as sediments and periphyton. Here, we analysed clonal differences in behaviour and morphology in relation to these alternative feeding strategies in *D. magna* using 40 clones from water bodies of different sizes distributed across the wide geographical range of the species. Knowledge of the historical genetic divergence between the clones allowed us to identify lineage-specific genetic variation for a morphological trait associated to feeding on submerged surfaces. We also found differences between habitats in the propensity of the clones to browse on bottom sediments. This work highlights the role of benthic feeding for habitat selection in a planktonic species in the context of both the local conditions experienced by the animals in their habitats and the broad geographical distribution of the species.

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Introduction

Environmental heterogeneity across geographical and habitat ranges has a great impact on species evolutionary, ecological and demographic dynamics (Byers 2005). On large geographical scales, physical and climatic barriers might, for example, affect dispersal and restrict or fragment the specie's range. On a more local scale, a species might encounter a variety of suitable and unsuitable habitats. While some species have evolved high specialization to certain habitats or micro-habitats, others live in a variety of different environments. Generalists that live on a wide range of conditions might be adapted to specific niches within the general habitat of the species (Piglucci 2001). Phenotypic divergence between populations can be caused by variation in selection between habitats and local adaptation might occur if selection is not counteracted by processes such as gene flow, mutation and genetic drift (De Meester 1996). Across spatial scales, patterns of genetic and phenotypic variation are therefore shaped by a combination of selection to habitat conditions and the spatial ecology of a species (e.g. dispersal, migration and habitat selection). Genetic and phenotypic studies that consider different levels of habitat spatial structure are essential to understand species' natural and evolutionary history.

The patchy distribution of inland freshwater habitats and their often well-defined boundaries generate limits for gene flow between populations, creating opportunities for local genetic differentiation (Slarkin, 1985). On the other hand, differences in geology, climate and habitat types across large geographic scales influence this processes by greatly impacting the dynamics of dispersal and gene flow. Freshwater invertebrate species that have wide geographical ranges and encounter a high variety of habitats are good model systems to study the interplay between local genetic differentiation/adaptation and geographic patterns of genetic structure (Fields *et al*. 2018). Zooplankton species have been extensively studied due to their great impact on freshwater ecosystem dynamics. Being primary consumers of phytoplankton (primary producers) and preys of many predators including fish and other invertebrates, zooplankton occupy a central position in freshwater food-webs (Miner *et al.* 2012). Zooplankton populations are often locally adapted to grazing and predation conditions (De Meester 1996; Cousyn *et al.* 2001). Studies using neutral markers have shown how zooplankton populations are genetically structured across local, regional and global scales. Importantly, many zooplankton species, such as cladocerans and rotifers, are able to disperse and resist local unfavourable conditions by producing resting eggs. These can passively disperse along long distances and colonize suitable habitats. Moreover, they can guarantee local population persistence in fluctuating environments. In cyclical parthenogenetic species, sexual resting eggs and males are produced when the environmental conditions deteriorate. In favourable conditions, asexual females resulting from clonal reproduction dominate the populations and often guarantee high population sizes. Local adaptation to habitat conditions and population genetic structure are therefore influenced by a combination of inter-clonal selection during the asexual phase of reproduction, periodical sexual shuffling of genetic variation and by the dynamics of passive dispersal between habitats (De Meester 1996).

Local adaptation and patterns of genetic variation at different spatial scales have been extensively studied in the order of fresh water zooplankton crustaceans *Daphnia*. Species of the genus are found in a variety of fresh water habitats from large lakes to ponds to ephemeral pools that periodically dry out or freeze. The genus is distributed worldwide in every continent (including one species in Antarctica) (Benzie 2005). While some species show moderately to highly restricted geographic distributions, others are found in several continents and their ranges encompass large climatic and latitudinal variation. The species *D. magna* has such a large, multi-continental range and it is found in all Eurasia, North America, North Africa and,

to a lesser extent, in East and South Africa. It inhabits water bodies of different sizes, from big lakes to small ephemeral pools in climatic conditions that span from temperate to subarctic. In *D. magna,* local adaptation to habitat conditions for physiological, life history and behavioural traits is widespread and extensively documented (De Meester 1996; Cousyn *et al.* 2001). Population genetic studies have also revealed genetic population structure at different spatial scales (De Meester 1996; Fields *et al.* 2015). Together, population genetic studies of neutral variation and studies of ecologically relevant traits have the potential to disentangle the effects of historical demography and natural selection determining adaptation to local conditions. For example, Roulin *et al.* (2013) found strong signals of local adaptation in resting egg and male production amongst 13 European populations sampled from ephemeral, permanent and seasonally freezing or drying environments. However, in another study of a rock pool metapopulation in Finland, no signal of local adaptation for sex induction was found, despite similar selective regimes defined by different degrees of water body persistence (Roulin *et al.* 2015). Small population sizes and consistent founder effects in ephemeral rook pools, may have a strong effect on limiting the evolution of locally adapted traits in these habitats. In support to this hypothesis, a recent phylogeographic study of Eurasian *D. magna* clones sampled from rock pools and more persistent habitats such as ponds (Fields *et al.* 2018) provides evidence of a strong effect of genetic drift in rock pool populations, possibly reducing the effect of local selection.

Albeit less frequently, *D. magna* is also found in large lakes (Benzie 2005). Similarly to what found for small rock pools, a comparison of ponds and lakes populations has revealed how drift is more a prominent feature of populations from smaller water bodies. In another species of the genus, *D. pulex* from North America, genetic differentiation between pond and lake populations has been revealed by the analysis of nuclear and mitochondrial markers. Due to its large size, *D. magna* is not able to survive in situations under intensive fish predation, a condition often found in big lakes. However, anti-predatory behaviours and morphological defences are often locally adapted to the type and magnitude of the predation regimes in lakes and ponds (De Meester 1993). Another difference between lakes and ponds is represented by their difference in the bottom environments. While in shallow ponds *D. magna* might live in close proximity to the bottom, in large deep lakes it might hardly come into contact with this habitat. Although being primarily planktonic, *D. magna* displays some behavioural and morphological traits that are associated with its trophic interaction with the bottom environment. When feeding conditions in the water deteriorate, this species adopts an alternative feeding strategy, termed sediment browsing behaviour (Horton *et al.* 1979; Arbore *et al.* 2016) (Chapters I and II of this thesis). The animals swim along a sediment surface, stirring up particles with movements of the second antennae; edible particles are then ingested by filter feeding. *D. magna* might also be able to feed on periphyton, the complex mixture of algae, cyanobacteria, heterotrophic microbes, and detritus that is attached to submerged surfaces, by scraping by means of a robust seta on its trunk limb II (Fryer 1991).

In relation to the benthic habits of *D. magna*, here we investigated phenotypic differences between 40 clones sampled throughout most of the species known range and from different environments namely ponds, small lakes and big lakes. The clones used in this study belong to a larger laboratory collection of clones whose genetic relationships are known from recent phylogeographic studies (Fields *et al*. 2015 and Fields *et al.* 2018). For individual replicates of each clone we measured browsing behaviour and morphological features of the setal apparatus of trunk limb II. We found genetic variation associated to each of the phenotypes, significant differences between habitat types and between genetic lineages and phenotypic correlations

with climatic conditions of the sites of origin of the clones. This study integrates some of the hitherto poorly investigated behavioural, functional and morphological traits associated with the interactions between *D. magna* and the bottom habitats across the wide geographical and habitat distribution of this species.

Materials and Methods

Daphnia clones

Daphnia magna reproduces by cyclical parthenogenesis and monoclonal populations can be cultivated in the laboratory by asexual reproduction. Here, we used 40 *D. magna* clones belonging to a larger collection of clones that were sampled throughout the northern hemisphere and are since then propagated asexually in the laboratory (the *Daphnia magna* Diversity Panel) (Figure 1). We selected the clones within four broadly defined macro-geographic regions: Canada (2), Mediterranean (14), East Europe (8), and Siberia (15, including one clone from Mongolia) and included one clone from China (1). Ongoing analyses of genetic diversity between *D. magna* clones shows that European, African, Middle-Eastern and Central-Siberian clones represent a distinct lineage from East-Asian clones (East-Siberia and China) and that the border between these lineages can be placed approximately at the western border of Mongolia (Peter Fields and Dieter Ebert, unpublished data, Fields *at al.* 2015). Moreover, these analyses are showing that Canadian clones likely belong to the East-Asian lineage. The present results were mostly mirrored when the Canadian clones were included in the East Asia lineage, but additional differences were found when contrasting these regions. For the present analyses, we therefore defined three distinct lineages by grouping clones from: Canada (the Canadian lineage), Europe, Africa, Middle East, and Central Siberia (the Western Eurasian lineage) and East Siberia and China (East Asian lineage).

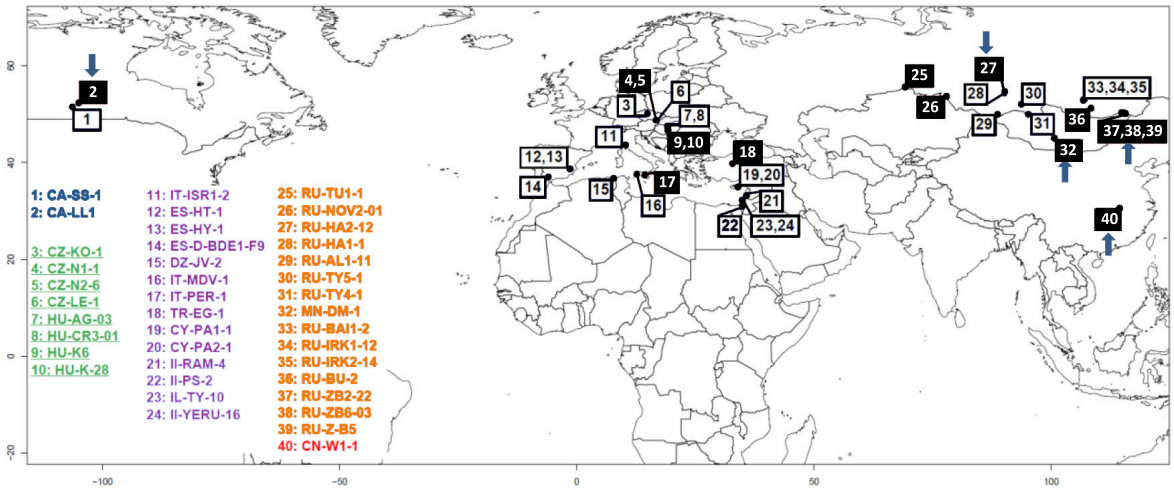

Figure 1: Sampling sites of the 40 *D. magna* clones included in this study. Blue: Canada; green: Central Europe; purple: Mediterranean; orange: Siberia; red: China. White boxes: ponds; Black boxes: lakes; Arrows: big lakes.

Habitat and climate data

The clones originated from lake (16) or pond (24) populations. Water bodies at least one km in length were considered as lakes and their surface areas were retrieved from shapefiles of inland waters for the countries of interest (www.diva-gis.org/gdata) using ArcGis v10.2.1 (ESRI 2011. Redlands, CA: Environmental Systems Research Institute). We further distinguished between small lakes (surface area < 10 km^2) and big lakes (surface area > 10 km^2). For the analyses we defined three habitat types: ponds, small lakes and big lakes. Climate data on the sampling locations were extracted from gridded climate geoTIFFS data files using the R package *raster*. This is a set of 19 bioclimatic variables on current conditions representing annual trends, seasonality and extreme or limiting environmental factors (WorldClim Version 1). A principal component analysis on the bioclimatic variables was performed excluding the Chinese site (clone CN-W1-1, showing distinct climatic conditions from all the other sites) and the principal components for the remaining sites were used in the analyses (Fig. S1).

Experimental animals

Individual replicate females for each clone were isolated from stock cultures, and used to establish the experimental populations. Each clone was propagated asexually in standardized conditions for three generations before the experiment. The animals were kept isolated in 100 ml-jars filled with 80 ml ADaM (Klüttgen *et al.* 1994) from four days old. When 12 days old the animals were transferred into fresh medium and thereafter every three/four days or when a clutch was released. The animals were fed daily with increasing amounts of chemostat-grown green algae *Scenedesmus sp.*: 1 x 10^6 algae cells/animal until day 5, 2 x 10^6 until day 8, 2.5 x 10^6 until day 10, 3 x 10^6 until day 12, and 5 x 10^6 onwards. Animals from the third or subsequent clutches were used to establish the next generation. All the animals were females and were kept in randomized positions with in incubators with a 16:8 light/dark cycle and constant temperature of 20 °C. For the experiment, we raised the offspring of two animals for each of the clones (2 x 6 replicates x 42 clones: n= 504 animals). These animals were kept in the same condition as before but were fed 2 x 10^6 algae twice a day from day 13 in order to minimise the accumulation of food on the bottom of the glass jars. Within 24 hours after releasing their second clutch, these animals were used in the behavioural assay. After the assay, the animals were transferred to fresh medium, maintained in the same conditions until they released their next clutch and then discarded. The exuviae of the second adult instars of these animals were kept within the glass jars at 10 °C for one day and then dissected for the morphometric analyses as described below.

Behavioural assay

The browsing behaviour of *D. magna* consists in the animals swimming along a surface of sediment and rapidly stirring up particles with movements of the second antennae. These particles are then processed by the filtering apparatus of the animals (Horton 1979, Arbore *et al.* 2016). The browsing activity of one animal on a layer of fine silt (loess), deposited on the bottom of glass jars, leaves steady traces which can be photographed and analysed after the animal is removed. This assay permits a quantification of the browsing behaviour of *Daphnia* and was used before in two studies (Arbore *et al.* 2016 and Mushegian *et al*. 2019; see Chapter I of this thesis for a detailed description of the method). Briefly, each animal was transferred into a cylindrical glass jar 20 cm tall and 6.5 cm wide with a 1 cm layer of loess. The surface of the loess was photographed before the animal was introduced using a ring light to ensure uniform illumination. For 30 mins, the animals were allowed to browse while the jar was kept into a

darkened carton tube and illuminated from the top by a neon light. At the end of the assay, the animal was removed and the surface of the loess was again photographed in the same position and light conditions as before. A new jar was used for each animal and a set of 12 jars was used in 45 minutes sessions, with each animal introduced at one minute intervals. The assays were conducted over a period of six days with the replicates of each clone distributed across days and sessions. Using the software ImageJ (http://rsb.info.nih. gov/ij/), the pictures were converted to grey scale, a central circular area was cropped to exclude shadows from the edge of the jar and the number of black pixels corresponding to the browsing traces was quantified after setting a high contrast threshold. Pictures of the same jars taken before the animal was introduced were processed in the same way and used to correct the measurements when irregularities on the loess surface were detected. The logtransformed pixel values [log10(X+1000)] are used to define the browsing index (1000 corresponds approximately to the number of pixels of one individual browsing trace). Since 45 animals were damaged or died before the experiment, a total of 459 animals were assayed. After the analysis, 16 measurements had to be discarded because the pictures were altered due to the handling of the jars, leaving n = 443 animals analysed in the behavioural assay. For each clone, we calculated the behavioural index as the mean of the measurements of the replicates (on average n=10 individual replicates per clone).

Morphological analyses

The exuviae of recently moulted animals (<24 h) were rinsed briefly in ADaM to remove attached material and transferred individually to a microscope slide with a glass pipette. Liquid in excess was removed with a piece of filter paper until a thin layer covered the exuvia; this prevented dehydration and kept the exuvia adherent to the slide facilitating dissection. The dissections were performed using two thin metal needles under a dissecting microscope with dark field illumination. First, by pulling the second antennae apart from the capapax, the two carapax valves were separated from the exuvia. Attached to the second antennae remained the residual exosceletron, including the armature of the trunk limbs and the post abdomen. One of the two trunk limbs II was isolated and all remaining parts removed. The setae of the limb were spread with attention on avoiding the overlapping with the stiff seta (anterior seta of the distal endite of limb II) and the gnatobase. A glass cover slip, covering the carapax valves and the trunk limb II, was then gently placed on the specimen, liquid in excess was removed with filter paper to make it adhere and its sides were sealed with nail polish to avoid dehydration. The specimen were kept covered in plastic boxes with water-soaked paper towels at 10 °C for not more than 2 hours before being photographed. The photographs of the specimen were taken. For each specimen, we documented the carapax and the 2nd stiff seta of the gnatobase and the stiff seta of the distal endite of trunk limb II at multiple magnifications. For the morphometric analyses, we defined fixed landmarks on the carapax and the setae and measured their coordinates for each specimen using the software ImageJ (Figure 2). The size of the animals was measured as the Cartesian distance, scaled in micrometers, between two landmarks (c1, c2) placed on the base of the carapax spine and on the distal margin of one of the carapax valves (Figure 2A). Ten landmarks were defined on the stiff seta (Figure 2B): one (1) at the extremity of the tip, one (2) at the emergence of the first spinules, four (3-6) at the base each 10th subsequent spinule, two (7, 8) at both sides of the end of the portion of the armature bearing spinules and two (9, 10) at both sides of the site of emergence of the seta from the endite (the exact position of emergence on the side opposite from the raw of spinules was often uncertain; landmark 10 was therefore instead placed in its proximity where the armature consistently displays a minute crease). Two semi-landmarks (W, Z) were then defined as the midpoints between landmarks 7 and 8 and between landmarks 9 and 10 respectively. Cartesian distances in micrometers of the segments between

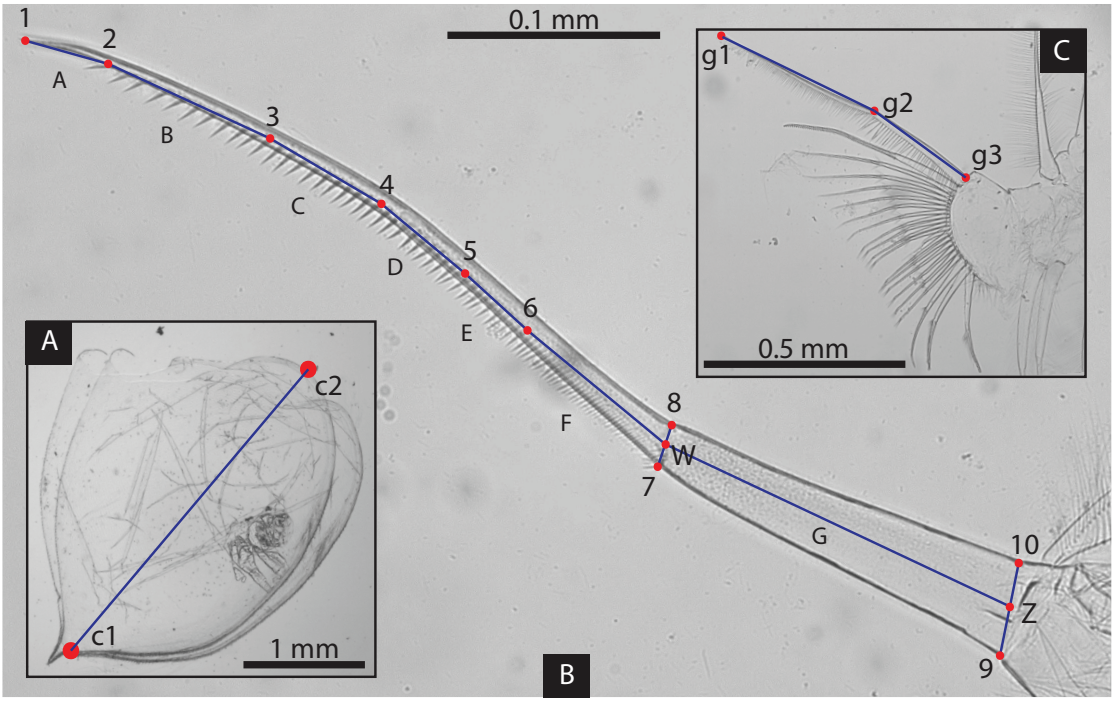

Figure 2: Photographs of the exuvia of an adult *Daphnia magna* female and landmarks on the stiff seta and the gnatobasic seta. A: Body size was measured as the distance from the base of the carapax spine (c1) and the top of the head of the animals (c2). B: Armature of the stiff eta of trunk limb II: ten landmarks were placed on the seta (1-10) and two semi-landmarks were defined as the midpoint between landmarks 7 and 8 (W) and between landmarks 9 and 10 (Z). Length of the seta was measured as the sum of segments A-G. Thickness of the seta was measured as the mean length of segments H and I. Robustness of the seta was defined as the ratio between stiff seta length and thickness. C: Armature of the gnatobase of trunk limb II and landmarks on 2nd stiff seta (g1-g3).

adjacent landmarks and semi-landmarks were then calculated to define length (A-G) and width (H, I) of different portions of the stiff seta. The total length of the seta was measured as the sum of segments A to G and its width as the mean of segments H and I. Three landmarks were placed on the 2nd stiff seta of the gnatobase (Figure 2C): at the tip (g1), at the junction between its distal portion and its base (g2) and at the emergence of the base from the endite. Specimen where the carapax or the setae revealed to be damaged or folded or where landmarks could not be placed were removed from the analysis. The dissection and the photographic documentation of 419 specimen were conducted on six consecutive days. At the end of the analysis we obtained size measurements for 389 specimen and stiff seta measurements for 366 specimens.

Statistical analyses

Linear mixed models were conducted in R (ver. 3.5.2; R Development Core Team, 2008 using the package Lme4 and the statistical significance of the fixed effect was estimated with Type II F tests with the function anova in the R package lmerTest (Kenward-Roger's approximation for denominator degrees of freedom). The adjusted intra-class correlation coefficient for the traits (equivalent to broad sense heritability) was calculated with a linear mixed effect (LMM) model, with size and habitat as fixed effects and clone as a random effect (R software package rptR developmental version; (Nakagawa & Schielzeth 2010). Confidence intervals and statistical significance were calculated using parametric bootstrapping with 5000 iterations and a randomization procedure with 5000 permutations. The principal component analysis has been performed using JMP (Version 13; SAS).

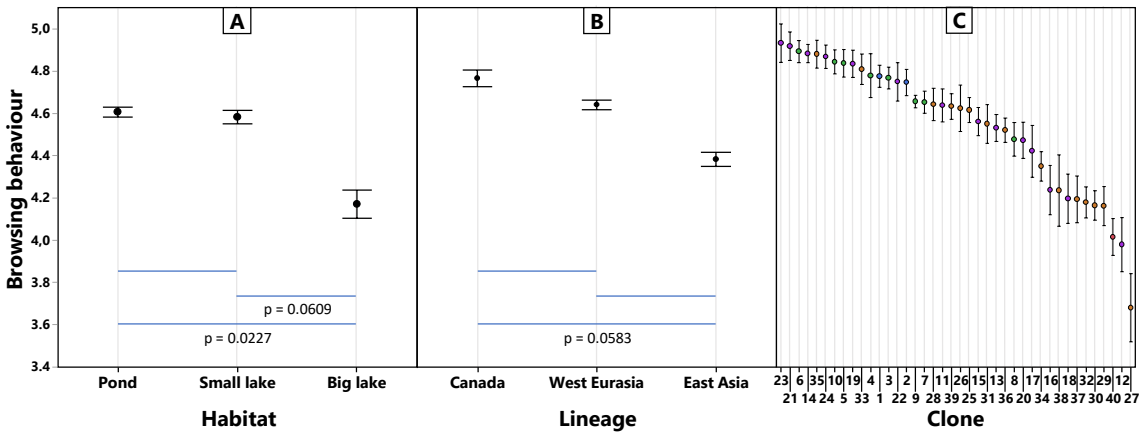

Figure 3: Habitat and lineage effects on browsing behaviour of 40 *D. magna* clones. A: habitat means and standard errors. B: lineage means and standard errors. C: clonal means and standard errors. Browsing behaviour was defined as the Log_{10} of the area of the browsing traces left by individual replicate animals browsing on a sediment surface for 30 minutes.

Results

Genetic and habitat effects on browsing behaviour

Browsing behaviour varied between clones, habitat type and lineages (Table 1; Fig. 3). Size of the animals showed no effects on behaviour and was excluded from the analyses ($F_{1,339.16}$ = 0.001, p = 0.97). The total phenotypic variance for browsing behaviour explained by clones corresponded to 53.1% (95% CI = [38.8, 64.1%], p < 0.0001) and 44% after correcting for habitat type and lineage effects (95% CI = [29.2, 56.2%], p < 0.0001). Clones from big lakes browsed less then clones from ponds (Tukey's HST p-value = 0.0227) and, albeit not significantly, then clones from small lakes (Tukey's HST p-value = 0.0609) (Fig. 3A). Accordingly, lake surface area also showed a significant effect on behaviour ($F_{1,38.503}$ = 5.18, p = 0.0284). Additionally, we found a trend effect of lineage: clones from East Asia browsed less than clones from Canada (Tukey's HST p-value = 0.0583, Fig. 3B) but no differences were found with West Eurasia.

Genetic variation in trunk limb II morphology

We analysed four morphological phenotypes of trunk limb II: length, thickness and robustness of the anterior seta of the distal endite ("stiff seta") and length of the 2nd stiff seta of the gnatobase (hereafter "gnatobase") (Figure 3). The lengths of the stiff seta and of the gnatobase jointly co-varied with size (R^2 = 0.507 and 0.541) while thickness of the stiff seta showed a weaker correlation (R^2 = 0.293) and robustness no correlation with size (R^2 = 0.02) (Fig. S2). No differences in body size of the animals were found between habitat types ($F_{2,\ 35.224}$ = 1.58, p = 0.22) or lineages ($F_{2,\ 35.372}$ = 0.93, p = 0.40). The effects of size, habitat type and lineage on the four morphological phenotypes are summarized in Table 1. Generally, trunk limb morphology varied between clones and lineages but not between habitat types. Moreover, we found no correlations between browsing behaviour and any of the morphological phenotypes. The total phenotypic variance explained by clones was: 60.1% for stiff seta length, 47.9% for stiff seta thickness, 60.2% for stiff seta robustness and 63.7% for gnatobase length (Table 1). We found significant effects of lineage on stiff seta thickness and robustness and for gnatobase length, but not for stiff seta length. Overall, clones from the West Eurasian lineage had a less thick stiff seta then East Asian clones (Tukey's HST p < 0.0001). Accordingly, stiff seta robustness was markedly lower in these clones than in East Asian ones (Tukey's HST p < 0.0001). Overall, gnatobase length was shorter in West Eurasian clones compared to East Asian ones (Tukey's HST p = 0.0321). Interestingly, the stiff seta of the two clones from Central Siberia, RU-NOV2-01 and RU-TUI1-1 (belonging to the western lineage) was as thick and robust as that of the other Siberian clones and the gnatobase as long.

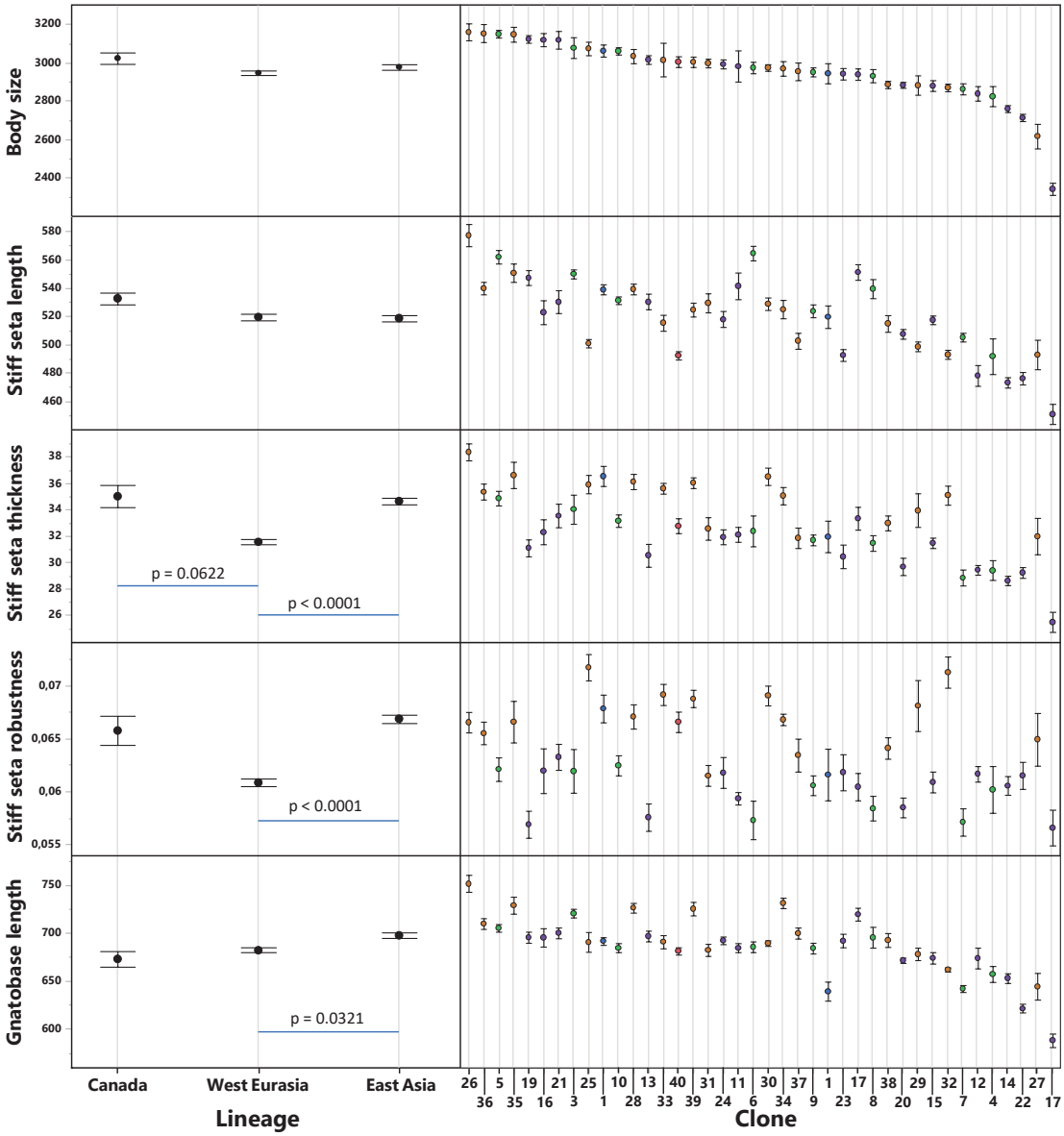

Figure 4: Lineage effects on stiff seta and the gnatobase 2nd seta morphology of 40 *D. magna* clones. Left panels: lineage means and standard errors; right panels: clonal means and standard errors.

Analysis of climate data

In the PC analysis of climate data, the sampling locations clustered within three groups: 1) Siberia/Canada, 2) Central Europe and 3) Mediterranean (the clone from China was previously excluded) (Fig. S1). The first three principal components explained 90% of variation and were considered in the statistical analyses. Major contributions to PC1 were temperature variables, while measures of variation in precipitation loaded on PC2 and measures of precipitation amounts on PC3 (Table S1). The effects of the first three principal components on browsing behaviour and on the morphological phenotypes are summarized in Table 2. Browsing behaviour was only marginally explained by PC1: browsing of the clones slightly increased from locations with low mean temperatures and high temperature variation (negative PC1 values; e.g. Siberia) to locations with more mild conditions (positive PC1 values; e.g. Mediterranean). As climate conditions group by geographic region, this result likely reflects the lineage effect previously found for browsing behaviour (Table 1). However, differences between lineages seem to better

Table 1: Results of the linear mixed models for browsing behaviour and four phenotypes of trunk limb II, mean values by lineage and clonal effects. Robustness was defined as the ratio between stiff seta length and thickness. Habitats: pond, small lakes and big lakes. Lineages: Canada, West Eurasia and East Asia. The intra-class correlation coefficient (ICC) corresponds to the percentage of the phenotypic variance explained by clone (random effect); size: covariate (for the phenotypes trunk limb II); habitat and lineage: fixed effects.

Phenotype (mean±SE)	Fixed effects	Num DF	Den DF	F-value	p-value	Clone ICC, 95% CI, p
Browsing behaviour	**Habitat**	2	35.445	3.8818	0.02989 *	0.440 [29.2, 56.2%] p<0.0001
Canada 4,764±0.17 **Western** 4.640±0.35 **East Asia** 4.381 ±0.40	**Lineage**	2	35.492	3.7534	0.03321 *	
Stiff seta length	**Body size**	1	329.52	198.789	<0.0001 ***	0.479 [33.3, 59.9%] p<0.0001
Canada 532.4±4.2 µm **Western** 519.46±2.43 µm **East Asia** 518.6±2.2 µm	**Habitat**	2	35.30	0.48	0.6223	
	Lineage	2	35.29	0.24	0.7825	
Stiff seta thickness	**Body size**	1	227.15	60.32	<0.0001 ***	0.601 [45.7, 70%] p<0.0001
Canada 35.02±0.84 µm **Western** 31.58±0.22 µm **East Asia** 34.65±0.24 µm	**Habitat**	2	35.341	1.84	0.1724	
	Lineage	2	35.647	13.34	<0.0001 ***	
Stiff seta robustness	**Body size**	1	216.659	0.52	0.4706	0.660 [46.0, 70.9% p<0.0001
Canada 0.0657±0.0013 **Western** 0.0608±0.0003 **East Asia** 0.0668±0.0004	**Habitat**	2	35.324	0.84	0.4382	
	Lineage	2	35.676	13.82	<0.0001 ***	
Gnatobase length	**Body size**	1	301.296	145.35	<0.0001 ***	0.637 [49.3, 73.7%] p < 0.0001
Canada 672.99±8.07 µm **Western** 682.31±2.62 µm **East Asia** 697.60±2.81 µm	**Habitat**	2	35.212	2.59	0.08856 .	
	Lineage	2	35.090	4.81	0.01419 *	

describe the distribution of behaviour among clones as Canadian and Western Siberian clones showed higher browsing levels than East Asian ones. Precipitation seasonality (PC2) showed a significant effect on stiff seta length and robustness. Differences in precipitation seasonality between regions (e.g. between East Europe and Mediterranean) allowed therefore to disentangle climatic and macro-geographic/lineage effects. Temperature conditions (PC1) showed a strong effect on stiff seta thickness and robustness. Clones from Siberia and Canada had thicker, and consequently more robust, setae then clones from East Europe and Mediterranean. Strong lineage effects were found on stiff seta thickness and robustness, but grouping by climatic conditions better described the variation we observed among the clones as Canadian and West and East Siberian clones had thicker setae that the other clones. Individually, several temperature variables, precipitation annual mean and precipitation seasonality showed significant effects on seta thickness and robustness (after correcting for multiple testing; Table S2). Finally, temperature conditions (PC1) also affected the length of the first stiff seta of the gnatobase, simply reflecting the lineage effect found before, as lineage and climatic effects could not be distinguished.

Table 2: Results of the linear mixed models for the effects of the bioclimate variables on browsing behaviour and trunk limb II morphology. PC1-3 are the principal components of variation of 19 bioclimatic variables (Table3). Fixed effects: PC-1, size; Random effect: clone.

Phenotype		Num DF	Den DF	F-value	p-value
Browsing behaviour	**PC1**	1	35.046	3.9340	0.0552 .
	PC2	1	35.458	34.548	0.4211
	PC3	1	35.790	34.790	0.1424
Stiff seta length	**PC1**	1	35.332	0.845	0.36412
	PC2	1	34.239	6.368	0.01643 *
	PC3	1	34.374	0.231	0.63381
	Size	1	305.589	206.048	<0.0001 ***
Stiff seta thickness	**PC1**	1	35.656	44.643	<0.0001 ***
	PC2	1	33.291	1.513	0.2273
	PC3	1	33.399	1.248	0.2719
	Size	1	185.996	61.542	<0.0001 ***
Stiff seta robustness	**PC1**	1	35.480	35.250	<0.0001 ***
	PC2	1	33.888	8.106	0.007443 **
	PC3	1	33.744	33.744	0.452389
Gnatobase length	**PC1**	1	35.206	5.051	0.03099 *
	PC2	1	33.960	0.975	0.33038
	PC3	1	34.169	0.013	0.91080
	Size	1	271.625	154.744	<0.0001 ***

Discussion

In this study, we investigated behavioural and morphological traits associated with two benthic feeding strategies of *D. magna*, namely sediment browsing and scraping of food items from submerged surfaces. The two behaviours might be interrelated under certain circumstances but, more generally, they might reflect different strategies for feeding on food sources other than seston (the living and non-living matter suspended in the water). Accordingly, we found no correlations between browsing behaviour and trunk limb II setal morphology. The stiff seta on trunk limb II is thought to play a role in scraping food from submerged surfaces, while browsing behaviour might be a specialization for feeding on particulate sediment settled on the bottom. Scraping might involve the collection of food items adherent on solid surfaces such as rocks or plants. *D. magna* is able to feed on a variety of food items including protozoa and bacteria but also on dead material and detritus. While seston constitutes the principal diet of *D. magna*, periphyton is also consumed (Siehoff *et al.* 2009). Therefore, this species does not display great selectivity in the quality or size of the particles that are collected on various ways and filtered (Smirnov 2005). The filtered food is then broken down by the mandibles and toxic or inedible material (such as blue-green algae and silt) is rejected from the feeding chamber by movements of the postabdominal claw. The scraping action of the seta on trunk limb II might therefore serve the non-selective collection of various food items. The fact that we did not find differences between habitat types for browsing behaviour but not for setal morphology might reflect the more specific circumstances in which feeding by means of browsing could be effective (i.e. the presence of soft, particulate sediments) and the non-specificity of setal morphology for collecting food items in various habitats.

Daphnia populations are often behaviourally adapted to their environment (De Meester 1996; Cousyn et al. 2001). In our study, clones from big lakes browsed less that clones from ponds (and small lakes too, albeit this difference was not statistically significant), suggesting that in

big lakes *D. magna* might dwell less frequently in proximity of the bottom. Predation by fish and invertebrates is generally regarded as the main selective pressure acting on the evolution of habitat selection behaviours in *Daphnia*. Local predation is a strong determinant of diel vertical migration, *i.e.* the movement to deep, dark water layers during the day to avoid visual predators and to the upper layers during the night (De Meester 1993). This behaviour commonly occurs in presence of fish predation and is less common in ponds without fish (De Meester 1993; Boersma, Spaak & De Meester 1998). *Daphnia* can also find visual protection from fish by hiding in littoral macrophyte beds during the day and feeding in open waters during the night (diel horizontal migration, DHM) (Burks, Jeppesen & Lodge 2001). Nevertheless, in environments where predatory fish are attracted by plant beds, *Daphnia* actively avoids plants and prefers to find refuge near or within the sediment (Nihan Tavşanoğlu *et al.* 2012). In a previous study, we found no differences in browsing behaviour between *D. magna* clones sampled from ponds with or without fish predation suggesting that predation might not be of overall importance for browsing behaviour (Arbore *et al.* 2016). In the present study, insufficient data about predation do not allow us to provide further evidence in this regard. Few studies have analysed zooplankton habitat selection behaviours in relation to alternative feeding strategies. Bottom foraging can maintain zooplankton population even in the absence of planktonic food. For example, in shallow high latitude ponds and lakes, nutrient limitations often reduce phytoplankton concentrations (Rautio & Vincent 2006). In these conditions, *D. middendorfiana* actively feeds on benthic substrates such as microbial mats. In laboratory experiments, *D. magna* switches from suspension to surface feeding on periphyton (notably altering its species composition) when the concentration of suspended food drops below a critical threshold (Siehof *et al*. 2009). In our study, no detailed information was available about the feeding ecology of *Daphnia* in the sampling sites. However, it is reasonable to assume that the larger dimensions of big lakes might favour more strictly pelagic habits than smaller and shallow lakes and ponds, where the bottom might represent an accessible and food-rich environment. A broader and dedicated sampling including multiple clones from natural populations from different habitat types distributed within a limited regional scale might provide additional insight about the distribution and significance of browsing behaviour in *D. magna* populations.

Stiff seta thickness and robustness (but not length) was significantly higher in East Asian clones. This might suggest a seta more suited for scraping hard material but the observed difference in thickness was in the order of few micrometres. Among all clones and specimen analysed, we never observed any variation in the general structure of the seta. The general morphology of the stiff seta varies between *Daphnia* species, where it is the most variable among the setae on trunk limb II. This variation has been suggested to reflect its possible function in the context of the feeding biology of the different species (Fryer 1991; Smirnov & Kotov 2010). A preliminary analysis in this regard is presented in Chapter V of this thesis. Seta thickness vary between species thereby suggesting a possible effect on setal mechanical properties and function. Despite its functional significance, seta thickness might however represent a neutral phenotypic marker expressing the genetic differences between lineages. However, the two Siberian clones included in the West Eurasia lineage had comparable thickness to the other Siberian clones. The stiff seta of the gnatobase was the longest in East Asian clones. No function in scraping or direct food collection has been suggested for this structure which is, instead, considered to serve as a filter cleaning spine as its position and orientation towards the filter plate of trunk limb III suggests (Fryer 1991). Here, we measured this gnatobasic seta in order to assess if any observed variation in the stiff seta would reflect changes at the level of the whole trunk limb II. Gnatobasic seta length was correlated with stiff seta length, simply suggesting overall larger trunk limb II in East Asian clones.

In the principal component analysis of climate data, the first PC1 corresponded to temperature variables, PC2 corresponded to measures of precipitation variability and PC3 to measures of precipitation amounts. We found a strong effect of temperature conditions on stiff seta thickness and, consequently, robustness. Clones from Siberia and Canada had thicker seta then clones from East Europe and Mediterrean clones. Grouping the clones by temperature conditions described the variation we observed among the clones better than by grouping the clones by lineage. The same was true for gnatobasic seta length. We also found that differences in stiff seta length and robustness were explained by precipitation variability conditions. Among the Western European clones a marked difference in precipitation regimes between Mediterranean and East European clones allowed to separate these groups. Despite the genetic similarities within the West Eurasian lineage, as defined by divergence between mitochondrial genomes (Fields *et al.* 2018), clones from different climatic regions showed some level of morphological variation in the seta. Taken together, these result suggest that morphological variation in trunk limb II cannot be exclusively attributed to neutral genetic differences between anciently diverged lineages, such as the Western Eurasian and the East Asian. In turn, this might provide the bases for a further analysis of the ecological features of *D. magna* habitats that vary between regions and that might influence setal evolution.

Conclusions

Here, we studied two hitherto poorly investigated aspects of the feeding biology of *D. magna* namely its propensity to feed on bottom sediments (browsing) and submerged surfaces (scraping). Browsing and scraping are displayed when food concentration in suspension in the water is limiting and permit the exploitation of nutrient-rich benthic food sources. The integration of these alternative feeding strategies might have profound influences on the ecology and evolution of *D. magna* and other species of the genus which, as "strong ecological interactors", have a profound impact in freshwater lentic ecosystems. In a previous study (Arbore et al. 2016; Chapter I of this thesis), we described the genetic architecture of sediment browsing behaviour of *D. magna* and found genetic variation in a set of 15 clones from natural populations. Here, using a larger sample of clones, we found evidence for an influence on behaviour of habitat type (ponds, small lakes and big lakes). In another study (Chapter II of this thesis), we analysed the potential for genetic variation in browsing behaviour to influence microbiota acquisition from sediments. Taken together, our analyses of browsing behaviour contributed to the understanding of the ecological genetics and functional implications of this ecologically relevant behavioural trait, offering further bases for the study of habitat selection in *D. magna*. We did not find differences between habitats in the morphology of a seta on the trunk limb II that was previously proposed to represent a specialization for surface scraping. Similarly, previous work on this structure (Chapter III of this thesis) did not find morphological variation associated to its functional role in different feeding conditions. However, in both studies we found extensive variation in setal morphology between clones, suggesting a strong underlying genetic component. Here, we found evidence for both lineage- and regional-specific genetic variation for this trait. Additional investigations are required to assess the functional significance of variation in the seta in the context of the ecology and distribution of *D. magna*. The clones employed in this study belong to a larger laboratory collection of clones sampled throughout the geographical range of the species. Extensive genomic information is available for these clones possibly allowing preliminary association analysis between genetic and phenotypic variation in setal morphology.

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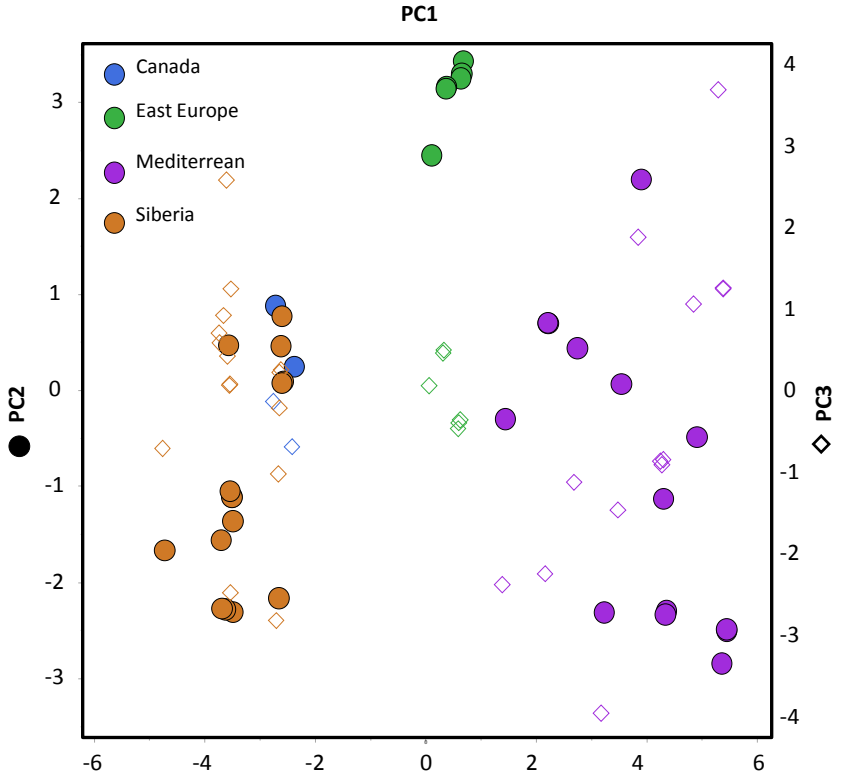

Figure S1: Principal component analysis of climate data for the sampling sites based on on 19 biolimatic variables (WorldClim). PC1 corresponds mainly to temperature variables, PC2 corresponds mainly to measures of precipitation variability and PC3 to measures of precipitation amounts.

Figure S2 (next page): Correlations among morphological measurements of 40 *D. magna* clones. Body size and four phenotypes of trunk limb II: stiff seta length, stiff seta thickness, stiff seta robustness and gnatobase 2nd stiff seta

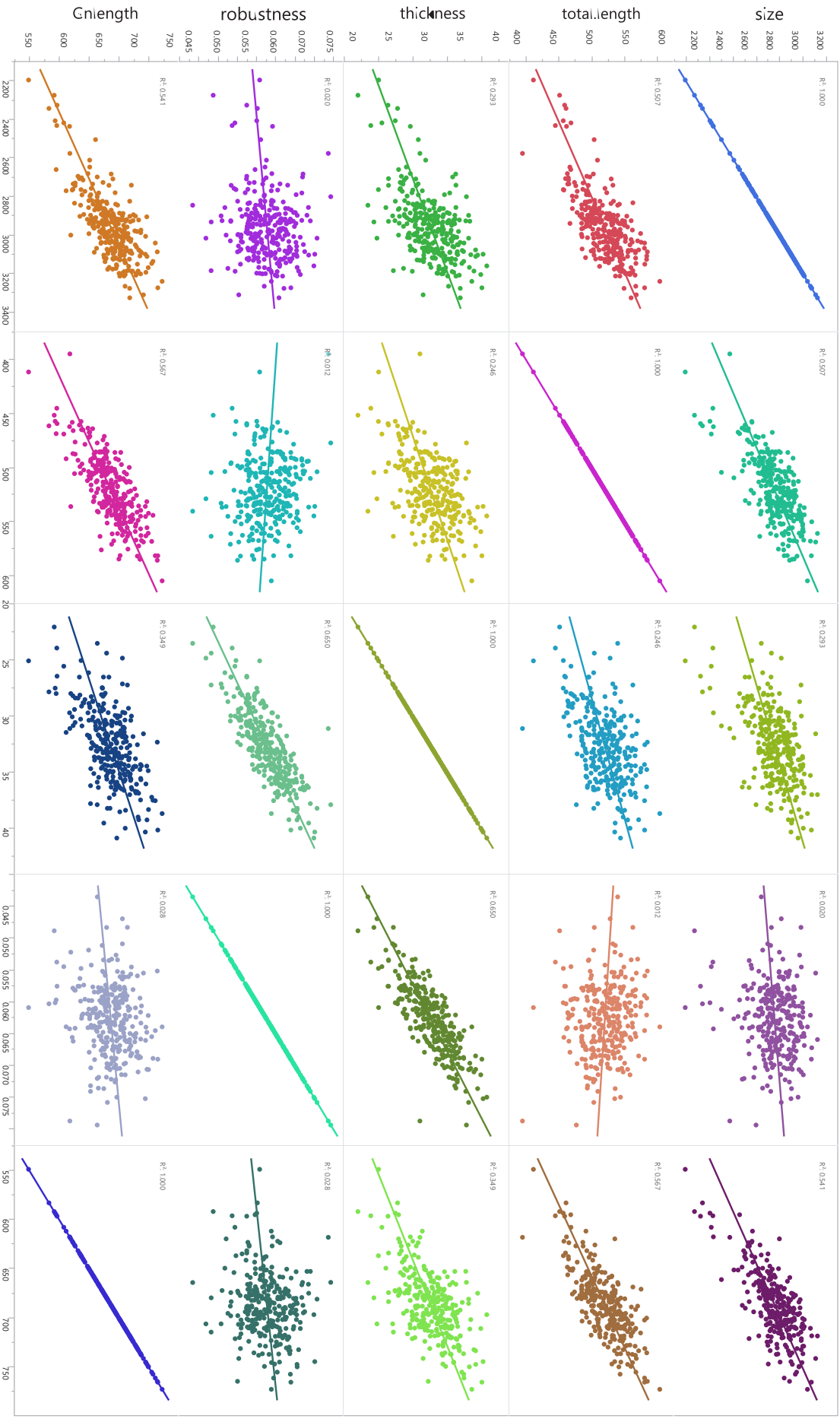
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Table S1: Principal component analysis of 19 bioclimatic variables for 39 sampling locations. The bioclimatic variables summarize year wise current temperature and precipitation conditions (interpolations of observed data, representative of 1960-1990). Loadings of the variables on the first 3 principal components (PC1-3) and % of variation explained by the PCs (clone CZ-N1-1 was excluded from the analysis).

Variable	Description	PC1	PC2	PC3
bio1	Annual Mean Temperature	**0.29842**	0.03181	-0.08384
bio2	Mean Diurnal Range	-0.18073	**-0.29425**	-0.05594
bio3	Isothermality (Bio2/bio7)	**0.27631**	-0.09403	-0.10400
bio4	Temperature Seasonality	**-0.28855**	-0.10736	0.09751
bio5	Max Temperature of Warmest Month	**0.26281**	-0.11228	-0.12416
bio6	Min Temperature of Coldest Month	**0.29464**	0.08701	-0.08890
bio7	Temperature Annual Range (bio5-bio6)	**-0.28557**	-0.13180	0.07495
bio8	Mean Temperature of Wettest Quarter	-0.15152	0.21943	0.07745
bio9	Mean Temperature of Driest Quarter	**0.29692**	-0.00951	-0.10959
bio10	Mean Temperature of Warmest Quarter	**0.28354**	-0.07372	-0.06879
bio11	Mean Temperature of Coldest Quarter	**0.29662**	0.06212	-0.09410
bio12	Annual Precipitation	0.17950	**0.21935**	**0.42049**
bio13	Precipitation of Wettest Month	0.13537	-0.17362	**0.54364**
bio14	Precipitation of Driest Month	-0.01278	**0.47475**	0.06576
bio15	Precipitation Seasonality	-0.02212	**-0.45579**	0.10187
bio16	Precipitation of Wettest Quarter	0.15901	-0.11357	**0.54872**
bio17	Precipitation of Driest Quarter	0.01031	**0.47308**	0.05060
bio18	Precipitation of Warmest Quarter	**-0.23502**	0.20609	**0.23751**
bio19	Precipitation of Coldest Quarter	**0.26443**	-0.08001	**0.24873**
		57.09%	**21.73%**	**11.69%**

Table S2: Table of p-values of linear mixed models with one bioclimatic variable as fixed effect, size as a covariate (not for behaviour and robustness) and clone as random effect (type II ANOVA). For each phenotype, the P-values were corrected for false discovery rate of 19 tests.

Bioclimatic variable	Browsing	SS length	SS thickn.	SS robustn.	Gn length	browsing	SS length	SS thickn.	SS robustn.	Gn length
	p-values					Corrected p-values (fdr)				
Annual Mean Temperature	0.0648 .	0.404	<0.0001 ***	<0.0001 ***	0.0208 *	0.1398	0.6001	<0.0001 ***	<0.0001 ***	0.0907 .
Mean Diurnal Range	0.0406 *	0.5054	0.5054	0.0004 ***	0.0312 *	0.1398	0.6001	0.5334	0.0008 ***	0.0990 .
Isothermality (Bio2/bio7)	0.149	0.1969	<0.0001 ***	0.0001 ***	0.1046	0.2177	0.5689	<0.0001 ***	0.0004 ***	0.1806
Temperature Seasonality	0.0692 .	0.6538	<0.0001 ***	<0.0001 ***	0.0238 *	0.1398	0.6901	<0.0001 ***	<0.0001 ***	0.0907 .
Max Temperature of Warmest Month	0.0639 .	0.2096	<0.0001 ***	0.0002 ***	0.16	0.1398	0.5689	<0.0001 ***	0.0005 ***	0.2338
Min Temperature of Coldest Month	0.0639 .	0.5848	<0.0001 ***	<0.0001 ***	0.0223 *	0.1398	0.6536	<0.0001 ***	<0.0001 ***	0.0907 .
Temperature Annual Range	0.0536 .	0.7344	<0.0001 ***	<0.0001 ***	0.0177 *	0.1398	0.7344	<0.0001 ***	<0.0001 ***	0.0907 .
Mean Temp. of Wettest Quarter	0.9391	0.4295	0.0709 .	0.1378	0.8055	0.9391	0.6001	0.1035	0.154	0.8055
Mean Temp. of Driest Quarter	0.1984	0.3885	<0.0001 ***	<0.0001 ***	0.0496 *	0.2692	0.6001	<0.0001 ***	<0.0001 ***	0.1047
Mean Temp of Warmest Quarter	0.1348	0.1813	<0.0001 ***	<0.0001 ***	0.0467 *	0.2134	0.5689	<0.0001 ***	0.0001 ***	0.1047
Mean Temp. of Coldest Quarter	0.0736 .	0.4799	<0.0001 ***	<0.0001 ***	0.0215 *	0.1398	0.6001	<0.0001 ***	<0.0001 ***	0.0907 .
Annual Precipitation	0.0585 .	0.3307	0.0133 .	0.0022 **	0.1485	0.1398	0.6001	0.021 *	0.0039 **	0.2338
Precipitation of Wettest Month	0.0809 *	0.428	0.2158	0.3903	0.6713	0.1398	0.6001	0.2733	0.3903	0.7085
Precipitation of Driest Month	0.2593	0.0102 *	0.548	0.0701 .	0.3589	0.3284	0.0971	0.480	0.083 .	0.4546
Precipitation Seasonality	0.8211	0.0153 *	0.3657	0.0326 *	0.4739	0.8667	0.0970	0.4087	0.0433 *	0.5296
Precipitation of Wettest Quarter	0.0405 *	0.5029	0.133	0.2395	0.4195	0.1398	0.6001	0.1800	0.5280	0.4981
Precipitation of Driest Quarter	0.4468	0.0131 *	0.3565	0.0341 *	0.344	0.5305	0.0970	0.4872	0.0431 *	0.4546
Precipitation of Warmest Quarter	0.5022	0.0544 .	<0.0001 ***	0.0236	0.0811 .	0.5612	0.2585	<0.0001 ***	0.0344 *	0.1541
Precipitation of Coldest Quarter	0.0202 *	0.3002	<0.0001 ***	0.0055 **	0.0488 *	0.1398	0.6001	<0.0001 ***	0.0087 **	0.1047

Chapter V

A preliminary analysis of trunk limb II setal morphology in the genus *Daphnia*

Abstract

The ancestors of the cladocerans were benthic species that collected food by scraping on bottom surfaces, probably with the use of their first two trunk limbs. In *Daphnia*, feeding is mostly achieved by filter feeding in the water column by a complex set of setae and setulae on trunk limb III and VI. While a lot is known about filtering functional morphology and diversification, analyses of the setal equipment of trunk limb I and II of *Daphnia* are largely missing. This is unfortunate as previous considerations, and the work presented in this thesis, pinpoint to an important role of trunk limb II in the feeding biology of *Daphnia* species. Specifically, it is hypothesised that, in *D. magna*, one stiff seta on this limb is involved in food acquisition by scaping from surfaces, a distinct feeding strategy from pelagic filter feeding and similar to that of truly benthic cladocerans. In turn, *Daphnia* species might present different trunk limb II setal morphologies in relation to their benthic habits and ecology, a hypothesis that was never tested. Here, I elaborate on the evolutionary significance of the benthic functional morphology of *Daphnia* by summarizing the scarce and fragmented reports of trunk limb II setal morphology in the genus and I present a preliminary investigation of its inter-specific variation supported by a phylogenetic analysis. Among the eleven species analysed, the stiff seta was the most variable, compared to other setae of the limb. Species known to dwell or not in benthic habitats show distinct morphologies suggestive of different uses of the structure. We defined five morphological traits that, in different combinations, can be used to classify the seta. The phylogenetic distribution of the different morphologies indicates a common origin of similar setae. However, two distantly related species known to dwell in benthic habitats, *D. magna* and *D. pulex*, showed high similarities suggestive of convergent evolution. An analysis on a larger number of species is required to support, and to build on, these preliminary insights.

Arbore, R., Fields, P. and D. Ebert.
A preliminary analysis of trunk limb II setal morphology in the genus *Daphnia*.

Introduction

Two studies presented in this thesis (Chapters III and IV) have analysed morphological variation in the stiff seta of the most distal endite of trunk limb II of *D. magna* at two levels: within individuals or genotypes (plasticity) and within a single species. Here, I elaborate on the functional significance of the stiff seta in terms of variation between species (mainly within the genus *Daphnia*) in relation to benthic feeding. I begin by introducing the features of the hypothetical benthic ancestor of the Anomopoda (the suborder of the order the Cladocera including the vast majority of species) from which the families *Daphniidae* and *Moinidae* diverged through the evolution of planktonic lifestyles (here, *Bosminae* are not considered). Then, I summarize what is known about the functional morphology of the stiff seta in *Daphnia* and related genera (e.g. *Scapholeberis* and *Simocephalus*). Finally, I present the results of a preliminary exploration of trunk limb II setal variation in several species of the genus *Daphnia* supported by a dedicated molecular phylogenetic analysis based upon coding and structural mitochondrial sequences.

Early attempts to derivate the anatomical features of *Daphniidae* from a hypothetical ancestor sharing most of the attributes with extant benthic macrothricids (Fryer 1995) have recently been confuted (Smirnov & Kotov 2010). However, there is broad consensus about the general aspect and habits of the ancestral anomopods. These were benthic species that collected food by scraping and moved by crawling or by briefly swimming using the second antennae for propulsion (Fryer 1995). These species were able to produce both parthenogenetic and sexual eggs depending on ecological conditions. Of primary importance here are the features of the feeding system. Food was collected by means of a uniseriate row of spines that extended from the tip of trunk limb I and II to their proximal region. The food collected was accumulated in a cage formed by the limbs and moved forward towards the mouth by anterior movements of the gnatobases of trunk limb III, IV and V. This system recalls the features of a primitive gnatobasic filtering device. During the evolution of the *Dapniid* line (*Daphnia* and *Moina*), the increase in size and efficiency of the gnatobasic filters eventually enabled efficient suspension filter feeding and, ultimately, the emancipation from the bottom environments. Other adaptations for pelagic habits, such as larger sizes and specialization of the antennae for efficient swimming are thought to have appeared later. Concerning the features of the setal apparatus, a general tendency in the evolution of the *Dapniid* line has been the increase in the number of gnatobasic filter setae on trunk limb III and IV and a reduction in the number of stiff setae on trunk limb I and II. These changes reflect the switch from surface feeding to suspension filter feeding. As a result of the evolution of pelagic habits, *Daphniidae* have been able to colonize a smaller number of niches compared to extant benthic anomopods. However, as in the case of *D. magna*, some species might have retained of secondarily acquired bottom feeding habits and the use of one stiff seta on trunk limb II for scraping. Smirnov and Kotov (2010) propose that the setae of anomopods display a “striking potential for morphogenesis” and that morphological radiation of the setal apparatus is at the basis of the adaptive radiation of the order. While critical for the early differentiation and evolution of the extant families of the order, this feature of the setal apparatus might as well have played a role in the evolution of substratum-utilizing habits of some species of *Daphnia*.

Among the setae on trunk limb II, the stiff seta shows the highest variability among species while all the soft setae show very similar morphologies in different species (Benzie 2005). Functional and morphological descriptions are scarce in the literature and are summarized here. Other species of the genus *Daphnia* other than *D. magna* might be able to exploit benthic food sources by employing the stiff seta on trunk limb II (Fryer 1991). The function of the seta in *D.*

magna has been the subject of Chapter III and IV of this thesis and will not be further discussed in this section. *D. obtusa* is also provided with a scraper-like stiff seta that differ from that of *D. magna* by displaying a continuous row of evenly spaced strong spines along its entire distal half portion. This species is able to perform surface scraping and might be more skilled than *D. magna* in this regard. *D. middendorffiana* can feed on periphyton and has a finely serrate stiff seta on trunk limb II but no direct observation of its action has been reported. *D. occidentalis* has a short, curved and somehow serrate seta and, as *D. middendorffiana,* inhabits shallow artic pools where benthic food sources might be of particular relevance as phytoplankton is often scarce at these latitudes (Rautio & Vincent 2006). It is worth noticing that species of the genera *Simocephalus*, *Scapholeberis* and *Megafenestra* (belonging to the family *Daphniidae*) display very different stiff seta morphologies from those of *Daphnia* and are associated with their specific feeding habits. *Simocephalus velutus* uses its antennal hooks to attach itself on its back to suitable vertical surfaces and feeds on suspended particles in this position. The stiff seta is present but is heavily reduced in size to a minute structure clearly unsuitable for scraping as the feeding habits of the species also suggest. *Scapholeberis* and *Megafenestra* attach themselves to the surface film in an inverted position where they filter small particles such as pollen. However, the same features of the carapax margin that allow for this specialization allow this species to attach to a variety of solid substrata. The spine on trunk limb II are robust and armed with spines and appear to be suited to transfer to the filter chamber large food particles such as flocculent pieces of periphyton collected from surfaces.

The functional significance of the stiff setae in different *Daphnia* species in the context of the species' ecology and habits has been so far poorly investigated. This is unfortunate since, as exemplified above, it can have an important role in the feeding biology of many species. Moreover, setal morphology has never been explicitly considered in relation to the phylogenetic relationship between different species. Speculations about the relative importance of convergent selection vs. developmental constrains in generating patterns of morphological variation for the seal apparatus of *Daphnia* have been raised but were supported by little evidence (Smirnov & Kotov 2010). In the following, we present the result of the first, albeit preliminary, analysis of stiff seta morphology in *Daphnia* from a phylogenetic perspective.

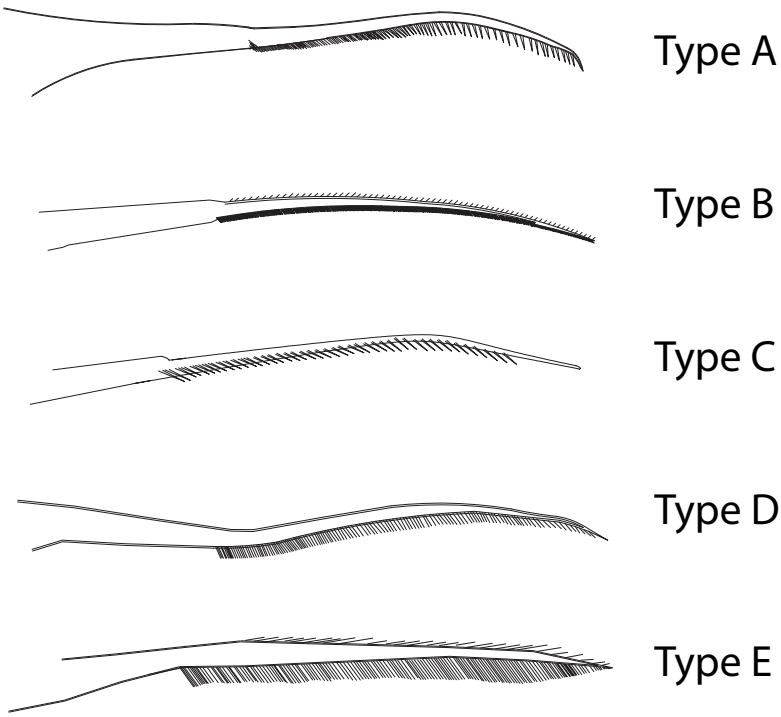


Figure 1 (previous page): Five morphological types of the most distal endite of trunk limb II of *Daphnia* identified in this study. Type A: *D. magna* and *D. pulex*. Type B: *D. similis*, *D. similoides* and *D. sinensis*. Type C: *D. lumholtzi*. Type D: *D. pulicaria*, *D. arenata* and *D. cf. "similis"* Type E: *D. galeata* and *D. longispina*. The morphological types are based on five characteristics of type and distribution of the setulae of the seta (see text and Figure 2).

Material and methods

Experimental animals

I documented stiff seta morphology from the exuviae of 2 individual females of each of eleven *Daphnia* species. I analysed two clones for *D. magna*, from Russia and Czech Republic (RUTY-4 and CZN1-1), as these are known to belong to different genetic linages. Two clones for *D. similis* from Israel and Russia (IL-SIM-A20 and RU-BU1-3), one of *D. sinensis* (sRU-NOV1-01) and one of *D. similoides* (RU-SZB3-2) from Russia were included. The clones of *D. sinensis* and *D. similoides* belong to the *D. similis* group and might be *D. similis*. One clone from Canada (CA-CBC-31) is now referred as *D. cf "similis"*, and probably belongs to the *D. exilis*-group. Finally, we included one clone of *D. lumholtzi* from Arizona, one clone of *D. pulicaria*, one of *D. arenata* from North America, one clone of *D. longispina* from Finland (FI-G-95-1) and one of *D. galeata* and one clone of *D. pulex* from Switzerland (CH-H-7). The clones belonged to monoclonal population normally maintained in the laboratory in the Ebert Lab. The clones originated either from field collected plankton samples or were hatched from field collected resting eggs. Field collected planktonic females were brought to the laboratory, and individual females were allowed to reproduce asexually. These lines were kept in the laboratory under conditions of continuous asexual reproduction. Information about the habitat of the different species (rock pools, ponds and lakes) were retrieved from the literature.

Morphological analyses

The exuviae of recently moulted animals (<24 h) were rinsed briefly in ADaM to remove attached material and transferred individually to a microscope slide with a glass pipette. Liquid in excess was removed with a piece of filter paper until a thin layer covered the exuvia; this prevented dehydration and kept the exuvia adherent to the slide facilitating dissection. The dissections were performed using two thin metal needles under a dissecting microscope with dark field illumination. First, by pulling the second antennae apart from the capapax, the two carapax valves were separated from the exuvia. Attached to the second antennae remained the residual exosceletron, including the armature of the trunk limbs and the post abdomen. One of the two trunk limbs II was isolated, and all remaining parts removed. The setae of the limb were spread with attention on avoiding the overlapping of setea with the stiff seta (anterior seta of the distal endite of limb II) and the gnatobase. A glass cover slip, covering the trunk limb II, was then gently placed on the specimen, liquid in excess was removed with filter paper to make it adhere and its sides were sealed with nail polish to avoid dehydration. The specimens were kept covered in plastic boxes with water-soaked paper towels at 10 °C for not more than 2 hours before being photographed.

mtDNA access, assembly, and sequence alignment

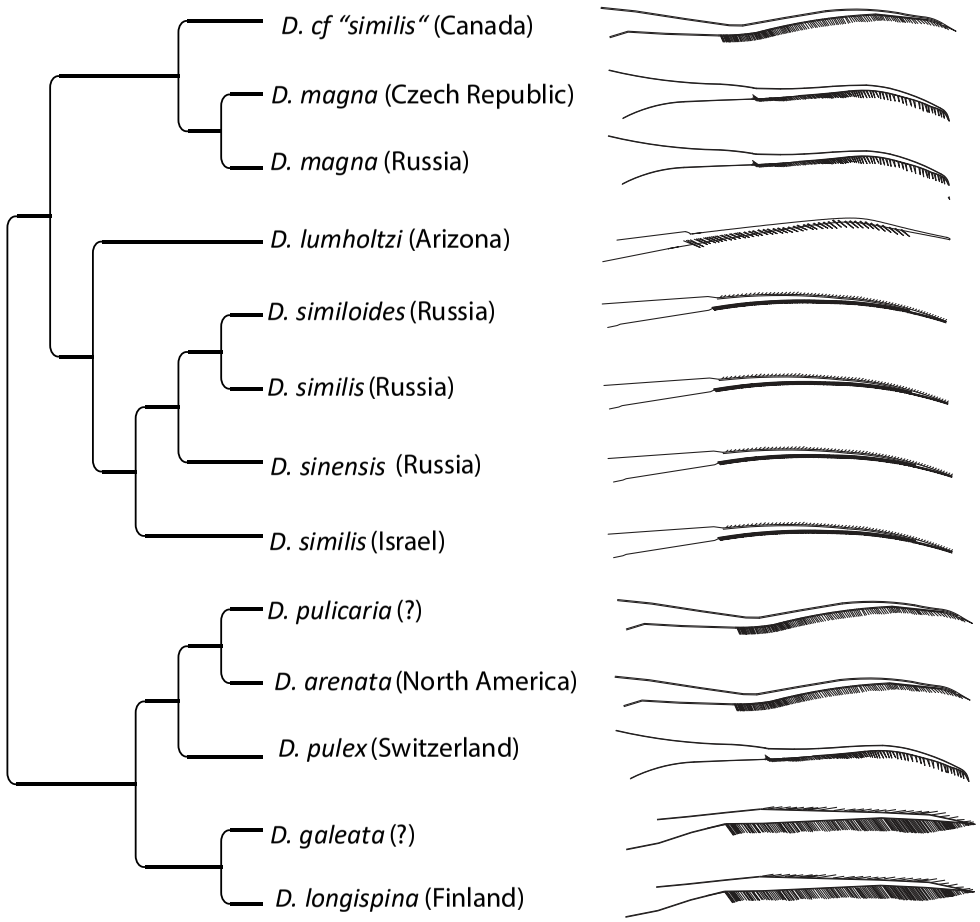
The following tasks, including the generation of the phylogeny of the species and clones used in this study, were performed by Dr. Peter Fields at the University of Basel. Requisite datasets for the assembly of the mitochondrial genomes of *D. arenata* derive from NCBI SRA accessions PRJNA247438, respectively. All additional mitochondrial genomes utilized in the present study

were generated with materials generated in the Ebert lab. In order to reduce non-focal DNA in the sequencing reactions (e.g. general microbiota components or algal food source DNA), individuals were treated for 72 h with three antibiotics (streptomycin, tetracycline, ampicillin) at a concentration of 50 mg/L each, wherein the treatment was refreshed every 24-hours. Clones were fed with dextran beads (Sephadex 'Small' by Sigma Aldrich: 50 lm diameter) at a concentration of 0.5 g/100 mL to aid in the expelling of gut contents which will also contribute to non-focal DNA sources. Animals were moved out of antibiotics and into 1.5-mL Eppendorf microcentrifuge tubes and excess fluids removed with a sterile pipette. Extraction buffer (Qiagen GenePure DNA Isolation Kit) was subsequently added to the tubes and tissue was disrupted using sterile and DNA free plastic pestles. The resultant solution was incubated overnight with Proteinase K at 55°C. RNA was degraded using RNAse treatment for one hour at 37°C. Protein removal and DNA precipitation, including the addition of glycogen to aid in DNA precipitation, were done using the Qiagen GenePure DNA Isolation Kit instructions. Resultant purified DNA was suspended in 40uL of Qiagen DNA hydration solution and subsequently tested for purity and concentration using a Nanodrop and Qubit 2.0, respectively. Libraries were prepared using Kapa, PCR-free kits. Paired-End 125 cycles sequencing was performed at the Quantitative Genomics Facility service platform at the Department of Biosystem Science and Engineering (D-BSSE, ETH), in Basel, Switzerland, on an Illumina HiSeq 2000.

Whole mitochondrial genomes were assembled *de novo* using the reference assisted approach MITObim (Hahn 2013). The reference mitochondrial genome derives from XINB3 individual genome (V2.4; *Daphnia* Genome Consortium). Individual PE read datasets were subsampled down to two million reads using SEQTK (https://github.com/lh3/seqtk) four different times, each time using a different seed in order to generate four different input datasets. Each assembly was then interrogated for assembly error and consistency using the following quality control steps. The full read dataset was aligned to each reference using BWA MEM (Li 2013), the resulting sam alignment file being subsequently converted to a bam and coordinate sorted using samtools (Li 2009). Alignments were then visualized using Tablet (Milne *et al*. 2013) in order to detect assembly and SNP errors by eye. All four individual mitochondrial assemblies were then aligned to one another using MAFFT v.7 (Katoh *et al.* 2002). Any assembly specific inconsistencies amongst the four were subsequently interrogated by comparing the alignments, resulting in a consensus assembly for each mitochondrial genome. Individual mitochondrial genomes were independently annotated using the MITOS webserver (Bernt *et al.* 2013), with the genetic code 'invertebrate' selected. Resultant annotations were then used to identify each of the 13 coding genes, two structural rRNA genes, and 22 tRNA genes. Individual genes as well as whole mitochondrial sequences were aligned to one another using the multiple sequence aligner MAFFT v.7 (Katoh *et al.* 2002).

Sequence partitioning and phylogeny construction

Partitionfinder v.2.1 (Lanfear *et al.* 2016) was used in order to partition the concatenated grouping of 13 coding and two structural rRNA gene alignment into appropriate mutation model groupings. The resultant best scheme included 27 separate partitions. BEAST2 (Bouckaert *et al.* 2014) was used to estimate the phylogenetic relationships amongst the utilized mitochondrial genomes. We included the aforementioned partitions to parameterize individual site models and specified that sites be constrained to a single linked tree. We specified a Yule model Prior with a MCMC chain length of 10000000. The resultant posterior was visualized with Tracer (Rambaut *et al.* 2014). The posterior median tree was estimated with the BEAST2 package TreeAnnotator (Bouckaert *et al.* 2014) after removing the first 50% of the posterior as burn-in.



	Type	Presence of strong setulae on inner margin	Uniform distribution of setulae on inner margin	Presence of a unique type of setulae	Presence of long setulae	Presence of setulae on outer margin	Habitat
D. cf "similis" (Canada)	D	0	0	0	0	0	Pond
D. magna (Czech Republic)	A	X	0	0	0	0	Pool, pond, lake
D. magna (Russia)	A	X	0	0	0	0	Pool, pond, lake
D. lumholtzi (Arizona)	C	X	0	0	0	0	Pond, lake
D. similoides (Russia)	B	0	X	X	0	X	Pond
D. similis (Russia)	B	0	X	X	0	X	Pond
D. sinensis (Russia)	B	0	X	X	0	X	Pond
D. similis (Israel)	B	0	X	X	0	X	Pond
D. pulicaria (?)	D	0	0	0	0	0	Pond, lake
D. arenata (North America)	D	0	0	0	0	0	Pool,pond
D. pulex (Switzerland)	A	X	0	0	0	0	Pond, lake
D. galeata (?)	E	0	X	X	X	X	Lake
D. longispina (Finland)	E	0	X	X	X	X	Rock, pool

Figure 2 (previous page): Phylogenetic relationships between the 11 *Daphnia* species considered and features of the most distal endite of trunk limb II. The phylogenetic analysis was based upon coding and structural mitochondrial sequences and is supported by posterior probabilities equal to 1 for every node. For *D. magna* and *D. similis*, two clones each were included. The schematic drawings of the setae are based on our reconstruction using five characteristics of type and distribution of the setulae of the seta (see text and Figure 1). Information about the habitat of the different species were retrieved from the literature.

Results and discussion

The results of the analyses are summarized in Figures 1 and 2. We recognized five distinct and recurrent phenotypes of stiff seta among the clones/species. We identified five features of setal morphology that are present in different combinations on the setae determining the morphological types (Figure 2). These are: the presence of strong or soft setulae on the margin of the seta, the distribution of the types of setulae which can be uniform or can vary along the distal half portion of the seta, the presence of a unique type or both types of setulae on the same seta, the length of the setulae and, finally, the presence of a row of spines on the outer margin of the seta.

The "*magna*" type (A) has strong spinules that become smaller, thinner and more tightly spaced towards the middle of the seta. The outer margin is smooth. This setal morphology is described by Fryer (1991) as suited for scratching. Indeed, it is found both in *D. magna* and in *D. pulex*, two species that have been described as able to feed on the bottom (Horton et al. 1979). It is notable that the morphology of the seta is so similar between these two distantly related species (Figure 1). The "*similis*" type (B) displays on its ventral margin a single type of short, soft setulae tightly spaced along the distal half of the seta. This morphology is found both *D. similis* clones, in *D. sinensis* and in *D. similoides* (the *D. similis* group species*)*. The "*lumholtzi*" type (C) (only found in this species) has strong setulae similar to those of the distal portion of the *magna* type but evenly distributed and regularly spaced along the entire seta. The outer margin is smooth. The "*pulicaria*" type (D) has soft short setulae that slightly increase in size towards the distal-most portion of the seta. Together with type A (*D. magna* and *D. pulex*) this seta shows a morphology that might be suited for scraping even if the strong spines are less numerous and smaller. The outer margin is smooth. This "*pulicaria*" type is found also in *D. arenata* and in the distantly related *D. cf "similis"* clone, probably belonging to the *D. exilis* group. Interestingly, all this species are pond dwellers and might therefore be equipped for benthic feeding in shallow ponds. However, more species and clones, and further ecological information, should be collected to corroborate this speculation. The "*galeata*" type (E), found also in *D. longispina*, is quite different from all the others being shorter and thicker. Its ventral margin displays a row of very long and soft setae evenly distributed along the seta. Fryer (1991) describes *D. galeata* as strictly planktonic and this species is mostly found in lakes. This peculiar morphology might reflect specific feeding conditions found in lakes. For example, *D. galeata* frequently ingests inorganic particles and might consume the films of organic matter adhering to the particles' surface. The *D. longispina* clone analysed came from a rock pool, but nevertheless it showed a very similar seta. These two species are sister species in our phylogeny and similarities between the setae might reflect their common origin. It remains to be further addressed whether these structures, very distinct from those found in other species, serve to a specific feeding mechanism shared by the two species in different environments. Again, a larger number of species and clones and detailed ecological information would be required for such analysis.

Given the limited number of species included in this analysis, is it not possible to derive strong conclusions about the phylogenetic patterns of diversity in stiff seta morphology in the genus *Daphnia*. However, we found consistent morphologies between closely related species suggestive of a shared common origin as between *D. longispina* and *D. galeata* and between the species of *D. similis* group. This evidence might be strengthened by the analysis of a larger number of species. In previous works (Chapter III and IV) I never observed any changes in the general morphology of the seta both between *D. magna* clones and between culturing conditions. Thereby, it is unlikely that the differences we observed here are the result of phenotypic plasticity or features of specific clones. Very similar morphologies have been found also between distantly related species such as *D. magna* and *D. pulex*. Moreover, *D. obtusa* (not included in the morphological analysis) also shows a setal morphology that, even if slightly different from that of the *magna* type, might be suited for scraping. Parallelism in the evolution of setal morphology might therefore be common. An analysis on a larger number of species might shed light on the relative contribution of convergent natural selection or of "design limitations" (Wake 1991) on setal evolution.

Conclusions

The functional morphology of the setal apparatus of *Daphnia* lies at the core of the adaptive radiation of the genus. Pelagic habits and suspension filter feeding represent key innovations and greatly contributed to shape the features of present-day fresh water ecosystems. I believe that a multi-level analysis of setal functional morphology in the genus (and in closely related taxa) deserves further investigation. The comparative analysis of trunk limb II setal morphology presented in this chapter is meant to be a preliminary exposition of the significance of this line of research. Further analysis of setal morphology and function in *Daphnia* and other crustaceans might provide additional knowledge of the evolutionary history of these fascinating organisms.

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Conclusions and final remarks

My PhD project focused on the study of the adaptations of the ecological and evolutionary model organism *Daphnia magna* to the benthic environments, with a focus on its feeding mechanisms. A benthic life style is ancestral in the Cladocera, with a planktonic life style being derived (Fryer 1991). In this regard, *D. magna* combines an ancestral with a derived life style. However, this aspect of *Daphnia* biology is still poorly integrated in eco-evolutionary studies of the genus. Reason for this are, to some extent, the acknowledgment that most species of the genus are key species in pelagic environments and the focus given to the mechanistic study of their sophisticated suspension filter feeding apparatus. However, as it has been pointed out by some authors (e.g. Horton *et al.* 1979 and Fryer 1991), some species of the genus, including *D. magna*, are by no means strictly pelagic and can engage in a number, and possibly ecologically important, interactions with the benthic environments. Recently, some authors have investigated the ecological relevance of these interactions in the light of the new focus considering the coupling of benthic and pelagic food webs (e.g. Siehoff et al. 2009, Cazzanelli *et al.* 2012). However, behavioural, morphological and functional studies in *Daphnia* species focusing on their benthic interactions are still largely missing. In this perspective, lies the work that I conducted during my PhD and I presented in this thesis.

Most of the work of my thesis has been inspired by the considerations of Fryer in his monography of *Daphnia* physiology (Fryer 1991). Also, a large conceptual contribution came from the recent insights of Smirnov and Kotov on the potential for morphogenesis of the setal apparatus of cladoceran crustaceans (Smirnov and Kotov 2010). Chapters I, II and IV of my thesis dealt with the behavioural interactions of *D. magna* with bottom sediments (i.e. browsing behaviour) from genetic and functional perspectives (Arbore *et al.* 2016 and Mushegian *et al.* 2019). Chapters III, IV and V focused on morphological variation in a previously poorly studied limb of *Daphnia* (trunk limb II) at different levels: plastic responses to food treatments within *D. magna* genotypes, genetic variation between *D. magna* clones spanning the geographical range and different habitats of the species and morphological comparisons between species of the genus. Altogether, I have approached different aspects of the benthic habits of *Daphnia*, behaviour and morphology, within a broad framework spanning from genetic adaptation to different environments, phanotypic plasticity, mechanistic analyses of environmental interactions and the evolutionary history of the species. The integration of the scarce and fragmented body of literature concerning the benthic habits of *Daphnia*, together with my original results, highlighted a perspective on the feeding biology of *Daphnia* where a traditionally neglected habitat is regarded as critical, and might contribute to a broader understanding of *Daphnia* ecology and evolution.

The work I conducted on browsing behaviour largely relied on the development of a behavioural assay to quantify sediment browsing.in *D. magna*. This assay has allowed, for the first time, to accurately quantify the sediment browsing activity of the animals, whose evidence has been previously almost entirely anecdotal (Arbore *et al.* 2016). The method has been proven robust: despite the high level of noise typically associated with behavioural measurements, the results showed high repeatability and the method could be used to clearly discern different clonal lines by their propensity to browse. Most importantly, the method is reasonably scalable and allows the screening of hundreds of individual replicates within few days. Over the course of my PhD,

the method has been applied to several sets of *D. magna* clones and each time provided better estimates of broad-sense heritability for browsing behaviour. This reflects the increased number of clones included in the studies and the technical optimization of the protocol. Most of what is known about *Daphnia* behavioural genetics comes from the analysis of phototactic behaviour. The proportion of phenotypic variance attributable to genetic differences between clones for phototactic behaviour vary considerably between studies, populations and environmental conditions (e.g. De Meester 1989, Cousyn *et al.* 2002). My estimates for browsing behaviour are within this range and suggest that genetic variation for sediment browsing might greatly influence the behavioural interactions between *Daphnia* and its environment. Arguably, sediment browsing should be considered as an important component of the behavioural repertoire of *D. magna* and, possibly, of other species of the genus. Future studies focusing on behavioural syndromes, suites of correlated behavioural traits such as, for example, habitat selection, should consider sediment browsing in order to describe more comprehensively the role of behaviour on *Daphnia* ecological interactions. In chapters I and IV of my thesis, I began to analyse the ecological determinants of variation in sediment browsing in *D. magna*. While no effect was detected for a hypothesised role of fish predation (Chapter I), further work (Chapter IV) showed how browsing levels can vary accordingly to the physical properties of the original habitat of different clones, namely water body surface area. Specifically, as clones sampled from big lakes tended to browse less, it is reasonable to assume that larger water body dimensions might favour more strictly pelagic habits than smaller and shallow lakes and ponds, where the bottom might represent an accessible and food-rich environment (Rautio & Vincent 2006, Cazzanelli *et al.* 2012). A broader and dedicated sampling including multiple clones from natural populations from different habitat types distributed within a limited regional scale might provide additional insight about the existence of local adaptation for browsing behaviour in *D. magna* populations. Additional assays should be conducted to broaden our understanding of sediment browsing. Beside its constitutive genetic component, variation in this behaviour is undoubtedly tightly linked to the immediate environmental conditions experienced by the animals (Horton *et al.* 1979). Although it is known that *D. magna* engages in browsing only when suspended food is scarce, this has never been tested quantitatively. It would be interesting to screen the browsing behaviour of different clones while manipulating the relative abundance of suspended and sediment-derived food. Additionally, an analysis of browsing behaviour in light and dark conditions might provide additional insights to the study of diel vertical migration, the phenomenon by which *Daphnia* dwells the deep, dark, layers of lakes and ponds during the day in the presence of diurnal fish predation in the water column (Destasio *et al.* 1993). In *D. magna*, there is a behaviourally mediated trade-off between the risk of predation by planktivorous fish and the risk of infection by parasite spores taken up from the sediment (Decaestecker et al. 2002). The degree to which *Daphnia* stay higher or lower in the water column, and thus farther from or closer to the sediment, is largely influenced by phototactic behaviour (De Meester 1989). Consequently, more negatively phototactic genotypes have a higher infection risk compared to more positively phototactic genotypes. However, how variation in browsing behaviour influences infection risk by affecting the intake of parasite spores is not known. Such a study is nevertheless made complicated by the existence of genetic variation for infection susceptibility and a large number of clones with similar susceptibility to parasite clonal lines (for example of the *Daphnia* obligate pathogen *Pasteuria ramosa*) but with different levels of browsing propensity would be needed. The finding that browsing behaviour consistently varies among *D. magna* clones has provided resource to test the important, and so far, poorly investigated, link between genetic variation in behaviour and microbiota acquisition from the environment. The work presented in Chapter II of my thesis (conducted in collaboration with Alexandra Mushegian at the University of Basel) found host-genotype-specific effects on *Daphnia*

microbiome mediated by genetic variation in browsing behaviour in an experimental setting (Mushegian *et al.* 2019). Importantly, this work highlighted how behaviour can be considered a genetic factor that shapes microbial exposure, and thus microbiome composition, in a given environment. The complexity of its ecological interactions, coupled with its genetic tractability, makes *Daphnia* an ideal model for ecoevolutionary studies. The focus on *Daphnia* behavioural interactions with the benthic environments, including microbiota and parasite encounter and acquisition, should provide novel inspirations in *Daphnia* eco-evolutionary research.

In chapters III, IV and V of this thesis, I presented three analyses of morphological variation of the stiff seta of the most distal endite of trunk limb II of *Daphnia*. As described in my thesis, this seta has probably a function in scraping food from submerges surfaces (e.g. epiphyton), an alternative feeding strategy adopted by the bottom-dwelling species *D. magna* (Fryer 1991). A most notable evidence in support of the role of the seta in scraping lies on its many analogies, in morphology and location, with the stiff setae of benthic anomopods (macrothricids) that indeed collect food by scraping by means of an elaborate assemblage of stiff setae on their first trunk limbs (Fryer 1991, Smirnov & Kotov 2010). Fryer therefore suggests that *D. magna* might be equipped to collect food in a similar, albeit less sophisticated, way. In general terms, trait function and morphology can be tightly correlated. Such a condition seems to apply to the setal equipment of crustacean and spiders (whose setae are often undistinguishable between the two taxa). In crustaceans, Garm and Watling (2013) classified limb setae into seven morphological categories each associated to a particular function. These authors use the term "serrate setae" for the type of setae that in cladoceran literature are called "anterior" or "stiff" (in contrast to "posterior" or "soft" setae) (Smirnov & Kotov 2010). Setal function in crustacean is best studied in decapods where serrate (stiff) setae serve a variety of functions including prey handling and grooming. Setal morphology appears remarkably various in crustaceans. Nevertheless, similar setal types are found even between distantly related taxa. The direct link between morphology and function in crustacean setae evidences the utility of functional morphology studies for expanding the knowledge of crustacean diversity and evolution. The comparative analysis of trunk limb II setal morphology presented in chapter V is meant to be a preliminary exposition of the significance of this line of research in *Daphnia*. Specifically, I have focused on the possible link between trunk limb II stiff seta morphology and *Daphnia* species' habitats, in relation to the possible role of the structure in benthic feeding in different environments. This analysis suggested that parallelism in the evolution of stiff seta morphology might be common. For example, distantly related species as *D. magna* and *D. pulex* presented similar setal morphologies and have very similar benthic habits (Horton *et al.* 1979). Further analysis of setal morphology and function in *Daphnia* species in relation to their benthic and pelagic habits might provide additional knowledge of the evolutionary history of the genus.

In his monography of functional morphology of *Daphnia*, Fryer (1991) indulges in highlighting the many adaptations of *D. magna* for inhabiting and exploiting the bottom environments. He also provides a description of the stiff seta of trunk limb II and of the surface scraping behaviour of *D. magna*. However, he could only hypothesize the function of the seta for scraping on the bases of its general morphology and position and orientation on the limb. Despite the sophistication of Fryer's observations, no clear evidence of the action of the seta was reported as such a task might be technically challenging. The work presented in chapters III and IV has attempted to provide indirect evidence to Fryer's insights by analyses of variation in the stiff seta of trunk limb II within clones (plasticity) and between clones. While no plastic response to two feeding treatments was observed in the morphology of the seta, a controlled feeding experiment showed that *D. magna* can fed on bottom algae, but that this has a negative impact

on its growth, a fact possibly relevant for the species' feeding biology. With only six clones included, this analysis was strongly suggestive of a relevant genetic component behind stiff seta morphological variation. Building on this evidence, the work presented in chapter IV identified high heritability for several stiff seta morphological measurements. 38 of the closes used in this study belong to two distinct genetic linages (Western Eurasia and East Asia). Largely, the morphological differences found for the seta were explained by this factor. In turn, no habitat effects for seta morphology were detected. However, testing whether or not variation in the stiff seta might have an adaptive value would require detailed ecological information for a large number of clones and remains to be clarified.

The use of moulted exuviae for the morphological analysis of *Daphnia* limbs been previously adopted by Pop (1991) who, however, reported the results only for few individuals and focused on trunk limb III and IV. After, two other studies adopted this method but again only on trunk limb III and IV (Macháček & Seda 2013, Macháček & Seda 2016). However, images of the setal apparatus of trunk limb I and II can be taken easily as the stiff setae are more resistant then the gnatobasic setae of the other limbs. This method might therefore be a useful tool for further functional morphology studies expanding the focus to the entire thoracic limb apparatus of *Daphnia*. The method allows for the measurement of hundreds of individuals and to collect repeated measurements of the same individual along its lifespan. Moreover, the method might have a utility in taxonomic identification. Here, the method allowed for the screening of several replicate individuals of many *D. magna* clones, the analysis of trunk limbs growth and the comparative analysis with other *Daphnia* species.

The multilevel analysis of morphological variation presented in this thesis represent the first work specifically dedicated to the second trunk limb of *Daphnia*, in relation to its feeding function. Most of what is known of *Daphnia* feeding functional morphology comes from studies of the gnatobasic filtering apparatus of trunk limbs III and IV. In my thesis, I argued on the importance of trunk limb II for the feeding biology of *D. magna* and, more generally, *Daphnia* species. I believe that a focus on the functional morphology of the benthic adaptation of *Daphnia* can greatly advance our understanding of its ecology and evolutionary history. This task will be greatly assisted by evolutionary developmental biology (evo-devo) studies aiming at uncovering the genetic bases of *Daphnia* trunk limb development and diversification within and between species. Together, my work on *Daphnia* benthic feeding functional morphology and on the ecological genetics and functional aspects of sediment browsing behaviour highlighted the interactions with the benthic environment as an important, yet often overlooked, aspect of the ecology of *Daphnia*. Recently, in ecological research, there has been increasing attention on the relevance of these interactions in the light of the new focus considering the coupling of benthic and pelagic environments. In this perspective, the work presented in my thesis might contribute to the integration of the benthic habitats into *Daphnia* ecoevolutionary models.

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