Universidade de Aveiro Departamento de Biologia
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ARIANA BORGES MOUTINHO

EFEITO DE FATORES AMBIENTAIS NA LONGEVIDADE DE *Daphnia magna*

EFFECT OF ENVIRONMENTAL FACTORS IN *Daphnia magna* LONGEVITY PARAMETERS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Isabel Maria Cunha Antunes Lopes, Investigadora Auxiliar do Departamento de Biologia e Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro e coorientação da Doutora Raquel Monteiro Marques da Silva, Investigadora Auxiliar do Instituto de Patologia e Imunologia Molecular da Universidade do Porto.

O júri

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palavras-chave

Temperatura, restrição alimentar, cobre, longevidade, *Daphnia magna*, sirtuínas, nicotinamidase.

resumo

Longevidade é a capacidade de um organismo sobreviver para além da idade média de morte para a espécie a que pertence. Pode ser modulada por vários parâmetros ambientais que influenciam o metabolismo celular, a oxidação, ou a integridade do ADN. Até ao presente já foram realizados alguns estudos com o objetivo de compreender a influência de vários fatores ambientais na longevidade dos organismos. No entanto, a maior parte destes estudos são limitados a um número reduzido de espécies modelo. Deste modo, é pertinente gerar mais conhecimento sobre a estrutura do genoma e respostas funcionais de genes às condições ambientais em espécies com ecologias bem conhecidas, de modo a promover uma melhor compreensão das interações gene-ambiente num contexto evolutivo. A metodologia mais direta para abordar esta questão é identificar genes ou intervenções que funcionam de forma semelhante de modo a se poder proceder à modulação do tempo de vida nos organismos. Componentes de insulina ou o fator de crescimento 1 da família da insulina (IGF-1), a cínase da rapamicina (TOR) e as sirtuínas (SIR) da família das desacetilases, reguladas pelo gene PNC1 (pirazinamidase e nicotinamidase), são alguns exemplos que apresentam essa característica. Levando isso em consideração, o principal objetivo deste trabalho foi avaliar a influência de diversos parâmetros abióticos na longevidade de duas linhagens clonais da espécie *Daphnia magna* Straus (K6 e BEAK), da sub ordem Cladocera. Para atingir este objectivo foram avaliados os efeitos da temperatura, da restrição alimentar e de diferentes níveis de cobre na reprodução (número total de neonatos produzidos) e no tempo de vida de *D. magna*. No caso de restrição calórica e cobre, foram ainda avaliados os efeitos na expressão génica e integridade (ocorrência de mutações) do gene PNC1, respetivamente. *Daphnia magna* foi selecionada como organismo para este estudo, por apresentar uma ecologia bem documentada, ser de fácil manutenção em laboratório e por se reproduzir por partenogénese, o que permite o estabelecimento de linhagens clonais, sendo desta forma uma ferramenta para discriminar diferenças genéticas (entre clones) do *background* experimental. Além disso, o genoma da espécie *Daphnia pulex* foi já sequenciado, permitindo a avaliação da relação entre a estrutura, expressão génica e respostas a nível populacional às alterações ambientais. De um modo geral, os resultados obtidos revelaram uma dependência entre linhagens clonais e a influência dos parâmetros ambientais na longevidade. O aumento da concentração de cobre e da temperatura provocou uma redução na produção de descendência e também na longevidade de *D. magna*. A restrição alimentar também induziu um decréscimo na produção de descendência nas duas linhagens clonais. A análise das sequências de ADN apenas revelou polimorfismos semelhantes em todas as amostras, o que sugere que não foram induzidas quaisquer mutações devido a exposição aos fatores ambientais em estudo. Também não foram identificados efeitos significativos de restrição calórica na expressão relativa do gene PNC1.

keywords Temperature, food restriction, copper, longevity, *Daphnia magna*, sirtuins, nicotinamidase.

Abstract

Longevity is the ability to survive beyond the average age of death for the species. It can be modulated by several environmental parameters that influence cellular metabolism, oxidation, or DNA integrity. A set of works has already been carried out in order to understand the influence of environmental factors in longevity. However, most of these studies are restricted to a narrow number of model species. It is then pertinent to generate more knowledge on the genome structure and on the functional responses of genes to environmental conditions within species with tractable ecologies, aiming to improve the understanding of gene-environment interactions in an evolutionary context. The most direct way to address this problem is to identify genes or interventions that function similarly to modulate life span in different organisms. Components of the insulin or insulin-like growth factor 1 (IGF-1) signaling pathway, the nutrient-responsive target of rapamycin (TOR) kinase and the sirtuin (SIR) family of protein deacetylases, regulated by PNC1 gene (pyrazinamidase and nicotinamidase), among others, have been found to have this property. Taking this into consideration, the main goal of this work was to assess the influence of several environmental parameters in the longevity of two clonal lineages of the cladoceran species *Daphnia magna* Straus (K6 and BEAK). To attain this purpose, the effects of temperature, food restriction and different copper levels were evaluated in the reproduction (total number of neonates produced) and life span of *D. magna*. The effects in the gene expression and genomic integrity of the PNC1 gene (occurrence of mutations) were assessed in the food restriction and copper assays, respectively. The model organism *Daphnia magna* was selected to address the purposed objective as its ecology is well understood, it is of easy maintenance and reproduces by parthenogenesis, which allows the creation of clonal lineages, thus providing a tool to discriminate genetically based differences (among clones) from the experimental background. In addition, the genome of the closely related *Daphnia pulex* has been sequenced, allowing the assessment of the relationship between structure, gene expression and population-level responses to environmental changes. In general, the obtained results revealed a clonal lineage dependency on the influence of the environmental parameters in longevity. An increased temperature and copper concentration provoked a reduction in the reproductive output and as well as in the longevity of daphnids. Food restriction also induced a decrease in the reproductive output of both clonal lineages of *D. magna*. After DNA sequencing, only similar SNPs were found in all samples and therefore, no mutational alterations were caused by exposure to the studied environmental factors. No significant effects occurred in the relative gene expression of PNC1 due to caloric restriction.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
APS	amonium persulfate
ASTM	American Society for Testing and Materials
BLAST	Basic Local Alignment Search Tool
bp	base pairs
cDNA	complementary DNA
DNA	deoxyribonucleic acid
CDS	coding sequence
DO	dissolved oxygen
DOE	U.S. Department of Energy
EDTA	ethylenediaminetetraacetic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IGF-1	insulin-like growth factor 1
ISO	International Organization for Standardization
mRNA	messenger RNA
NA	nicotinic acid
NAM	nicotinamide
NCBI	National Center for Biotechnology Information
OECD	Organisation for Economic Co-operation and Development
PCR	polymerase chain reaction
PNC1	pyrazinamidase and nicotinamidase
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIR	sirtuin
SNP	single nucleotide polymorphism
Stdev	standard deviation
TBE	Tris/Borate/EDTA
TOR	target of rapamycin

1. INTRODUCTION

1. INTRODUCTION

1.1. *Longevity and aging*

Longevity is the ability to survive for longer than the species average age of death (De Benedictis and Franceschi, 2006), expressed in a cohort measure (Carey and Judge, 2001). So, the definition includes not only the individual capacity to reach old age but also the population-level mortality (Canudas-Romo, 2010). This term is often mistaken for life expectancy, which is the average period of time an organism (or a population) is expected to live, as determined by statistics (Sullivan and Sheffrin, 2003; The American Heritage Medical Dictionary, 2008). Still, another remark can be made for the term life span, which is the genetically endowed limit to life an individual of a species can live (Segen, 2006).

The consequence of an extended period of life is aging, an unavoidable part of life history of an individual (Partridge and Barton, 1993; Gems and Partridge, 2012) that can be observed in all species (De Benedictis and Franceschi, 2006). It consists of an intricate process of cumulated changes in molecular and cellular structures, resulting in the decline of the ability to maintain biochemical and physiological functions, thus increasing susceptibility to disease and death (Fontana et al., 2010). According to McDonald and Ruhe (2011), aging and longevity are different concepts, even though its discrimination may be difficult to apprehend. The rate of aging may affect the length of the life span, although longevity is *per se* independent from the biological aging process.

The longevity of an individual is a complex feature, dependent on many components that modulate multifactorial phenotypes. Such components can be both biological, for instance genes (Finch and Tanzi, 1997; Puca et al., 2001; Browner et al., 2004; Rose et al., 2008), hormonal and growth factor signaling (Coschigano et al., 2000; Flurkey et al., 2001) body weight (Piantanelli et al., 2001), body fat content (Blüher, 2003) and non-biological (extrinsic factors), such as environmental (Coschigano, 2000; Masoro, 2000) and stochastic elements (De Benedictis and Franceschi, 2006). The potential life span of an individual can be

greatly determined by the gene-environment interplay, which ultimately modulates the rates of molecular and cellular deterioration during aging (Finch, 1990; Finch and Tanzi 1997; Pedersen et al., 2012).

The role of genetics in determining longevity is intricate and ambiguous (Finch and Tanzi, 1997). Some mutations significantly have positive and negative impacts, modifying the life span of nematodes and fruit flies (Finch and Tanzi, 1997 and references therein). Nevertheless, the heritability of life span has a relatively minor contribution, accounting for approximately 25% of its variance, in short-lived invertebrates such as nematodes (Johnson and Wood, 1982) and the fruit fly (Promislow et al., 1996) and in mammals, like mouse (Gelman et al., 1988) and humans (Herskind et al., 1996; Mitchell et al., 2001). The remaining 75% are due to environmental influences and chance (Browner et al., 2004), thus modifying the outcomes of longevity.

1.2. *Model organism*

Simple eukaryotic organisms have been used to analyze the pathways that regulate the aging and life span processes (Kaeberlein, 2007). Of these, the budding yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*, have been the most extensively used and best characterized model organisms regarding longevity (Fontana et al., 2010). Invertebrate model organisms are important tools for this kind of research because they are relatively short-lived, have profuse information on databases and are of ease genetic analysis (Partridge, 2009). However, little is known in other model species, namely aquatic ones, such as the freshwater planktonic crustacean *Daphnia magna* Straus, 1820. *Daphnia magna* has a well studied ecology and a sentinel role within freshwater ecosystems, turning this species into a particularly amenable system for ecological genomics that aims to understand the genetic mechanisms underlying responses of organisms to their natural environments (Eads et al., 2008; Ungerer et al., 2008).

Daphnia is commonly used as a model system for cyclical parthenogenesis in ecological and evolutionary research. Cyclically parthenogenetic organisms,

which reproduce both sexually and asexually, are common in nature, in both the animal and plant kingdoms (Hebert, 1987; Ellstrand et al., 1996). This mode of reproduction, especially its asexual (clonal) component, has attracted considerable interests among population geneticists and evolutionary biologists (Yin et al., 2012).

Under favourable environmental conditions, *Daphnia* reproduces by parthenogenesis, forming all-female clones (Eads et al., 2008). This life cycle enables the creation of lines for mapping studies, and provides a tool to discriminate genetically based differences (among clones) from experimental or developmental noise by measuring replicates within a clone (Eads et al., 2008; Tollrian et al., 2010). The genome of the closely related *Daphnia pulex* has already been sequenced, allowing us to assess the relationship between genome structure, gene expression and population-level responses to environmental change in *Daphnia magna* as well (Colbourne et al., 2011).

Many abiotic and biotic factors determine the natural fluctuations of zooplankton population densities in general, and *Daphnia* in particular: climatic parameters (temperature and light, hydrogen ions concentration (pH), oxygen conditions, and other water quality parameters) quantitative and qualitative food conditions, essential nutritional elements and compounds as well as predation by fish or invertebrates (Boucknak et al., 2010 and references therein).

1.3. *Longevity associated genes*

As reviewed by Browner et al. (2004), most biological pathways are evolutionarily conserved, through mechanisms shared by common effects on metabolism and gene expression. Many of the genes that affect aging and longevity have homologues between species. Key pathways that link nutrient and growth factor cues with longevity associated genes, assuming such genes as conservative, can be studied with that purpose. The most direct way to address this problem is to identify genes or interventions that function similarly to modulate life span in different organisms (Kaeberlein, 2007). Components of insulin or insulin-like growth factor 1 (IGF-1) signaling pathway, the nutrient-responsive

target of rapamycin (TOR) kinase and the sirtuin (SIR) family of protein deacetylases, among others, have been found to have this property (Kaeberlein, 2007). Many others can be related to longevity modulation (de Magalhaes et al., 2009).

1.3.1. *Insulin-like growth factors*

The insulin-like growth factors (IGFs) are metabolic proteins along the insulin signaling pathway (Guarente and Kenyon, 2000; Tatar et al., 2003) and their receptors and binding proteins constitute a family of cellular modulators that play essential roles in the regulation of growth and development (LeRoith et al., 1995). Mutations that decrease IGF-1 like signaling increase life span in worms, mice and flies (Fontana et al., 2010).

1.3.2. *Target of rapamycin*

The target of rapamycin (TOR) kinases are evolutionarily conserved proteins that function to mediate mRNA translation, cell growth, metabolism, degradation, and stress resistance (among other processes) in response to nutrient and growth factor cues (Arsham et al., 2006; Martin et al., 2005). Mutations that decrease TOR activity have also been reported to increase life span in both *C.* elegans (Jia et al., 2004; Vellai et al., 2003) and *D. melanogaster* (Kapahi et al., 2004), suggesting an evolutionarily conserved link between TOR signaling and aging. In yeasts, it is considered an important key nutrient-responsive factor as a primary determinant of replicative longevity (Kaeberlein, 2007).

Many of the mutations that extend life span, decrease activity of nutrient-signaling pathways, such as the IGF-1 and the TOR pathways, suggesting that they may induce a physiological state similar to that resulting from periods of food shortage (Fontana et al., 2010). Indeed, dietary restriction, a reduction in food intake without malnutrition, extends life span of diverse organisms, including yeast,

flies, worms, fish, rodents, and rhesus monkeys (Mair and Dillin, 2008; Fontana et al., 2010).

1.3.3. *Sirtuins and PNC1*

The sirtuin (SIR) family of protein deacetylases has been implicated in influencing aging and regulating transcription, apoptosis and stress resistance (Taylor et al., 2008). The role of sirtuins in aging was discovered in budding yeast, where overexpression of SIR2 increases replicative life span (Kaeberlein et al., 1999). It was then reported that elevated sirtuin activity increases life span in *C. elegans* (Tissenbaum and Guarente, 2001; Viswanathan et al., 2005; Berdichevsky et al., 2006), the fruit fly *Drosophila* (Rogina and Helfand, 2004) and in yeasts (Anderson et al., 2003), indicating an evolutionarily ancient role of sirtuins in longevity assurance (Guarente, 2007).

The activity of yeast SIR2 is known to be regulated by PNC1 (pyrazinamidase and nicotinamidase), an important metabolic enzyme that hydrolyzes nicotinamide (NAM) to nicotinic acid (NA) (Anderson et al., 2003; Silva et al., 2009). SIR2 is NAD-dependent, thus, NAD is consumed in the deacetylation reaction producing nicotinamide, a natural sirtuin inhibitor (Gallo et al., 2004). PNC1 influences sirtuin activity in two ways, by converting NAM to NA it relieves sirtuin inhibition and contributes to recycle the NAD cofactor through the NAM salvage pathway (Gallo et al., 2004). In this study, the PNC1 gene was selected to assess a possible relationship between the environment and *Daphnia* longevity. To control the experimental variations in the amount of mRNA, parallel measurement of a housekeeping gene has been used for control and for the normalization of target gene expression data: glyceraldehyde-3-phosphate dehydrogenase, GAPDH (Barber, 2005).

1.4. *Environmental factors*

The life span of model organisms can be modulated by environmental conditions that influence cellular metabolism, oxidation, or DNA integrity (Sinclair, 2005; Balan et al., 2008). Several factors are known to influence longevity, including temperature (Dudycha, 2003), caloric restriction (Kenyon, 2001) and oxidative stress (e.g. copper) (Anderson, 2003; Enesco et al., 1989; Kirchman and Botta, 2007).

1.4.1. *Temperature*

Temperature affects essentially every aspect of the biology of poikilothermic animals, including the energy, activity, growth, and reproduction (Nelson and Cox, 2004). While thermal effects in daphnids have been intensively studied at the ecosystem level, much less is known about the molecular mechanisms underlying the acclimation to different temperatures (Schwerin et al., 2009). *Daphnia magna,* for instance, has been shown to have genetic ability to tolerate high temperatures (Stephenson et al., 1984; MacIsaac et al., 1985; Lagerspetz, 2000). Experiments made with other daphnids (*D. gessneri*, *Diaphanosoma sarsi* and *Moina reticulata*) showed that although temperature exerted the most powerful influence on embryonic development (Hardy and Duncan, 1994), there was also a smaller food effect (Weglenska, 1971; Korinek, 1970).

1.4.2. *Food restriction*

For almost 80 years, caloric restriction has been known to significantly extend the average life span of rodents (McCay, 1935). This restriction refers to a dietary regimen low in calories with no undernutrition, to a limitation level below that of control animals fed *ad libitum* (Koubova, 2003). In many other animal models (including yeast, worms and fruit flies), caloric restriction is the most effective environmental method of increasing life span (Masoro, 2000; Mair et al., 2003).

Daphnia longevity is also strongly influenced by food levels (Ingle et al., 1937; Martínez-Jerónimo et al., 1994; Muñoz-Mejía and Martínez-Jerónimo, 2007) and can be twofold extended by dietary restriction (Pietrzak et al., 2010). Many authors have shown that cladocerans cultured at high food concentrations have a shorter duration of post-embryonic development than those cultured at low food levels (De Bernardi et al., 1978; Gras and Saint-Jean, 1978; Lei and Armitage, 1980; Vijverberg, 1980; Kankaala and Wulff, 1981; Rocha, 1983; Orcutt and Porter, 1984; Jayatunga, 1986). However, a study made with *D. similis*, indicated that animals reared at the same temperature, exhibited a higher mean longevity when exposed in natural water at high food concentration of algae (Pedrozo and Bohrer, 2003).

1.4.3. *Copper*

Metals are extremely important elements in aquatic ecosystems. They are naturally present as a result of weathering of soil and rocks (Bradl, 2005). Albeit essential in small concentrations, many are toxic at high levels, and can bioaccumulate and biomagnify in food webs (Rodgher et al., 2005). Numerous studies have reported that copper induces toxicity (Conradi and Depledge, 1998; Koivisto and Ketola, 1995) including genotoxicity (Eicchorn and Shin, 1968; Lloyd and Phillips, 1999) to several species. According to Atienzar et al. (2001), genotoxins can shorten the life expectancy of organisms and can also result in population dynamics alterations (Koivisto and Ketola, 1995; Meador, 1991; Soares et al., 1992). Because copper can also trigger oxidative stress (Lushchak, 2011), it was chosen to assess its effect in longevity, as it is an essential metal, namely being a constitutive of many proteins associated with oxygen processing in organisms (Siegel et al., 1999).

1.5. *Research aims*

According to the above mentioned, the purpose of the present study was to evaluate the effects of three environmental factors (temperature, food restriction

and copper) in the longevity parameters of the cladoceran species *Daphnia magna* Straus. To attain this goal, longevity related parameters were monitored at the individual (total reproduction and life span) and molecular (protein expression and mutations in the case of exposure to copper, and gene expression and genomic integrity of the PNC1 gene in the case of exposure to food restriction) levels.

2. MATERIAL AND METHODS

2. MATERIAL AND METHODS

2.1. *Daphnia magna breeding*

Clones K6 and BEAK of *D. magna*, were kept under laboratory-controlled conditions for several generations as pure parthenogenetic cultures. They were reared in a room at 20ºC±1ºC, with a 16:8 light:dark photoperiod (provided by fluorescent light tubes) in groups of 15 to 20 female adults per 800mL of medium, in glass vessels. ASTM synthetic hard water (ASTM, 1988) was used as culture medium, according to the OECD recommendations (OECD, 1998). ASTM was prepared with Milli-Q® ultrapure water (Merck KGaA, Darmstadt, Germany) with resistivity of 18.2 MΩ.cm and the vitamins thiamine hydrochloride (B1), cyanocobalamin (B12) and biotin (H), from Sigma-Aldrich, were added to provide optimal development conditions (ASTM, 1980).

The culture medium was supplemented with 4.8mL of an organic additive at 0.9%, the Marinure® seaweed algae extract (Glenside Organics Limited, Stirlingshire, GB), from the brown alga *Ascophyllum nodosum* (Baird et al., 1989). The combination of ASTM medium and seaweed extract is appropriate for the long-term maintenance of viable cultures and testing of daphnids in the laboratory (Sims et al., 1993; OECD, 1997).

The cultures were fed on suspensions of the unicellular green freshwater algae *Pseudokirchneriella subcapitata* (Korshikov) F.Hindák, at the non limiting-concentration of 300000 cells/mL, corresponding to approximately 25µg/mL of carbon (*ad libitum*), measured in a total organic carbon analyzer (TOC-V CPH, Shimadzu, MD, USA). Algal suspensions were obtained from a batch culture, which had been kept continuously in the laboratory (Stein, 1973), harvested in the exponential phase of growth, centrifuged and then resuspended in ASTM water. The number of algal cells was calculated from absorbance measurements at λ=440nm (Jenway 6505 UV/vis spectrophotometer), by using a standard calibration curve, previously obtained for the algae culture. *Daphnia* were fed everyday and the medium was changed every other day.

2.2. *Experimental procedure*

The experiments were carried out with neonates (less than 24hrs old) from the third, fourth or fifth clutches from the aforementioned clones (OECD, 1998). For each assay, 15 replicas were performed per treatment and all daphnids were individually placed in tall form glass vessels, containing 50mL of test solution. The experiments were carried out in similar conditions as those for the cultures, except when otherwise mentioned. The daphnids were fed everyday and the medium was changed every other day. An experiment ended when all daphnids died.

2.2.1. *Temperature exposure*

The neonates of *D. magna* were exposed to 15 and 25º±1ºC, in two different climate chambers (KBWF720, Binder, Germany). The control group was exposed at 20ºC in the same acclimatised room used for the cultures. The temperature of the fresh medium was adjusted in the respective climate chamber prior to the daphnids' transfer.

2.2.2. *Copper exposure*

The test solution used in this assay was prepared by dilution of copper (II) sulphate pentahydrate ($CuSO4.5H_2O$, with a purity degree ≥99.6%, p.a. ACI, ISO 5, supplied by Merck KGaA Darmstadt, Germany) in ultrapure water (Milli-Q®). It was prepared in advance and the nominal concentration was 100mg/L. The following test concentrations were attained by diluting the stock solution with ASTM hard water: 11.1, 16.6, 25.0, 37.5 and 56.3µg/L, in a geometric progression series of 1.5. The choice of these values was based on previous experiments made with *Daphnia* (Atienzar et al., 2001; Agra et al., 2011). The control group (no metal) for this assay was the same as used for the temperature control, described above.

2.2.3. *Food restriction exposure*

The treatments consisted in providing *D. magna* with different amounts of food. The concentrations used were the following: 75000, 97500, 126750 and 164775 cells/mL of the green algae *P. subcapitata*, in a geometric progression of 1.3. These values correspond respectively to 6.25, 8.13, 10.56 and 13.73 µg/mL of C. The control group was fed with 300000 cells/mL (25µg/mL of C).

2.2.4. *Water monitoring*

Measurements of pH, dissolved oxygen (DO) and conductivity were observed at medium change, both in old and fresh media, to control abrupt differences in the transition and for test validation purposes. The devices used to carry out these measurements were: 330i/SET-2 (WTW, Weilheim, Germany) and HI 8314 (HANNA Instruments, RI, USA) membrane ph-meters, Oxi 330i/SET (WTW) DO meter, Cond 330i/SET with TetraCon® 325 and 3110/SET1 (WTW) conductivity meters. SympHony SP90M5 (VWR) was also used for pH and DO measurements.

2.2.5. *Collection of organisms for molecular analysis*

The exposed animals were checked everyday for mortality and their offspring were collected daily, counted and moved to 50mL vessels containing ASTM water only, where they remained for 24hrs to lessen algal contamination. Afterwards, the juveniles were passed through distilled water and the count was repeated to ensure accuracy. Then, each brood was flash frozen in liquid nitrogen and stored at -80ºC. Dead neonates were rejected prior to freezing. Each experiment ended when all parent daphnids died.

Overall, 28 different assays were executed, yielding a total of 420 *D. magna* replicates, from which longevity and reproduction parameters were assessed.

2.3. *Statistical analysis*

To test for statistical significant differences among treatments, all data were verified for normality and homoscedasticity of variances and then analyzed through parametric one-way ANOVA, using SigmaStat v3.5 (Systat® Software, Cranes Software International). For statistically significant ANOVAs, post-hoc Dunnett multicomparison tests were performed to discriminate significantly different treatments relatively to control (Zar, 2010). When the normality or the homoscedasticity of variance tests failed, non-parametric Kruskal-Wallis one-way ANOVA by ranks (Kruskal and Wallis, 1952) was used and differences, relatively to the control, determined by the Dunn's multiple tests for post-hoc comparisons. Variations were calculated as standard error of the mean. Variances were considered significant at $p<0.05$, indicated by *.

2.3.1. *Selection of organisms for molecular analysis*

Replicas from groups significantly different from control in both life span and reproduction parameters were then selected for molecular analysis, by choosing samples whose values were above mean+sdv (μ+σ), under mean-sdv (μ-σ) and within the median values.

2.4. *Molecular analysis*

2.4.1. *DNA, RNA and protein extraction*

Genomic DNA, total RNA and total denatured protein from the same undivided samples were extracted with the IllustraTM triplePrep kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions, except proteins were resuspended in DeStreak™ Reagent (GE Healthcare).

In samples where RNA was degraded, the phenol-chloroform method was used for DNA extraction only. Each brood was disrupted with 300μL of STE buffer solution, containing 1M NaCl, 100mM Tris and 10mM EDTA pH 8.0 (G-

Biosciences, St. Louis, MO, USA), 25µL of SDS at 20% (Eurobio, Courtaboeuf, France), 20µL of proteinase K at 20mg/mL (Thermo Scientific) and 20µL of DTT (DL-dithiothreitol) 1M from Sigma-Aldrich. After the addition of 200µL of STE, the samples were incubated in a thermostatic cabinet (GFL 3032) at 56ºC for 48hrs, with gentle mixing. Afterwards, Phase-Lock Gel™ Light tubes (5 PRIME) were centrifuged at 12000g for 20-30s (Eppendorf 5418R refrigerated microcentrifuge), and 20µL of a previously heated NaCl solution at 5M were added, followed by addition of 575µL of phenol:chloroform:isoamilic alcohol (25:24:1) from AppliChem, Germany. Then, the samples were transferred to those tubes and the volumes were completed with nuclease-free water. The mixtures were homogenized and centrifuged at 14000g for 3min. The aqueous phases were moved to new Phase-Lock Gel™ tubes with 575µL of 24:1 chloroform:isoamilic alcohol (AppliChem), inverted and centrifuged at 14000g for 3min. The aqueous phases were transferred to DNase-free tubes, mixed with 1mL of 96% (v/v) ethanol at -20ºC and placed at -80ºC for 15min. The microtubes were centrifuged at 14000g for 15min at 4ºC and the ethanol was discarded. Then, 1 mL of ethanol at 70% was added, followed by a centrifugation step at 14000g for 5min. The ethanol was discarded and the tubes were left to dry at room temperature. The DNA precipitates were resuspended in 7µL of nuclease-free water.

In both extraction procedures, samples were disrupted with a pellet pestle (Z359971-1EA, Sigma-Aldrich).

Nucleic acid concentrations were measured with a NanoDrop® ND-1000 UV/vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and analyzed with the software ND-1000 v3.3.

2.4.2. *cDNA synthesis*

RNA samples selected for reverse transcription were firstly subjected to DNase I, RNase-free (Fermentas) treatment as described in the manufacturer's procedure. The reverse transcription reactions were made using RETROscript® Kit (Ambion/Life Technologies, Austin, TX), according to the manufacturer's protocol. Negative control (-RT) samples were performed at the same time, substituting the

MMLV-RT reverse transcriptase enzyme (Moloney Murine Leukemia Virus Reverse Transcriptase) with 1µL of nuclease free water.

2.4.3. *Primers*

Primer sequences were manually designed or retrieved from available literature, as indicated. The OligoCalc web interface (Kibbe, 2007) was used to evaluate the properties of each oligonucleotide, as well as to detect potential hairpin formation and primer dimers. The complete primer sequences and amplicon sizes can be found in Table 1.

Table 1 - Primers used in PCR amplifications. The sequences are specific to *Daphnia magna* (Dm) and *D. pulex* (Dp). Forward and reverse sequences and expected amplicon sizes (bp) are displayed. All primers had an annealing temperature of 54ºC. GAPDH primer sequences were retrieved from Haag et al. (2009).

Primer name	Primer sequences (5'-3')	Amplicon size (bp)
Dm_GAPDH_F	F CAATGGTCACCACATTCAGG	442
Dm_GAPDH_R	R AATATTTTGGGCAGCACCAC	
Dp_Pcds_F1	F GTAGCAGCAGCAGTGACC	444
Dp_Pcds_R1	R TCTTGACCATTCTGCTGAGC	
Dm_P3UTR_F2	F CATCCGGACATCGATTCCTATTCTGC	423
Dp_Pcds_R2	R GATGACGGAATCGCGAGG	

2.4.4. *PCR conditions*

Each PCR reaction was carried out with 5µL QIAGEN Multiplex PCR Master Mix (containing HotStarTaq® DNA polymerase, Multiplex PCR buffer and dNTP mix), 1µL of Q-solution (QIAGEN), 1µL of each primer at 2µM (Thermo Scientific), 1µL of DNA of varying concentrations and 1µL of nuclease free water, to a final volume of 10µL. A no-template control was always included. All reactions were performed with 2720 Thermal Cycler and GeneAmp® PCR System 2700

thermocyclers (Applied Biosystems) and with MyCycler™ Thermal Cycler (Bio-Rad). The PCR cycles for all primers had a 15min initial denaturation step at 95ºC, followed by 40 cycles of denaturation at 94ºC for 30s, annealing at 54ºC for 1min and extension at 72ºC for 1min30s. A final hold occurred at 72ºC for 15min. The same cycle was used for Dm_GAPDH and Dm_P3UTR_F2/Dp_Pcds_R2 primers, except the amplification step had 35 cycles. An additional cycle was performed for GAPDH primers, as described for the later, but with an extension step of 1min and a final extension of 10min.

2.4.5. *Electrophoresis*

PCR products were separated by polyacrylamide gel electrophoresis. The gels were made from a solution of 8.42% (w/v) 19:1 AccuGel (Acrylamide to Bisacrylamide Stabilized Solution, National Diagnostics, Atlanta, GA), 1.5M Tris-HCl Buffer pH 8.8 (Bio-Rad), glycerol at 7.37% (Merck) and distilled water. The gels were prepared with 3mL of the previous solution, 170µL of 2.5% APS (ammonium persulfate) from Promega and 7µL of TEMED (N,N,N',N'-tetramethylethylene diamine) from National Diagnostics. The molecular size marker used was O'GeneRuler™ 100bp Plus DNA Ladder (Fermentas). Runs were performed in Pharmacia LKB Multiphor II Electrophoresis Unit, with a power supply from Desatronic 500/500 (DESAGA, Germany).

DNA bands on the gels were visualized through a silver staining process, consisting of a series of steps in the following order: 10% ethanol for 10min, 1% nitric acid for 5min, two washings with distilled water for 10s, silver nitrate (0.2%) for 20min in the dark and two washing steps again. The images were developed by a solution of formaldehyde 0.02% (Merck) in sodium carbonate 0.28M, from AppliChem (Merril and Goldman 1984). To prevent overdevelopment, the reactions were stopped with 10% acetic acid for 2min, ending with a final water wash. The DNA samples that presented single, well-defined bands with the expected size were sequenced.

cDNA PCR products were subjected to an electrophoresis of 1% agarose gel (BIORON, Germany) in 0.5x TBE buffer (Tris, borate and EDTA). The samples

were prepared for the run by the addition of 2µL of loading buffer (containing 9% glycerol and 2.5mg/mL of 0.25% bromophenol blue in distilled water) to 9µL of PCR product. GelRed™ (Biotium, CA, USA) was used to stain the gel and the molecular size ladder was the same as previously mentioned. The run was made with a Sub-Cell® GT system with PowerPac 300 power supply (Bio-Rad). cDNA bands were observed through a Molecular Imager® ChemiDoc™ XRS Imaging System (Bio-Rad). Relative quantification of the cDNA bands was made with Quantity One® 1-D Analysis Software version 4.6.8 (Basic), from Bio-Rad.

2.4.6. *Sequencing*

For sequencing reactions, the DNA products were purified with ExoSAP-IT® PCR (USB Corporation) by adding 1U (2µL) to 5µL of PCR product. Reactions were incubated at 37ºC for 15min followed by additional 15min at 85ºC for enzyme inactivation, and used as a template for sequencing, performed with the ABI Prism® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. The following programme was used: an initial denaturation step at 96ºC for 5min and 35 cycles of 96ºC for 20s, 54ºC for 10s (52ºC for the copper samples) and 60ºC for 1min30s; then, the mixture was subjected to an extension step of 10min at 60ºC.

The DNA was then purified with Sephadex® G-50 Fine (GE Healthcare) and denatured with 10µL HiDi™ formamide (Applied Biosystems). The samples were separated by capillary electrophoresis on a 3130*xl* Genetic Analyzer (Applied Biosystems) and the results were analyzed using Geneious software, v5.6.5 (Drummond et al., 2011).

2.4.7. *Sequence analysis and bioinformatics*

After sequencing, the DNA fragments were aligned with each other and with reference sequences from the National Center for Biotechnology Information (NCBI) database, to search for nucleotidic alterations.

Human sirtuin (Hs) aminoacid sequences (NP) from the NCBI database were compared to the *D. pulex* protein database (*Daphnia pulex* v1.0 all gene models) using BLASTp (Basic Local Alignment Search Tool for proteins) analysis software, to identify regions of local similarity between sequences (Altschul et al., 1990). BLASTp was used via the *D. pulex* genome portal, from the DOE Joint Genome Institute (Grigoriev et al., 2011). The corresponding *D. pulex* sirtuin sequences were retrieved, aligned and phylogenetic relationships between them and human sirtuins were assessed, utilizing Geneious software, v5.6.6 (Drummond et al., 2011). Bayesian inference analyses were performed with MrBayes v3.2 (Huelsenbeck and Ronquist, 2001) available in Geneious, using the Blosum62+I+G+F model of protein evolution, as determined by ProtTest v2.4 server (Abascal et al., 2005) and the program's default parameters.

2.4.8. *Protein quantification*

Following protein extraction, the Bradford method (Bradford, 1976) was used in selected samples to estimate protein concentration, using Quick Start™ Bradford 1x dye Reagent (Bio-Rad). Measurements were made at λ=595nm in an Ultraspec 1100 pro (Amersham Biosciences) spectrophotometer.

2.4.9. *SDS-PAGE*

To separate proteins according to their electrophoretic mobility, an SDS-PAGE was performed (Laemmli, 1970), using a 12.5% resolving acrylamide gel. It was made with 1.6mL of distilled water, 50µL of SDS at 10%, 1.875mL of Tris-Cl (1M pH 8), 1.5mL of AccuGel (Acrylamide to Bisacrylamide Stabilized Solution) 29:1 at 40% (w/v), from National Diagnostics, 25µL of APS 10% and 5µL of TEMED. The stacking gel contained 1.732mL of water, 25µL of SDS 10%, 500µL of Tris (0.625M pH 6.8), 250µL of acrylamide and the same quantities of APS and TEMED as above. 10µL of protein sample with 10µL of sample buffer, consisting of Laemmli buffer (Bio-Rad) with 2-mercaptoethanol (Bio-Rad), were loaded on the

gel. The molecular size marker used was Precision Plus Protein™ WesternC™ Standards (Bio-Rad) and the running buffer was Tris/Glycine/SDS (Bio-Rad). After running the gel on a Bio-Rad Mini PROTEAN® Tetra Cell, it was washed three times in distilled water for 5min, stained with Bio-Safe™ Coomassie G-250 stain (Bio-Rad) for 1hr and destained with distilled water. Bands that presented a different pattern from the control were cut and sent to mass spectrometry for identification.

3. RESULTS

3. RESULTS

Following a continuous exposure to the aforementioned environmental factors, the results at both individual and molecular levels are described beneath.

3.1. *Temperature*

3.1.1. *Physicochemical parameters*

Mean values from the water quality measurements are displayed in Table 2. Over the course of the experiments, values did not exhibit high variations between old and new media; nonetheless, pH from the old medium was slightly higher comparing to the fresh one, with no assumed consequences for the test's purpose (OECD, 1998).

Table 2 - Water quality parameters, measured during the temperature exposure experiments with *Daphnia magna* clonal lineages K6 and BEAK. Values are the mean±standard deviation. The control group corresponds to 20ºC.

	pH		Dissolved Oxygen (mg/L)		Conductivity (µS/cm)	
	K6	BEAK	K6	BEAK	K6	BEAK
20ºC	8.02±0.28	8.18±0.25	6.55±1.69	7.61±0.95	518±26	531±23
15ºC	8.25±0.36	8.30±0.35	8.17±1.57	7.83±0.98	548±34	554±34
25ºC	8.09±0.32	8.22±0.36	7.60±1.63	7.13±1.05	579±55	615±94

3.1.2. *Age at first reproduction*

An increase or decrease in temperature, relatively to the temperature of rearing conditions (control: 20ºC), significantly influenced the age of release of the first brood for both *D. magna* clones (K6 and BEAK). A significant increase of 6 days and decrease of 1.6 days in age at first reproduction was observed for *D. magna* K6 exposed to 15ºC and 25ºC, respectively, in comparison to the control (Kruskal-Wallis test: H_2=40.8, p<0.001, followed by the Dunn's test: p<0.05; Fig. 1). While for the clone BEAK, exposure to 15ºC induced a delay in the release of the first brood of 1.2 days and at 25ºC it was hastened for 4 days (Kurskal-Wallis test: H_2=43.1, p<0.001, Dunn's test: p<0.05; Fig. 1).

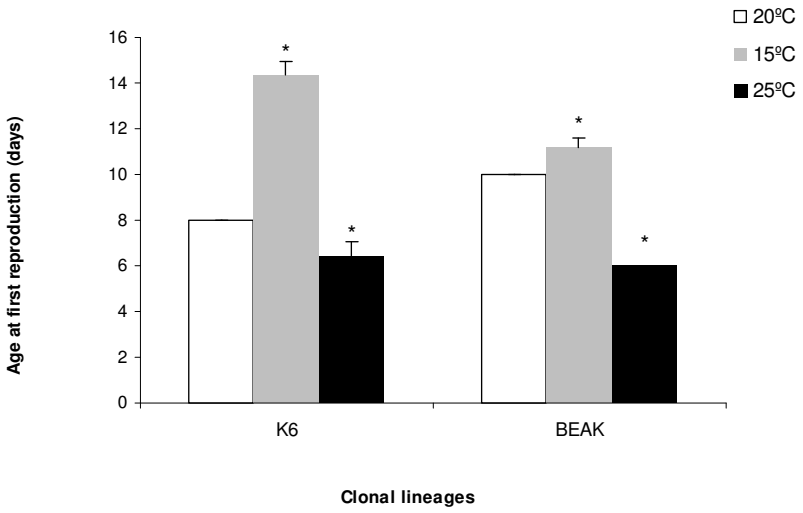

Figure 1 - Average of age at first reproduction (days) for clonal lineages K6 and BEAK, of *Daphnia magna*, exposed to different temperatures. Error bars represent the standard deviation. Significant differences from control are indicated by *(p<0.05), following Kruskal-Wallis and Dunn's post-hoc tests.

3.1.3. *Life span and reproduction parameters*

Daphnia magna that were exposed to 25ºC exhibited a significant decreased life span, both for clone K6 (Kruskal-Wallis: H_2=18.8, p<0.001, followed by the Dunn's test: p<0.05) and clone BEAK (Kruskal-Wallis: H_2=7.65, p<0.022, Dunn's test: p<0.05), comparatively to the control daphnids (Fig. 2). The same occurred for *D. magna* exposed to 15ºC, but only for clone K6 (Kruskal-Wallis: H_2=18.8, p<0.022, Dunn's test: p<0.05; in Fig. 2). A reduction of 29.8% (at 15ºC) and 45.7% (at 25ºC) in life span of *D. magna* K6 was registered relatively to the control, while for *D. magna* BEAK a reduction of 6.21% and 24.1%, relatively to the respective control, was observed at 15ºC and 25ºC, respectively.

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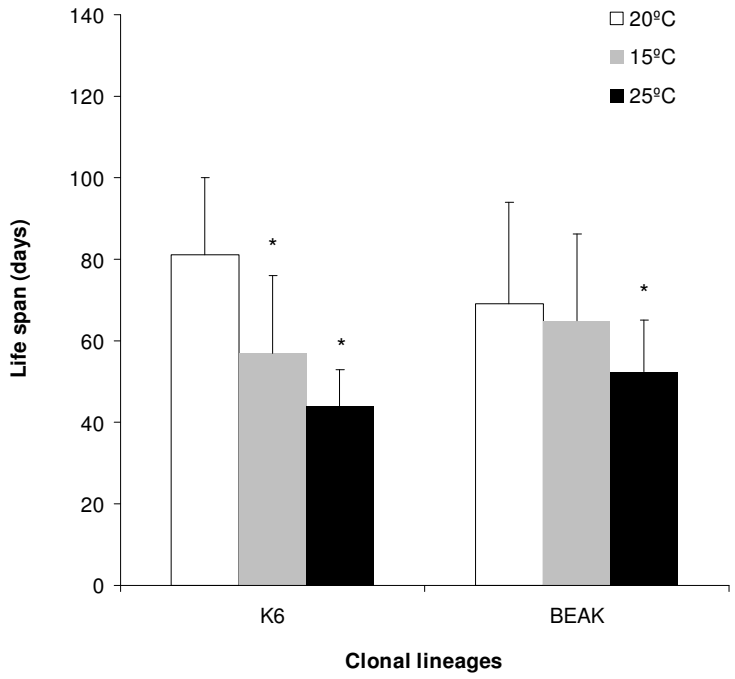

Figure 2 - Average of life span (days) for clonal lineages K6 and BEAK, of *Daphnia magna*, after being exposed to different temperatures. Error bars represent standard deviation. Significant differences from control are indicated by *(p<0.05), following Kruskal-Wallis and Dunn's post-hoc tests.

Survival curves representing the temperature effect evidence the relative rate at which the females died (Fig. 3). Even though differences were found in K6 life span, until the 25th day all groups exhibited a similar survivorship. Survival curve of K6 clone exposed at 25ºC had a faster decline than when exposed to the control (20ºC) and 15ºC. Similarly, BEAK's life span was identical in all groups up to the 25th day.

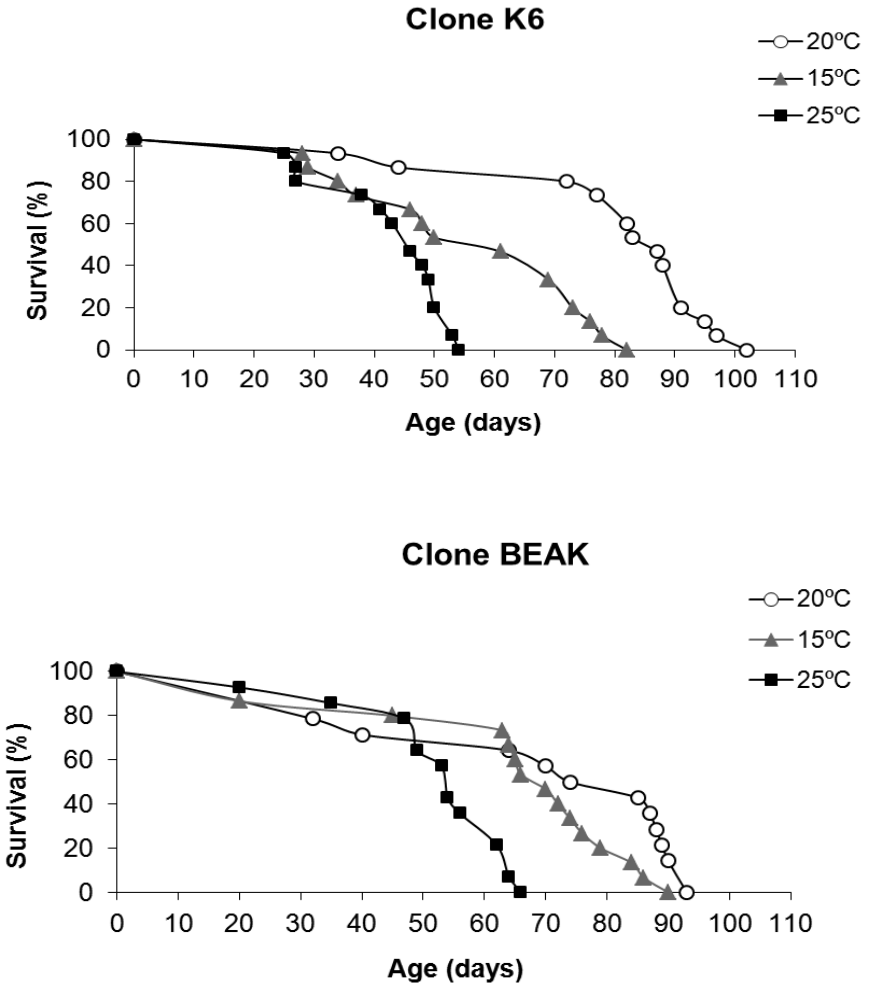

Figure 3 - Survival curves (%) for females of clonal lineages K6 and BEAK, of *Daphnia magna*, after being exposed to different temperatures (20ºC, 15ºC and 25ºC).

Considering reproduction, temperature influence was only observed in K6 clone exposed at 25ºC, since it released a significantly lower number of neonates

than the control (Kruskal-Wallis: H_2=8.31, p=0.016, followed by the Dunn's test: p<0.05) (Fig. 4). The increase or decrease in temperature treatment did not exert statistically significant effects on BEAK clone concerning reproduction (Kruskal-Wallis: H_2=5.07, p=0.079 (Fig. 4).

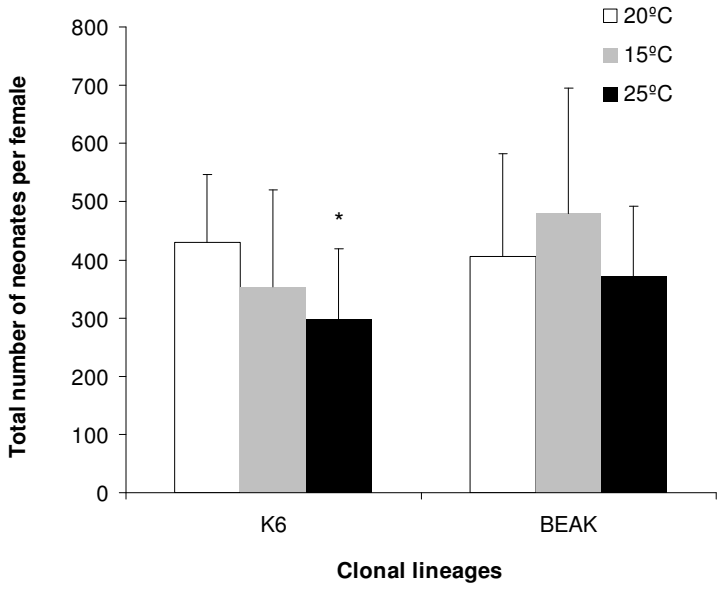

Figure 4 - Average of the total number of neonates produced per female of clonal lineages K6 and BEAK, of *Daphnia magna*, after being exposed to different temperatures. Error bars represent standard deviation. Significant differences from control are indicated by *(p<0.05), following Kruskal-Wallis and Dunn's post-hoc tests.

Despite the significant life span decrease in K6 at 15ºC and in BEAK at 25ºC (Fig. 2), it did not influence their reproduction (Fig. 4).

3.1.4. *Molecular analysis*

During this study, the first samples to be collected were from the temperature assay. Even though significant differences were found, preservation problems occurred with the samples and therefore could not be further processed.

For that reason, no data from temperature exposed daphnids could be presented here.

3.2. *Copper*

3.2.1. Physicochemical parameters

The water quality measurements during medium changes did not exhibit large variations within assays and between old and new media. Mean values of those measurements are displayed in Table 3.

Table 3 - Water quality parameters, measured during the copper exposure experiments of clonal lineages K6 and BEAK of *Daphnia magna*. Values are the mean±standard deviation. Control data (0µg/L) here presented are the same as for the temperature assay.

	pH		Dissolved Oxygen (mg/L)		Conductivity (µS/cm)	
	K6	BEAK	K6	BEAK	K6	BEAK
0µg/L	8.02±0.28	8.18±0.25	6.55±1.69	7.61±0.95	518±26	531±23
11.1µg/L	8.34±0.31	8.24±0.34	7.65±0.92	7.72±0.99	582±40	584±42
16.6µg/L	8.40±0.35	8.47±0.44	7.67±0.93	7.74±0.85	574±30	569±32
25.0µg/L	8.00±0.27	8.42±0.39	7.08±1.18	7.73±0.96	515±27	573±34
37.5µg/L	7.96±0.24	8.37±0.32	6.87±1.13	7.56±0.80	510±10	571±36
56.3µg/L	7.93±0.22	8.27±0.24	6.89±0.91	7.53±0.80	513±20	576±44

3.2.2. Age at first reproduction

The two lowest tested copper concentrations (11.1 and 16.6µg/L) induced an increase in the age of the first brood release for clonal lineage K6 (Kruskal-Wallis: H_5= 69.9, p<0.001, followed by the Dunn's test: p<0.05; Fig. 5), which occurred, on average, 3 days later than in the control. Regarding clonal lineage

BEAK, only the concentration of 16.6 µg/L caused a significant increase in age at first reproduction (Kruskal-Wallis: H_5=14.5, p=0.013, Dunn's test: p<0.05; Fig. 5); on average, organisms released the first brood 1 day later than the control.

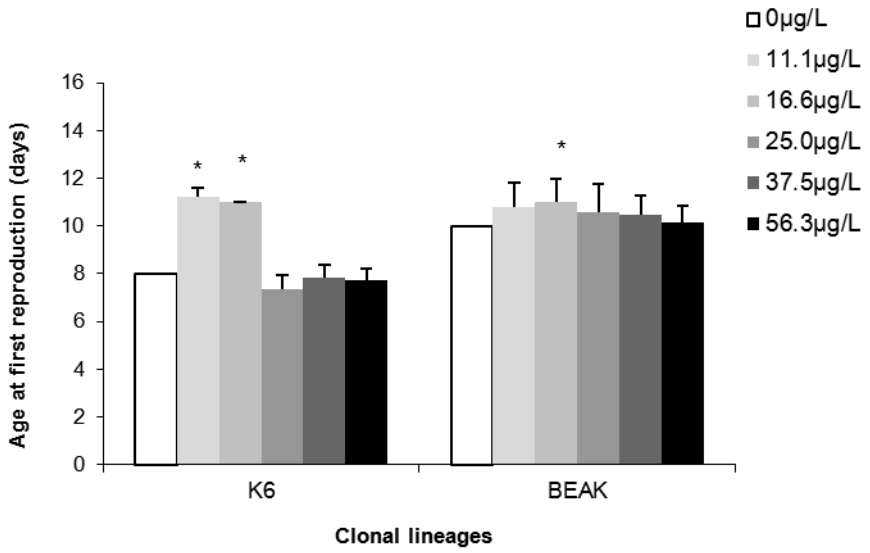

Figure 5 - Average of age at first reproduction (days) for clonal lineages K6 and BEAK, of *Daphnia magna*, exposed to different copper concentrations. Error bars represent the standard deviation. Significant differences from control are indicated by *(p<0.05), following Kruskal-Wallis and Dunn's post-hoc tests.

3.2.3. Life span and reproduction parameters

The continuous exposure to copper in both *D. magna* clones resulted in a significant reduction in life span in all tested concentrations, excepting at the lowest one (11.1µg/L) (K6 - Kruskal-Wallis: H_5=55.7, p<0.001, followed by the Dunn's test: p<0.05 and BEAK - Kruskal-Wallis: H_5=45.6, p<0.001, Dunn's test: p<0.05, Fig. 6). A reduction in life span, relatively to the control, of 43.4% to 75.7% and of 43.8% to 69.5%, with increasing copper concentrations, was observed for clonal lineage K6 and BEAK, respectively.

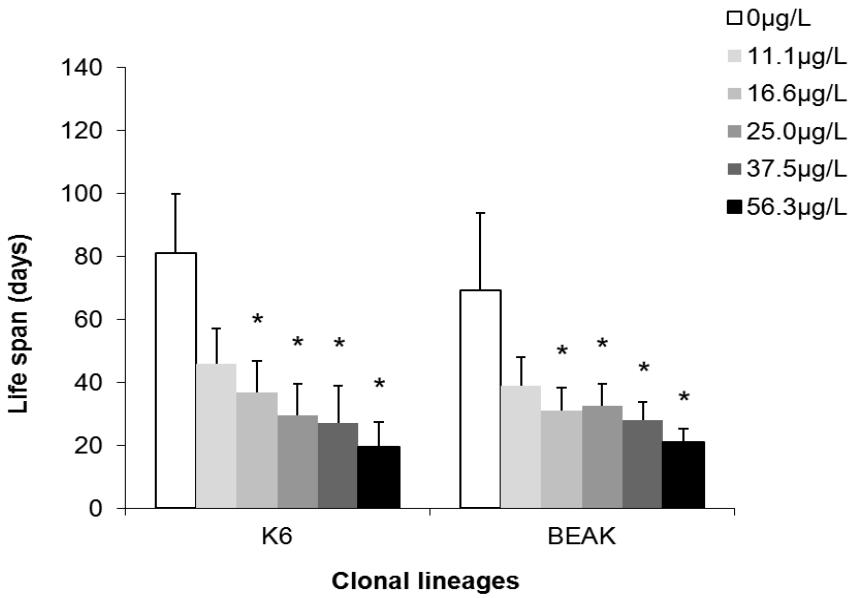

Figure 6 - Average of life span (days) for clonal lineages K6 and BEAK, of *Daphnia magna* after being exposed to different copper concentrations. Error bars represent standard deviation. Significant differences from control are indicated by *($p<0.05$), following Kruskal-Wallis and Dunn's post-hoc tests

By observing the survival curves of the two clones of *D. magna* exposed to the control and five copper concentrations (Fig. 7), clones present a similar survival pattern. BEAK at 16.6µg/L had a lower survival percentage than 25.0, 37.5 and 56.3µg/L until around the 35th, 25th and 15th days considering their life span until the end of the assays.

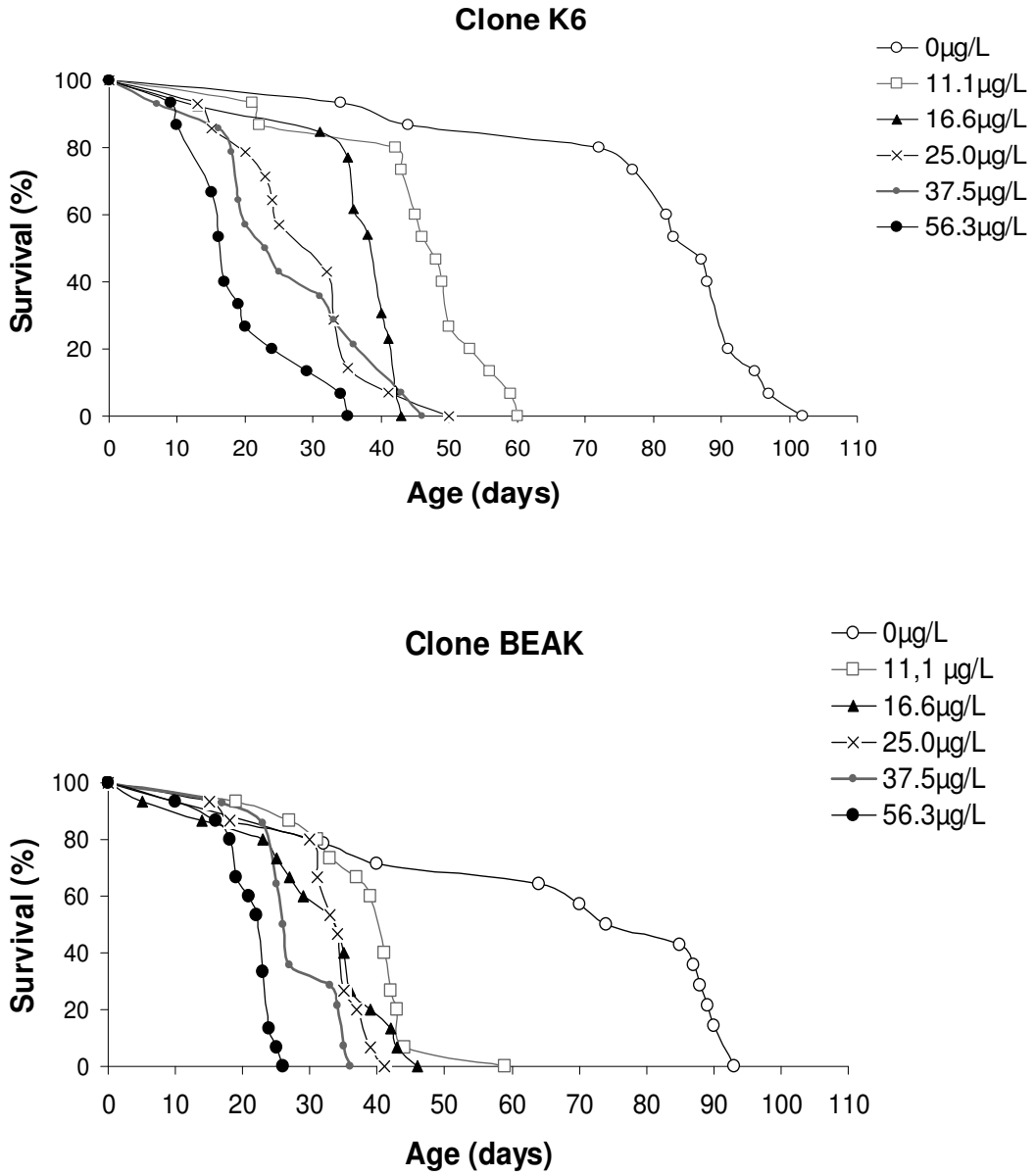

Figure 7 - Survival curves (%) for females of clonal lineages K6 and BEAK, of *Daphnia magna* after being exposed to different copper concentrations.

Regarding clone K6, a significant reduction in the number of neonates released per female occurred for organisms exposed to all tested copper concentrations (ANOVA: $F_{5,79}$=29.1, p<0.001; Dunnett: p=0.002 for 11.1µg/L, p<0.001 for the rest) (Fig. 8). The total number of neonates produced by clone BEAK decreased in all tested copper concentrations, excepting for 11.1µg/L (Kruskal-Wallis: H_5= 45.4, p<0.001, followed by the Dunn's test: p<0.05).

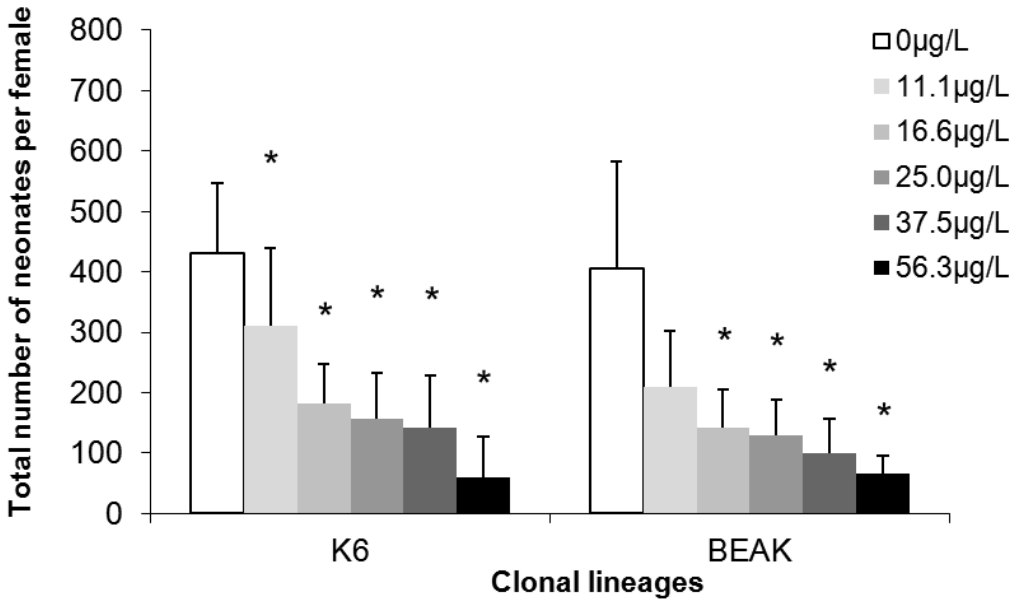

Figure 8 - Average of the total number of neonates produced per female of clonal lineages K6 and BEAK, of *Daphnia magna*, after being exposed to different copper concentrations. Error bars represent standard deviation. Significant differences from control are indicated by *($p<0.05$), following ANOVA and Dunnett's (K6) and Kruskal-Wallis and Dunn's (BEAK) post-hoc tests.

Even though the mean life span of clone K6 exposed to 11.1µg/L was not affected, a significant decrease in the total number of released newborns was observed (14.0±9.1 neonates per brood, *N*=14; control: 23.4±5.32 neonates per brood, *N*=22).

3.2.4. Molecular analysis

Even though significant differences were found for copper treatments, analysis of these samples was conditioned as an unfortunate thawing impaired RNA analysis due to degradation. Therefore, these samples were screened for DNA mutations and protein expression profiles instead.

3.2.4.1. DNA sequence analysis

We have amplified and sequenced fragments of *D. magna* K6 of each PNC1 and GAPDH genes from samples of the control and copper experiments (25.0 and 56.3µg/L). For all samples tested, similar SNPs were present and are shown in Figures 9 and 10 and in Table 4.

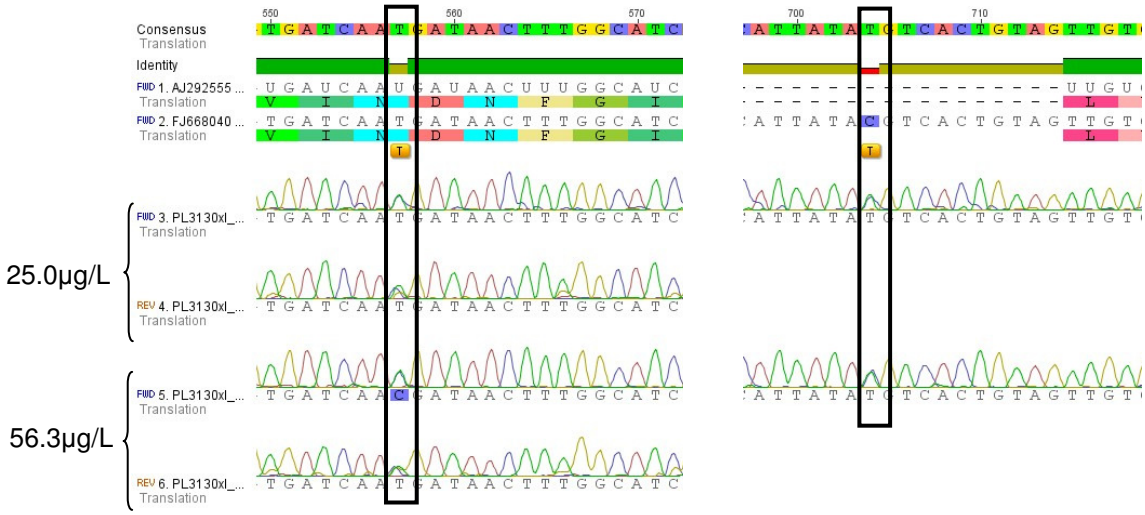

Figure 9 - Electropherograms showing the heterozygous substitutions 557T>C and 704T>C (highlighted in frames), in the GAPDH gene from copper samples of *Daphnia magna* K6. The alignment was made with a partial mRNA (AJ292555, GenBank) and a partial CDS of GAPDH (FJ668040, GenBank).

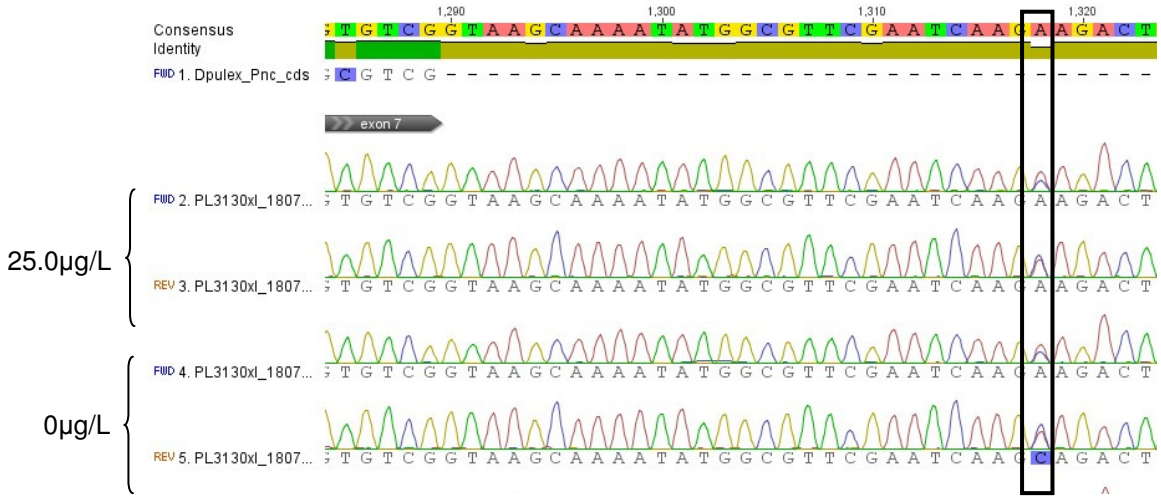

Figure 10 - Electropherograms showing the heterozygous substitution 1318A>C (highlighted in frames) in the PNC1 gene from both control and copper samples of *Daphnia magna* K6. The alignment was made with a *D. pulex* CDS.

Table 4 - Localization of the SNPs observed in GAPDH and PNC1 sequences of *Daphnia magna*.

Gene	SNP position	Nucleotide alteration	Localization
GAPDH	557	T>T/C	Exon
	704	T>T/C	Intron
PNC1	1318	A>A/C	Intron

Since *D. magna*'s genome has not been fully sequenced to date (*Daphnia* Genomics Consortium, DGC, https://wiki.cgb.indiana.edu/display/DGC/Home, last visited 7 December 2012), a partial mRNA reference sequence from the GAPDH gene (AJ292555, GenBank accession) and 81 partial coding sequences (CDSs) of the GAPDH gene were retrieved from the NCBI database, and compared to the consensus sequence of our *D. magna* samples. The sequences used are from several clonal lineages and strains, collected from different geographic regions. The naturally occurring variability can be observed through existing single nucleotide polymorphisms (SNPs). It was observed that 36 out of 81 sequences

had SNPs, in 16 different positions, 5 of them in an intronic region. The alignment is depicted in Figure 11, showing a few examples.

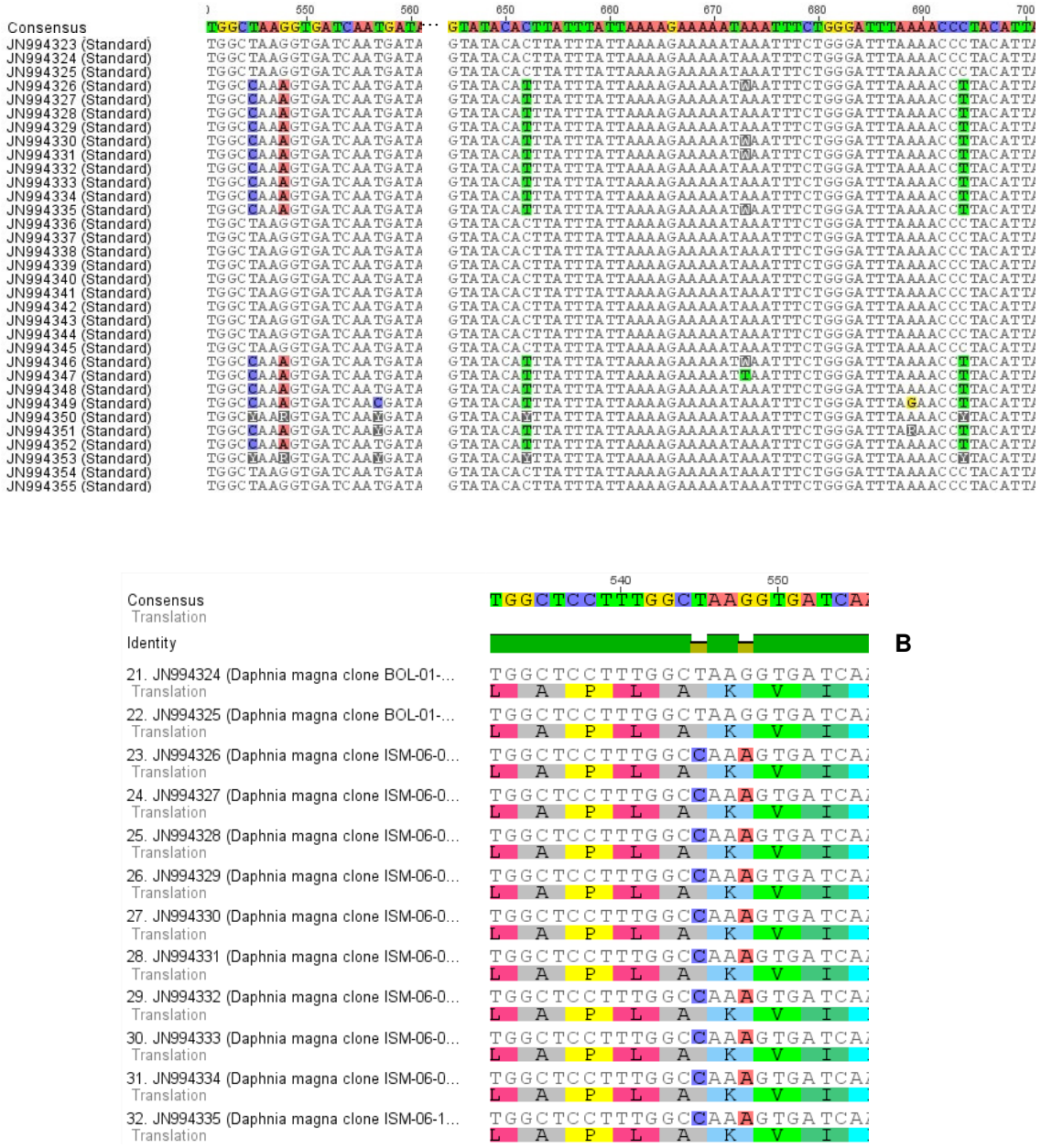

Figure 11 - A - Alignment of *Daphnia magna* partial coding sequences (JN994323 to 55, GenBank, NBCI database) of the GAPDH gene, with our consensus sequence. B - Partial translation of a selected sequences from figure A, showing the SNPs 545T>C and 548G>A. These correspond to synonymous alterations, as the aminoacid remains unchanged.

Other primer combinations for PCR were tested, although unsuccessfully, namely for the ultra spiracle (USP) gene, Dm_USP_F/R (F 5'-ACAATTCCAATGGCTCCAAG/ R 5'-TTGCACTCGACACGTTTCTC), retrieved from Haag et al. (2009), Dm_USP_F1/R1 (F 5'-GGAGGCAAGTTCCCAATACG/ R 5'-CCATCTGCAGGCATTTCTGG) and a primer for *D. longispina* mitochondrial rRNA 12s gene, Dl_12s_F/R (F 5'-ACGGGCGATATGTACACAC/ R 5'-CGGTTAGACGAAGAATCCG).

3.2.4.2. Protein expression profile

Protein extracts from both control and copper samples were separated by SDS-PAGE (Fig. 12). The protein profile observed for control and copper exposed daphnids is different, with a stronger band in the organisms exposed to 56.3µg/L of copper (marked in the figure). The band was cut and sent to mass spectrometry analysis; however, no positive identification was made. To further address differences in protein expression, 2D-PAGE analysis will be performed.

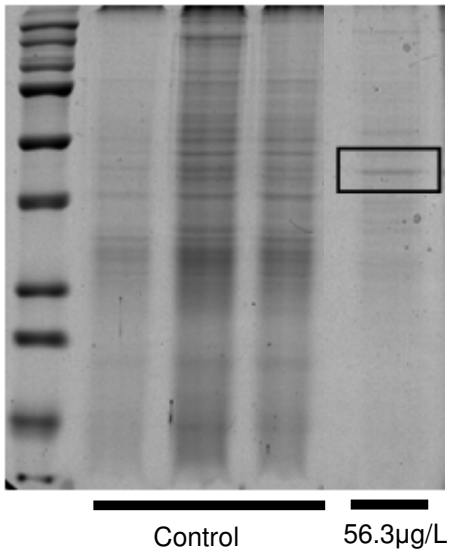

Figure 12 - SDS-PAGE with a sample from the highest tested copper concentration treatment (56.3µg/L), comparing to control. The molecular size marker is Precision Plus Protein™ WesternC™ Standards (Bio-Rad).

3.3. *Food restriction*

In this assay, additional food concentrations of 5000 and 19000 cells/mL of the green microalgae *P. subcapitata* were tested. However, the scarce number of neonates produced (1 to 2 neonates at 5000 cells/mL and 2 to 3 neonates per brood at 19000 cells/mL), insufficient for the subsequent analysis, led to the rejection of these groups. Therefore, they were not included in the statistical analysis.

As samples from the food restriction experiments were preserved, they were used for gene expression analysis by RT-PCR.

3.3.1. Physicochemical parameters

Mean values from the medium quality measurements are shown in Table 5. Values of the parameters from the old and new media did not exhibit great differences.

Table 5 - Water quality parameters, measured during the food restriction experiments of *Daphnia magna* clonal lineages K6 and BEAK. Values are the mean±standard deviation. The control group corresponds to 300000 cells/mL.

	pH		Dissolved Oxygen (mg/L)		Conductivity (µS/cm)	
	K6	BEAK	K6	BEAK	K6	BEAK
300000 cells/mL	8.41±0.46	8.39±0.47	8.05±2.14	8.07±2.13	526±69	532±59
164775 cells/mL	8.29±0.54	8.41±0.51	8.62±1.90	8.56±1.33	498±22	521±36
126750 cells/mL	8.22±0.52	8.37±0.52	8.67±1.98	8.49±1.42	498±21	520±35
97500 cells/mL	8.42±0.57	8.47±0.54	8.90±1.79	8.62±1.33	547±42	539±40
75000 cells/mL	8.31±0.37	8.30±0.38	7.70±2.04	7.71±1.96	531±62	532±61

3.3.2. Age at first reproduction

Only the lowest tested food concentration (75000cells/mL) increased the age at first reproduction for both clone K6 (Kruskal-Wallis: H_4=24.7, p<0.001, followed by the Dunn's test: p<0.05) and clone BEAK (Kruskal-Wallis: H_4=49.1, p<0.001, Dunn's test: p<0.05) (Fig. 13). Both clones released the first brood 1.5 (for K6) and 1 day later (BEAK) than the control.

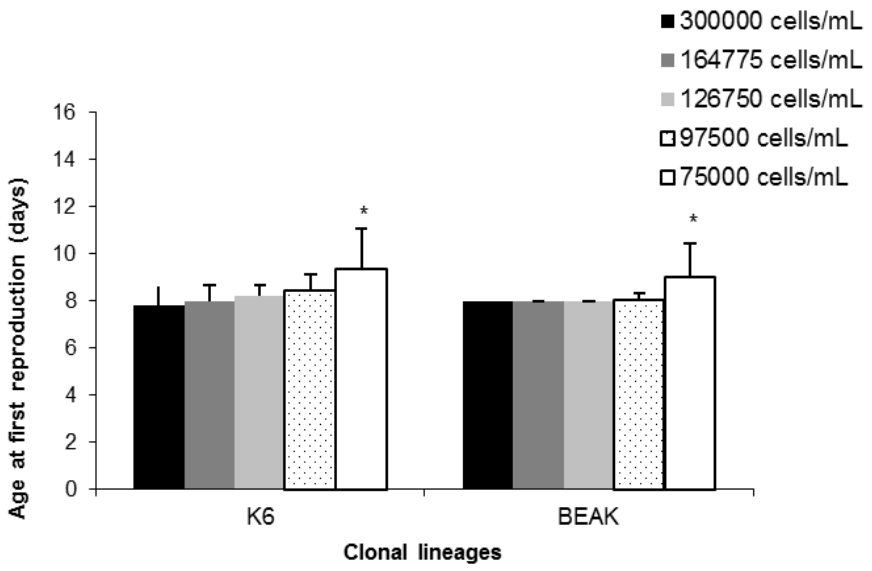

Figure 13 - Average of age at first reproduction (days) for clonal lineages K6 and BEAK, of *Daphnia magna*, exposed to food restriction. Error bars represent the standard deviation. Significant differences from control are indicated by *(p<0.05), following Kruskal-Wallis and Dunn's post-hoc tests.

3.3.3. Life span and reproduction parameters

The life span of both clonal lineages (K6 and BEAK) did not significantly differ from the control in any treatment (K6 - Kruskal-Wallis: H_4=4.26, p=0.372 and BEAK - Kruskal-Wallis: H_4=17.4, p=0.002, followed by the Dunn's test: $p \geq 0.05$; Fig. 14.

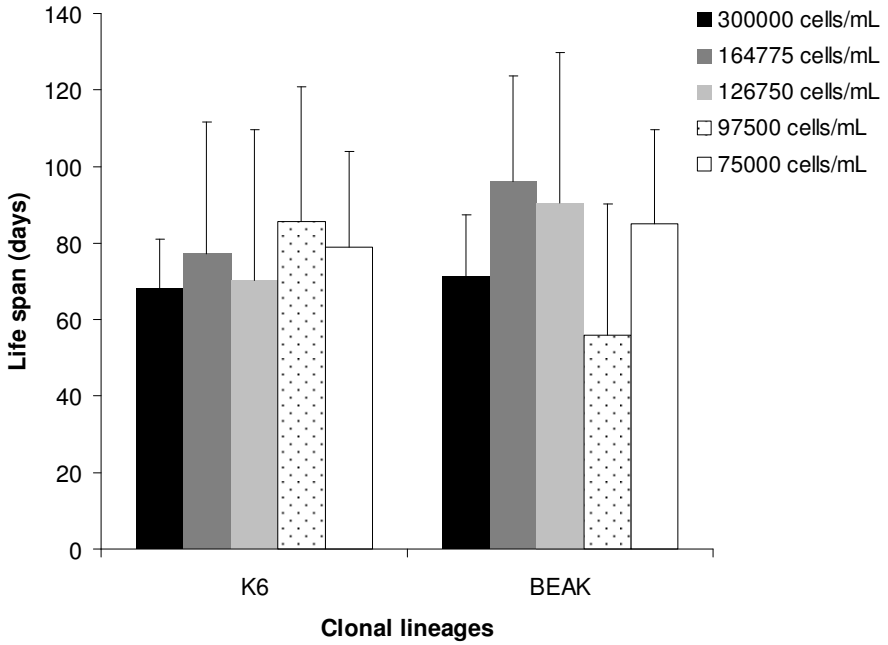

Figure 14 – Average of life span (days) for clonal lineages K6 and BEAK, of *Daphnia magna* after being exposed to food restriction. Error bars represent standard deviation.

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Regardless of the non-significant differences in the mean life span, there is a trend in survival rates, where a tendency for prolonged survival of some females in the food restriction groups is observed comparatively with the control (Fig. 15). All food restriction groups lived approximately 20% more than the corresponding controls. Individuals from clone K6 exposed to the control exhibited a slightly higher survival rate until approximately the 65th and the 75th days when it had an abrupt descend (about 75%). BEAK control survival decreased more progressively. The BEAK group fed with 97500 cells/mL had, over the course of the assay, a relative lower survival, yet the longest lived daphnid (137 days).

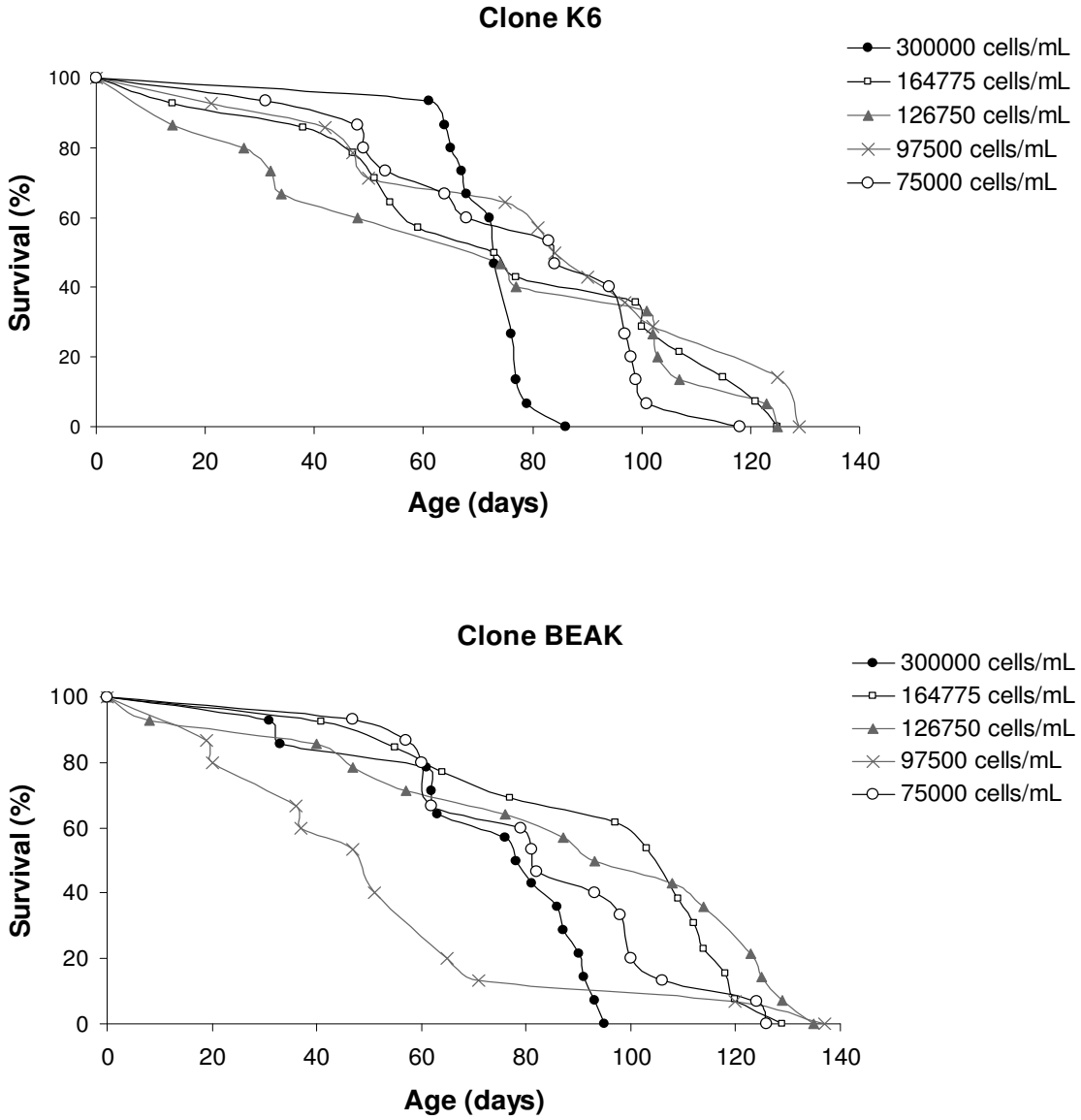

Figure 15 - Survival curves (%) for females of clonal lineages K6 and BEAK, of *Daphnia magna* after being exposed to food restriction.

Regarding reproduction, a significant decrease in the total number of neonates was observed for daphnids exposed to all tested food levels, except for clone BEAK exposed at 164775 cells/mL (K6, Kruskal-Wallis: H_4=53.7, p<0.001, followed by the Dunn's test: p<0.05 and BEAK, Kruskal-Wallis: H_4=61.4, p<0.001, Dunn's test: p<0.05). Clone BEAK presented a smaller number of neonates at the second lowest tested food concentration (97500 cells/mL) than at the lowest (75000 cells/mL) (Fig. 16), but the difference is not significant.

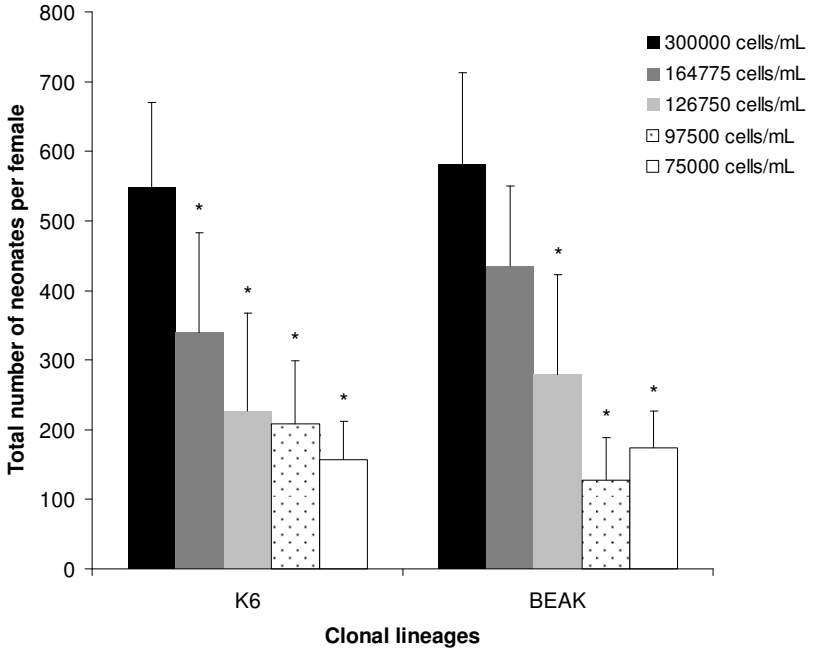

Figure 16 - Average of the total number of neonates produced per female of clonal lineages K6 and BEAK, of *Daphnia magna*, after being exposed to food restriction. Error bars represent standard deviation. Significant differences from control are indicated by *($p<0.05$), following Kruskal-Wallis and Dunn's post-hoc tests. in food concentration.

3.3.4. Gene expression analysis

PNC1 and GAPDH gene expression levels were determined in samples from control (300000 cells/mL) and food restriction (75000 cells/mL) experiments.

Results from the agarose gel electrophoresis are displayed in Figure 17. Bands from the PNC1 gene were compared to those of GAPDH by measuring band intensity (excluding the intensity of the image background) and calculating the resulting PNC1/GAPDH ratios. Data were normalized to a sample from the control group (within median values for both longevity and reproduction parameters, marked on the figures by •). One sample from 75000 cells/mL using Dm_GAPDH primers was not successfully amplified. All bands from 75000 cells/mL group and one from control presented pixel saturation; the exposure length was not optimal, so the number of saturated pixels was surpassed (less than 1% is desirable).

Consequently, the relative quantifications are underestimated (marked as ‡ on Figure 17).

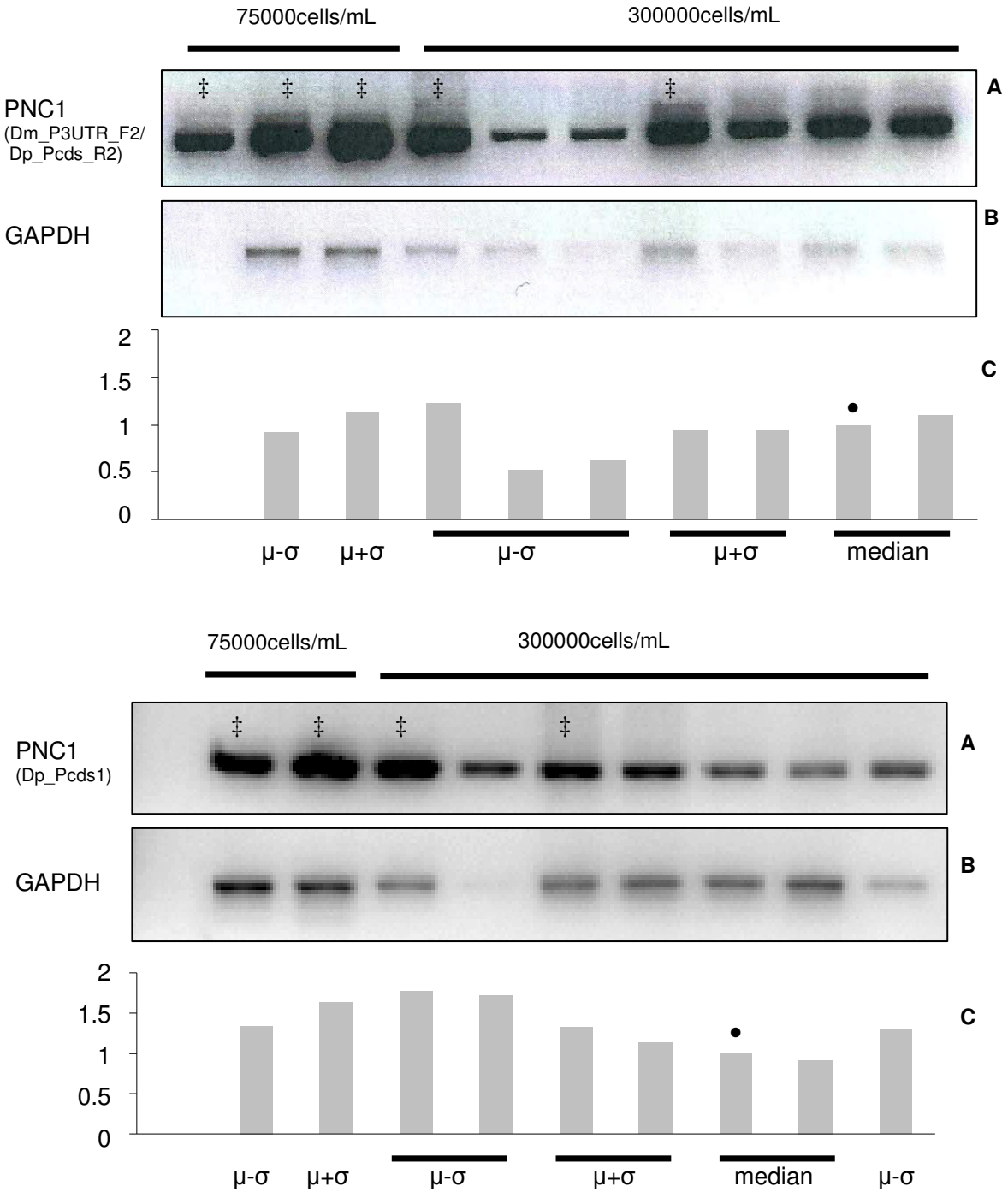

Figure 17 - cDNA PCR products of PNC1 (amplified with the primers Dm_P3UTR_F2/Dp_Pcds_R2 and Dp_Pcds1) (A) and GAPDH (B) genes of *Daphnia magna* clonal lineage K6 fed with 75000 cells/mL. GAPDH was used as control (housekeeping gene). The first bands on the left could not be compared due to their non-amplified samples; ‡, pixel saturation. C - Relative mRNA expression in function of a median control (●) by normalization of the signal

intensity ratios. The samples are from daphnids whose life span and reproduction values were above mean+stdev (μ+σ), under mean-stdev (μ-σ) or around the median values for each group.

3.4 Sirtuin phylogeny

As PNC1 is a regulator of sirtuin activity, we have further characterized *Daphnia* sirtuins. Through BLAST searches, we have identified 5 *D. pulex* sequences corresponding to the 7 human sirtuins. In order to correctly match each sequence, a reciprocal best hit approach was applied (Table 6).

Table 6 - The sirtuin family of protein deacetylases with the subcelullar location and correspondent *Daphnia pulex* protein ID code.

Human Sirtuin	Localization	Protein ID
SIRT1	Nucleus, PML body, cytoplasm	196125
SIRT2	Cytoplasm, cytoskeleton	227006
SIRT3	Mitochondrion matrix	227006
SIRT4	Mitochondrion matrix	194758
SIRT5	Cytoplasm, mitochondrion	195468
SIRT6	Nucleus (nucleoplasm)	199958
SIRT7	Cytoplasm, nucleolus (nucleolus)	199958

Sirtuin aminoacid sequences were aligned and used to build a phylogenetic tree, using the available Mr.Bayes running in Geneious (Fig. 18). The tree is well resolved, except for *SIRT1* and Dp_196125 that surprisingly were not grouped. As expected, *SIRT2* and *SIRT3* grouped together with Dp_227006, and the same was observed for *SIRT6* and *SIRT7* that are both close to Dp_199958.

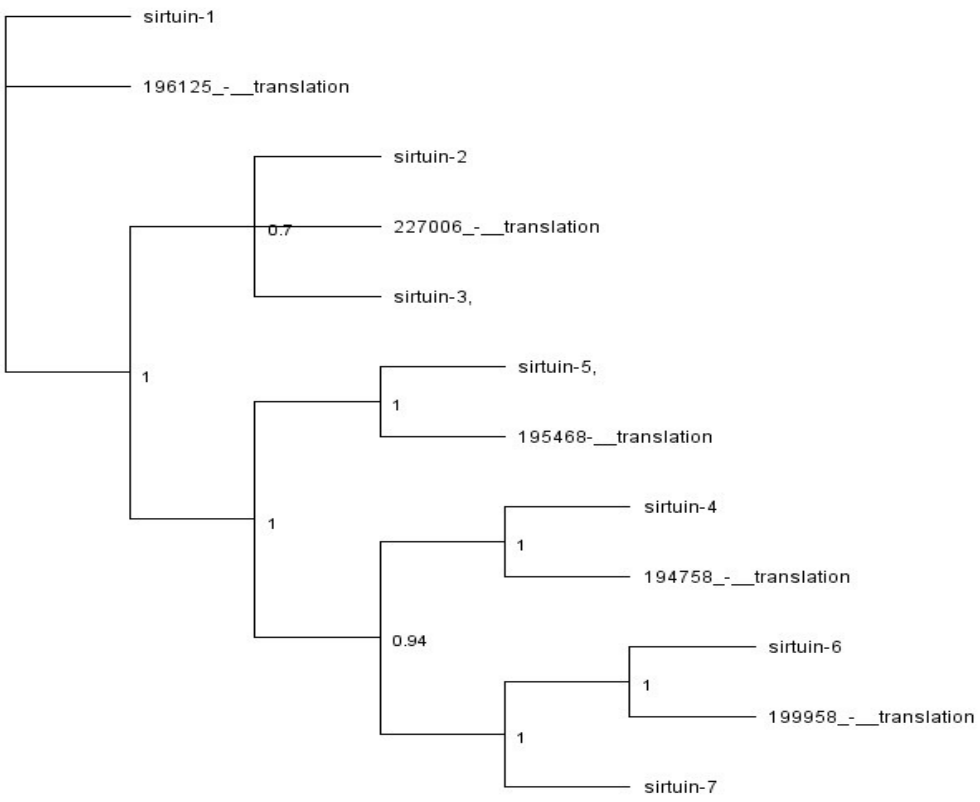

Figure 18 - Bayesian phylogenetic analysis of the sirtuin family of protein deacetylases, based on the aminoacid sequence alignment of 12 sirtuins (7 human and 5 from *Daphnia pulex*).

4. DISCUSSION

4. DISCUSSION

The outcomes of the present work showed that, with a few exceptions, the variations in the tested environmental parameters (temperature, copper levels and food restriction) caused a significant effect at the individual level, both in age at first reproduction, reproduction and life span. Molecular data, nevertheless, did not reflect those differences, as discussed further ahead in the text.

4.1. Temperature

Several studies in literature have addressed the effects that an increase or decrease in temperature may cause in many species. Specifically for poikilothermic organisms (with no effective physiological mechanisms for thermoregulation), as *Daphnia spp.,* temperature is a key factor (Ebert, 2005). Experiments carried out with poikilothermic species, such as the zebra mussel *Dreissena polymorpha* (Rao and Khan, 2000), the crayfish *Orconectes immunis* (Khan et al., 2006) and the vertebrate gilthead sea bream *Sparus aurata* (Guinea and Fernandez, 1997), reported an intensified metabolic activity with increasing temperature, measured as the oxygen consumption rates. It is also known that cladoceran developmental rates (both embryonic and post-embryonic) are faster with increasing temperatures (Brown, 1929; Korinek, 1970; Munro and White, 1975). Therefore, increased metabolic and developmental rates may have consequences in the reproduction and life span of *D. magna*. Within the present work, a significant increase, of 6 to 1.2 days, and decrease, of 1.6 to 4 days, in age at first reproduction was observed for clone K6 and BEAK of *D. magna* after being exposed to 15ºC and 25ºC, respectively, in comparison to the control. These results are in line with other published works with several other species (e.g. Kryuchkova, 1973; Goss and Bunting, 1983; Huey et al., 1995). For example, Huye et al. (1995) reported that *D. melanogaster* laid eggs 1 day earlier when exposed at 25ºC comparatively to those flies exposed to 18ºC. In addition, Goss and Bunting (1983) also observed for *D. magna* a delay of 6.3 days in the first newborn release for animals exposed to 15ºC and a precocious release of 3.5

days of the first reproduction for animals exposed at 25ºC, comparatively with daphnids exposed to 20ºC. The number of days until the first brood was equally comparable to the same authors' study (15ºC: 14.3 and 11.2 days for K6 and BEAK, respectively, compared to 16.3 days in Goss and Bunting (1983); 25ºC: 6.4 and 6 days, for K6 and BEAK, compared to 6.5 days in Goss and Bunting (1983); 20ºC: 8 and 10 days, for K6 and BEAK, compared to 10 days in Goss and Bunting (1983). These results can be explained, for the females exposed at 25ºC, by the increase of the metabolic rates, as abovementioned. Likewise, a temperature decrease to 15ºC led to a prolongation of the development, in accordance with a previous report by Hardy and Duncan (1994), due to a metabolic slowdown (Gillooly et al., 2006).

Overall, temperature had more influence in the life span than in reproduction. The average life span for *D. magna* K6 living at 20ºC was 81.1 days, which is in accordance with data obtained by Robertson (1988) (though no information is given in the paper, concerning the clonal lineage of *D. magna* used in the work) and 56.9 days at 15ºC, which corresponds to a much lower life span than that observed by the same author: 100 days as the average life span for *D. magna* at 15ºC. Furthermore, previous works carried out with *D. magna* referred an average life span at 25ºC of 40 (Anderson and Jenkins, 1942) and 60 days (Robertson, 1988). The life span observed in the present study at 25ºC was 44 days (for K6) and 52.5 days (for BEAK), within the range of those reported in the studies previously mentioned.

Most studies regarding total reproduction in *Daphnia* only account for the first broods. However, Goss and Bunting (1983) estimated the total reproduction in *D. pulex* at 20ºC to be 230 neonates in average, far less than the values observed here (approximately 400); nevertheless, these values are different probably because they relate two different species. Higher temperatures (in the sublethal range) were related to the production of a reduced number of neonates (Green, 1956) of *D. magna*. The present work showed the same pattern, only for the K6 clone. Therefore, BEAK clone appears to be more resistant to temperature stress than K6. Despite the decrease in the average life span, both clones K6 and BEAK exposed at 15ºC and 25ºC, respectively, exhibited a similar reproduction output

comparatively to the respective control. Possible explanations may reside in a general increase of the newborn number per clutch (for K6), and a tendency for an increase of the brood frequency (for BEAK). However, these hypotheses are based in observed trends and cannot be assumed as significant measurements, since many broods were not susceptible to be accurately individualized.

As referred in the introduction, works by Stephenson et al. (1984), MacIsaac et al. (1985) and Lagerspetz (2000) demonstrated *D. magna* to be genetically able to tolerate high temperatures. However, in the current work, both clones of *D. magna* were more affected by 25ºC exposure than by 15ºC: the high temperature affected the life span of both clones (15ºC only affected K6) and the effect of temperature in the total number of newborns produced was only observed at 25ºC, in clone K6. It is also possible to suggest that 25ºC is a more critical stress temperature for the K6 clone than for BEAK: 25ºC affected both life span (decreased 45.7% relatively to the control) and reproduction in K6, whereas it only affected the life span in BEAK.

In the present study, no molecular information was obtained regarding the effects induced by temperature variation. Therefore, no comparisons or conclusions could be withdrawn.

4.2. ***Copper***

The importance of copper as a trace element can be observed through its role in multiple cellular enzymes (Siegel et al., 1999). However, the redox nature of copper, although essential to many cellular processes, is also a powerful toxicant (Bossuyt and Colin, 2005), if the concentration in the environment is higher. The maximum recommendable value in water for human consumption is 3000µg/L (MA, 1998). The World Health Organization has established a 2mg/L copper guidance level (Zeitz et al., 2003). This order of magnitude was far from being attained in the assays performed in the present work. *Daphnia* is very sensitive to this metal so the tested concentrations were very low, still environmentally realistic and within the range of many copper toxicity studies (Atienzar et al., 2001; Bossuyt and Colin, 2005; Lopes et al., 2006; Agra et al. 2011).

The two lowest concentrations (11.1 and 16.6µg/L) induced an increase in the age of the first brood release for clonal lineage K6. The same was observed for BEAK exposed to 16.6µg/L. Higher copper concentrations did not affect the time of the first brood release. Interestingly, a study by Atienzar et al. (2001) reported an increase of the mean period for *D. magna* to become ovigerous (of 1 day) at the highest copper concentration (120µg/L).

Regarding the effects of copper in life span, Kirchman and Botta (2007) found that the supplementation of glycerol-containing media with copper (to a final concentration of 62µM), resulted in an increase of the life span (from 17 to 72%) in yeast. Furthermore, these authors also found that adding copper to glucose medium had no effect on yeast life span. In the present study, a significant reduction in life span for all tested copper concentrations was observed, except in the lowest (11.1µg/L). These results showed that these clones are more sensitive to copper exposure than clone 5 of *D. magna*, in which longevity was only affected at 120 µg/L (Atienzar et al., 2001). However, regarding reproduction, the clonal lineage BEAK was in agreement with the same report, where a reduction in the number of eggs per female occurred in a range of 15 to 120µg/L. In the current study, 11.1µg/L of copper did not affect the number of total neonates produced per female of clone BEAK. Regardless, K6 clone was affected in all concentrations.

In the present study, the effect of copper was also evaluated at the molecular level. Though it is known that copper may induce DNA damage (mutagenicity) (Tkeshelashvili et al., 1991), the copper concentrations here tested did not cause any mutations in the PNC1 gene, neither in the control (GAPDH). However, all sequenced samples presented SNPs. These alterations are a consequence of the naturally occurring variability (Colbourne et al., 2011). Sequences of *D. magna* from different geographic locations (that possibly combine sexual and asexual reproduction), showed equally synonymous variations, possibly resulting as well from the influence of their habitat.

4.3. Food restriction

Food supply is highly variable in aquatic habitats and the planktonic herbivores often live at different food levels (Gliwicz and Guisande, 1992). It was recognised previously that both quantity and quality of food are important for daphnids (OECD, 1997). The dietary treatment here experimented had slightly lower food concentrations than the ratios tested in other studies. Richardson (1985) and Weindruch et al. (1986), for instance, used between 25% to 60% less than the *ad libitum* quantities. In the present study, there was a reduction of 45% to 75% relatively to the *ad libitum* quantity available in the control. Still, the lowest tested food concentration (75000 cells/mL) was the only one to cause a delay in age at first reproduction of the females of both clones. On the other hand, a study in which *D. similis* were subjected to a low food content (20% of the correspondent high food concentration), did not exhibit differences in the age at first reproduction (Pedrozo and Bohrer, 2003).

The significant decrease of the total reproduction was expected, as females grown at low food levels produced smaller clutches. This pattern of response was also reported in several previous works with *Daphnia* spp. (Gliwicz and Guisande, 1992; Pietrzak et al., 2010).

The life span extension (longevity) under food restriction is a biological phenomenon well identified in many organisms (Sinclair, 2005,) as well as in *Daphnia* spp. (e.g. Latta et al., 2011). The results of the present study were not expected, as no significant differences were found in the life span of daphnids fed with a lower food concentration; despite that, the trend in survival rates (especially in K6 clone), evidences a prolonged survival of some females in the food restriction groups comparatively with the control. On the other hand, Pedrozo and Bohrer (2003) reported an increase in life span of *D. similis* reared at high food concentration. Nevertheless, those studies use different species of algae as food and the information relating its carbon content is not always provided, so comparisons could be difficult to establish.

An increased expression of PNC1 is both necessary for replicative life span extension by caloric restriction and low-intensity stress (Anderson, 2003).

Therefore, it would be expected for *Daphnia* under caloric restriction to present an overexpression in the PNC1 mRNA levels. However, observing the ratios of the corresponding cDNA band intensities (Fig. 17 C), no differences could be found, because the samples from the control group (300000 cells/mL) presented both high and low relative mRNA expression levels. Nevertheless, the individuals under food restriction with increased life span and reproduction values (above mean+stdev) seem to present a higher relative expression than those from the same group, but under mean-stdev, regarding the same parameters. Still, more replicates must be carried out in the present study in order to confirm the abovementioned for the daphnids.

Though not being a goal of the present work, a phylogenetic tree comparing human and *D. pulex* sirtuin aminoacid sequences was carried out in order to assess if *D. pulex* sirtuins were equally conserved as those of other model species. This phylogenetic tree presented high levels of confidence (probability values between 0.97 and 1). Human sirtuins 2 and 3 were grouped together with the *D. pulex* sequence Dp_227006, but presented a lower score (0.7). Sirtuin 1 and the sequence Dp_196125 were not grouped and therefore can be considered here as outgroups. The sirtuin phylogeny here presented may reinforce the conservative charactistics of this family of deacetylases and more specifically observe the aminoacid homology between *D. pulex* and human sirtuins.

5. CONCLUSIONS AND FINAL REMARKS

5. CONCLUSIONS AND FINAL REMARKS

With a life-time exposure of *Daphnia magna* to particular environmental parameters, this work intended to assess their influence on the individual longevity parameters, genetic outcome, in terms of mutations, gene expression levels of the PNC1 gene and protein expression. At the individual level, the copper exposure had a more intense effect than the other environmental factors in this study, because more significant differences were found here, comparing to temperature and food restriction influences. Copper and food restriction exposure had a stronger influence than temperature, regarding reproduction, as can be observed, again, by the levels of significance, greater than for temperature. Nevertheless, temperature was more influent than the other parameters at the age *D. magna* females became primiparous, for both temperatures had influence. At the molecular level, the differences here obtained were not reflected in the pyrazinamidase and nicotinamidase gene, PNC1: there were no mutations that could be attributed to copper influence or gene expression level alterations associated to food restriction influences. However, daphnids with an increased life span and reproduction presented higher relative mRNA expression of PNC1 than those with values under the mean-stdev. Comparing the responses of the clonal lineages here tested, K6 was more sensitive to temperature, copper and caloric restriction than BEAK.

5.1. Future perspectives

As more *D. magna* genetic information is sequenced, further research with other longevity associated genes could be performed, to confirm or refute the effects reported on the present study. Equally, other factors could be tested for their influence in *D. magna* life span and consequently, longevity. Furthermore, other parthenogenetic model species could be used to assess the same issues here presented, for instance, artemia and the collembolan *Folsomia candida* (Browne, 1992; Tully et al., 2006). The concept of the experiment could as well be

modified to approach this theme from another perspective: an intergenerational study. It would be valuable to follow the consequences of a permanent exposure in the forthcoming generations of daphnids, by observing its reproduction and life span implications and possible maternal effects in the newborns. Part of the cladocerans would be allowed to grow and reproduce, as others would, in parallel, be used for molecular assessments.

6. REFERENCES

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