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Effects of Respiratory Perturbations on Aging and Healthspan in *Daphnia magna*

A thesis
presented to
the faculty of the Department of Biological Sciences
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Master of Science in Biology

by
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May 2021

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Keywords: *Daphnia magna*, hypoxia, healthspan, 2, 4-dinitrophenol, lifespan, RNA-Seq

ABSTRACT

Effects of Respiratory Perturbations on Aging and Healthspan in *Daphnia magna*

by

Millicent Nkiruka Ekwudo

Aging is a degenerative process characterized by a decline in physiological functions and cellular activities. Environmental and pharmacological interventions affecting longevity pathways have been extensively studied in model organisms. This study investigated the effect of chronic mild intermittent hypoxia (4 mg O₂/L) or mild mitochondrial uncoupling with three doses of 0 (control), 0.1, 1, and 5 μM of 2,4-Dinitrophenol (DNP), on life history and gene expression in four clones of *Daphnia magna*. Interestingly, clones from intermittent ponds displayed better tolerance to hypoxia and DNP. Although neither treatments extended longevity, hypoxia increased fecundity and body size, and decreased food consumption and respiration rate. We uncovered 12 candidate genes that were differentially expressed in hypoxia-tolerant and sensitive clones in response to hypoxia. Unexpectedly, DNP increased fecundity and mitochondrial membrane potential without affecting food intake. This work opens up an opportunity for genomic determination of the potentially important phenotypes in a model organism.

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DEDICATION

To little girls who were forced to draw swords in battles

Little hands holding on to big dreams

Little voices refusing to be silenced

To the Unbroken

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I was never an isle on this project; this work came to fruition because of various people whom I would like to appreciate. I would like to begin with my advisor, Dr. Lev Yampolsky; thank you for taking me under your wing, for creating a conducive research environment for my muse to thrive, for your incredible supervision and invaluable counsel. I owe so much to you.

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CHAPTER 1. INTRODUCTION

Mitochondria and Mitochondrial Membrane Potential

The mitochondria are vital self-replicating organelles that can no longer be solely described as the powerhouse of the cell since recent studies have demonstrated that they play a vital role not just in bioenergetics and metabolism, but also in the maintenance of calcium levels, cell death, autophagy and production of reactive oxygen species (ROS) (Akbari et al. 2019; Belenguer et al. 2019). The electron transport chain (ETC), which occurs in the mitochondrial inner membrane, provides eukaryotes with significant amounts of adenosine triphosphate (ATP) necessary for the maintenance of biological activities. In the ETC, oxygen acts as the final electron acceptor. The ETC involves five complexes, of which complexes I, III and IV are H⁺ pumps that largely generate the mitochondrial membrane potential (Zorova et al. 2018).

Mitochondrial membrane potential ($\Delta\Psi_m$) is created largely by the proton concentration gradient, and is used to drive ATP synthesis in the mitochondrial matrix. Mitochondrial membrane potential is fairly stable under standard conditions and is an indicator of cellular health and viability. Increased $\Delta\Psi_m$ tends to elevate ROS, which could result in oxidative damage, whereas a large decrease in $\Delta\Psi_m$ could lead to a decline in ROS production and reductive stress. Oxidative and reductive stress may trigger apoptotic mechanisms or even cause various pathologies (Peris et al. 2019).

During the early stress response, $\Delta\Psi_m$ can be significantly affected, possibly being the reason why $\Delta\Psi_m$ is a good measure for assessing cellular stress and apoptotic processes (Witte and Horke 2011; Barbour and Turner 2014). Mitochondrial membrane potential can be modulated endogenously by mitochondrial uncoupling proteins or exogenously by

pharmacological uncouplers such as carbonyl cyanide m-chlorophenylhydrazone (CCCP), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and 2,4-Dinitrophenol (DNP).

Rhodamine 123 is a cationic green fluorescent dye used for the detection of the electrochemical potential across the mitochondrial inner membrane, the greater the fluorescence, the greater the mitochondrial membrane potential. The diffusion of rhodamine 123 from the mitochondrial matrix into the cytoplasm results in a decrease in the amount fluorescence emitted by the dye in the treated tissues and this may serve as evidence of mitochondrial dysfunction (Chazotte, 2011). The rhodamine 123 probe is preferred for its commercial availability, affordability, minimal interference with cellular processes, desirable quantum yield, and easy detection (Forster et al. 2012).

Aging and Mitochondria

Aging is a degenerative process often characterized by a decline in physiological functions and cellular activities and can lead to tissue failure and ultimately, death. Mitochondrial biogenesis, which entails the formation of new mitochondria from preexisting ones, frequently begins with Peroxisome Proliferator-Activated Receptor-gamma (PPAR γ) coactivator-1alpha (PGC-1 α) (Li et al. 2017; Akbari et al. 2019; Bouchez et al. 2019), with the phosphorylated or deacetylated form of PGC-1 α being active and can activate the transcription factors nuclear respiratory factor 1 (NRF1) and NRF2 (Li et al. 2017). Ultimately, through both factors, the expression of mitochondrial transcription factor A (TFAM) is induced, with TFAM translocating into mitochondria and inducing the expression of mtDNA encoded genes. Other nuclear-encoded mitochondrial proteins are also induced by NRF1 and NRF2, leading to mitochondrial division and new mitochondria being formed (Bouchez et al.2019). Mitochondrial

function and mitochondrial biogenesis decline with age whereas the frequency of mitochondrial DNA mutation increases with age (Akbari et al. 2019).

While the mitochondrial free radical theory of aging proposes ageing is induced by oxidative damage arising from the accumulation of ROS, which are products of cellular metabolism (Pomatto et al. 2018), the mitochondrial theory of aging considers aging as a consequence of mitochondrial damage and mutations accumulated in mtDNA (Wei et al. 2001). Thus, both theories implicate oxidative damage as a predisposition to senescence (Yeo 2019).

Hypoxia and Hypoxia-Mediated Responses

Hypoxia in an aquatic environment occurs when the dissolved oxygen (DO) level is less than 2.8 mg O₂/L (Lai et al. 2016) and can result in mass mortality not just in fishes but macroinvertebrates as well. For most animals, suffocation can occur at low oxygen levels, but hypoxic effects begin to set in when the level of dissolved oxygen drops to 2 mg/L, which is a huge deviation from the normal 8 mg O₂/L (Collins, 2019). Chronic mild intermittent hypoxia (CMIH) refers to daily exposure to 50% dissolved oxygen (4 mg O₂/L). Responses to hypoxia have been well characterized in *Daphnia*, including acclamatory gene expression adjustments (Pirow et al. 2001; Bäumer et al. 2002; Zeis et al. 2003; Klumpen et al. 2017) that can extend epigenetically across parthenogenetic generations (Lai et al. 2016). Also, hypoxia increases hemoglobin concentration and mortality rate, and decreases body size and fecundity (Seidl et al. 2005; Lyu et al. 2015). Coggins et al. 2017, reported that exposure of *D. magna* to mild hypoxia at 5 mg O₂/L for 90 minutes significantly increased acute heat tolerance, possibly through activation of antioxidant pathways.

Hypoxia mediated responses are controlled by a family of heterodimeric transcription factors, the hypoxia-inducible factors (HIFs). The HIF family includes three genes; HIF1A,

HIF2A, and HIF3A (Wang et al. 1995; Yeo 2019). The HIF pathway interacts with other pathways such as SIRT1, AMPK, mTOR, and NF- κ B pathways (Ruderman et al. 2010; Hong et al. 2014; Antikainen et al. 2017; Pan et al. 2017). Hypoxic responses at the cellular level are maintained mostly by HIF1 α (Wang et al. 1995; Iyer et al. 1998). Hypoxia response elements (HREs) are located in the promoter region of hypoxia-responsive genes and HIF1 α exerts transcriptional control over roughly 100 target genes during hypoxia (Yang et al. 2015) especially genes associated with oxygen homeostasis such as Vascular Endothelial Growth Factor (*VEGF*), Erythropoietin (*EPO*), and hemoglobin that are vital for increasing tissue perfusion and oxygenation as adaptive responses to hypoxia (Zeis et al. 2009; Yeo 2019).

HIF1 α upregulates pyruvate dehydrogenase kinase and consequently the conversion of pyruvate to lactate when low oxygen hinders ETC function, thereby decreasing NAD⁺: NADH ratio. This ratio is vital for intracellular redox homeostasis especially in the mitochondria and nucleus as well as cellular signaling and the regulation of metabolic activities. A decline in NAD⁺: NADH ratio is indicative of slower oxidative metabolism. Furthermore, HIF1 α upregulates the expression of glucose transporters GLUT1 and GLUT 3 to improve glucose absorption and other glycolytic enzymes, thus promoting anaerobic glycolysis (Yeo 2019). Importantly, HIF1 α modulates c-MYC and IGF2 signaling, the cell cycle, and apoptotic pathways (Yeo 2019). Hypoxia induces inflammation and the immune response by increasing the expression of tumor necrosis factor-alpha (TNF- α). Interestingly, HIF-1 α is constitutively expressed under normoxia, although the protein is degraded, and expression is highly upregulated under hypoxic conditions and by increased ROS through NF- κ B (a redox-sensitive transcription factor) (Bonello et al. 2007).

Hypoxia-induced AMPK-associated pathways and aging. The *FOXO* (forkhead box O) genes, most notably FOXO1 and FOXO3A in mammals, exert protective effects on cells and increase resistance to oxidative stress by upregulating the activities of catalases, thus promoting longevity (Wang et al. 2017). Invertebrates such as *Daphnia* possess one *FOXO* homolog, which may promote longevity like was shown for the *C. elegans FOXO* homolog DAF-16 (Murtaza et al. 2017).

Sirtuins are a family of seven mammalian proteins (SIRT 1-7) homologous to the silent information regulator 2 (*sir2*) protein of yeast; orthologs of several sirtuins are also present in *Daphnia* genome. They are NAD⁺-dependent protein deacetylases (and deacylases) whose activities have been greatly investigated because they oversee the regulation of biological processes such as inflammation, apoptosis and senescence. Since several of these processes are associated with aging, *SIRT1* is often described as the “longevity gene”. However, aging decreases NAD⁺: NADH ratio in organisms, thereby repressing the activity of sirtuins.

When activated, SIRT1 protects cells against age-associated pathologies by promoting DNA stability, oxidative stress alleviation, and regulation of glucose and lipid metabolism (Guarente 2011; Kilic et al. 2015). SIRT1 stimulates the expression and activity of FOXO (earlier described) and it also prevents the acetylation of the p53 gene, which can trigger cell apoptosis (Alcendor et al. 2004, 2007). SIRT1 enhances mitochondrial biogenesis by deacetylating the p53 and PGC-1 α genes (Lee and Gu 2013).

The AMP activated protein kinase pathway regulates oxidative energy metabolism and responds to a variety of stresses such as a decrease in glucose levels, ischemia, heat shock and hypoxia (Yeo 2019). Previous studies have implicated this pathway in slowing the rate of aging. Salminen et al. 2012, suggest that activation of the AMPK pathway decreases oxidative stress by

increasing thioredoxin levels and the autophagic degradation of protein aggregates (such as observed in Alzheimer's disease with the amyloid-beta peptide). AMPK also attenuates endoplasmic reticulum (ER) stress and inflammatory diseases.

Hypoxia can directly trigger the AMPK (Figure 1) pathway when there is an elevation in the AMP: ATP ratio. It can also activate SIRT1, which in turn triggers the AMPK pathway through phosphorylation of AMPK by *LKB1* (Tumor suppressor serine/threonine-protein kinase a regulator of AMPK). Activation of *LKB1* by SIRT1 leads to the phosphorylation and activation of p53 (Jones et al. 2005). Following this activation, cyclin dependent protein kinase inhibitor turns on genes that induce cell death (p21 and *Bax*). These genes arrest the cell cycle and initiate apoptosis (Yeo 2019). Additionally, the phosphorylation of p53 by AMPK and the inactivation of SIRT1, increases the acetylation of p53 and consequently the proapoptotic genes are further upregulated. (Lee et al. 2012).

AMPK also plays a role in the regulation of mitochondrial biogenesis through PGC-1 α . In skeletal muscles, activation of AMPK during exercise increases the activity of PGC-1 α , which augments mitochondrial biogenesis and promotes the transcription of mitochondrial genes (Jäger et al. 2007). When PGC-1 α is acetylated, mitochondrial biogenesis is greatly reduced (Jornayvaz and Shulman 2010; Fernandez-Marcos et al. 2011).

Gui et al. (2017) suggest that hypoxia is accompanied by cellular autophagy. Protein kinase mTOR can decrease lifespan (Pan et al. 2017). ULK1 is a protein kinase that promotes autophagy during nutrient depletion. Activated AMPK can phosphorylate ULK1 directly or indirectly through the mTOR protein by activating the TSC2 gene, a negative regulator of mTOR. AMPK can thus exert an inhibitory effect on mTOR, decreasing cell growth and

proliferation. MTOR phosphorylates ULK1 and inhibits autophagy (Kim et al. 2011; Shang et al. 2011).

AMPK suppresses NF- κ B signaling by alleviating ER and oxidative stress as well as by phosphorylating proteins such as SIRT1, p53, PGC-1 α , and FOXO3A, which in turn inhibits NF- κ B-mediated inflammation (Salminen et al. 2011). This anti-inflammatory activity of AMPK helps to enhance healthspan and lifespan (Yeo 2019).

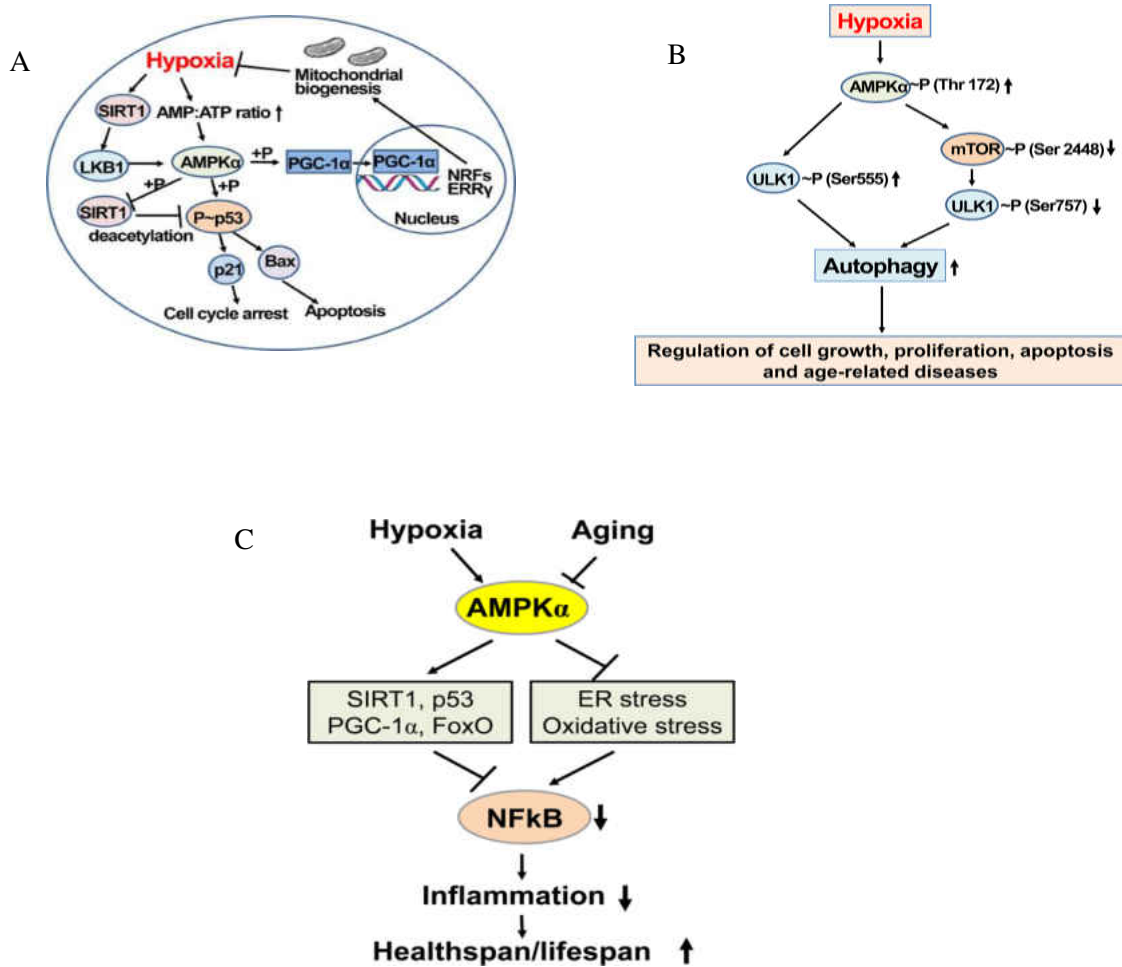


Figure 1. Hypoxia-induced AMPK-associated pathways and aging. A. Hypoxia mediated activation of AMPK through AMP: ATP ratio and SIRT1. B. Hypoxia-mediated autophagy through AMPK’s crosstalk with mTOR-ULK1. C. Relationship between hypoxia, AMPK, aging and NF-KB signaling. Yeo, EJ. (2019) Hypoxia and aging. *Exp Mol Med.* 51, 1–15 (creative commons license 4.0).

2,4-Dinitrophenol

2,4-Dinitrophenol (DNP) is a protonophore that carries protons down their concentration gradient across lipid bilayers. In mitochondria protonophores dissipate the proton gradient uncoupling oxidative phosphorylation, thus inhibiting ATP synthesis. DNP was taken by humans

as an anti-obesity agonist (Tainter 1935) before its ban by the FDA in the 1930's. (Caldeira da Silva et al. 2008). The lipolytic effects of DNP have been suggested to occur independently of lipolytic enzymes, but instead occur through autophagy since endolysosomal vesicles engulf lipid droplets in adipocytes (Demine et al. 2017). Sun and Zemel (2003), showed DNP increased the expression of fatty acid synthase but suppressed its activities in an intracellular Ca^{2+} -independent manner. They also reported that DNP inhibited lipolysis in a calcium-dependent manner.

Nile red (NR) is a remarkable histological stain known to fluoresce brightly in lipophilic environments (Hornum et al. 2021). Thus, the NR probe has been commonly used to assess intracellular lipid droplets in diverse organisms such as yeast, microalgae, *C. elegans*, and *Daphnia magna* (Alemán-Nava et al. 2016; Escorcía et al. 2018; Song et al. 2020; Gosalawit et al. 2021).

Despite being banned by the FDA, over the last couple of decades DNP has been increasingly used in animal studies. Interestingly, the neuroprotective role of DNP in neurodegenerative and aging-associated disorders has become a “hot” area of research. Recent studies have considered DNP prodrugs with the goal to optimize DNP's rapid pharmacokinetics, and attenuate its toxicity and thermogenicity, while enjoying the benefits of mild mitochondrial uncoupling (Khan et al. 2017; Geisler 2019).

Liu et al. (2015) demonstrated a neuroprotective of DNP in cortical neurons where the uncoupler suppressed the mTOR and insulin receptor-PI3K-MAPK pathways. DNP also upregulated CREB (cAMP-response element-binding protein) signaling, autophagy-related genes, as well as proteins involved in maintaining synaptic plasticity and stress response. Mitochondrial uncoupling with DNP decreases mitochondrial membrane potential, ROS

production, and tissue and protein oxidation (Cho et al. 2017; Ulgherait et al. 2020; Caldeira da Siva et al. 2008). DNP increases intracellular calcium and the AMP:ATP ratio. The latter stimulates the AMPK pathway whose important role in aging has been described above. Samaiya et al. (2018) provided evidence that a seven day administration of DNP increased antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) in post-anoxia neonatal rats. DNP also increased the respiration rate and decreased the expression of proapoptotic proteins, thus preventing mitochondrial dysfunction.

The lifespan-extending potential of DNP has been demonstrated in various species including mice, toad, *Drosophila*, and *C. elegans* (Caldeira da Silva, 2008; Salin et al. 2012; Ulgherait et al. 2020, Cho et al. 2017). DNP did not increase lifespan in zebra finches. DNP also caused a decline in inflammatory immune response and failed to decrease oxidative stress (Stier et al. 2014). Surprisingly, there is dearth of literature on the effect of mitochondrial uncoupling with DNP on longevity in the reputable model organism *Daphnia magna*. Additionally, clone-specific responses to chronic DNP exposure in *D. magna* are yet to be understood. This research seeks to bridge the knowledge gap.

Daphnia magna as a Model Organism

Considered one of NIH's recognized model organisms (Edison et al. 2016) *Daphnia magna* are plankton crustaceans that are primary consumers and thus are an integral part of trophic webs. They are short-lived and easy to culture (Lai et al. 2016). *Daphnia* reproduce by cyclic parthenogenesis, giving rise to clonal offspring that are genetically identical, thus, allowing experimental work with genetically uniform individuals. The *Daphnia* genome is responsive to environmental stressors (Coulborne et al. 2011). In the presence of environmental stressors (predation, temperature, low food), male offspring and haploid resting eggs are

produced (Khudr et al. 2019). These eggs require fertilization and thus *Daphnia* populations are outbred even when genetically uniform. These make *D. magna* a great model for investigating the effects of genetics, various environmental factors, and pharmacological treatments on lifespan (Constantinou et al. 2019) and healthspan.

Daphnia magna clones are found in geographically diverse habitats (Table 1). Clones IL and FI are from intermittent ponds/ pools that are short-lived. As the water dries up, particularly in the summer/fall, dissolved oxygen presumably becomes less available. Hence these clones are better accustomed (tolerant) to hypoxic conditions (unpublished data). On the other hand, their conspecifics GB and HU are from permanent lakes/ponds that are all year round. Hence these clones are hypoxia naïve (sensitive).

Hypotheses and Specific Goals

The goal of this study is to investigate the effects of hypoxia and mitochondria uncoupling on lifespan in four different clones of *Daphnia magna*. Specifically, we expected that prolonged exposure to mild intermittent hypoxia or DNP might improve longevity and healthspan. Additionally, we aimed to test the predictions that the aging-decelerating effects of chronic mild intermittent hypoxia (CMIH thereafter) will be more pronounced in hypoxia-adapted clones of *Daphnia* than in hypoxia naïve ones and that acclimation to CMIH would improve tolerance to acute severe hypoxic stress. To test these hypotheses, we conducted the following experiments:

1. We evaluated age-related changes of $\text{NAD}^+:\text{NADH}$ ratio (using lactate:pyruvate as a proxy) in *Daphnia* from geographically distinct clones in hypoxic and normoxic conditions.

2. We assessed the effects of hypoxia and mitochondrial uncoupling using the mild uncoupler DNP, on life history traits such as survival, respiration rate, feeding rate, body length and fecundity, mitochondrial membrane potential, and neutral lipid storage.
3. We measured transcriptomic changes in response to CMIH (18 days) and acute (12 h) severe hypoxia (<1 mg O₂/L) in all the four clones.

CHAPTER 2. MATERIALS AND METHODS

Daphnia Cultures

Daphnia magna from four clones, two with low hypoxia naivety (IL, FI) and two with high hypoxia naivety (GB, HU) (Table 1) were maintained on a 12:12 light dark cycle at 20 °C in 100 mL glass jars containing artificial pond (COMBO) medium (Kilham et al. 1998) at the density of n=5. Daily, each individual was fed approximately 100,000 *Scenedesmus obliquus* cells/ mL augmented with essential minerals and vitamins. Artificial pond medium and jars were replaced every 3 days to avoid waste buildup and to maintain desired density. Adult female *Daphnia* were maintained for three generations before use. The goal of three-generation acclimation was to annul maternal effects (Yampolsky et al. 2014). Therefore, for all experiments, third generation (G3) female adults were used.

CMIH Longevity Experiments

Seven days old G3 individuals were transferred to TetraO 10 L glass tanks organized in 2 consecutive experimental blocks consisting of three tanks in block 1 and five tanks in block 2. Each tank contained an array of eight plastic containers (Appendix A) equipped with 1 mm plastic mesh, each of which had a capacity to house 20 individuals. Cups were equitably assigned to all four clones, thus creating a common garden experiment, with *Daphnia* of different clones sharing the same tank (Figure 2). The mesh allowed water to circulate between containers (facilitated by regular raising and lowering the containers into the tank) as well as removal of neonates produced inside each container. Thus, each tank contained 160 individuals from 4 clones, 40 individuals per clone, as the start of each cohort. Tanks were maintained at 20°C until all individuals in a cohort died or were censored from the experiment with water level and food quantity adjusted every three days to maintain 20 mL of water per *Daphnia* and

100,000 *Scenedesmus* cells per mL as food. Water change was done every 3 days to prevent the accumulation of waste products (especially ammonia) and to prevent competition for food between mothers and neonates. At the time of each water change, adult individuals in each cup/tank were counted, neonates removed, and volume of fresh COMBO water adjusted to suit the current number of individuals alive. *Daphnia* aged 15-20 days as well as 55-60 days old were frozen for future assays.

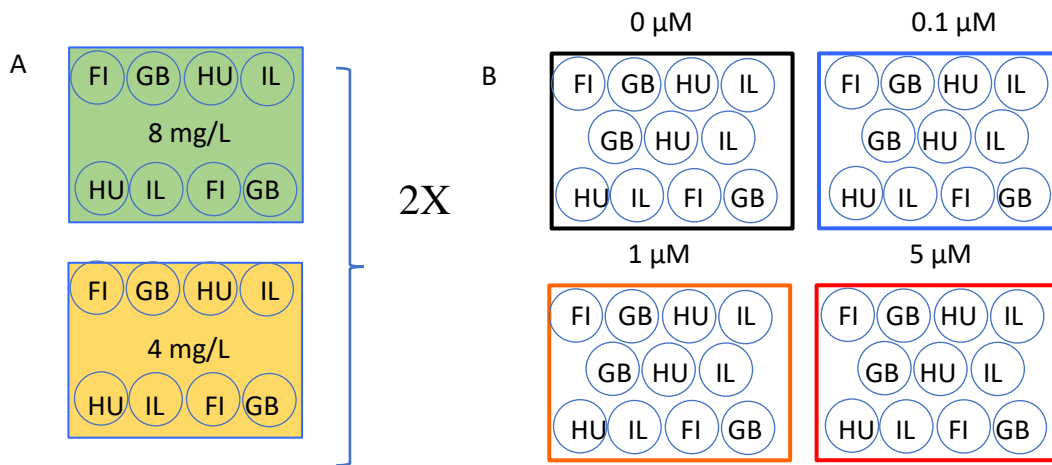


Figure 2. Schematic of hypoxia and DNP experiments. Concentrations of O₂ mg/L (A) and DNP, μM (B) are shown. Colors correspond to color code on figures thereafter.

Oxygen levels in each tank were adjusted twice daily by bubbling either compressed nitrogen gas at a flow rate of approximately 25 mL per second through COMBO water (Butler et al. 1994) until the oxygen concentration decreased to 4 mg O₂/L for the hypoxic conditions, or ambient air until the concentration 8 mg O₂/L was attained. The dissolved oxygen concentration was measured with either YSI Pro20 or Extech DO210 dissolved oxygen probes (Appendix D).

Fecundity in Hypoxia Experiments

To assess fecundity in normoxic and hypoxic conditions, snapshot measurements were taken, such that individuals with clutches, ovaries, and ephippium were counted and recorded. Also, the number of eggs in three randomly selected individuals from each cup were counted.

Respiration in Hypoxia

Respiration rates were measured using Loligo® 24-channel fluorometer (Loligo Systems, Viborg, Denmark; sensor software: MicroResp™) equipped with SDR SensorDish® sensor (PreSens Precision Sensing, Regensburg, Germany). To investigate total metabolic rate, 1700 µL of hypoxic/normoxic COMBO water were pipetted into each well of Loligo sensor plate with one *Daphnia* per well and with each plate containing 4 blanks (without *Daphnia*) and the other twenty wells containing 20 *Daphnia*, 10 from each hypoxia treatment. The wells were sealed with PCR film avoiding air bubbles. The well plates were placed on Loligo sensors and oxygen readings were logged for 45 minutes with the first 15 minutes discarded. A similar procedure was implemented to measure basal metabolic rate (i.e. in immobilized individuals) except that it was done in 200 µL Loligo plates containing 1% urethane solution in hypoxic/ normoxic water. The same individual *Daphnia* from the total metabolic rate measurements were transferred to the 1% urethane-containing plate. Afterwards, *Daphnia* were thoroughly rinsed with COMBO water, body length was measured, and individuals turned back to the tanks in which they were sampled.

Feeding Rate in Hypoxia

To measure filtering rate in normoxia and hypoxia, we placed *Daphnia* from either normoxic or hypoxic treatments individually into plastic cell culture flasks containing 20 mL of COMBO water with the same oxygen concentration as in *Daphnia*'s tanks of origin containing 200,000 cells/mL of *Scenedesmus* food (i.e., twice the regular food concentration). A total of

fifty 20 mL flasks were used, 25 flasks per treatment. Out of these 50 flasks, 10 were blanks (had no *Daphnia*, just COMBO water with food). The other 40 flasks were distributed evenly between the two treatments and four clones, resulting in five replicate individuals per clone per treatment. The flasks were placed in the dark in a 20°C incubator on a shaker set to 60 rpm.

Food concentration with chlorophyll fluorescence as a proxy was measured with Loligo fluorometer (set to amplitude) using plastic 24-well plates (not Loligo sensor plates) at the beginning of the experiment as well as after 17 and 24 hours by taking 3 mL samples from each flask, with replacement. Pilot experiments demonstrated that there was not enough sensitivity to detect consistent decreases of food concentration during shorter durations. Fluorescence readings were logged for 5 minutes.

At the end of the 24-hour experiment, body length of all individuals, from the top of the head to the posterior edge of the carapace, excluding the spine, was measured using an ocular micrometer and *Daphnia* were returned to the tanks from which they were sampled.

Measurement of Hemoglobin

We assessed hemoglobin concentration in *Daphnia* tissues by either measuring the intensity of red coloration in optical microscopy or by absorption at 575 nm using a Nanodrop spectrophotometer. First, whole bodies of individuals sampled from the hypoxia experiment, one *Daphnia* per clone, and four per treatment (n=8) were photographed using a 10X lens on a Leica compound microscope equipped with color camera, upper body, and abdomen separately. The image was split into RGB channels and the intensity of the red channel was measured across the whole image using ImageJ. Next, the same *Daphnia* individuals were homogenized in 10 µL of PBS, the homogenates centrifuged at 13,000 rpm for 4 min and the supernatant absorption measured using a Nanodrop spectrophotometer at 541 and 575 nm.

Lactate and Pyruvate Measurements

To compare lactate and pyruvate levels in hypoxic and normoxic individuals, pyruvate and lactate assays were conducted using CellBiolab kits. 15-20 days old and 55-60 days old *Daphnia* were sampled from the experimental tanks, stored frozen at -80°C until assay time. Each *Daphnia* was homogenized in 100 µL ice-cold PBS with a pestle and the homogenates were centrifuged at 4°C. 25 µL of supernatant were pipetted into each of the lactate and pyruvate assay plates using the manufacturer's protocol (CellBiolab CAT IDs 101820174 & 82320181). Additionally, 25 µL of the supernatant were used to quantify soluble proteins by Bradford assay with 175 µl of Bradford colorimetric reagent added to each well. Water served as blanks for some of the wells and BSA concentrations from 0.125 to 2 mg/mL used for calibration. All assay well plates were analyzed using BIOTEK plate reader, lactate and pyruvate fluorescence assay sensitivity was set to 35 and the Bradford assay absorption was measured at 595 nm.

Acute Hypoxia Experiments and Sample Collection for RNA-Seq

Daphnia were sampled from each of the four tanks, two tanks per hypoxia treatment (see above) at the age of 25 days (i.e. after 18 days at either normoxic or mild intermittent hypoxic conditions) and moved into 70 mL cell culture flasks filled with COMBO water with the concentration of oxygen at or below 1 mg/L, sealed without a bubble, 7 *Daphnia* per flask. Control groups included *Daphnia* exposed to normoxia or chronic hypoxia but not acute hypoxia. Flasks were kept at 20°C. The acute hypoxia experiment was set up at 9:00 p.m. and mortality was recorded 12 hours afterwards and every hour subsequently. Individuals for RNA sequencing were frozen at the beginning at the experiment and after 12 hours exposure, sampling 2 individuals from each flask. Flasks were then topped with 1 mg O₂/L water and sealed again.

At the end of the 48 hours of exposure, the collective wet weight of adult individuals and any neonates born in each flask was measured.

Table 1. Location of Geographically Diverse *Daphnia* Clones Used. Clone ID, habitat and geographic coordinates are displayed (Yampolsky et al. 2014). IL-MI-8 and FI-FSP1-16-2 have the least hypoxia naivety while GB-EL75-69 and HU-K-6 have the highest (Yampolsky, unpublished).

CLONE ID	LOCATION	LATITUDE	LONGITUDE	HABITAT
IL-MI-8	Jerusalem, Israel	318° 42' 52"	358 3' 03"	Intermittent Mediterranean pond
FI-FSP1-16-2	Suur-Pellinki, Finland	608 ° 10' 04''	258 47' 41''	Intermittent summer rock pool
GB-EL75-69	London, UK	518 ° 30' 26''	208 7' 39''	Permanent pond
HU-K-6	Fülöpszállás, Hungary	46° 47' 33.3"	19° 10' 53.84"	Permanent lake

RNA Sequencing

Two individuals from each of the four clones (IL, FI, GB, and HU) and from each of the hypoxia treatments were frozen during the acute hypoxia experiment (see above). The four treatments were: *Daphnia* reared at normoxia (8 mg O₂/L), *Daphnia* reared at normoxia and exposed to acute hypoxia (<1 mg O₂/L) for 12 hours, *Daphnia* reared at mild intermittent hypoxia (4-6 mg/L O₂), and *Daphnia* reared at mild intermittent hypoxia and exposed to acute hypoxia for 12 hours. RNA extraction was performed using Qiagen RNAeasy kit (Cat ID: 74134). RNA was quantified by a Qubit (ThermoFisher) fluorometer.

Following extraction, RNA were reverse transcribed and sequencing libraries were constructed from the cDNAs as prescribed by Oxford Nanopore Technology PCR-cDNA

Barcoding kit protocol (SQK-PCB109), with 3 biological replicates per clone per treatment, each replicate consisting of RNA extracted from two *Daphnia*. Barcoded samples from the 4 treatments within each clone were pooled together into 3 replicate libraries purified separately and pooled together immediately before adding the sequencing adapter. Libraries were then sequenced using Oxford Nanopore MinION for 24-48 hours per sequencing run obtaining 2-4 Gb of reads in each run.

Gene Expression and Transcript Analysis

Once sequencing was completed, base calling and quality filtering were done using OxfordNanopore *guppy* basecaller. The reads were then demultiplexed and barcodes trimmed using *guppy* barcoder and aligned to a reference transcriptome (ver. DM3.0) obtained from Dieter Ebert and Peter Fields (Basel University, Switzerland). Following alignment, BAM files obtained were merged and indexed using with *samtools* and read counts per transcript exported for further analysis. Transcripts were filtered to retain only those that had at least 60 reads in at least one sample. Reads per kilobase per million reads (RPKM) and Transcripts per kilobase per million (TPM) in each sample were calculated and used as the response variables in downstream statistical analysis. Principal components analysis and heatmap clustering were performed to evaluate clustering of samples in the space of transcripts abundances.

Differential gene expression in acute hypoxia was analyzed in *JMP*® using a full factorial general linear model with clones, chronic hypoxia levels and acute hypoxia levels as factors, and RPKM as the response variable. False discovery rate was calculated for each transcript to correct for multiple testing. An *a priori* determined set of transcripts with expected changes in response to hypoxia was analyzed with respect to significant effects of chronic hypoxia or their interaction. In order to identify transcripts that respond to hypoxia differently in

clones from permanent habitats (sensitive) and clones from intermittent habitats (resistant), we analyzed generalized model of the effects of habitat and clones nested within habitats on the abundance of reads mapped to each transcript, with the assumption of binomial distribution and with logit link function (JMP, SAS Institute, 2012)

DNP Longevity Experiments

Ten days old *Daphnia* from the four clones were placed, in groups of 10 into plastic inserts with the bottom opening covered with 1 mm nylon mesh, each of which were placed into 200 mL jars (Appendix E) containing 0, or 0.1, or 1, or 5 μM concentration of DNP (Figure 2). The LC50 for *Daphnia magna* is 4.1 mg/L (Sigma 2013) so the highest dose of DNP used (5 μM) was about 4.5 times below the LC50. The jars were maintained at 20°C with 100,000 algae cells per mL per day supplied as food and water changed every 3 days. At each water change the surviving individuals and offspring produced were counted blindly and water volume and food supply adjusted to maintain 20 mL of water per *Daphnia*.

Rhodamine 123 Assays

On day 10 and 30, one individual per clone per treatment (a total of 16 individuals per assay) were sampled. Each individual was kept in 0.5mL of 4 μM rhodamine 123 and 0.5 mL of the concentration of COMBO water with or without DNP and left in the dark for 24 hours at 20°C. Post incubation, individuals were washed twice with COMBO water to get rid of excess rhodamine 123. *Daphnia* body regions of interest (head and epipodite) were photographed with a Leica confocal microscope at 10X, cube 3 (blue-wide green-red) exposure filter 50% at gain 1 (Appendix B).

Nile Red Assay for Lipids

Following photographing *Daphnia* after rhodamine 123 exposure, the COMBO water was removed from the vials and individual daphnids were incubated in 1 $\mu\text{g/mL}$ of NR for two hours in the dark at 20°C. The vials were left to sit in the dark incubator at 20°C for two hours after which they were rinsed thrice with COMBO water. Then, the body regions, abdomen, and mid-sections (base of antenna and heart) were photographed (Appendix C).

Food Consumption with DNP Treatment

The feeding rate was measured in a similar way as in the hypoxia feeding experiment. A two-point calibration was created such that *Daphnia* were in water containing 0 and 5 μM DNP with food (200,000 cells) and without food.

Statistical Analyses

Statistical analyses for this study were performed using the software JMP (SAS Institute, Cary, North Carolina). Data are shown as mean \pm SE. Lifespan/ survival in chronic mild and acute severe hypoxia was analyzed using proportional hazard and parametric survival. 3-way ANOVA analysis was performed to assess the effect of hypoxia, age, and clone on the lactate/pyruvate. Three-way ANOVA was done to investigate the effects of O₂ concentration and clones on the respiration rate, with wet weight as a covariable. A 2-way ANOVA was used to investigate the effect of O₂ and clone on body length. One-tailed student's t test was used to analyze the mean absorbance of hemoglobin at 541 and 575 nm, as well as the median red intensity of the upper body and abdomen. Two-way ANOVA was used to determine the effect of O₂ and clone on clutch size and the number of eggs per female.

For DNP experiments, longevity analysis was done using parametric survival platform and effects likelihood ratio tests, additional survival comparisons were done using log rank and

Wilcoxon tests. Two-way ANOVA was used to investigate the effect of DNP concentration and clones on sum fecundity in early, mid, and late life respectively. Two-way ANOVA was employed to assess the effect of DNP and clones on feeding rate normalized by wet weight.

Image analysis was performed with *ImageJ*. Nile Red fluorescence and rhodamine 123 fluorescence intensities (total number of pixels and sum of intensities weighted by the number of pixels) were log-transformed to achieve normality of residuals of linear regression over DNP concentration.

CHAPTER 3. RESULTS

Contrary to the expectations, neither CMIH nor prolonged exposure to DNP increases lifespan in *Daphnia magna*. Both respiratory perturbations decreased lifespan in clones originating from permanent habitats, but not in clones from hypoxia-prone intermittent ones. We observed clone by environment interactions for some of the traits assessed. Also, we uncovered genes showing strong habitat-by-hypoxia interactions. The results of the hypoxia and DNP experiments are summarized in Table 2.

Table 2. Summary of Results for Hypoxia and DNP Experiments

Measurements	Treatments	
	Hypoxia (8 vs. 4 mg O ₂ /L)	DNP (0, 0.1, 1, 5 μM)
Longevity (lifespan)	Reduced for clones from permanent ponds (Figure 3, Table 3)	Reduced for clones from permanent ponds (Figure.14, Table 9)
Fecundity	Increased at older ages (Figure 4)	Increased in a dose dependent manner (Figure 16, Table 10)
Body length	Significantly increased in a clone- specific manner (Figure 5, Table 4).	No effect. Analysis not shown
Feeding rate	Significantly increased (Figure 6, Table 4)	No effect on food intake (Figure 17, Table 11)
Respiratory rate	No effect.(Figure 8, Table 6)	Analysis not performed
Lactate:Pyruvate ratio	Significantly increased especially in older <i>Daphnia</i> (Figure 9, Table 7).	Analysis not performed
Hemoglobin	Significantly increased (Figure 10)	Analysis not performed
Mitochondrial membrane potential (MMP)	Analysis not performed	Unexpectedly increased (Figure 18, Table 12)
Lipid storage	Analysis not performed	Significantly decreased (Figure 19, Tables 13)

Effect of CMIH on Longevity

CMIH did not extend lifespan in any of the clones (Figure 3). In fact, it significantly ($p=0.0084$) reduced lifespan in hypoxia naïve clones originating from permanent ponds/ lakes (GB and HU) and had no significant ($p=0.9579$) effect on hypoxia- adapted clones from intermittent ponds (IL and FI). While there was a significant effect of clone (0.0007) on the survival of clones from intermittent habitats in hypoxia, we provide no such evidence for clones from permanent ponds (Table 3).

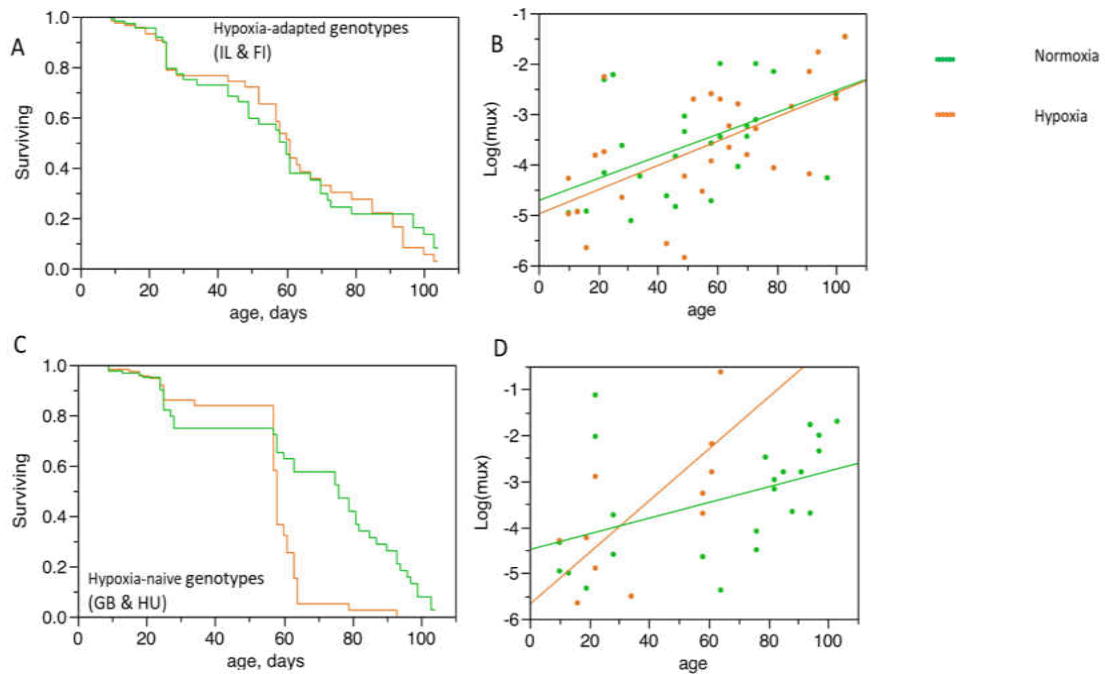


Figure 3. Effect of chronic mild intermittent hypoxia on lifespan. A-D Kaplan-Meier survival and mortality rate among four different *D. magna* clones from intermittent (hypoxia adapted) and permanent habitats (hypoxia-naïve). Probability of survival/mortality rate on the vertical axes and age on the horizontal axes. Orange lines and dots represents hypoxia, green normoxia, here and throughout. Statistical analysis is shown in Table 3.

Table 3. Parametric Survival Fit Effect Likelihood Ratio Tests for *D. magna* Clones from Intermittent and Permanent Habitats

Habitat	Source	DF	L-R ChiSquare	Prob > ChiSq
Intermittent	O ₂	1	0.00279	0.96
	Clone	1	11.3744	0.0007
	O ₂ *Clone	1	0.15468	0.69
Permanent	O ₂	1	6.95623	0.0084
	Clone	1	1.24407	0.26
	O ₂ *Clone	1	0.00074	0.98

Effect of Hypoxia on Fecundity

Though hypoxia had no significant effect ($p=0.9439$) on early age (18 days) fecundity, and fecundity in middle age (38 days) was lower ($p=0.9675$), there was a significant ($p=0.0021$) increase in fecundity in older *Daphnia* (66 days). Additionally, there was no significant interaction between clones and oxygen levels in all ages (Figure 4).

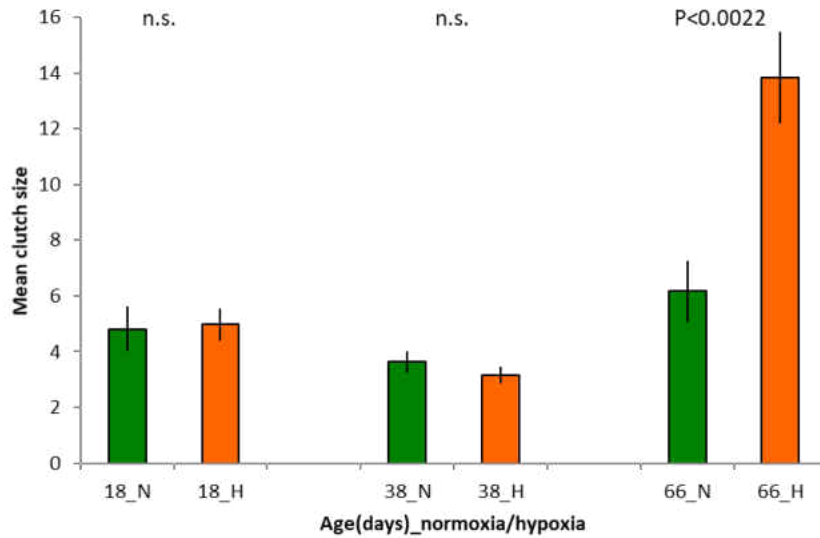


Figure 4. Fecundity in hypoxia. Mean clutch size of hypoxia and normoxia acclimated *Daphnia*. Green bars represent normoxia, orange bars represent hypoxia, here and throughout. Vertical bars represent standard error.

Effect of Hypoxia on Body Length

Exposure to CMIH resulted in a significant ($p=0.0377$) increase in body length across all clones compared to controls, except in GBs, where there was a decrease in body length (Figure 5). There was also a significant interaction between clones and hypoxia (Table 4).

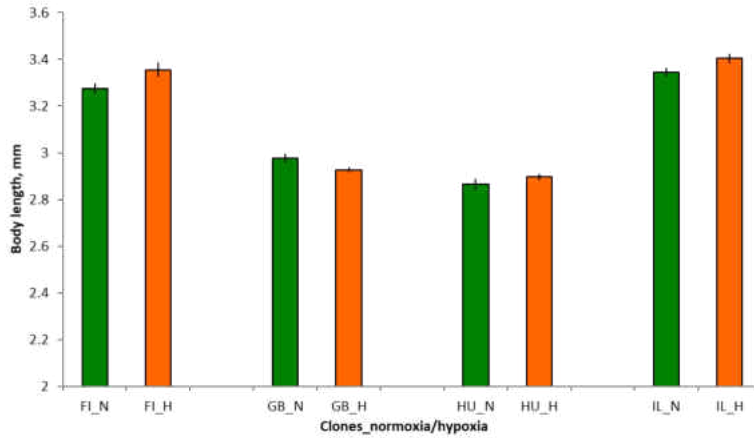


Figure 5. Body length of clones acclimated to hypoxia. Green and orange bars represent mean body length (mm) in normoxic and hypoxic clones and vertical bars represent standard error. P-values are shown in Table 4.

Effect of Hypoxia on Feeding Rate

Food intake declined across all clones in hypoxic conditions compared to controls with IL and FI consuming the most amount of food respectively, and GB remarkably consuming the least number of algal cells (Figure 6). Although hypoxia and clone had a significant effect ($p=0.0014$ and $p =0.003$, respectively) on feeding rate, there were no significant interactions with each other (Table 4).

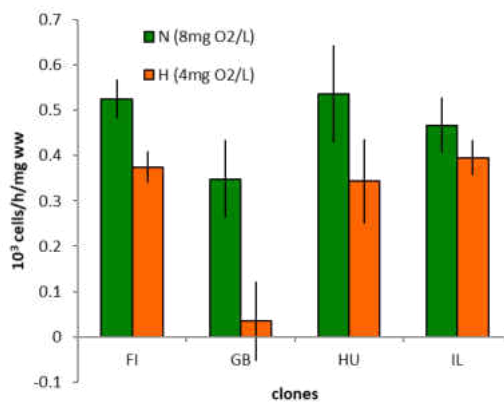


Figure 6. Feeding rate in hypoxia. Green and orange bars represent mean cells consumed per hour normalized by body wet weight (10^3 cells/h/mg ww). Vertical bars represent standard error.

P-values are shown in Table 4.

Table 4. ANOVA Model of the Effects of Hypoxia on Feeding Rate and Body Length

Response: feeding rate				
Source	DF	MS	F Ratio	Prob > F
Hypoxia	1	0.331	12.24	0.0014
Clones	3	0.155	5.73	0.003
Hypoxia*clones	3	0.026	0.95	0.43
Error	32	0.027		
Response: body length				
Source	DF	MS	F Ratio	Prob > F
Hypoxia	1	0.037	4.4	0.0377
Clones	3	2.479	298.35	<.0001
Hypoxia*clones	3	0.034	4.1	0.0079
Error	151	0.008		

Survival in Acute Severe Hypoxia

Acclimation to CMIH significantly decreased survival in acute severe hypoxia (Figure 7). It wasn't surprising to observe poor survival of hypoxia/ normoxia acclimated clones from permanent ponds in acute severe hypoxia, but it was unexpected to observe this in hypoxia-tolerant clones from intermittent ponds. Normoxia acclimated clones from intermittent ponds had the highest survival in these experiments. Overall, habitat, treatment and clone nested within habitat had significant effects on survival and a significant interaction between clone and treatment nested within habitat was recorded (Table 5).

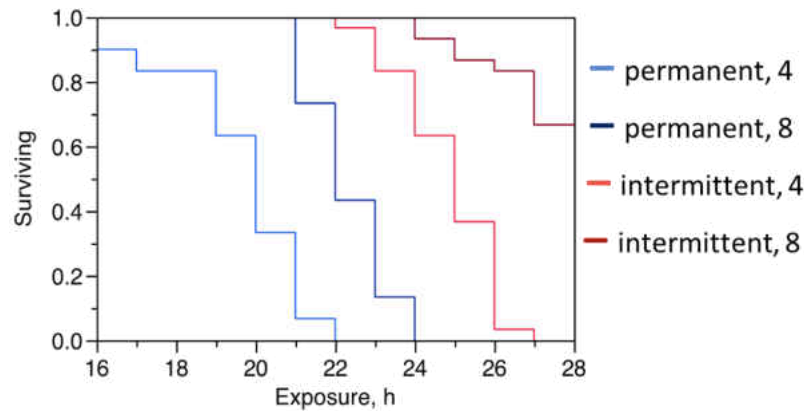


Figure 7. Parametric survival plot of all clones and treatments following exposure to severe acute hypoxia. Survival is plotted on the vertical axis and hours of exposure is plotted on the horizontal axis. The light blue line represents hypoxia-acclimated clones from permanent ponds. The dark blue line represents normoxia-acclimated clones from permanent ponds. The light red line represents hypoxia-acclimated clones from intermittent ponds. And the dark red line represents normoxia-acclimated clones from intermittent ponds. Statistical analysis is shown in Table 5.

Table 5. Effect Likelihood Ratio Tests for Survival in Acute Severe Hypoxia Tolerance. cHyp84 represents *Daphnia* acclimated to normoxia (8 mg O₂/L) or hypoxia (4 mg O₂/L). Habitat is permanent or intermittent ponds.

Source	DF	L-R ChiSquare	Prob > ChiSq
Habitat	1	204.168028	<.0001
Clone[habitat]	2	6.3965041	0.0408
cHyp84	1	98.4017384	<.0001
habitat*cHyp84	1	3.14979839	0.0759
Clone*cHyp84[habitat]	2	7.98079205	0.0185

Effect of Hypoxia on Respiration Rate

There was no statistically significant difference in the respiratory rate between hypoxia- and normoxia-acclimated *Daphnia* measured in either condition (Figure 8, $p < 0.13$), with no interactions detected (Table 6).

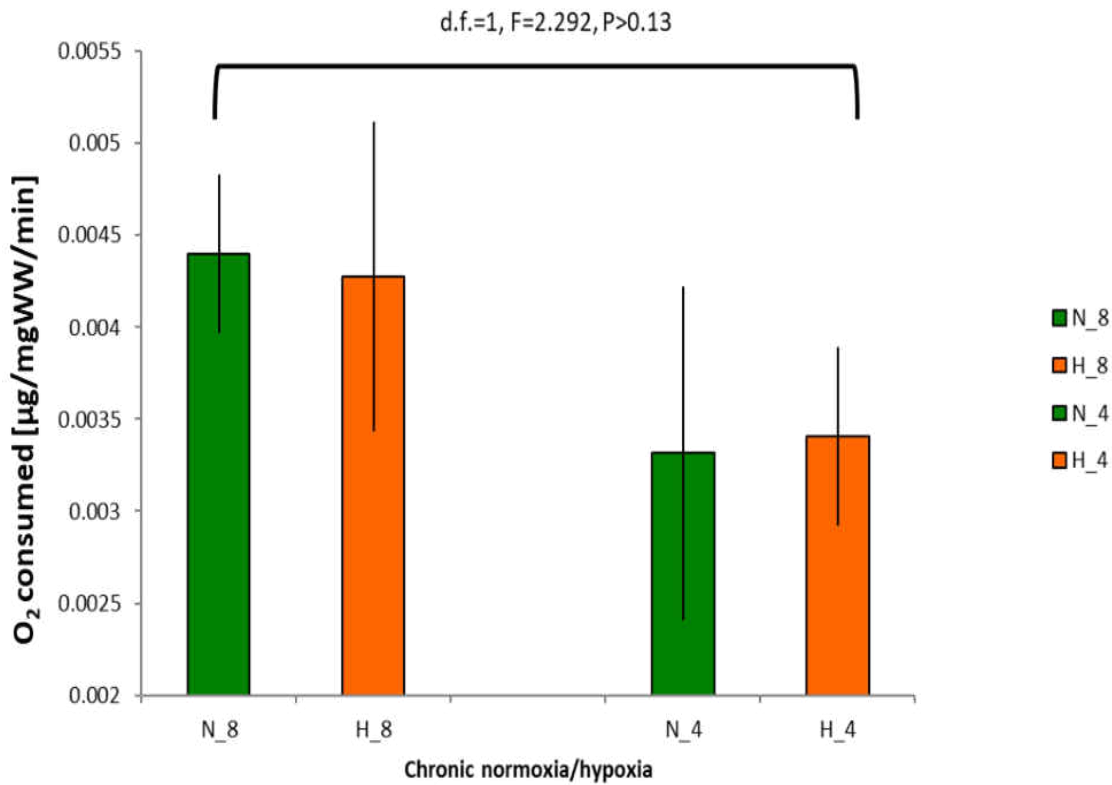


Figure 8. Respiratory rate in hypoxia. Oxygen consumed normalized by wet weight. N_8 is normoxia acclimated *Daphnia*, assay O₂ =8, H_8 is normoxia acclimated *Daphnia* (assay O₂=4), N_4 is hypoxia acclimated *Daphnia* (assay O₂=8), H_4 is hypoxia acclimated *Daphnia* (assay O₂=4). Vertical bars represent standard error. Statistical analysis is shown in Table 6.

Table 6. Three-way ANOVA for the Effect of Hypoxia on Respiratory Rate. Respiratory rate is normalized by wet weight.

Source	DF	MSx10 ³	F Ratio	Prob > F
Assay O ₂	1	0.023	2.048	0.16
Acclimation O ₂	1	1.2E-04	0.011	0.92
Assay O ₂ * Accl. O ₂	1	1.2E-04	0.01	0.92
Clone	3	0.003	0.243	0.87
Assay O ₂ *Clone	3	0.001	0.075	0.97
Accl. O ₂ *Clone	3	0.003	0.236	0.87
Assay O ₂ * Accl. O ₂ *Clone	3	0.012	1.099	0.35
Error	100	0.237		

Effect of Hypoxia on Lactate:Pyruvate Ratio

Lactate and pyruvate concentrations in two clones, IL and GB were assessed using younger *Daphnia* (15-20 days old) and older *Daphnia* (55-60 days). CMIH hypoxia caused a significant increase in lactate concentration and a decline in pyruvate levels in older *Daphnia* (Figure 9). Thus, a significant increase in lactate: pyruvate ratio was seen. While there was no significant three-way interaction, there was a significant effect of oxygen, age, and an interaction between oxygen and age (Table 7).

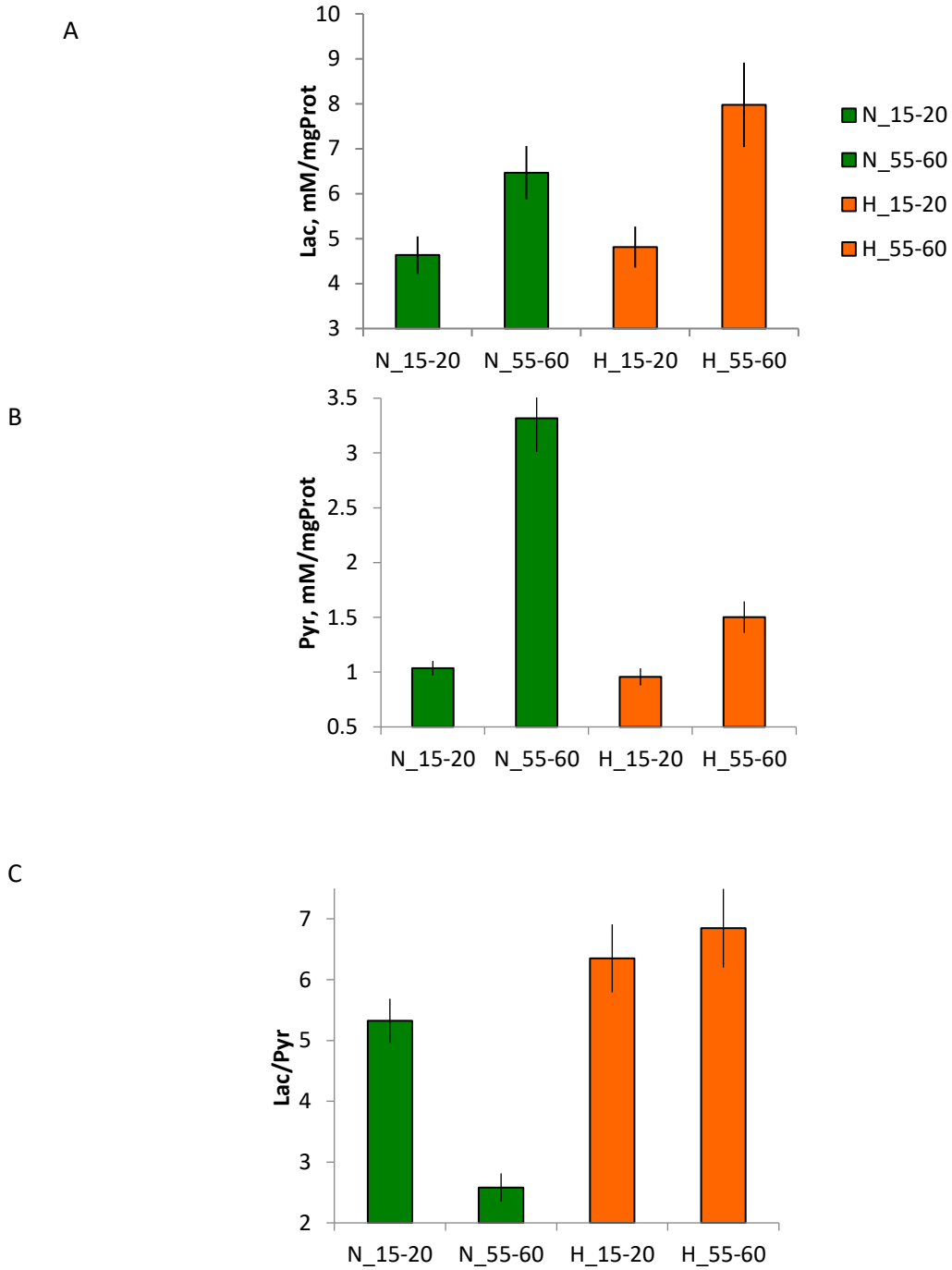


Figure 9. Lactate and pyruvate concentration in young and old *Daphnia*. A. Mean lactate concentration normalized by total proteins (mM/mgProt) B. Mean pyruvate concentration normalized by total proteins (mM/mgProt). C. Lactate: pyruvate ratio. Green bars: normoxia; orange bars: hypoxia. Vertical bars represent standard error. See Table 7 for statistical analysis.

Table 7. Three-way ANOVA of the Effects of Hypoxia on Lactate:Pyruvate Ratio. Clone, age, and block are random factors.

Source	DF Num	MS Num	F Ratio	Prob > F
O2	1	252.	51.31	<.0001
Clone	1	21.6	4.38	0.0389
O2*Clone	1	1.26	0.26	0.61
Age	1	137.4	27.89	<.0001
O2*age	1	112	22.75	<.0001
Clone*age	1	0.21	0.04	0.84
O2*Clone*age	1	0.06	0.01	0.92
Block	1	107.8	21.88	<.0001
Error	103	4.93		

Effect of Hypoxia on Hemoglobin Expression

Daphnia exposed to hypoxic conditions had increased levels ($p < 0.0011$ and $p = 0.0626$) of hemoglobin measured as mean absorbance (Figure 10) or by median red intensity, respectively.

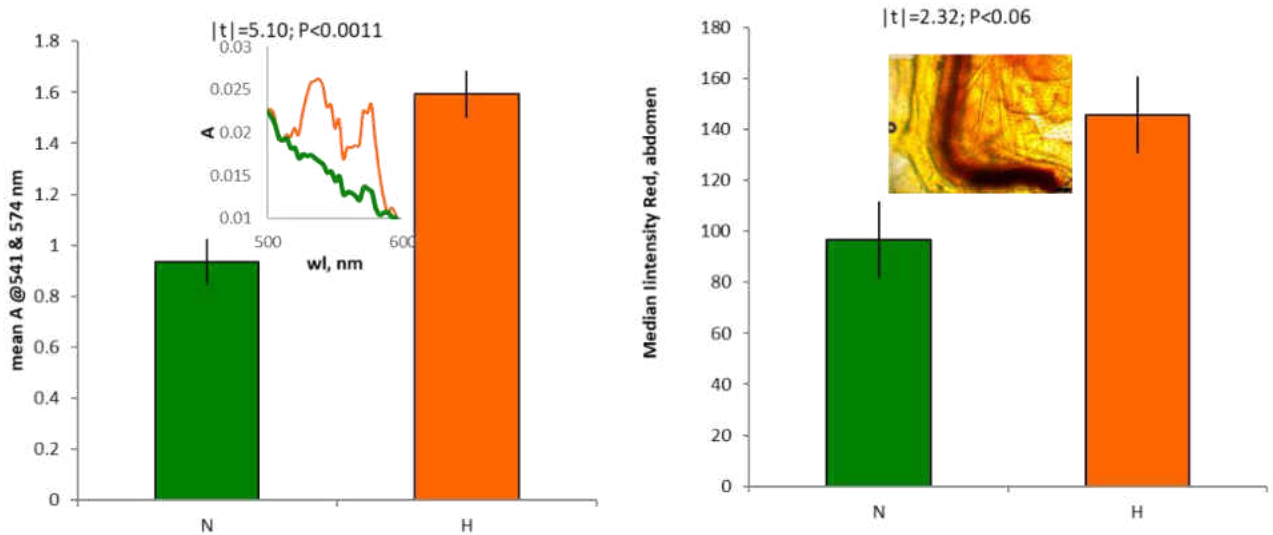


Figure 10. Effect of hypoxia on hemoglobin level. A is a plot of mean absorbance at 541 and 574 nm of hemoglobin in hypoxic and normoxic clones. The insertion in A is a plot of absorbance against wavelength (nanometers) in both treatments. B is a graph of the median red pigment intensity of the abdomen.

Gene Expression in Hypoxia

Preliminary assessment of the overall effect of hypoxia on gene expression in all 48 RNA samples revealed clustering patterns and differential expression of the 65 *a priori* genes. (Figure 11). Principal component analysis of the samples showed that FI (the most tolerant to acute hypoxia) is central and also the only clone for which acute hypoxia and controls are separated by PC1 (Figure 12). In order to identify transcripts that respond to CMIH differently in clones from permanent habitats (sensitive) and clones from intermittent habitats (tolerant) we analyzed a generalized model of the effects of habitat and clones nested within habitats on the abundance of reads mapped to each of the 65 *a priori* transcripts of interest. We identified 12 transcripts with a significant habitat effect surviving FDR correction with $p < 0.05$ (Table 8). Four of these transcripts were up-regulated in (intermittent) hypoxia tolerant clones (Figure 13), but down-regulated in hypoxia sensitive clones (permanent).

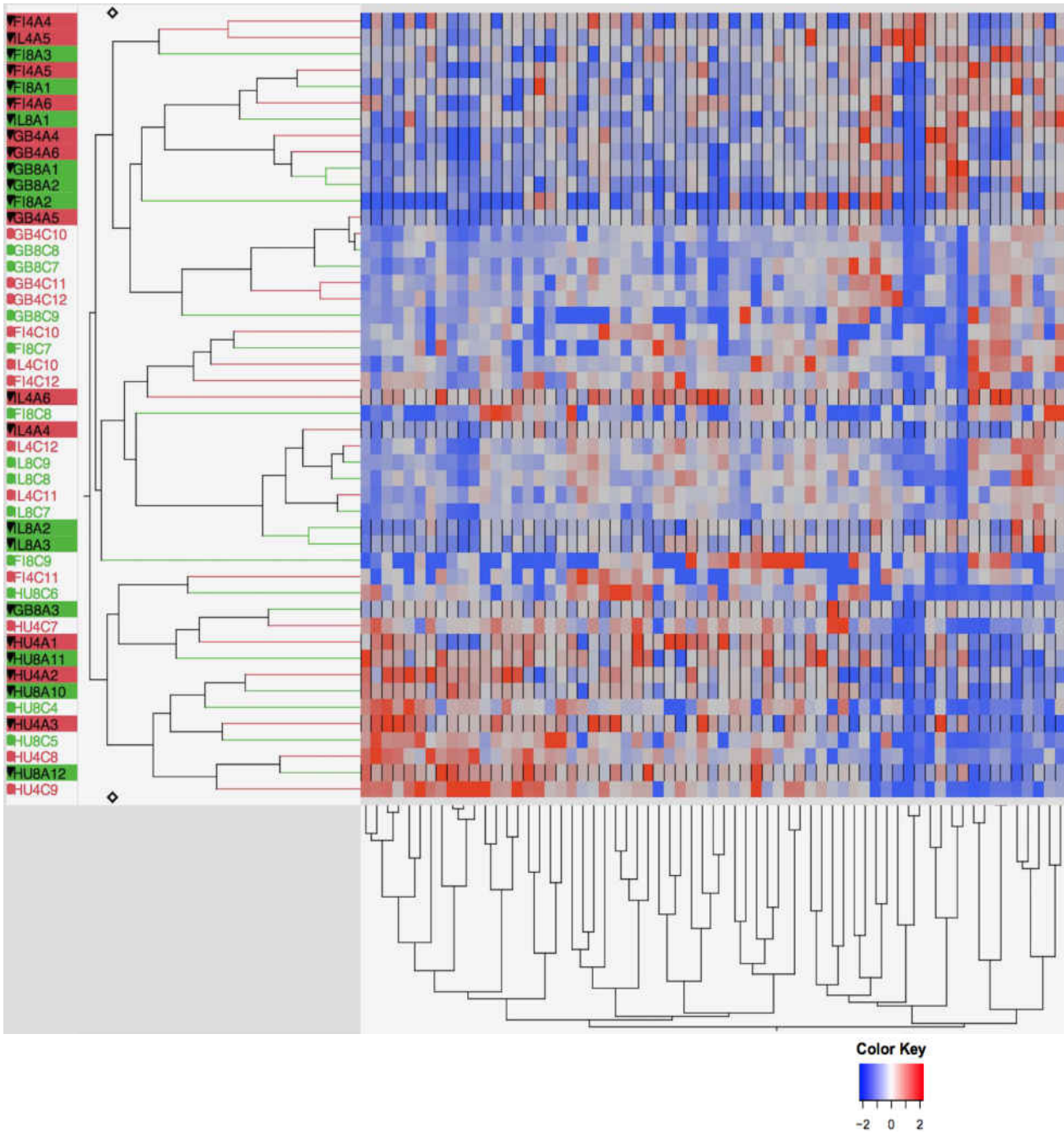


Figure 11. Gene expression in hypoxia. A: Two-way clustering heatmap of 48 samples and 65 “genes of interest”. IDs are displayed as clone, treatment (A=acute, C= chronic) and barcode (1-12). Sample IDs in format “Clone/CMIH/AcuteHypoxia/barcode”, where 8 (green) and 4 (red) are oxygen concentrations in CMIH treatments and A and C are acute hypoxia and control treatments in the acute hypoxia experiment; samples from acute hypoxia treatment highlighted by background color (controls = white background). Color code on the heatmap: red- up-regulated; blue down-regulated (log fold change).

Samples from acute hypoxia treatment clustered together, along with some of the GB control samples and with the exception of some of the IL and HU samples. There was no evidence of samples from the same CMIH treatments clustering together. This is consistent with differential response to CMIH treatment observed in different clones (see below).

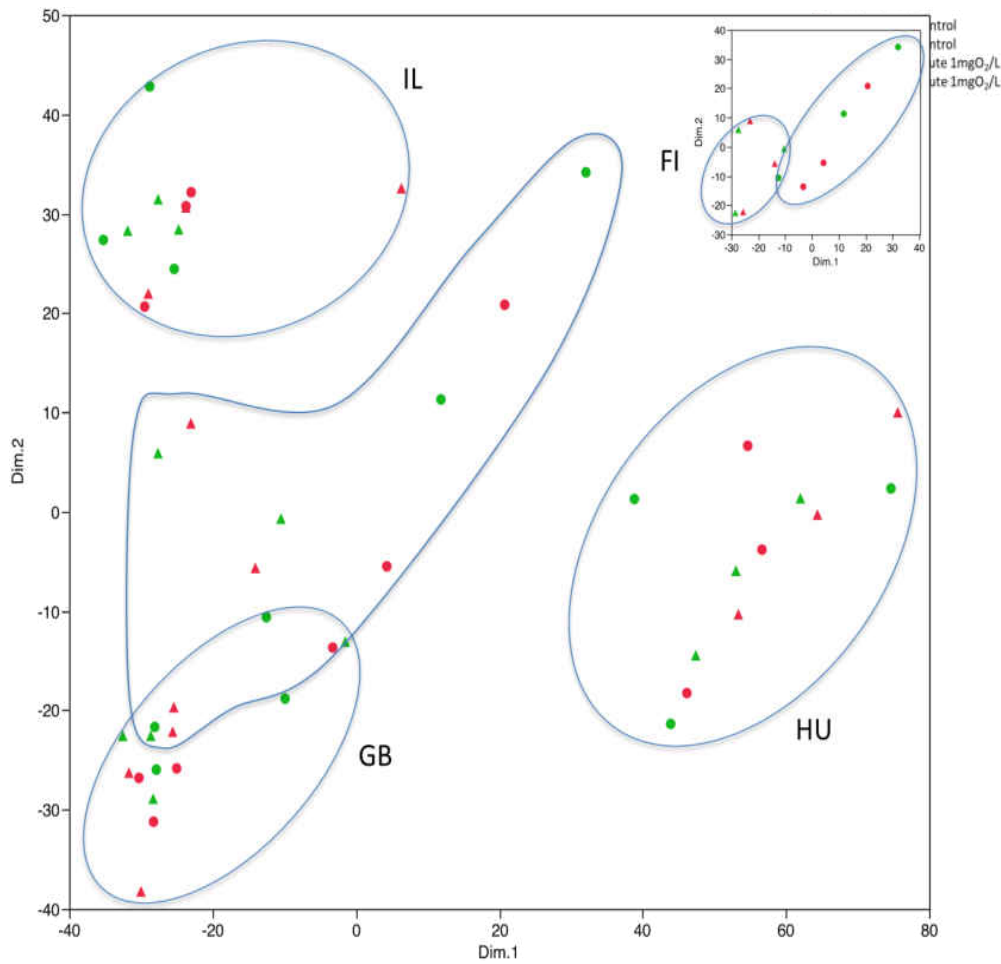


Figure 12. Principal component analysis of 48 RNA samples. Four clones in 4 hypoxia treatments with 3 replicates in each, represented by 6050 transcripts with at least 60 reads and present in at least one sample.

Table 8. Transcripts with a Significant Habitat Effect in Chronic Mild Intermittent Hypoxia. The table shows transcript description, false discovery rate (FDR), habitat with most expressed transcripts in normoxia and differential expression of transcripts in chronic hypoxia by clones from intermittent and permanent habitats.

Transcript	FDR	Habitat of most expressed in normoxia:	Response to CMIH	
			intermittent	Permanent
di-domain hemoglobin precursor	5.00E-13	permanent	no change	UP
RICTOR	1.15E-07	permanent	UP	DOWN
Tumor necrosis factor ligand	6.91E-07	intermittent	DOWN	UP
TP53-regulated inhibitor of apoptosis	0.0004	permanent	UP	DOWN
Cytoglobin-2	0.0004	intermittent	DOWN	UP
Vitellogenin precursor 1F36.70	0.0004	intermittent	DOWN	UP
Cu-Zn superoxide dismutase	0.0019	permanent	UP	UP
Carbonic anhydrase	0.0089	intermittent	DOWN	UP
Autophagy-related protein 16-1	0.0116	intermittent	DOWN	UP
Vitellogenin precursor 16F1.38	0.0104	intermittent	UP	DOWN
L-lactate dehydrogenase A chain	0.0154	permanent	UP	DOWN
NAD-dependent protein deacetylase sirtuin-2	0.0179	permanent	UP	DOWN

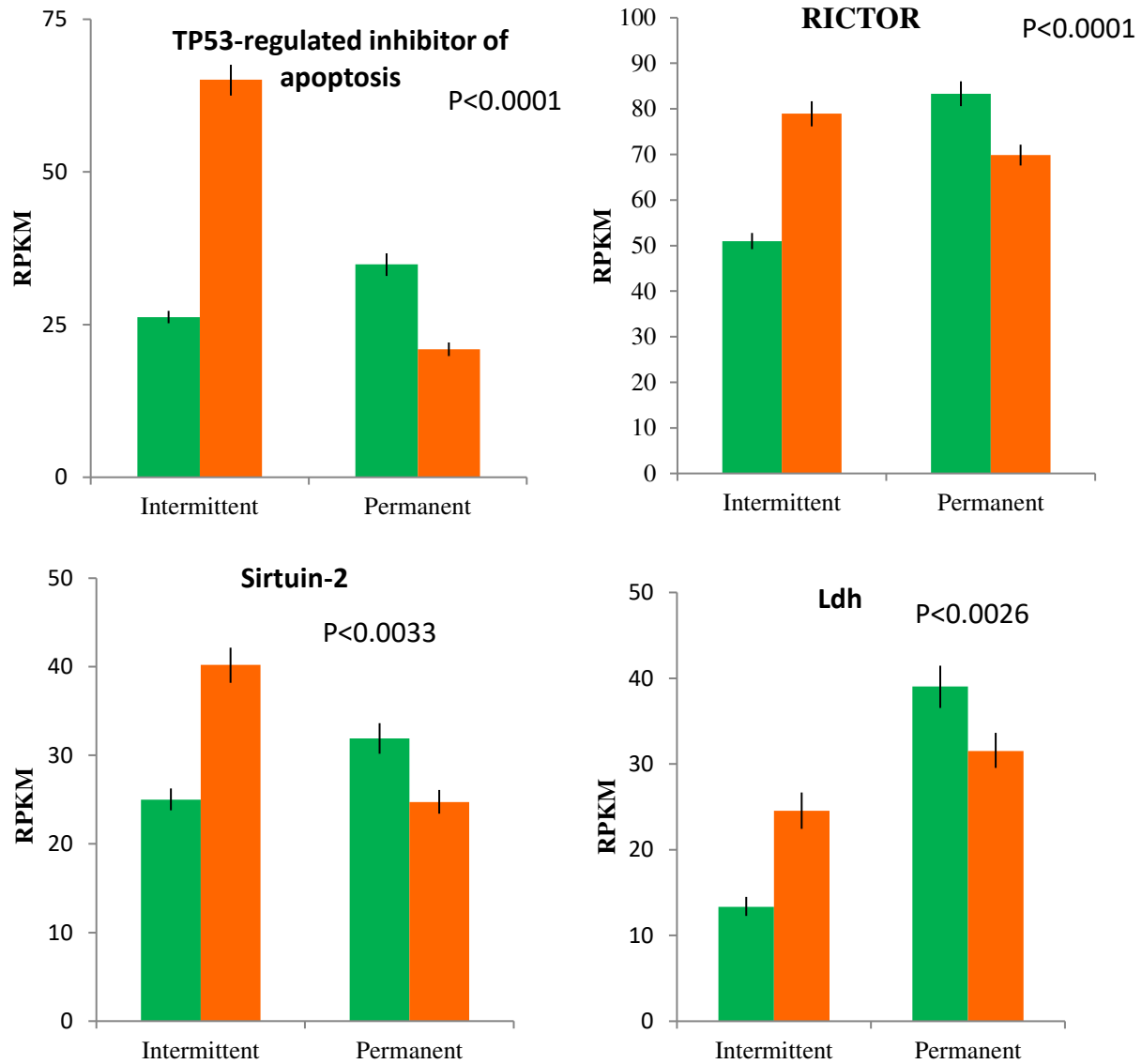


Figure 13. Genes up-regulated in hypoxia-tolerant clones. P-values are from a generalized linear model of the effect of habitat type on abundance of reads in the two hypoxia treatments. Vertical bars represent standard error.

Effects of DNP on Lifespan

Chronic DNP exposure did not increase lifespan, but we observed remarkable clone-environment interactions similar to CMIH (described earlier). DNP significantly ($p < 0.0001$) reduced longevity in clones from permanent ponds and had no significant effect on clones from intermittent ponds (Figures 14-15 and Table 9).

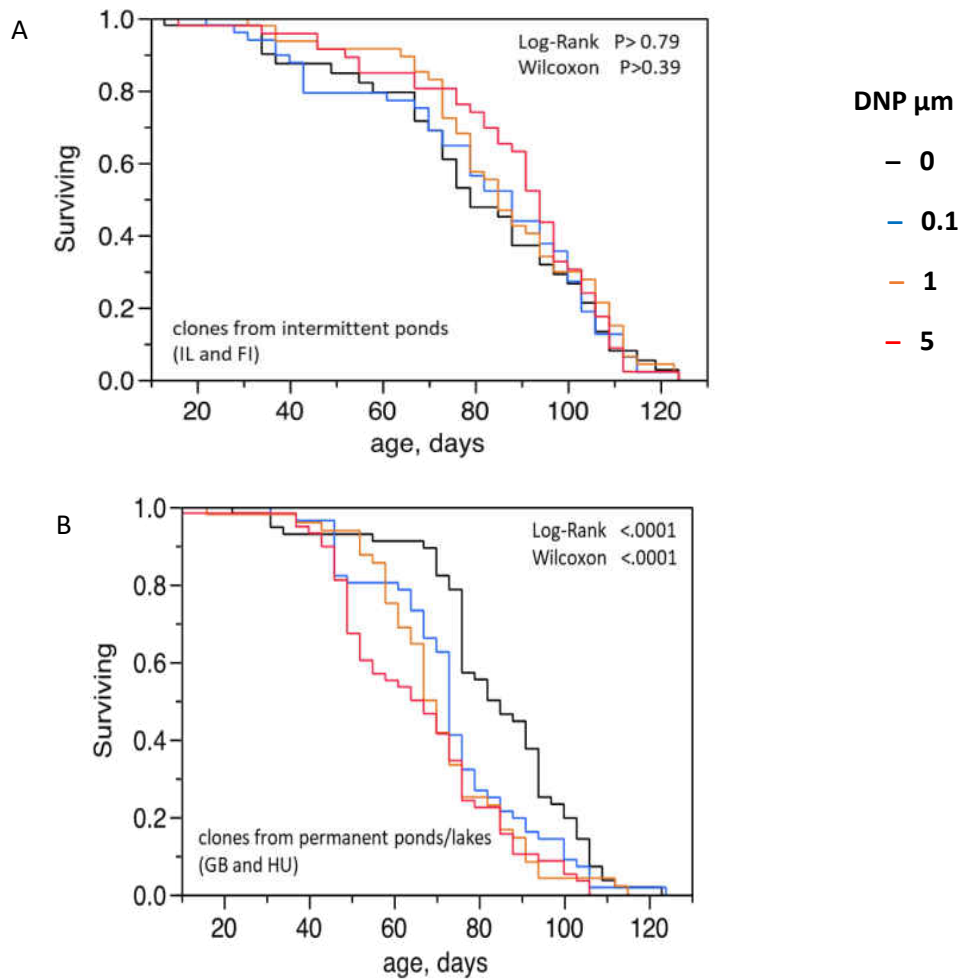


Figure 14. Kaplan-Meier survival curves of *Daphnia* clones treated with DNP. Survival is plotted on the Y-axes, and age is plotted on the X-axes. A: survival curves of clones from intermittent ponds. B: survival curves of clones from permanent pond. Black lines represent unexposed controls. Blue, orange, and red lines represent *Daphnia* exposed to 0.1, 1 and 5 μM DNP, respectively. See Table 9 for statistical analysis.

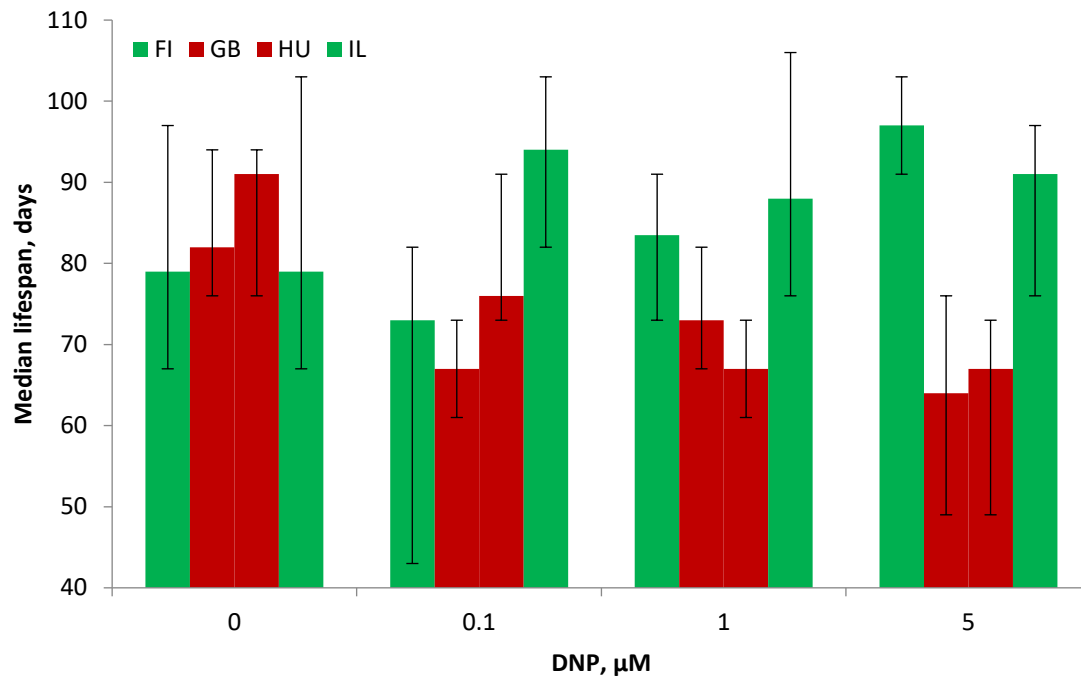


Figure 15. Median lifespan of all four clones treated with DNP. Vertical axis represents median lifespan in days. Horizontal axis represents concentrations of DNP (0, 0.1, 1, and 5 μM , respectively). Green bars represent clones from intermittent habitats and red bars represent clones from permanent habitats. Vertical bars represent 95% confidence intervals.

Table 9. Likelihood Ratio Test for Longevity in Chronic DNP Exposure. Habitat is permanent or intermittent ponds.

Source	DF	L-R ChiSquare	Prob > ChiSq
Habitat	1	35.753	<.0001
Clone[habitat]	2	5.616	0.06
DNP	3	6.275	0.1
habitat*DNP	3	13.194	0.0042
Clone*DNP [habitat]	6	13.816	0.03

Effect of DNP on Fecundity

DNP increased lifetime fecundity in all clones and in a dose-dependent manner, with some saturation observed at 5 μM (Figure 16). Further analysis showed that DNP significantly

increased fecundity at early and middle ages, but had no significant effect on fecundity late in life (Table 10). Different clones had no significant effect on fecundity in the different life stages.

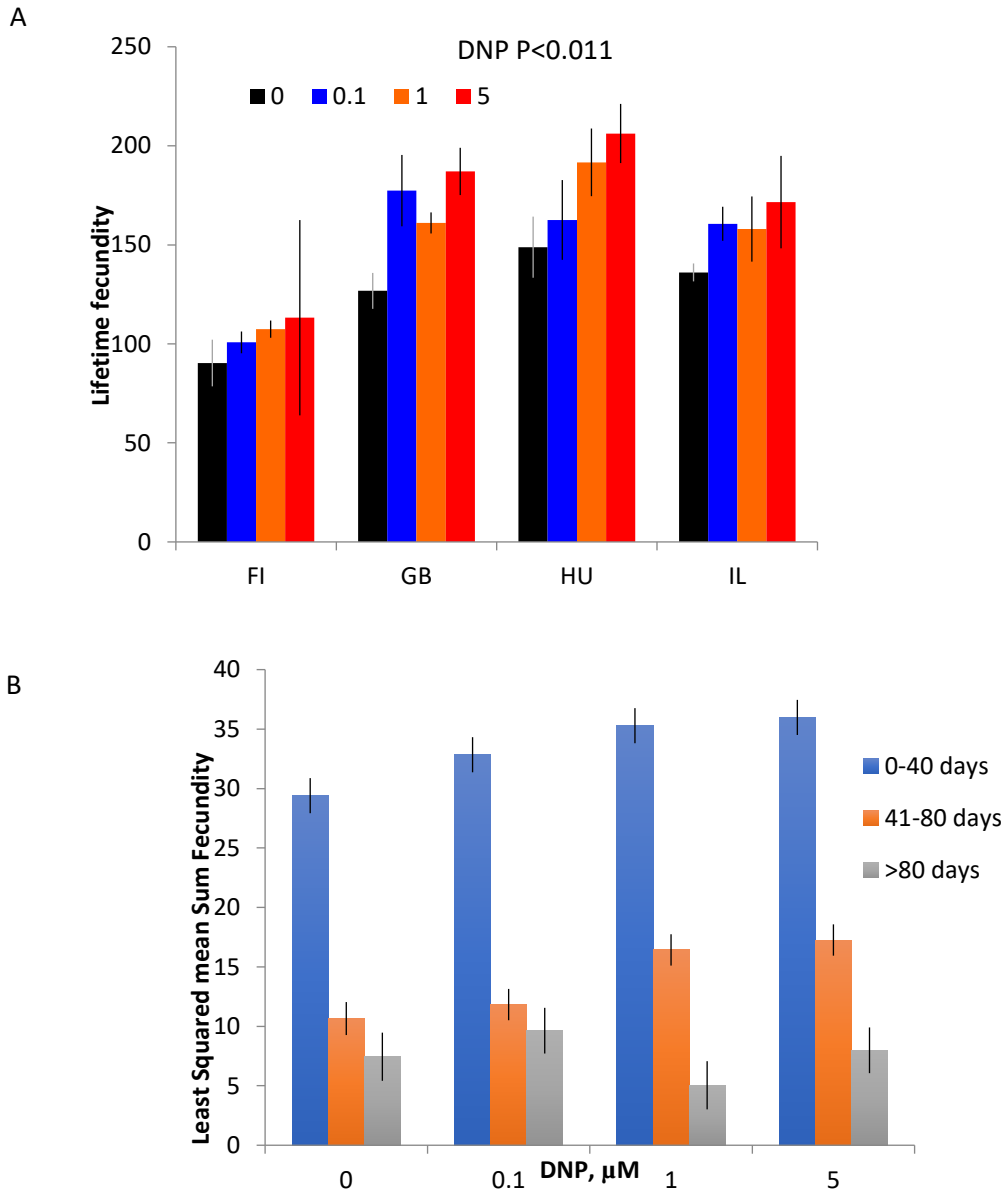


Figure 16. Fecundity during DNP Treatment. A: Lifetime fecundity across all clone, black, blue, orange, and red represent 0, 0.1, 1, and 5 μM of DNP respectively. B. Least squared mean sum fecundity when treated with DNP. Blue, fecundity between 0-40 days, orange fecundity between 41- 80 days, and gray, fecundity beyond 80 days. Vertical bars represent standard error. See Table 10 for statistical analysis.

Table 10. Two-way ANOVAs of the Effect of DNP Concentration on Sum Fecundity. Sum fecundity (per female) during early (0-40), mid-life (41-80), and late life (>80 days) periods are shown.

Age		0-40 days		
Source	DF	MS	F Ratio	Prob > F
Clone	3	55.73	2.4	0.09
DNP	3	94.26	4.06	0.02
Clone*DNP	9	9.97	0.43	0.91
Error	28	23.23		
Age		41-80 days		
Source	DF	MS	F Ratio	Prob > F
Clone	3	13.55	0.73	0.54
DNP	3	111.03	6	0.0029
Clone*DNP	9	12.21	0.66	0.74
Error	27	18.5		
Age		>80 days		
Source	DF	MS	F Ratio	Prob > F
Clone	3	31.09	0.79	0.51
DNP	3	36.24	0.92	0.45
Clone*DNP	9	27.05	0.69	0.71
Error	26	39.4		

Effect of DNP on Feeding Rate

Chronic DNP exposure had no significant effect on food consumption (Figure 17). However, report a significant effect of clones as well as clone by environment interactions (Table 11).

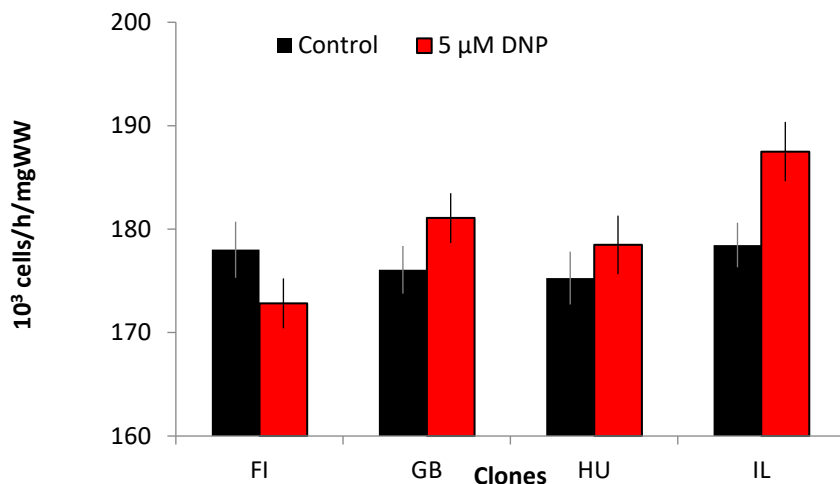


Figure 17. Feeding Rate in DNP. Black and red bars represent mean cells consumed per hour normalized by body wet weight (10^3 cells/h/mgWW) in controls and in DNP treated *Daphnia*. Vertical bars represent standard error. P-values are shown in Table 11.

Table 11. Two-way ANOVA of the Effect of Clone and DNP on Feeding Rate. Wet weight is a covariable.

Source	DF	MS	F Ratio	Prob > F
Clone	3	90.66	4.544	0.011
DNP	1	79	3.96	0.057
Clone*DNP	3	74.85	3.75	0.023
CalcWW	1	10.49	0.53	0.47
Error	27	19.95		

Effect of DNP on Mitochondrial Membrane Potential

Tissue rhodamine 123 fluorescence intensity (regions of interest- brain, optical lobe, and epipodite) was significantly ($p=0.0063$) higher in all clones exposed to DNP compared to controls (Figure 18, Table 12). Interestingly, tissue fluorescence in younger controls (day 0) was greater than that of the older controls (30 days), although this comparison may be misleading,

because these two measurements were not done in a common garden experiment. Since tissue fluorescence is an indicator of MMP, the latter was significantly increased in DNP treated *Daphnia*.

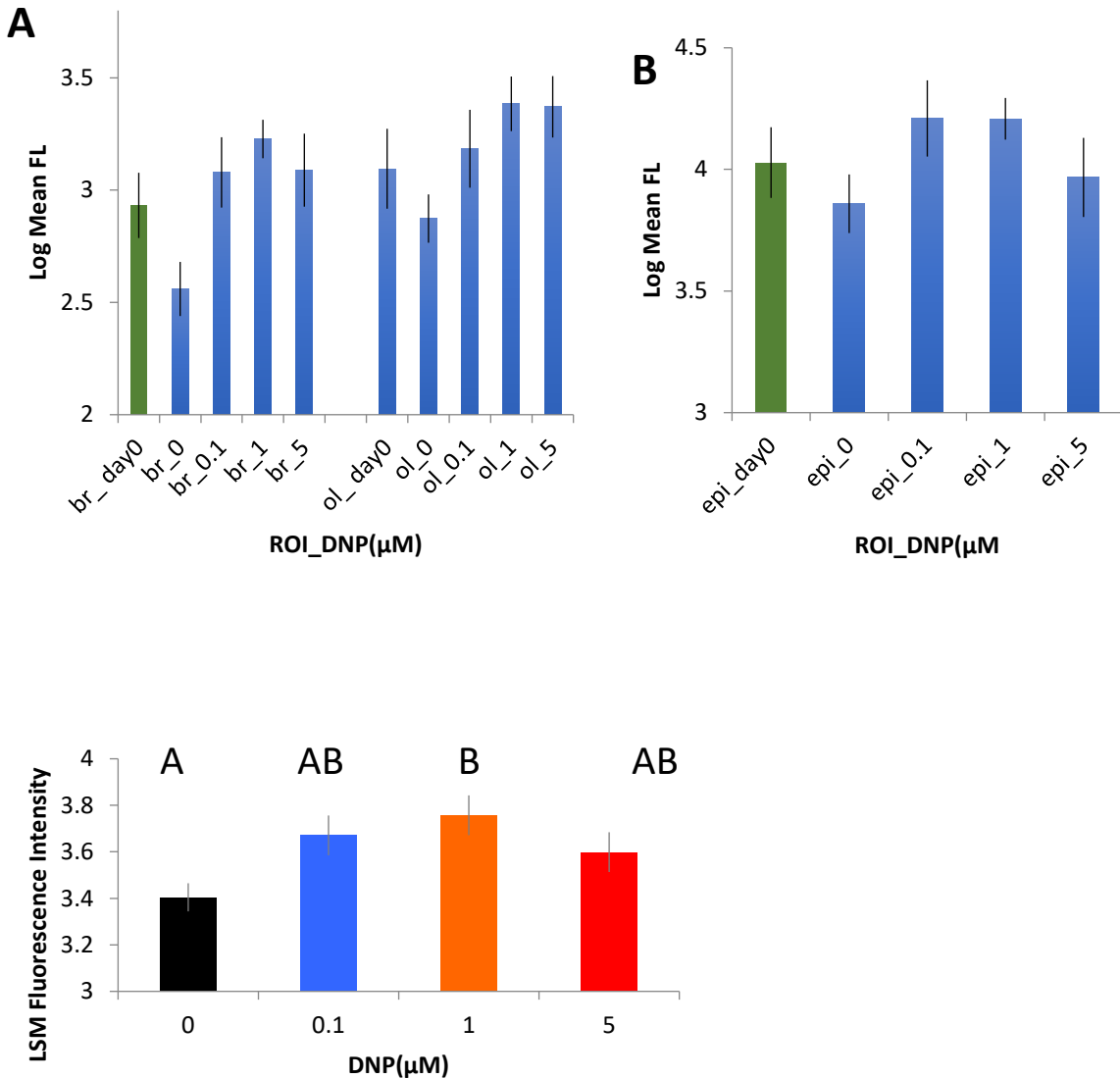


Figure 18. Effect of DNP on Mitochondrial membrane potential. Plots A and B represent log mean fluorescence of brain, optical lobe and epipodite in varied concentrations of DNP (0, 0.1, 1 and 5 μM) before the start of the experiment (green bars) and after thirty days (blue bars). Plot C represents the least squares means (LSM) of the mean fluorescence. Letters over the bars depict significant differences (at $\alpha=0.05$) among DNP concentrations, based on Tukey-Kramer HSD. See Table 12 for statistical analysis.

Table 12. Two-way ANOVA of the Effect of DNP and ROI on Rh123 Mean Fluorescence

Source	DF	MS	F Ratio	Prob > F
DNP	3	0.358	4.5907	0.0063
ROI	1	10.974	140.5647	<.0001
DNP*ROI	3	0.083	1.0695	0.3701
Error	52	0.07807		

Effect of DNP on Neutral Lipid Storage

Chronic exposure to DNP led to a significant decrease (regression coefficient =0.744951, SE = 0.141696, p<0.0001) in lipid droplets as indicated by the lower fluorescence intensity of abdomen and mid-body compared to controls (Figure 19). Clones also had a significant (p=0.0202) effect on lipid storage, but there were no significant interactions between DNP and other groups of the model (Table 13). Interestingly, it appears that lipid storage declines with age when considering the starting point as day 0. DNP =0 and Day 30 (as described above).

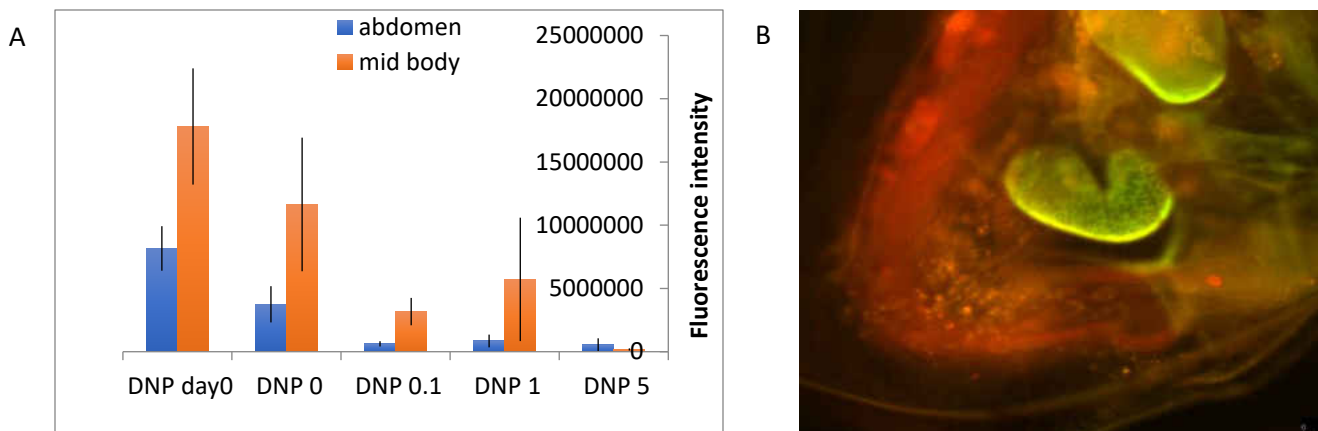


Figure 19. Effect of DNP on Neutral Lipid Storage. Vertical axis represents mean fluorescence intensity, horizontal axis represents varied food and DNP concentration in abdomen (blue) and mid-body (orange). Error bars represent standard error. DNP day 0 represents before the start of the experiment. B: Nile Red-positive small neutral lipid droplets in cells (red) and rhodamine 123-positive mitochondria (green), bar = 100 μm. See Table 13 for statistical analysis.

Table 13. Three-Way ANOVA Model for the Effect of DNP on Lipid Storage. Body region includes mid body and abdomen.

Source	DF	MS	F Ratio	Prob > F
Clone	3	10.918	4.34	0.02
DNP	1	74.174	29.52	<.0001
Clone*DNP	3	5.308	2.11	0.14
body region	1	11.061	4.40	0.052
Clone*body region	3	0.0851	0.034	0.99
DNP*body region	1	0.555	0.22	0.64
Clone*DNP*body region	3	1.684	0.67	0.58
Error	16	2.512		

CHAPTER 4. DISCUSSION

In this study, the effects of two respiratory function perturbations, CMIH and mitochondrial uncoupling, on aging, healthspan, and gene expression were investigated in a model organism, *Daphnia magna*.

Effect of CMIH on Aging and Health span

We observed several effects of hypoxia on the physiology of *Daphnia*. Prolonged exposure of *D. magna* to CMIH for a period of 68 days significantly increased total hemoglobin content in tissues. Under normoxic conditions, *Daphnia* are pale, which is often thought to have evolved to reduce visibility to predators (Pirow et al. 2001). However, during chronic hypoxia, the pertinent need to optimize aerobic metabolism through increased tissue perfusion and oxygen carrying capacity, contributes to increased hemoglobin synthesis and consequently a reddish color (Zeis et al. 2003). Hypoxia-induced hemoglobin synthesis is dependent on HIF expression. Thus, it was unsurprising to see elevated hemoglobin transcripts levels and this served as a plausible positive control in the transcriptomics study (Figure 10). Different hemoglobin isoforms (paralogs) are known to be expressed in response to acute vs. chronic hypoxia (Zeis et al. 2003). Consistently with that we observe some, but not other hemoglobin transcripts to be differentially expressed (see below).

We observed a significant decrease in feeding rate in hypoxia-acclimated clones and no significant interaction between clone and environment (Figure 6), which was surprising considering the elevated hemoglobin levels that occurred. Increased hemoglobin allows *Daphnia* to filter during oxygen depletion. Animals with elevated hemoglobin levels consume food 2.5 faster when compared to individuals with less or lack of hemoglobin (Kring and Brien 1976).

The feeding rate of rainbow trout (*Oncorhynchus mykiss*) chronically exposed to hypoxia (4.5 mg O₂/L), decreased by 6% compared to controls (Magnoni et al. 2018).

Our findings revealed that hypoxia increased body length and, in 3 out of the 4 clones studied (the clone that showed a decrease in body length in hypoxia, GB, was also the one that showed the most reduced feeding rate and had the shortest lifespan in the hypoxic condition). The increase of body length in hypoxia, at least in hypoxia-tolerant clones was surprising, considering that previous studies have demonstrated that during oxygen depletion, *Daphnia* have smaller body size (Seidl et al. 2005; Lyu et al. 2015). The decrease in body length is suggested to increase hypoxia tolerance due to their larger surface area to volume ratio that promotes diffusive oxygen transport. The observed increase in body length could not be explained by filtering rate, considering the latter was significantly decreased in hypoxic conditions in all 4 clones.

We found no significant difference in the respiratory rate between normoxia- and hypoxia-acclimated *Daphnia*, measured either in normoxic or hypoxic conditions, overall, or in any of the four clones studied separately. This finding contradicts Seidl et al. (2005), who demonstrated that hypoxia-acclimated *Daphnia* had 22% lower mass-specific oxygen consumption rate in normoxia compared to normoxia-acclimated controls. We hypothesize that the difference between the two studies is in the degree of hypoxia treatment (10–19% oxygen saturation in Seidl et al. 2005 vs. ~50% of saturation in this study). This result thus confirms that our goal of achieving mild, non-damaging level of hypoxia had been met.

In contrast, despite no significant differences in respiratory rate, we showed that the lactate:pyruvate ratio increased in hypoxia-acclimated *Daphnia*. We attribute this increase to the accumulation of lactate and decrease in pyruvate in hypoxia especially with age (Figure 9). The results of our gene expression analysis further confirm the upregulation of LDH in hypoxia

(Figure 12). Hypoxia is often characterized by increased conversion of pyruvate to lactate as a result of HIF-stimulated increased expression of pyruvate dehydrogenase kinase and lactate dehydrogenase (Yeo 2019). Webster (1987) reported 2-5-fold higher expression of genes such as LDH and pyruvate kinase in the skeletal muscles of rat, following a 72 hr chronic hypoxia exposure. During sustained hypoxia, lactate dehydrogenase was upregulated in killifish (Rees et al. 2001). Moreover, the increase in lactate: pyruvate ratio is indicative of an increased NADH: NAD⁺ ratio.

Increased heat tolerance had previously been reported in heat-acclimated *Daphnia* (Yampolsky et al. 2014; Loureiro et al. 2015). We posited that acclimation to CMIH would increase tolerance to severe acute hypoxic stress. Our findings do not support this hypothesis since we observed that normoxia-acclimated individuals have better tolerance and survival under acute severe hypoxic stress. We suggest this stronger tolerance might be due to healthier mitochondria and lower oxidative stress.

Earlier, we hypothesized that CMIH extends lifespan in *Daphnia magna*. We also posited better hypoxic stress tolerance in hypoxia-adapted clones (ILs and FIs). Our findings support the latter hypothesis. So, while CMIH did not significantly increase lifespan in any of the clones, we recorded better survival of clones with lower hypoxia naivety (Figure 3). We hypothesize that *D. magna* hypoxia-naive clones GB and HU are locally adapted to permanent lakes/ ponds that are less prone to develop hypoxic conditions, unlike their hypoxia-adapted counterparts IL & FI from short lived/intermittent ponds, with possibilities of poorer oxygen availability especially during the dry season. Overall, we observed remarkably significant clone-environment interactions in CMIH that reflects local adaptation and poses questions about evolutionary trade-offs that might be underlying such local adaptations.

In this study, we reported increased fecundity in older hypoxia-acclimated *Daphnia*. However, we are wary of interpreting this data considering our previous studies (unpublished data) have shown that brief late-life spikes in fecundity occur in these clones, often after a period of reproductive senescence. More detailed fecundity data are needed to ensure that this observation is not an artifact of snap-shot fecundity measurements. Future investigations will involve detailed assessments of fecundity using three-day measurement intervals.

Effect of CMIH on Gene Expression

We focused on genes that show a significant effect of the habitat of origin (intermittent vs. permanent ponds) on transcript abundance in chronic mild hypoxia; this analysis uncovered 12 candidate genes (Table 8). This list should be interpreted with caution, because in most of these cases, clones nested within habitats also had a significant effect on transcript abundance and we do not have statistical power, due to low number of clones per habitat type, to test whether the habitat effect is independent from the effect of individual clones. Interestingly, we identified two patterns of differential response of transcript abundance in different hypoxia treatments between habitat types. The pattern with a transcript's expression increasing in sensitive clones but decreasing in tolerant clones may be indicative of an induced response to a higher damage or stress caused by hypoxia in these clones (such transcripts include cytoglobin, hemoglobin, TNF, SOD, carbonic anhydrase, and autophagy related protein). This pattern identifies genes functionally related to the hypoxia response, but not necessarily providing protection against hypoxia, as the lifespan of clones from permanent ponds was still lower than those from intermittent ponds, despite this, presumable adaptive, up-regulation. The other pattern is characterized by up-regulation of a transcript in tolerant clones, but down-regulation in the

sensitive clones, which is consistent with the hypothesized protective action (transcripts such as *RICTOR*, *Ldh*, tp53-regulated apoptosis inhibitor, and *SIRT2*).

Effect of DNP on Aging and Healthspan

As expected, the results of lipid storage analysis revealed that daphnids who had been exposed to DNP for thirty days, had lower lipid reserves. Additionally, we observed that lipid reserves in these individuals, declined with age. As a caloric restriction mimetic, although DNP reduces ATP generation and increases NAD⁺: NADH ratio, it does not restrict energy uptake (Barger et al. 2017). Sustained release of an anti-lipogenic uncoupling compound reduced plasma triacylglycerol, liver triglyceride, and total cholesterol in rat (Wei et al. 2017) and supports our findings.

Decreased lipid accumulation was not associated with decreased food intake. Although we observed significant interactions between the feeding rate of clones and DNP- acclimation, we interpreted this cautiously because we know clones such as ILs and FIs have bigger sizes and arguably eat more compared to their counterparts GB's and HUs with relatively smaller body size. Overall, *Daphnia* did not increase food consumptions to make up for inefficient ATP production following mitochondrial uncoupling. These findings agree with the Salin et al. (2012) study using frogs; Goldgof et al. (2014) and Alexopoulos et al. (2020) studies using mice; and the Takahashi et al. (2009) study using rat, and are in contrast with Stier et al. (2014) study using zebra finches and Toyomizu et al.'s study using broilers (100 mg DNP/day diet).

The “uncoupling to survive” hypothesis proposes mitochondrial uncoupling extends lifespan by decreasing mitochondrial membrane potential and consequently ROS production (Brand 2000). Contrary to previous studies by Caldeira da Silva et al. (2008); Salin et al. (2012); Ulgherait et al. (2020), depicting the lifespan extension potential of the mitochondrial uncoupler

DNP in mice, fruitflies and frogs respectively, our findings indicate DNP does not extend lifespan in *Daphnia magna*. Interestingly, while pharmacological mitochondrial uncoupling had no significant effect on longevity of clones from intermittent ponds; ILs and FIs, it significantly decreased lifespan in clones such as GB and HU from permanent ponds. This clone-environment interaction suggests mitochondrial uncoupling might have already been optimized in the latter clones and further uncoupling had no effect. Alternatively, we suspect these clones from intermittent ponds are better equipped to handle respiratory perturbations, judging from their exceptional tolerance to CMIH demonstrated earlier (Figure 3).

As a mild uncoupler, DNP increases respiratory rates and reduces ROS accumulation (Barros 2004). Caldeira da Silva et al. (2008) reported decreased hydrogen peroxide production as well as decreased oxidative damage in DNP-treated fruit flies. DNP treatment of zebra finches for 32 months had no effect on lifespan or total reproductive capacity and Stier et al. (2014) additionally reported a decline in optimum immune function in the DNP-treated birds. They suggested a healthy amount of ROS was important for triggering an inflammatory response, supporting earlier proposed ROS-dependent signaling to improve stress tolerance and consequently longevity (Schulz et al. 2007; Brys et al. 2010). Wada and Heidinger (2019) suggest the pharmacological effect of DNP is dose, species, duration, as well as context dependent. Their argument considered Stier et al.'s (2014), findings as described before and Padalko's (2005) finding where DNP increased lifespan in well-nourished *Drosophila*, and decreased lifespan in calorie restricted flies. In this study *Daphnia* were exposed 0.1, 1, or 5 μM of DNP and provided food *ad libitum*, yet we provide no evidence for lifespan extension.

We showed that DNP increased fecundity in a dose dependent manner, contrary to the findings of Stier et al. (2014) that showed that DNP impacted reproductive capacity specifically

by reducing the number of eggs produced by female zebra finches with uncoupled mitochondria. Wulff et al. (1935); and Takahashi et al. (2009) provided evidence that DNP treatment did not significantly affect the number of offspring produced by rats. They did however report a significant decrease in the viability and survival of pups. While we cannot certainly explain the mechanisms behind the increased fecundity observed in DNP treated *Daphnia*, we suspect it may be due to upregulation of vitellogenin fused with SOD. This would require future investigation to confirm. We also hypothesize that upregulation of lipid catabolism caused by DNP indirectly resulted in increased deposition of lipids into the ovaries, resulting in higher fecundity.

Finally, we observed one more unexpected result of DNP exposure. The mitochondrial depolarization potential of exogenous and endogenous uncouplers have been extensively demonstrated (Cho et al. 2017; Khailova et al. 2020; Ulgherait et al. 2020). Unexpectedly, thirty days exposure to DNP increased MMP in our experiment, at least in neural tissues, possibly due to some sort of compensatory mechanisms that operated during prolonged exposure to the DNP treatment.

Future Research

A follow up study should evaluate mitochondrial membrane potential, mitochondrial ROS, lipid peroxidation, antioxidant capacity, and lipid storage/mobilization in CMIH exposed clones. These tests would provide deeper insight as to how clones from intermittent ponds were more tolerant to hypoxic stress and also why prolonged exposure to CMIH does not increase tolerance to severe acute hypoxic stress. Furthermore, it is pertinent to assess transcriptomic changes in response to chronic DNP treatment. This would provide invaluable understanding of differentially expressed genes among clones that may have influenced survival and fecundity. Similar to hypoxia, measurement of mitochondrial ROS, antioxidant capacity, lipid peroxidation,

antioxidant capacity, sex ratio of neonates in DNP and even hemoglobin levels will enhance our understanding of the effects of chronic DNP exposure.

Conclusion

This work demonstrates that neither CMIH nor mitochondrial uncoupling with DNP increased longevity in *Daphnia magna*. On the other hand, we observed clone-specific changes in gene expression in response to hypoxia that are consistent with regulation of respiratory metabolism, apoptosis, cellular proliferation, and anti-inflammatory responses. Such clone-specific differential expression together with clone-specific reduction of lifespan in hypoxia and uncoupler treatments, is consistent with the prediction that hypoxia-adapted clones from intermittent ponds would be more tolerant to hypoxic stress than their conspecifics from permanent ponds. These findings notwithstanding, acclimation to CMIH does decrease tolerance to severe acute hypoxic stress.

Overall, our findings elucidate clone-specific physiological and transcriptomic responses to respiratory perturbations (CMIH and DNP treatment) and contribute to our understanding of how longevity is altered as a function of environment in *Daphnia* clones from geographically diverse habitats. Knowledge of how *Daphnia magna* clones from intermittent habitats are able to cope with respiratory perturbations could be geared towards the development of pharmacotherapeutic strategies to promote healthspan.

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APPENDICES

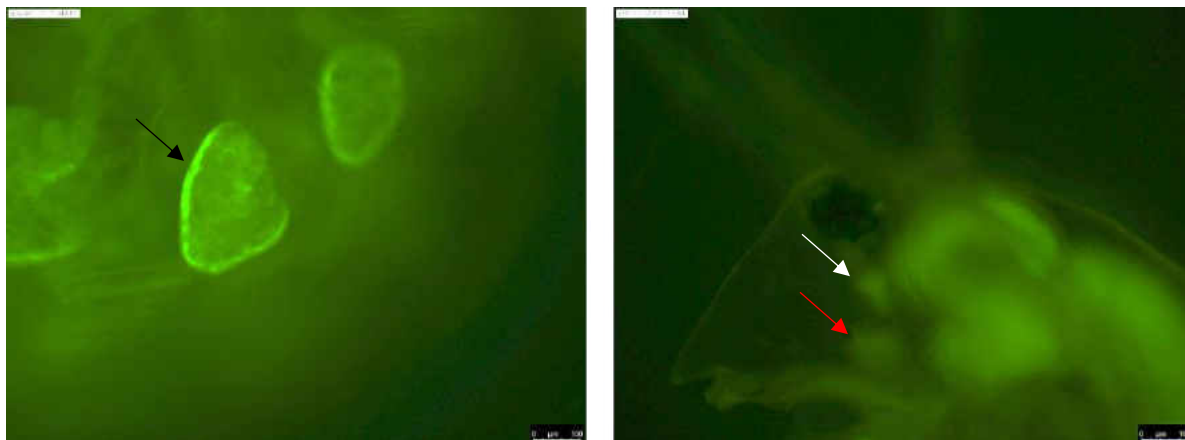
Appendix A: Tanks Used for the Hypoxia Experiment



Appendix B: Rhodamine 123 Stained Epipodite and Head of Daphnia magna.

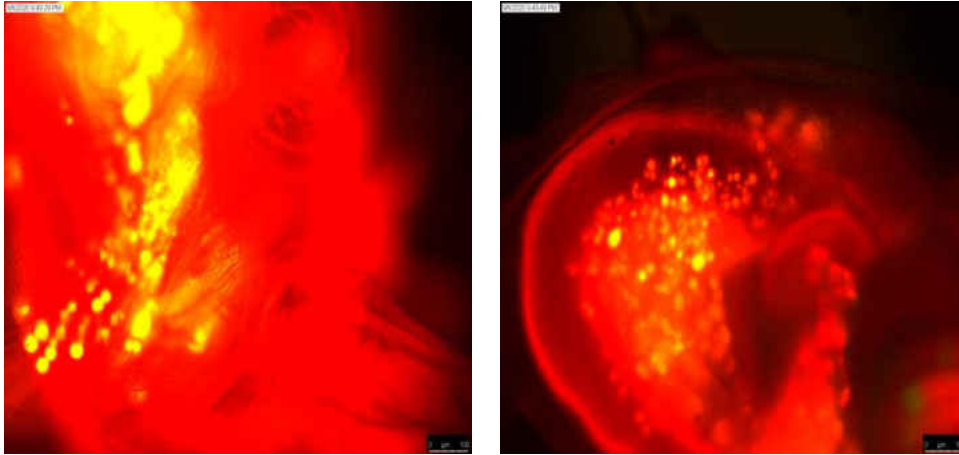
Black arrow indicates epipodite, red arrow, brain and white arrow, optical lobe.

Bar = 100 μ m here and thereafter

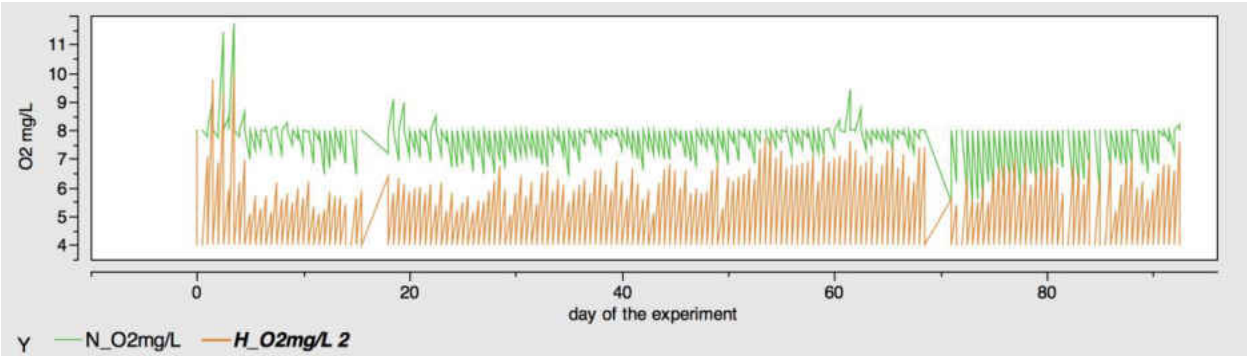


Appendix C: Nile Red stained Mid-section and Abdomen of Daphnia magna.

Yellowish portions are neutral lipid droplets in mid-body (left) and abdomen (right).



Appendix D: Daily Changes in Oxygen Concentration



Appendix E: Jars and Inserts Used for DNP Experiment



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Professional Presentations: Millicent N Ekwudo. March 2021. Effects of Chronic Mild Intermittent Hypoxia, and 2,4-Dinitrophenol on Longevity and Gene Expression in *Daphnia magna*. Appalachian Student Research Forum, Johnson City, Tennessee.