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Effects of Maternal and Neonatal Hypoxia on the Future Life History of *Daphnia magna*

By

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Abstract

Early exposure to hypoxia is related to a variety of physiological and metabolic changes that have lasting effects on organisms' physiology and life history. We measured the effects of maternal and embryonic mild, intermittent hypoxia on the life history of four clones of microcrustacean *Daphnia magna,* an emerging model organism for the studies of senescence and longevity. *Daphnia* individuals were produced parthenogenically, maintained in individual vials, and fed standard algal concentration daily. The cohort consisted of 189 individuals. We measured body size at first reproduction, fecundity (including late-life fecundity peak), offspring sex ratio, and longevity. We found no effect of maternal and embryonic hypoxia on body size and longevity; however, there was a slight but statistically significant increase in age-specific mortality in the early hypoxia treatment cohort. *Daphnia* from the hypoxia group showed higher early fecundity which disappeared by the age of 100 days. A late-life spike in fecundity was observed at the age of 100 days when hypoxia group individuals showed significantly lower fecundity. There was little evidence of a trade-off between early- and late-life fecundity. Finally, early hypoxia affected mid-life male production in one of the four clones, and we discuss possible physiological changes triggered by maternal and embryonic exposure to hypoxia.

Introduction

Daphnia is an aquatic organism that is highly valued by researchers because they are easy to grow, translucent, easy to view under a microscope, and quite sensitive to environmental stressors and pharmaceuticals (Tkaczyk et al., 2020). Daphnids make up part of the phytoplankton that fish use for food in lakes and ponds (Ebert, 2005) making them important as a keystone species (Miner et al., 2012). Reproduction in nature is accomplished through sexual and asexual methods (Ebert, 2005) while reproduction in a laboratory is mostly parthenogenic. Asexual reproduction is best for laboratory purposes because this reduces the genetic variability of clone lines. Some clones are breed specifically for longevity or shortness of lifespan with the normal lifespan being around 60 days (Cai et al., 2019). Individuals living in temporary environments often experience periods of drying in their habitats. As the dry periods nears, daphnids reproducing asexually react to these environmental cues and activate the terpenoid signaling pathway transitioning the population back to a sexual lifecycle which produces eggs for a period of diapause. These eggs produced are able to withstand desiccation within their pond. When the water returns, these diapause eggs hatch and start the cycle over again preserving the population (Rider et al., 2004).

In this experiment, four different clones were used (Table 1). The FI-FSP1-16-2 and IL-MI-8 clones come from bodies of water that experience dry periods, while the GB-EL75-69 and HU-K-6 clones come from permanent ponds. The FI-FSP1-16-2 clones live in summer rock pools along the edge of Löksskär Island in Finland. The GB-EL75-69 clones live in a year-round pond called Regents Pond in a metropolitan area of London, England. The IL-MI-8 clones live in a Mediterranean pond called Mamilla Pond located in Jerusalem, Israel, that is similar to a swimming pool sized area that dries out each year. The HU-K-6 clone lives in a permanent lake called Kelemen-Szék Lake in Hungary. Each of the climates and ecosystems in which these daphnia reside has a unique effect on their life histories that change the way each species exists in other environments.

Clone ID	**Origin**	**Type of Habitat**	**Latitude**	**Longitude**
FI-FSP1-16-2	Löksskär Island, Finland	Intermittent summer rock pool	60° 10' 40"	25° 47' 45"
GB-EL75-69	Regents Pond, London, UK	Permanent pond	51° 31' 39"	-0° 9' 29"
IL-MI-8	Mamilla Pond, Jerusalem, Israel	Intermittent summer-dry pond	31° 46' 40"	35° 13' 14"
HU-K-6	Kelemen-Szék lake, Hungary	Permanent lake	46° 47' 33"	19° 10' 54"

Table 1: Locations from which each daphnia clone used in the experiment originated.

Clones are greatly affected by the environment that their mothers live within. Mothers change the hormones they produce based on cues they obtain from their environment affecting the development of neonates. These cues change the gender of the daphnia produced. In milder environments, mothers tend to produce females while males are produced in more harsh environments which may also promote periods of diapause (Ye et al., 2019). It has been shown that methyl farnesoate determines male gender by way of the methyl farnesoate pathway. Specific environmental cues acting on pregnant female daphnia cause this pathway to become active which changes the gender of her neonates. A copper or orange color is present in many females who produce male offspring due to external stimulation because of a buildup of hemoglobin allowing the mothers to circulate more oxygen through their tissues (Rider et al., 2004).

Respiratory gas exchange in daphnids is conducted through multiple systems which include the gills, intestines, and integument. The gills on daphnia are called epipodites located in the thoracic region. Epipodites are similar to vesicles or bronchial sacs. Intestinal respiration occurs by water uptake in the anus and gas exchange in the intestines. Integumentary respiration is thought to occur largely in the filtering chamber where the animal filters out water and food. It was found that water going into this chamber had a higher oxygen content than that of the water leaving the chamber demonstrating that daphnia extract oxygen from the water as they filter feed. The blood hemolymph is another source of oxygen utilization that circulates diffused oxygen molecules throughout the body (Pirow et al., 1999). Daphnids exposed to hypoxia have been found to produce hemoglobin to compensate for normal oxygen levels they are not receiving. Hemoglobin production is induced by way of the hypoxia signaling pathway which is activated by the binding of the hypoxia inducible factor to areas in the promoter region in the genetic sequence called the hypoxia response element (Rider et al., 2004).

Maternal changes in the environment effect the way neonates interact with the same environment. When mothers are subjected to stressors, they prepare their offspring for similar environmental conditions. This can have both positive and negative effects for offspring by helping them survive adverse conditions or preparing them for a stressful event that they never experience. Mothers living in hypoxia pass on traits allowing their neonates to better withstand hypoxia. When these neonates spend the rest of their lives in normoxic conditions, the change causes a negative environmental correlation where neonates are prepared for one condition but live within the opposing one (Coakley et al., 2018).

As temperatures rise due to climate change, water temperatures would be expected to rise as well. Warming waters do not contain as high an oxygen concentration as cooler waters which affects the ecosystems living within them (Rider et al., 2004). Due to greater sensitivity to environmental changes, daphnids are one of many major species to demonstrate the effects of climate change earlier in their life history than other species. Exposure to hypoxia can be detrimental because of increased production of reactive oxygen species (Yeo, 2019) damaging cellular structures and proteins. However, exposure to mild hypoxia has the potential to activate the hypoxia signaling pathway leading to hemoglobin production and a variety of other protective responses (Rider et al., 2004; Yeo, 2019). Hypoxia is also known to alter the rate of oxygen consumption slowing their metabolism (Martins et al., 2007). Rising water temperatures would be likely to alter their metabolic rate by reducing the level of free oxygen in the water creating an environment of hypoxia.

Hypoxic exposure in early life influences metabolic rates and physiologic conditions of *Daphnia magna* individuals. Oxygen reduction produces physiologic changes through pathways that cause production of clutches composed primarily of males, the transition from asexual to sexual reproduction, and creation of hemoglobin (Rider et al., 2004). It is predicted that changes in the life history of *Daphnia magna* will be seen when mothers and offspring, as embryos and neonates, are exposed to mild hypoxia that activates metabolic and physiological pathways affecting the populations lifespan and fecundity. Alternatively, there will be no changes seen in the life histories of mothers and the offspring who are exposed to mild early hypoxia as embryos and neonates.

Methods

The mothers were grown in either normoxic (oxygen concentration 8 mg/L) or hypoxic (oxygen concentration 4 mg/L) conditions at 20°C for their entire life, and neonates were collected in the same conditions. Hypoxic conditions were created by pumping nitrogen gas into the water through an air stone for a short period of time twice each day. The level of hypoxia was measured with an oxygen meter allowing hypoxia to be maintained in jars with multiple mothers per jar. The neonates born in a hypoxic treatment and a normoxic control were the individuals used for this study. All of the neonates were transferred into normoxic water medium at the age of 48 hours and maintained in normoxic conditions through their lives. A 189 individual blind sample of daphnia from each clone, FI-FSP1-16-2, IL-MI-8, GB-EL75-69, and HU-K-6, was used. Each daphnia was placed in individual vials filled with 20 mL of COMBO water (Kilham et al. 1998). They were fed 200 µL of algae to the standard concentration (100,000 cells/mL). Neonates were removed from vials containing mothers and counted daily. The sex of neonates was determined daily until the maternal age of 25 days. Later on, they were sexed intermittently at maternal ages 45-50 and 65-70 days. Life history data were then recorded and analyzed using JMP analysis software. Two-way ANOVA tests with clones and early hypoxia exposure as factors were used for body size. Fecundity, sex-ratio data, and the proportional hazards model were used for longevity data. Data of particular importance in this study was mortality, clutch size variation throughout life, sex ratios of babies from young and old daphnia, and body size at first reproduction.

Results

The findings of this experiment showed minimal changes among clones born in hypoxia throughout their lifespan. They all produced many offspring early in life, slowed offspring production in midlife, and greatly increased offspring production one last time before reducing production again and dying. There were some individuals of each clone that out lived the majority of the cohort and continued producing broods until death. The analyses showed some statistically significant differences comparing lifespan, clutch size, and sex ratios in terms of hypoxia and normoxia among clone lines.

Body size at first reproduction

Body size (Figure 1) was similar among clones exposed to hypoxia and normoxia with slight differences. Differences in data for FI clones showed statistical significance while GB, HU, and IL clones were not statistically different comparing normoxia and hypoxia. Another noticeable difference between the measurements is that IL and FI clones have significantly larger body size, almost twice the size, compared to GB and HU clones. These differences among individual clone lines were statistically significant (Table 2). The life histories of each clone have a part to play in these differences. HU and GB clones are found in permanent bodies of water while IL and FI clones come from ponds that experience seasonal drying periods.

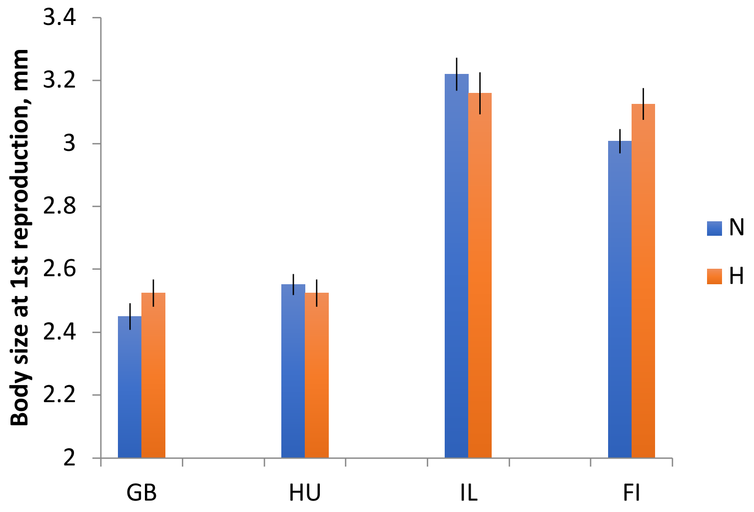

Figure 1: Body size of *Daphnia magna* at their first reproduction in clones exposed to normoxia (N) and hypoxia (H).

Effect Tests

Source	DF	Sum of Squares	F Ratio	Prob > F
Clone	3	12.2	113.88	<.0001
H/N	1	0.02	0.62	0.43
Clone*H/N	3	0.17	1.57	0.20
Error	141	5.04		

Table 2: Two-way ANOVA test demonstrating how body size of clones at first reproduction is affected by early hypoxia and the clone lines.

Longevity

The data (Figure 2) shows that the survivorship of female daphnids from normoxia control verses early hypoxia exposure group is only slightly greater in daphnids living in normoxic conditions. Hypoxic conditions tended to have decreased survivorship over the life of the daphnia. After age 40 days, fewer daphnia in hypoxia survived than in normoxia throughout the rest of the lifespan. All daphnia experienced a significant increase in mortality between 95 to 105 days, followed by a marked drop in mortality for the rest of their lifespan (Figure 5 and Figure 6A). Table 3 shows that these data are highly significant for clones, but not significant for hypoxia versus normoxia.

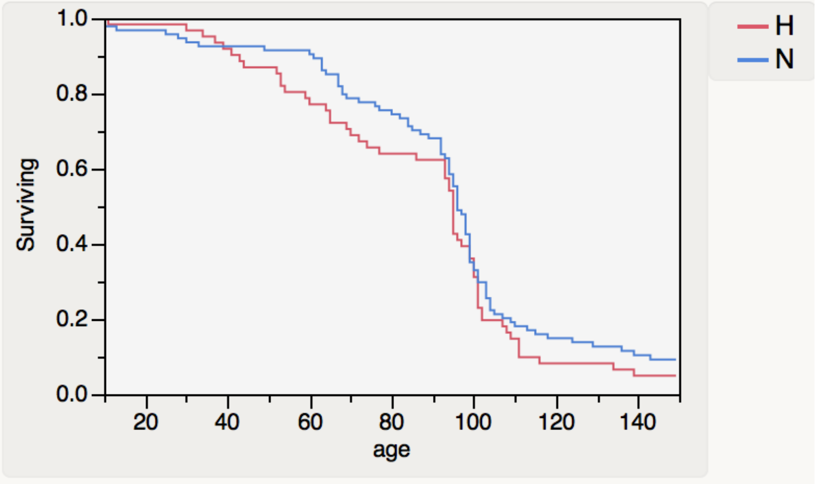

Figure 2: The survivorship of female clones exposed to normoxia (N) and hypoxia (H).

Effect Likelihood Ratio Tests for females

Source	DF	L-R ChiSquare	Prob>ChiSq
Clone	3	16.998	0.0007
H/N	1	3.493	0.062
Clone*H/N	3	3.1167	0.372

Table 3: The maximum likelihood fitting of the Proportional Hazards model to longevity data with Chi-square analysis used to compare between groups. Data: Females from four clones exposed to maternal and embryonic hypoxia (H) and normoxia (N).

Most of the clones demonstrated differing lifespans, but they all exhibited the same trends throughout their life (Figure 3). The HU and GB clones were longest lived. The IL clone lifespan was less than the HU and GB clones but longer than FI clones. All clones demonstrated some initial decline then a marked decline around the 90-to-100-day mark. The population of GB, HU, and IL clones leveled off until death between 135 and 150 days while the FI clone was very short lived after the 100-day mark.

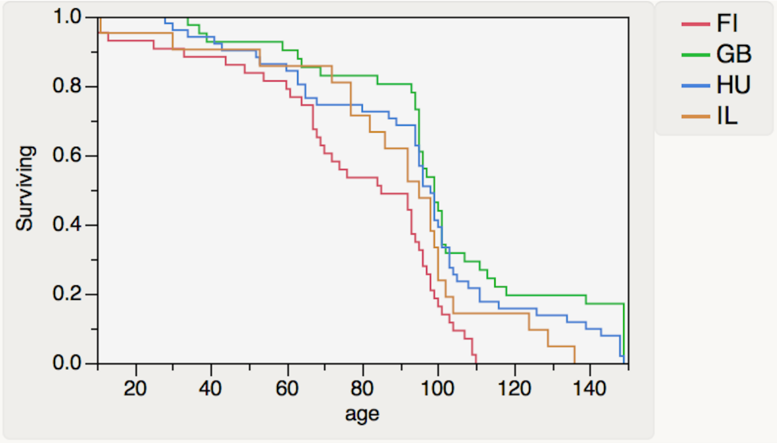

Figure 3: The survivorship of each clone line over the daphnid lifespan.

Males had no difference in life span in hypoxic or normoxic environments (Figure 4). Males born in normoxia and hypoxia lived nearly the same number of days. Trend lines demonstrating survivorship were very similar to one another. None of these results shown in Table 4 were different enough to be considered significant based on statistical analysis.

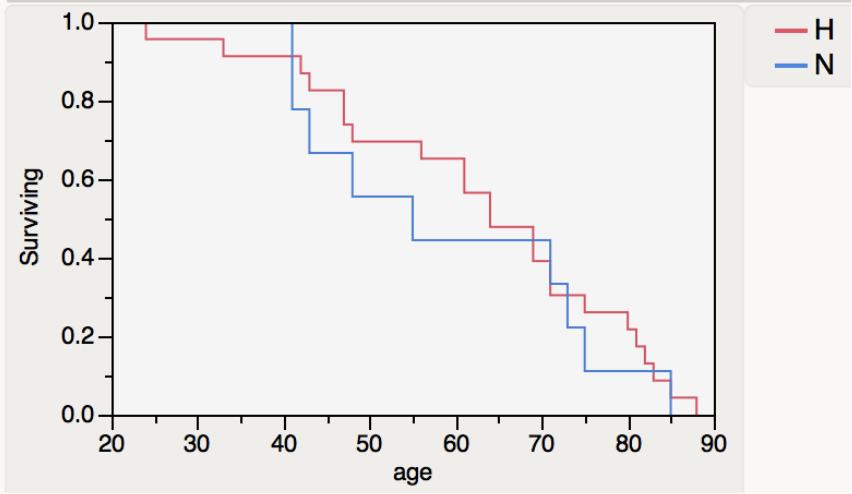

Figure 4: The survivorship of male clones exposed to normoxia (N) and hypoxia (H).

Effect Likelihood Ratio Tests for males

Source	DF	L-R ChiSquare	Prob>ChiSq
Clone	3	3.230	0.35
H/N	1	1.675	0.19
Clone*H/N	2	1.915	0.38

Table 4: The maximum likelihood fitting of the Proportional Hazards model to longevity data with Chi-square analysis used to compare between groups. Data: Males from four clones exposed to maternal and embryonic hypoxia (H) and normoxia (N).

Fecundity

Mortality among clones (Figure 5) was observed to increase with age in both normoxia and hypoxia. In both groups, the rate of aging is similar because the slopes of both exponential regression (Gompertz model expectation) lines are similar. The intercepts of both regression lines were different between the hypoxia versus normoxia groups, indicating that in some age classes daphnids with early exposure to hypoxia experienced higher mortality. Upon analysis shown in Table 5, the data were found to be statistically significant where the age of clones at death is observed. Trends observed between individuals in hypoxia and normoxia were found to be very mildly statistically significant.

As mortality increased with age (Figures 5, 6A), clutch size decreased (Figure 6B). This pattern continued until the age of approximately 105-115 days, at which age there was a spike in fecundity and a marked drop in mortality. Not all daphnids surviving to this age exhibited a spike in fecundity.

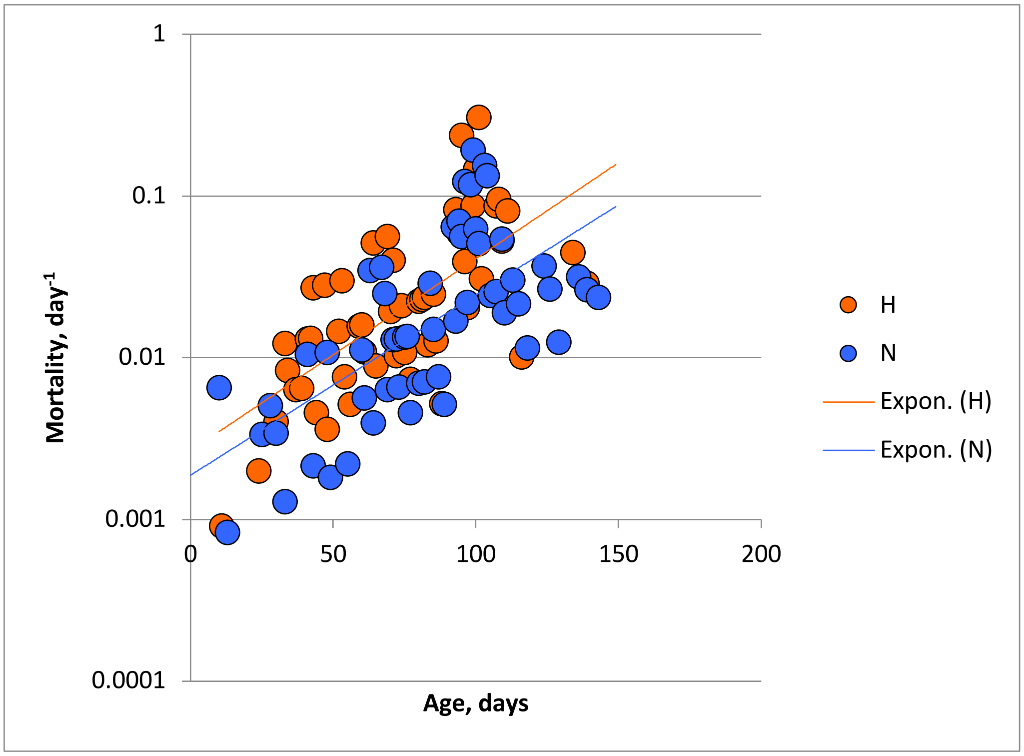

Figure 5: Mortality of individuals in hypoxia (H) and normoxia (N).

Effect Tests

Source	DF	Sum of Squares	F Ratio	Prob > F
age	1	12.669	80.73	<.0001
N/H	1	1.124	7.16	0.009
age*N/H	1	0.013	0.08	0.78
Error	103	16.164		

Table 5: Two-way ANOVA Test of individual clone mortality in hypoxia (H) versus normoxia (N).

The analysis of fecundity data revealed the following patterns. Early fecundity (age range 8-15 days) showed significant differences between both hypoxia and normoxia and among clones. Clutch sizes were significantly smaller (Figure 7A) for GB and HU than in IL and FI clones. The IL and FI clones generally had greater clutch sizes than GB and HU clones. Overall, *Daphnia* exposed to maternal hypoxia experienced greater clutch sizes than those that had only experienced normoxia (Table 6). In contrast. daphnids in the age class 95-105 days had higher fecundity in the control group (normoxia) than in the early hypoxia treatment (Figure 7B), with no difference among clones (Table 6).

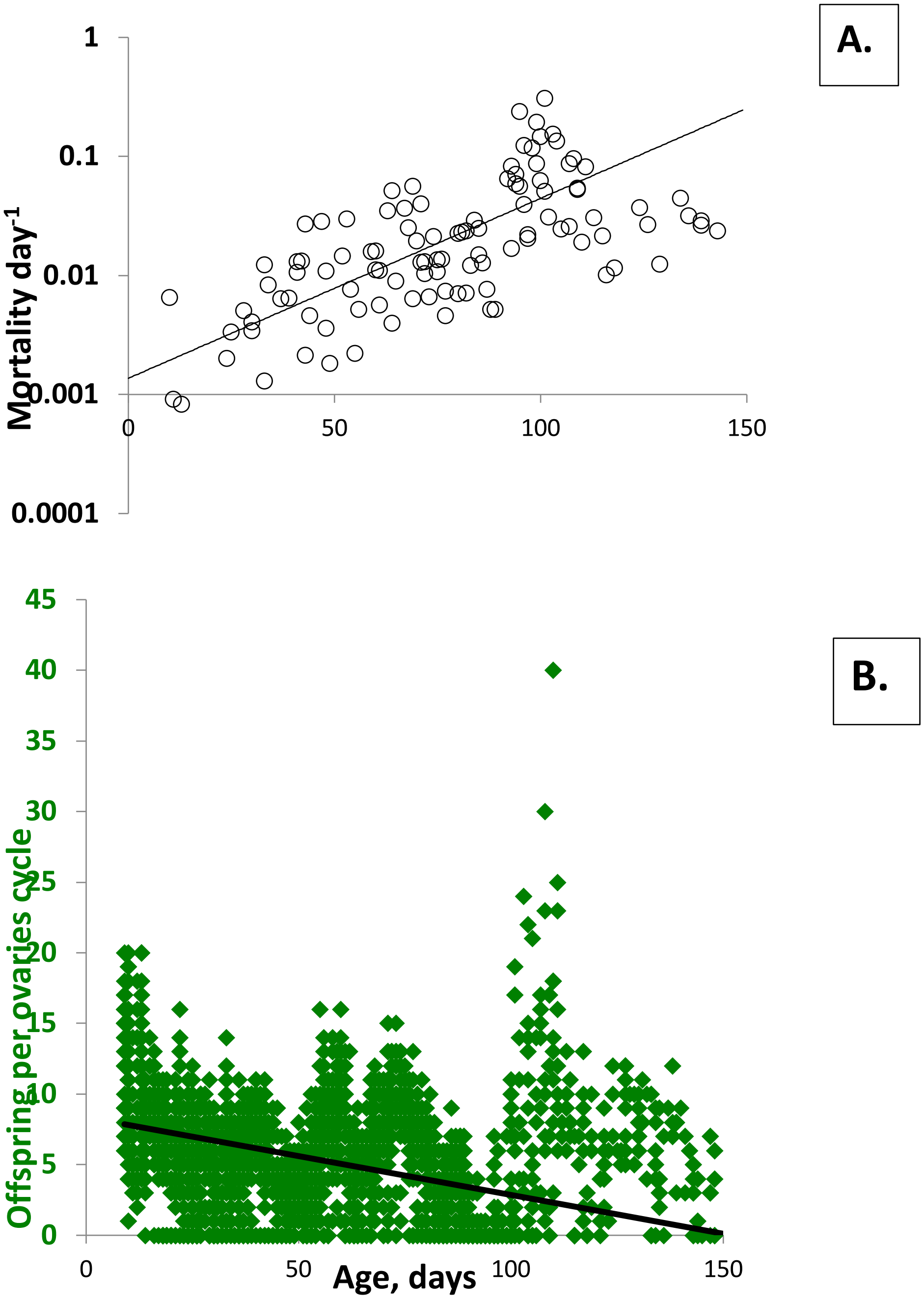

Figure 6: (A.) The mortality of individuals throughout the lifespan. (B.) Numbers of offspring per clutch throughout the lifespan of daphnia.

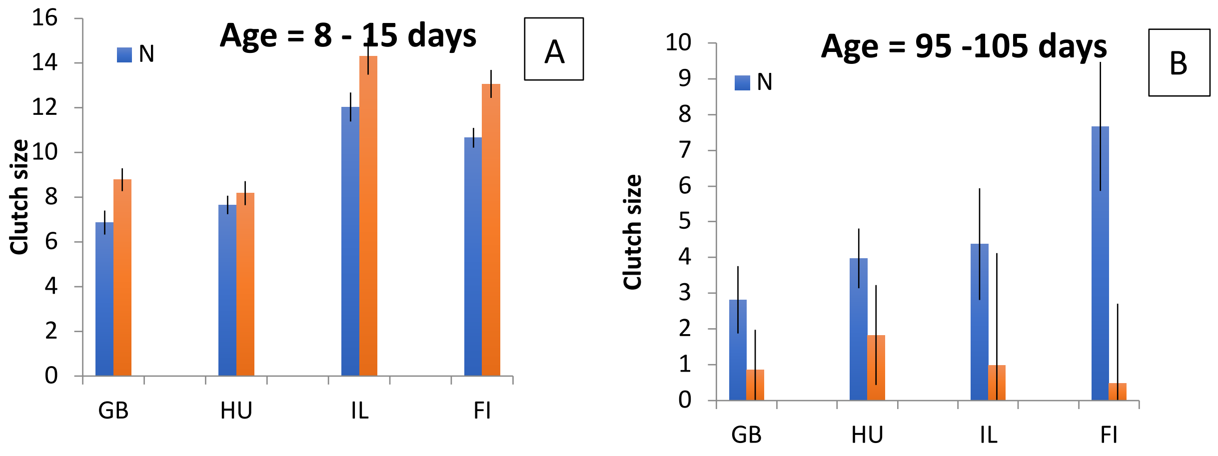

Figure 7: (A) Effect of early hypoxia (H) and normoxia (N) on fecundity of daphnids at age 8-15 days. (B) Effect of early hypoxia (H) and normoxia (N) on fecundity of clones at age 95-105 days.

Effect Tests: maternal age class 8-15 days

Source	DF	Sum of Squares	F Ratio	Prob > F
Clone	3	704.780	42.89	<.0001
H/N	1	103.076	18.82	<.0001
Clone*H/N	3	23.044	1.40	0.24
Error	146	799.686		

Effect Tests: maternal age class 95-105 days

Source	DF	Sum of Squares	F Ratio	Prob > F
Clone	3	22.37	0.76	0.52
H/N	1	83.23	8.53	0.006
Clone*H/N	3	28.09	0.96	0.42
Error	40	390.36		

Table 6: Two-way ANOVA tests on the fecundity of *Daphnia* in different age classes.

Offspring sex ratio

Sex ratios in the offspring produced in early and late maternal age classes (10–25 and 65-70 days) (Figure 8 A and C) showed no significant effects from early maternal hypoxia treatments. At age 45-50 days, there was a significant difference in offspring sex ratios (Figure 8B) with mothers exposed to early hypoxia producing significantly more male offspring; however, this effect was entirely due to the difference between the hypoxia groups in just one clone, IL. In contrast, the sex ratio of offspring in all maternal age classes was significantly different among clones (Table 7). The clones from temporary habitats (IL and FI) demonstrated the largest proportion of males in clutches in both normoxia and hypoxia while clones from permanent habitats (GB and HU) had the smallest proportions of male offspring.

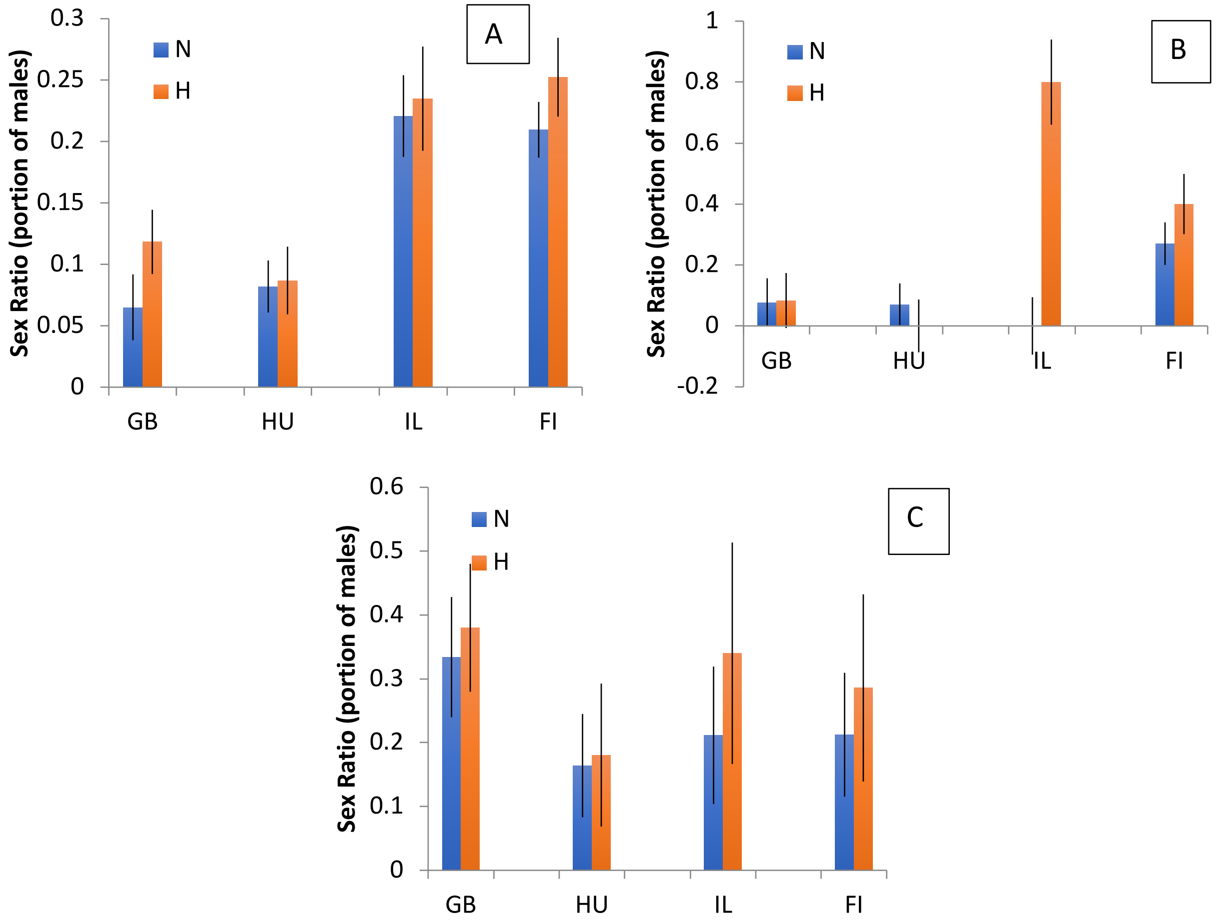

Figure 8: Sex ratios as a proportion of males in clutches of each clone at age 10-25 days (A), age 45-50 days (B), and 65-70 days (C) under normoxic (N) and hypoxic (H) conditions.

Effect Tests: maternal age class <25 days

Source	DF	Sum of Squares	F Ratio	Prob > F
Clone	3	0.703	16.27	<.0001
H/N	1	0.027	1.87	0.17
Clone*H/N	3	0.016	0.37	0.78
Error	147	2.118		

Effect Tests: maternal age class 45-50 days

Source	DF	Sum of Squares	F Ratio	Prob > F
Clone	3	2.245	7.66	0.0001
H/N	1	1.049	10.74	0.0014
Clone*H/N	3	1.975	6.74	0.0003
Error	100	9.766		

Effect Tests: maternal age class 65-70 days

Source	DF	Sum of Squares	F Ratio	Prob > F
Clone	3	0.547	1.21	0.31
H/N	1	0.095	0.63	0.43
Clone*H/N	3	0.033	0.07	0.97
Error	100	15.051		

Table 7: Two-way ANOVA tests demonstrating the effects of hypoxia (H) and normoxia (N) on clutch sex ratios in offspring born to mothers of different ages.

To investigate the early versus late-life fecundity trade-offs, the number of neonates born alive at maternal age of 8-15 days was compared to that at maternal age of 90 and 105 days (Figure 9 A and B) to see how clutch fecundity changed over the lifespan. Data are spread evenly across the graph with tend lines for normoxia and hypoxia having a negative correlation. The trend lines cross near the mean of 8 neonates alive at birth at maternal age 15 days. The regression coefficient shows that by increasing fecundity by 10 individuals early in life you reduce fecundity by 1 individual late in life. Although small in magnitude, the difference is statistically significant for the hypoxia group and all data analyzed together. This correlation completely disappeared in the comparison with fecundity at maternal age 105 days indicating that early reproductive effort did not affect late-life restoration of fertility.

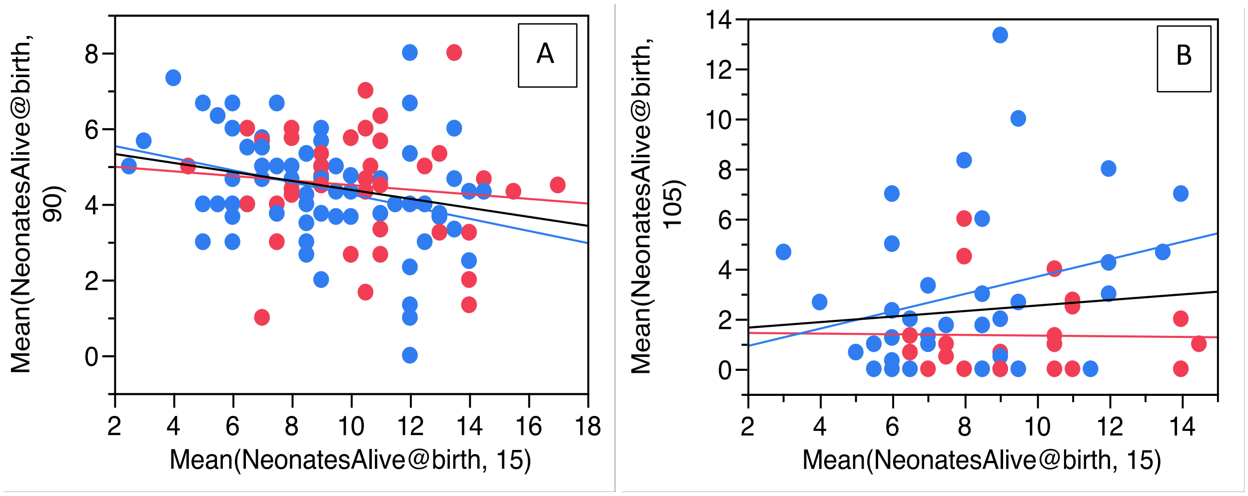

Figure 9: The Correlation between the number of neonates born alive at maternal age of 90 days versus 15 days (A) and 105 days versus 15 days (B). Data: Blue represents born in normoxia, and red represents born in hypoxia. The black line is linear regression for all points, and blue and red lines are linear regression for normoxia and hypoxia, respectively.

Discussion

Maternal and embryonic exposure to mild intermittent hypoxia did not have radical effects on events occurring later in life. The only traits in which such effect had been observed were early fecundity (the hypoxia group showing higher fecundity than the controls) and late-life fecundity (the hypoxia group showing a significantly lower values than the controls). The early fecundity differences may be a nutritional rather than epigenetic effect (perhaps hypoxic mothers were provisioning embryos with higher level of resources). On the other hand, late life fecundity, corresponding to the late file fecundity spike, could not possibly be explained by maternal provisioning and most likely represents long-term plasticity and/or epigenetic effect of maternal hypoxia. The nature of this epigenetic signal remains to be investigated. The effect of early hypoxia on longevity was equivocal.

There was no significant difference in proportional hazard test between the two groups, although at least in some ages the hypoxia group showed higher mortality. When offspring are raised in an environment they were not adequately prepared for, it can have negative effects on the life history of those offspring (Coakley et al., 2018) as was seen here. The mothers prepared them for a hypoxic environment because that is what the mothers lived in; however, the neonates were transferred to a normoxic environment that their mothers had never experienced, leaving the offspring at a slight disadvantage.

The *Daphnia* used in this study originated from four distinct clones with varying life histories. The GB and HU clones coming from permanent ponds experience similar responses to environmental stimuli as do IL and FI clones originating in intermittent ponds. The lifespans of each of the four clones shared similar trends. Each clone experienced a different lifespan corresponding to its unique life history. Data describing body size at first reproduction, fecundity, and sex ratios from GB and HU clones differed significantly from IL and FI clones. Other research has shown that when mothers are exposed to one set of environmental conditions, they prepare their offspring to live within the same environment.

Reproductive changes were seen in each clone as age progressed. The body size of mothers at the first clutch was significantly different between clones that came from permanent ponds verses those that originated in temporary ponds. Mothers had larger clutches later in life after decreasing offspring numbers around midlife. This is consistent with findings from other studies linking advanced maternal age to neonatal and reproductive changes (Pirow et al., 1999). *Daphnia* at approximately age 100 days experienced severe decline followed by an extreme increase in clutch size by a few individuals. This rejuvenation seen in daphnids occurs after a significant drop in fecundity before the age of 100 days. It is not understood why or how rejuvenation happens, but further studies plan to investigate this observation. The day 95-100 age group encompasses the same period in which a major decline across all clone lines was seen as the population grew more elderly. Daphnids of age 8-15 days were close enough to hypoxia to show physiological effects from it such as larger clutches in hypoxia groups. Older individuals from the 95-105 day age group had larger clutches in normoxia demonstrating that the effects of hypoxia on clutch size are only limited to early life reproduction. Further analyses also supported these trends demonstrating that reproduction early in life has no effect on the rejuvenation seen after the age of 100 days.

Sex ratios demonstrated similarities to trends seen in other data showing IL and FI clone fitness to be greater than that of GB and HU clones. Data from the sex ratios of *Daphnia* in age groups 10-25 days, 45-50 days, and 65-70 days demonstrate the effects of maternal hypoxia well. This data was obtained before the major population decline between 95-105 days allowing trends to be better seen with a larger population. The sex ratio as a proportion of males was only significant in the 10-25 day group among clones which is around the time of the first reproduction. This significance disappeared as the cohort of mothers advanced in age. These trends are inconsistent with findings by other researchers who observed that an increase in male offspring production often results from environmental cues such as hypoxia (Rider et al., 2004), and harsher environments such as those found in hypoxia tend to promote higher proportions of male offspring during physiological stress (Ye et al., 2019).

The sensitivity of daphnids to their environment enhances their ability to adjust to climactic changes. As a keystone species, *Daphnia* play an important role in the ecosystem which allows scientists to understand the health of the environment (Miner et al., 2012). Research into hypoxia enhances our understanding of how aquatic environments may change and shift their populations as the climate and water warms. This study demonstrated that early hypoxia does not promote more male offspring production early in life and shortens the lifespan a little rather than lengthening it. Further research into hypoxia with a greater number of clone lines and more individuals per clone would clarify the findings of this research and our understanding of the effects of hypoxia across larger populations and more varied life histories of *Daphnia magna.*

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