

Article

The Effects of Hypoxia on Threshold Food Concentrations in Different *Daphnia* Species

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Abstract: Numerous studies have revealed a negative correlation between the body size and temperature among a variety of aquatic ectotherms. Many studies at individual and population levels indicated that this mechanism may be explained by the decrease of competitive abilities of larger-over smaller-bodied individuals, as the production of larger-bodied individuals is more limited due to greater susceptibility to decreased oxygen concentrations (i.e., environmental hypoxia) at elevated temperatures. However, this hypothesis is still not tested at the community level. To test this, we performed several experiments on the food thresholds (which is a proxy for competitive ability) of 6 zooplankton (*Daphnia*) species varying in body size, at high or low oxygen concentrations. Contrary to the hypothesis tested, hypoxia increased threshold food concentrations to a relatively greater extent in smaller species than in larger ones. This may be attributed to the better evolutionary adaptations of larger-bodied daphnids to oxygen-poor environments manifested in higher production of haemoglobin. The results obtained in this study cannot exclude the possibility that environmental hypoxia is responsible for the temperature-size pattern in aquatic ectotherms, as our experiments did not take into account the long-term energetic costs of expedited haemoglobin synthesis, which could shift size-dependent competitive power.

Keywords: Bergmann's rule; *Daphnia*; hypoxia; interspecific competition; oxygen demands; oxygen limitations; threshold food concentrations; T-S rule



Citation: Wilczynski, W.; Babkiewicz, E.; Pukos, S.; Wawrzeńczak, J.; Zebrowski, M.L.; Banasiak, L.; Kudriashov, M.; Maszczyk, P. The Effects of Hypoxia on Threshold Food Concentrations in Different *Daphnia* Species. *Water* **2022**, *14*, 3213. <https://doi.org/10.3390/w14203213>

Academic Editors: Gilberto Pambianchi and Matteo Gentilucci

Received: 2 September 2022

Accepted: 9 October 2022

Published: 13 October 2022

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1. Introduction

Body size in ectothermic animals is one of the most important determinants of their ability to cope with various biotic and abiotic environmental conditions and many of them (e.g., reptiles, insects or crustaceans) are known to adapt to environmental cues by modifying their final body size [1–3]. In some cases, this can even be visible at every level of biological organization, e.g., the body size of individuals, as well as the mean body size of individuals in populations and species in communities of ectotherms tend to decrease in warmer as compared to cooler environments [4–6]. The pattern is consistent with the temperature-size rule at the individual level [5] and with Bergmann's rule at the population and community levels [4]. In populations and communities, the pattern can be observed both in temperature gradients of geographical clines (i.e., along latitude and altitude; [3,7–9]), as well as in temporal warming events (e.g., seasonal or due to climate change; [3,10,11]), particularly in aquatic environments (e.g., [12,13]). In line with the shift in species proportion along latitudes and altitudes [14] and seasonal shifts in life-history events [15], reductions in body size are seen as a third ubiquitous ecological response to global warming in aquatic environments [16].

Elevated temperatures could reduce the mean body size in communities, due to processes operating at different levels of biological organization. At the individual and population levels, they consist of changes in an individual's life history (concerning its body size and reproduction). At the community level, they consist of increasing the proportion of small-bodied species in relation to large-bodied ones [16]. The literature provides numerous mechanistic (proximate) and evolutionary (ultimate) explanations of the life history traits changes in the context of this pattern [17]. At the community level, these specific explanations can be grouped into three general and non-exclusive explanations [3,7,18]. The first, is due to the direct effect of temperature. This may occur during catastrophic events when the temperature rises above the thermal tolerance limit of larger- but not smaller-bodied species. Although this explanation is theoretically possible, it is rather unlikely. Remaining explanations concerning indirect effects of temperature on the species composition through interspecific interactions are more plausible. The second, is that higher water temperature may increase mortality due to predation, which selectively eliminates larger species from the community [19,20]. Finally, the warming might increase the competitive capacity of smaller species over larger ones (causing the elimination or decline of the latter) due to their physiological adaptation to higher temperatures [3,21]. The last explanation is based on the argument that larger bodied ectotherms are relatively more sensitive to elevated temperatures [22] or temperature-associated factors such as increased oxygen demand [23,24], decreased oxygen availability in water [25] or changes in food quality [18,26].

Although the inverse correlation of body size and temperature in aquatic ectotherms is ubiquitous, it remains unknown what mechanisms and underlying environmental factors are universally responsible for this pattern [17,27–29]. Among the most extensively studied and feasible factor is hypoxia. At elevated temperature, it can occur in the water as environmental hypoxia (due to the decrease in gas solubility in water, and therefore dissolved oxygen depletion) and in animals, as tissue hypoxia (due to increased metabolic rate, and thus oxygen demand). Hypoxia could be the main factor responsible for the inverse correlation of body size and temperature in aquatic ectotherms for at least three reasons, that is: (i) the respiratory surface area for oxygen uptake in a smaller body is challenged to a lesser degree in supplying oxygen due to its greater surface-to-body volume ratio [21], (ii) once inside a smaller body, oxygen is transported over shorter distances [30,31], and (iii) smaller cell size improves diffusion within tissues [32], because oxygen diffuses through a cell membrane more efficiently, than it does through the cytoplasm [31,33].

While body-size related effects of the tissue and environmental hypoxia on the life history traits have been extensively studied experimentally, usually confirming the predictions of the TSR (e.g., [3,5]), the studies concerning its effects on the species composition are much less numerous and concern mainly the tissue rather than environmental hypoxia associated with elevated temperature. The majority of studies concerning the effect of elevated temperature (i.e., the effect of tissue hypoxia) on the species composition are only correlational (e.g., [8,11,34]), therefore they do not exclude the possibility that the combinations of extrinsic factors other than elevated temperature may provide a better explanation of the observed trends [8]. This is further supported by the fact that the majority of experimental studies addressing this found no effect of temperature on competitive power [26,35] or even found inverse relationship to the expected one, i.e., elevated temperature accelerated competitive exclusion of smaller-bodied species [36].

Literature provides several examples of correlative studies between environmental hypoxia and the body size of species in different geographical clines (e.g., [12,37,38]). For instance, an analysis of 2092 species of benthic amphipods has shown a positive correlation between oxygen concentration and maximum body size along a latitudinal cline in freshwater and marine habitats [37]. However, according to our knowledge, the literature does not provide any example of an experimental study testing whether environmental hypoxia affects the relative increase of competitive abilities of small- over large-bodied species. There are no such studies even for planktonic animals, including *Daphnia*, which

are keystone organisms for the functioning of lentic ecosystems. This is surprising as numerous studies at the individual level revealed that environmental hypoxia favours smaller body size [31,39].

One of the indirect methods of studying the effects of different environmental factors on the competitive abilities of different *Daphnia* genotypes is to compare the effect of that factor on their threshold food concentration (C_0), the concentration at which production = 0, as assimilation equals respiration [40]. It has long been known that the body size of zooplankton is inversely related with C_0 [41], although some later studies have not fully confirmed this relationship (e.g., [42–44]). Regardless of whether or not the C_0 in *Daphnia* depends on body size, if the environmental hypoxia associated with elevated temperature affects competitive strength according to the pattern, it will have a relatively greater effect of raising C_0 in larger- than smaller-bodied species. The aim of our study was to test whether environmental hypoxia increases the C_0 of larger zooplankton species to a greater extent than it increases the C_0 of smaller species. To do this, we performed growth experiments on several closely related *Daphnia* species differing in body size.

2. Materials and Methods

2.1. Experimental Animals

We have conducted experiments using 6 species of *Daphnia* characterised by different body size (ranked from largest to smallest): *D. magna*, *D. pulicaria*, *D. pulex*, *D. galeata*, *D. longispina*, *D. cucullata*. The first three species are typical for fishless pond ecosystems, whereas the rest are typical for lake ecosystems. Each species was represented by 4 clones (Table S1, Supplementary Material). The stock and experimental animals were fed unicellular green algae *Chlamydomonas reinhardtii*. Adult *Daphnia* cultivated for the production of juveniles for the experiments were fed *ad libitum* every day ($1.2 \text{ mg } C_{\text{org}} \times \text{L}^{-1}$). An optimal culture density was constantly maintained in the cultivation stocks ($\approx 5 \text{ } D. magna$, $9 \text{ } D. pulicaria$, $10 \text{ } D. pulex$, $13 \text{ } D. galeata$, $15 \text{ } D. longispina$, $17 \text{ } D. cucullata \text{ ind.} \times \text{L}^{-1}$) and half of the stock cultivation medium was changed every two days (tap water aerated for 24 h before use). The cultivation stocks were located in a temperature-controlled room ($22 \text{ }^\circ\text{C}$) with a 16:8 (L:D) photoperiod with a light intensity of $2.6 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (measured by a photometer, Li-COR 89).

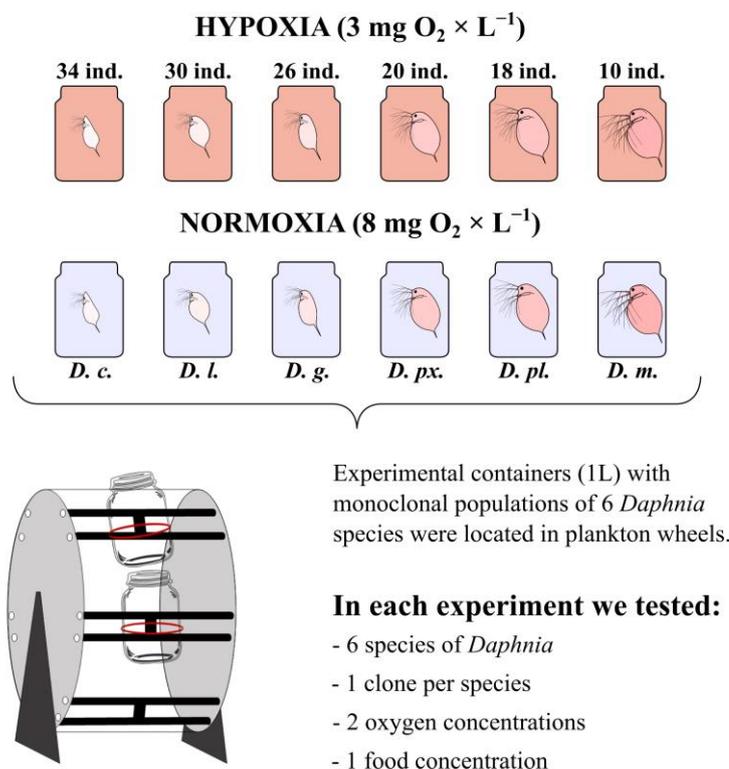
2.2. Experimental System

The system consisted of twin plankton wheels, each with 6 autoclaved and hermetically sealed (using lids and parafilm) glass experimental containers filled to the brim with media (the same water, as used for the cultivation stocks). The wheels were programmed to make a full turn every minute, which allowed heterogeneous distribution of algal food in the containers. They were located in a sarcophagus, evenly illuminated by low intensity LED bands ($0.4 \mu\text{mol m}^{-2} \text{ s}^{-1}$) with a photoperiod of 16:8 (L:D). The experimental system was located in a temperature-controlled room ($22 \text{ }^\circ\text{C}$). The temperature and oxygen concentration in the experimental media was measured by an optical dissolved oxygen probe (hand-held dissolved oxygen meter, YSI ProODO, YSI Incorporated, OH, USA). The algal food concentrations were determined by AquaFluor Handheld Fluorometer (Turner designs, San Jose, CA, USA).

2.3. Experimental Design

The experiments were performed at the Hydrobiological Field Station of University of Warsaw in Pilchy between March and August 2021. During each experiment, 12 experimental containers were used. Each experiment included 6 species, and each container consisted of one species (a single clone per experiment), either in hypoxia ($3 \text{ mg } \times \text{O}_2 \text{ L}^{-1}$), or normoxia ($8 \text{ mg } \text{O}_2 \times \text{L}^{-1}$). The experiments were conducted in one algal food concentration at a time: 0.05, 0.15 or $0.45 \text{ mg } C_{\text{org}} \times \text{L}^{-1}$ and the total number of clones studied was 4 and each experiment was conducted in 2 replications ($3 \text{ food concentrations} \times 4 \text{ clones} \times 2 \text{ replications} = 24 \text{ experiments}$) (Figure 1). For

haemoglobin measurements, some experimental variants were missing (e.g., there are no measurements of C4 clone), either due to high mortality or due to loss of animals during the handling procedure.



Experimental parameters:

Temperature	22°C
Photoperiod	16:8 (L:D)
Food concentrations	0.05, 0.15, 0.40 mg $C_{\text{org}} \times \text{L}^{-1}$ (tested separately)
Clones (experimental replications)	4 per species (tested separately)
Technical replications	2 per clones
Media exchange	every 24h
Experiment duration	96h

Data collected:

- Dry mass
- Haemoglobin concentration

Figure 1. A diagram highlighting the experimental plan.

Aerated (24 h) tap water was filtered through 0.20 μm membrane filters (Sartorius™, Göttingen, Germany). Filtered water was then mixed with algal suspension (to one of the three experimental food concentrations, calculated by measuring chlorophyll fluorescence on the AquaFluor Handheld Fluorometer (Turner designs, San Jose, CA, USA), calibrated to organic carbon concentration). Then, two different experimental media were prepared, either normoxic or hypoxic. Hypoxia was obtained by nitrogen purging (ultra-pure grade 99.9%) until reaching the desired oxygen concentration. The media were distributed into the experimental containers. The concentration of oxygen in each of the containers was maintained at the desired level using media with a very low ($\approx 0.5 \text{ mg O}_2 \times \text{L}^{-1}$) or very high ($\approx 10.5 \text{ mg O}_2 \times \text{L}^{-1}$) oxygen concentration. Synchronized *Daphnia* neonates (0–12 h) were introduced either to the hypoxic or normoxic treatments. In order to account for the

different filtration rates (and therefore, the rate of algal food depletion), the number of *Daphnia* in the experiment was different for each of the species (10 *D. magna*, 18 *D. pulicaria*, 20 *D. pulex*, 26 *D. galeata*, 30 *D. longispina*, 34 *D. cucullata*). Additional fifteen individuals were rinsed, divided into five technical replicates (each with 3 individuals), dried (48 h at 60 °C) and weighted on a microbalance scale (Orion-Cahn C-35) to determine the average initial body mass. The experiments were started by hermetically sealing of the containers and by placing them onto the plankton wheels.

The experimental media were prepared and replaced every 24 h. The algal food concentration, temperature and oxygen concentration in each of the containers was measured twice a day, before and after media exchange. Experimental animals were gently isolated from the old containers and transferred into a prepared set of new containers with fresh media using a glass pipette. The containers were randomly placed on different plankton wheels every day. The duration of each experiment was 96 h. At the end of the experiments, *Daphnia* in each treatment were randomly divided into two groups. *Daphnia* from the first group ($75 \pm 5\%$ of individuals) were divided into 3 technical replicates and then weighed to obtain final mass using the same procedure as for initial individuals. *Daphnia* from the second group were used to assess the Hb concentration ($25 \pm 5\%$ of individuals). The % of weighted individuals slightly varied between treatments due to clone- and species-specific mortality.

2.4. Data Analysis

The individual growth rate (of each clone in each treatment) was calculated from the initial and final dry masses according to the formula: $gr = (ln_{C_t} - ln_{C_0})/t$, where C_0 is the mean body mass of newborn neonates and C_t is the mean body mass of individuals of a given clone at the end of the experiment.

The haemoglobin content in *Daphnia* was measured using a standard spectrophotometric method [45]. The final mean concentration was divided by the number of individuals in the sample and then related to the mean body mass of an individual *Daphnia* belonging to a given clone.

We fitted to the data GLM mixed-effect models predicting growth rate and haemoglobin content based on a set of fixed (species, oxygen concentration, food concentration) and random (clone) variables. We assumed that the growth rate is proportional to the logarithm of food concentration incorporated to the model as a continuous variable. From the other hand, food concentration was treated as a simple categorical variable when explaining the haemoglobin content. The interaction between model terms was assessed by the likelihood-ratio chi-squared test. For all the model parameters, approximate 95% confidence intervals (95% CI) were calculated based on profile likelihood. Multiple comparisons of growth rate and haemoglobin content between species were performed using Tukey HSD test. Threshold food concentration (TFC) for each species and oxygen level was calculated based on the point estimates of the growth rate model parameters. In order to assess TFC statistical uncertainty, 1000 bootstrap replicates of the growth rate model were performed. All the calculations were performed using R statistical software with 'lme4', 'lmerTest' and 'multcomp' packages.

3. Results

Although by introducing different numbers of *Daphnia* belonging to the studied species in each experimental container, we have, to some extent, normalized the algal food depletion due to species-specific filtration rates, there was still variability in the algal concentrations between the experiments. For that reason, we conducted the growth rate and TFC analysis for the mean food concentrations calculated based on the actually measured initial and final food concentrations and not the fixed levels assumed in the experimental design.

3.1. Growth Rate

The model was well fitted to the data with marginal R^2 equal to 0.80. The individual growth rate of *Daphnia* (Figure 2) was affected by both food and oxygen concentrations. Doubling food concentration increased the growth rate of *Daphnia* by 0.097–0.108 d^{-1} (95% CI; $p < 2 \times 10^{-16}$). Although we have not detected any interaction between oxygen concentration and species ($LRT = 6.44$; $p = 0.2656$, likelihood-ratio chi-squared test), upon grouping the species into two clusters: lake cluster (comprising smaller species; *D. cucullata*, *D. longispina* and *D. galeata*) and pond cluster (comprising larger species; *D. pulex*, *D. pulicaria* and *D. magna*), the statistical analysis has shown that decreased oxygen concentrations had different effect on the groups. Hypoxia hampered growth rate by 0.034–0.079 d^{-1} (95% CI; $p < 9.11 \times 10^{-7}$) in the case of lake species, and the effect of decreased oxygen concentration was less prominent by 0.004–0.063 d^{-1} for pond species (95% CI; $p = 0.0255$). The growth rate was also significantly different between most of the studied species; it was the highest in the largest bodied species and gradually lower in the smaller species (Figure S1).

3.2. Threshold Food Concentrations

The TFC of *Daphnia* (Figure 3) was significantly different between most of the studied species; it was the lowest in the largest bodied species and gradually higher in smaller species (normoxia; Figure S2, hypoxia; Figure S3). Although we have not detected any interaction between oxygen concentration and species ($LRT = 6.44$; $p = 0.2656$, likelihood-ratio chi-squared test), upon grouping the species into two clusters: lake cluster (comprising smaller species; *D. cucullata*, *D. longispina* and *D. galeata*) and pond cluster (comprising larger species; *D. pulex*, *D. pulicaria* and *D. magna*), the statistical analysis has shown that decreased oxygen concentration has increased the TFC of the lake cluster more than the pond cluster by 3.8–57.9% (95% bootstrapped CI).

3.3. Haemoglobin Production

Although the marginal R^2 was rather low (0.26), we could determine that the haemoglobin production in *Daphnia* (Figure 4) was significantly affected by both food and oxygen concentrations. As compared to the lowest food concentration (0.05 $mg C_{org} \times L^{-1}$), increased food concentrations have lowered the production of haemoglobin at both 0.15 and 0.45 $mg C_{org} \times L^{-1}$ by 1.240–18.709 and 11.393–26.249 $\mu g \times mg^{-1}$ respectively (95% CI; corresponding $p = 0.0254$ and $p = 2.41 \times 10^{-6}$). Decreased oxygen concentration has expedited the production of haemoglobin by 8.096–21.040 $\mu g \times mg^{-1}$ (95% CI; $p = 1.66 \times 10^{-5}$). Moreover, the haemoglobin production was different between some of the species; it was the highest in the largest bodied species and gradually lower in the smaller species (Figure S3). We have not detected any interaction of oxygen concentration and species ($LRT = 8.03$; $p = 0.1545$, likelihood-ratio chi-squared test) or different clusters ($LRT = 2.95$; $p = 0.0861$, likelihood-ratio chi-squared test).

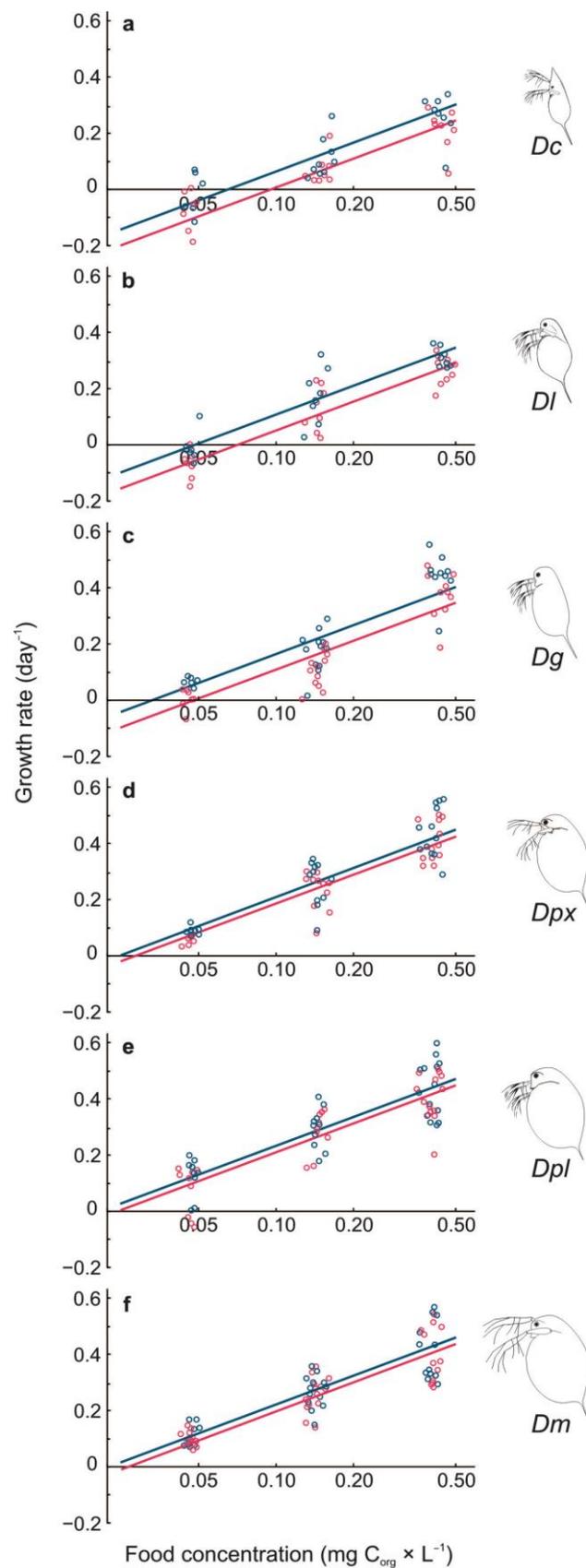


Figure 2. Regression lines of growth rate (d^{-1}) vs. food concentration ($\text{mg C}_{\text{org}} \times \text{L}^{-1}$) for six species of *Daphnia* at different oxygen concentrations (normoxia = blue lines; hypoxia = red lines). Each line represents the mean growth rate calculated from four clones (experimental replicates).

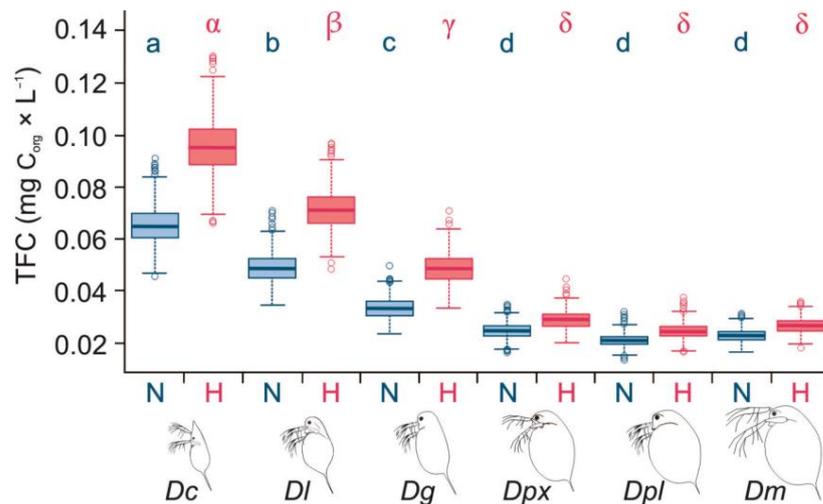


Figure 3. Boxplots showing the bootstrapped threshold food concentration (TFC, $\text{mg C}_{\text{org}} \times \text{L}^{-1}$) for six *Daphnia* species at different oxygen concentrations (normoxia = blue bars; hypoxia = red bars; whiskers = min. and max. excluding outliers; boxes represent quartiles). The species that were significantly different from the others are denoted with different letters (latin letters for normoxia and greek letters for hypoxia).

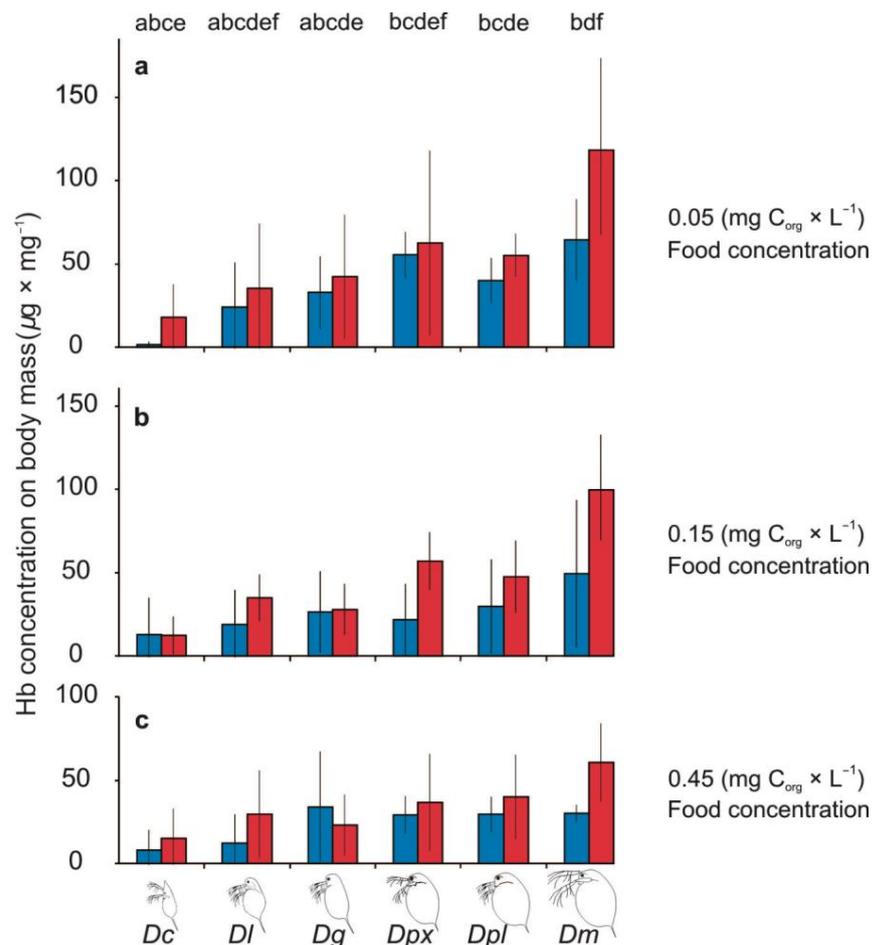


Figure 4. Haemoglobin concentration on dry body mass ($\mu\text{g} \times \text{mg}^{-1}$, mean \pm SD) at three different food concentrations: (a) 0.05, (b) 0.15 and (c) 0.45 $\text{mg C}_{\text{org}} \times \text{L}^{-1}$ for six *Daphnia* species at different oxygen concentrations (normoxia = blue bars; hypoxia = red bars). Statistically significant differences are denoted with different letters.

4. Discussion

In this study, the effects of environmental hypoxia on the growth rate and TFC in *Daphnia* were apparent, however, contrary to our hypothesis, hypoxia had decreased the growth rate and increased the threshold food concentrations to a relatively greater extent in the smaller bodied species, than it did in the larger bodied species. Potentially, this could be attributed to evolutionary adaptations of the larger species—in our study, the production of haemoglobin was greater in larger- than in smaller-bodied species. This is consistent with the results of earlier studies on the inter-specific differences in haemoglobin concentrations in *Daphnia* [46–48], which indicated that larger-bodied species have higher basal levels of haemoglobin.

The results are in contradiction to the studies performed at the individual and population levels, which revealed that environmental hypoxia favours smaller body size [31,39]. In such studies, for example, isopods only grew in accordance with the temperature-size rule when reared in hypoxic waters [39]. Our results however, do not exclude the possibility, that oxygen deficiencies could favour smaller body size (as well as favour smaller-bodied species at the community level). As opposed to the previous studies, in our case, the observed effects could also be attributed to great species-specific phenotypic plasticity of *Daphnia* (expressed in, e.g., differences in haemoglobin production). Nevertheless, Our results suggest that hypoxic conditions associated with elevated temperature alone cannot be responsible for the temperature-size pattern observed in zooplankton, although we cannot exclude the possibility that they play a significant role when combined with other size-selective factors such as increased oxygen demands and fish predation at elevated temperature.

Moreover, the results obtained in our study are also in contradiction with the correlational studies between environmental hypoxia and the body size in ectotherms communities [12]. However, it should be pointed out that the presence of the given correlation does not exclude the possibility that the combinations of extrinsic factors other than compared ones may provide a better explanation of the observed trends.

Lastly, hypoxia could possibly favorise smaller body size if the energetic costs of haemoglobin production in larger *Daphnia* outweighed the competitive advantages gained by expediting its synthesis at oxygen deficiencies. As our experiments were short-term, we could not observe such an effect, however a possibility cannot be excluded that it would shift the competitive strengths between larger- and smaller-bodied species over time.

We obtained several interesting additional results, e.g., the negative effect of hypoxia on the growth rate of *Daphnia*, which is consistent with several earlier studies demonstrating, that oxygen deficiencies hamper the growth rates of aquatic biota, e.g., fishes [49,50] or invertebrate species [51]. These results, however, are in contradiction with the studies questioning the importance of oxygen supply as a determinant of body size, which challenge the claim that oxygen supply could limit growth and body size under most conditions, at least for gill breathing ectotherms, such as fish [52]. Indeed, the current view among physiologists is that oxygen uptake can be easily modulated by organisms and therefore reflects oxygen demand rather than the other way around.

In this study we have also found consistent relations of both growth rate and TFC to body size. In general, the largest species had the highest growth rate and the lowest TFC, while the smaller species had gradually lower growth rate and higher TFC. This pattern has long since been described [41], although it was not apparent in another studies concerning the species-specific growth rate and TFC in *Daphnia* [42–44].

Although the increase in the individual growth rates of *Daphnia* along increasing food concentrations was quite obvious, interestingly, we have also found a significant effect of food concentrations on haemoglobin production. Because the haemoglobin production seemed to decrease along with increasing food concentrations, we hypothesize that *Daphnia* were producing more haemoglobin at lower food levels because it allows them to gather more food [53].

5. Conclusions

Our study revealed that environmental hypoxia has a greater negative effect on the growth rate and a greater positive effect on the threshold food concentrations in the smaller than in larger-bodied *Daphnia* species, which suggests that its presence results in the decrease of relative competitive abilities of the latter more than the former. Therefore, the study suggests that environmental hypoxia associated with an elevated temperature cannot be responsible for the decrease in relative proportion of larger-bodied species in zooplankton communities at an elevated temperature. In order to gain a deeper insight on the potential role of oxygen deficiencies in this pattern, further studies on the effects of hypoxia coupled with elevated temperatures and other environmental factors are necessary.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w14203213/s1>, Figure S1: 95% confidence intervals for the differences in growth rate (d^{-1}) between each studied species; Figure S2: 95% bootstrapped confidence intervals for the differences in the threshold food concentration ($mg\ C_{org} \times L^{-1}$) between each studied species in normoxia. Figure S3: 95% bootstrapped confidence intervals for the differences in the threshold food concentration ($mg\ C_{org} \times L^{-1}$) between each studied species in hypoxia. Figure S4: 95% confidence intervals for the differences in haemoglobin production ($\mu g \times mg^{-1}$) between each studied species. Table S1: *Daphnia* species and clones used in our study, as well as their body size at first reproduction (mm), date of their isolation and their location of origin.

Author Contributions: Conceptualization, P.M.; methodology, P.M. and M.K.; validation, P.M., E.B. and W.W.; formal analysis, Ł.B. and W.W.; investigation, W.W., E.B., S.P., J.W., M.L.Z. and P.M.; resources, P.M.; data curation, P.M. and W.W.; writing—original draft preparation, W.W. and P.M.; writing—review and editing, W.W., Ł.B. and P.M.; visualization, P.M.; supervision, P.M.; project administration, P.M.; funding acquisition, P.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Science Centre, Poland, grants number 2014/15/B/NZ8/00245, 2016/23/D/NZ8/03532. E.B. was supported by the National Science Centre, Poland, grant number 2018/31/N/NZ8/03269. P.M. was supported by the National Science Centre, Poland, grant number 2019/35/B/NZ8/04523.

Institutional Review Board Statement: All applicable institutional and national guidelines for the care and use of animals were followed.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The study was conducted with the help of the infrastructure of the Hydrobiological Station of the University of Warsaw in Pilchy. We are thankful to Mirosław Ślusarczyk for valuable insights into the manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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