water

MDPI

Article

Effect of Temperature Rising on the Stygobitic Crustacean Species *Diacyclops belgicus*: Does Global Warming Affect Groundwater Populations?

Tiziana Di Lorenzo [1,*] and Diana Maria Paola Galassi [2]

[1] Institute of Ecosystem Study of the CNR—National Research Council of Italy, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Italy

[2] Department of Life, Health and Environmental Sciences, University of L'Aquila, Via Vetoio 1, 67100 Coppito, Italy; dianamariapaola.galassi@univaq.it

* Correspondence: tiziana.dilorenzo@ise.cnr.it; Tel.: +39-055-522-5918

Received: 27 October 2017; Accepted: 4 December 2017; Published: 7 December 2017

Abstract: The average global temperature is predicted to increase by 3 °C by the end of this century due to human-induced climate change. The overall metabolism of the aquatic biota will be directly affected by rising temperatures and associated changes. Since thermal stability is a characteristic of groundwater ecosystems, global warming is expected to have a profound effect on the groundwater fauna. The prediction that stygobitic (obligate groundwater dweller) species are vulnerable to climate change includes assumptions about metabolic effects that can only be tested by comparisons across a thermal gradient. To this end, we investigated the effects of two different thermal regimes on the metabolism of the stygobitic copepod species *Diacyclops belgicus* (Kiefer, 1936). We measured the individual-based oxygen consumption of this species as a proxy of possible metabolic reactions to temperature rising from 14 to 17 °C. We used a sealed glass microplate equipped with planar oxygen sensor spots with optical isolation glued onto the bottom of 80-µL wells integrated with a 24-channel fluorescence-based respirometry system. The tests have provided controversial results according to which the *D. belgicus* populations should be prudently considered at risk under a global warming scenario.

Keywords: metabolism; respirometry; copepods; crustaceans; groundwater; porous aquifer

1. Introduction

Global climate change is expected to seriously alter the supply of aquatic ecosystem services that are crucial for human wellbeing. Mediterranean ecosystems appear the most vulnerable to global change in Europe, with potential impacts related primarily to increasing temperatures, reduction in precipitation, water scarcity, concentration of economic activities in coastal areas and climate-sensitive agriculture [1]. Groundwater will play a fundamental role in sustaining ecosystems and enabling human adaptation to climate change. The strategic importance of groundwater will intensify as climate extremes become more frequent and intense. However, understanding climate-change effects on groundwater ecosystems is not an easy task because global warming may affect groundwater ecosystems both directly, through changes in temperature and replenishment by recharge, and indirectly, through changes in groundwater use [2,3]. Accordingly, the IPPC (Intergovernmental Panel on Climate Change) [4] stated that a lack of necessary data has made it impossible to determine the magnitude and direction of groundwater change due to climate change [5]. Multidisciplinary collaboration is needed to study changes in groundwater chemistry, temperature, hydro-geophysical properties and biology over a range of spatial scales [6].

An air temperature increase of 2–3 °C is expected in the Mediterranean region by 2050 and a rise of 3–5 °C is expected by 2100 [4,7]. Global warming will result in an increase of groundwater temperatures as well. In the extreme cases, groundwater temperatures in shallow porous aquifers (depth < 15 m below ground level) are expected to increase by up to 4–5 °C in some temperate climate regions in the 45° northern latitude, depending on the degree of urbanization [8]. Climate warming will affect the aquifers that are recharged by surface water through riverbank infiltration [9] as well as those that are directly recharged by precipitation and those affected by ground surface heating [8]. In shallow porous aquifers temperatures are not constant. Seasonal temperature cycles at the ground surface drive seasonal temperature fluctuations in the subsurface down to depths of 10–15 m [10]. Diurnal temperature fluctuations are typically found at depths of less than 1 m, but seasonal fluctuations may be detectable at depths up to 15 m [10]. The annual amplitude of temperature is typically less than 2 °C in porous aquifers that are deeper than 5 m below the soil surface [11–15]. Yet, shallow porous aquifers are not immune to global warming, because the annual mean temperature of groundwater closely tracks the ambient air temperature [11]. This means that groundwater temperatures in these aquifers might reach high values in the warm seasons under the expected global warming scenarios.

While the effects of global warming on groundwater chemistry, hydro-geophysical properties and resources have been studied in recent years, the assessment of the biological responses to the groundwater temperature increases due to climate change is in its infancy. Metabolic rates of obligate groundwater (stygobitic) species are expected to be highly temperature-dependent. Groundwater ectotherm species reside in habitats where temperatures may vary seasonally but only one or two degrees throughout the year [16,17]. In such stable thermal environments, natural selection should favor stenothermal species, i.e., organisms that maximize their performance along a very narrow range of temperatures [18]. Few experiments that deal with the impact of temperature on the physiology of obligate groundwater species are available [19–23], however none of them was specifically designed to test the predictions of the climate change hypothesis.

In this study, we determined the thermal tolerance of a stygobitic species, *Diacyclops belgicus* (Kiefer, 1936), to increasing groundwater temperatures. We selected *D. belgicus* because it is an obligate groundwater species that has been collected from caves, porous aquifers and hyporheic zones of Europe and the former USSR (the Union of Soviet Socialist Republics) [24]. The species shows a wide geographic distribution, and no marked habitat and microhabitat preferences, even if it has been more frequently collected from porous aquifers and the hyporheic zone. *D. belgicus* also resides in a Mediterranean shallow porous aquifer in Tuscany which is characterized by annual temperature amplitude of about 2 °C. Since an exponential relationship between oxygen consumption and temperature was observed in some other obligate groundwater species [23], we expected a change of the metabolic rates of *D. belgicus* under rising temperatures. We explored the likelihood of this prediction by measuring the oxygen consumption of *D. belgicus* under two different temperatures, namely 14 and 17 °C. Fourteen degrees is the lowest annual temperature that has been measured in the shallow porous aquifer where the individuals of *D. belgicus* that were used in our trials were collected. The mean annual temperature of the collection site of *D. belgicus* in this study is 15 °C. Seventeen degrees is 1.1 °C above the highest annual temperature recorded (15.9 °C). The aquifer temperature was measured monthly by a multiparametric probe (ECM MultiTM; Lange GmbH, Düsseldorf, Germany). We selected 14 °C as the reference value because this temperature appeared to be an important threshold for some stygobitic crustaceans, namely the amphipods *Niphargus rhenorhodanensis* Schellenberg, 1937 and *N. virei* Chevreux, 1896: the ventilatory activity increased largely above this temperature in these organisms [20]. The temperature of 17 °C was selected because a 2 °C increase in the mean annual groundwater temperature is anticipated by 2050 due to global warming.

2. Materials and Methods

2.1. Animal Collection and Rearing

The specimens of *D. belgicus* (Figure 1) were collected from a phreatic well in Tuscany, Italy (coordinates: 43°49′02.61″ N; 11°11′59.79″ E) in June 2017. The well (depth: 14 m) is situated in the shallow Quaternary porous aquifer of Medio Valdarno. Prior to the collection of the animals, some groundwater samples were withdrawn from the well and analyzed for dissolved organic carbon (DOC), prokaryotic cell count and 32 chemicals (ammonium, nitrites, nitrates, heavy metals, inorganic pollutants, PAHs (polycyclic aromatic hydrocarbons), pesticides and organochlorines). None of the tested chemicals were detected at concentrations higher than the European and national threshold values. Dissolved organic carbon (DOC) was 1.1 mg/L. The collection of copepods was performed in June 2017 when the temperature of groundwater was 15.3 °C, i.e., very close to the mean annual value (15 °C).

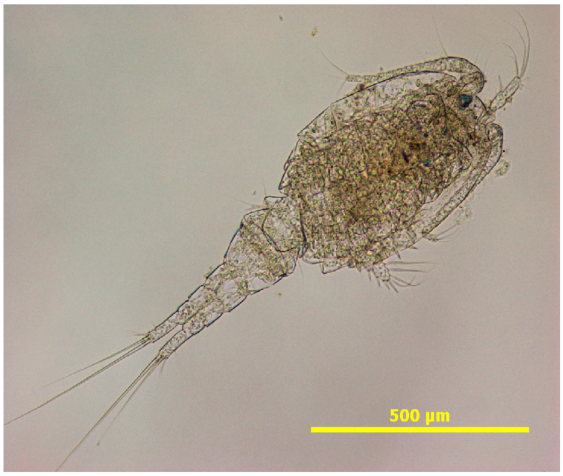

Figure 1. Female of *Diacyclops belgicus* at the optical microscope.

A phreatobiological net-sampler (mesh size: 60 µm) was used to collect copepods from the bottom and the water column of the well. After the collection, the samples were transferred to the laboratory within 10 min. In the laboratory, the individuals of *D. belgicus* were picked up by a glass pipette and pooled in a 100-mL glass vial filled with the groundwater collected from the well (hereafter called bore water). The culture was kept in the dark in a thermostatic cabinet (Mod. ST 3, Pol-Eko-Aparatura, Wodzisław Śląski, Poland) at 14 °C for 7 days. The bore water was not renewed and no additional food was offered. Microbes occurred with about 1.8×10^6 prokaryotic cells/mL in the bore water (Flow Cytometer A50-micro, Apogee Flow System, Hertfordshire, UK). Eighteen hours before the trial at 14 °C the copepods were rinsed in standard water (1 L of MILLIPORE MILLI-Q® (Elix®, Merck KGaA, Darmstadt, Germany) deionized water remineralized with the following reagent grade chemicals: 0.06 g of $MgSO_4$, 0.096 g of $NaHCO_3$, 0.004 g of KCl, 0.06 g of $CaSO_4 \cdot 2H_2O$; [25]) and kept in a 10-mL glass vial with standard water and in the darkness at the testing temperature. No food was offered during acclimation in order to allow gut-emptying [26,27]. After the acclimation, only the actively swimming individuals were selected for testing. The test individuals (juvenile stages, i.e., copepodids) were picked up by a glass pipette under a Leica M80 stereomicroscope at 20× magnification. The pick-up of fecal pellets was carefully avoided so to exclude the overshoot in oxygen consumption due to digestive metabolism [20,26,27]. The respirometric measurements were started on the day 8th for the trials at 14 °C. The whole procedure (collection of new animals from the field, acclimation in bore water and in standard water and testing) was repeated for the trial at 17 °C at the day 9th. A sampling effect on the trials was not considered likely since groundwater habitats

have a strong physical inertia on a monthly scale [11–15]. The animals that had been not used in the tests were kept in the bore water in the thermostatic cabinet.

2.2. Measurement of Oxygen Consumption

Standard respiration rates (SRRs) were measured in a sealed glass microplate equipped with planar oxygen sensor spots with optical isolation glued onto the bottom of 80-µL wells (Loligo Systems, Viborg, Denmark). The microplate was integrated with a 24-channel fluorescence-based respirometry system (SDR Sensor Dish Reader) (PreSens Precision Sensing GmbH, Regensburg, Germany) that allows simultaneous measurement of 20 replicates and 4 controls (wells without animals). This respirometer is known for its simplicity, high throughput, and high temporal resolution and sensitivity [28,29]. The reader was placed inside the thermostatic cabinet at the appropriate testing temperatures (either 14 or 17 °C; accuracy: ±0.1 °C) 18 h before the beginning of the trials to bring the equipment and the standard water to the same temperature prior to measurements [20,29]. Twenty copepodids of *D. belgicus* were then individually loaded in the microwells that were in turn inspected for air bubbles under a stereomicroscope (Leica M80, Leica Microsystems Srl, Wetzlar, Germany) at 20× magnification. The microwells were overfilled with oversaturated standard water and sealed with parafilm before the plate was placed on the SDR reader. The well inspection was repeated after the sealing with the parafilm. Four microwells were kept without animals for controls and they were filled with the standard water used for copepod acclimation. A 10 cm × 7 cm × 0.5 cm rectangular silicone layer was put on top of the microplate for a further sealing. Finally, a weight was posed on top of everything. The preparation of the microplate was conducted under the stereomicroscope at room temperature within 20 min. Nevertheless, when the sealed microplate was put back into the thermostatic cabinet after copepod loading, the device temperature was always about 2 °C higher than the respective test temperature (15.9 °C vs. 14 °C; 18.8 °C vs. 17 °C). The measurements were started as soon as the device temperature had reached the equilibrium with the test temperature, by the SDR v4 Software (PreSens Precision Sensing GmbH, Regensburg, Germany). From preliminary experiments, we set at 120 min the time needed to temperature re-equilibration. Afterwards, the oxygen consumption (% air saturation) was recorded every 2 min during 2 h after the temperature re-equilibration. The plate was not shaken allowing the animals to respire without stress. Total oxygen consumption (µg O_2 per individual per hour) was calculated while correcting for observed changes in oxygen readings in the control wells (mean values of the 4 controls). The percentage of air saturation was above 80% in each well at the end of the trials at both test temperatures.

At the end of each trial, the microwells were opened and inspected under the microscope at 50× magnification. The copepods were individually picked-up by a glass pipette and loaded in a Petri dish and checked for mortality (no movement after gentle stimulation by means of a sorting needle). No dead individuals were detected at the end of the trials. Some drops of carbonated water were added to the Petri dish in order to narcotize the individuals. Pictures of each individual were taken by a HD camera (MC 170, Leica Microsystems Srl, Wetzlar, Germany) that was integrated to the microscope. The camera was connected to a notebook and the pictures were elaborated by LAS EZ vs. 3 software (Leica Microsystems Srl, Wetzlar, Germany) and the free vector graphic software Inkscape (https://inkscape.org/it/). The prosome length (L, mm) and width (W, mm) of each individual were measured using the methods described in our previous studies [26,27]. Dry mass was calculated according to [30]:

$$\text{wet mass (WM)} = K \times (\text{prosomal length}) \times \text{width}^2 \quad (1)$$

where K is a constant, being 0.705 for cyclopoids [31]. A conversion factor of 0.25 was used to convert the wet mass to the dry mass [32], i.e.,:

$$\text{dry mass (DM)} = 0.25 \times \text{WM}. \quad (2)$$

To describe the relationship between temperature and performance we used the metric Q_{10} that is the rate of change of a biological system (in this case, oxygen consumption) as a consequence of a temperature increase of 10 °C. This value was calculated between 14 and 17 °C using the formula:

$$Q_{10} = (R_2/R_1)^{10/(T_2-T_1)} \quad (3)$$

where R_1 and R_2 are the oxygen consumptions at temperatures T_1 and T_2 and $T_2 > T_1$ [20,33]. We used both the mean and the median values of the oxygen consumption.

2.3. Statistical Analyses

To test for differences in size (L, W and DM) between the two trials (at 14 and 17 °C) we used three different one-way permutational analyses of variance (PERMANOVA) [34], one per each variable, with the factor "temperature" (two levels: 14 and 17 °C). To test for differences in the oxygen consumption in μg O_2 per individual per hour, we used a one-way permutational analysis of covariance (PERMANCOVA) [34], with individual DM as the covariate. The permutational analyses were performed on the basis of a Euclidean Distance similarity matrix and using either non-transformed or log(x + 1) transformed data, after performing a Levene's test on the original dataset to check for variance homogeneity. PERMANOVA was run under Type III Sum of Squares and unrestricted permutation of raw data. PERMANCOVA was run under Type I Sum of Squares and permutation of residuals under a reduced model [34]. The number of permutations required to derive *p*-values was set at 9999. Whenever the number of unique permutations was lower than 100, Monte Carlo *p*-values were preferred over the permutation *p*-values.

Prior to the analyses, all data were checked for outliers based on the following fences: lower inner fence (Q1 − 1.5 × IQ), upper inner fence (Q3 + 1.5 × IQ), lower outer fence (Q1 − 3 × IQ) and upper outer fence (Q3 + 3 × IQ), where Q1 and Q3 are the first and the third quartiles respectively and IQ is the interquartile range (i.e., Q3 − Q1). We assumed as outliers those data that were either lower than the lower fences or higher than the upper fences. PERMANOVAs and PERMANCOVA were run on both the raw data and on the raw data set deprived of outliers.

All the statistical tests were performed using PRIMER v.6 & PERMANOVA + routines for PRIMER (PRIME-E, Auckland, New Zealand) [35]. The level of significance was set to $p < 0.05$.

3. Results

SRR, L, W and DM of the individuals used in the trials at 14 and 17 °C are shown in Table 1. L, W and DM of the individuals used in the trial at 14 °C were not significantly different from those at 17 °C (PERMANOVA; L: Pseudo-$F_{1,38}$ = 1.06, *p*-value = 0.3074, perms = 9834; W: Pseudo-$F_{1,38}$ = 0.45, MonteCarlo *p*-value = 0.5269, perms = 67; DM: Pseudo-$F_{1,38}$ = 0.003, *p*-value = 0.9580, perms = 9823). The results of the PERMANCOVA showed that there was not a significant relationship between the dry mass and the SRR of the individuals (PERMANCOVA; DM: Pseudo-$F_{1,36}$ = 1.74, *p*-value = 0.1941, perms = 9817). Nevertheless, even given the non-occurrence of variation of the SRRs due to the dry mass, no significant variability was detected between the trials at 14 and 17 °C (PERMANCOVA; SRR: Pseudo-$F_{1,36}$ = 1.82, *p*-value = 0.1878, perms = 9839).

Table 1. Prosome length (L), prosome width (W), dry mass (DM) and standard respiration rates (SRR) of the 40 individuals of *Diacyclops belgicus* (Kiefer, 1936) involved in the respirometric trials at two temperatures (T). Mean, standard deviation (SD) and minimum and maximum values are also shown. SRR are in µg of oxygen per individual per hour. Outliers above the upper inner fence are in bold and underlined.

Microwell	T (°C)	L (mm)	W (mm)	DM (mg)	SRR (µg O_2/ind. ×h)
W1	14	0.2521	0.1123	0.0006	0.0030
W2	14	0.2356	0.1151	0.0006	0.0030
W3	14	0.2630	0.0959	0.0004	0.0148
W4	14	0.2356	0.0877	0.0003	0.0131
W5	14	0.2795	0.0904	0.0004	0.0059
W6	14	0.3069	0.1206	0.0008	0.0089
W7	14	0.2274	0.1014	0.0004	0.0148
W8	14	0.2795	0.0959	0.0005	0.0059
W9	14	0.3206	0.1452	**0.0012**	0.0119
W10	14	0.2411	0.1123	0.0005	0.0163
W11	14	0.2795	0.0904	0.0004	0.0074
W12	14	0.2713	0.0795	0.0003	0.0074
W13	14	0.2384	0.0932	0.0004	0.0074
W14	14	0.2987	0.0849	0.0004	0.0074
W15	14	0.2356	0.1151	0.0006	0.0030
W16	14	0.2795	0.0904	0.0004	0.0059
W17	14	0.3206	0.1452	**0.0012**	0.0119
W18	14	0.2411	0.1123	0.0005	0.0163
W19	14	0.2713	0.0795	0.0003	0.0074
W20	14	0.2987	0.0849	0.0004	0.0074
W1	17	0.2411	0.1233	0.0006	0.0037
W2	17	0.2521	0.1206	0.0006	0.0120
W3	17	0.2630	0.1178	0.0006	0.0037
W4	17	0.2411	0.1096	0.0005	0.0037
W5	17	0.2630	0.1151	0.0006	**0.0232**
W6	17	0.2658	0.1233	0.0007	0.0037
W7	17	0.2302	0.1069	0.0005	0.0037
W8	17	0.2795	0.1233	0.0007	0.0042
W9	17	0.2822	0.0986	0.0005	0.0014
W10	17	0.2576	0.0986	0.0004	0.0014
W11	17	0.2576	0.1014	0.0005	0.0014
W12	17	0.2685	0.1014	0.0005	0.0065
W13	17	0.2822	0.0904	0.0004	0.0037
W14	17	0.2548	0.1069	0.0005	0.0148
W15	17	0.2493	0.0932	0.0004	0.0148
W16	17	0.3014	0.1233	0.0008	0.0148
W17	17	0.2767	0.1041	0.0005	0.0037
W18	17	0.2740	0.0986	0.0005	0.0093
W19	17	0.2439	0.0932	0.0004	0.0037
W20	17	0.2192	0.0740	0.0002	0.0015
Statistics	**T (°C)**	**L (mm)**	**W (mm)**	**DM (mg)**	**SRR (µg O_2/ind. ×h)**
Mean	14	0.2688	0.1026	0.0005	0.0090
Mean	17	0.2602	0.1062	0.0005	0.0067
SD	14	0.0298	0.0193	0.0003	0.0044
SD	17	0.0198	0.0135	0.0001	0.0060
Max	14	0.3206	0.1452	0.0012	0.0163
Max	17	0.3014	0.1233	0.0008	0.0232
Min	14	0.2274	0.0795	0.0003	0.0030
Min	17	0.2192	0.0740	0.0002	0.0014

Two dry mass and 1 SRR values were above the respective upper inner fences and were thus excluded from the analyses (Table 1). The dry masses of the individuals used in the trial at

14 °C were not significantly different from those at 17 °C (PERMANOVA; DM: Pseudo-$F_{1,36}$ = 3.20, *p*-value = 0.0805, perms = 9842). The results of the PERMANCOVA showed that there was not a significant relationship between the dry mass and the SRRs of the individuals (PERMANCOVA; DM: Pseudo-$F_{1,33}$ = 9.91, *p*-value = 0.7584, perms = 9835). In addition, no significant variability in the SRRs was detected between the trials at 14 and 17 °C (PERMANCOVA; SRR: Pseudo-$F_{1,33}$ = 3.77, *p*-value = 0.0616, perms = 9853).

No outliers higher or lower the outer fences were found.

Q_{10} was 0.39 considering the mean values of the oxygen consumptions and 0.99 considering the median values.

4. Discussion

Groundwater ecosystems are generally poorer in nutrients and oxygen than surface water ecosystems [12–15,36–40]. In order to reduce energetic costs, groundwater ectotherms have evolved metabolic rates that are lower than those of their close epigean relatives [20,41–44]. The results of the one and only study on the metabolism of a stygobitic copepod species [26], in which we compared the SRRs of the stygobitic *D. belgicus* and the epigean *Eucyclops serrulatus* (belonging to the same family Cyclopidae), were consistent with this statement. The SRRs of *E. serrulatus* was 7 and 5 fold the SRRs of *D. belgicus* juveniles and adults, respectively. Albeit measured with a different device and protocol and under different temperatures, the individual-based measurements of the SRRs of *D. belgicus* of the present study are of the same order of magnitude (nanograms of O_2 per individual per hour) as those observed in [26]. Details are provided in Table S1. The scarcity of available data and the difficulty in performing experiments with stygobitic species have frequently led to the sensitivity of groundwater taxa to environmental stressors being inferred from data obtained for epigean relatives. This approach has resulted in the assessment of threshold values for groundwater ecosystem quality that are not protective of the biota which live in this environment [14,45,46]. There are severe constraints that make difficult to perform metabolic, physiological and ecotoxicological studies with groundwater microcrustaceans in laboratory, most of them related to the low metabolism of these species. To our knowledge, no researcher has determined how to make groundwater copepods reproduce in the laboratory. We ourselves kept cultures of *D. belgicus* in laboratory for one year before this study and we did not observe any ovigerous female in the cohorts. This limits the number of replicates and sometimes prevents performing a trial. Most importantly, the abundances are very low and "the one well to one species" is the rule of thumb [12,14,15,40].

Very few studies have investigated the importance of temperature variations for obligate groundwater species [19,20,23]. In this study, the copepod *D. belgicus* did not significantly varied its SRRs between 14 and 17 °C after 7-days acclimation at the testing temperatures. During exposure to a range of temperatures, the relationship between the body temperature and metabolism can be described by an asymmetric bell-shaped function, where the metabolic rate is maximized at an intermediate temperature: the thermal optimum [18,47]. As found for many other species [20,48–52], acclimation to temperatures that are different from the thermal optimum has a significant impact on crustaceans physiology that may change in order to confer protection against the injuries produced by temperature variation. Under acclimation, hemolymph pH may vary [53], as well as enzyme properties [54] and hemoglobin affinities [55], as response to temperature variations. In this study, we did not measure specifically these physiological changes, however we used the respirometric metabolism as a proxy. Nevertheless, the SRRs of *D. belgicus* at 17 °C were not significantly different from those at 14 °C thus indicating a non-significant variation in the physiological processes. Similarly, the SRRs at 14, 17 and 21 °C of the stygobitic amphipods *N. renorhodanensis* and *N. virei* were very close to each other, although differences had been not tested by statistical analyses [20]. Moreover, the mode of the bell-shaped curve of oxygen consumption and temperature fell around 17 and 21 °C for *N. rhenorhodanensis* which consequently has led it to be characterized as a eurythermal species [20]. Accordingly, individuals of a population of the stygobitic isopod *Proasellus valdensis* (Chappuis, 1948)

which survived in the laboratory at temperatures up to 22 °C, no longer increased their metabolic rates above 16 °C [23].

The thermal tolerance of *D. belgicus* is relevant to the habitat where this species was collected for this study, that is a Mediterranean shallow aquifer characterized by annual temperature amplitude of about 2 °C. The individuals of *D. belgicus* that reside in the Medio Valdarno porous aquifer are adapted to temperatures that vary between 14 and 15.9 °C and do not change its respiratory metabolism under a further increasing of 1.1 °C, at least as far as the juvenile stages are concerned and under 7-days acclimation. As high metabolic rates are expected to increase the influx rate of toxicants in aquatic crustaceans [27,44,56], the invariability of the SRRs between 14 and 17 °C may be considered protective to this species. In addition, the absence of SRR variability suggests that the survival of *D. belgicus* at a temperature of 1.1 °C above the highest measured in the Medio Valdarno porous aquifer does not depend on the ability of this species to satisfy its aerobic metabolic demand [57]. This is clearly an advantage in oligotrophic environments.

Acclimation timing also matters. An organism might easily cope with an individual short-term heat shock but be compromised if the same shock is applied repeatedly or continuously. On the other hand, repeated sub-lethal heat shocks can lead to a form of acclimation in which the body's tolerance becomes more robust [58]. Thus, we cannot exclude the possibility that an early increase in the respirometric metabolism of *D. belgicus* might occur few hours after the acclimation and be recovered after some days.

The Q_{10} of *D. belgicus* between 14 and 17 °C was 0.39 considering the mean value of oxygen consumption. Q_{10} values between 21 and 28 °C were also smaller than 1 for the amphipod *Gammarus fossarum* Koch, 1836 (Q_{10} = 0.66), *N. rhenorhodanensis* (Q_{10} = 0.68) and *N. virei* (Q_{10} = 0.36) [20]. $Q_{10} < 1$ indicates that a temperature change of 10 °C respect to the thermal optimum is likely to damage the metabolic system of these species leading to what may be an irreversible loss of functions [33]. It also suggests a low capacity to maintain optimal enzymatic activities and a limited survival as soon as the environmental temperature goes out of the optimal thermal range of the species. An irreversible denaturation of enzymes/proteins seems to begin for *D. belgicus* between 14 and 17 °C according to the Q_{10} = 0.39. However, the Q_{10} metric is computed with the mean of the SRR values whose partition between 14 and 17 °C did not differ significantly. In addition, when computed with the median SRR values, the Q_{10} is 0.99 for *D. belgicus* and a Q_{10} of approximately 1, supports optimal physiological performance and thermal tolerance. The wide geographical range of this species appears to support the thermal tolerance hypothesis [59], which likely favors dispersal, especially via the porous medium. However, further experiments at temperatures above and below those tested in this study are required to assess the optimal thermal range of this species.

5. Conclusions

The results of this study derive from an experimental increase of temperature that is consistent with the foreseen increase in the next 30 years due to global warming. The populations of *D. belgicus* of the Medio Valdarno porous aquifer did not show significant variations in the oxygen consumptions under a temperature change of 3 °C. This result is in agreement with the wide geographical range of occurrence of this species. The result is supported by a thermal coefficient Q_{10} ~1, computed on the median values of the oxygen consumptions, that suggests a thermal tolerance of this species. Conversely, the value of the thermal coefficient Q_{10} was far below 1 when the mean values of the oxygen consumptions were considered. This value indicates the probable beginning of an irreversible denaturation of enzymes/proteins at a temperature increase of 10 °C from the thermal optimum. These controversial results do not provide full certainty about the fate of this species under a global warming scenario. Hence, *D. belgicus* should be prudently considered at risk. Further experiments at temperatures above and below those tested in this study are required to assess the optimal thermal range of *D. belgicus*. In addition, new studies are required with groundwater species exhibiting narrow

distributions. The physiological capacity to respond to temperature changes is expected to be very low in narrowly distributed species, thus facing a higher risk of extinction due to global warming.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4441/9/12/951/s1.

Acknowledgments: The project, including the costs to publish in open access, was funded by the European Community (LIFE12 BIO/IT/000231 AQUALIFE). We thank Giuseppe Messana (ISE-CNR) and Sigurd Einum (NTNU) for the tips for the use of the respirometric device, Claudio Sili (ISE-CNR) for the picture of *D. belgicus* and Stefano Amalfitano (IRSA-CNR) for the flow cytometry. We would like to thank three anonymous reviewers who provided comments to improve the manuscript.

Author Contributions: Both authors conceived and designed the experiments; Tiziana Di Lorenzo performed the experiments; both authors analyzed the data and wrote the paper.

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

References

1. Gamvroudis, C.; Dokou, Z.; Nikolaidis, N.P.; Karatzas, G.P. Impacts of surface and groundwater variability response to future climate change scenarios in a large Mediterranean watershed. *Environ. Earth Sci.* **2017**, *76*. [CrossRef]
2. Dettinger, M.D.; Earman, S. Western Ground Water and Climate Change—Pivotal to Supply Sustainability or Vulnerable in Its Own Right? *Ground Water* **2007**, *4*. Available online: http://tenaya.ucsd.edu/~dettinge/agwse07.pdf (accessed on 23 October 2017).
3. Taylor, R.G.; Scanlon, B.; Döll, P.; Rodell, M.; van Beek, R.; Wada, Y.; Longuevergne, L.; Leblanc, M.; Famiglietti, J.S.; Edmunds, M.; et al. Ground water and climate change. *Nat. Clim. Chang.* **2007**, *3*, 322–329. [CrossRef]
4. Intergovernmental Panel on Climate Change (IPCC). The physical science basis. In *Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*; Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M., Miller, H.L., Eds.; Cambridge University Press: Cambridge, UK; New York, NY, USA, 2007.
5. Kundzewicz, Z.W.; Mata, L.J.; Arnell, N.W.; Doll, P.; Kabat, P.; Jimenez, B.; Miller, K.A.; Oki, T.; Sen, Z.; Shiklomanov, I.A. Freshwater resources and their management. In *Climate Change 2007: Impacts, Adaptation and Vulnerability*; Parry, M.L., Canziani, O.F., Palutikof, J.P., van der Linden, P.J., Hanson, C.E., Eds.; Cambridge University Press: Cambridge, UK; New York, NY, USA, 2007; pp. 173–210.
6. Green, T.R.; Taniguchi, M.; Kooi, H.; Gurdakd, J.J.; Allen, D.M.; Hiscock, K.M.; Treidel, H.; Aurelig, A. Beneath the surface of global change: Impacts of climate change on groundwater. *J. Hydrol.* **2011**, *405*, 532–560. [CrossRef]
7. Intergovernmental Panel on Climate Change (IPCC). Climate Change (IPCC). Climate Change 2013: The physical science basis. In *Contribution of Working Group I to the fifth Assessment Report of the Intergovernmental Panel On Climate Change*; Stocker, T.F., Qin, D., Plattner, G.-K., Tignor, M.M.B., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M., Eds.; Cambridge University Press: Cambridge, UK; New York, NY, USA, 2014. [CrossRef]
8. Menberg, K.; Blum, P.; Kurylyk, B.L.; Bayer, P. Observed groundwater temperature response to recent climate change. *Hydrol. Earth Syst. Sci.* **2014**, *18*, 4453–4466. [CrossRef]
9. Figura, S.; Livingstone, D.M.; Hoehn, E.; Kipfer, R. Regime shift in groundwater temperature triggered by the Arctic Oscillation. *Geophys. Res. Lett.* **2011**, *38*, L23401. [CrossRef]
10. Taylor, A.C.; Stefan, H.G. Shallow groundwater temperature response to climate change and urbanization. *J. Hydrol.* **2009**, *375*, 601–612. [CrossRef]
11. Freeze, R.A.; Cherry, J.A. *Groundwater*; Prentice-Hall, Inc.: Englewood Cliffs, NJ, USA, 1979; p. 604.
12. Galassi, D.M.P.; Stoch, F.; Fiasca, B.; Di Lorenzo, T.; Gattone, E. Groundwater biodiversity patterns in the Lessinian Massif of northern Italy. *Freshwat. Biol.* **2009**, *54*, 830–847. [CrossRef]
13. Di Lorenzo, T.; Brilli, M.; Del Tosto, D.; Galassi, D.M.P.; Petitta, M. Nitrate source and fate at the catchment scale of the Vibrata River and aquifer (central Italy): An analysis by integrating component approaches and nitrogen isotopes. *Environ. Earth Sci.* **2012**. [CrossRef]

14. Di Lorenzo, T.; Cifoni, M.; Lombardo, P.; Fiasca, B.; Galassi, D.M.P. Ammonium threshold value for groundwater quality in the EU may not protect groundwater fauna: Evidence from an alluvial aquifer in Italy. *Hydrobiologia* **2015**, *743*, 139–150. [CrossRef]
15. Di Lorenzo, T.; Galassi, D.M.P. Agricultural impact on Mediterranean alluvial aquifers: Do groundwater communities respond? *Fundam. Appl. Limnol.* **2013**, *182*, 271–282. [CrossRef]
16. Eckert, R.; Randall, D.; Burggren, W.; French, K. *Animal Physiology: Mechanisms and Adaptations*; Freeman and Company: New York, NY, USA, 1979; p. 120, ISBN 10:0716738635.
17. Peck, L.S.; Webb, K.E.; Bailey, D.M. Extreme sensitivity of biological function to temperature in Antarctic marine species. *Funct. Ecol.* **2004**, *18*, 625–630. [CrossRef]
18. Huey, R.B.; Kingsolver, J.G. Evolution of thermal sensitivity of ectotherm performance. *Trends Ecol. Evol.* **1989**, *4*, 131–135. [CrossRef]
19. Issartel, J.; Renault, D.; Voituron, Y.; Bouchereau, A.; Vernon, P.; Hervant, F. Metabolic responses to cold in subterranean crustaceans. *J. Exp. Biol.* **2005**, *208*, 2923–2929. [CrossRef] [PubMed]
20. Issartel, J.; Hervant, F.; Voituron, Y.; Renault, D.; Vernon, P. Behavioural, ventilatory and respiratory responses of epigean and hypogean crustaceans to different temperatures. *Comp. Biochem. Physiol.* **2005**, *141*, 1–7. [CrossRef] [PubMed]
21. Colson-Proch, C.; Renault, D.; Gravot, A.; Douady, C.J.; Hervant, F. Do current environmental conditions explain physiological and metabolic responses of subterranean crustaceans to cold? *J. Exp. Biol.* **2009**, *212*, 1859–1868. [CrossRef] [PubMed]
22. Colson-Proch, C.; Morales, A.; Hervant, F.; Konecny, L.; Moulin, C.; Douady, C.J. First cellular approach of the effects of global warming on groundwater organisms: A study of the HSP70 gene expression. *Cell Stress Chaperon.* **2010**, *15*, 259–270. [CrossRef] [PubMed]
23. Mermillod-Blondin, F.; Lefour, C.; Lalouette, L.; Renault, D.; Malard, F.; Simon, L.; Douady, C.J. Thermal tolerance breadths among groundwater crustaceans living in a thermally constant environment. *J. Exp. Biol.* **2013**, *216*, 1683–1694. [CrossRef] [PubMed]
24. Pesce, L. The genus *Diacyclops* Kiefer in Italy: A taxonomic, ecological and biogeographical up-to-date review (Crustacea Copepoda Cyclopidae). *Arthropoda Sel.* **1994**, *3*, 13–19.
25. Cifoni, M.; Galassi, D.M.P.; Faraloni, C.; Di Lorenzo, T. Test procedures for measuring the (sub)chronic effects of chemicals on the freshwater cyclopoid *Eucyclops serrulatus*. *Chemosphere* **2017**, *173*, 89–98. [CrossRef] [PubMed]
26. Di Lorenzo, T.; Di Marzio, W.D.; Spigoli, D.; Baratti, M.; Messana, G.; Cannicci, S.; Galassi, D.M.P. Metabolic rates of a hypogean and an epigean species of copepod in an alluvial aquifer. *Freshw. Biol.* **2015**, *60*, 426–435. [CrossRef]
27. Di Lorenzo, T.; Cannicci, S.; Spigoli, D.; Cifoni, M.; Baratti, M.; Galassi, D.M.P. Bioenergetic cost of living in polluted freshwater bodies: Respiration rates of the cyclopoid *Eucyclops serrulatus* under ammonia-N exposures. *Fundam. Appl. Limnol.* **2016**, *188*, 147–156. [CrossRef]
28. Szela, T.L.; Marsh, A.G. Microtiter plate, optode respirometry, and inter-individual variance in metabolic rates among nauplii of *Artemia* sp. *Mar. Ecol. Prog. Ser.* **2005**, *296*, 281–289. [CrossRef]
29. Yashchenko, V.; Fossen, E.I.; Kielland, Ø.N.; Einum, S. Negative relationships between population density and metabolic rates are not general. *J. Anim. Ecol.* **2016**, *85*, 1070–1077. [CrossRef] [PubMed]
30. Svetlichny, L.S.; Khanaychenko, A.; Hubareva, E.; Aganesova, L. Partitioning of respiratory energy and environmental tolerance in the copepods *Calanipeda aquaedulcis* and *Arctodiaptomus salinus*. *Estuar. Coast. Shelf. Sci.* **2012**, *114*, 199–207. [CrossRef]
31. McKinnon, A.D.; Duggan, S. Summer copepod production in subtropical waters adjacent to Australia's North West Cape. *Mar. Biol.* **2003**, *143*, 897–907. [CrossRef]
32. Reiss, J.; Schmid-Araya, J.M. Feeding response of a benthic copepod to ciliate prey type, prey concentration and habitat complexity. *Freshw. Biol.* **2011**, *56*, 1519–1530. [CrossRef]
33. Hochachka, P.; Somero, G. *Biochemical Adaptation, Mechanism and Physiological Evolution*; Oxford University Press: New York, NY, USA, 2002; p. 480, ISBN 9780195117035.
34. Anderson, M.J. A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* **2001**, *26*, 32–46. [CrossRef]
35. Anderson, M.J.; Gorley, R.N.; Clarke, K.R. *PERMANOVA + for PRIMER: Guide to Software and Statistical Methods*; PRIMER-E Ltd.: Plymouth, UK, 2008.

36. Galassi, D.M.P.; Lombardo, P.; Fiasca, B.; Di Cioccio, A.; Di Lorenzo, T.; Petitta, M.; Di Carlo, P. Earthquakes trigger the loss of groundwater biodiversity. *Sci. Rep.* **2014**, *4*. [CrossRef] [PubMed]
37. Galassi, D.M.P.; Fiasca, B.; Di Lorenzo, T.; Montanari, A.; Porfirio, S.; Fattorini, S. Groundwater biodiversity in a chemoautotrophic cave ecosystem: How geochemistry regulates microcrustacean community structure. *Aquat. Ecol.* **2017**, *51*, 75–90. [CrossRef]
38. Di Lorenzo, T.; Stoch, F.; Galassi, D.M.P. Incorporating the hyporheic zone within the river discontinuum: Longitudinal patterns of subsurface copepod assemblages in an Alpine stream. *Limnologica* **2013**, *43*, 288–296. [CrossRef]
39. Stoch, F.; Barbara, F.; Di Lorenzo, T.; Porfirio, S.; Petitta, M.; Galassi, D.M.P. Exploring copepod distribution patterns at three nested spatial scales in a spring system: Habitat partitioning and potential for hydrological bioindication. *J. Limnol.* **2016**, *75*, 1–13. [CrossRef]
40. Iepure, S.; Rasines-Ladero, R.; Meffe, R.; Carreno, F.; Mostaza, D.; Sundberg, A.; Di Lorenzo, T.; Barroso, J.L. The role of groundwater crustaceans in disentangling aquifer type features—A case study of the Upper Tagus Basin, central Spain. *Ecohydrology* **2017**, *10*, e1876. [CrossRef]
41. Hervant, F.; Mathieu, J.; Messana, G. Locomotory, ventilatory and metabolic responses of the subterranean *Stenasellus virei* (Crustacea, Isopoda) to severe hypoxia and subsequent recovery. *C. R. Acad. Sci.* **1997**, *320*, 139–148. [CrossRef]
42. Hervant, F.; Renault, D. Long-term fasting and realimentation in hypogean and epigean isopods: A proposed adaptive strategy for groundwater organisms. *J. Exp. Biol.* **2002**, *205*, 2079–2087. [PubMed]
43. Simčič, T.; Lukančič, S.; Brancelj, A. Comparative study of electron transport system activity and oxygen consumption of amphipods from caves and surface habitats. *Freshw. Biol.* **2005**, *50*, 494–501. [CrossRef]
44. Simčič, T.; Pajk, F.; Brancelj, A. Electron transport system activity and oxygen consumption of two amphibious isopods, epigean *Ligia italic* Fabricius and hypogean *Titanethes albus* (Koch), in air and water. *Mar. Freshw. Behav. Physiol.* **2010**, *43*, 149–156. [CrossRef]
45. Di Lorenzo, T.; Di Marzio, W.D.; Sáenz, M.E.; Baratti, M.; Dedonno, A.A.; Iannucci, A.; Cannicci, S.; Messana, G.; Galassi, D.M.P. Sensitivity of hypogean and epigean freshwater copepods to agricultural pollutants. *Environ. Sci. Pollut. Res.* **2014**, *21*, 4643–4655. [CrossRef] [PubMed]
46. Di Lorenzo, T.; Di Marzio, W.D.; Cifoni, M.; Fiasca, B.; Baratti, M.; Sáenz, M.E.; Galassi, D.M.P. Temperature effect on the sensitivity of the copepod *Eucyclops serrulatus* (Crustacea, Copepoda, Cyclopoida) to agricultural pollutants in the hyporheic zone. *Curr. Zool.* **2015**, *61*, 629–640. [CrossRef]
47. Angilletta, M.J.; Niewiarowski, P.H.; Navas, C.A. The evolution of thermal physiology in ectotherms. *J. Therm. Biol.* **2002**, *27*, 249–268. [CrossRef]
48. Holmstrup, M.; Costanzo, J.P.; Lee, R.E., Jr. Cryoprotective and osmotic responses to cold acclimation and freezing in freeze-tolerant and freeze-intolerant earthworms. *J. Comp. Physiol.* **1999**, *169*, 207–214. [CrossRef]
49. Bale, J.S.; Block, W.; Worland, M.R. Thermal tolerance and acclimation response of larvae of the sub-Antarctic beetle *Hydromedion sparsutum* (Coleoptera: Perimylopidae). *Polar Biol.* **2000**, *23*, 77–84. [CrossRef]
50. Renault, D.; Vernon, P.; Nedved, O.; Hervant, F. The importance of fluctuating thermal regimes for repairing chill injuries in the tropical beetle *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) during exposure to low temperature. *Physiol. Entomol.* **2004**, *29*, 139–145. [CrossRef]
51. Simčič, T.; Brancelj, A. Electron transport system (ETS) activity in five *Daphnia* species at different temperatures. *Hydrobiologia* **1997**, *360*, 117–125. [CrossRef]
52. Simčič, T.; Pajk, F.; Vrezec, A.; Brancelj, A. Size scaling of whole-body metabolic activity in the noble crayfish (*Astacus astacus*) estimated from measurements on a single leg. *Freshw. Biol.* **2012**, *57*, 39–48. [CrossRef]
53. Tanaka, K.; Udagawa, T. Cold adaptation of the terrestrial isopod, *Porcellio scaber*, to subnivean environments. *J. Comp. Physiol.* **1993**, *163*, 439–444.
54. Mulkiewicz, E.; Zietara, M.S.; Stachowiak, K.; Skorkowski, E.F. Properties of lactate dehydrogenase from the isopod, *Saduria entomon*. *Comp. Biochem. Physiol.* **2000**, *126*, 337–346. [CrossRef]
55. Paul, R.J.; Lamkemeyer, T.; Maurer, J.; Pinkhaus, O.; Pirow, R.; Seidl, M.; Zeis, B. Thermal acclimation in the microcrustacean *Daphnia*: A survey of behavioural, physiological and biochemical mechanisms. *J. Therm. Biol.* **2004**, *29*, 655–662. [CrossRef]
56. Gutierrez, F.M.; Gagneten, A.M.; Paggi, J.C. Copper and chromium alter life cycle variables and the equiproportional development of the freshwater copepod *Notodiaptomus conifer* (SARS). *Water Air Soil Pollut.* **2010**, *213*, 275–286. [CrossRef]

57. Pörtner, H.-O. Climate variations and the physiological basis of temperature dependent biogeography: Systemic to molecular hierarchy of thermal tolerance in animals. *Comp. Biochem. Physiol.* **2002**, *132*, 739–761. [CrossRef]
58. Dowd, W.W.; King, F.A.; Denny, M.W. Thermal variation, thermal extremes and the physiological performance of individuals. *J. Exp. Biol.* **2015**, *218*, 1956–1967. [CrossRef] [PubMed]
59. Eme, D.; Malard, F.; Colson-Proch, C.; Jean, P.; Calvignac, S.; Konecny-Dupr, L.; Hervant, F.; Douady, C.J. Integrating phylogeography, physiology and habitat modelling to explore species range determinants. *J. Biogeogr.* **2014**, *41*, 687–699. [CrossRef]