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Oxygen consumption rates and respiratory carbon losses in three species of copepods (*Acartia clausi*, *Calanus helgolandicus* and *Limnocalanus macrurus*) during starvation

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Abstract: Calanoid copepods with different life cycle strategies demonstrate varied velocity in the utilization of energy resources during starvation. This study analyzes the influence of multiday starvation on the rate of total, basal and active energy metabolism in adult females of two Marmara Sea and one Baltic Sea species of copepods; epiplanktonic *Acartia clausi* unable to accumulate lipid reserves and mesoplanktonic *Calanus helgolandicus* with minor lipid reserves in the oil sac (approximately 7% of body volume) and the Baltic Sea copepod *Limnocalanus macrurus* in quasi-diapausing state with large lipid reserves in the oil sac (approximately 19% of body volume). Initially, total weight specific respiration rates were about 0.12 $\mu\text{g C}$ per $\mu\text{g C}$ body weight per day (d^{-1}) for both Marmara Sea species at 20 °C and 0.079 d^{-1} in the Baltic *L. macrurus* at 14 °C, decreasing significantly by 1.6–2.5 times with starvation, while basal metabolic rates, measured in the same anesthetized individuals, was nearly constant during starvation. In this regard, the active metabolic rates, defined as the difference between the respiration rate of active and anesthetized individuals, decreased during starvation by 1.7 times in *C. helgolandicus* and by 4.7 and 6 times in *A. clausi* and *L. macrurus*, respectively. In *A. clausi* and *C. helgolandicus* with minimal lipid reserves, total daily energy expenditure (5.1% of initial body carbon content) was twice as high as in *L. macrurus* (2.2% of initial body carbon content daily), which has a large oil sac. Overall, the trend demonstrates that despite different energy reserves, a decrease in the total energy metabolism in copepods occurs due to a reduction in energy expenditure for locomotor activity.

Key words: Calanoid copepods, starvation, metabolic rates

1. Introduction

Many marine holoplanktonic copepods are forced to adapt to periods of food deprivation due to the extreme temporal and spatial heterogeneity of phytoplankton distribution and seasonal cycles of composition and abundance (Mullin and Brooks, 1976; Dag and Grill, 1980).

The starvation response manifests itself as a set of adaptive behavioral and physiological changes that reduce the need for energy in the absence of food. With short-term fasting, not associated with significant loss of body weight, both lack of response to food deprivation and variations in the change of the respiration rate of copepods may appear (Conover and Corner 1968; Ikeda 1971; Le

Borgne 1979), however, many researchers have observed a reduction in oxygen consumption rates (Skjoldal et al., 1984; Abau Debs 1984; Kiørboe et al., 1985; Lehette et al., 2016). One of the important reasons for the reduction in energy consumption at the onset of starvation is associated with the cessation of search, capture, and digestion of food. Oxygen consumption due to the digestion of food is termed “specific dynamic action of the food” (Kiørboe et al., 1985; Hiromi 1994; Thor 2000; 2002; 2003).

During extensive starvation periods, a decrease in the rate of oxygen consumption of copepods (Tsuda, 1994) correlates with total loss of body weight and especially, loss of reserve lipids (Båmstedt and Holt, 1978). Many

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epiplanktonic copepods are able to withstand starvation for many days, consuming stored lipids and body proteins (Helland et al., 2003; Koussoroplis et al., 2014). Copepod species of cold and temperate latitudes are capable of experiencing a prolonged seasonal lack of food in a state of diapause (Baumgartner and Tarrant, 2017). They primarily accumulate large amounts of reserve lipids (mainly in the form of high-energy wax esters) for their subsequent long-term consumption under conditions of low temperature and oxygen concentration, that leads to reduction in locomotion and acts to offset the decrease in respiration rate (Vinogradov et al., 1992; Svetlichny et al., 2000, 2017; Maps et al., 2014). Starvation tolerance can be attributed to foraging strategies, i.e. the type and efficiency of copepod feeding (Lee et al., 2006; Holm et al., 2019).

Many of the above field and experimental observations characterize trends in the rate of total energy metabolism during starvation, but little is known about adaptive changes in its structural components.

The total respiration rate of actively feeding copepod individuals under normal conditions (R_{TOT}) is an integral indicator of energy metabolism, which includes three main components: i) expenditure on locomotion and manipulative activity of the limbs (R_{ACT}), ii) basal energy, so called “basal metabolism”, (Ikeda et al., 2000) required to maintain muscle and other body tissues (R_{BAS}), and iii) energy costs for food digestion also called “Specific dynamic action of food” (R_{SDA}) (Kiørboe et al., 1985).

Observations on changes in the R_{TOT} of copepods in the presence or absence of food have led to the conclusion that R_{SDA} is the main component in energy metabolism (Kiørboe et al., 1985; Hiromi 1994; Thor 2000; 2002; 2003). On the other hand, a number of experimental studies in which simultaneous monitoring of motor activity and respiration rates of both feeding and nonfeeding individuals were performed, shows that predominantly, energy is spent on muscle activity of copepods during swimming and grazing (Buskey 1998; Svetlichny and Hubareva, 2005). There is a scarcity of data on R_{BAS} (Buskey 1998; Minkina and Pavlova 1990; Svetlichny et al., 2021) and even less is known about the ratio and possible adaptive redistribution of energy metabolism components during the starvation of copepods.

In previous studies aimed at examining the effect of microplastics on the energy metabolism of copepods, the multiday observations of respiration rates of the Marmara Sea mesoplanktonic *Calanus helgolandicus* adult females (Isinibilir et al., 2020) as well as epipelagic *Acartia clausi* (Svetlichny et al., 2021) were performed. Since part of these experiments was carried out on animals completely deprived of food or sediment in water, in this publication these data are analyzed with respect to the starvation of copepods, presenting the amount of oxygen consumed in comparable units of carbon catabolized by a

unit of body mass. In addition, within the framework of a similar project carried out by the Finnish Environment Institute in Helsinki (SYKE), studying the effects of microplastics on the planktonic community of the Baltic Sea, the 11-day observations on respiration rates in sexually mature females of mesoplanktonic *Limnocalanus macrurus* were carried out. Adults of this species, collected at the end of summer, were probably in the pre-diapause state typical for this time of the year (Mäkinen et al., 2017) which is confirmed by the fact that during the entire 11-day experiment did not consume any of the components of plant or animal food available in the mesocosm. Therefore, these data were interpreted in the aspect of starvation, thus adding a new variant (copepods exhibiting natural refusal to eat) to data regarding long-term artificial starvation of copepods. Based on the dynamics of the respiration rates of active and anesthetized individuals, converted into units of respiratory carbon, the rate of loss of body carbon for total and basal energy metabolism of copepods in terms of starvation strategies of short and long life cycle species with low and high lipid reserves was analyzed.

2. Materials and methods

2.1. Copepod sampling and culture

Starvation experiments with adult *Acartia clausi* and *Calanus helgolandicus* females collected from the northwestern Marmara Sea near the Bosphorus (40°51'71N – 28°57'90E), were carried out in April of 2018 and 2019, while the experiments with *Limnocalanus macrurus* collected from the central region of the Gulf of Finland, Baltic Sea (59°39'03N–24°07'01E) were conducted from 27th August to 6th September 2019.

2.2. Microalgae culture

The Marmara Sea copepods were preadapted for 24 h in filtered seawater (salinity 20) at a temperature of 17 °C, containing *ad libitum* microalgae *Rhodomonas salina*. The original strain of the cryptomonad algae *Rhodomonas salina* (5–10 µm size range) was obtained from the Culture Collection of Algae and Protozoa (CCAP), Scotland, UK. Initial microalgae cultures were started in test tubes (30 mL) containing f / 2 + Si medium previously sterilized at 121 °C for 15 min. All subcultures were maintained at 23 °C at a salinity of 32 psu under a 12 h light – 12 h dark photoperiod in laboratory conditions. The *R. salina* culture volumes were upscaled from 30 mL test tubes to 250 mL Erlenmeyer flasks, followed by 1 L, 3 L, and 30 L culture containers in photobioreactors, continuously. Illumination was maintained at 200 µmol/m²/s at the culture surface. Population growth was determined daily by cell counting using Neubauer chambers under Leica DM 1000 microscope.

2.3. Experimental design and respiration rate

After preadaptation of the Marmara species with food *ad libitum* during one day, copepods were placed in 1-L jars

with filtered (mesh size 0.2 μm) seawater free of food or sediments, with a maximum of 20 specimens per vessel for an exposure period of 4 days for *Acartia clausi* and 7 days for *Calanus helgolandicus* at 17 °C. During exposure, the filtered seawater exchange ratio was adjusted 100% daily. The photoperiod was 16L:8D during day. Transition of light and dark period was slowly adjusted.

Females of *Limnocalanus macrurus*, after capture in the Baltic Sea and preliminary acclimation in 80 L buckets (in situ water), were placed in 12 mesocosms (300 L tanks) with natural unfiltered brackish water (salinity 5), containing microalgae and zooplankton at typical concentrations. The water temperature in the tanks was maintained at 13 °C during all 11 days of exposure period. The photoperiod mimicked natural diurnal rhythms.

Respiration rates in both Marmara Sea species were measured at 20 °C: in *Acartia clausi* on one day before and 2, 4 days of starvation, while in *Calanus helgolandicus* on one day before and 1, 3, 5, and 7 days of starvation. Respiration rates of Baltic Sea *Limnocalanus macrurus* were measured at 14 °C on 1, 3, 5, 7, 9 and 11 days of starvation. For measuring of copepods oxygen consumption the closed, sealed chamber method (Ikeda et al., 2000) was used with glass experimental and control syringes of 2.0 mL as respirometers filled with filtered seawater to 1 mL. On each of the indicated days of the experiment, 10 active individuals of *A. clausi*, 3 individuals of *L. macrurus* and one individual of *C. helgolandicus* were selected from the vessels in which copepods were kept and gently transferred by pipette to each of the syringes in 8–12 replicates. Incubation periods averaged 2 h and 3 h for active and anesthetized females, respectively. As shown earlier, crowding up to concentrations of 10 ind ml^{-1} does not affect the behavior and respiration rate of *Acartia* (Nilsson et al., 2017).

Oxygen concentration in the syringes were determined using a luminescent dissolved oxygen sensor Hach LDO™, combined with a small 0.3 mL flow chamber, into which water was injected from each of the syringes closed during exposure in several consecutive 0.2 mL portions to remove contaminants from the flow chamber. Oxygen concentrations were measured in the last portion of water. A similar method was used by earlier when measuring the respiratory rate of many copepods (Svetlichny and Hubareva 2005; Svetlichny et al., 2012, 2017, 2018; Isinibilir et al., 2020; Svetlichny et al., 2021). The decrease in oxygen concentration in syringes with copepods during incubation did not exceed 70% of its initial value.

The amount of oxygen associated with motor activity was determined by the difference in respiration rates between active and anesthetized individuals (Minkina and Pavlova, 1990; Svetlichny et al., 2000, 2012; 2018; 2021; Svetlichny and Hubareva, 2005). The methods of anesthesia

differed for the Marmara and Baltic species due to the variation in their ambient water salinity. For anesthesia of the Marmara copepods water in the syringes containing active individuals at the end of treatment exposure was initially replaced by a solution of magnesium chloride of isoosmotic concentration 15 g L^{-1} (Svetlichny et al., 2020), and following immobilization of copepods, the solution was replaced with oxygen-saturated seawater containing half the initial concentration of magnesium chloride and distributed equally between test and control syringes in order to obtain identical initial oxygen, chemical and seston content. This was achieved by pumping water between syringes through a connecting tube. The animals in the syringes were kept in good condition due to the wide 100- μm sieve located in the upper part of the syringe.

Since the use of magnesium chloride in concentration of 15 g L^{-1} for anesthesia of *Limnocalanus macrurus*, which lives in the Baltic Sea with a practical salinity of about 5, was impossible, the copepods were immobilized with 0.05% MS-222 solution (Svetlichny et al., 2016) adopting the same procedure as for magnesium chloride. Results of anesthesia experiments were analyzed only for the copepods that remained inactive during the incubation but recovered their activity after being placed in clean water. Individuals that underwent anesthesia were not used in further experiments.

The amounts of oxygen consumed by active (R_{tot}) and anesthetized (R_{bas}) individuals were determined by the differences in oxygen concentrations of test and control syringes expressed as $\mu\text{g O}_2 \text{ ind}^{-1} \text{ h}^{-1}$. These values of individual oxygen consumption rates were converted to carbon units for the estimation of carbon requirements of the copepods assuming that the consumption of 1 mL oxygen mobilizes 0.38 mg of organic carbon by using a respiratory quotient (RQ) of 0.7 for catabolism of lipids in the case of *L. macrurus*, which has large reserves of lipids in the oil sac (Figure 1). For Marmara Sea adult female copepods which lack an oil sac i.e. *A. clausi*, or possess a small oil sac, i.e. *C. helgolandicus* (Svetlichny et al., 2006), we used a conversion factor of 0.44, corresponding to a respiratory quotient (RQ) of 0.82 for mixed substrate catabolism (Auel and Werner, 2003).

To compare the starvation energy budget of the studied copepods with different body weights, we converted the obtained values of oxygen consumption rates into respired carbon and attributed them to the carbon weight of metabolically active body tissues ($CRCW_{tot}$ and $CRCW_{bas}$ for active and anesthetized individuals, respectively), taking the respiratory quotient (RQ) of 0.82 at mixed composition of metabolic substrate for *Acartia clausi* and *Calanus helgolandicus*, and RQ of 0.7 for *Limnocalanus macrurus* catabolized lipid reserves (Omori and Ikeda, 1984).

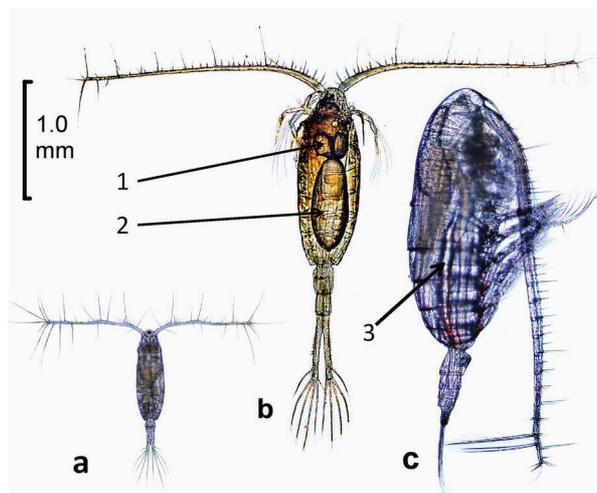


Figure 1. *Acartia clausi* (a), *Limnocalanus macrurus* (b) and *Calanus helgolandicus* (c). Arrows indicate anterior (1) and posterior (2) oil sacs of *L. macrurus* and oil sac of *C. helgolandicus* (3).

2.4. Body volume and weight of copepods

After experiments, the total length (L_{tot}), and length and width of the prosoma (l_{pr} and d_{pr} , respectively) in mm were determined in anesthetized copepods, from which the body volume V_b (mm^3) was calculated in accordance to the empirical formula $V_b = 0.47 \times L_{tot}^{0.2} \times l_{pr}^{0.93} \times d_{pr}^{1.86}$ (Svetlichny et al., 2012). In *L. macrurus*, the length and width of the posterior oil sac and diameters of anterior sacs were also determined, by which their volume (V_{os}) was determined using the formulas of an ellipsoid and a sphere, respectively (Figure 1).

Body wet weights (WW , mg) were determined according to the equation $WW = V_b \times \rho_b$, where $\rho_b \approx 1.05 \text{ mg mm}^{-3}$ (Mauchline, 1998).

2.5. Carbon body content

Total carbon body weight (CW_{tot} , μg) of copepods (Table 1) was calculated as $CW_{tot} = (1000 \times WW \times 0.2) \times 0.45$, taking into account that for most calanoid copepods, which do not have large fat reserves, average dry weight (DW , mg) is equal to 20% of WW and CW_{tot} averages 45% of DW (Mauchline, 1998).

Despite such an artificial calculation method, CW_{tot} in *Acartia clausi* ($6.02 \mu\text{g C}$) (see Table 1) was calculated as similar to the body carbon weight of *A. clausi* females obtained by other authors as a result of direct measurements (Durbin and Durbin, 1978; Kenitz et al., 2017) and generalized dependences of CW_{body} on copepod body length (Martynova et al., 2009).

Lipid stores in the body of *Acartia clausi* are very low, about 3% dry weight, however *Calanus helgolandicus* and *Limnocalanus macrurus* differ in their ability to accumulate large amounts of lipids (in the form of wax) in oil sac (see

review by Lee et al., 2006). Because waxes are the least metabolically active tissue in the body, we determined the lipid-free carbon content of copepods (CW_{body}^{bodyf}) to correlate respiration rates with metabolically active body mass.

In *Calanus helgolandicus* ($V_b = 0.953 \pm 0.11 \text{ mm}^3$), collected for the experiments in April 2019 near the Bosphorus Strait, the volume of lipids in the oil sac was $0.065 \pm 0.046 \text{ mm}^3$, in accordance with its average lipid content in females of this species of the same size studied in the area of the Bosphorus in April 2003 (Svetlichny et al., 2009). Thus, in *C. helgolandicus* CW_{body}^{bodyf} was estimated to be $80 \mu\text{g ind}^{-1}$, which is also close to the measured CW_{body}^{bodyf} values known for *C. helgolandicus* according to literature data. For example, the highest carbon weights of about $90 \mu\text{g ind}^{-1}$ in *C. helgolandicus* females from the Celtic Sea in April–March was found in specimens with a prosome length of 2.8 mm and a dry weight of up to $200 \mu\text{g}$ (Bottrell and Robins, 1984).

In *L. macrurus* CW_{body}^{bodyf} was calculated as $CW_{body}^{bodyf} = (V_b / 140.0) \times 0.45$, taking into account that the concentration of nonlipid body substance, expressed as lipid-free dry mass per unit body volume in *L. macrurus* females in the August period equaled to ca $140 \mu\text{g } \mu\text{L}^{-1}$ (Vanderploeg et al., 1998). The CW_{tot} was defined as the sum of CW_{body}^{bodyf} and CW_{sac} , where $CW_{sac} = V_{os} \times \rho_{sac} \times k$, where ρ_{sac} is the density of wax esters equal to $0.86 \mu\text{g mm}^{-3}$ (Lewis, 1970), and k is wax ester weight / carbon content coefficient ≈ 1 . The total volume of the anterior and posterior oil sacs V_{os} and V_b at the beginning of starvation, in *L. macrurus* with body length of 1.86 ± 0.04 equal to $0.083 \pm 0.026 \text{ mm}^3$ and, respectively 0.428 ± 0.037 (Table 1), when the oil sac

Table 1. Summary of the characteristics of the Marmara Sea copepods *Acartia clausi* and *Calanus helgolandicus* at 20 °C and the Baltic Sea copepod *Limnocalanus macrurus* at 14 °C. Number of replications are shown in parentheses, L_{pr} is the average prosome length in mm; V_b is the average body volume in mm³; V_{os} is the average oil sac volume in mm³; CW_{tot} and CW_{bodysf} are total and oil sac free (respectively) body carbon weights, µg; R_{tot} and R_{bas} are total and basal (respectively) respiration rates, µg O₂ ind h⁻¹; $CRCW_{tot}$, $CRCW_{bas}$ and $CRCW_{active}$ are total, basal and active (respectively) respiratory carbon loss per unit of body carbon weight without carbon in oil sac, d⁻¹; CL_{tot} and CL_{bas} are total and basal (respectively) cumulative respiratory carbon loss for total starvation period, µgC, and DCL_{tot} and DCL_{bas} are total and basal (respectively) daily average weight specific body carbon losses during starvation, % of initial total carbon body weight. Mean ± standard deviation SD; low-case letters (a, b) denote significant variable differences among each group using Duncan's multiple range test, $p < 0.05$.

Characteristics	<i>Acartia clausi</i>		<i>Limnocalanus macrurus</i>		<i>Calanus helgolandicus</i>	
Day of starvation	0	4	1	11	0	7
L_{pr}	0.99 ± 0.034 (18)	1.02 ± 0.03 (19)	1.86 ± 0.04 ^a (39)	1.76 ± 0.08 ^b (33)	2.66 ± 0.08 (21)	2.68 ± 0.09 (23)
V_b	0.061 ± 0.007 (37)		0.428 ± 0.037 (39)	0.39 ± 0.041 (33)	0.953 ± 0.11 (44)	
V_{os}	-		0.083 ± 0.026 (11)	0.060 ± 0.018 (12)	0.065*	
CW_{tot}	6.0		98	-	115	
CW_{bodysf}	6.0	-	31	-	80	-
R_{tot}	0.094 ± 0.014 ^a (8)	0.037 ± 0.004 ^b (12)	0.363 ± 0.053 ^a (12)	0.189 ± 0.031 ^b (12)	1.15 ± 0.094 ^a (9)	0.70 ± 0.19 ^b (9)
R_{bas}	0.032 ± 0.006 ^a (4)	0.022 ± 0.003 ^b (5)	0.141 ± 0.0152 (12)	0.152 ± 0.036 (12)	0.497 ± 0.11 ^a (8)	0.249 ± 0.08 ^b (4)
R_{act}	0.052 ± 0.008 ^a (4)	0.011 ± 0.003 ^b (5)	0.223 ± 0.057 ^a (12)	0.038 ± 0.032 ^b (12)	0.652 ± 0.186 ^a (8)	0.391 ± 0.118 ^b (4)
$CRCW_{tot}$	0.123 ± 0.017 ^a	0.048 ± 0.006 ^b	0.079 ± 0.011 ^a	0.039 ± 0.008 ^b	0.114 ± 0.009 ^a	0.072 ± 0.014 ^b
$CRCW_{bas}$	0.041 ± 0.008 ^a	0.028 ± 0.004 ^b	0.031 ± 0.008	0.035 ± 0.007	0.069 ± 0.019 ^a	0.025 ± 0.008 ^b
$CRCW_{active}$	0.082 ± 0.012 ^a	0.017 ± 0.006 ^b	0.048 ± 0.012	0.004 ± 0.003	0.065 ± 0.005 ^a	0.045 ± 0.013 ^b
CL_{tot}		1.3**		22.3		42.0**
CL_{bas}		0.6**		12.0		15.2**
DCL_{tot}	5.1 ± 2.1**		2.2 ± 0.7		5.1 ± 1.1**	
DCL_{bas}	2.9 ± 0.3**		1.2 ± 0.1		2.0 ± 0.4**	

· Data from Svetlichny et al. (2009).

** Values are reduced from 20 to 17 °C using the temperature coefficient $Q_{10} = 2$ (Svetlichny et al., 2018).

free body volume was 0.345 mm³ and CW_{bodysf} was 31 µg C while CW_{tot} was 98 µg C. For comparison, in *L. macrurus* from Lake Michigan with the same prosoma length (1.73–1.80 mm) and body volume of 0.29–0.33 µL the maximum lipid concentration in the body (also noted at the end of August), was approximately 280 µg µL⁻¹, which corresponds to approximately 81–92 µg C ind⁻¹, relatively close to the concentration of lipids (67 µg C) accumulated in the sacs by *L. macrurus* females in our experiments.

2.6. Statistical analysis

All data were tested for normality with the Shapiro–Wilk test and homogeneity of variances by Levene's test, and treated using one-way ANOVA. Means were compared by the two-tailed Student's t-test ($p < 0.05$) and Duncan's multiple range test (DMRT) using SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA). Duncan's multiple range test (DMRT) is a posthoc test to measure differences between pairs of means (shown as a, b, c, etc., in relevant figures below). In this test, whilst same letters

denote no significant difference, different letters indicate significant differences among pairs of means. Values presented in the figures and tables are means ± standard deviations SD. All values presented as percentage were arc cosine transformed before performing any statistical test.

3. Results

3.1. Body size and volume

In the Marmara Sea copepods *Acartia clausi* and *Calanus helgolandicus* subjected to enforced starvation for 4 and 7 days, respectively, body length and volume (Table 1) did not have significant changes. On the contrary, in *Limnocalanus macrurus* during the 11 days of starvation, L_{pr} significantly decreased (by 5%) from 1.86 ± 0.049 to 1.75 ± 0.089 mm ($n = 48$, $p < 0.05$), however, the corresponding 9% decrease in the body volume from 0.43 ± 0.04 to 0.39 ± 0.04 mm³ was not significant. Almost 30% decrease in the total volume of the anterior and posterior oil sacs from 0.083 ± 0.026 to 0.060 ± 0.018 mm³ turned out to be non-significant. Only

the volume of the two anterior sacs significantly ($p < 0.05$) decreased (2.7 times) from 0.017 ± 0.012 to 0.0062 ± 0.006 mm^3 .

3.2. Respiration experiment

The results of respiration experiments indicate that R_{tot} ($\mu\text{g O}_2$ ind $^{-1}$ h $^{-1}$) in all studied species decreased evenly during the starvation (Figure 2).

For the Marmara Sea copepods *A. clausi* and *C. helgolandicus*, R_{tot} at the end of starvation significantly ($p < 0.01$) decreased by 2.5 and 1.6 times, respectively, compared to the R_{tot} of individuals feeding on microalgae before starvation 0.094 ± 0.014 and 1.15 ± 0.094 (Table 1).

In *L. macrurus*, R_{tot} decreased by 1.9 times during the 11 days of starvation. Simultaneously, R_{bas} in *L. macrurus* had no significant differences, as well as in other species during the period of starvation. However, starving individuals of *A. clausi* and *C. helgolandicus*, displayed an R_{bas} approximately 30% lower ($p < 0.01$) than in specimens fed before starvation. Due to the significant decrease in R_{tot} and insignificant changes in R_{bas} , R_{act} calculated as $R_{tot} - R_{bas}$, in *A. clausi* and *L. macrurus* decreased during starvation by 4.7 and 6.0 times, respectively, and in *C. helgolandicus* R_{act} decreased only by 1.7 times (Table 1). The metabolic range of activity, calculated as the $R_{tot} : R_{bas}$ ratio, also decreased greatly by 1.7 times in *A. clausi* and 2.1 times in *L. macrurus* ($p < 0.01$), with the exception of *C. helgolandicus*, in which no significant changes of $R_{tot} : R_{bas}$ ratio were observed during 7 days of starvation (Figure 2).

The $CRCW_{tot}$ were very similar in *A. clausi* and *C. helgolandicus* individuals (Table 1) that consumed food prior to starvation (0.123 and 0.114 d^{-1} , respectively) but decreased significantly ($p < 0.001$) 2.5 times to 0.048 d^{-1} in *A. clausi*, while in *C. helgolandicus* $CRCW_{tot}$ decreased to a lesser extent from 0.114 to 0.07 d^{-1} . In *Limnocalanus*

macrurus, $CRCW_{tot}$ decreased significantly ($p < 0.001$) almost to the $CRCW_{bas}$ level from 0.079 to 0.039 d^{-1} .

The $CRCW_{bas}$ showed no significant correlation with starvation time in *L. macrurus* while $CRCW_{active}$, calculated as $CRCW_{tot} - CRCW_{bas}$ decreased during starvation by 12 times. In *A. clausi* and *C. helgolandicus* $CRCW_{bas}$ decreased during starvation in comparison with feeding period by 1.5 and 2.8 times, respectively, while $CRCW_{active}$ decreased by 4.7 and 1.7 times (Table 1).

The average values of CL_{tot} and CL_{bas} and $DC_{L_{tot}}$ and DCL_{bas} calculated on the basis of respiration rates of active and anesthetized individuals as well as cumulative losses of carbon during starvation normalized to the temperature of keeping three species (17 °C for *A. clausi* and *C. helgolandicus* and 13 °C for *L. macrurus*) are shown in Table 1, and the dynamics of the average daily carbon losses during starvation in % of its initial carbon content in copepod bodies is shown in Figure 3. The total average daily carbon loss during starvation in *L. macrurus* was 2.2 $\mu\text{g C}$, while in both Black Sea species (*A. clausi* and *C. helgolandicus*) they were equal to 5.1 $\mu\text{g C}$.

4. Discussion

4.1. Comparative evaluation of respiration rate of fed copepods before starvation

The R_{tot} of the studied copepods from the Marmara Sea; *Acartia clausi* and *Calanus helgolandicus* measured the day prior to the starvation period at a temperature of 20 °C were equal to 0.094 ± 0.014 and 1.15 ± 0.094 $\mu\text{g O}_2$ ind $^{-1}$ h $^{-1}$, respectively, which is within the range of results obtained by other authors at the same temperature of 20 °C (Hubareva et al., 2008; Svetlichny et al., 2000; 2010).

R_{tot} of the copepod *Limnocalanus macrurus* on the first day was equal to 0.363 ± 0.053 $\mu\text{g O}_2$ ind $^{-1}$ h $^{-1}$ at 14 °C. It can be assumed that, like in the Marmara Sea species,

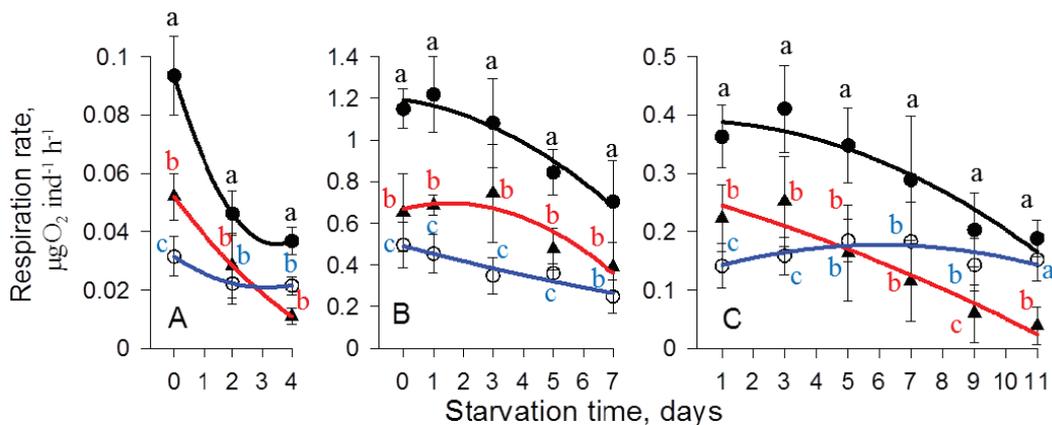


Figure 2. Changes in Total (●, black lines), Basal (○, blue lines) and Active (▲, red lines) respiration in the Marmara Sea copepods *Acartia clausi* (A) and *Calanus helgolandicus* (B), and the Baltic Sea *Limnocalanus macrurus* (C) during starvation. Low-case letters (a, b and c) are the significant variable differences from Duncan's multiple range test (DMRT), $p < 0.05$.

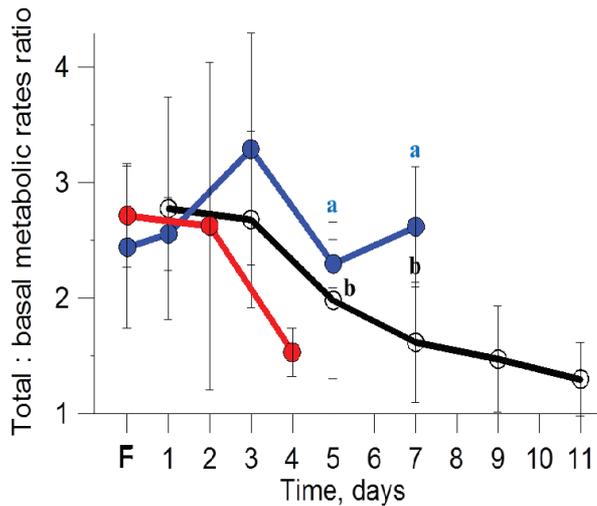


Figure 3. Total to basal respiration rate ratio in *Acartia clausi* (red line), *Limnocalanus macrurus* (black line) and *Calanus helgolandicus* (blue line). Note, the $R_{tot} : R_{bas}$ ratio values may differ from values given in Table 2, since they were calculated from the results of the experiments in which the values of R_{tot} and R_{bas} were obtained from the same individuals (see details in Materials and Methods section). Low-case letters (a, b) signify the significant variable differences from Duncan's multiple range test (DMRT), $p < 0.05$.

R_{tot} of starving *L. macrurus* was significantly less than for actively feeding specimens. However, the R_{tot} values obtained in our study were close to or even greater than previously estimated by other authors. Thus, in adult *L. macrurus* from Arctic lakes, the respiration rate was $0.165 \mu\text{g O}_2 \text{ ind}^{-1} \text{ h}^{-1}$ at 15°C (Roff, 1973). In adult *L. macrurus* from the Kara Sea, the respiration rate was $5.02 \mu\text{L O}_2 \text{ ind}^{-1} \text{ day}^{-1}$ at 8°C (Drits et al., 2016) which means approximately $0.27 \mu\text{g O}_2 \text{ ind}^{-1} \text{ h}^{-1}$. If this value to adjust to 14°C using the temperature coefficient $Q_{10} = 2.2$ (Roff, 1973), then the R_{tot} of these individuals will be approximately $0.43 \mu\text{g O}_2 \text{ ind}^{-1} \text{ h}^{-1}$. This value turned out to be higher than our result of $0.363 \mu\text{g O}_2 \text{ ind}^{-1} \text{ h}^{-1}$; however, if we take into account differences in the dry weights of individuals from the Kara Sea (i.e. 0.226 mg) (Drits et al., 2016) and our experiment (approximately 0.14 mg), the weight-specific respiration rate of *L. macrurus* from the Kara Sea would then be equivalent to $1.9 \mu\text{g O}_2 \text{ mg}^{-1} \text{ h}^{-1}$. So, it is lower than for adult females at the beginning of our experiment ($2.6 \mu\text{g O}_2 \text{ mg}^{-1} \text{ h}^{-1}$).

4.2 Effect of starvation on copepod respiration rate

During starvation, all copepod species studied here demonstrated a significant decrease in R_{tot} (Figure 1). The decrease in R_{tot} was fastest in species with a short life cycle i.e. *Acartia clausi* (15% of initial level d^{-1}) during 4 days of starvation, while in both *Limnocalanus macrurus* and *Calanus helgolandicus* with a long life cycle, the daily

decrease in R_{tot} was 4.4 and 5.5%, respectively, of the initial oxygen consumption level during 11 and 7 days of starvation, respectively. For comparison, similar trends in decreasing respiration rates were obtained earlier for *A. clausi* by Conover (1956) (about 10% per day from baseline) and about 5% per day in copepods with a long development cycle, *Calanus cristatus* (Ikeda, 1971), *Calanus plumchirus* (Ikeda, 1977) and *Pseudocalanus newmani* (Tsuda, 1994), during 11, 12 and 15 days of starvation, respectively.

The decrease in the respiratory rate of copepods deprived of food is usually explained by a decrease in their feeding activity i.e. R_{sda} , not attaching importance to the possible effect of a change in locomotor activity. For example, respiration rates of feeding copepods *Acartia tonsa* (Kiorboe et al., 1985) and *Oithona davisae* (Hiromi, 1994) were about 1.4 times higher than those of unfed animals. In feeding *A. clausi* and *Calanus finmarchicus* respiration rates exceeded levels of starved copepods by 1.6–2.8 times (Thor, 2000, 2002, 2003). In the experiments of Lehetter et al. (2016), a decrease in the respiration rate of *Pseudodiaptomus annandalei* within 8 h was also interpreted as a result of starvation. However, in studies that accounted for the motor activity of copepods, a high R_{sda} value of up to 85% R_{tot} was found only in individuals with very low motor activity, while in actively swimming individuals the R_{SDA} value decreased to 10% of R_{tot} (Svetlichny and Hubareva, 2005).

However, as shown by experiments (Thor, 2000, 2002, 2003) the effect of SDA lasts no more than a day after eating. This can probably explain the fact that R_{bas} in *A. clausi* and *C. helgolandicus* feeding before starvation was 30% higher than during the period of starvation. It is important to note that during multiday starvation, R_{bas} of studied species varied within a very narrow range. This may indicate the maintenance of the amount of body tissues that consume energy at rest.

Consequently, the revealed features of the R_{tot} trends (Figure 2) of copepods during starving period can only be explained by a decrease in R_{acr} , namely locomotor activity. This was partially confirmed by the study by Isinibilir et al. (2020) in which the change in the respiration rate of *C. helgolandicus* correlated with the change in the motor activity of these copepods; however, more complete data with respect to the effect of starvation on copepod behavior were obtained by Holm et al. (2019). Starvation has been shown to have a greater effect on the locomotor activity of foraging species such as *Acartia*. From a physiological point of view, such a response to starvation by copepods can be explained by the rapid depletion of short-term energy substrates that provide muscle activity. In *A. clausi* $R_{tot} : R_{bas}$ ratio (2.7), as an indicator of their locomotor activity, during 4 days of starvation decreased by 1.7 times (Figure 3).

Unlike *A. clausi*, the long lifespan *L. macrurus* are capable of accumulating significant lipid reserves, up to 67% of the dry body weight in oil sacs (Vanderploeg et al., 1998). At the beginning of our experiment, *L. macrurus* females displayed an average lipid content of 51 % of body dry weight. Probably due to the large reserves of lipids, the $R_{tot} : R_{bas}$ ratio in this species decreased more slowly than in *A. clausi* (Figure 3). Nevertheless, within 11 days of starvation, R_{tot} in *L. macrurus* decreased to the R_{bas} level due to an almost 6-fold decrease in R_{act} (Table 1).

Probably, such a change in the respiration rate of *Limnocalanus macrurus* can also be associated with a natural seasonal change in their activity. In the Baltic Sea in late summer and early September, adult *L. macrurus*, which accumulate many lipids, sink into the deep layers, where they can “suffer from starvation” in quasi-diapause state (Mäkinen et al., 2017). The same changes in the activity of *L. macrurus* were noted by Hirche et al. (2003) in the Kara Sea in August–September 1999.

The life cycle of *Calanus helgolandicus* also includes a diapause phase in the summer, the least productive period at sea throughout the year. However, in contrast to *L. macrurus*, only preadult copepodites of *C. helgolandicus* that have accumulated sufficient lipid reserves undergo diapause. Females, on the other hand, can only use up lipid reserves remained for egg production. A feature of *C. helgolandicus* females in the Black Sea (from which they enter the Sea of Marmara) is that they undertake daily vertical migrations from the near-surface layer, where they actively feed in the dark, into deep cold and hypoxic layers above the hydrogen sulfide zone in the daytime (Nikitin and Malm, 1932). During daytime they do not eat (Flint, 1989), but must maintain sufficient locomotor activity to avoid descending in hydrogen sulfide layers and to be able to rise to the surface of the sea in the evening (Svetlichny et al., 2018). This probably explains the absence of significant changes in the $R_{tot} : R_{bas}$ ratio in this species during starvation (Figure 3).

4.3. Carbon budget change during starvation

Conversion of oxygen values respired by copepods to respiratory carbon loss per unit of body carbon weight oil sac led to unexpected results. Despite the 15-fold difference in body weight (Table 1), the $CRCW_{tot}$ values were very similar in Marmara copepods fed before starvation (about 0.12 d^{-1}), as well as for $CRCW_{bas}$ during the entire starvation period (Table 1). In *Limnocalanus macrurus*, $CRCW_{tot}$ at 14°C was almost 1.5 times lower than for Marmara Sea copepods (0.08 d^{-1}). However, if we convert this value to a temperature of 20°C applying a temperature coefficient of $Q_{10} = 2.2$ (Roff, 1973) the $CRCW_{tot}$ values in *L. macrurus* also increase by about 1.5 times to the values 0.12 d^{-1} obtained for the Marmara Sea species at 20°C .

As a result, $CRCW_{tot}$ was in an isometric relationship with CW_{bodysf} with an exponent of about 1 (Figure 4). This

differs from the theoretically substantiated allometric dependence of copepod energy metabolism on body mass, in the form of a power equation with exponent 07–08 (Ikeda et al., 2001). However, the slope of the $CRCW_{tot}$ regression line for the total carbon content in the body of copepods, including the mass of lipid reserves, turned out to be 0.83 (Figure 4). This observation deserves special attention to the possible differences in the allometric dependences of the respiratory rate on the body weight of animals with different contents of metabolically inert components, which is however beyond the scope of our study.

To compare the rates of body carbon loss during the starvation period in the studied species, we calculated the cumulative increases in their total and basal respiratory carbon expenditures (Figure 5), and the average daily carbon loss in total and basal metabolism over the entire starvation period DCL_{tot} and DCL_{bas} , respectively (see Table 1). Thus, we were able to distinguish two types of starvation responses.

The first type is typical for such species as *Acartia clausi*, which do not have large lipid stores (approximately 3% of dry mass, see review data Lee et al., 2006), after 4 days of starvation, while total loss of body carbon was expected to be about 20.7% and basal 10.4%, respectively, of the initial quantity (Figure 5) the average daily carbon losses were in fact found to be equal of app 5.1 and 2.6%, respectively (Table 1). Similar values for the daily rate of loss of body carbon (3.8% of initial weight) or dry weight (4.8% of initial weight) were established during experimental starvation at days 15 and day 10 for *Pseudocalanus newmani* (Tsuda, 1994) and *Eudiaptomus gracilis* (Koussoroplis et al., 2014), respectively. Copepods, including some marine coastal and estuarine species, are not adapted to the economical use of energy resources and generally cannot withstand long-term starvation (Lee et al., 2006).

During our experiments, a high rate of body carbon expenditure (5.1% of the initial daily rate) was also found in *Calanus helgolandicus* females. During 7 days of starvation at 17°C , estimated carbon loss was calculated as $42.0 \mu\text{g C}$, approximately 36.5% of the total body carbon (Figure 5), or 75.1% of carbon content in reserved lipids. It should be noted that in the Black Sea, *C. helgolandicus* spend most of the day in deep mixed layers at a temperature of about 8°C , which allows them to efficiently regulate the daily energy balance in order to restore energy resources even with the relatively low productivity of this sea. But in the Sea of Marmara, where large females ($2.5 < \text{Lpr} < 2.8 \text{ mm}$) enter via the Black Sea current, the lower layers are formed by Mediterranean waters at a constant temperature of 15°C . Due to the higher temperature, the expenditure portion of the copepod energy balance increases. Therefore, as shown (Svetlichny et al., 2006), *C. helgolandicus* in the Sea of Marmara cannot accumulate lipids in the oil sac and

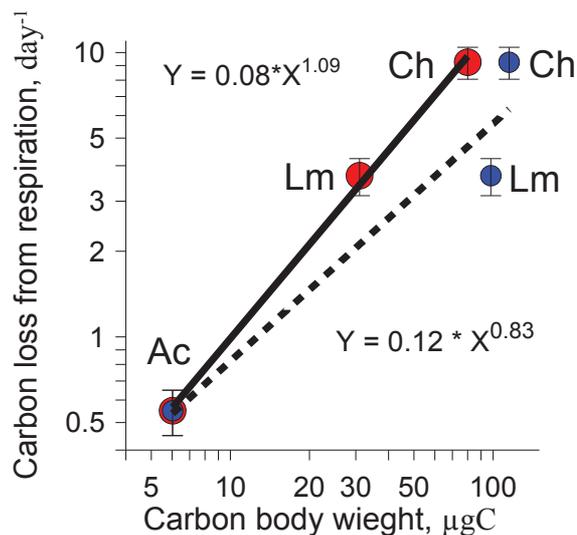


Figure 4. Relationship between $CRCW_{tot}$ of *Acartia clausi* (Ac), *Limnocalanus macrurus* (Lm) and *Calanus helgolandicus* (Ch) on the first day of experiments with carbon body weight excluding oil sacs (●, —) and total carbon content in the body of copepods (●, ----).

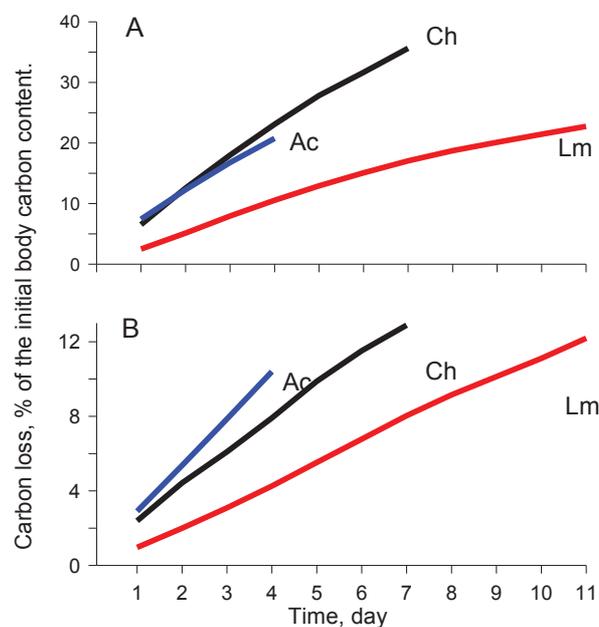


Figure 5. Respiratory carbon cumulative losses in accordance with the respiration rates of active (A) and anesthetized (B) individuals of *Acartia clausi* (Ac) and *Calanus helgolandicus* (Ch) during starvation, recalculated for a temperature of 17 °C, and a similar increase in respiratory losses of carbon in *Limnocalanus macrurus* (Lm) at 13 °C.

individuals of the generations developing in the Sea of Marmara exhibit reduced body sizes (Isinibilir et al., 2009).

The second type of starvation was found in *Limnocalanus macrurus*, which has large reserves of lipids, in which the total respiratory energy expenditure during the 11 days of starvation at 13 °C was measured as 22.3 µg C, of which 12 µg C was spent for basal needs (Figure 5). Thus, the total loss of carbon amounted to 33% of oil sac content. Oil sacs were consumed at an average rate of 2.2% day⁻¹ of the original carbon content in the body. Our preliminary statistically unconfirmed data indicate a possible decrease in sac volume during 11 days of starvation by 0.023 mm³, or about 20 µg C, which is close to the respiratory carbon consumption rate (Table 1), but this data require additional verification.

Compared to starvation of other lipid rich copepods, the rate of daily carbon loss per day in *Limnocalanus macrurus* (2.2% of initial content day⁻¹) is higher than the rates of daily lipid consumption in *Calanus cristatus*, which lost about 1.6% C day⁻¹ (Ikeda 1971), and in *Calanus glacialis*, which lost 1.3% (Hirche and Bohrer, 1987) and 1.6% (Båtmstedt and Tande, 1985) body C d⁻¹. During a 60-day starvation period, *Gaussia princeps* lost 1.7% of total lipids or approximately 0.8% of initial body C day⁻¹ (Lee and Barnes, 1975). In the wintering *Euchaeta norvegica* and *Calanus glacialis*, starvation during 60 and 77 days was accompanied by a loss of 0.8 and 1.05% of initial body C day⁻¹ (Lee, Barnes 1975 and Hirche and Kattner, 1993) respectively, while during the 4 months of summer-fall diapause in *Calanus finmarchicus*, stored fat decreased at a rate of about 0.4% of body carbon per day (Pasternak et al., 2001). Note, however, that all the above observations by other authors describe starvation of copepods at temperatures much lower than in our experiments (13 °C).

However, for the stenothermic glacial relict *Limnocalanus macrurus*, 13 °C is similar to the upper limit of temperature tolerance. According to literature data, the optimal habitat temperature of this species is 1–8 °C, the maximum can reach 17–18 °C, but usually during daily migrations, animals do not cross the 13 °C isotherm (Hutchinson, 1967; Vezhnovets, 1984; Aispure, 2006) and can be present in deep layers (Mäkinen et al., 2017) at a temperature of approximately 4–5 °C (Vuori et al., 2015). Considering the ability of *L. macrurus* during starvation to reduce total levels of energy metabolism to basal, at a temperature of 5 °C, the daily carbon loss in this species can decrease, according to our estimates, to 0.7%, which is quite consistent with the regimes of nonlethal starvation of Arctic calanoid copepods (Hatlebakk et al., 2019). With such low energy requirements, the energy reserves of lipids in oil sac of *L. macrurus* would be enough for 98 days of starvation without the need to consume the main tissues of the body. Thus, our results indicate that species capable of accumulating energy substrates for surviving prolonged food scarcity, such as *Limnocalanus macrurus*,

can use up reserves more economically during starvation, in comparison to epipelagic copepods *Acartia clausi* with short life cycles, or *Calanus helgolandicus* females in the spawning phase. However, regardless of their ecology, variations in energy budget expenditure of copepods occur due to modifications in utilization for locomotor activity at a very conservative level of basal metabolism. This is probably due to the fact that the duration of our experiments did not exceed the sublethal limits of starvation duration of the studied species. Longer-term experiments enable the determination of the thresholds at which adaptive changes in the basal metabolism of copepods arise.

Abbreviations

L_{pr} : prosome length, V_b : body volume, V_{os} : oil sac volume, V_{bsf} : sac free body volume, WW : wet weight, DW : dry weight, CW_{tot} : total body carbon weight, CW_{sac} : carbon weight of wax in oil sac, CW_{bodysf} : oil sac free body carbon weight, R_{tot} : total respiration rate, R_{bas} : basal respiration rate, R_{act} : active metabolic rate, "scope of activity" ($R_{tot} - R_{bas}$), $CRCW_{tot}$: daily total respiratory carbon loss per unit of body carbon weight oil sac, $CRCW_{bas}$: daily basal respiratory carbon loss per unit of body carbon weight

weight oil sac, $CRCW_{active}$: daily basal respiratory carbon loss per unit of body carbon weight oil sac, CCL_{tot} : total cumulative respiratory carbon loss during starvation, CCL_{bas} : basal cumulative respiratory carbon loss during total time of starvation, DCL_{tot} : total daily average weight specific body carbon losses during starvation, % of initial total carbon body weight, DCL_{bas} : basal daily average weight specific body carbon losses during starvation, % of initial total carbon body weight.

Contribution of authors

L.S., M.I. and A.K. designed the experiment. L.S., M.L., O.S., L.S., E.T., K.M.E. conducted the experiment. K.M.E. performed the statistical analysis of the data. L.S., Y.G., O.S., A.L., M.L., K.M.E., G.C. prepared the manuscript.

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