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Lipid storage consumption and feeding ability of *Calanus glacialis* Jaschnov, 1955 males

Maja Hatlebakk[a,b,*], Martin Graeve[c], Lauris Boissonnot[c,1], Janne E. Søreide[a]

[a] University Centre in Svalbard, Pb. 156, 9171 Longyearbyen, Norway
[b] Faculty of Biosciences and Aquaculture, Nord University, Pb. 1490, 8049 Bodø, Norway
[c] Alfred Wegener Institute Helmholtz Centre for Polar and Marine Science, Am Handelshafen 12, 27570 Bremerhaven, Germany

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ABSTRACT

Calanus is one of the best studied genera of Arctic zooplankton, but still we know very little about the males since they are short-lived and mainly present in winter. Their short life-span compared to females is assumed to be a combination of high mating activity, no feeding and consequential depletion of lipid stores. In this study we tested 1) if the life span of male *Calanus glacialis* is limited by their lipid storage reserves and 2) if males are capable of feeding and utilize food if present. We ran two separate experiments from January to March; one on starvation and one on feeding. In the 39-days long starvation experiment we followed the lipid sac size of individually incubated males until their time of death. On average the total lipid (TL) content decreased by 2.6 to 4.5 μg day^{-1}, but despite this males had substantial amounts of lipids left (131.4 μg, SD 44.0) when they died. This strongly suggests that the depletion of lipid reserves is not the main reason for males' short life span which in this study was measured to be up to 73 days. In the feeding experiment, we fed both *C. glacialis* males and females ad libitum with ^{13}C labelled microalgae. Both males and females were capable of feeding and assimilate the diatom monoculture, but females responded faster to the sudden favourable food conditions, and produced more and larger fecal pellets than the males. Assimilation of ^{13}C labelled 20:5(n-3), an essential polyunsaturated fatty acid (PUFA), from the diatom diet was traceable in both males and females on day 21, and then with a higher enrichment in females than males. Morphological investigations of the feeding appendages showed some differences between sexes, suggesting males to be more omnivorous than females. In conclusion, lipid storage depletion is not the cause of death for male *C. glacialis*, and males may even compensate for some of the mating energy costs by feeding. In future, we recommend further studies on the role of essential fatty acids (FA) for sperm formation and aging as determining factors for males' relatively short life span.

1. Introduction

The mesozooplankton community of Arctic and Sub-Arctic seas, in terms of biomass, are dominated by copepods of the genus *Calanus* (Kosobokova and Hirche, 2009). In Svalbard shelf seas and fjords this genus comprise up to 80% of the mesozooplankton biomass (Blachowiak-Samolyk et al., 2008; Søreide et al., 2008), and they are key actors in the Arctic marine Ecosystem. When light returns in spring, it triggers first an ice algae bloom followed by a short and intense pelagic bloom which lasts for a couple of weeks before fading into a smaller summer and fall production (Leu et al., 2015). *Calanus* spp. plays a major role in harvesting and transferring the energy from these primary producers to higher trophic levels (Falk-Petersen et al., 2009).

Copepods have developed three different foraging strategies: ambush feeders which waits passively for prey to come within range (Kiørboe et al., 2009), cruise feeders which encounter and catch prey as they swim through the water (Kjellerup and Kiørboe, 2011) and feeding-current feeders which create a feeding current and harvest their catch in the current (Koehl and Strickier, 1981). The different foraging strategies entails different levels of activity, which has consequences for encounter rates with food, predators and mating partners and trade-offs between these. The passive ambush feeders have lower risk of being eaten, but at the cost of lower feeding efficiency (Henriksen et al., 2007; Kiørboe et al., 2010) while the active feeders benefit from high feeding efficiency, but at the cost of higher risk of encountering predators (Gonçalves et al., 2014; Van Someren Gréve et al., 2017). *Calanus* spp.

* Corresponding author at: University Centre in Svalbard, Pb. 156, 9171 Longyearbyen, Norway.
E-mail addresses: majah@unis.no (M. Hatlebakk), martin.graeve@awi.de (M. Graeve), lauris@aqua-kompetanse.no (L. Boissonnot), janne.soreide@unis.no (J.E. Søreide).
[1] Present affiliation: Aqua Kompetanse, Havbruksparken, Storlavika 7, 7770 Flatanger, Norway

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belongs to the feeding-current feeders, using their antennules and feeding appendages to create currents around the body (Koehl and Strickier, 1981; Huntley, 1988). They can be very effective in filtering water for food, though it varies with season and size of available prey (Tande and Båmstedt, 1985; Levinsen et al., 2000).

In the European Arctic three species of *Calanus* co-exist; the big Arctic *C. hyperboreus* with core distribution in the Greenland Sea (Conover, 1988), the intermediate sized *C. glacialis* which mainly inhabit the Arctic shelf seas, and the slightly smaller *C. finmarchicus*, a north Atlantic expatriate species carried to the Arctic with Atlantic water currents (Falk-Petersen et al., 2009). *Calanus* spp. efficiently accumulates and de novo biosynthesises lipids from its microalgae diet, storing the surplus in a lipid sac which can fill up to 80% of the body cavity of older copepodite stages (Vogedes et al., 2010). Especially essential polyunsaturated fatty acids (PUFA), which are important for physiological processes and reproduction (Sargent and Falk-Petersen, 1988; Kattner and Hagen, 2009; Koski et al., 2012) are preferably retained from the diet (Graeve et al., 2005). This efficient accumulation of lipids makes *Calanus* spp. key species in Arctic marine food webs, ensuring efficient energy transfer of essential fatty acids (FA) and other lipids to higher trophic levels (Falk-Petersen et al., 2007).

The three *Calanus* species are morphologically similar, and are primarily separated to species level by their differences in body size (Daase and Eiane, 2007) despite that recent molecular studies show that *C. glacialis* and *C. finmarchicus* have overlapping size distributions (Gabrielsen et al., 2012; Choquet et al., 2017). At high latitudes, however, live specimens can be distinguished with a high degree of confidence to species level by size (prosome length) combined with red pigmentation (Choquet et al., 2018). Females of *C. glacialis* tend to have red antennules and genital segment, and *C. finmarchicus* tend to have pale antennules and genital segment (Choquet et al., 2018). Live males of *Calanus* are not pigmented, but genetic studies confirm that males appearing in January in Svalbard with prosome lengths between 2.9 and 3.4 mm are *C. glacialis* (Daase et al., 2018). Males of *C. finmarchicus* are smaller (2.4–3.1 mm) and first seem to appear in mid-February/March (Daase et al., 2018). *C. hyperboreus* are only found in low numbers in Svalbard fjords (Scott et al., 2000; Arnkværn et al., 2005), hence we focused on *C. glacialis* in this study.

C. glacialis efficiently build up its lipid storages during the short, but intensive spring bloom period. The females utilize previous summer's stored lipid resources to fuel early maturation and egg production the following year to enable the offspring to hatch and develop in time for the phytoplankton bloom (Søreide et al., 2010). The young copepods develop and grow to the overwintering copepodite stages CIV or CV and descend to deeper waters in autumn as soon as they have built up sufficient lipid storages. The primary storage lipids in *Calanus* spp. are wax esters (WE), characterised by the long-chain monounsaturated fatty acids (MUFA) and fatty alcohols (FAlc) 20:1 (n-9) and 22:1 (n-11) (Lee et al., 1971; Graeve and Kattner, 1992; Lee et al., 2006). As the microalgae season narrows down towards higher latitudes the proportions of WE in copepods increases (Lee and Hirota, 1973; Lee et al., 2006), and may constitute > 90% of the TL content in the Arctic *C. glacialis* (Conover, 1988; Lee et al., 2006). This extensive energy storage combined with reduced metabolism during fall and winter makes it possible for *C. glacialis* to survive the long food-poor Arctic winter. Individuals spending the winter as CIV will first be capable to moult to CV after one more feeding season in spring for so to overwinter as CV the following winter (Kosobokova, 1999). The individuals which overwinter as CV will moult to adults and mate during the winter to ensure early reproduction. Males moult a few weeks earlier than females, and are only present for a few months with peak abundance in December–January (Kosobokova, 1999; Daase et al., 2018). In comparison, females of *C. glacialis* may be iteroparous i.e. being able to survive another winter and reproduce again (Kosobokova, 1999).

The shorter life span for males compared to females is typical among calanoid copepods (Gilbert and Williamson, 1983). Short life span may not necessarily be due to predation or energy depletion, but also due to the physiological aging being faster in males than females (Rodríguez-Graña et al., 2010; Kiørboe et al., 2015). Kiørboe et al. (2015) found that trade-offs between various life history traits and behaviours of small, copepods are consistent with the disposable soma theory (Kirkwood, 2002). In short it states that the investment in maintaining somatic (non-reproductive) tissue in good health should not exceed the life expectancy in the wild. Van Someren Gréve et al. (2017) demonstrated the relationship between behaviour and predation risk in pelagic copepods. Higher risk means shorter life expectancy. As males are typically the most active part in mate search, they are generally at higher predation risk (Kiørboe, 2008). Thus male copepods benefit most on investing any spare resources in reproduction rather than self-maintenance and growth (Hirst and Kiørboe, 2014). Even at high latitudes when the light is practically absent in winter, the predation risk may be considerable since recent studies found unexpectedly high activity levels in pelagic communities during the polar night (Berge et al., 2015a).

The live-fast-die-young approach and trade-off between mate search and food search have led some calanoid males to not invest in fully developed mouth parts, rendering them less efficient feeders or unable to feed (Schnack, 1989). However, this has not been observed within the *Calanus* genus (Bradford and Jillett, 1974), so they may potentially capture food particles they encounter (e.g. Cleary et al., 2017). Due to the lack of microalgae during the dark Arctic winter, it is hypothesized that males most likely do not actively feed and that their short life span is due to lipid storage depletion (Raymont and Gross, 1942; Kosobokova, 1999). However, we know very little about the ecology of *Calanus* males, since Arctic winter data is scarce (Kosobokova, 1999; Madsen et al., 2001; Daase et al., 2018). Recent studies have shown that the long, dark winter is not void of biological activity as previously assumed (Berge et al., 2015b and references therein), and more winter-studies are urgently needed to better understand polar zooplankton life strategies and their capability to adapt to a rapidly changing Arctic (e.g. Madsen et al., 2001; Berge et al., 2012; Zamora-Terol et al., 2013).

To increase our knowledge on *Calanus* life strategies we investigated lipid storage consumption and feeding capability of male *C. glacialis* in parallel with the much better known *C. glacialis* female as a reference. Following research questions were targeted: (1) is the life span of males determined by their lipid storage reserves? We know their life span is shorter than females, but there are uncertainties to why. Do they die because they run out of energy in the unproductive winter or are there other factors connected to their life strategy? (2) do males actively feed? And finally (3) can males utilize this food? Males don't need to make a trade-off between mate search and feeding, since actively searching for females increase their food encounter rate. It may therefore be beneficial for males to take advantage of present food, especially if they encounter algal blooms which may start as early as March (Leu et al., 2015). These questions were addressed in two experimental studies. First, a starvation experiment where the lipid sac sizes of individually incubated males were followed by image analyses from early winter until time of death. Secondly, a feeding experiment with males and females offered ad libitum ^{13}C labelled microalgae, to study their capability to ingest and assimilate dietary lipids.

2. Materials and methods

2.1. Sampling

Copepods for the experimental work were collected in Billefjorden (78° 39′N, 016°44′E) and Rijpfjorden (80°17′ N, 022°18′E) (Fig. 1) in January 2013 and 2016 onboard R/V Helmer Hanssen. Billefjorden is situated in the innermost part of Isfjorden in Western Spitsbergen. It is a threshold fjord with a sill depth of 50 m and an inner basin of 190 m depth, which restricts the influence of the warmer and saltier Atlantic water from the West Spitsbergen Current to enter the fjord (Nilsen et al., 2008). Local cold water formation due to winter cooling and sea

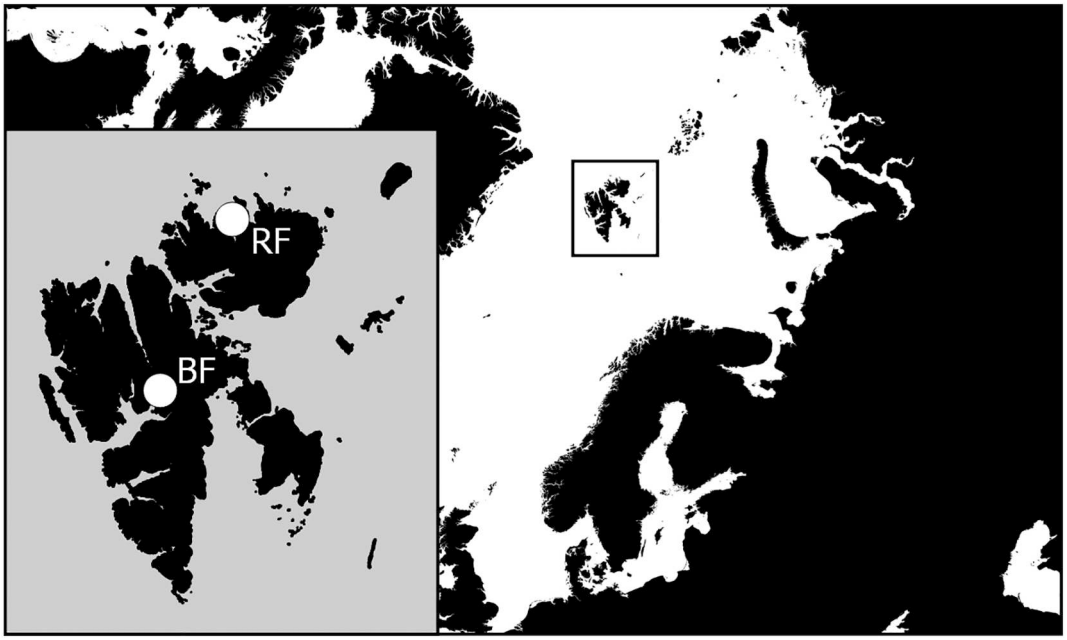

Fig. 1. Map showing the sampling stations Billefjorden (BF) and Rijpfjorden (RF) in Svalbard archipelago (Map data: Norwegian Polar Institute and thematicmapping.org).

ice formation result in water masses similarly cold (< -1 °C) and saline ($\leq$ 34.8 psu) as Arctic water year-round below the threshold depth in Billefjorden (Nilsen et al., 2008). Rijpfjorden is a north facing fjord on Nordaustlandet in NE Svalbard. It is considered to be a high-Arctic fjord with its extensive seasonal sea ice cover 6–8 months a year (Wallace et al., 2010). Detailed knowledge on the bathymetry of Rijpfjorden is sparse, but there are at least two basins (Howe et al., 2010) with a maximum depth of 250 m, and no sill separating the fjord from the wide shelf outside (Leu et al., 2011).

For experiments, copepods were collected vertically by a WP3 net (1000 µm, 1 m^2 opening) with a large non-filtrating cod end at slow speed (0.5 m s^{-1}). Samples were quickly and gently transferred to 30 L barrels, diluted with surface sea water and kept in the dark in a cold room (2 °C) until start of the experiments.

2.2. Starvation experiment

The copepods for the starvation experiment were collected from bottom to surface in Billefjorden (180 to 0 m) and Rijpfjorden (270 to 0 m) in January 2013 and kept in large 30 L barrels in a temperature regulated cold room (2 °C) on board RV Helmer Hanssen for eight and six days respectively before being transferred to the cold room laboratory at the University Centre in Svalbard (UNIS). There they were kept in 1 µm filtered sea water (Sartopure PP2 capsule, Sartorius stedim biotech) in 30 L barrels at −1 °C for another 28 days until the experiment was started at February 15. Five males from Billefjorden and 38 males from Rijpfjorden were sorted out from the barrels, giving a total of 43 males that were incubated individually in incubation chambers of 150 mL filled with GF/F filtered sea water (Whatman GF/F: 0.7 µm, GE Healthcare). The incubation was done in darkness at −1 °C to simulate the in situ winter conditions. Every three to four days, when the water was renewed, the males were photographed under a dissecting microscope at 16× magnification from lateral view for lipid sac size estimates. This interval was chosen as a compromise between handling stress and relatively frequent lipid sac size measurements. TL content was estimated from the area of the lipid sac following Vogedes et al. (2010). Images were taken with a SONY video camera (HDR-HC7) with an ocular adapter and analysed using ImageJ software 1.48v (Rasband 1997–2009).

2.3. Feeding experiment

Males and females were collected from Rijpfjorden (100 to 0 m) during the polar night cruise with R/V Helmer Hanssen in January 2016. The feeding experiment was running for three days on board before it was transferred to the cold lab at UNIS where it was run for another 18 days.

The ^{13}C labelling of the microalgae culture started 2 weeks prior to start of feeding experiment to ensure the microalgae to be sufficiently labelled (Boissonnot et al., 2016). Algae culture of *Porosira glacialis* (Grunow) Jörgensen, 1905 *(size class 30–35 µm ⌀*, calculated carbon content 774 pg $cell^{-1}$ (Menden-Deuer and Lessard, 2000)) was kept at ~5 °C in a light:dark cycle of 16:8. *P. glacialis* is a centric diatom with bipolar distribution (Hasle, 1976) and it is in the food size range typically preferred by *Calanus* (Levinsen et al., 2000). The algae culture was diluted regularly to keep it growing in an exponential phase with Guillards f2 medium dissolved in sterile sea water, enriched with silicate (0.1 µmol L^{-1}) and labelled with ^{13}C sodium bicarbonate (1.5 mg L^{-1}). On day 0, 11 and 21 of the feeding experiment, triplicates of the algal cultures (100 mL each) were filtered on burnt GF/F filters and quickly frozen at −80 °C for later FA and compound specific stable isotope analyses at Alfred Wegener Institute, Bremerhaven, Germany.

The copepods were incubated in 1 L borosilicate bottles: 4 bottles with 20 females each and 4 bottles with 20 males each. This number was a compromise between space on the plankton wheel and sufficient number of individuals for lipid samples throughout the feeding experiment. Incubations were done in darkness at 2–3 °C, slightly higher than the in situ sea temperature in Rijpfjorden (mean 0 °C; range − 1 to 1 °C). The incubation bottles with the copepods were kept on a rotating plankton wheel (1.5–2 RPM) to keep the microalgae in constant suspension. The copepods were fed with ^{13}C labelled *P. glacialis* at a concentration of 1200 cells mL^{-1} (~929 µg C L^{-1}), and water and microalgae were renewed every 2–3 days by diluting parts of the culture in filtered sea water to the right concentration. When water was changed, the content of each bottle was filtered through a 90 µm mesh. The animals were inspected and the number of dead animals was counted. Live animals were transferred back to the bottle with renewed water and microalgae. The fecal pellets collected on the mesh were transferred to a petri dish and counted under dissecting microscope, and the fecal pellet production was used as a relative indicator of ingestion rate. Fecal pellet volume was measured on day

7 by image analyses using ImageJ software 1.48v (Rasband 1997–2009). In total, 46 fecal pellets from males and 168 fecal pellets from females were collected in petri dishes and photographed at 40× magnification with a SONY video camera (HDR-HC7) with an ocular adapter. Length and width of the fecal pellets were measured and volume calculated assuming a cylindrical shape. At day 0, 2, 11 and 21, copepods from each bottle were collected for lipid composition and compound specific stable isotope analyses. On day 0 triplicates of 10 random males and females were sampled, on day 2 and 11 five individuals were collected from each incubation bottle giving quadruplicates for both males and females, and on day 21 the remaining animals were collected as one replicate per bottle (1, 2 and 3 individuals for male triplicate, and 3, 4 and 6 individuals for female triplicate). Animals were rinsed in filtered seawater and frozen in glass vials at −80 °C.

Of specific FA we were particularly interested in the diatom FA markers 16:1(n-7) and the essential PUFA 20:5(n-3) (Dalsgaard et al., 2003). In addition, the most abundant saturated FA; 16:0 and 18:0 and the essential PUFA 22:6(n-3), known to be an appropriate dinoflagellate fatty acid trophic marker (FATM) were focused upon.

2.3.1. Lipid composition and stable isotope analyses

TL was extracted by homogenizing animal tissues and filters in a solution of dichloromethane:methanol (2:1, v:v), modified after Folch et al. (1957). As internal standard, a known amount of the tricosanoic acid methyl ester (23:0) was added to each sample. A 0.88% solution of KCl (potassium chloride) was added to easily differentiate the biphasic system. Transesterification of the lipid extracts was performed by heating the samples with 3% sulfuric acid H_2SO_4 in methanol for 4 h at 80 °C under nitrogen atmosphere. Fatty acid methyl esters (FAME) were extracted with cyclohexane. FAME and FAlc were determined using a gas chromatograph (HP 6890 N, Agilent Technologies Deutschland GmbH & *Co*. KG) equipped with a 30 m × 0.25 mm i.d. wall-coated open tubular capillary column (film thickness: 0.25 μm; liquid phase: DB-FFAP), a split/splitless injector (250 °C) and a flame ionization detector (280 °C), according to the method of Kattner and Fricke (1986). The oven program was set from 60° to 160 °C with a rate of 30 °C min^{-1}, reaching a final temperature of 240 °C at 1.5 °C min^{-1}. Helium 5.0 was used as carrier gas at a flow rate of 1.0 mL min^{-1}. To identify unknown peaks, additional GC-mass spectrometry runs were carried out. The chromatograms were evaluated using the ChemStation software from Agilent. TL mass per individual was calculated by summing up FA and FAlc masses. The percentage of WE in TL was calculated from the proportion of FAlc on a mole basis, assuming that copepods contain no free FAlc (Kattner and Krause, 1989).

2.3.2. Carbon isotopic ratios

The ^{13}C isotopic enrichment in FA and FAlc was measured using a Thermo GC-c-IRMS (gas chromatography-combustion-isotope-ratio-mass spectrometry) system, equipped with a Trace GC Ultra gas chromatograph, a GC Isolink operated in combustion mode at 1000 °C and a Delta V Plus isotope ratio mass spectrometer connected via a Conflo IV interface (Thermo Scientific Corporation, Bremen, Germany). FAME and FAlc, dissolved in cyclohexane, were injected (1 μL) in splitless mode and separated on a DB-FFAP column (60 m, 0.25 mm I.D, 0.25 μm film thickness). The column flow was set to constant flow mode. Helium 5.0 was used as carrier gas at a flow rate of 1.6 mL min^{-1}. Injector and detector temperature was set to 250 °C. Temperature programming started at 80 °C for 2 min, increased by 20 °C min^{-1} to 160 °C, and with 2 °C min^{-1} to the final temperature of 240 °C, with a final hold for 15 min.

Linearity and precision of the mass spectrometer were checked with a series of reference gas pulses (CO_2). The isotopic composition of different amounts of reference gas (CO_2, δ 35.08 vs PDB) within a concentration interval resulting in a response of mass 44 from 400 to 6000 mV were measured in five to seven repetitions per concentration step. For each analytical run, two reference gas pulses were used for data calibration at the start and at the end together with the internal 23:0 FAME (δ −32.50 vs PDB). The chromatographic peak areas and carbon isotope ratios were obtained with the instrument-specific software (Isodat 3.0) and the reference standards 14:0 and 18:0 FAME (Iowa University) were used with known δ-values for further calculations.

Isotopic ratios of each FA and FAlc are normally expressed in δ notation according to the formula (1).

$$\delta^{13}C\,(‰) = [(R_{sample}/R_{standard}) - 1] \times 1000 \quad (1)$$

where R is the ratio $^{13}C/^{12}C$, and the commonly used standard is Vienna Pee Dee Belemnite (V-PDB): $R_{standard} = 0.0112372$.

For this study, δ-values of labelled samples were converted to atom percent, which is more appropriate than relative values to express isotope data in terms of isotope concentrations. Conversion was made according to the following Eq. (2):

$$AT\,(atom\ percent) = \frac{R_{sample}}{R_{sample} + 1} \times 100 \quad (2)$$

This equation's result includes the atom percent of enriched samples as well as their natural background (Brenna et al., 1997).

2.3.3. Morphology of mouthparts

Because differences in ingestion of food between males and females were observed during the feeding experiment in this study, dissection of mouthparts was done to compare the morphology for a possible explanation of these observations. Males and females collected in Rijpfjorden in January 2016 were picked from 4% formaldehyde preserved community samples and dissected under a stereo microscope in a 1:1 glycerol:distilled water solution on watch glass. Due to limited material only one male and one female was successfully dissected for further analysis. Mouthparts were mounted in glycerol and photographed using a Canon EOS 750D camera mounted on a Leica DM 1000 LED light microscope at 20× magnification. Pictures of the mandibular gnathobases were measured using ImageJ software 1.48v (Rasband 1997–2009) to calculate Itoh's edge index (Ie) (3) for the male and female (Itoh, 1970; Giesecke and González, 2004).

$$\mathrm{Ie} = \sum \left(\frac{w_i}{W} \times \frac{h_i}{H} \times 10^4\right)/N \quad (3)$$

Where w_i is the distance between adjacent cusps, W is the total length of the cutting edge, h_i is the depth of inter-cusp depression, H is the height of the ventral tooth and N is the number of teeth on the mandible blade. Based on the calculated Ie the copepods could be categorized as either herbivorous (Ie < 500), omnivorous (500 < Ie > 900) or carnivorous (Ie > 900) (Itoh, 1970).

2.4. Statistical analyses

Statistical analyses were done in Sigmaplot (14.0, Systat Software, San Jose, CA). One way ANOVA was applied to test the null-hypothesis of no variation between time points in the experiments. If the null-hypothesis was rejected, a post hoc pairwise comparison using Holm-Sidak method was applied to test for when a significant change from time 0 had occurred. When the data failed to pass the equal variance test (Brown-Forsythe), Kruskal-Wallis was applied instead of ANOVA to test for significant differences, and the post hoc pairwise comparison of the groups was done using the Dunn's method. For comparison of the fecal pellet production of males and females over time, a two way repeated measure ANOVA was applied, followed by Bonferroni *t*-test comparing males vs. females for each day of the experiment. When comparing only two groups, a student's t-test was applied. Significance level was set to $p \leq .05$ in all tests.

3. Results

3.1. Starvation experiment

In total, 43 males were incubated individually without food, of which seven males died soon after the start of the experiment (day <

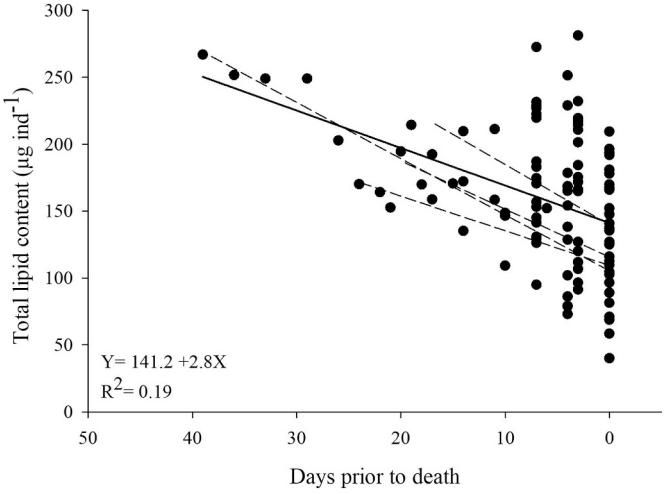

Fig. 2. Total lipid content of males of *Calanus glacialis* during the starvation experiment run at constant temperature (2–3 °C) in the dark from February to March. The time of death (TOD) is set as Day 0. The longest lived male survived for 38 days. The solid line shows the regression line for all data points. Dashed lines show regression lines for the four individuals with six or more time points (R^2 = 0.73 to 0.85).

3). These were not included in the calculations since we could not rule out the effect of handling as cause of death. The male starvation experiment was run until the last male died, in total 39 days from February to March. The prosome length of the males varied little 3.3 mm (SD 0.3) and was within the core size range of *C. glacialis* males (Daase et al., 2018). During the experiment, the TL content decreased by 2.8 µg day^{-1} per individual (linear regression, $R^2 = 0.19$, $p < .001$), and at the time of death the males had on average 131.4 (SD 44.0) µg TL per individual (Fig. 2). TL decrease rate differed between individuals, hence individual linear regression was run for the four individuals with six or more time points. Lipid decrease rates for these four individuals varied between 2.6 and 4.5 µg (mean 3.7 µg) day^{-1} per individual (linear regressions, $R^2 > 0.73$, $p < .03$) (Fig. 2). The longest survival of *C. glacialis* males in this study was 73 days, which was calculated from the day of capture to date of death. The average life span was 23 days (SD 7.3) for the males incubated, calculated from their day of capture.

3.2. Feeding experiment

During the feeding experiments in January–February 2016 both males and females were found to ingest and assimilate the microalgae, and green guts were observed (Fig. 3). Remaining microalgae in the incubation water was observed when exchanging water. From a visual comparison of the male and female side by side their mouthparts appeared to be very similar, but it was observed that the setae of the maxillipeds were longer for the female than for the male (Supplementary Fig. S1). The cutting edge of the mandible were also slightly different as indicated by Itoh's edge index which was calculated to Ie = 659 for the male and Ie = 508 for the female, categorizing them as omnivorous and borderline herbivorous-omnivorous, respectively (Itoh, 1970). The mandibular gnathobase from both the male and female (supplementary, Fig. S2) had two relatively big ventral teeth clearly separated from the other teeth. On the male gnathobase, another seven teeth were identified, with the dorsal most tooth being slightly longer than the others. On the female gnathobase another eight teeth were identified and like the male, the dorsal most tooth was slightly longer. The widths of the gnathobases were 164 µm for the male and 197 µm for the female. The teeth of the male gnathobase appeared more distinct than those at the female gnathobase.

Fig. 3. Male *Calanus glacialis* with clearly visible green gut at day 21 of the feeding experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2.1. Fecal pellet production

Both males and females had low fecal pellet (FP) production in the beginning (day 0 to 2) of the feeding experiment (0.16 FP ind^{-1} day^{-1}, SD 0.15 and 0.74 FP ind^{-1} day^{-1}, SD 0.26, respectively) but it steadily increased with time (Fig. 4). Two-way repeated measures ANOVA found a difference between sexes over time, with the males consistently producing fewer fecal pellets (Fig. 4, supplementary, Table S2) and fecals of smaller size than females (Fig. 5). The fecal pellet volume measured on day 7 was on average 2.6 $\times 10^5$ µm^3 (SD 0.9) for males and 9.3 $\times 10^5$ µm^3 (SD 5.2) for females. Females reached a maximum fecal pellet production on day 16 (Fig. 4). On day 21 males and females had similarly high fecal pellet production, but by the end of the experiment the number of individuals in each incubation bottle was very low (1–3 ind. per bottle for males and 3 to 6 ind. per bottle for females).

3.2.2. FA composition and stable isotope analyses of male and female copepods

To follow the incorporation of dietary lipids, the copepods were fed ^{13}C labelled diatoms, a monoculture of *P. glacialis*. The microalgae appeared to be in a healthy condition throughout the experiment with high Chl *a* values, comparable to spring bloom concentrations (4–10 µg Chl *a* L^{-1}) with modest Chl *a*: phaeophytin ratios (~ 1:1) (Supplementary, Table S1). Dominant (> 10%) FA in *P. glacialis* was 16:0, 16:1(n-7), 18:0 and 20:5(n-3) (Table 1). For the ^{13}C labelling, the

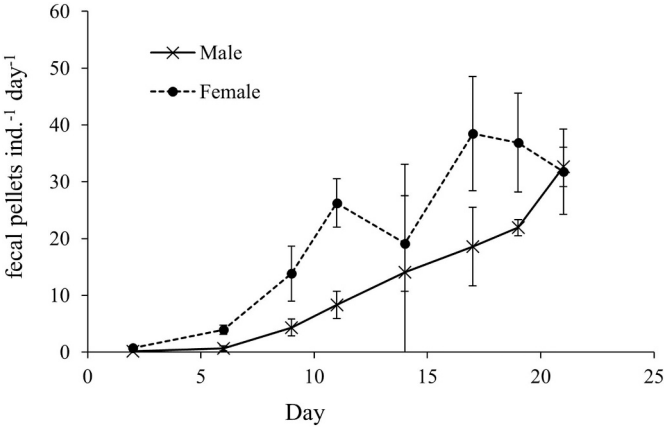

Fig. 4. Fecal pellet production (mean ± SD) of *Calanus glacialis* males and females fed ad libitum with a diatom monoculture of *Porosira glacialis* in winter.

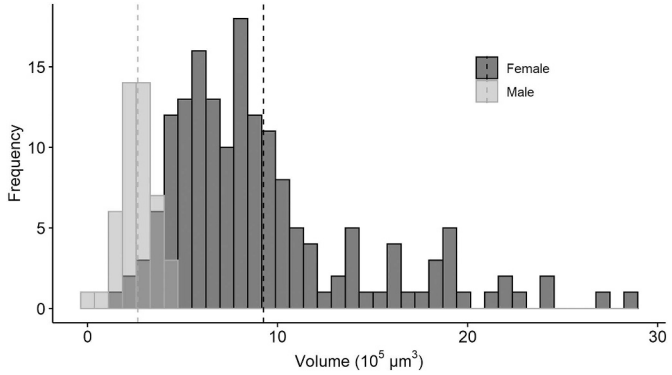

Fig. 5. Histogram showing the volume distribution of fecal pellet of *Calanus glacialis* males (light grey) and females (dark grey) at day 7 of the feeding experiment. Overlapping data in medium grey.

analyses showed enrichment, albeit poorly for the microalgae (Supplementary Fig. S3). However, it was sufficient to prove incorporation of the most important algae FA in the lipids of the copepods. On Day 11, the highest ^{13}C enrichment in algal lipids was found in 20:5(n-3) at 1.3 AT.%, followed by 14:0 (1.2 AT.%), 16:0 (1.1 AT.%) and 16:1(n-7) (1.2 AT.%) (Supplementary, Fig. S3).

For the male *C. glacialis* the TL content kept stable throughout the feeding experiment (Kruskal-Wallis, $H_3 = 2.9$, $p = .41$) with an average TL weight of 147.3 µg ind^{-1} (SD = 23.4). Major FA (> 10%) in the males were, in descending order, 20:1(n-9), 16:1(n-7), 22:1(n-11) and 14:0. Major FAlc were 20:1(n-9) and 22:1(n-11) which constituted 83–84% of the FAlc for the duration of the experiment (Table1). The proportion of WE did not change, but were stable at an average 87.3% (One way ANOVA, $F_{3,9} = 2.35$, $p = .14$) for the duration of the feeding experiment.

The TL content in *C. glacialis* females were stable around 160 µg ind^{-1} (SD 16.4) throughout the feeding experiment (One way ANOVA, $F_{3,11} = 1.84$, $p = .20$) (Table 1). Major FA in the females were, in descending order, 20:1(n-9), 16:1(n-7), 22:1(n-11), 14:0 and 16:0. Another important FA was 22:6(n-3) which increased from 3.6% (3.2 µg ind^{-1}) on day 0 to 6% (5.0 µg ind^{-1}) on day 21. Major FAlc were 20:1(n-9) and 22:1(n-11) which constituted 82–85% of the FAlc for the duration of the experiment. The proportion of WE in females varied through the experiment (One way ANOVA, $F_{3,11} = 10.21$. $p = .002$) (Table 1) fluctuating between 74.9% (SD 2.3) and 90.0% (SD 5.0) with no clear trend throughout the experiment.

Feeding on ^{13}C labelled diatoms resulted in a significant increase of this heavier C isotope in the PUFA 20:5(n-3) in both males and females. On day 0 the natural background of ^{13}C of 20:5(n-3) in the copepods was $\delta\ ^{13}C = -27.1$ for males and $\delta^{13}C = -28.3$ for females. Both for the males and the females a ^{13}C enrichment in 20:5 (n-3) was found with time on day 21 (Kruskal-Wallis, $H_3 > 8.0$, $p < .046$) (Fig. 6) and the ^{13}C enrichment was higher in females ($\delta\ ^{13}C = 29.5$, SD 13.0) than males ($\delta\ ^{13}C = -4.2$, SD 10.5) on day 21 (Student's *t*-test, $t_4 = 3.5$, $p = .025$).

4. Discussion

4.1. The life span of male Calanus glacialis

In the Arctic, *Calanus* males are mainly present during the winter. The lack of feeding combined with active mating behaviour and rapid depletion of lipid resources have been regarded as the most likely reason for the short life span of *Calanus* males (Raymont and Gross, 1942; Kosobokova, 1999). From the starvation experiment we observed that *C. glacialis* males had considerable amount of lipids (131.4 µg ind^{-1}) left at time of death. In contrast, similar experiment with females by Hatlebakk (2014) showed that females completely depleted their lipid sac and still survived. Respiration rates of *Calanus* males in January in Svalbard suggest a carbon demand equivalent to a consumption of 6.3 µg carbon day^{-1} (Daase et al., 2018), which is twice as high as the estimated lipid decrease rate of 2.8 µg TL day^{-1} (range 2.6–4.5 µg TL day^{-1}) in this study. However, high individual variability in the respiration rates was found and was only measured for the most active males in the study to Daase et al. (2018). It could be that males in our lipid decrease study had lower metabolic activity as it has been found that male copepods kept away from females tend to live longer, which may be connected to lower activity levels (Burris and Dam, 2015). Assuming roughly 10% of TL to be structural lipids (Lee, 1975), these rates suggest that males had on average 19 to 42 days' worth of lipids left at the time of death. This indicated that their death was not a direct cause of lipid reserve depletion. The longest life span of males recorded in this study was 73 days (average 43 days, SD 6), from capture in January to death at the end of March. To our knowledge, this is the first record of *C. glacialis* male life span under laboratory conditions. Peak male abundance has been recorded to be December/January (Bailey, 2010) in a seasonal study in Billefjorden, Svalbard with end of male appearance in late February. This suggests that the life span of *C. glacialis* males do not exceed 100 days and that most males live much shorter.

4.2. Feeding activity of male and female Calanus glacialis

In this study we fed our *Calanus* unrealistically high microalgae concentrations for being the winter season, although ice algae may start to grow as early as beginning of March in Svalbard (Hegseth, 1998). Reasons for these high algal concentrations were to ensure data above detection limit for studying males' capability of feeding and to assimilate ingested food. Feeding ability to *Calanus* females, on the other hand, are well-known. Females manage to utilize the early growing sea ice algae which may precede the phytoplankton bloom by 2 months (Søreide et al., 2010). Inclusion of females in our feeding experiment thus gave valuable comparative data and a “female-reference” knowing that females are capable to quickly respond to changes in the algal food environment during the winter-spring transition (Wold et al., 2011; Daase et al., 2013). Clearly visible green guts in both males and females showed that both sexes were capable of ingesting the microalgae they were offered in mid-winter. Moulting and onset of feeding characterise the final phase of diapause for the sibling species *C. finmarchicus* (Hirche, 1996). The slow start and the steadily increase in ingestion rate throughout the feeding experiment showed that both females and males in this study were in a transition state and had not completely terminated diapause at the start of the feeding experiment. Our results for the females are in agreement with Toxværd et al. (2018) who measured fecal pellet production in *C. glacialis* females in March–April when they were fed after an overwintering period without food. Also in this experiment the FP production started out low and increased with time, but the initial response in Toxværd et al. (2018) was steeper and the copepods here reached a stable high FP production quicker than in our study. Main reason for this may be the difference in timing of these two experiments with females' being less “dormant” in March–April than in February (Freese, 2015).

Compared to the females, males needed more time to adjust and optimize to the new, sudden favourable food conditions in our study. They had consistently lower fecal pellet production, with the exception of the very last day of the experiment. The volume of the fecal pellets was also smaller for males than females with a female to male ratio of 3.5 to 1. The fecal pellets appeared to be intact, hence we did not consider fragmentation (coprorhexy) and consumption (coprophagy) of fecal pellets to have influenced the results. Our results are in agreement with the experimental findings from Raymont and Gross (1942) for the sibling species *C. finmarchicus*. Through several feeding experiments they found that females produced 2–10 times as many fecal pellets as males and that the volume of the fecal pellets in the experiments with the diatoms *Skeletonema* and *Ditylum* had a female to male ratio of 5.5 to 1 and 6.5 to 1, respectively. Fecal pellet size has been connected to food concentration, with low concentrations leading to smaller fecal pellets since the copepods are not able to ingest sufficient food to fill the

Table 1
Fatty acid and alcohol composition per individual *Calanus glacialis* female and male at days 0, 2, 11 and 21 of the feeding experiment run in January–February 2016. Values given as mass% of total fatty acids and alcohols, respectively, unless otherwise specified.

Time(day)	Females				Males			
	00 (n = 3)	02 (n = 4)	11 (n = 4)	21 (n = 3)	00 (n = 3)	02 (n = 4)	11 (n = 4)	21 (n = 3)
Fatty acids								
14:0	11.4	10.9	11.4	7.1	10.2	11.0	13.1	7.8
15:0	2.0	1.6	1.4	1.4	1.5	1.7	2.0	1.6
16:0	9.7	9.0	8.0	10.4	8.7	9.1	9.5	8.8
16:1(n-5)	0.6	0.2	0.6	0.7	0.6	0.6	0.6	0.4
16:1(n-7)	13.0	14.0	17.8	10.3	15.0	12.6	12.3	9.7
16:2(n-4)	0.9	0.5	0.9	0.8	0.9	0.7	0.8	0.7
16:3(n-4)	0.4	0.1	0.2	0.3	0.3	0.3	0.3	0.3
16:4(n-1)	0.7	0.1	0.2	0.1	0.2	0.2	0.1	0.2
17:0	–	–	–	0.2	–	–	–	–
18:0	0.9	0.7	0.8	1.0	0.7	0.9	0.9	1.8
18:1(n-5)	0.8	0.7	0.6	1.0	0.8	0.8	0.8	0.9
18:1(n-7)	1.0	1.0	1.0	1.4	0.9	1.0	0.8	0.9
18:1(n-9)	7.6	7.6	5.9	8.4	6.6	7.1	7.4	8.2
18:2(n-6)	–	1.9	1.3	1.7	1.3	2.0	1.7	1.6
18:3(n-3)	2.0	1.8	1.2	1.8	1.4	1.3	1.4	1.4
18:3(n-6)	0.4	0.1	0.2	0.1	–	–	0.2	–
18:4(n-3)	3.8	2.8	1.4	0.6	1.8	2.0	1.4	0.7
20:0	0.3	0.1	0.3	0.1	0.0	–	0.2	0.1
20:1(n-7)	1.0	0.3	0.5	0.7	0.9	1.0	0.8	0.5
20:1(n-9)	15.3	21.3	20.1	20.7	20.8	20.9	20.0	22.9
20:2(n-6)	1.1	0.7	0.2	2.5	0.6	1.5	0.8	–
20:3(n-6)	0.7	0.3	0.4	0.1	0.3	0.5	0.5	1.2
20:4(n-6)	–	0.1	0.2	0.1	–	–	0.1	0.1
20:3(n-3)	–	–	–	–	–	–	–	–
20:4(n-3)	1.1	0.5	0.8	1.1	0.9	0.9	1.0	1.1
20:5(n-3)	7.4	7.0	7.3	8.3	6.2	5.5	5.0	5.9
22:1(n-7)	–	0.2	0.3	0.1	–	–	0.2	0.3
22:1(n-9)	1.9	2.2	2.0	1.8	1.9	1.8	1.8	2.0
22:1(n-11)	10.8	10.6	10.4	9.8	11.9	11.5	11.3	14.4
22:5(n-3)	0.4	0.1	0.2	0.1	0.0	0.2	0.4	0.2
22:6(n-3)	3.6	3.0	3.3	6.0	4.3	3.7	3.5	5.0
24:1(n-9)	1.2	0.6	1.1	1.5	1.1	1.2	1.1	1.3
Alcohols								
14:0	1.6	2.1	1.7	1.1	1.5	1.7	1.5	1.5
16:0	8.6	9.4	7.9	7.1	7.7	7.7	7.5	7.7
16:1(n-7)	1.8	2.5	2.7	1.2	2.2	2.2	1.7	1.3
18:1(n-9)	2.9	3.0	2.4	2.7	2.7	2.8	2.7	3.0
18:1(n-7)	2.0	2.2	2.3	2.2	1.9	2.1	1.8	1.6
20:1(n-9)	54.2	52.6	54.1	57.0	53.6	55.3	53.1	57.7
22:1(n-11)	27.9	25.7	28.1	28.1	29.7	27.6	30.7	26.5
22:1(n-9)	0.9	2.6	0.9	0.5	0.8	0.6	0.9	0.7
Sum								
Total (μg ind^{-1})	161.4	157.6	181.2	134.9	135.5	147.8	184.5	121.6
MUFA	53.1	58.6	60.4	56.3	60.5	58.7	57.1	61.4
PUFA	22.5	18.9	17.6	23.4	18.3	18.6	17.2	18.2
SFA	24.3	22.4	21.9	20.2	21.2	22.7	25.6	20.2
FAlc (μg ind^{-1})	72.8	59.8	76.1	50.3	60.7	62.8	78.0	54.6
Wax ester %	90.2	75.8	84.0	74.6	89.6	85.0	84.5	89.9

n = number of replicates; – = below detection limit.

gut before defecating (Dagg and Walser Jr, 1986). This argues for females being more efficient than males in grazing on the *P. glacialis* in our study. Reasons for this may be related to sex differences in physiological state (e.g. Hallberg and Hirche, 1980) or possibly due to females being more herbivorous than the males (see below).

In this feeding experiment the number of individuals per 1 L bottle was rather high with potential negative impacts on the individual clearance rates (e.g. Levinsen et al., 2000). However, we feel confident that sufficient microalgae concentrations were provided. The low grazing rates as suggested by the low fecal pellet production in the beginning of the feeding experiment was more likely a result of the copepods needing time to adjust to the new favourable food conditions than reduced clearance rates due to food limitation (Morata and Søreide, 2015; Toxværd et al., 2018). This was also indicated by the increase in number of fecal pellets with no distinct reduction in number of copepods per bottle the first 11 days. Females of *C. glacialis* ingest approximately 40 μg C fem^{-1} d^{-1} in a bloom setting when their feeding activity is at the highest (Levinsen et al., 2000). We provided the copepods with approximately 929 μg C bottle^{-1} which would support 8–12 actively feeding females for 3–2 days assuming these max bloom ingestion rates determined by Levinsen et al. (2000). Based on Seuthe et al. (2007) we estimated that males egested between 0.002 and 0.41 μg C ind^{-1} day^{-1} and females egested between 0.03 and 1.69 μg C ind^{-1} day^{-1} during the feeding experiment. Assuming the same and constant respiration rates as measured by Daase et al. (2018), this means a minimum carbon demand of 6.71 μg C ind^{-1} day^{-1} and 4.49 μg C ind^{-1} day^{-1} for males and females respectively. Since the TL levels remained stable throughout the experiment the net carbon budget should be close to I = E + R, where I is ingestion, E is egestion and R is respiration. If we apply this to the longest sampling interval, where algae was not replenished for four days, and assume maximum number of individuals in a bottle (n = 20), the total carbon demand

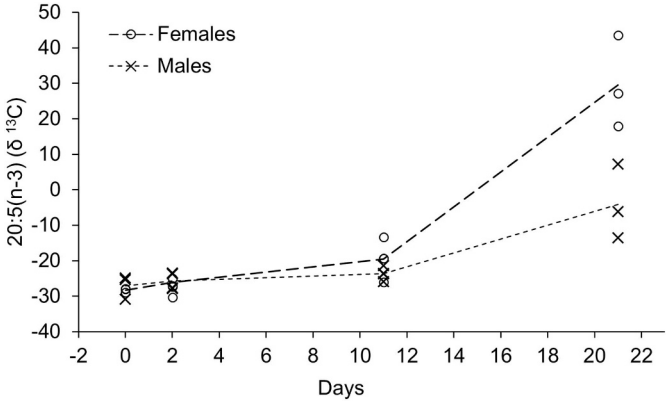

Fig. 6. ^{13}C enrichment of the essential polyunsaturated fatty acid (PUFA) 20:5(n-3) in male and female *Calanus glacialis* throughout the feeding experiment run in winter.

amounts to 536.8 µg carbon or 58% of the carbon they were offered. In addition, microalgae were still abundant (M. Hatlebakk pers. observation) when water was renewed. We are therefore confident that the copepods grazing rates were not limited by the experimental set up.

4.3. Utilization of ingested microalgae

The ^{13}C labelling of the *P. glacialis* algae culture in the feeding experiment was poor. It was detectable only in the measurements from day 11 of the experiment at a maximum enrichment of 1.3 AT.% (20:5(n-3)) which is low compared to similar experiments, which reached an average enrichment of 15.3 AT.% (Boissonnot et al., 2016) and 37 AT.% (Graeve et al., 2005). Reasons for this is not known, but something may have happened with the storage and shipment of the lipid samples since the FA composition of the microalgae samples showed various signs of being degraded with its high proportions of 18:0 (up to 60%) and poor PUFAs (Dalsgaard et al., 2003) despite that the Chl *a* concentrations measured were high (4.1–9.5 µg Chl *a* mL^{-1}; Supplementary Table S1). Despite the apparently poor labelling of the microalgae, we were able to follow an increase in the ^{13}C enrichment in copepods' FA with time. The essential PUFA 20:5 (n-3) is selectively retained by the copepods (Graeve et al., 2005), and a ^{13}C enrichment of this important PUFA was detected with time both in the females and the males by the end of the feeding experiment. The females appear to have been quicker to assimilate the microalgae dietary lipids and showed a higher enrichment in 20:5 (n-3) on day 21 than the males. The reason for this slow or delayed assimilation of the algal food most likely corresponds to the time it takes for the copepods to respond to the change in food conditions and up-adjust their digestive enzyme activity as shown by Freese (2015). As mentioned above, the females and males were most likely not fully awake from the winter dormancy at the start of the feeding experiment, meaning that the metabolism was still reduced. Freese (2015) studied the regulation of digestive enzyme activity in CV of *C. glacialis* under different light and food conditions in autumn, and even if the copepods were offered surplus algal food it took minimum 10 days before there was a significant increase in digestive enzyme activity. This delay was explained by the copepods already being in diapause when captured in field and that they needed time to mobilize again. Though it took some time before the copepods in our feeding experiment incorporated dietary ^{13}C into their lipids, they did appear to sustain their basic metabolic needs during the experiment since no reduction in TL content was observed in either females or males from day 1 to day 21. Based on the lipid consumption rates estimated for males from the starvation experiment (Sections 3.1 and 4.1), the males should have spent approximately ~65 µg of their lipid reserves over the course of the feeding experiment (21 days), almost half of the measured TL at day 0. Looking at the carbon budget (Section 4.2) we see that the copepods were offered more carbon than they spent and the stable TL content over the duration of the feeding experiment thus reflects that they were able to sustain themselves on the food offered. Even if the TL content of the females kept stable, the amount of FAlc decreased over the course of the experiment, from 45.1% to 37.3%, indicating a 16% reduction in the amount of WE of TL. WE are the primary long-term storage lipids of copepods and these are used extensively by the females during gonad maturation, even if they are actively feeding (Hirche and Kattner, 1993). For female *C. glacialis* the WE content was reduced by 20% when fed and 31% when starved, with the steepest WE decline seen during gonad maturation and not during egg production (Hirche and Kattner, 1993). In our study several of the females had well developed gonads (Hatlebakk, pers. obs.) at the end of the feeding experiment in February, which is rather early, but access to food had probably sped up the gonad maturation (Rey-Rassat et al., 2002).

The twice as high respiration rates in males versus females in winter (Daase et al., 2018) confirms that males are the most active part in mating, spending energy searching for the females (Kiørboe, 2008). Higher swimming activity results in higher risk of predator encounter, but also higher chance of encountering food particles. From the feeding experiment we observed that females had a higher feeding efficiency than males, which made us investigate potential sexual dimorphism in the mouth parts as part of the explanation. Though it was not originally planned in this study, we were able to investigate the mouthparts of a few specimens collected at the same time and site as those used in the feeding experiment. When we studied the mouth parts of males and females the setae on the maxillipeds of males were slightly shorter than those for the females, a trait that has been connected to omnivorous copepods (Schnack, 1989). In addition, Itoh's edge index categorized the males as omnivores, while it for females suggested females to be less omnivorous, on the borderline herbivores-omnivore (Itoh, 1970). Itoh's index is considered a simple indicator of diet (Giesecke and González, 2004). Nevertheless, it is interesting that the Arctic *Calanus* males may be better equipped for an omnivorous diet than the females. Though access to microalgae is poor during the polar night, the waters are not void of food particles for filter feeders like *Calanus*. The winter protist community in Svalbard are typically made up of *Gymnodinium* (Dinophyceae) and unidentified nanoflagellates, as well as a clear presence of Bacillariophyceae cells, most likely introduced from the sediments through strong vertical mixing. Even if the overall biomass (0.001–0.1 g C m^{-2}) in winter is low (Kubiszyn et al., 2017), there is a presence of organisms that could potentially be consumed by e.g. *C. glacialis* males.

4.4. Aging - another potential cause of death?

Kirkwood (2002) identifies extrinsic mortality as the principal driver for length of life, meaning that if external factors greatly limit the life expectancy, there will be no selection for maintaining costly body processes to counter act aging. Male *C. glacialis* are dependent of stored resources since they are only present in winter, and they expose themselves to higher risk of predator encounter by actively searching for females (van Duren and Videler, 1996), factors that contribute to high extrinsic mortality. Other male copepods, e.g. *Paraeuchaeta norvegica*, which primarily utilize stored resources, have been shown to only produce a limited amount of high quality spermatophores (Hopkins, 1978; Burris and Dam, 2015). These males do not benefit from a longer life, but are better served by quick investment in reproduction (Bonduriansky et al., 2008; Ceballos and Kiørboe, 2011). This suggest that even though the males have not depleted their lipid storage, important FA, such as essential PUFAs (e.g. Docosahexaenoic acid (DHA) or Eicosapentaenoic acid (EPA)), could have been limited because they have been prioritized for sperm production and thus hindered the copepod from maintaining bodily functions. DHA and EPA are vital components of the cell membrane and play various roles in mediating and controlling several physiological processes (Sargent and Falk-Petersen, 1988; Ahlgren et al., 2009). Unfortunately, the FA composition of the males at day 0 and time of death was not analysed in

the starvation experiment so further studies are needed to investigate this hypothesis closer.

4.5. Concluding remark

Depletion of the energy storage, the oil sac, was not the main cause for the short life span of *C. glacialis* males. Further, males were also found to be capable to feed, although less efficiently on microalgae than females, which may be due to males being more omnivorous combined with slower digestive enzyme recovery than females. Main reasons for males short life span is thus not resolved and we recommend more research on the role of essential FA and aging as determining factors for males' life span.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jembe.2019.151226.

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