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Membrane lipid sensitivity to ocean warming and acidification poses a severe threat to Arctic pteropods

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Ocean warming and acidification will be most pronounced in the Arctic. Both phenomena severely threaten thecosome pteropods (holoplanktonic marine gastropods) by reducing their survival (warming) and causing the dissolution of their aragonitic shell (acidification). Lipids, particularly phospholipids, play a major role in veligers and juveniles of the polar thecosome pteropod *Limacina helicina* comprising more than two-thirds of their total lipids. Membrane lipids (phospholipids) are important for the temperature acclimation of ectotherms. Hence, we experimentally investigated ocean warming and acidification effects on total lipids, lipid classes, and fatty acids of Arctic early-stage *L. helicina*. The temperature and pCO₂ treatments chosen resembled Representative Concentration Pathway model scenarios for this century. We found a massive decrease in total lipids at elevated temperatures and at the highest CO₂ concentration (1,100 μatm) of the *in situ* temperature. Clearly, temperature was the overriding factor. Total lipids were reduced by 47%–70%, mainly caused by a reduction of phospholipids by up to 60%. Further, based on pH_T development in the incubation water of pteropods during the experiment, some evidence exists for metabolic downregulation in pteropods at high factor levels of temperature and pCO₂. Consequently, the cell differentiation and energy balance of early-stage larvae were probably severely compromised. Comparison of our experimental with 'wild' organisms suggests phospholipid reduction to values clearly outside natural variability. Based on the well-known significance of phospholipids for membranogenesis, early development, and reproduction, negative warming effects on such a basal metabolic function may be a much more immediate threat for pteropods than so far anticipated shell dissolution effects due to acidification.

KEYWORDS

ocean warming, ocean acidification, arctic, pteropods, lipids

Introduction

Ocean warming (OW) and ocean acidification (OA), both in isolation and in combination, impact marine life in various ways, ranging from cellular- and physiological- to population-, community-, and ecosystem-level responses (Harvey et al., 2013; Kroeker et al., 2013; Hammill et al., 2018; Riebesell et al., 2018). Ocean warming and acidification (OWA) is caused by rising atmospheric CO₂ concentrations resulting from fossil burning and land use changes since the beginning of industrialization (Sabine et al., 2004). Because the Arctic responds more rapidly to global warming and because of the high solubility of CO₂ in cold waters, expected physical and chemical changes in seawater are particularly pronounced in polar regions (Doney, 2010; Spielhagen et al., 2011; Bopp et al., 2013; Rhein et al., 2013). Depending on the Representative Concentration Pathway (RCP) model scenario, projected ocean surface temperature and pH changes in the European Arctic vary between +1°C and +4°C, and between −0.1 and −0.45 pH units at the end of the century relative to the nineties of the last century. Concomitantly, aragonite undersaturation ($\Omega_{ar}<1$) in the surface Arctic Ocean may have reached its maximum before 2050 (Bopp et al., 2013).

The tolerance of organisms to environmental variability and change is determined by physiological performance (Doney et al., 2012), whereas optimal physiological performance in terms of fitness and survival is defined by species-specific thermal windows (Pörtner, 2002). Among the major types of biomolecules, lipids are of utmost physiological importance, for example as membrane components (Lee et al., 1970; Hagen and Auel, 2001; Kattner et al., 2007). Temperature acclimation of ectotherms includes rapid modification of membrane lipids to a higher average amount of unsaturated phospholipid fatty acids to maintain adequate physical functioning of membranes at lower environmental temperatures (Farkas and Nevenzel, 1981; Chapelle, 1986). The effects of temperature acclimation can be amplified by elevated pCO₂ through narrowing thermal tolerance windows (Pörtner, 2008; Hofmann and Todgham, 2010). Consequently, living under future OWA conditions may alter the lipid metabolism of marine ectotherms. Polar ectotherms, that possess a narrower thermal tolerance window than temperate and tropical ectotherms, may be particularly affected (Pörtner, 2002).

A few studies so far have examined combined OWA effects on the lipid composition of marine metazoa. In some experiments that showed effects on either lipid class or fatty acid compositions, temperature was the overriding factor (Rivest and Hofmann, 2015; Valles-Regino et al., 2015; Garzke et al., 2016), while OWA had no effects on the lipids of corals except for one species (out of four) where elevated pCO₂ induced higher lipid concentrations (Schoepf et al., 2013). Moreover, pronounced genetic changes in response to OA in genes for lipid metabolism have been demonstrated (Pespeni et al., 2013).

Thecosome pteropods, marine holoplanktonic gastropods with aragonite shells, are highly sensitive to OWA, responding with reduced growth, calcification, development, and survival as well as with shell dissolution (Comeau et al., 2009; Lischka et al., 2011; Bednaršek et al., 2012; Manno et al., 2012; Bednaršek et al., 2019). The effects on metabolic rates vary with the developmental stage (Comeau et al., 2010; Lischka and Riebesell, 2017). Early life stages are particularly sensitive to OA and this sensitivity is further amplified with exposure duration and the concurrent impact of OW (Bednaršek et al., 2019). Hence, they have been suggested as candidate indicator species for the combined effects of OA and OW (Bednaršek et al., 2016).

Specifically, at higher latitudes, pteropods can dominate zooplankton communities, at times contributing more than 20% of zooplankton biomass in Arctic waters (Gannefors et al., 2005; Blachowiak-Samolyk et al., 2008; Mackas and Galbraith, 2012). They are important members of the epipelagic food web both as consumers and prey (Gilmer and Harbison, 1991; Levasseur et al., 1994; Falk-Petersen et al., 2001; Hunt et al., 2008; Karnovsky et al., 2008; Renaud et al., 2012). *Limacina helicina* (Phipps 1774) is also a regular member of the meso-/macrozooplankton community in Kongsfjord (Spitsbergen), usually constituting <1%–11% in terms of abundance (Hop et al., 2002; Walkusz et al., 2009; Lischka and Hagen, 2016; Hop et al., 2019), and in summer, occasionally dominating the macrozooplankton with up to 47% (Wesławski et al., 2000). Its natural temperature range extends from −4°C to 4°C and occasionally up to 7°C (van der Spoel, 1967). Lipids play a crucial role in the development and life cycle of *L. helicina* (Gannefors et al., 2005) and can make pteropods highly nutritive prey (Boissonnot et al., 2019).

To investigate whether and, if so, how the lipid metabolism of Arctic pteropods is impacted by OWA, we subjected early offspring of Arctic *L. helicina* at the transition from the veliger to the juvenile stage to elevated temperature and pCO₂ conditions projected to occur within this century (Bopp et al., 2013; Rhein et al., 2013) for seven days. During this time of active development and growth of larvae, lipids, in particular phospholipids, are of exceptional importance (63%–88% of total lipids in juveniles and veligers) (Gannefors et al., 2005). Hence, any harmful impact of OWA on lipid metabolism would directly transfer to population and food web dynamics and may accompany more evident response signs such as shell dissolution (Bednaršek et al., 2019). Based on the physicochemical nature of lipids, we hypothesized a pronounced decrease, particularly of the polyunsaturated fatty acid and phospholipid compositions, primarily due to temperature.

Materials and methods

Sampling of pteropods

On 21 August 2013, early-stage *Limacina* spp. (Pteropoda, Thecosomata) were collected in the deepest part of Kongsfjord

(northwest Spitsbergen) integrated from 300 m using a plankton net (100 μm mesh size, 0.2 m² mouth opening, 1 L cod end) onboard *R/V Teisten*. Onboard, freshly collected samples were stored in 5 L containers filled to the top with ambient seawater to accommodate the live zooplankton until further processing in the Kings Bay Marine Laboratory (Ny Ålesund). To characterize abiotic parameters of the natural habitat of the pteropods, temperature and salinity were recorded with a STD/CTD model SD-204 (SAIV A/S, Bergen, Norway), and water samples for the determination of the *in situ* carbonate system [pH_T, total alkalinity (A_T), inorganic nutrients] were taken with a Niskin bottle from 50 m depth. Back in the lab, early-stage pteropods (veliger larvae, early juveniles) were immediately picked up from the zooplankton samples using a pipette and placed in 2 L beakers filled with seawater provided in the lab at a mean *in situ* temperature (3.5°C, see below) allowing for a 24 h acclimation phase of the pteropods before the experiment was set up on the next day (22 August). Seawater from Kongsfjord was pumped from 80 m depth to the Marine Laboratory *via* 20 μm filters, thus leaving particles <20 μm as food for incubated pteropods. The CO₂ partial pressure at 50 m depth of pteropod collection was 258 μatm on 23 August.

Temperature and pCO₂ perturbation experiment

For perturbation experiments, present and future carbonate chemistry conditions were simulated by bubbling the seawater provided in the Marine Laboratory with CO₂ using Wösthoff gas mixing pumps (Wösthoff, Germany). Target values for the three CO₂ treatments were 400 μatm (target value = ambient CO₂), 750 μatm (target value = medium CO₂), and 1,100 μatm (target value = high CO₂). According to the RCP 8.5 scenario (Rhein et al., 2013), CO₂ levels refer to the present day, years 2080, and >2100. CO₂ levels were established in storage containers placed in two temperature-controlled rooms adjusted to two different temperature levels (3.5°C and 5.5°C). The 400 μatm treatment seawater was bubbled with ambient compressed air from the same outlet used for the production of the gas mixtures. 3.5°C was chosen as the *in situ* temperature and corresponds to the mean temperature averaged over the 300 m depth range where pteropods were collected from (= *in situ* T). The elevated temperature level of 5.5°C was chosen according to a projected 2°C increase for the Arctic Ocean in the upper 100–200 m (= high T) (Steinacher et al., 2009).

For the pteropod incubation experiment, six replicates were set up for each of the six treatment combinations. Before pipetting 200 early-stage juvenile *Limacina* spp. in each of the 300 ml glass dishes (one dish = one experimental unit = one replicate), 10 ml of the seawater provided in the laboratory was pipetted into the dishes to accommodate pteropods until the dishes were filled with the experimental seawater and covered with a glass lid. The whole procedure was performed in a crushed-ice bath to keep pteropods at an approximate *in situ*

temperature during the experiment preparation. This procedure was performed in the temperature-controlled rooms where the prepared seawater was stored. Before filling the dishes with the experimental seawater, the manipulated seawater was sampled for carbonate system parameters [total alkalinity (A_T), nutrients] and pH on the total scale (pH_T), which was measured directly (see next section). A_T samples were poisoned with 10 μl HgCl₂ and stored in a fridge for subsequent analysis in the home laboratory. Nutrient samples were immediately frozen (−20°C) (Dickson et al., 2007). The experiment started on 22 August.

The glass lids used did not close the dishes airtight, thus did not prevent gas exchange with the ambient air, but head-space was equally minimal. For this reason, and also to assure that oxygen concentrations in the dishes did not fall below critical saturation levels of oxygen partial pressure for marine mollusks due to respiration of the incubated pteropods (Childress and Seibel, 1998; Maas et al., 2011), the experimental seawater was changed on 24, 26, 27, and 28 August. Following this approach concurrently included a generally accepted metabolic acclimation phase of pteropods of 24 h (e.g., Comeau et al., 2010; Maas et al., 2012; Seibel et al., 2012) and minimized the impact of pH fluctuations by providing continuously fresh target conditions. Before each water exchange, the pH in each dish and in the fresh manipulated seawater was measured and water samples for the determination of the carbonate system (A_T, nutrients) were taken from the storage containers before the dishes were refilled with the fresh manipulated seawater (note: in order to keep the duration of water exchanges at a minimum and also to reduce the risk of losing pteropods (see below), no A_T and nutrient samples were taken from the water in the dishes). The experiment was terminated after 7 days (on 29 August). Before the juvenile pteropods were retrieved from the dishes, the pH was measured in the dishes and water samples for the determination of the carbonate system were taken (A_T, nutrients). Subsequently, specimens were collected from the dishes, and inspected for survival/mortality under a stereomicroscope (Lischka et al., 2011). All dead and live pteropods were counted. The number of pteropods recovered from the replicate dishes at the end of the experiment did not match the number initially incubated. This was most likely due to the loss of some individuals during seawater exchange. Therefore, we show mortality as the percentage of dead organisms of the total number of recovered individuals (see Supplementary Figure S1 online). Live pteropods were then carefully transferred into sample vials and stored at −80°C for later lipid analyses (total lipids, lipid class, and fatty acid compositions) in the home laboratory.

Carbonate chemistry analysis

pH measurements were done with a pH Mobile 826 pH meter (Metrohm, Germany). pH on the total scale (pH_T) was

calculated from voltage readings (Dickson et al., 2007). The precision was within 1.1 mV units. As standard, certified reference material (CRM) with a known pH calculated from known A_T and C_T (A. Dickson, Scripps Institution of Oceanography, La Jolla, California) was used.

A potentiometric titration device (Titrand 808, Metrohm) was used to determine total alkalinity (A_T) (Bradshaw et al., 1981) calculated from the Gran function (Dickson et al., 2003). The same CRM used for pH measurements was used for A_T determination. Specifically, precision (max. difference between two duplicate measurements) was within $4.9 \mu\text{mol kg}^{-1}$ and accuracy was between 6.7 and $13.5 \mu\text{mol kg}^{-1}$ (differences between measured CRM and target CRM values, depending on prepared solutions and temperatures, etc.). The remaining carbonate system parameters were calculated from measured A_T and pH_T with the free software CO2SYS (Pierrot and Wallace, 2006) using the constants from Mehrbach et al. (1973) refitted by Dickson and Millero (1987). Dissolved inorganic nutrient concentrations (PO_4^{3-} , $(\text{SiOH})_4$) were analyzed with an autosampler (XY2 autosampler, SEAL Analytical) and a continuous flow analyzer (QuAAtro AutoAnalyzer, SEAL Analytical), respectively (Mullin and Riley, 1955; Murphy and Riley, 1962). The correction of all colorimetric methods was with the refractive index method (Coverly et al., 2012). Salinity was measured using a WTW 340i conductivity meter.

Lipid class and fatty acid analysis

Total lipids were extracted three times with 3 ml of dichloromethane:methanol (2:1, v/v) from pooled (per replicate), lyophilized (24 h) and homogenized individuals using a Potter homogenizer (Sartorius, Göttingen, Germany) (Folch et al., 1957). The solvent was washed with 2 ml of 0.88% KCl solution before the mixture was centrifuged at low speed (2,000 rpm). The lipid-containing organic phase was separated and evaporated under nitrogen. The lipid extract was re-dissolved in dichloromethane and taken for analysis. All extracts were kept at -20°C for further analysis. The lipid classes were separated and identified on a monolithic silica column (Chromolith[®] Performance-Si) using high performance liquid chromatography (HPLC, LaChromElite HPLC system) with an evaporative light scattering detector (ELSD) (Graeve and Janssen, 2009). A gradient program with a combination of three solvent mixtures was used: Eluent A consisted of isooctane:ethylacetate (99.8:0.2, v/v), eluent B was a mixture of acetone and ethyl acetate (2:1, v/v) containing acetic acid (0.02% (v/v)) and eluent C 2-propanol–water (85:15, v/v) (with acetic acid and ethanolamine, each 0.05% (v/v)). The total lipid content was calculated using the sum of the neutral and polar lipids.

For the gas–liquid chromatographic analyses of the fatty acids and alcohols, aliquots of the total lipid extract were taken.

Methyl esters of fatty acids were prepared by transesterification under a nitrogen atmosphere with 3% concentrated sulfuric acid in methanol for 4 h at -80°C . The resulting fatty acid methyl esters and fatty alcohols were extracted with hexane and their composition determined using a Hewlett Packard 6890 gas liquid chromatograph on a 30 m wall-coated capillary column (i.d. 0.25 mm; film thickness: $0.25 \mu\text{m}$; liquid phase DB-FFAP) using temperature programming (Kattner and Fricke, 1986). Fatty acids and fatty alcohols were quantified with an internal tricosanoic acid methylester standard (FAME, Sigma-Aldrich, USA) and identified by comparing the retention time with those of known standard mixtures.

Raw effect sizes

For assessing the impact of an effect, effect sizes are more critical than the significance of p-values because effect sizes quantify the difference between two response values measured at two different treatment levels. P-values strongly depend on the underlying assumptions and the number of observations (Greenland et al., 2016) and are often not helpful to evaluate the importance of their associated effects (Di Stefano, 2014). Placing the focus on effect sizes rather than p-values raises greater awareness of the metrics used and how they relate to the natural state (Claridge-Chang and Assam, 2016). For example, a significant p-value determined by a response measure between two groups must not necessarily mean an effect large enough to be ecologically important on the species or population level. Therefore, we calculated effect sizes for mortality, lipid classes, dominant fatty acids, fatty acid classes (SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid), and total lipids based on the raw mean difference (D) to characterize the relevance of possible temperature and pCO_2 effects between the different treatment levels (Table 1). In contrast to common standardized effect size measures such as Cohen's d , raw effect sizes have the advantage of being in the units of the response variable and, thus, are more intuitive to interpret (Borenstein et al., 2009; Di Stefano, 2014). We calculated effect sizes for all possible combinations (contrasts) of factor levels and always subtracted the higher level response from the lower level response (Supplementary Tables S2, S3 online). Hence, positive effect sizes indicate that the response (percent contribution or amount of mortality, total lipid, a lipid class or fatty acid) at the lower factor level was larger, while negative effect sizes indicate that the higher factor level response was larger. Raw effect sizes are given together with their 95% confidence intervals. Statistics and plots were done with "R" using the packages nlme, lattice, ggplot, and effsize (R Core Team, 2014).

The raw effect size D was estimated from two contrasting group means (m_1 , m_2):

TABLE 1 Raw mean difference *D* with 95% confidence intervals (CI) in parentheses caused by the two factors (temperature [°C], pCO₂ [μatm]) at the different treatment levels on survival of pteropods (% mortality), on the %-contribution of each lipid class, on total lipids [μg ind⁻¹], and on the %-contribution of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and of dominant fatty acids (mean contribution >5% of total lipids).

Contrast

Temperature pCO ₂ Effect size	3.5	3.5	3.5	5.5	5.5	5.5	3.5/5.5	3.5/5.5	3.5/5.5
	400/750	400/1100	750/1100	400/750	400/1100	750/1100	400	750	1100
	<i>D</i> (CI)	<i>D</i> (CI)	<i>D</i> (CI)	<i>D</i> (CI)	<i>D</i> (CI)	<i>D</i> (CI)	<i>D</i> (CI)	<i>D</i> (CI)	<i>D</i> (CI)
Mortality	-0.05 (-0.75/0.64)	-0.58 (-2.25/1.08)	-0.53 (-2.25/1.19)	-0.32 (-1.30/0.66)	-1.82 (-2.79/-0.85)	-1.50 (-2.81/-0.20)	0.99 (0.49/1.48)	0.72 (-0.40/1.81)	-0.25 (-2.11/1.62)
Lipid Class									
WE	1.60 (0.32/2.87)	3.07 (1.80/4.34)	1.48 (0.72/2.23)	-0.88 (-1.79/0.03)	-0.35 (-0.98/0.37)	0.58 (-0.56/1.71)	3.45 (2.29/4.60)	0.97 (-0.08/2.03)	0.07 (-0.80/0.93)
TAG	4.73 (-1.78/11.23)	-3.83 (-10.50/2.85)	-8.55 (-15.05/-2.05)	2.62 (-6.23/11.47)	0.29 (-8.82/9.40)	-2.33 (-6.92/2.26)	-5.00 (-14.62/4.61)	-7.11 (-12.42/-1.80)	-0.89 (-6.81/5.04)
ST	0.24 (-0.86/1.34)	-9.60 (-14.77/-4.43)	-9.84 (-15.08/-4.59)	-6.23 (-8.75/-3.71)	-2.12 (-5.27/1.03)	4.12 (0.71/7.51)	-8.69 (-10.29/-7.08)	-15.16 (-17.40/-12.92)	-1.21 (-7.04/4.63)
FFA	-3.27 (-8.71/2.16)	-14.78 (-22.11/-7.43)	-11.50 (-19.96/-3.05)	-4.06 (-8.89/0.77)	-7.21 (-13.55/-0.86)	-3.15 (-8.49/2.20)	-17.50 (-22.34/-12.65)	-18.28 (-23.70/-12.86)	-9.93 (-18.33/-1.52)
PE	-2.78 (-6.47/0.91)	3.86 (-0.13/7.85)	6.65 (2.65/10.67)	1.26 (-1.61/4.13)	3.36 (-0.16/6.88)	2.10 (-0.67/4.86)	6.70 (3.06/10.35)	10.75 (7.82/13.68)	6.20 (2.32/10.08)
PI	0.36 (-0.86/1.59)	0.99 (-1.10/3.08)	0.63 (-1.36/2.62)	3.85 (-0.03/7.73)	3.85 (-0.03/7.73)	0 (0/0)	3.26 (-0.74/7.26)	6.75 (6.01/7.48)	6.12 (4.27/7.97)
PS	0.33 (-0.25/0.90)	-0.30 (-4.22/3.62)	-0.63 (-4.53/3.27)	5.93 (3.12/8.74)	0.74 (-0.94/2.43)	-5.19 (-8.17/-2.20)	-2.66 (-3.74/-1.60)	2.94 (-0.28/5.59)	-1.62 (-5.75/2.51)
CL	-0.52 (-2.26/1.21)	0.74 (-1.82/3.30)	1.26 (-1.82/4.34)	-1.7 (-3.93/0.46)	0.25 (-0.20/0.69)	1.98 (-0.21/4.17)	1.73 (1.33/2.13)	0.52 (-2.26/3.30)	1.24 (-1.33/3.81)
PC	-0.68 (-3.16/1.80)	19.83 (17.89/21.77)	20.51 (17.92/23.11)	-0.76 (-2.81/1.30)	1.14 (-1.25/3.52)	1.89 (-0.85/4.64)	18.71 (17.05/20.36)	18.63 (15.87/21.38)	0.01 (-2.58/2.60)
PL	-3.29 (-10.38/3.79)	25.12 (18.95/31.29)	28.42 (20.47/36.36)	8.55 (-0.56/16.54)	9.34 (-0.06/18.61)	0.77 (-5.20/6.77)	27.74 (19.40/36.08)	39.58 (32.91/46.25)	11.95 (4.57/19.34)
Total Lipid									
TL	-0.013 (-0.060/0.035)	0.105 (0.061/0.149)	0.117 (0.071/0.164)	0.039 (0.009/0.07)	0.022 (-0.012/0.056)	0.017 (-0.045/0.011)	0.089 (0.048/0.13)	0.141 (0.102/0.180)	0.006 (-0.031/0.044)
Fatty Acids									
SFA	0.66 (-9.38/8.05)	6.23 (-0.67/13.12)	6.89 (-3.17/16.95)	-1.14 (-8.53/6.26)	-7.31 (-16.03/1.42)	-6.17 (-11.22/-1.11)	4.32 (-3.53/12.16)	3.84 (-4.49/12.18)	-9.21 (-16.86/-1.56)
16:0	-0.22 (-1.61/1.17)	1.83 (-0.14/3.51)	2.05 (-0.04/4.14)	-1.61 (-3.42/0.21)	-3.75 (-6.24/-1.26)	-2.14 (-4.12/-0.17)	0.76 (-0.88/2.45)	-0.60 (-2.19/1.00)	-4.79 (-7.21/-2.37)
18:0	-1.40 (-7.79/4.99)	2.55 (-3.15/8.25)	3.95 (-3.75/11.65)	2.36 (-1.51/6.23)	2.50 (-2.02/7.02)	0.14 (-1.89/2.18)	1.33 (-3.37/6.02)	5.08 (-0.70/10.87)	1.28 (-4.29/6.84)
MUFA	4.37 (-3.96/12.70)	5.15 (-2.43/12.72)	0.78 (-2.79/4.35)	-2.62 (-6.20/0.96)	-0.58 (-2.50/1.34)	2.04 (-1.75/5.82)	4.04 (-3.82/11.90)	-2.94 (-7.69/1.81)	-1.69 (-3.72/0.35)
20:1(n-9)	0.80 (-0.41/2.01)	0.94 (-0.91/2.79)	0.14 (-1.92/2.19)	-0.67 (-1.10/-0.23)	-0.33 (-0.66/0.01)	0.34 (-0.06/0.75)	0.12 (-0.48/0.72)	-1.35 (-2.46/0.24)	-1.14 (-2.95/0.67)
PUFA	-3.71 (-11.53/4.12)	-11.37 (-18.68/-4.07)	-7.67 (-15.22/-0.10)	3.75 (-3.86/11.36)	7.89 (10.11/15.66)	4.14 (-1.16/9.44)	-8.36 (-16.96/0.24)	-0.91 (-7.75/5.94)	10.90 (4.67/17.13)
20:5(n-3)	-1.22 (-5.31/2.87)	-5.04 (-8.30/-1.77)	-3.82 (-7.46/-0.17)	1.56 (-2.06/5.17)	3.58 (-0.29/7.44)	2.02 (-0.03/4.06)	-3.10 (-7.45/1.26)	-0.32 (-3.68/3.04)	5.52 (3.02/8.02)
22:6(n-3)	-1.60 (-4.89/1.69)	-4.42 (-7.23/-1.58)	-2.82 (-5.64/0.01)	1.85 (-1.35/5.05)	2.68 (-0.62/5.97)	0.83 (-1.22/2.87)	-3.25 (-6.99/0.49)	0.20 (-2.54/2.94)	3.84 (1.68/6.00)

Effect sizes with their CIs not crossing zero suggest for a significant treatment effect and are indicated in bold. WE, wax ester; TAG, triacylglycerol; ST, Sterols; FFA, free fatty acids; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipine; PC, phosphatidylcholine; PL, phospholipids; TL, total lipids.

$$D = m_1 - m_2$$

For the calculation of the 95% confidence intervals, the pooled standard error (se) was estimated from the standard deviation ($sd_1, 2$) and the number of replicates ($n_1, 2$) of both groups:

$$se_{pooled} = \sqrt{\frac{sd_1 * sd_1}{n_1} + \frac{sd_2 * sd_2}{n_2}}$$

The lower and upper boundaries of the 95% confidence intervals were then calculated for small samples based on the t -distribution (<https://www.cyclismo.org/tutorial/R/confidence.html>) as follows:

$$CI_{lower/upper} = D - / + (t_{(n_1+n_2)-2}) * se_{pooled}$$

ANOVA statistics

Additionally to raw effect sizes, the effect of temperature and pCO_2 on lipid class contributions, contributions of major fatty acids, and fatty acid classes (SFA, MUFA, PUFA) were determined by a linear model applied to a two-way ANOVA. Before the test, all percentage data were arcsin transformed. In case of resulting variance heterogeneity, a generalized least square approach (GLS with restricted maximum likelihood method, REML) was applied with the varIdent function on one or both of the factor(s) to stabilize the variance structure of the residuals to apply a two-way ANOVA afterward (Table 2). α significance level was 0.05. The homoscedasticity and normality of the residuals were inspected graphically (Zuur et al., 2007). All statistics and plots were done with “R” using the packages nlme, lattice, and ggplot2 (R Core Team, 2014).

Multivariate statistics

Co-correspondence analysis (CoCA) (ter Braak, 2004; Greenacre, 2016) was used to relate the fatty acid and lipid class compositions to a joint analysis. In addition, CoCA has previously been used to relate benthos and fish community compositions in the Barents Sea (Johannesen et al., 2017). The results of CoCA are two co-varying ordinations, one of the fatty acids and the other of the lipid classes. Each of the two tables, the fatty acid composition and the lipid class composition, could be analyzed separately by correspondence analysis, each identifying the principal dimensions of the respective table, but that would ignore the fact that there is a relationship between the two. The “co-” of co-correspondence analysis focuses on that relationship and identifies dimensions that explain the covariation (= linear relationships) between the two tables, i.e., it identifies the dimensions common to the two tables. The biplots resulting from multivariate analysis show the main features of the

relationships between the variables (in this case, the lipid classes, respectively, the fatty acids) and the samples (in this case, the experimental units according to the two treatment factors, temperature and pCO_2 level). In our ordinations, the contribution biplot scaling was used (Greenacre, 2013), where the more outlying variables (fatty acids or lipid classes) are those that contribute most to the solution and thus to the co-variation. Labels of the variables with contributions of less than the average are de-emphasized to make the ordinations more legible. Added to each ordination biplot are the three standardized environmental variables: pCO_2 , pH, and Ω_{ar} . These supplementary variables have not been used in determining the solution but are added as biplot vectors afterwards, using as coordinates their regression coefficients on the two axes of the respective ordinations (Greenacre, 2016).

Comparison with “wild” pteropods

To evaluate whether the treatment effects found are large enough to be important for the pteropod population, we followed an approach suggested by Di Stefano (2014). This approach contrasts the magnitude of estimated treatment effect sizes and their associated errors (confidence intervals) with an effect size specified as being important in an ecological context *a priori*. In our study, phospholipids were the dominant lipid class in early-stage pteropods, and they showed the strongest response to temperature and CO_2 . To evaluate whether the observed changes in phospholipids are of ecological importance, we calculated the effect sizes between the phospholipid contribution found in pteropods from our experiment and published data of *L. helicina* from Kongsfjord by Gannefors et al. (2005). These authors report phospholipid contributions of between 88% (SD 8.8, SD = standard deviation) in veliger larvae collected in September and 62% (SD 9.5) in juveniles collected in May. The most conservative approach to judging the outcome of such a comparison was to assume the lowest average phospholipid contribution (62%) to be the minimum required amount to assure normal performance of *L. helicina*. Thus, we contrasted the %-contribution of phospholipids determined in our experiment at the different factor-level combinations each separately with the 62% of juveniles and, moreover, the 88% mean phospholipids described for veligers (Gannefors et al., 2005):

$$D_{field\ comparison} = mean_{present\ study} - mean_{Gannefors}$$

with:

* $D_{field\ comparison}$ = Difference (raw effect size) between % phospholipid at each factor level combination of present experiment and % phospholipid of juveniles/veligers of Gannefors et al. (2005)

* $mean_{present\ study}$ = mean % phospholipids at each factor level combination of present study

TABLE 2 Summary of two-way ANOVA statistics and applied model for each response variable (LM, linear model; GLS, Generalized Least Squares).

FACTOR	Temperature			pCO ₂			Temperature*pCO ₂			Model
	df	F	p	df	F	p	df	F	p	
RESPONSE										
Mortality	1	3	0.0820	2	3	0.0561	2	3	0.0539	LM
Lipid Class										
WE	1	31.96	<0.0001	2	8.30	0.0014	2	10.33	0.0004	LM
TAG	1	6.22	0.0183	2	3.42	0.0458	2	1.31	0.2885	LM
ST	1	524.83	<0.0001	2	6.68	0.004	2	21.46	<0.0001	GLS
FFA	1	89.87	<0.0001	2	16.35	<0.0001	2	3.76	0.035	LM
PE	1	74.38	<0.0001	2	8.90	0.001	2	1.76	0.1893	LM
PI	1	1707.83	<0.0001	2	3.02	0.0638	2	2.43	0.1052	LM
PS	1	52.17	<0.0001	2	1.68	0.2038	2	7.33	0.0026	GLS
CL	1	170.34	<0.0001	2	2.35	0.1125	2	0.91	0.4135	GLS
PC	1	680.00	<0.0001	2	70.52	<0.0001	2	47.83	<0.0001	GLS
PL	1	171.55	<0.0001	2	28.04	<0.0001	2	14.86	<0.0001	LM
Total Lipid										
TL	1	60	<0.0001	2	14	<0.0001	2	15	<0.0001	LM
Fatty Acids										
SFA	1	0.49	0.4899	2	3.99	0.0319	2	12.28	0.0002	GLS
16:0	1	13.98	0.001	2	35.08	<0.0001	2	9.57	<0.0001	GLS
18:0	1	3.20	0.0865	2	0.90	0.4186	2	2.65	0.0909	LM
MUFA	1	0.57	0.456	2	2.29	0.123	2	0.35	0.708	LM
20:1(n-9)	1	2.20	0.1511	2	29.51	<0.0001	2	6.46	0.0057	GLS
PUFA	1	0.22	0.6423	2	1.31	0.2876	2	5.52	0.0107	LM
20:5(n-3)	1	0.75	0.3949	2	1.13	0.3403	2	4.11	0.0293	LM
22:6(n-3)	1	0.29	0.5985	2	1.88	0.1752	2	3.39	0.0507	LM

Percent contributions of lipid classes and dominant fatty acids were arcsin transformed for analysis, total lipid mass was untransformed. For all applied GLS models, the varIdent function was applied on both factors, i.e. temperature and pCO₂. WE, wax ester; TAG, triacylglycerol; ST, Sterols; FFA, free fatty acids; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipine; PC, phosphatidylcholine; PL, phospholipids; TL, total lipids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Significant effects are indicated in bold.

* $\text{mean}_{\text{Gannefors}} = \text{mean \% phospholipids of juveniles (62\%)} \text{ and veligers (88\%), respectively, of Gannefors et al. (2005)}$

The 95% confidence intervals for $D_{\text{field comparison}}$ were estimated like above using the standard deviation given in Gannefors et al. (2005) for juveniles and veligers.

Finally, calculated effect sizes not larger than one standard deviation reported for the *in situ* phospholipid values of juveniles and veligers by Gannefors (i.e., SD 9.5 and SD 8.8; so-called '1-SD-line' in Figure 8) were defined as sustainable, whereas effect sizes larger than one negative SD were defined as unsustainable for the pteropod population.

Results

Carbonate system in the experimental treatments

Carbonate chemistry analysis indicated clear distinctions among treatments also with respect to the saturation states of

aragonite (Ω_{ar}). At the start of the experiment, for both temperature treatments, Ω_{ar} was oversaturated at ambient CO₂ (1.66, 1.86), it was close to the saturation threshold at medium CO₂ (1.18, 1.35), and under-saturated at high CO₂ (0.85, 0.97) (Supplementary Table S1 online). Accordingly, initial pH values in the experimental dishes (black dots in Figure 7) were well separated between the three pCO₂ treatment levels, i.e., decreasing from the ambient to the high pCO₂ level.

Lipids

Total lipids and lipid class composition

Total lipids ranged between 0.06 $\mu\text{g ind}^{-1}$ and 0.20 $\mu\text{g ind}^{-1}$ in all treatment combinations and constituted an average of 0.19 $\mu\text{g ind}^{-1}$ in the control treatment (*in situ* T/ambient CO₂) (see Supplementary Table S2 online). Phospholipids were the predominant lipid class of juvenile pteropods in all treatments and at all factor levels, contributing between 26% and 66% of

total lipids (Figure 1, see Supplementary Table S2 online). Free fatty acids (FFAs, 12%–36%) and triacylglycerols (TAGs, 9%–19%) were the second and third most important lipid classes. Sterols (STs) contributed on average 8%–23% to total lipids, wax esters (WEs) played a minor role (0%–3%) (Figure 2).

Treatment effects on total lipids and lipid classes

Based on calculated effect sizes, increased temperature induced clear negative effects on the amount of total lipids at ambient CO₂ and medium CO₂ (effect sizes between 3.5°C and 5.5°C: 0.089 and 0.141 μg ind⁻¹, respectively) (Table 1 and Figure 1). Moreover, at the high CO₂ level, total lipids were drastically reduced at 3.5°C (effect size up to 0.117 μg), indicating a strong CO₂ effect at *in situ* T.

The proportion of phospholipids (= the sum of phosphatidylcholine (PC) + phosphatidylethanolamine (PE) + phosphatidylinositol (PI) + phosphatidylserine (PS) + cardiolipine (CL)) was significantly (i.e., clearly separated CIs) lower at high T with a maximum effect size of 40% between the two temperatures at medium CO₂. The factor CO₂ initiated distinct effects with CIs separate from zero only at *in situ* T (25%–28%). The loss of phospholipids was largely compensated by a concomitant increase in FFAs and STs with higher factor levels (see further below).

Among phospholipids, effect sizes (partly 18%) were largest for PC: proportions were generally higher at *in situ* T and a CO₂ impact was only visible at *in situ* T (Figure 2). PE, PI, PS, and CL revealed effect sizes of up to <11% in response to the factor levels. CIs are quite narrow for all phospholipids, suggesting consistent responses.

Proportions of FFAs at all CO₂ levels were higher at the higher T level (effect sizes of 10%–18%). TAGs and STs occurred in higher proportions at high T, except for the high CO₂ level, where proportions were very similar at both temperatures. The largest temperature and CO₂ effects for TAGs and STs ranged between 7% and 15%. WEs had minor contributions at all factor levels, and the effect sizes ranged between 3.5% and ≤1.6%.

Generally, based on two-way ANOVA, temperature was the overriding factor shaping the total lipid and lipid class composition of early-stage pteropods exposed to OWA conditions ($F_T > F_{CO_2}$, p -values usually <0.0001, Table 2). An additional significant CO₂ effect was determined in the case of TAGs and PEs, and significant synergistic effects in the case of WEs, STs, FFAs, PSs, PCs, PLs, and TLs, which were highly significant in the case of STs, PCs, PLs, and TLs ($p < 0.0001$).

Fatty acid composition

The dominant fatty acids (defined as mean percent contribution to total lipid amount >5% across treatments and all factor levels) were the SFAs 16:0 and 18:0, the MUFA 20:1(n-9), and the PUFAs 20:5(n-3) and 22:6(n-3) (The (n-x) notation

indicates the position of the first double bond from the terminal methyl group of a fatty acid, i.e., in the case of (n-3), the first double bond is three C-atoms away from the terminal methyl group) (Figures 3–5, and Supplementary Table S3 online). The sum of SFAs varied between 28.5% and 38%. The share of MUFAs was between 19.4% and 14.2%. The sum of PUFAs had the highest contributions at all factor levels, varying between 46% and 57.3% at *in situ* T, and between 46.4% and 54.3% at high T.

Treatment effects on fatty acids

Among the dominant fatty acids, the largest effect sizes associated with well-separated CIs were determined for the SFA 16:0 and the PUFA 20:5(n-3) (effect sizes of 4.8% and 5.5%, respectively) for the temperature contrast at high CO₂ (Table 1 and Figures 3, 5). The PUFA 22:6(n-3) was also distinctly affected, first along the CO₂ gradient (effect size 4.4% between ambient/high CO₂) and second largest by temperature at high CO₂ (effect size 3.8%). The MUFA 20:1(n-9) showed only for the ambient/medium CO₂ contrast at high T a distinct but small difference with non-overlapping CIs (0.34%) (Figure 4).

SFAs and PUFAs showed pronounced interactive effects along the temperature/CO₂ gradient with partly clearly separated CIs but opposite effect directions and particularly pronounced effects at high T/high CO₂ (effect sizes up to >9% for SFA, and up to >11% for PUFA) (Table 1). In the case of MUFAs, all CIs of identified effect sizes overlapped, suggesting a non-consistent response nature.

Consistently with effect sizes, two-way ANOVA confirmed significant synergistic effects for SFA ($F_{T \times CO_2} = 12.28$, $p = 0.0002$), 16:0 ($F_{T \times CO_2} = 9.57$, $p < 0.0001$), PUFA ($F_{T \times CO_2} = 5.52$, $p = 0.0107$), and 20:5(n-3) ($F_{T \times CO_2} = 4.11$, $p = 0.0293$), at which for 20:1(n-9) a significant pCO₂ effect was determined ($F_{CO_2} = 29.51$, $p < 0.0001$).

Multivariate analysis

The joint analysis of the fatty acid and lipid class compositions using co-correspondence analysis (CoCA) is shown in the two ordination plots of Figure 6. The CoCA identifies the most important common axes in the two compositions that maximize the co-variation between the samples in the fatty acid and lipid class spaces. The fatty acids and lipid classes that contribute to these common axes are thus identified as strongly associated.

These two common dimensions are highly significant ($p < 0.001$ and $p = 0.009$, respectively, using a permutation test with 999 permutations). The first dimension generally contrasts the CO₂ treatment levels at *in situ* T, with fatty acids such as 22:1 (n-11) and 20:1(n-7) being higher than average in the low CO₂ group (Figure 6A), as well as lipid classes PC and WE (Figure 6B). The second dimension contrasts the CO₂ levels at high T, with fatty acids such as 14:0, 15:0, and 16:0 higher in the low CO₂ treatment compared to 18:0, 18:2(n-6), 22:6(n-3), and

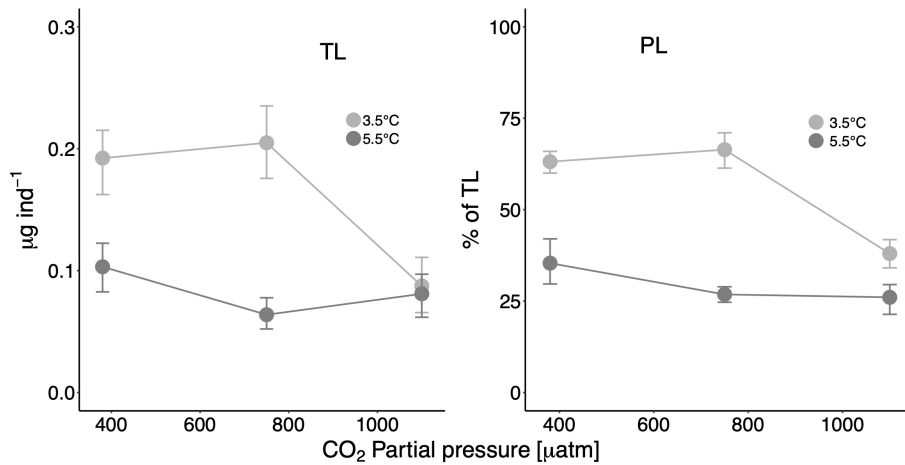


FIGURE 1
Total lipid (TL) mass ($\mu\text{g ind}^{-1}$) and percent contribution of phospholipids (PL = %PC + %PE + %PI + %PS + %CL) on TL at the different treatment and factor levels. Error bars depict 95% confidence intervals.

20:5(n-3) higher than average in the high CO_2 treatment (Figure 6A), as well as the lipid class PI (Figure 6B).

The three supplementary variables added to each ordination are significantly correlated with the second dimension ($p < 0.05$ in all cases), showing they are relevant to the contrast in CO_2 levels in the high T treatment group.

Thus, the results clearly show the differences between the treatment groups and which fatty acids are responsible for those differences. pCO_2 contrasts both the low (*in situ*) temperature

treatment group as well as the higher temperature group, but involving different fatty acids.

pH_T development in the experimental dishes

The experimental dishes were not covered gas-tight and had minimal head space (see *Materials and methods*). Thus, pH

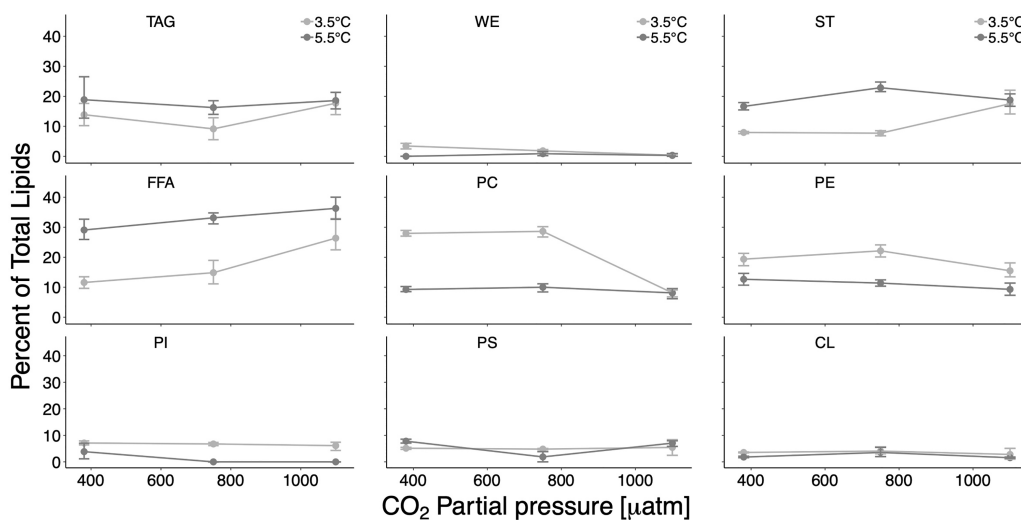
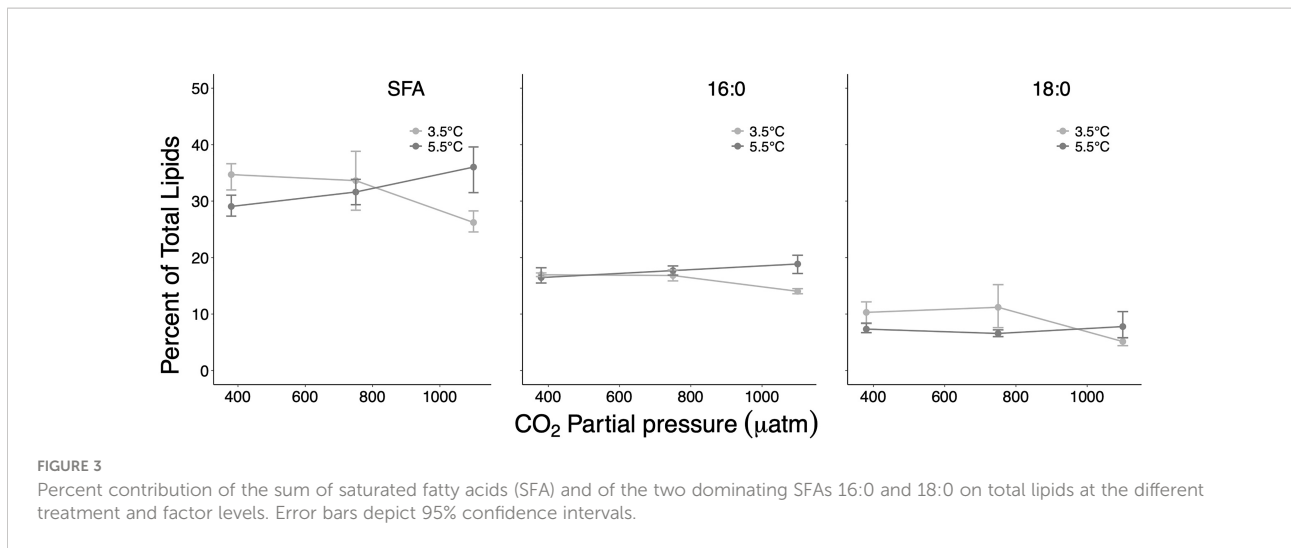


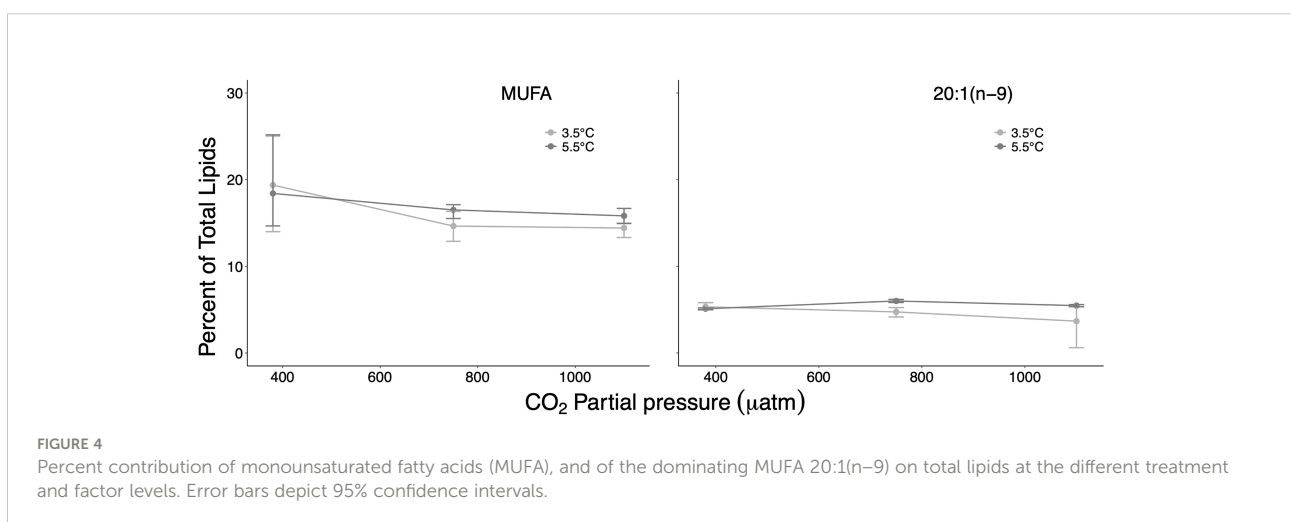
FIGURE 2
Percent contribution of each lipid class on the total lipid content (total lipids) per individual. TAG, triacylglycerol; WE, wax ester; ST, sterols; FFA, free fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipine. Error bars depict 95% confidence intervals.

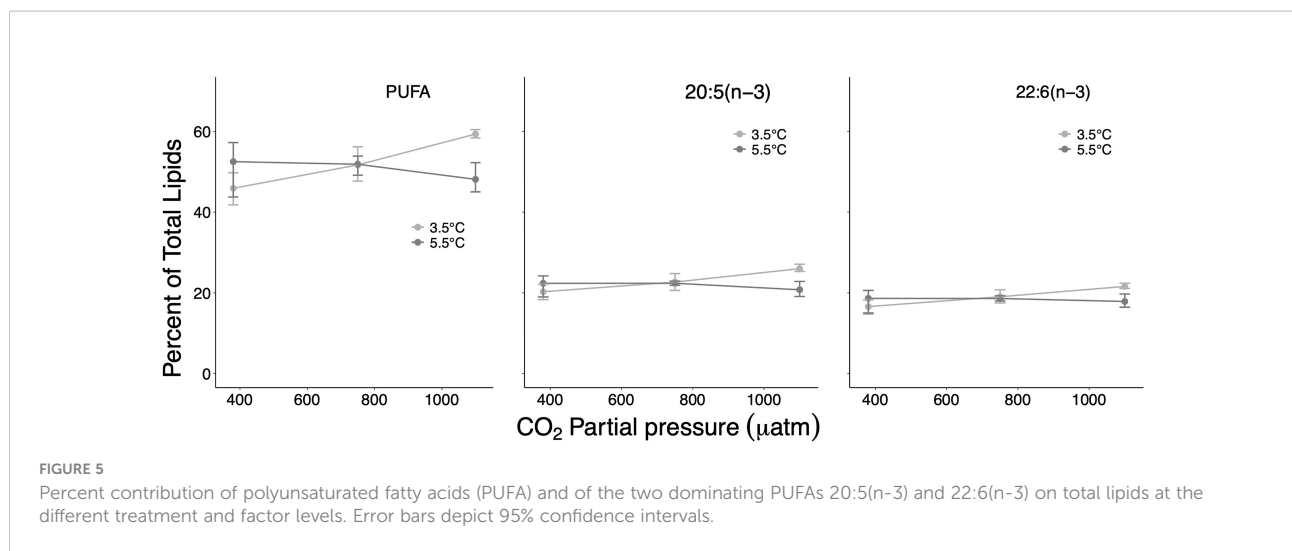


development in the dishes was presumably driven by a physical and a biological process both occurring simultaneously: gas exchange with the outside air (CO₂-outgassing, increasing the pH), and respiration of incubated pteropods (CO₂-production, decreasing the pH). In total, net pH development was determined by the process dominating at a time (Figure 7). Although we cannot completely constrain CO₂-/pH-development, considering CO₂-outgassing is identical for all dishes within each treatment, suggests that the respiration of pteropods determined pH_T development under control conditions (*in situ* T, high T/ambient CO₂). At a medium CO₂ level, after some initial outgassing, pteropods seemed to upregulate respiration rates first at both temperatures. Strikingly, though, on the last experiment day at high T, pteropods decreased respiration (note: mortality of pteropods was equally low in both treatments; see [Supplementary Figure S2](#) online), thus they presumably downregulated their metabolism. Relatively strong outgassing characterized the first four

experiment days at the high CO₂ levels. Later, in *in situ* T respiration dominated, suggesting for metabolic upregulation in pteropods. At the high T treatment, outgassing seemed to dominate almost consistently throughout the experiment. Only on 27 and 28 August, outgassing was almost balanced for only a short time period probably due to upregulated respiration of pteropods. The subsequent pH increase points to metabolic downregulation (shutdown?) toward experiment termination. Generally, the incidence of metabolic upregulation is particularly striking since we switched from every other day to everyday water exchange after four days of incubation, i.e., less time for the genesis of respiratory CO₂ to dominate over CO₂-outgassing and determine net pH development.

Although our experimental design does not allow us to fully support these assumptions, the described pH-development well resembles oxygen consumption rate patterns in response to very similar OWA conditions reported in a previous study (Lischka and Riebesell, 2017): At elevated temperatures and high CO₂,





oxygen consumption rates decreased, at which at high T and medium CO₂ respiration rates increased (= hormesis type response), supporting the reliability of the variable metabolic response direction determined of the pteropods in this study through the pH-development.

Contrast of phospholipids of experimental and *in situ* ('wild') organisms

Raw effect sizes between phospholipid contents of our experimental organisms and phospholipids measured directly on 'wild' veliger and juvenile *L. helicina* by Gannefors and co-authors (Gannefors et al., 2005) are shown in Figure 8. At *in situ* T, effect sizes calculated between experimental and wild juveniles did not deviate much from zero at ambient CO₂ and medium CO₂ (0.7%; 4%), whereas CIs of mean effect sizes calculated for veliger larvae (-24.6%; -21.3%) were clearly separate from zero but not from the 1-SD-line. At the highest pCO₂ treatment, phospholipid content of both stages was significantly reduced compared to that measured in wild individuals, with the effect sizes being larger in veligers (-49.7%) than in juveniles (-24.4%), and CIs of both stages to the left of the 1-SD-line. This pCO₂ effect disappeared at the high T treatment, where all calculated contrasts deviated from the 1-SD-line and mean effect size increased compared to *in situ* T for both stages. Moreover, contrasts between veligers (effect sizes: -52.3% to -61.7%) were clearly larger than those of juveniles (effect sizes: -27% to -36.4%).

Mortality

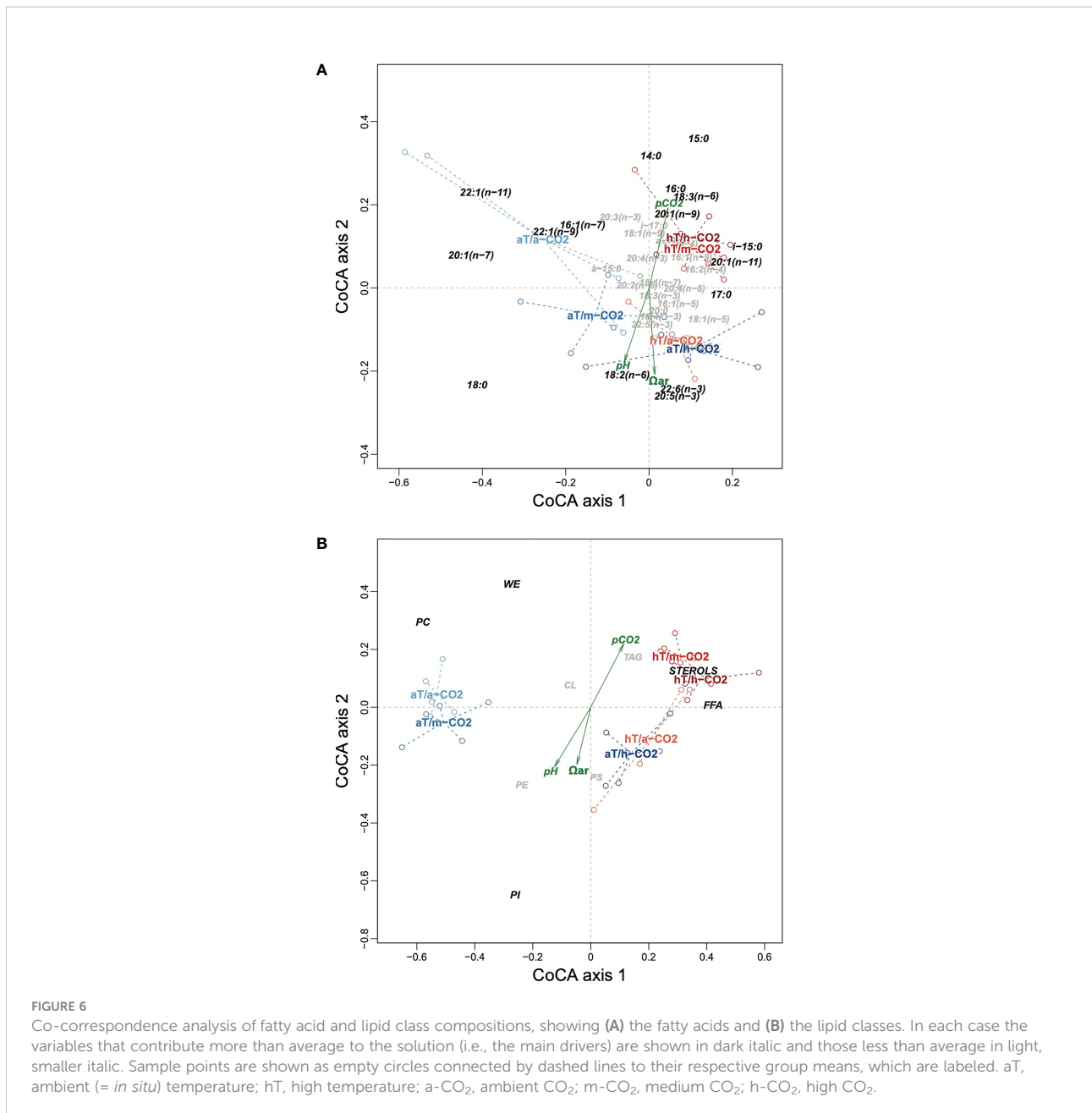
Mortality of pteropods was very low, thus we assume that stress experienced by pteropods was negligible (see Supplementary Figure

S1 online). At *in situ* T, effect sizes (raw mean differences) were -1.82% and -1.50% between ambient/high CO₂ and medium/high CO₂, respectively. Their confidence intervals (CIs) did not cross zero. The effect size of the temperature contrast (3.5°C/5.5°C) at ambient CO₂ was 0.99% with also distinct separate CIs. All other pairwise comparisons revealed effect sizes of <1%, and CIs overlapped the zero line (Table 1). Overall, no significant factor effects were determined by ANOVA statistics (Table 2).

Discussion

At the experiment termination, lipids, and in particular phospholipids, of our early-stage experimental organisms incubated under control conditions (*in situ* T/ambient CO₂) resemble at large those measured of veliger larvae and juveniles from Kongsfjord *in situ* (Gannefors et al., 2005). Thus, cultivation caused no major modification to lipid metabolism over the seven-day experiment, which is further supported by the fact that total lipid mass and phospholipids at control conditions did not differ from *in situ* T/medium CO₂. Slight differences can most likely be attributed to some differences in the developmental stage of our individuals compared to Gannefors et al. Beyond that, lipid class and fatty acid composition are in the range of what is currently known for members of the genus *Limacina* in both polar regions (Phleger et al., 1997; Kattner et al., 1998; Falk-Petersen et al., 2001).

Our results show a massive decrease in the total lipid amount by 47%–70% of early-stage *L. helicina* under future OW scenarios that will be further amplified by increasing levels of seawater pCO₂ projected at the end of the century due to OA. The higher sensitivity of lipid metabolism to temperature than CO₂ changes is in accordance with previous results shown for planula larvae of the coral *Pocillopora damicornis* (Rivest and Hofmann, 2015). Temperature-/pCO₂-induced modifications of



the lipid metabolism were mainly due to a reduction of the major phospholipid classes PC and PE that showed almost identical response curves as total lipids and a concomitant increase of FFAs with higher factor levels. Phospholipids are particularly vulnerable to temperature increases (Farkas and JC, 1981; Chapelle, 1986). The massive decrease in total lipids and phospholipids is probably due to increased lipid catabolism to fuel higher metabolic costs at higher temperatures (Monaghan et al., 2009). Moreover, oxidative lipid degradation due to increased lipid peroxidation toxicity at elevated pCO₂ levels (Zenebe et al., 2007; Murphy, 2008) might have been a contributing factor. Because lipid peroxidation is an

autocatalytic self-propagating process, it can induce great damage, particularly to phospholipids (Hulbert et al., 2006).

Stress adaptation and tolerance imperatively require energy balance, i.e., energy gain from the environment must be sufficient to cover metabolic loss and allow for allocation to somatic growth and reproduction (Sokolova et al., 2012). PC is a major component of lipovitellin that is part of the egg yolk. Its content is important for the survival of many zooplankton embryos and early larvae and is used for energy and membranes (Lee et al., 2006). During this actively growing life phase of our incubated pteropods, a large portion of phospholipids is surely associated with membrane buildup.

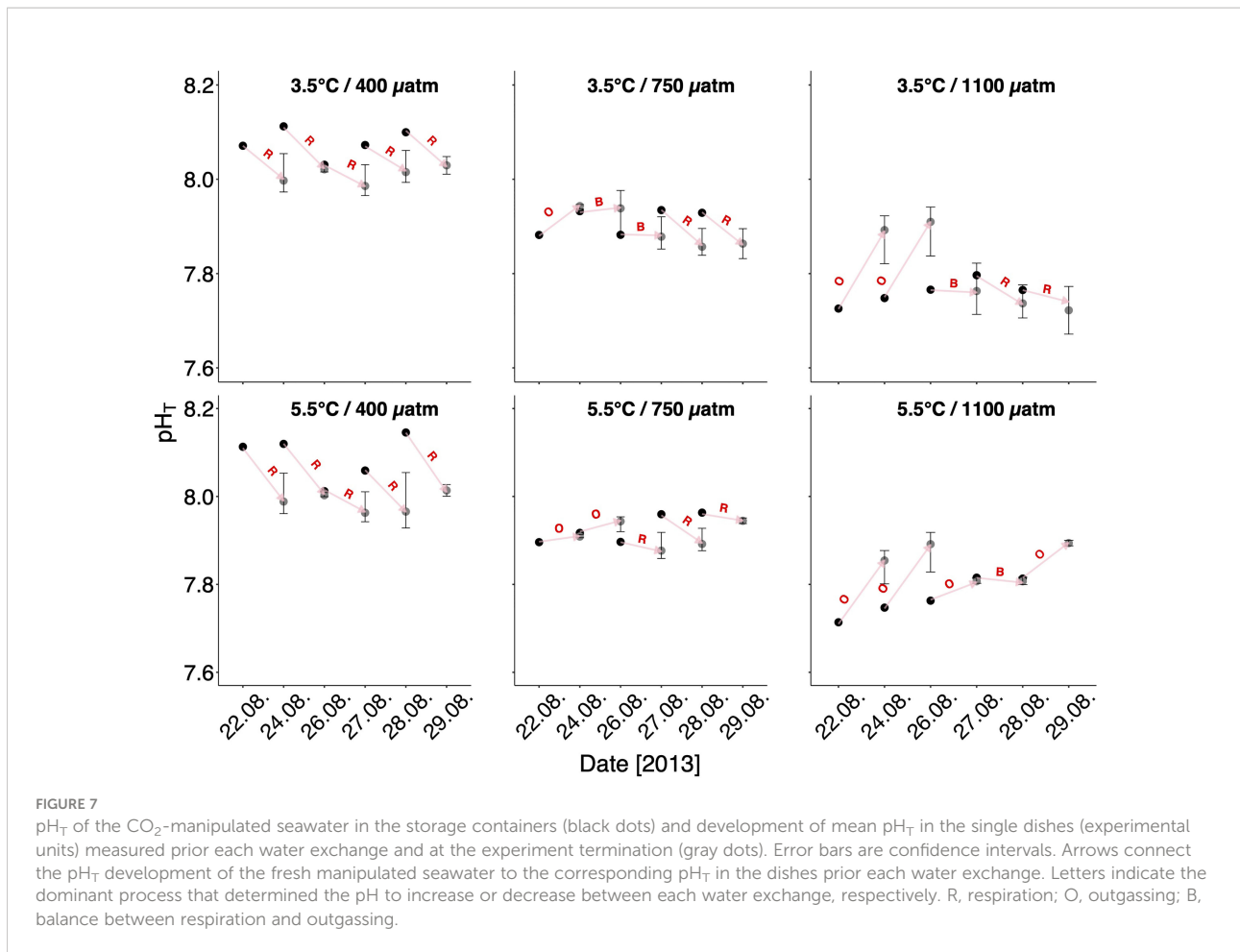


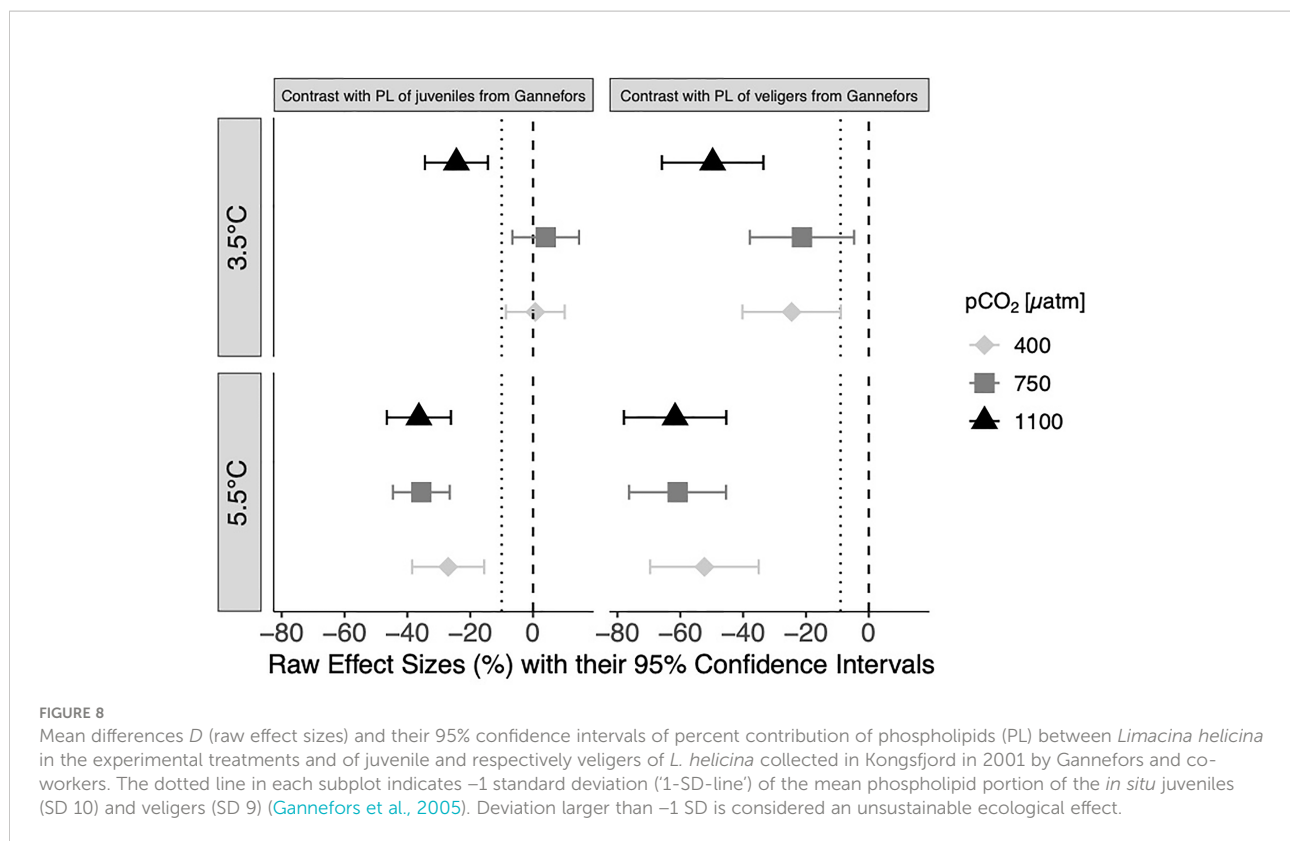
FIGURE 7

pH_T of the CO₂-manipulated seawater in the storage containers (black dots) and development of mean pH_T in the single dishes (experimental units) measured prior each water exchange and at the experiment termination (gray dots). Error bars are confidence intervals. Arrows connect the pH_T development of the fresh manipulated seawater to the corresponding pH_T in the dishes prior each water exchange. Letters indicate the dominant process that determined the pH to increase or decrease between each water exchange, respectively. R, respiration; O, outgassing; B, balance between respiration and outgassing.

Beyond that, part of the large phospholipid content present in pteropod eggs (Gannefors et al., 2005) may still be available as lipovitellin and used to cover increased energetic demands at high T as well as at high CO₂ of the *in situ* T treatment. Increased proportions of FFAs at high T and varying proportions of SFAs, MUFAs, and PUFAs further foster the notion of enhanced catabolic and compensatory processes to maintain cellular and metabolic functioning (Kattner et al., 2007). Also, investigation of gene expression patterns of adult *Limacina retroversa* from the Gulf of Maine in response to elevated CO₂ revealed that lipid catabolic processes become substantially influenced after 14 days of exposure (Maas et al., 2018).

The pH-development in the incubation dishes could point to increased energetic demands of pteropods at all elevated factor levels. In particular, the strong decrease in total lipids and phospholipids at *in situ* T/high CO₂ would support this idea. Further, the membrane fatty acid composition is not only essential for membrane functionality but also impacts on metabolic rate (Hulbert et al., 2007; Monaghan et al., 2009). The pH-development, corresponding to the assumed metabolic upregulation of pteropods, also indicates some smaller scale physiological constraints at *in situ* T/medium CO₂, which,

however, were not (yet?) reflected in the lipid development. It is possible that, in this case, pteropods could cover moderately increased metabolic costs from food particles <20 μm available in the incubation water by ciliary feeding (Howes et al., 2014; Boissonnot et al., 2019). In contrast, a strong metabolic shut-down or collapse of pteropods may have occurred at high T/high CO₂ after a short period of metabolic upregulation. This could mean pteropods initially tried to withstand or counteract CO₂ stress at high T, but finally failed, possibly because remaining lipids were already too depleted or degraded. The metabolic shutdown under the most severe low pH and Ω_{ar} conditions is also expected for *L. helicina* under thermal and upwelling-related OA stress in the California Current System (Bednaršek et al., 2018). Such interpretation of our results further corresponds to an increasing impact of CO₂ inducing complex changes on the respiration of *L. retroversa* with severity and duration of exposure (Maas et al., 2018) as well as with hormesis-type response of oxygen consumption rates under OWA reported by Lischka and Riebesell (2017) for *L. helicina*. However, as outlined in the previous section, this interpretation comes with some reservation.



In principle, lipid biosynthesis is highly susceptible to oxidative stress (Monaghan et al., 2009). Temperature is a highly potent factor to induce the production of reactive oxygen species that *via* lipid peroxidation initiates among others significant oxidative degradations to lipids (Zenebe et al., 2007; Murphy, 2008). Phospholipids are important membrane components, serve cell differentiation and have high PUFA proportions (Kattner et al., 2007). PUFAs are much more susceptible to lipid peroxidation than SFAs and MUFAs (Hulbert et al., 2007). Low pH and Ω_{ar} can also result in elevated oxidative stress (Sokolova et al., 2012). Thus, oxidative stress may at least partly have contributed to the large total lipid and phospholipid decrease at high T compared to *in situ* T and the amplification of this effect at elevated pCO_2 as revealed in our study. Further, our results may support conclusions on lipid metabolism drawn indirectly from measured lipid peroxidation activities (positively correlated with low Ω_{ar} , low pH, and high pCO_2) of *L. helicina* in the California Current System exposed to multiple abiotic stressors during an El Niño event coupled with upwelling-related OA (Bednaršek et al., 2018). But this can only remain speculative as we have not measured antioxidant capacity and/or lipid peroxidation.

Considering the massive lipid reduction (47%–70% of total lipids, up to 60% of phospholipids) and the assumed metabolic shutdown together with the natural temperature range of *L. helicina* from $-4^\circ C$ to $4^\circ C$ (van der Spoel, 1967), a $2^\circ C$

temperature increase may have pushed this species close to its possible thermal tolerance limits or even beyond critical temperature. Moreover, as suggested from the sudden and drastic change of lipid class composition at *in situ* T/high CO_2 , pCO_2 scenarios expected for this century may narrow the natural thermal tolerance window also at *in situ* temperature (Pörtner, 2002).

The effect sizes identified on the fatty acids were generally smaller than those identified on the lipid classes. This can be explained by the independent response behavior of lipid classes and fatty acids; i.e., the number of lipid classes as a whole (which are composed of different fatty acids) is more strongly affected than their individual constituting fatty acids. As long as the relative change in a lipid class is accompanied by the same relative change of all its constituting fatty acids (meaning a 50% change in a lipid class and a 50% change of all its fatty acids), no fatty acid signal would be detected. A change in the fatty acid composition is revealed only if the response of the individual fatty acids to the stressors varies from that of the lipid class as a whole.

Based on ANOVA statistics, temperature and pCO_2 synergistically affected PUFAs and the PUFA 20:5(n-3), with higher contributions in total lipids at *in situ* T/high CO_2 than at high T/high CO_2 . Also, for 22:6(n-3) confidence intervals were clearly separated between these treatment levels. Apparently, at the high pCO_2 level of the *in situ* temperature treatment, proper membrane physical functionality (fluidity) could only be assured

by increased metabolic production of phospholipid PUFAs. The (n-3) and (n-6) PUFAs are essential fatty acids and constitute most of *Limacina*'s PUFAs (between 63% and 88% of total lipids in juveniles and veligers, respectively, (Gannefors et al., 2005), and present study). They are not entirely synthesized by heterotrophic organisms but need to be incorporated with food. Thus, if a large/additional number of PUFAs are lost/required, they can only be substituted if a specific food (diatoms, flagellates) is available (Graeve et al., 1994; Lee et al., 2006). Additionally, *L. helicina* has relatively low lipid turnover rates (Boissonnot et al., 2019). The vast majority of pteropod populations in Kongsfjord overwinter as juveniles with ceased growth and reduced metabolism, most likely fueled by continuous lower level feeding and possibly by internal lipid reserves (TAGs) (Gannefors et al., 2005; Lischka and Riebesell, 2012; Lischka and Riebesell, 2017). Ultimately, developmental delay and decreased fitness would result in a lower chance of survival of individual pteropods and thus a reduced overwintering population that forms the seed for reproduction next summer.

As mentioned above, lipid class and fatty acid compositions measured for our early-stage pteropods incubated under control conditions (*in situ* T/ambient CO₂) resembled those measured for juveniles and veliger larvae from Kongsfjord *in situ* well (mean phospholipid of our early juveniles: 63%, of the Gannefors juveniles: 62%, and of Gannefors veligers: 88%), indicating that cultivation had no major effect, thus overall justifying our comparison, though we cannot completely rule out the possible existence of some physiological differences between experimental and *in situ* organisms (e.g., with respect to background temperature, food availability, energetic status). However, in further support of our comparison is the fact that the effect sizes estimated for the comparison with the Gannefors juveniles did not deviate from zero at *in situ* T/ambient CO₂ and even at *in situ* T/medium CO₂. But at high CO₂ of the *in situ* T treatment as well as at all pCO₂ levels of the high T treatment, effect sizes calculated between phospholipid proportions of our experimental organisms and *in situ* organisms from Kongsfjord (Gannefors et al., 2005) show a clear discrepancy of more than one standard deviation from that of *in situ* organisms. That is, the phospholipid content of early-stage pteropods exposed to temperature and pCO₂ stress was reduced to levels clearly outside the natural variability (with a reduction of up to 62% in veligers and up to 37% in juveniles). Based on the low lipid turnover rates of *L. helicina* (Boissonnot et al., 2019), the prominent role of phospholipids in biomembranes, for reproduction and early development (Kattner et al., 2007), we suppose phospholipid proportions clearly outside the natural range may entail unsustainable populations and ecological drawbacks.

Stress response depends on species, habitat, individual adaptation, etc. (Seibel et al., 2007; Comeau et al., 2010; Lischka et al., 2011; Maas et al., 2012; Seibel et al., 2012; Lischka and Riebesell, 2017). Sensitivity of lipid metabolism to OA and OWA stress in other marine organisms indicates that results scale from the apparent ability of functional (positive)

adaptation to the point of harmful effect. For example, total lipid content and fatty acid composition of adult Antarctic krill were unaffected under exposure to future pCO₂ levels (up to 2,000 μatm), while only extreme pCO₂ levels elicited changes (Ericson et al., 2019). The total lipid of Australian whelk was significantly reduced by temperature but not pCO₂ (Valles-Regino et al., 2015). Purple sea urchin early larvae collected in the upwelling region of the California coast did not exhibit differences in lipid usage rates (total lipids, TAGs, phospholipids, STs) when exposed to varying pCO₂ conditions (Matson et al., 2012), whereas elevated pCO₂ (856 μatm) reduced total lipids and phospholipids of larval oysters by 13%–39% (Gibbs et al., 2021). None of these examples approximates the extent of OWA effects induced in lipids of early-stage pteropods. Three major reasons may account for the high sensitivity: *i*) Early larval stages are particularly vulnerable to OA stress, at which most of the mentioned published studies exposed older developmental stages to experimental conditions; *ii*) we applied two stressors, at which the referred studies were single-stressor (OA) experiments; and *iii*) in the case of Matson et al. (2012), experimental organisms were naturally adapted to the highly variable carbonate chemistry conditions in upwelling regions. Nonetheless, our study emphasizes that Arctic early-stage pteropods must be ranked at the right end of a corresponding response scale (i.e., higher risk of harmful effect) and underlines their particular vulnerability to OWA.

Further, under RCP 8.5, much of the Arctic Ocean is projected to become undersaturated with respect to aragonite by 2040 (Ciais et al., 2013), whereas the sea surface temperature of the Arctic Ocean may rise by up to 4°C by 2090–2099 (Bopp et al., 2013). Thus, widespread shell dissolution due to OA (Lischka et al., 2011; Bednaršek et al., 2012; Bednaršek et al., 2019) may occur earlier than massive OW effects on the lipid metabolism of Arctic pteropod populations. As basic modules for cell growth and differentiation, lipids serve multiple vital functions at the molecular level. Consequently, negative OW effects on lipid metabolism may carry over to calcification processes at the cellular level and diminish the ability of pteropods to counteract dissolution. Therefore, given the short experimental duration and magnitude of initiated effects on pteropod lipid dynamics, detected alterations in lipid metabolism may pose a much more immediate and momentous threat to the fitness of pteropods, and hence population development, than anticipated so far of shell dissolution effects (Lischka et al., 2011; Bednaršek et al., 2012; Bednaršek et al., 2019).

Conclusion

The massive lipid changes in response to OWA observed in our study were most likely due to increased lipid catabolism, and may have been co-triggered by increased lipid peroxidation

toxicity. As a consequence, both would lead to severe energetic imbalance and constraints, thus decreasing stress tolerance and fitness (Sokolova et al., 2012). Finally, negative OWA effects on lipids could directly translate into population and ecosystem-level effects due to developmental delay (Thabet et al., 2015) or failure depending on the emerging mismatch with food availability in high-Arctic regions the longer development time is prolonged toward autumn/winter (Gannefors et al., 2005; Lischka and Riebesell, 2012). In consequence, Arctic *Limacina* populations would diminish and elicit manifold changes in zooplankton community structure, food-web interactions as well as carbon transport (Lalli and Gilmer, 1989; Bathmann et al., 1991; Hunt et al., 2008). As OWA is primarily a surface phenomenon, sooner or later epipelagic pteropods will be confronted with the anticipated future temperature and pCO₂ changes because they must effectively feed in the surface layer. Accordingly, in Kongsfjord, most of the summer population is found in the upper 50 m (Gluchowska et al., 2016). Avoiding such adverse conditions in deeper layers could only come at the cost of effective feeding, contributing to (further) energetic imbalance. However, as the projected temperature increase of water masses occurs only gradually, likewise, we would expect *in situ* changes in the lipid metabolism of pteropods to occur gradually/continuously.

Data availability statement

Data are available via the PANGAEA data repository at <https://doi.org/10.1594/PANGAEA.936072>.

Author contributions

SL conceived and performed experiments. SL, MG, and MJG analyzed the results. SL wrote the paper with contributions of MG, MJG, and UR. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.920163/full#supplementary-material>

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