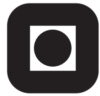

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Interaction Effects of Ocean Acidification and Warming on the Fecundity of the Marine Copepod *Calanus finmarchicus*

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Marine Coastal Development
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Abstract

This study provides the first information on the combined effect of ocean acidification and elevated temperature on the fecundity of the marine pelagic copepod *Calanus finmarchicus* (Gunnerus, 1770). The copepod is considered to have a key role in the transfer of planktonic production to fish, seabirds and marine mammals, and is stated as a key species in the North Atlantic food web and ecosystem. To obtain a better understanding of the links between physical forcing and the population dynamics of *C. finmarchicus* in order to predict responses to climate change, it is fundamental to observe the reproduction, which sets the upper limit on recruitment. To study interactive effects of acidification and warming, ovigerous females (that had developed from eggs under the same conditions) were incubated at 380 ppm (ambient) and 2080 ppm (future predictions), and temperatures at 11 and 14˚C in a 2x2 factorial design. A significant reduction in egg production (79%) and hatching success (75%) was observed in the warming treatment alone (+3˚C) compared to the ambient treatment, also a positive antagonistic effect of acidification was when combined with warming was observed. No significant interaction was seen in the lipid storage; however, elevated temperature alone caused a significant reduction (95%) compared to the ambient treatment. The body volume and the sex ratio were not affected by acidification and warming combined, or separately. The observed effects suggest that *C. finmarchicus* may be robust to ocean acidification predicted to occur within year 2300 when combined with elevated temperature.

Sammendrag

Denne studien gir den første informasjonen om den kombinerte effekten av havforsuring og forhøyet temperatur på reproduksjon hos den marine kopepoden *Calanus finmarchicus* (Gunnerus, 1770). Denne arten ansees å ha en sentral rolle i overføring av primær produksjon til fisk, sjøfugl og marine pattedyr, og er oppgitt som en nøkkelart i den nordatlantiske næringskjeden. For å få en bedre forståelse av sammenhengen mellom fysiske faktorer og populasjonsdynamikken hos *C. finmarchicus,* og for å kunne forutsi eventuelle responser til klimaendringer, er det grunnleggende å observere reproduksjon som angir den øvre grensen for populasjonsvekst. For å studere interaksjonseffekter av forsuring og oppvarming ble eggbærende hunner (som hadde utviklet seg fra egg under samme forhold) inkubert ved 380 ppm (nåtid) og 2080 ppm (forsuring), og temperaturer ved 11 og 14˚C i et 2x2 faktorielt design. En signifikant reduksjon i eggproduksjon (79 %) og klekkesuksess (75 %) ble observert i behandlingen eksponert med forhøyet temperatur (+3˚C) sammenlignet med kontrollbehandlingen, og en positiv antagonistisk effekt av forsuring kombinert med oppvarming ble også observert. Ingen signifikant interaksjon ble sett i lipidlagringen; imidlertid forårsaket økt temperatur alene en signifikant reduksjon (95 %) sammenlignet med kontrollbehandlingen. Kroppsvolum og kjønnsfordelingen ble ikke påvirket av forsuring og oppvarming kombinert, eller separat. De observerte effektene antyder at *C. finmarchicus* kan være robust mot havforsuring som er forventet å skje innen år 2300 i kombinasjon med økt temperatur.

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B.1 Raw data from the biometric measurements

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C.1 Results from the Levene's tests

Abbreviations

Abbreviation	Meaning
μatm	Micro atmospheres
μg C/L	Microgram Carbon per Liter
˚C	Degrees Celsius
AIC	Akaike Information Criterion
CO_2	Carbon dioxide
CO_3^{2-}	Carbonate
CS	Clutch size
DIC	Dissolved Inorganic Carbon
d.f.	Degrees of Freedom
EPR	Egg production rate
Glmer	Generalized linear mixed model
H_2CO_3	Carbonic acid
HCO_3^-	Bicarbonate
HS	Hatching success
mm^3	Cubic millimeter
MS	Mean square
MS-222	Tricaine methansulfonate
NTNU	Norwegian University of Science and Technology
OA	Ocean acidification
pCO_2	Partial pressure of CO_2
pg C/cell	Picogram Carbon per cell
pH	Power of Hydrogen
pH_{tot}	Total power of Hydrogen
Permanova	Permutional Multivariate Analysis of Variance
PID	Proportional Integral Derivate control
ppm	Parts per million
rpm	Rounds per minute
SD	Standard deviation
SE	Standard error
SPSS	Statistical Package for the Social Sciences
SS	Sum of squares
T_A	Total alkalinity

1. Introduction

Carbon dioxide (CO_2) is a component of the natural gas in the earth's atmosphere and takes part in essential processes such as photosynthesis and respiration. Emission of CO_2, caused by human activity, such as burning of fossil fuels is referred to as anthropogenic CO_2 emission. Since the industrial revolution, in the late 1700s, the global atmospheric CO_2 – level has increased by 36%, which is equal to about 100 parts per million (ppm) (Solomon et al., 2007). The oceans, which cover 70% of the earth's surface, have absorbed approximately half of the anthropogenic CO_2 emissions in the last 200 years (Sabine et al., 2004) and have thereby acted as a natural buffer of atmospheric CO_2, due to its chemical composition and large volume (Pörtner, 2008). The increase in global atmospheric CO_2 is mainly due to anthropogenic CO_2 emissions from the combustion of gas flaring and cements productions. Since the anthropogenic CO_2 emissions seems to have an effect on global warming, that already have caused shifts in marine ecosystems (Pörtner, 2008), the subject has been highly discussed within scientific communities the last decades. The present atmospheric CO_2 content, now exceeding 380 ppm, is the highest level during the last 420 000 and possibly more than 10 million years (Houghton et al., 2001; Solomon et al., 2007), and unless significant reductions in emissions are achieved this number will rise (Gattuso and Hansson, 2011). Future prognoses, based on a "business as usual" scenario, estimates that the CO_2 concentrations may exceed 1900 ppm by the end of 2300 (Caldeira and Wickett, 2003), and 8000 ppm CO_2 has been put forward as a "worst-case" scenario (Caldeira and Wickett, 2005).

1.1 Ocean acidification

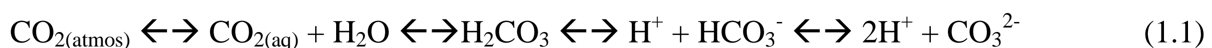
Ocean acidification refers the oceans uptake of CO_2, which can cause a reduction in pH and alteration in the seawater chemistry (Caldeira and Wickett, 2003; 2005; Doney et al., 2009; Orr et al., 2005). A global decrease in ocean pH of 0.1 pH units indicates a 30% increase in H^+ ions in current surface waters. A future scenario model based on a "business as usual" principle has estimated a 0.2-0.4 unit pH decrease by the end of this century (Caldeira and Wickett, 2003; Solomon et al., 2007). For several million years the pH value has been relatively constant at approximately 8.2 (Widdicombe and Spicer, 2008), mainly due to the oceans buffer capacity (Revelle capacity). Buffers are compounds which act to minimize perturbations in pH, by reacting with exogenous H^+-ions (Roos and Boron, 1981). The

chemical changes are practically irreversible on a time scale of centuries due to the naturally slow turnover of biogeochemical cycles in the ocean (Falkowski et al., 2000).

1.2 Carbon dioxide and the chemical balance in seawater

Like all other dissoluble gasses, CO_2 dissolves in water until the equilibrium with the atmosphere is reached. Henry's law states that *the solubility of a gas in a liquid is directly proportional to the partial pressure of the gas over the solution* (Henry, 1803). The exchange between CO_2 in seawater and the atmosphere is a naturally exchange and the concentration of CO_2 in the ocean surface increases in line with the atmospheric CO_2. The inorganic carbon system is one of the most important chemical equilibria in the ocean and is responsible for controlling the pH of the seawater.

When the atmospheric CO_2 is exchanged in water, aqueous CO_2 reacts with water (H_2O) and forms the transient product carbonic acid (H_2CO_3). Since H_2CO_3 is a weak acid most of it will dissociate quickly by losing hydrogen to form bicarbonate (HCO_3^-), carbonate (CO_3^{2-}) and hydrogen ions.



$$CO_{2(atmos)} \leftrightarrow CO_{2(aq)} + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \quad (1.1)$$

Therefore, the net effect of adding CO_2 to seawater is an increase in the concentrations of H_2CO_3, HCO_3^-, and H^+, a decrease in the concentration of CO_3^{2-} and a reduction of the pH level (Fabry et al., 2008). At a pH of 8.2, temperature at 25˚C and salinity at 35‰ the majority of dissolved inorganic carbon (DIC) in the ocean is present as bicarbonate ions (~88%), 11% as carbonate, and only ~0.5% of carbon is in the form of dissolved CO_2, including H_2CO_3 (Fabry et al., 2008). Carbon dioxide has a high solubility in water, but the solubility of CO_2 is known to decrease with increasing salinity and temperature (Williams and Follows, 2011). Due to these properties of CO_2 colder regions are more vulnerable to acidification than warmer ones (Gattuso and Hansson, 2011). The effects of global warming and ocean acidification have been shown to increase more quickly near both poles (Orr et al., 2005). The open ocean surface water has a pH value varying between 7.9 and 8.3 (Bindoff et al., 2007), while the surface water near shore varies between pH 7.5 and 8.5 depending on the habitat (Hinga, 2002), and shows much larger seasonal and diel fluctuations (Middelboe and Hansen, 2007). Low mixing rates and thermoclines are some of the reasons that over 90% of all CO_2 absorption takes place currently from 0 to 1500 meter depth, this depth layer includes the most abundant zones of marine organisms (Sabine et al., 2004). Physical or biological

activity can remove or add CO_2 and thereby change the ocean pH (Dickson, 2010). Photoautotrophic CO_2 depletion can cause significant daily pH fluctuations (Middelboe and Hansen, 2007; Yates et al., 2007), with lowest pH values during the night when CO_2 is released due to respiration and higher values during the afternoon due to photosynthesis (Bensoussan and Gattuso, 2007).

1.3 Potential effects of increased CO_2 concentrations on marine organisms

Increased acidity due to reduction in ocean pH is stated to have a range of possible harmful consequences, the scale and magnitude of CO_2 induced stress depends on concentration and duration of exposure (Pörtner, 2008). One of the first responses to exposure of high CO_2 concentrations is shifts in the acid-base status and a drop in extracellular pH (Pörtner, 2008). Even though the majority of species studied have shown negative effects, high CO_2 concentrations may benefit some species (Dupont and Thorndyke, 2009), and a broad variation of responses have been found (Whiteley, 2011). Generally, marine organisms exposed to changes in environmental conditions will be vulnerable for stress that could lead to changes in reproduction, growth and development (Hendriks et al., 2009). Several studies (Shirayama and Thornton, 2005; Byrne, 2010) have revealed adverse effects on marine life due to elevated CO_2 at levels that are within projections for near future (Caldeira and Wickett, 2003; 2005).

1.3.1 Hypercapnia

Elevated partial pressure of CO_2 (pCO_2) in seawater (also known as hypercapnia) can impact marine organisms both via decreased calcium carbonate ($CaCO_3$) saturation, which affects calcification rates, and via acid-base (metabolic) physiology (Fabry et al., 2008). When the pCO_2-levels increases in seawater, dissolved CO_2 will easier diffuse across animal surfaces and equilibrate in both intra- and extracellular spaces. An internal CO_2-level rises until a new value is reached, sufficient to restore CO_2 excretion against the elevated environmental level. As in seawater, CO_2 reacts with internal body fluids causing an increase in hydrogen ions and, therefore, pH decreases (Fabry et al., 2008). Organisms with low buffering capacity will experience greater fluctuations in intracellular pH during hypercapnia than the organisms with higher capacity (Fabry et al., 2008). An increase in CO_2 concentration in seawater sufficient to reduce intracellular pH by for example 0.2 in a slow benthic fish may cause only a 0.02 pH unit drop in an active epipelagic fish such as tuna (Seibel and Walsh, 2003). Also, a state of hypoxia (low oxygen concentrations) can cause decreased pH regulation capacity and set-

point for acid-base regulation (Pörtner, 2008). Earlier studies have shown that copepods have a relatively high tolerance when exposed to CO_2-induced hypercapnia, unlike other invertebrates, such as corals and molluscs (Whiteley, 2011).

1.3.2 Reduction in calcification rates

The studies on impacts of acidification on marine invertebrates have been focused on the calcifying organisms, while researchers have paid less attention to the non-calcifying organisms, such as copepods. Present ocean surface is saturated with respect to calcium carbonate, but increasing atmospheric pCO_2 are reducing ocean pH and carbonate ion concentrations, and thus the level of calcium carbonate saturation (Orr et al., 2005). Recent studies have stated that the combined effect of elevated pCO_2 and higher temperature can explain the reduction in calcification rates (Pörtner, 2008), other studies have suggested that the calcification rate in corals is controlled by the $CaCO_3$ saturation state (Gattuso et al., 1998), rather than pH or other parameters of the seawater CO_2 system. The most documented and global observed biological effect of increased pCO_2 is the reduction in calcification rates (Stumpp et al., 2011). Among marine organisms studied, results indicating adverse effects have been demonstrated in groups, such as corals (Gagnon et al., 2013) and mussels (Gazeau et al., 2007). Due to reduction in pH, carbonate becomes less available, which makes it more difficult for calcifying organisms to secrete calcium carbonate (Fabry et al., 2008). The concentration of Ca^{2+} in the ocean today is approximately constant and changes in the CO_3^{2-} concentration are therefore reflected directly as changes in the $CaCO_3$ saturation state (Fabry et al., 2008). Still, the effects of chronic exposure to increased pCO_2, and long-term implications of reduced calcification rates within individual species and communities are unknown. Decreased calcification rates would presumably compromise the fitness of the calcifying organisms and could shift the competitive advantage towards non-calcifies (Fabry et al., 2008).

1.4 Temperature

The increase in ocean temperature is occurring concurrently with the phenomenon of ocean acidification (Caldeira and Wickett, 2003), this trend is also observed in the atmospheric temperature. The increase in atmospheric temperature may cause changes in the global evaporation- and precipitation pattern, which may lead to salinity variations in the oceans (increased salinity at lower latitudes and reduced salinity at higher latitudes (Solomon et al.,

2007)). The surface ocean temperature is expected to be two to four degrees celsius warmer than the present temperature by the end of year 2100 (Caldeira and Wickett, 2003). Oceans have a large heating capacity and have absorbed more than 80% of the energy released from natural and anthropogenic radiation the last 50 years, this, enhanced by increasing snow- and ice melting, leads to an elevated volume of the oceans. Copepods and other marine organisms must adjust their vital rates to changing temperatures (Møller et al., 2012), so the influence of temperature on individual copepods and populations must be considered as critical (Yang and Rudolf, 2010; Forster and Hirst, 2012). Earlier studies have shown that each degree of warming has been shown to decreases the body size by 0.5-4 % in marine invertebrates (Williamson et al., 2002; Daufresne et al., 2009; Irie and Fischer, 2009). Temperature effects on reproduction and development are widely recognized as a key issue for understanding population dynamics of copepods (Mauchline, 1998; Dam, 2013). Increasing temperature threatens to change species specific geographical distributions, including local extinction of previously common species (Parmesan and Yohe, 2003; Thomas et al., 2004).

1.5 Calanus finmarchicus (Gunnerus, 1770)

The marine calanoid copepod *Calanus finmarchicus* is considered as a keystone species in the pelagic food web of the North Atlantic where it is widely distributed, as well as in the Artic basin and the Barents Sea (Vadstein, 2009). It is a highly abundant copepod and seasonally, due to feeding on phytoplankton blooms, it contributes to more than half of the total copepod biomass (Planque and Batten, 2000). Due to their synthesis and accumulation of lipids, *Calanus* species represent an important energy link between the phytoplankton and higher trophic level predators, including important commercial fisheries species like the North Atlantic Cod (*Gadus morhua*) and Herring (*Clupea harengus*) (Beaugrand and Kirby, 2010).

Together with other calanoid copepods they constitute an important part of the total vertical carbon flux in the ocean through the production of fecal pellets (Bathmann et al., 1987). Bathymetrically, the species has been reported from near-surface waters down to 4000m, but it is most dominant within 200m from the surface. In recent years the need of alternative marine lipid sources for use in fish feed in the aspiring aquaculture industry has been prominent (FAO, 2012). This has led to an interest to use the n-3 highly unsaturated fatty acids contained in the lipid storage in *C. finmarchicus.* The progressive warming of the North Sea and the Northeast Atlantic is causing major shifts to the biogeographical distribution of *C. finmarchicus* and *Calanus helgolandicus* (Claus, 1863) (Beaugrand et al., 2002). The *C.*

finmarchicus is an oceanic copepod and inhabits thermally stable environments; however, through advection and seasonally changes they experience temperature fluctuations (Marshall and Orr, 1972).

1.5.1 Life strategies of C. finmarchicus

The life cycle of *C. finmarchicus* consists of six naupliar stages (NI-NVI) and five copepodite stages (CI-CV), at the end the sexually male and female emerge (Mauchline, 1998; Marshall and Orr, 1955). The two first stages are non-feeding stages and their only energy source comes from the maternal lipid-sac (Marshall and Orr, 1952; 1955).

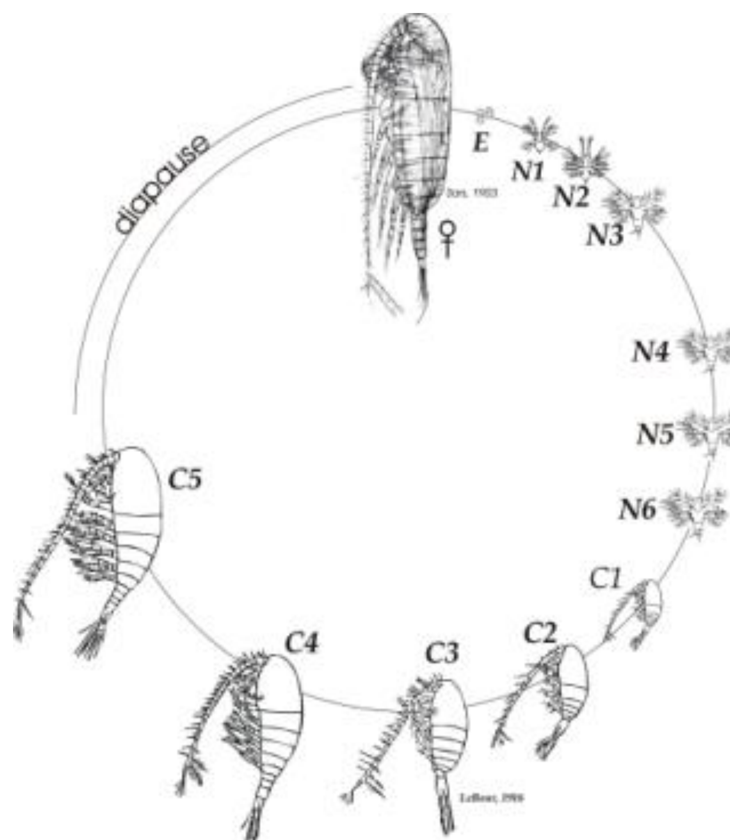


FIGURE 1.1: Life cycle of the copepod *C. finmarchicus.* A full-grown copepod starts spawning of egg (usually during springtime). Then follows 6 naupili stages (N1-N6) before it moults into its copepodite stage, consisting of 5 stages (CI-CV), before the adult stage is reached. Individuals at this stage have built up an adequate lipid reserve and may migrate to deeper water for overwintering (diapause). Credit: Baumgartner, M. at Woods Hole Oceanogrphic Institution (Baumgartner, 2009)

Like most pelagic copepods, *C. finmarchicus* is a broadcast spawner and releases eggs freely into surrounding seawater (Kiørboe, 2008; Mauchline, 1998). Usually, the eggs are released in near-surface water from March to April and nauplii starts emerge within 24-72 hours after

spawning (Kiørboe and Sabatini, 1994; Mauchline, 1998). Free-spawning species generally have shorter intervals between clutches of eggs than those species which produce egg masses, for *C. finmarchicus,* clutches of eggs are spawned every 24 hours in British areas, but every 2-3 days in higher latitude (Diel and Tande, 1992). This regional difference in spawning rate may occur due to variations in temperature, depending on the temperature; *C. finmarchicus* can produce 11-70 eggs each day per female (15˚C) (Hirche, 1990; Ohman and Runge, 1994).

For *Calanus* sp. a skewed sex ratio is common finding (Mullin and Brooks, 1970; Hirche, 1980). An extreme reflection of this was found by Peterson (1986) during a laboratory study where it was only one male and 136 females, even though this finding was extreme it indicates that females are generally more abundant (Svensen and Tande, 1999). Based on female dimorphism, Fleminger (1985) suggested a possible sex change from genotypic CV males to adult females, but findings done by Irigoien (2000) suggests that the change happens between CIII-CV and is determined at stage CV. In addition to a possible sex change, several authors have indicated that the environment may influence the sex ratio through factors as food quality and quantity and temperature (Hopkins, 1982; Irigoien et al., 2000).

The life strategy of *C. finmarchicus* includes two patterns of vertical migration in the water column. The *diel* vertical migration involves ascending the surface at sunset to feed on phytoplankton that occurs there during the night and then return to deep waters (Edvardsen et al., 2006). This migration pattern is typical, but variable, among zooplankton and varies both within and among years (Baumgartner et al., 2011), it is believed to be a response to the trade-off between the need for growth and the necessity of avoiding visual predators (Edvardsen et al., 2006).

This species also performs seasonal vertical migrations. These migrations, includes ascent of CV and adult individuals from deep waters towards the surface prior to the spring phytoplankton bloom, and descend to deep waters the subsequent fall (Edvardsen et al., 2006). These seasonal migrations has been found irregular, but the timing of ascent, mating and egg release is assumed to be linked to cues that precede the on-set of the phytoplankton spring bloom, ensuring favorable feeding conditions for the first feeding stages of their life cycle (Mauchline, 1998; Kiørboe, 2008). In the northern distribution, the copepodite stages starts to migrate to deeper waters during the late summer time and forms a dense layer near the bottom of basins and continental slope waters (400-1000 m). During the winter months they enters diapause and stay dormant, without feeding, reducing energy consumption and

living on lipid reserves in their bodies (Conover, 1988). If *C. finmarchicus* experience elevated temperatures during this resting stage due to ocean warming, the lipid stores may be faster depleted and, thus, the maximum duration of the diapause may be shortened (Saumweber and Durbin, 2006). At the end of winter the surviving CV complete their final molt to the adult stage, and migrate to surface waters for feeding (Conover, 1988). Each cycle, from egg production to adult and their egg production, takes about two months under favorable conditions. The number of generations produced per year varies between regions, in the northern distribution they usually complete only one generation, while they in the southern regions can produce two and sometimes three generations (Mauchline, 1998).

1.6 Previous studies of the effects of OA and elevated temperature on copepods

The oceans will gradually become warmer and more acidic and it is therefore a need for long-term (months) and multistressor experiments to better understand the consequences of this phenomenon (Pörtner, 2008). Most of the existing studies on the effects of CO_2 have involved high concentration levels (>2000 µatm) and have lasted over short exposure periods (<10 days), whereas pCO_2 -levels predicted for the end of the century seem to have no impact on most of the copepod species yet studied (Kurihara et al., 2004; Mayor et al., 2007; Kurihara and Ishimatsu, 2008; Zhang et al., 2011). Although unrealistic, such studies can provide indications on when the effects may set in and how organisms respond to changing environmental factors. To the authors knowledge the present study represent one of the first studies on the long-term effect of elevated pCO_2 and temperature.

A 70% decline in the total biomass of *Calanus* spp. over the past 50 years has been documented (Edwards et al., 2006). Short-term studies on wild-caught *C. finmarchicus* have indicated that this species may be robust towards the predicted outcome of OA scenarios (Mayor et al., 2007; 2012; Pedersen et al., 2014). However, the same studies also reveal that the response to elevated CO_2 concentrations varies between life stages and depends of the degree of acidification (Mayor et al., 2007; Pedersen et al., 2014). A study that used extreme high exposure of pCO_2 (7300 and 9700 ppm) revealed significant reduction of survival (reduced by approximately 50%) and indicated slow development of the animals (Pedersen et al., 2013). Generally, it seems like CO_2 values at approximately 2000 ppm sets the limit for when animal survival is not influenced directly (Mayor et al., 2012; Pedersen et al., 2013).

A recent study by Hildebrandt et al. (2014) on *Calanus glacialis* (Jaschnov, 1955) and *Calanus hyperboreus* (Kroyer, 1838) did not show any effect of elevated pCO_2 (3000 ppm) on respiration rates, body mass or mortality in both species and life stages (Hildebrandt et al., 2014). To detect synergistic effects, *C. hyperboreus* females were kept at different pCO_2 and temperatures (0, 5, 10˚C). The highest incubation temperature (10˚C) induced sub-lethal stress, something that may have overruled potential effects of pCO_2 (Hildebrandt et al., 2014). The overall conclusion was; the copepods, can tolerate pCO_2 predicted for the future, but the combined effect with temperatures could make them sensitive to OA (Hildebrandt et al., 2014).

1.7 Environmental effects on egg production and hatching success of copepods

Studies on egg production (EP) of herbivorous copepods have become a widely used tool in copepod ecology, and require knowledge of the regulatory mechanisms involved and the response to changes in food concentrations, acidity and temperature. In *C. finmarchicus* spawning is a short and discrete event and results in clutches of eggs (Marshall and Orr, 1955). Most previous studies have investigated effects of OA on copepods with CO_2 concentrations beyond those expected in the next 100 years by the IPCC, but they are still relevant as they emulate potential Carbon Capture & Storage (CCS) conditions (Kurihara et al., 2004; Kurihara and Ishimatsu, 2008). The term CCS represents a number of methods by which anthropogenic-generated CO_2 is collected at source and released into mesopelagic waters, seafloor depressions or geological sub-surface formations (Liro et al., 1992; Parson and Keith, 1998). Estimates suggest that CO_2 concentrations of 20,000 ppm and pH-levels as low as 5.8 are likely to occur near the point of release, in case of carbon leaks (Herzog et al., 2003).

A medium-term laboratory study on *C. finmarchicus* showed no effect on hatching success (HS) or later survival at CO_2 concentrations of 3300 ppm (one generation, 28 days), but the same study found negative effects on growth and development at the same exposure concentration (Pedersen et al., 2013).

A study contradicting the perception that copepods can manage elevated CO_2- levels (≤ 2000 ppm (Mayor et al., 2012; Kurihara et al., 2004)) was performed by Fitzer et al. (2012). They revealed a negative effect on growth and reproductive rates of the harpacticoid copepod *Tisbe battagliai* (Volkmann-Rocco, 1972) exposed to pCO_2 well below 1000 ppm (Fitzer et al., 2012). Also, another pelagic copepod, *Centropages tenuiremis*, showed reduction in respiration and grazing rates during a 90h experiment with a pCO_2 of only 1000μatm (Li and Gao, 2012).

1.8 Aim of the present study

The aim of this study was to investigate if potential long-term effects of OA and increased ocean temperature could have a synergistic impact on the fecundity on *C. finmarchicus.* To get a comprehensive view of the impact, egg production, hatching success, biometric data and sex ratio were examined by exposing *C. finmarchicus* to ambient and elevated pCO_2 (380 and 2080 ppm) and ambient and elevated temperature (11 and 14˚C) for one generation (eggs to adults). This was done in a fully crossed design during 62 days. The goal was to provide a picture of how this species can handle the environmental changes that could occur within year 2300.

2. Materials and methods

This experiment was conducted at NTNU Center of Fisheries and Aquaculture (SeaLab) in the period December 2012 to February 2013. The experiment consisted of two parts, a growth phase and an egg production phase.

The CO_2-concentrations used were 380 ppm as a control (i.e. present day scenario) and 2080 ppm which is considered a worst case scenario in year 2300 (Caldeira and Wickett, 2005), and the temperatures used were 11˚C and 14˚C to simulate the expected ocean warming.

Both parts of the experiment were done in a climate controlled room (10˚C), but to control the temperature in the experimental tanks custom developed temperature units were used. To maintain stable CO_2-levels a custom-made gas mixing system dispersed the gas to the 12 tanks via four equilibrium columns. During the experiment period the animals were fed with an algae mixture which consisted of three different algae species. To verify that the target levels of CO_2, temperature and feed were maintained in the different tanks, pH, salinity, total alkalinity, temperature and concentration of algae were measured at regular intervals.

2.1 Spawning and collection of eggs

Adult *C. finmarchicus* from a maintained culture (36th generation) were transferred to eight polyethylene tanks (50 L) filled with seawater (10˚C), 500 individuals per tank. After 24 hours the produced eggs were collected by first reducing the water in each tank to about 2-3 L by the siphon principle, and then the remaining water was poured through a filtering cup (mesh size 200 µm) and into a glass bowl (i.e. removing the large copepods). Finally, a filtering cup (mesh size 46 µm) was used to collect the eggs.

The collected eggs were pooled, diluted in seawater (2 L) and gently inverted to ensure a homogenous mixture, before the density of eggs were measured from subsamples. Based on the density measurements the total number of eggs was calculated to be approximately 58.000. The homogenous mixture (2 L) was divided between 12 beaker glasses (166 ml in each). Each beaker glass contained about 4800 eggs and these represented the starting quantity of eggs in each exposure tank. The eggs transferred to the tanks with ambient CO_2 concentration (380 ppm, tanks 1 and 2) were washed in seawater from these tanks three times, this washing was done by gently submerging a filter cup (mesh size 46 µm) containing eggs

in three different glass bowls with the ambient pCO_2. After this the eggs were transferred to marked glass bottle (0.5 L) with screw cap. The same procedure was done to the eggs to be transferred to exposure tanks 3 and 4 (2080 ppm), except that here the eggs were washed with seawater with elevated pCO_2 (2080 ppm). Finally, the bottles were submerged in the respective exposure tanks to equilibrate slowly to the temperature. The eggs were released into the exposure tanks when the pH and temperature were stable.

2.2 Experimental set up

Eggs from an indoor cultivated culture (originally collected from Trondheimsfjorden in 2004) were transferred to 12 cylindro-conical-shaped polystyrene tanks (90 L) where the growth phase of the experiment took place. The cohorts were exposed to two different regimes of CO_2 combined with two different temperature regimes. These two parameters were crossed to provide four unique treatment combinations (2x2 factorial design), and each treatment combination had three replicates (block A, B and C).

TABLE 2.1: Overview over the experimental set up, describing the treatments with the different pCO_2 and temperature, and triplicates for each treatment

Treatment ([CO_2]/temp.)	Block A	Block B	Block C
ambient (380 ppm/11˚C)	A1	B1	C1
warming (380 ppm/14˚C)	A2	B2	C2
warming*acidified (2080 ppm/14˚C)	A3	B3	C3
acidified (2080 ppm/11˚C)	A4	B4	C4

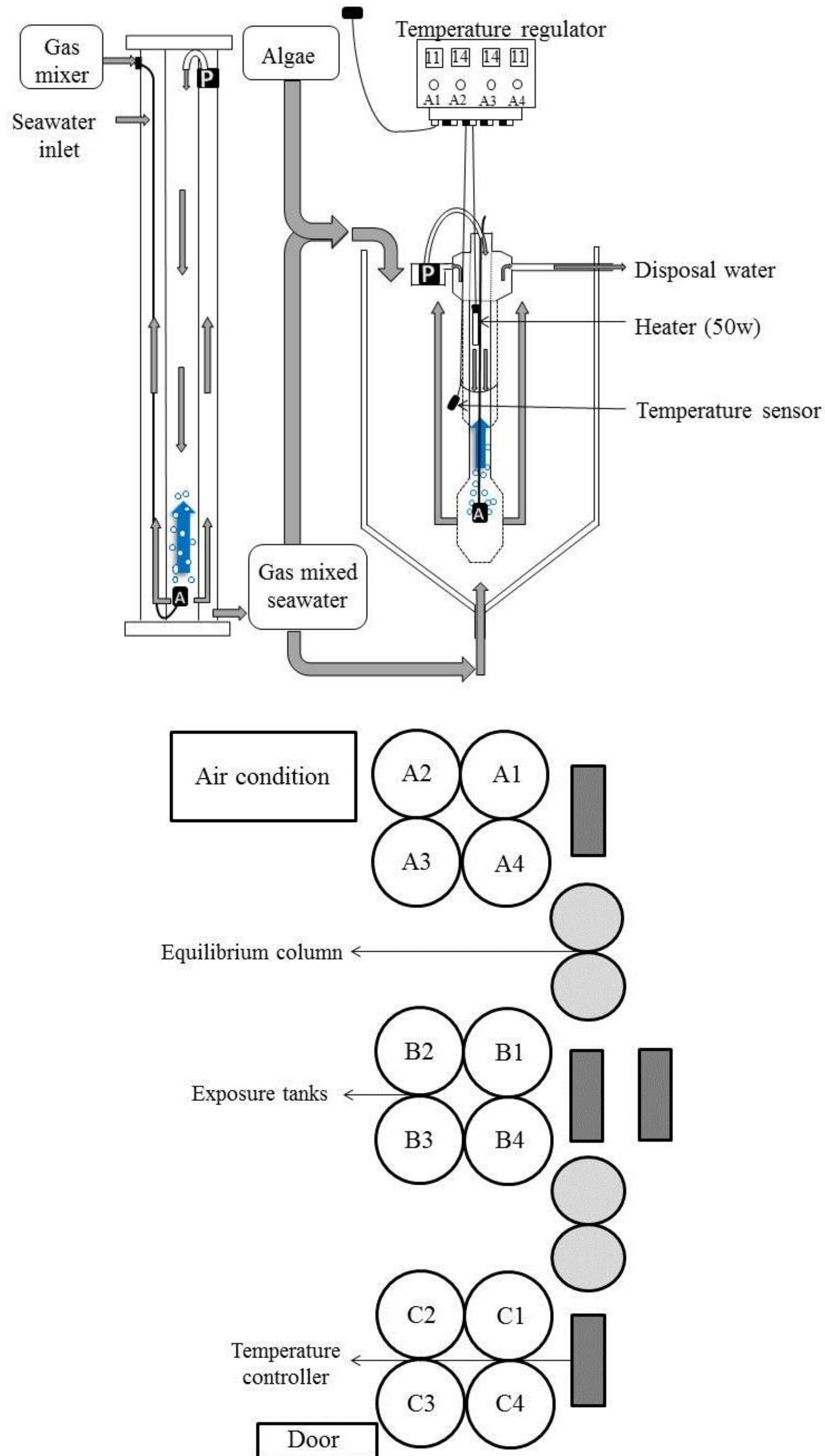


FIGURE 2.1: The experimental set up of the climate room is shown in the figure at the bottom, with four equilibrium columns and temperature regulators, and 12 exposure tanks. The figure at the top shows a detailed illustration of the equilibrium column and the exposure tank. The pumps are marked with a P and the air stones are marked with an A. Illustrations: A. Hanssen

2.2.1 Temperature control

The climate room had a set point at 10˚C in addition to the four (one to each block, A, B, C and one for the equilibrium columns) custom made temperature control units that were used to maintain the target temperatures, 11˚C and 14˚C, within each tank. These units consisted of four separate SYL-2372 PID temperature controllers (Auber instruments, Alpharetta, Georgia, USA) built into a waterproof plastic housing. The PID controllers comprised an auto-tune feature that improved accuracy and stability within one degree of accuracy (Auber Instruments, Instruction Manual, version 1.1). Before the experiment started, each PID controller was calibrated using ice-water solution (i.e. 0˚C). To each unit a feed line was integrated to provide power, and to each of the four PID controllers a temperature sensor (Platinum RTD. Pt100) measured the water temperature, while a heat element (Newatt Eco Therm Aquarium Heater, 50 W) maintained the chosen set point temperature. An integrated electrical fuse made it possible to shut down the temperature system separately in the four exposure tanks. The heat element was submerged about 10 cm down in the inner tube of the equilibrium system and the temperature sensor was placed in the middle of the tank, and kept at a stable position by a silicon rubber band strapped around the equilibrium system. The same temperature control system used in the exposure tanks was also applied to the two equilibrium columns that provide water to the treatment with 14˚C, so the inflowing water was pre heated to 13˚C before reaching the exposure tanks. This procedure was not necessary for the equilibrium column that provided water to the 11˚C treatment since the difference between the climate room (10˚C) and the exposure tanks only was 1˚C. The water was equilibrated to the different gas mixtures one degree below the target temperature to avoid potential oxygen super-saturation of the water.

2.2.2 Perturbation of the CO_2 concentrations

Seawater from 70m depth of Trondheimsfjorden was filtered and pumped into a reservoir. To produce different CO_2 concentrations in the different experiment tanks a custom build gas mixer was used to mix pressurized air with 100% CO_2 (Mapcon, Yara Praxair, Norway). In the columns seawater and pre-mixed CO_2 gas had an opposite flow to provide optimal conditions to equilibrate the water to different CO_2 concentrations. Inside these columns a cylinder with an air stone (lime wood, Aqua medic) in the bottom ensured that the gas mixtures entered the water in form of small bubbles. At the top of the column a submersible aquarium pump (Micro-Jet MC 450, Aquarium systems) made sure that the water from the column was lifted into the cylinder. Together this created a counter current system that

transferred gas into the water with high efficiency. From the bottom of the column the equilibrated water entered the experiment tanks to a water inlet at the top and bottom. To neutralize CO_2 loss due to air-water exchange, CO_2 enriched air was also applied directly into the experiment tanks by the same principle as in the equilibrium column. The water outlet was located at the top of the tank. To get a total water exchange in the experiment tanks during 24 hours the flow rate of introduced water was set to approximately 3.75 liter per hour in the tanks. To prevent animals from escaping the outlet the circulation system was covered with a nylon mesh (mesh size 120 µm), which was washed once during the experiment to prevent clogging due to algae and particles. The actual pCO_2 values were estimated from measurements of total alkalinity (T_A), pH and salinity, using the software calculating program CO2SYS-v2.1.xls (Pelletier et al., 2007)

2.2.3 Feeding

The cohorts were fed with a mixture of the unicellular algae *Isochrysis galbana* (Parke, 1949), *Dunaliella tertiolecta* (Bucher, 1959) and *Rhodomonas baltica* (Karsten, 1898) during both parts of the experiment. Even though it have been shown that *C. finmarchicus* do not use *I. galbana* active as a food source (Båmstedt et al., 1999), this algae mixture has proven to be successful during previous culturing of *C. finmarchicus* (Hansen et al., 2007).The algae mixture was continuously supplied from a stock solution by the aid of peristaltic dosing pump (Watson-Marlow 520S). Based on earlier feeding experiments, *I. galbana* was added with a concentration of 25μg C/L in addition, the combined target concentration of *D. tertiolecta* and *R. baltica* was set to 225μg C/L. The combined target concentration was chosen to be limiting (enough to maintain physiological processes), as surplus supply of food may mask a combined effect of CO_2 and temperature, or an effect of one of the parameters. To provide an equal distribution, the inflow of algae was located at the top of each exposure tank, together with the water inflow from the equilibrium column.

2.3 Experimental conditions

To maintain the environmental conditions close to the target levels during the experimental period (53 days for the animals exposed to 14˚C, and 62 days for the animals exposed to 11˚C), pH, salinity, total alkalinity (T_A), temperature and concentration of algae was measured, following a specific schedule.

2.3.1 Spectrophotometric pH measurements

Every day throughout the experiment period, spectrophotometric pH measurements of the 12 exposure tanks were performed according to a method modified from (Dickson et al., 2007). The method exploits the pH-dependent coloration of an indicator dye, *m*-cresol purple in this case, and it is also based on the total hydrogen ion concentration pH scale. The measurements were performed by using a Cary 50 Bio UV-visible Spectrophotometer (Varian, Inc., Mulgrave, Australia), Cary Single cell pettier accessory (Varian, Inc., Mulgrave, Australia) and a Micro-Jet MC450 multi-use micro-pump (Aquarium Systems, Sarrebourg, France).

The water samples were collected by placing a filtered cup (64 µm) at the edge of each exposure tanks (used to shield the animals from be collected), and then a 50 ml borosilicate glass sample bottle was rinsed twice inside the cup before filling the bottle so that a positive meniscus was obtained. Then a ground glass stopper was placed in the opening so that excess water was removed and the bottle sealed. After the sampling the bottles were placed in a water bath set (25˚C). The samples were then transferred to a special gas-tight optical glass cuvette (3.7 ml) creating a positive meniscus, before a Teflon cap was applied so that excess water and air bubbles were removed. 50 µl of *m*-cresol purple solution was pipetted into the cuvette for indicator dye, followed by adding sample water from the same sample until a positive meniscus was obtained (this procedure was first done without the indicator). The absorbance was measured at the wavelengths; 578, 434 and 730 nm using the RNA-DNA sub-menu of the Cary WinUV software. The values were typed into an excel sheet where pH of the samples were calculated (Dickson et al., 2007).

The measured pH_{tot}-values were compared to the target values that were expected at the different combinations of pCO_2 and temperature. The software program CO_2-Calc was used to calculate the target pH_{tot}-values and these results were used as target values in the exposure tanks at a combination of CO_2 and temperature (table 2.2). The CO_2 concentration in the gas mixture was fine-tuned to reach the proper pH-values in the different exposure tanks. The target pH-values in the 11˚C treatment were a bit lower than the 14˚C treatment, within the same CO_2 concentrations, due to the fact that dissolution of carbon dioxide in water increase with decreased temperature (Gattuso and Hansson, 2011).

TABLE 2.2: Calculated pH_{tot} values at 25ºC used as target values in the four different treatments. There are some differences in the pH-values at the different CO_2 concentrations as a consequence of carbon dioxides dilutions abilities at different target temperatures.

Treatment		pH_{tot} at 25˚C
$[CO_2]$	Temperature	
380 ppm	11˚C	7.833
380 ppm	14˚C	7.879
2080 ppm	14˚C	7.247
2080 ppm	11˚C	7.205

2.3.2 Salinity measurements

The salinity was measured daily from one of the exposure tanks (collected the same way as for the pH measurement) using a refractometer (H_2Ocean ATC salinity refractometer). To calibrate the instrument 2-3 drops of certified seawater (Scripps Institution of Oceanography, La Jolla, CA, USA) with known pH, salinity and A_T were placed on the sample surface. The salinity of the sample was measured the same way as the calibration.

2.3.3 Total alkalinity measurements (T_A)

The total alkalinity (T_A) was measured daily in one sample from one of the tanks (different tank each day), collected with the same sampling method as described for the pH measurements. Before analyzing the sample, an evaluation was done by measuring the T_A of certified seawater (Scripps Institution of Oceanography, La Jolla, CA, USA) with known T_A. The T_A was determined by using an automatic poteniometric titrator (TitraLab TIM860 Titration Manager, Radiometer Analytical SAS; Villeurbanne, France) with a water-jacked titration cell, additional thermostat bath and PHC2001-8 pH electrode. The following procedure was done as described by (Xiaowan et al., 2009), except that the ion strength in the titrant was regulated by sodium chloride (NaCl) instead of certified seawater.

2.3.4 Temperature measurements

The temperature in each exposure tank was measured every day by using a glass thermometer (VWR®Precision Thermometer, accuracy ± 0.3˚C). To get the most accuracy and to make sure that the temperature was measured the same way each time, the thermometer was placed in the middle of the exposure tank, attached to the top of the gas mixing system, and left to equilibrate for one to two minutes before reading. This measurement was done as an additional control to the temperature given by the PID controller.

2.3.5 Algae concentration measurements

The concentration of algae was measured every second day or twice a week during the experiment period by using a coulter counter (Multisizer^{TM}3 Coulter Counter®Beckman Coulter inc., USA). Water samples from the tanks were collected into a 25 ml cell counter cup, with the same procedure as for the pH measurements. The samples were placed on a stirring table (150rpm) (Labotron 960742, Infors, HT, Bottmingen) prior to coulter counter measurements to prevent sedimentation of algae. The amount of algae was measured by Multisizer^{TM}3 (software program) which extract and analyze three consecutive measurements (1 ml). *D.tertiolecta* and *R. baltica* have approximately the same size so their concentration (number of cells ml^{-1}) was measured together by integrating the size range between 5.333 to 9.561 µm.

2.3.6. Carbon content measurements

To measure the carbon content of the algae used in the feed mixture, stock solution of each algae was set up with air supply (to avoid sedimentation), in the dark at 10˚C for about 12 hours to reproduce the conditions during the experiment period. Then the stock solution was diluted 1:10 (10 ml stock solution and 90 ml filtered seawater), and coulter counted (same procedure as the algae concentration measurement) to determine the cell density. The solutions were then vacuum filtrated (50 ml) using a vacuum pump (Vacuumbrand GMBH, Wertheim Germany), with three replicates for each algae. The algae was collected on a standard filter (Whatman® glass microfiber filters, binder free, Grade GF/C, 24 mm, 1.2 µm) for algae filtration, folded into individual tin capsules (5x9 mm, Mikro Kemi AB, Sweden) and stored in a 96- well plate. The samples was stored in a freezing room (-20˚C) for 24 hours and then dried in a heating cabinet (Termarks TS4115, 60˚C) for another 24 hours, finally, the

tin capsules were compressed to small balls before they were delivered for carbon analysis (CHN Elemental Analyser 1106 , Carlo Erba Instruments, Italy).

2.4 Egg production

The treatments exposed to 14˚C was sampled in the first round, and the treatment exposed to 11˚C after. Six females were randomly chosen from the four different exposure regimes (one female from each of the three replicates), and incubated individually in special crafted egg production chambers (0.5 L) as presented in figure 2.2. For all treatments the first 24 hours were exclued from the results and used only as an indicator if the females had started spawning. After a female started her egg production, the female was observed and eggs were collected every 24 hours for five consectuive days. If a female died during course of the five days period a new female was incubated. The eggs produced by each female were collected in a plastic bucket through daily filtration (mesh size 300 µm). For the 11˚C treatments the eggs were incubated for nearly 26 hours and for the 14˚C treatments the incubation time was 50 hours. The incubation time was calculated by using equation 2.1 described by (Campbell et al., 2001).

$$D=a(T-\alpha)^b \qquad (2.1)$$

Development time (D) for any one stage, where a, b and α are constants and T is the temperature.

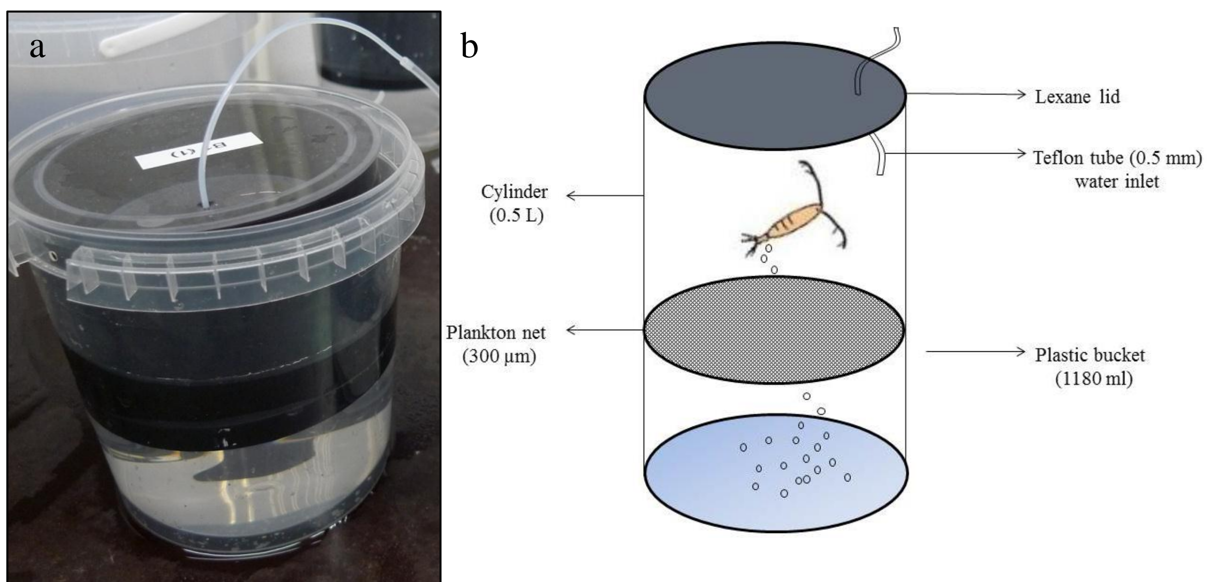


FIGURE 2.2: Picture (a) and graphical illustration (b) of the egg production chamber (0.5 L) used during the experiment. This was a special crafted chamber made for a previous experiment. Photo and illustration: A. Hanssen

2.4.1 Experimental set up

The chamber was formed as a cylinder (0.5 L) and a nylon filter(mesh size 300 µm) was mounted at the bottom of the cylinder, making sure that produced eggs fall through to avoid the mother feeding on them. At the top of the cylinder a plastic lid made sure that the cylinder was sealed (figure 2.2). To provide flow through, seawater entered through a Teflon tube (0.5 mm). Beneath the cylinder a plastic bucket (1180 ml, 13.3 cm i.d.) collected eggs and seawater, and when the bucket was filled up the seawater floated over to a plastic tank (72x72x12 cm) with a water outlet. This tank helped maintain the temperature in the chambers stable and avoided large temperature gradients. The chambers were also placed in a block design to avoid temperature gradient bias (figure 2.3). The environmental conditions within the incubation chambers, CO_2 concentration, temperature, salinity, T_A and algae concentration, were measured and controlled the same way as in the exposure tanks.

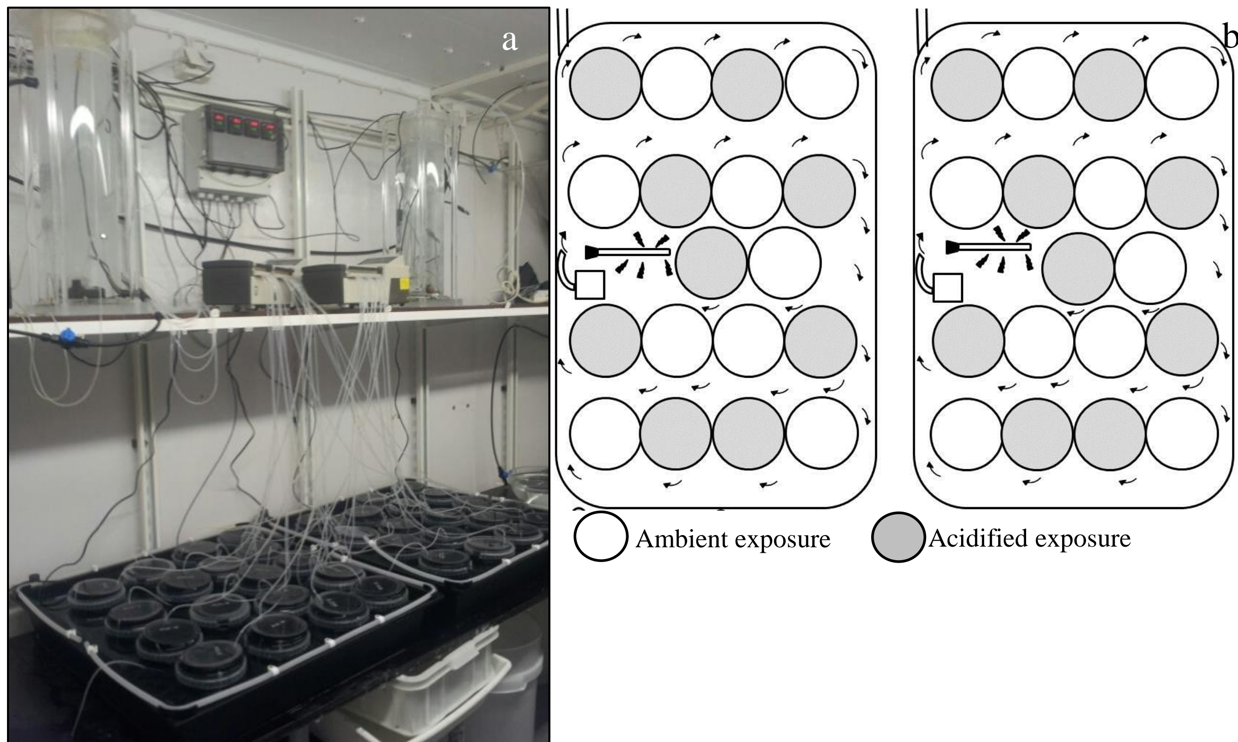


FIGURE 2.3: The picture at the top (a) shows an overview over the whole experimental set up for the egg production experiment. It includes a temperature regulator, water columns (with 380 ppm and 2080 ppm), pumps and all 36 chambers. The graphical block design (b) displays how the egg chambers were rearranged to avoid temperature gradient bias. Photo and illustration: A. Hanssen.

2.4.2 Egg sampling

The eggs were collected by daily exchanging the plastic bucket beneath the egg production chamber, and then filtrating the content through a funnel with removable 64 µm nylon filter in the bottom. Following the filtration, the filter was transferred to an 8 ml glass bottle and the initial water was added. The bottles were stored at the relevant temperature during an incubation time (50h for 11˚C and 26h for 14˚C) to allow the eggs to hatch, and the nauplii to develop. During this procedure the stress to the females were minimize due to the watertight integrity of the chamber, holding the water inside while the bucket was replaced with a new one.

2.5 Biometry

After each female had produced eggs during five consecutive days they were sedated with some drops of MS-222 (Finquel, Agent Chemical Laboratories Inc.). Then they were transferred gently in a petri dish with water and placed with their left body side up (figure 2.1). Pictures of the females were taken under a stereo dissection microscope (Leica MZ125, Leica microsystems, Wetzlar, Germany) with a camera (Sony DFW-SX900, color digital camera). After this the areal and length of the whole animal and lipid storage were measured on the pictures; this was done with the software program ImageJ (National Institutes of Health, Bethesda MD, USA) and a pen-on-screen graphical tablet (Wacom Intous3, Wacom CO., LTD., Saitama, Japan). Before starting the ImageJ software was calibrated to known pixel magnification of the photographs. Length measurement of the body was performed by drawing a line from the anterior dome of the head to the rear thoracic segment and the length of the lipid-sac was measured by drawing a line from the anterior part to the posterior part of the lipid-sac (figure 2.1). The area was measured by following the outline of both body and lipid-sac. From these measurements volume of the body and the lipid-sac, and lipid-sac percent were calculated in an excel-sheet according to (Miller et al., 2000)

FIGURE 2.4: A photograph taken of a female (acidified treatment) after ended experiment. The picture was taken for use in measuring of biometric data. The length was measured by drawing a line from the anterior part to the posterior part of the body or lipid-sac, and the area was measured by following an outer line along the circumference (yellow line). Photo: A. Hanssen

2.6 Sex ratio determination

After finalized experiment the exposure tanks were emptied through an opening at the bottom of the tanks. A filtering cup (mesh size, 64 µm) was placed beneath the plastic tube to collect all the remaining individuals. Further, the individuals were separated, the females from the males; also, stage determination of the copepodites was performed. The ratio was determined by dividing number of males over females.

2.7 Staining and storage

To separate living eggs and nauplii from dead a method based on neutral red staining was chosen. The stain (stock solution) was made pre experiment by adding 1 g neutral red powder (Neutral Red High purity biological stain; Acros Organics) to every 100 ml deionized water and stored in the dark until use. Before staining procedure the stock solution was incubated (15 min.) in *in situ* temperature (11˚C and 14˚C).

After the incubation each sample was stained one hour with 0.12 ml vital stain neutral red (1:100), measured with a pipette (Eppendorf research, 1000 µm). The stain was washed of by filtration through a removable nylon filter (mesh size 64 µm) in the bottom of a funnel. Finally, the two filters were stored with a conserving agent with phosphate buffer (Apotekerproduksjonen AS, Oslo) in the same bottles as the incubation. The samples were stored in a cooling room (4˚C) prior to visual examination. This was done under a microscope (Leica M205C) to count total number of individuals (eggs and nauplii) and also to determine the life stages of the nauplii.

2.8 Statistic analysis

The statistical analyses were performed by using SPSS (IMB® SPSS® Statistics, Version 21) and R (Rx64, version 3.03). SPSS was used to conduct *post hoc* tests and tests for homogeneity variations (Levene's). The level of significance was set to 0.05 in all tests.

The EP and HS data was analyzed by using a generalized linear mixed model fitted by maximum likelihood, with Laplace approximation (glmer from the package lme4 in R). The AIC-value was used to identify the most proper model for further analysis. Further, *post hoc* LSD tests were performed.

The biometric and ratio data were analyzed by performing a permutational multivariate analysis of variance (PERMANOVA) in R, using the function Adonis from the Vegan package. This test performs analysis of multivariate (or univarate) data in context of more complex sampling structures, experimental designs and models. By adopting a more parametric approach it allows i.e. measurements or tests of interactions among factors, and where a normal distribution is not demand. Further, if a significant interaction was present, the data sets were tested for variations within the sets with a Levene's test and if they satisfied the criteria for equal variance *post hoc* LSD tests were conducted.

3. Results

3.1 Water parameters

The mean values for the water parameters measured daily throughout the entire experiment (62 days) are listed in table 3.1. The mean pCO_2 concentration in the ambient-,warming-, acidified- and the warming and acidified combined was respectively 474±25.4 (380 ppm), 514±30.7 (380 ppm), 2021±237.4 (2080 ppm) and 2065±154.2 ppm (2080 ppm). The mean temperature (measured in ˚C) was 11.1±0.04 (ambient, 11˚C), 14.0±0.04 (warming, 14˚C), 11.1±0.05 (acidified, 11˚C) and 14.0±0.04 (warming*acidified, 14˚C), respectively.

TABLE 3.1: Experimental water parameters measured during the experiment period (62 days). The calculated values are the overall mean±SD for the tanks (n=3). Calculated pCO_2 (ppm) and $pH_{T,in\ situ}$ are also included to implement the experimental temperature (11 and 14°C). In addition are the calcite and aragonite saturation included. The treatments are named ambient (380 ppm/11˚C), warming (380 ppm/14˚C), acidified (2080 ppm/11˚C) and warming*acidified (2080 ppm/14˚C).

Treatment	$pH_{T,25˚C}$	Temp (˚C)	T_A	Salinity (ppt)	pCO_2 (μatm)	$pH_{T,in\ situ}$	Ω_{Ca}	Ω_{Ar}
Ambient	7.77±0.01	11.1±0.04	2234±85	33.4±0.4	474±25.4	7.97±0.02	2.71±0.17	1.72±0.11
Warming	7.78±0.02	14.0±0.04			514±30.7	7.94±0.02	2.84±0.19	1.82±0.12
Acidified	7.22±0.01	11.1±0.05			2021±237.4	7.39±0.03	0.79±0.05	0.50±0.03
warming*acidified	7.25±0.02	14.0±0.04			2065±154.2	7.38±0.03	0.87±0.08	0.55±0.05

3.2 Feeding conditions

The overall mean concentrations of algae feeding (*R. baltica, D. tertiolecta* and *I. galbana* (*µg* C/L)) in the four treatments (mean ± SD, n=3) during the exposure period (62 days) are shown in figure 3.1. The algae concentrations follow the same pattern until day 11, but after this point the concentrations in tanks at 14˚C were lower compared to the tanks at 11˚C. After day 11, the concentrations of algae decreased steadily in all treatments until day 23 was reached. Further, there was a sudden increase in the algae concentration in the acidified exposure group and also the acidified and warming combined, followed by a decrease in the subsequent measurement. Between day 43 and 53 there were some fluctuations between the four treatments, and the tanks exposed to higher temperature (14˚C) generally showed higher concentrations than the tanks maintained at 11˚C. At day 55 all four treatments were gathered into a peak and from this point they followed the same general pattern.

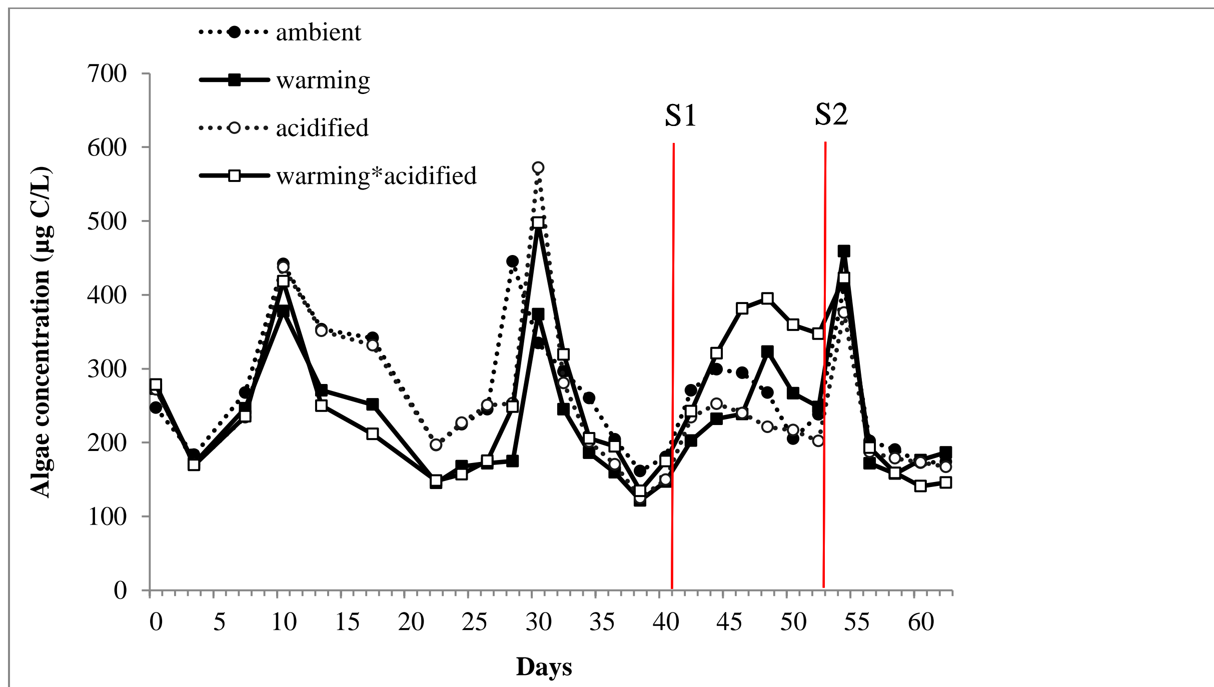


FIGURE 3.1: Measured algae concentrations (μ*g* C/L) in the four different treatments. The algae concentrations were measured during the experimental period of 62 days (mean±SD, n=3). Ambient (380 ppm/11˚C), warming (380 ppm/14˚C), acidified (2080 ppm/11˚C) and warming*acidified (2080 ppm/14˚C). S1 indicates the sampling start to the egg production for the treatments exposed to 14˚C (day 43) and S2 for the 11˚C treatments (day 54). The algae concentration is marked on the y-axis while the days are on the x-axis.

The mean carbon content (μg C/L) for the ambient-, warming-, acidified-, and the warming and acidified combined treatment was respectively 263.3±79.8, 227.7±79.4, 246.1±95.0, 256.5±101.8. The target value was 250 μg C/L.

The results of the carbon analysis done post-experiment showed that *D.tertiolecta* had the highest carbon content (74.3±1.3 pg C/cell). The carbon content of *R.baltica* was 54.6±1.2 pg C/cell and *I. galbana* had 15.1±0.2 pg C/cell. Further, the carbon content in the algae was used to calculate the total carbon concentrations (μg C/L).

3.3 Egg production experiment

3.3.1 Water parameters

Table 3.4 shows pH, temperature, total alkalinity and salinity values that were measured in three egg production chambers from each treatment. The mean pCO_2 concentration in the ambient-,warming-, acidified- and the warming and acidified combined was respectively 439±15.6 (380 ppm), 474±57.7 (380 ppm), 1621±201.9 (2080 ppm) and 1724±155.8 ppm (2080 ppm). The mean temperature (measured in ˚C) was 11.1±0.01 (ambient, 11˚C), 14.0±0.01 (warming, 14 ˚C), 11.1±0.03 (acidified, 11˚C) and 14.0±0.04 (warming*acidified, 14 ˚C), respectively

TABLE 3.2: Water parameter values (mean ± SD, n=3) from the egg production experiment (21 days), calculated from samples taken from two new egg production chambers every day within each treatment (except A_T and salinity which were measured from the exposure tanks during the egg production part). Also included are the calculated pCO_2 (ppm) and $pH_{tot,in\ situ}$ values, where experimental temperatures are taken into consideration (11 and 14°C). In addition the calcite and aragonite saturation state are included. The treatments are named ambient (380 ppm/11˚C), warming (380 ppm/14˚C), acidified (2080 ppm/11˚C) and warming*acidified (2080 ppm/14˚C).

Treatment	$pH_{T,25°C}$	Temp (˚C)	A_T	Salinity (ppt)	pCO_2 (μatm)	$pH_{T,in\ situ}$	Ω_{Ca}	Ω_{Ar}
ambient	7.79±0.01	11.1±0.01	2215±106	33.3±0.3	439±15.6	7.99±0.01	2.58±0.18	1.64±0.11
warming	7.80±0.01	14.0±0.01			474±57.7	7.96±0.02	2.70±0.19	1.73±0.12
acidified	7.30±0.03	11.1±0.03			1621±201.9	7.47±0.06	0.87±0.11	0.56±0.07
warming*acidfied	7.31±0.01	14.0±0.04			1724±155.8	7.45±0.02	0.82±0.05	0.52±0.03

3.3.2 Feeding conditions

The algae concentrations that prevailed during the egg production experiment are shown in figure 3.2. Some variations in algae concentration between the days for all the exposure groups can be observed; still, they all follow the same general pattern during the incubation time of 8 and 11 days. The mean carbon content (μg C/L) for the ambient-, warming-, acidified-, and the warming and acidified combined treatment was respectively 144.2±18.8, 170.6±22.7, 153.1±40.9, 167.7±35.0. The target value was 250 μg C/L.

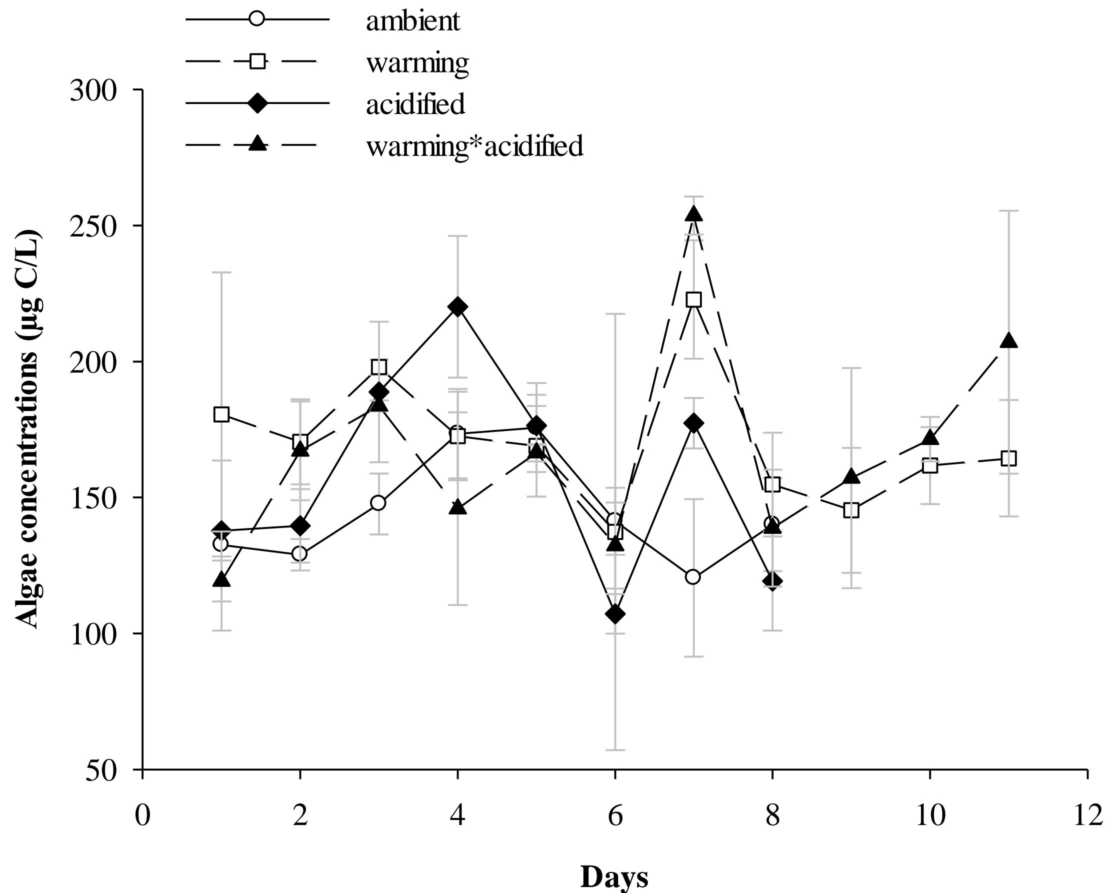


FIGURE 3.2: Algae concentration (µg C/L) measured during the egg production experiment (mean ± SD, n=3). The first eight days include the chambers treated at 11˚C, while the animals exposed to 14˚C were incubated for a total of 11 days. The y-axis represents the algae concentration and the x-axis represents the days.

3.3.3 Egg production

The EP results showed some variations both within and between groups (figure 3.3). The ambient exposure group (11˚C, 380 ppm) displayed the highest mean EP during the five days (~7.5), while the lowest rate (~1) was observed in the treatment with elevated temperature only (warming). The mean rate in the acidified only and warming and acidified combined was 5.6 and 4.8, respectively. All treatments, except the ambient treatment, displayed a drop in the production on the final day of incubation. The statistical analysis (table 3.6) revealed a significant interaction between elevated temperature and pCO_2 on the EP ($F_{3,55}$=5.610, p=<0.001). Further analysis showed that warming alone caused significant reductions in EP by 83% compared to the ambient treatment (p=0.004) and 79% compared to the acidified treatment (p=0.020). No significant differences between the acidified-, or warming and acidified combined treatment were observed compared to the ambient treatment.

TABLE 3.3: Statistic outcome from a generalized linear mixed model with poisson distribution conducted on the EP results (from five days). The significant effects ($p<0.05$) are bold and indicated with an asterisk (*). The intercept model had an AIC-value of 353.4. The notation d.f = degrees of freedom. Each replicate is the mean value EP from six spawning females.

Factors	d.f.	AIC	F-value	*p*-value
Temperature	**3,55**	**-**	**-7.20**	**6.00E-13***
CO_2	3,55	-	-2.14	0.33
Temperature* $\mathbf{CO_2}$	**3,55**	**282.5**	**5.61**	**2.02E-08***

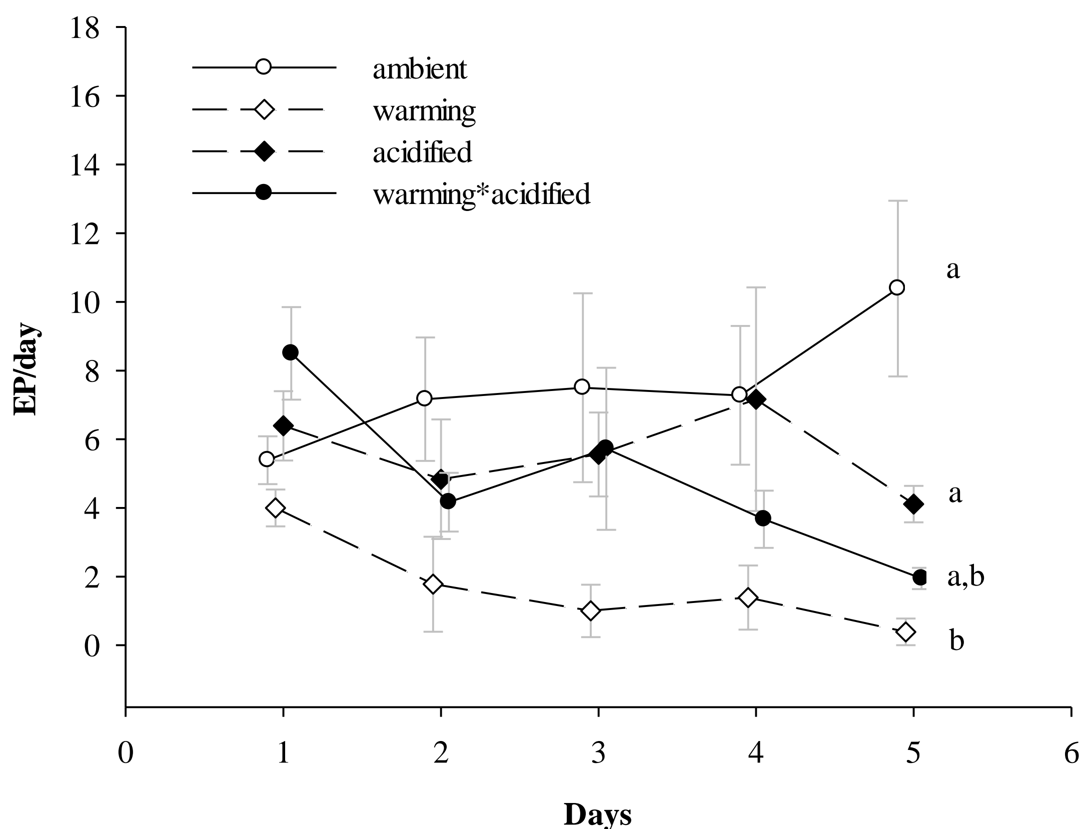


FIGURE 3.3: EP over five subsequent days from the four exposure treatments (mean ± SE, n=3). Ambient (380 ppm/11˚C), warming (380 ppm/14˚C), acidified (2080 ppm/11˚C) and warming*acidified (2080 ppm/14˚C). The significant differences ($p<0.05$) are marked with different letters (a, b). Each replicate is the mean value EP from six spawning females. The y-axis represent number of eggs per day and the x-axis represent each day.

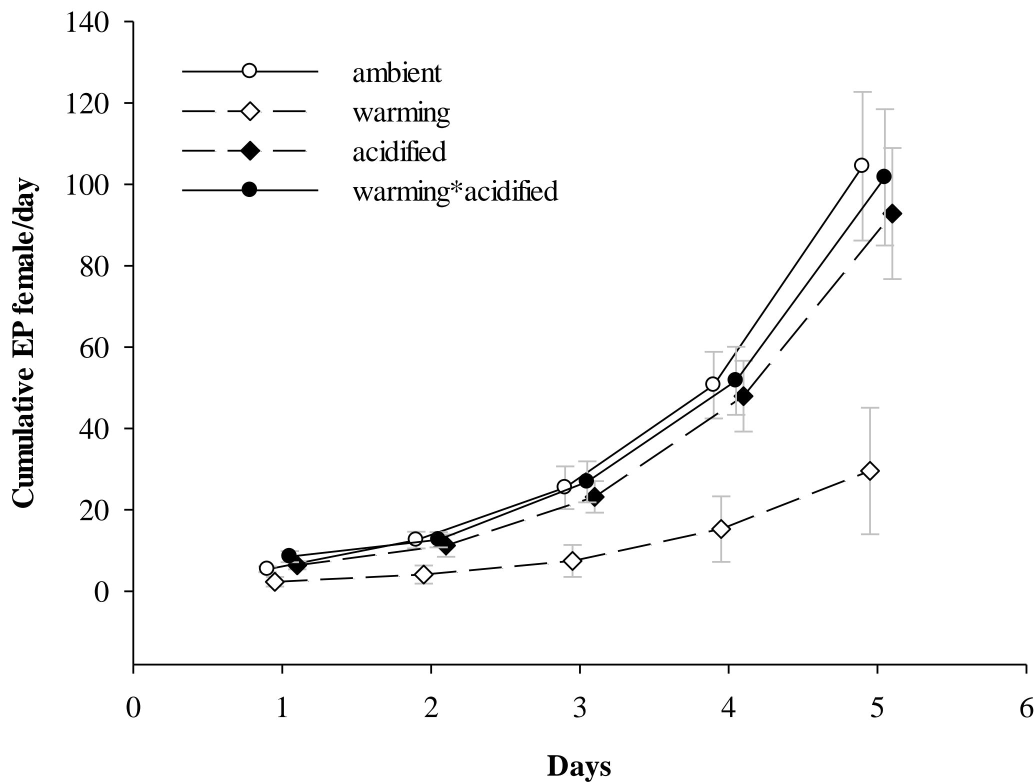


FIGURE 3.4: Cumulative egg production per female (mean ± SE, n=3) in the different experimental treatments during the incubation period. Each replicate is the mean value from six spawning females.

The cumulative egg productions in the females (figure 3.4) are another way of presenting the EP data. Here, the significant reduction in EP of the warming treatment compared to the ambient- and acidified treatment is more evident compared to figure 3.3.

3.3.4 Hatching success

Statistical analysis (table 3.7) showed a significant interaction between elevated temperature and pCO_2 on the HS ($F_{3,55}$=14.0, *p*=<0.001). Further analysis showed no significant differences between the ambient, acidified and warming and acidified combined. However, a significant reduction in HS (75% reduction) (*p*=<0.001) was observed in the warming treatment compared to the ambient treatment. Also, significant increases in HS was observed in the acidified-(77% increase) (*p*=<0.001), and the combined (warming*acidified) treatment (71% increase) (*p*=0.001) compared to the warming treatment.

TABLE 3.4: Statistic outcome from a generalized linear mixed model with poisson distribution conducted on the EP results from five days. The significant effects (p<0.05) are bold and indicated with an asterisk (*).The intercept model had an AIC-value of 1436. Each replicate is the mean value EP from six females.

Factors	d.f.	AIC	F-value	P-value
Temperature	**3,55**	**-**	**1.9**	**<2.00E-16***
CO_2	3,55	-	-19.9	0.057
Temperature*CO_2	**3,55**	**758**	**14.0**	**<2.00E-16***

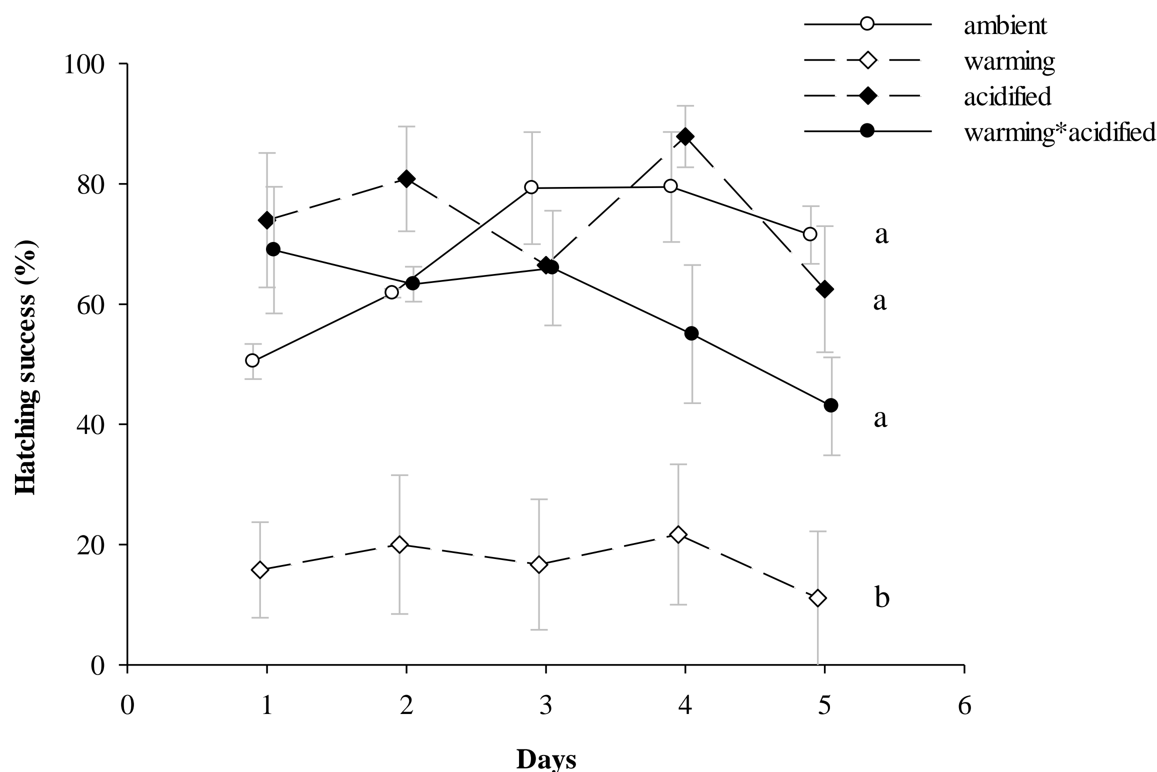


FIGURE 3.5: HS for five subsequent days from the four exposure treatments (mean ± SE, n=3). Ambient (380 ppm/11˚C), warming (380 ppm/14˚C), acidified (2080 ppm/11˚C) and warming*acidified (2080 ppm/14˚C). The significant differences (p<0.05) are marked with different letters (a, b). Each replicate indicate the mean EP from six females. The y-axis represents the HS in percent and the x-axis is the number of days.

3.4 Body volume and lipid storage

TABLE 3.5: The statistic outcome from a permanova analysis of the biometric data (retrieved after egg production) on body volume and lipid storage in the different treatments. MS=mean square, SS=sum of squares and the degrees of freedom is noted d.f.. The significant effects (p<0.05) are bold and marked with an asterisk (*).

Variables	Factors	d.f.	SS	MS	F-value	R^2	p-value
Body volume (mm^3)	Temperature	1	0.00257	0.00257	0.5702	0.0440	0.483
	CO_2	1	0.01253	0.01253	2.7788	0.2143	0.121
	Temperature*CO_2	1	0.01179	0.01179	2.6158	0.2018	0.157
	Residuals	7	0.03156	0.00451	-	0.5399	-
Lipid-sac volume (mm^3)	**Temperature**	**1**	**0.00211**	**0.00211**	**9.0924**	**0.4108**	**0.005***
	CO_2	1	0.00034	0.00034	1.4731	0.0665	0.280
	Temperature*CO_2	1	0.00082	0.00082	3.5678	0.1612	0.063
	Residuals	7	0.00185	0.00023	-	0.3614	-
Lipid-sac volume percent	**Temperature**	**1**	**16.3100**	**16.3100**	**15.8673**	**0.6281**	**0.006***
	CO_2	1	0.18500	0.18500	0.1800	0.0071	0.692
	Temperature*CO_2	1	1.2480	1.24810	1.2142	0.0481	0.312
	Residuals	7	8.2232	1.02790	-	0.3167	-

3.4.1 Body volume

The mean body volume for the ambient-, warming-, acidified-, and warming and acidified combined treatment was respectively 0.8±0.13, 0.7±0.06, 0.6±0.13 and 0.7±0.11 mm^3. No significant interaction between temperature and pCO_2 ($F_{3,7}$=2.62, p=0.157) was observed (table 3.8). However, reductions in body volume in the warming- (13% reduction), acidified- (21% reduction), and warming and acidified combined treatment (19% reduction) were observed compared to the ambient treatment.

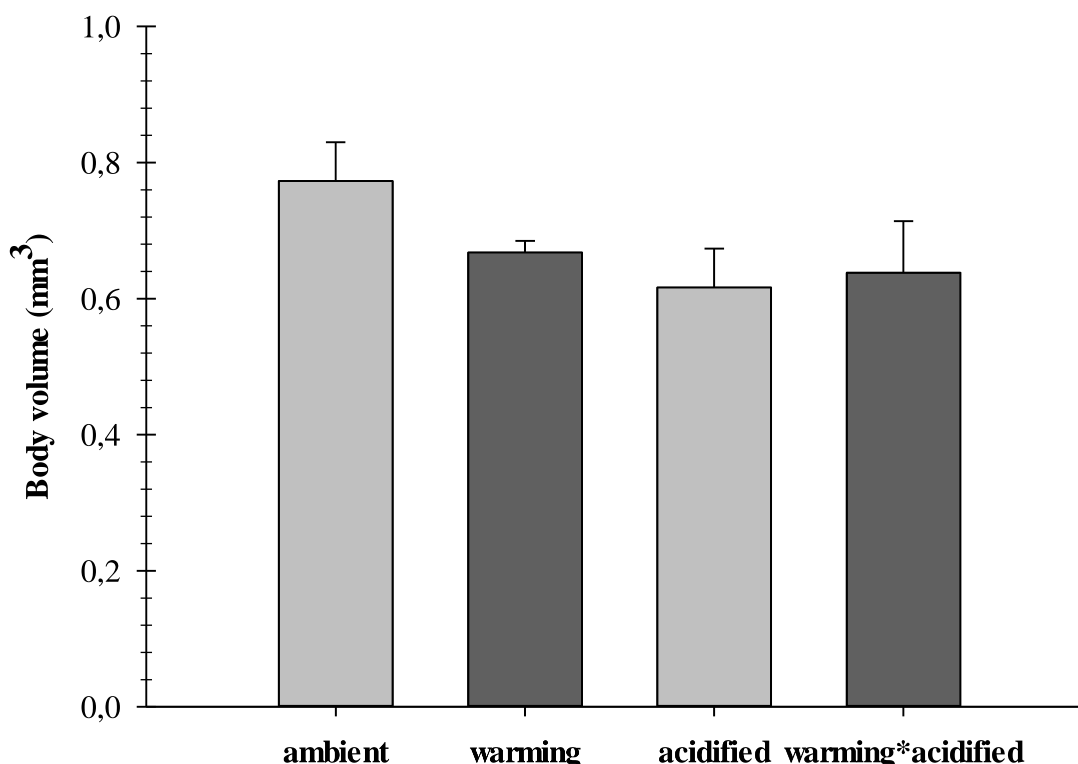


FIGURE 3.6: Body volume (mm^3) from the four different treatments (ambient (380 ppm/11˚C) (16 females), warming (380 ppm/14˚C) (4 females), acidified (2080 ppm/11˚C) (18 females) and warming*acidified (2080 ppm/14˚C) (17 females)). The treatments are marked on the x-axis and the body volume is represents the y-axis, measured as mm^3. The bars indicate mean ± SD, except for the warming treatment (n=2) n=3.).

3.4.2 Lipid storage

The mean lipid-sac volume for the ambient-, warming-, acidified-, and warming and acidified combined treatment was respectively 0.05±0.05, 0.00±0.00, 0.02±0.02 and 0.01±0.01 mm^3. For the percent lipid-sac volume the mean values was respectively 3.40±2.79, 0.16±0.20, 2.60±.3.41 and 1.09±2.02 %.

The statistical analysis revealed no significant interaction between pCO_2 and temperature (table 3.8) on the lipid-sac volume ($F_{3,7}$=3.57, *p*=0.063) or the percent lipid-sac volume($F_{3,7}$=1.21, *p*=0.312). However, temperature showed a significant effect on the lipid storage ($F_{3,7}$=9.09, *p*=0.005 for the lipid-sac volume and $F_{3,7}$=15.87, *p*=0.006 for the percent lipid-sac volume). No significant differences between warming, acidified, and warming and acidified combined was observed. The ambient treatment, however, was significant higher than the other three treatments.

Figure 3.7 show significant reductions in lipid-sac volume in the warming-, (95% reduction) (p=0.018), acidified- (60% reduction) (p=0.019), and the warming and acidified combined treatment (85% reduction) (p=0.048) compared to the ambient treatment.

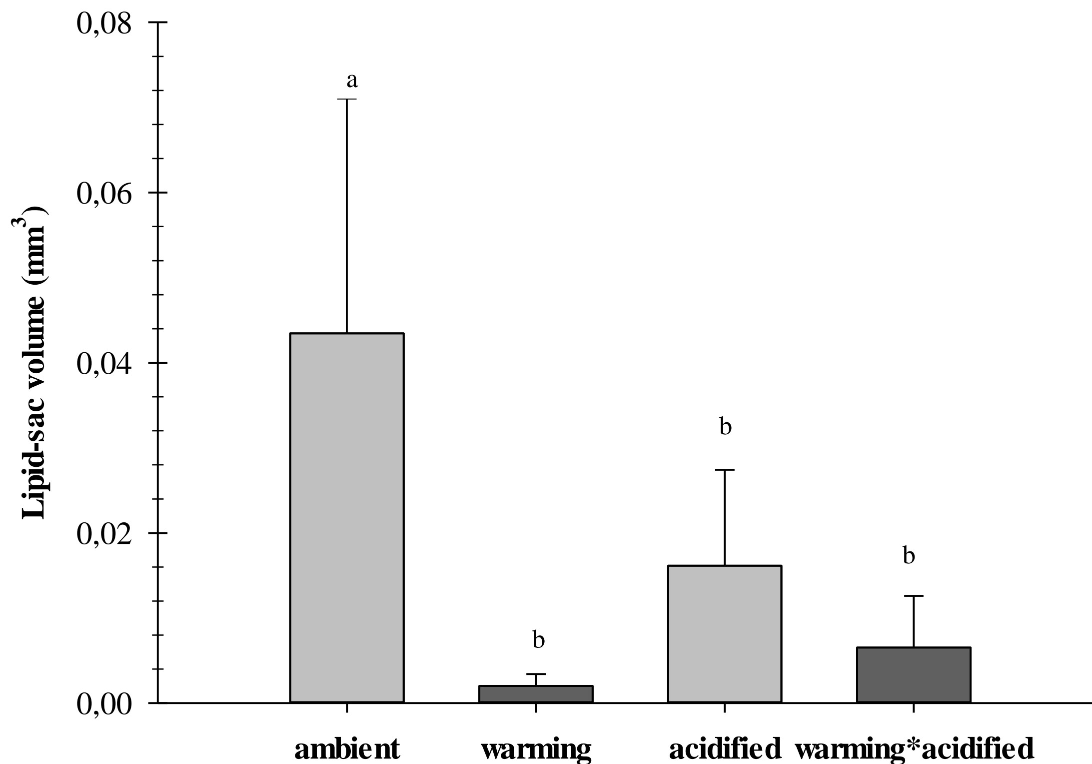


FIGURE 3.7: Mean lipid-sac volume (mm^3) from the four different treatments (ambient (380 ppm/11˚C) (16 females), warming (380 ppm/14˚C) (4 females), acidified (2080 ppm/11˚C) (18 females) and warming*acidified (2080 ppm/14˚C) (17 females)). The treatments are marked on the x-axis. The bars indicate mean ± SD, except for the warming treatment (n=2) n=3. Significant differences ($p<0.05$) between the different groups are indicated by different letters (a, b). The y-axis represents the lipid volume measured in mm^3.

The volume percent lipid-sac (of the whole body) of the female copepods after egg production from the different treatments is shown in figure 3.8. A similar trend as for lipid-sac volume was observed. The statistical analysis showed the similar results (table 3.8) as for the lipid-sac volume; no significant interaction between temperature and pCO_2 was seen. Further analysis revealed that the warming treatment showed significant reductions in the volume percent lipid-sac (98.3% reduction) compared to the ambient treatment (p=0.007) and to the acidified treatment (88% reduction) (p=0.014). A significant reduction in the warming and acidified combined treatment (67.3% reduction) (p=0.045) compared to the ambient treatment was also seen. However, no other differences were observed.

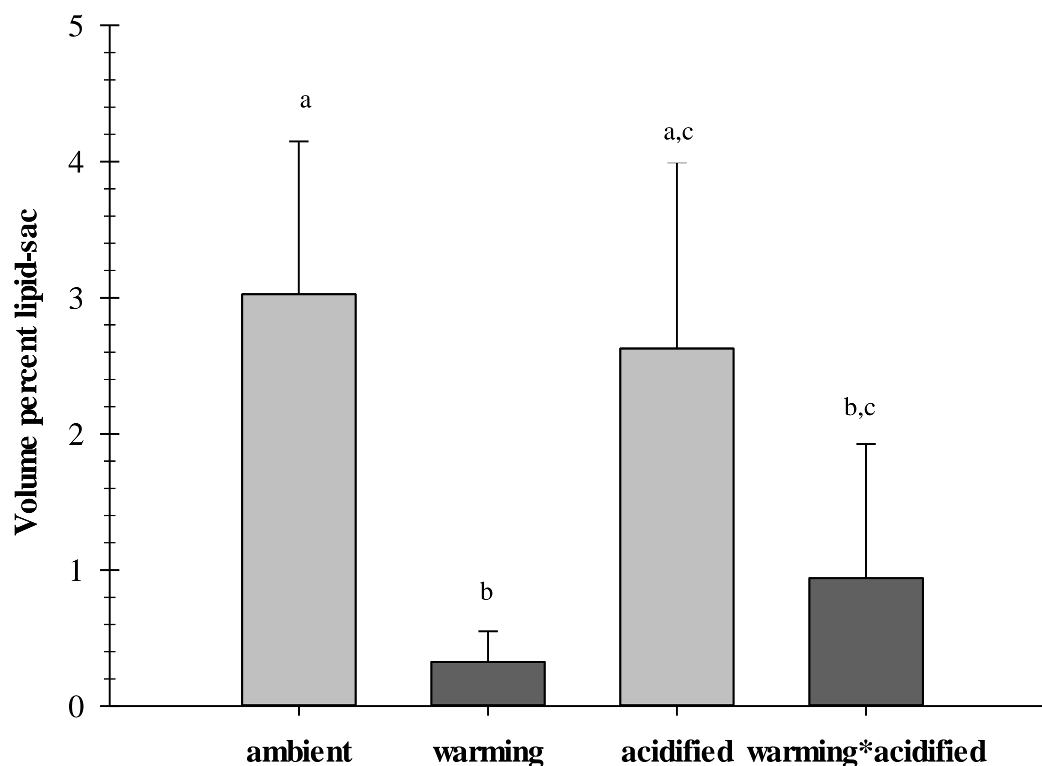


FIGURE 3.8: Mean lipid-sac percent (volume as percentage of the whole body) from the four different experiment groups (ambient (380 ppm/11˚C) (16 females), warming (380 ppm/14˚C) (4 females), acidified (2080 ppm/11˚C) (18 females) and warming*acidified (2080 ppm/14˚C) (17 females)). The treatments are marked on the x-axis. The bars indicate mean ± SD, except for the warming treatment (n=2) n=3. The significant differences ($p<0.05$) are marked with different letters (a-c). The y-axis represents the volume lipid-sac measured as percent relative to body size.

3.5 Sex ratio

Some variations with respect to the total number of animals left in the exposure tanks following the egg production experiment were observed. The mean number in the ambient-, warming-, acidified-, and warming and acidified combined treatment was respectively 441±91, 260±34, 660±119 and 654±109. In spite of the variation in number of adults per tank, no significant effect of either elevated CO_2 or temperature alone was discovered (table 3.9). The lowest observed mean ratio (figure 3.8) was in the warming treatment (0.28) and the highest ratio in the warming and acidified combined treatment (0.43).

TABLE 3.6: An analysis of variance table that shows the outcome from a permanova conducted on the sex ratio results from the four exposure treatments (mean ± SD, n=3). No significant effects of CO_2 concentration or temperature on the sex ratio were observed. d.f.= degrees of freedom. SS= sum of squares and MS= mean square.

Factors	d.f.	SS	MS	F-value	R2	*p*-value
Temperature	1	0.019200	0.019200	2.7560	0.1850	0.138
CO_2	1	0.019200	0.019200	2.7560	0.1850	0.139
Temperature*CO_2	1	0.009633	0.009633	1.3828	0.0928	0.258
Residuals	8	0.055733	0.006967	-	0.5371	-

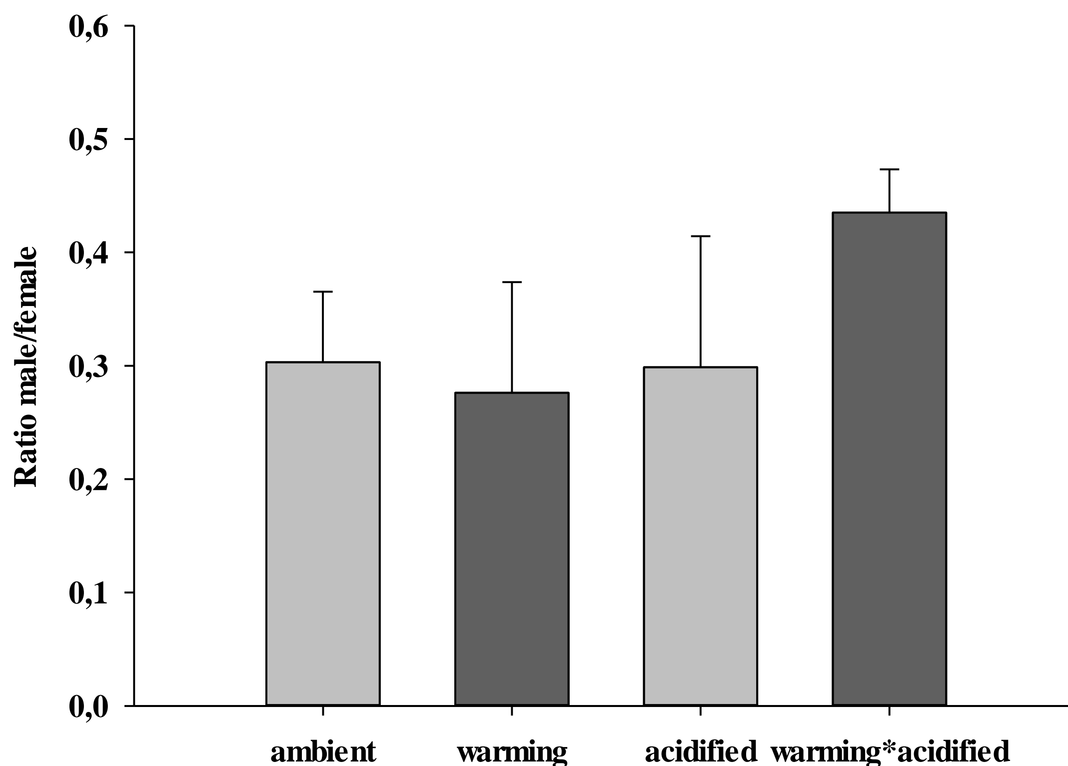


FIGURE 3.9: Sex ratio from the four different treatments (ambient (380 ppm/11˚C), warming (380 ppm/14˚C), acidified (2080 ppm/11˚C) and warming*acidified (2080 ppm/14˚C)), the treatments are marked on the x-axis. The bars indicate mean ± SD, n=3. The y-axis represents the ratio, male/female.

3.6 Staining with neutral red

All samples were stained with neutral red to separate the living and dead individuals. Figure 3.10 shows some examples of the results from the samples (picture c) and also from a test done post-experiment (picture a and b). A range of different red nuances was observed (figure 3.10 a), even though all the eggs were dead after exposing them to hot freshwater for one hour before staining. Also, in picture b) it can be observed different shades of red, and the eggs in the picture are stained right after collection.

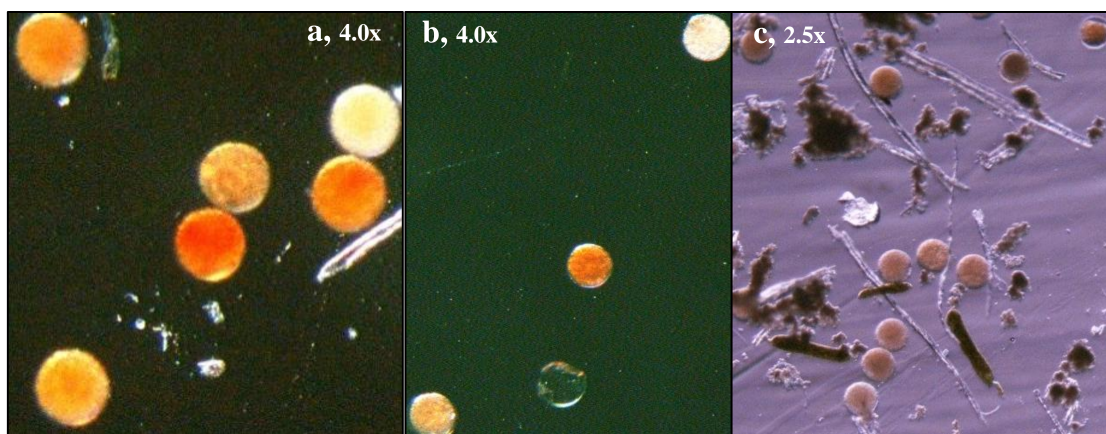


FIGURE 3.10: Staining with neutral red, pictures are taken during the experiment and also from tests done after the experiment. Picture a (4.0x) represent a test where all the eggs were killed on forehand, while in picture b (4.0x) the eggs were alive. Picture c (2.5x) are taken during the experiment.

4. Discussion

4.1 Environment- and feeding conditions

The preparations done pre-experiment and the work done during the experiment had high priority on maintaining the different environmental parameters as close to target levels as possible. The small deviations observed in temperature are not considered to be large enough to have caused any significant changes between the treatments. In the egg production experiment the pCO_2 values diverged quite substantially from the target values, especial for the acidified-, and warming and acidified combined treatment (1621±201 and 1724±155, see table 3.2). For the ambient- and warming treatment the observed values were 439±15and 474±57, respectively.

The measured algae concentrations in the different treatments show that the animals received a fairly stable carbon concentration (248.64 ± 89.03 μg C/L) that was close to the targeted value of 250 μg C/L (see figure 3.1). During the experiment the concentration in the treatments that were exposed to elevated temperature was lower than the tanks exposed to the ambient temperature and may be due to more intensive grazing. Temperature has been shown to affect growth through regulation of development time (Campbell et al., 2001), thus the animals in the treatments exposed to 14˚C reached the active feeding stage (NIII) before the treatments at ambient temperature. Although development rates were not measured in the present study, indications of increased development rates at the higher exposure temperature were observed.

For *R. baltica* previous studies have, based on a linear relationship (R^2 =0.976) between algal cell density and carbon biomass, estimated that the carbon content is 45 pg/cell (Skogstad, 2010). This content is close to the same as the value (54 pg C/cell) obtained from the carbon analysis performed in relation to this study. For *D. tertiolecta* no literature on the carbon content has been found, but the C/N-analysis revealed that in the present study the content was approximately 74 pg C/cell. Båmstedt et al. (1999) claimed that *C. finmarchicus* do not use *I. galbana* as a food source, which is also in line with another study that reported that the grazing impact of *C. finmarchicus* on cells < 20µm was negligible (*I. galbana* is ~5µm) (Gifford et al., 1995). Including *I. galbana* in the algae mixture was still preferred in the present study as a mixed diet of the three listed algae species have been shown to be successful upon previous culturing of *C. finmarchicus* (Hansen et al., 2007).

For the egg production experiment the average carbon concentration was lower (~159 μg C/L) than the concentration in the exposure tanks; this, in combination with previous literature on EP and food supply, may explain why the mean egg production over five days (~5, n=72) was low. These findings corresponds with data retrieved from Hirche et al. (1997) where animals fed with 150 μg C/L had a spawning interval of four days and a EP rate equal to seven (n=7). It has been suggested that egg production is related to the feeding conditions two to three days prior to spawning and that the females need these days to adapt to new conditions (Hirche et al., 1997; Irigoien et al., 2000). This may explain the apparent drop in EP at day two for all the treatments, except the ambient one (see figure 3.3).

4.2 Biometric measurements

4.2.1 Body volume

The experiment revealed no significant interaction between elevated temperature and pCO_2 on the body volume. Although no significant differences were observed between the different treatments, the animals in the warming- (13% reduction), acidified- (21% reduction) and the warming and acidified combined treatment (19% reduction) were somewhat smaller compared to the ambient treatment (see figure 3.6).

The temperature-size rule is a well-known phenomenon, which describes the phenotypic plastic response of size to temperature. A smaller size results due to the fact that the increase in growth rate does not often match the increase in development rate that occurs when the temperature is elevated (Van der Have and De Jong, 1996). Breteler and Gonzalez (1988) reported that the prosome length of the copepod *Pseudocalanus elongatus* (Boeck) was inversely related to temperature, and similar results were found in a study on *C. finmarchicus* (Campbell et al., 2001). Also, the latter study revealed that the algae concentrations had a significant negative effect on prosome length (Campbell et al., 2001), a result that earlier has been found by Breteler and Gonzalez (1982). As organisms must divide energy between physiological maintenance, growth and reproduction, they might limit growth in favor of the other two factors (Sheridan and Bickford, 2011).

Based on previous literature a reduction in body size of the animals in the warming treatment was expected. However, in the present study temperature alone had no discernible effect on the body volume. It has been stated that body size, among other factors, is related to egg

production (Diel and Tande, 1992; Plourde and Runge, 1993). In the present study only the females that completed their spawning were measured. Due to this it is possible that a size selection has occurred, and thereby obscured the effect of elevated temperature on the body volume.

4.2.2 Lipid storage

The females exposed to ambient condition showed a significant higher lipid content compared to the warming- (95% reduction), acidified- (60% reduction), and warming and acidified combined treatment (85% reduction) (see figure 3.7 and 3.8). While no significant differences were observed between the other treatments, the pattern observed suggest a negative additive effect on lipid volume when acidification is combined with elevated temperature.

Rising temperature increases the metabolism and leads to a higher demand for energy (Mauchline, 1998; Koski and Kuosa, 1999) and increased filtering rates (Irigoien et al., 1996). However, another study have reported that increased filtering rates do not necessary lead to higher food intake, since copepods also feed by tactile encounter (Cushing, 1968). The increased development rate at warmer temperature could give the animals less time two grow and build up their lipid storage (Campbell et al., 2001). Reduced lipid storage also correlates with body size; maximum lipid-sac volume increases with prosome length and body volume, and it has been suggested that organs other than the lipid-sac have a constant size (Miller et al., 2000). Lipid stores are used by female copepods to form reproductive tissue but is also used for growth and physiological functions, and energy reserves at times of starvation or fluctuating food supply (Mauchline, 1998). Thus, a reduction in lipid stores may affect the population size of this species trough reduced reproduction and a reduced ability to cope during periods of low food supply. An increase in temperature (+3˚C) is projected to reduce the biological primary activity by ~35% (Gröger et al., 2012). This may lead to more food limited conditions for marine species by the end of this century, compared to the already limited conditions in present time.

Several of the females that completed spawning for five consecutive days had no visible lipid-sac left (20/54), or such a small amount that it was difficult to detect (figure 4.1) compared to the females with large lipid-sac (figure 4.1). This could indicate that the food supply has not been sufficient during spawning or that the energetic costs of spawning under warm conditions have been in excess due to a reduction in the available energy for reproduction. In the two treatments exposed to 14˚C only half of the females had a visible lipid-sac. This indicates that animals exposed to warmer temperature have a higher demand for food due to increased metabolism, and may be more vulnerable to food limitation compared to animals exposed to ambient temperature. However, elevated pCO_2 seems to moderate the negative effects of elevated temperature as the animals in the warming and acidified combined treatment seems to be less food limited compared to the warming treatment. This may be explained by the results of a previous study that has shown induced developmental delay in some of the development stages in *C. finmarchicus* during exposure of elevated pCO_2 (2080 ppm) (Håkedal, 2013). Consequently, it is possible that elevated pCO_2 might, to some extent, counteract the acceleration effect of increased temperature, thus providing the animal with more time to grow during each developmental stage.

FIGURE 4.1: The female in the picture at the top (a) has no visible lipid-sac left (warming treatment, 380 ppm/14˚C). The prosome length is 2.24 mm. The female in the bottom picture (b) has a large lipid-sac (acidified treatment, 2080 ppm/11˚C). The prosome length is 2.43 mm and the lipid-sac is 9.53% of the total body size. Photo: A. Hanssen

4.3 Egg production

The results in the present study show that daily EP during the incubation of females were quite small (4.6±2.07), and clutches >20 were only observed in 11 of the 54 females. Also, it was observed that warming alone caused a significant reduction of the EP compared to the ambient- (83% reduction), acidified- (78% reduction), and warming and acidified combined treatment (79% reduction). No differences were observed between any of the other treatments. A significant interaction between elevated temperature and pCO_2 in the present study shows that acidification had a positive antagonistic effect on EP when combined with elevated temperature, and thereby caused the fecundity to return to a normal level.

Other studies have shown similar interactions between elevated temperature and pCO_2 on EP. Including a study done on *Acartia* spp (Vehmaa et al., 2012), here, elevated temperature (+3˚C) led to an increased EP. However, a smaller increase was observed when the animals were exposed to the elevated temperature and acidified conditions combined, indicating an antagonistic effect. Another study on a Mediterranean copepod *Acartia clause* (Zervoudaki et al., 2013) showed a significant synergetic interaction between elevated pCO_2 (pH=7.83) and temperature (+4˚C) on egg production, however, acidification alone had no discernible effect, whereas warming, food and duration of the exposure were more significant for the reproduction.

Copepod fecundity is in general dependent on temperature (Runge and Plourde, 1996; Hirche et al., 1997). This is confirmed in most studies done on reproduction and temperature on *C. finmarchicus* and similar species, and the majority of them states that the EP increases with increasing temperature under favorable feeding conditions (ad libitum feeding) (Hirche et al., 1997; Pasternak et al., 2013).

Earlier studies have established that the reproduction of *C. finmarchicus,* in addition to temperature, body size and lipid content, is dependent on food abundance and quality (Diel and Tande, 1992; Plourde and Runge, 1993; Hirche et al., 1997; Pasternak et al., 2013). Previous laboratory experiments and field observations have found a close relationship between food concentration and EP (Hirche et al., 1997; Irigoien et al., 1998). A study done on EP at different temperatures and food concentrations in *C. finmarchicus* showed that females managed to produce eggs with only 15 μg C/L of food (5˚C). The spawning interval was then 12 days and the average EP was the smallest one observed (0.9±1.6). On the other hand, feeding rates equal to 250 μg C/L resulted in a spawning interval of approximately 2

days and larger mean EP (9.9±6.8) (Hirche et al., 1997). The correlation between feeding rates and EP is also supported by a previous study done by Tagliati (2013) at SeaLab, where spawning females fed with algae at *ad libitum* concentrations (1087±370 μg C/L) had a higher EP compared to the females fed with limited food concentrations (i.e. 200 μg C/L).

Based on the biometric data, it appeared to be a strong correlation between egg production and lipid content. Although no significant effects of elevated temperature and pCO_2 on body volume were observed, body size and EP seemed to follow the same pattern. This is illustrated by the fact that the largest female also had the largest mean clutch (18.6 eggs), and the smallest female had among the smallest mean clutches (2.8 eggs). The low lipid content, in addition or combination with limited food supply may therefore be assumed to be the main explanation for the reduced average EP. In the present study no significant effect of acidification on EP was observed. An earlier study performed at SeaLab on *C. finmarchicus* animals obtained from the same original culture as used in the present study support these findings. Results from the supporting study showed a significant effect of acidified treatment at 3080 ppm on the EP. Further, no effect of the acidification level equal to the present study (2080 ppm) was observed on the EP (Tagliati, 2013).

An interesting result in the present study is the significant interaction between temperature and $pCO_{2,}$ which shows that elevated $pCO_{2,}$ has an antagonistic effect on EP in combination with elevated temperature. This suggests that acidified conditions may potentially moderate the negative effects of elevated temperature on EP, and thereby reduce the negative effects of elevated temperature on the overall fitness of the species. An explanation for this may be that elevated pCO_2 could reduce the development rate (caused by elevated temperature) and thereby give the animals additional time to growth and lipid accumulation, two functions central for the fecundity and HS in *C. finmarchicus.*

4.4 Hatching success

The results from the HS displayed a similar pattern as observed for the EP. Warming conditions alone caused a significant reduction of the HS compared to the ambient- (75% reduction), acidified- (77% reduction), and warming and acidified combined treatment (71% reduction). A significant interaction was also observed, and indicates a positive antagonistic effect of elevated pCO_2 on HS when combined with elevated temperature. This is supported by the observation that the highest HS was found in the warming and acidified combined treatment (74.5%), and the lowest HS was observed in the warming treatment with only 17.2%. No significant effects of acidification alone were seen.

A previous study done on HS of *C. finmarchicus* from the same culture as in the present study revealed no significant effect on HS in an acidified exposure equal to the present study (2080 ppm), however, a tendency of a concentration dependent decrease in HS was observed (Tagliati, 2013). Several short-term studies (Kurihara et al., 2004; Kurihara and Ishimatsu, 2008; Pedersen et al., 2014) shows results that are in agreement with the results found by Tagliati, even at higher exposure of pCO_2 (2400 and 2800 ppm) compared to the present study. Temperature studies on several copepod species (*Acartia spp., A. tonsa, C. helgolandicus* and *C. finmarchicus*) have in general showed a higher HS with increasing temperature (Castro-Longoria, 2003; Holste and Peck, 2006; Cook et al., 2007), but it is important to note that food was not limited in any of these studies.

In the present study no significant reduction in HS was observed in the acidified treatment. It is possible that the embryo inside the eggs might be less vulnerable to high pCO_2 exposure, at least compared to the first nauplii stages (Pedersen et al., 2014). The HS show a strong correlation with the EP in the present study, suggesting that the females that produced fewest eggs also had the poorest prerequisite for hatching. This correlation, and the fact that the HS result is contradictory compared to earlier studies, could be explained by the females condition and duration of exposure. The female condition, influenced by elevated temperature which further affects factors related to reproduction (lipid accumulation and body size), seem to have a significant impact on the reproductive outcome. In addition to elevated temperature, the food concentration also affect the female size and lipid content (Deevey, 1960; Breteler and Gonzalez, 1988), which in turn have an impact the EP and HS (Hirche, 1992; Runge and Plourde, 1996).

4.5 Sex ratio

For the present study there was no significant interaction observed on sex ratio, nor of acidification or warming alone, indicating that the environmental factors, including the limited food concentration and quality, have been satisfactory with regards to development of both male and females. The results also shows a reduced average proportion in the warming treatment (0.27), however in the warming and acidified combined exposure the highest proportion of males was observed (0.43), indicating that pCO_2 could alter the effects of temperature on sex ratio.

Previous laboratory studies have revealed that sex ratio in *Calanus* spp. or related species is depended on food quality and quantity, as well as container size. Low proportions of males have been found in small containers (<5 L) (Hirche, 1980; Peterson and Painting, 1990), and in large containers (19 L) with only single food types (Mullin and Brooks, 1967). A higher proportion of males have been found in larger container and reared with mixed diets or better food quality (Marcus and Alatalo, 1989). A study by Campbell et al. (2001) observed a reduced proportion of males treated under 8˚C with medium (50 μg C/L) and low feeding (25 μg C/L) conditions compared to the treatments with high feeding concentrations (500 μg C/L). This is also in line with results from a study done by Irigoien et al. (2000) where a higher ratio of males with increased food concentration were observed.

Reduced average proportion of males in the elevated temperature exposure compared to the warming and acidified combined treatment is consistent in a study on the copepod *Pseudocalanus newmani*, where the sex ratio, as proportion of males, decreased with 0.28 at increasing temperature (15˚C) (Lee et al., 2003). The results from the present study indicates that elevated pCO_2 in combination with elevated temperature could change the distribution in sex ratio. This is however, is only speculations, as no explanation for the high proportion of males in this treatment is found.

4.6 Staining with neutral red

Staining with neutral red did not work as a method to distinguish living individuals from dead. During verification of the method dead eggs displayed a range of different shades of red. This might be explained by the fact that the embryos have an extra diffusion barrier, the egg fluid and the gastrula. The amount of staining that crosses the barriers may possibly vary depending on which stages of development the eggs have reached. For the nauplii, the differences between living and dead animals during verification of the method seemed more promising. However, during analyses this did not prove to be the case, so the mortality measurements on eggs and nauplii could not be used.

4.7 Observed effects in an ecological perspective

Findings in the present study indicate that *C. finmarchicus* may be among the more resilient copepod species against near future predicted acidification. It appears that a temperature increase within what is expected for year 2100, could affect the fecundity and HS due to both reduced body size and lipid content. However, the observed positive antagonistic effect of acidification combined with elevated temperature may possibly moderate some of the negative effects from elevated temperature. The rise in ocean temperature has already led to significant shifts in distribution of different species (Brodie and Russell, 1999; Parmesan and Yohe, 2003; Biro et al., 2010). E.g. the distribution of *C. finmarchicus* has shifted north in favor of the related species, *C. helgolandicus,* which prefers a higher water temperature. The observed antagonistic effects in the present study suggest that this distribution shift might be reduces, due to the ameliorating effect acidification seem to have on elevated temperature. Further, changes in environmental conditions may lead to alteration in the dynamics of natural populations whose life cycle is finely tuned to environmental windows of opportunity. Additionally, since the main periods of egg production and growth of *C. finmarchicus* is tuned to match algae blooms, a change in timing or density in this phenomenon may result in a mismatch of the timing between higher trophic levels. This may lead to changes in structure or function of the marine ecosystem, e.g in the Norwegian sea which is one of the principal regions of *C. finmarchicus* in the North Atlantic, here, it is considered as the most vital zooplankton species (Bagøien et al., 2012). Due to its high lipid content it is one of the most important energy sources for several commercial fish species e.g. the Norwegian Spring Spawning Herring (*C. Harengus*) (Planque and Batten, 2000). A general reduction of total lipid content or population size of *C. finmarchicus* will have major implications for the Norwegian commercial fisheries.

4.8 Future research

Rising ocean temperature is occurring concurrently with ocean acidification and due this, for future research, it would be interesting to investigate the potential effects of elevated temperature (+3˚C) in combination with acidification levels relevant for year 2100 (800 ppm), as the pCO_2-level for the present study is predicted to occur within year 2300. Also, an important part would be to investigate cultured *C. finmarchicus* compared to free living animals to verify previous and future research. The culture at NTNU SeaLab has been operative for 36 generations and it is, to some extent, unknown whether cultured animals react different to elevated pCO_2 and temperature compared to natural living copepods, particularly with respect to food limitations and duration of exposure.

5. Conclusion

The results from the present study indicates that negative effects of warming alone may be overestimated in experiments with temperature as the only factor. The combination of warming and acidification at the investigated levels showed an antagonistic effect on EP and HS and may therefore reduce the negative effects of warming, and potentially offset the negative fitness effects on *C. finmarchicus*, at least with regard to the fecundity. Climate changes will not occur isolated and due to this it is important to perform experiments with two- or more environmental factors to understand how species will respond to the predicted changes in the marine biota.

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A. Appendix

TABLE A.1: Raw data from the EP experiment. The dark grey areas represent those females that did not spawn during the incubation period. Animals are named: treatment-female-day. Treatment 1=ambient (380 ppm/11˚C), treatment 2=warming (380 ppm/14˚C), treatment 3=warming*acidified (2080ppm/14˚C) and treatment 4=acidified (2080ppm/11˚C).

Animal	Eggs	N1	N2	Total	Animal	Eggs	N1	N2	Total	Animal	Eggs	N1	N2	Total
A1-1-1	0	3	6	9	B1-1-1	0	5	6	11	C1-1-1	0	0	0	0
A1-1-2	0	2	9	11	B1-1-2	0	3	6	9	C1-1-2	1	1	2	4
A1-1-3	0	3	8	11	B1-1-3	0	2	10	12	C1-1-3	0	0	1	1
A1-1-4	0	0	0	0	B1-1-4	3	7	13	23	C1-1-4	0	5	8	13
A1-1-5	0	2	6	8	B1-1-5	0	4	17	21	C1-1-5	3	11	34	48
A1-2-1	1	0	2	3	B1-2-1	2	2	3	7	C1-2-1	0	1	2	3
A1-2-2	0	4	14	18	B1-2-2	0	3	7	10	C1-2-2	1	5	16	22
A1-2-3	0	0	0	0	B1-2-3	1	2	5	8	C1-2-3	1	3	12	16
A1-2-4	0	0	3	3	B1-2-4	0	1	3	4	C1-2-4	0	3	4	7
A1-2-5	0	0	2	2	B1-2-5	0	0	0	0	C1-2-5	0	0	0	0
A1-3-1				0	B1-3-1				0	C1-3-1	0	0	0	0
A1-3-2				0	B1-3-2				0	C1-3-2	11	0	0	11
A1-3-3				0	B1-3-3				0	C1-3-3	0	3	9	12
A1-3-4				0	B1-3-4				0	C1-3-4	0	0	1	1
A1-3-5				0	B1-3-5				0	C1-3-5	0	0	0	0
A1-4-1	2	2	4	8	B1-4-1	0	1	5	6	C1-4-1	3	7	18	28
A1-4-2	2	1	3	6	B1-4-2	0	0	2	2	C1-4-2	1	5	19	25
A1-4-3	0	2	7	9	B1-4-3	1	2	2	5	C1-4-3	0	3	9	12
A1-4-4	0	0	2	2	B1-4-4	1	0	2	3	C1-4-4	0	0	1	1
A1-4-5	0	2	5	7	B1-4-5	0	0	1	1	C1-4-5	0	0	0	0
A1-5-1	0	1	3	4	B1-5-1	0	0	0	0	C1-5-1	0	0	0	0
A1-5-2	0	2	3	5	B1-5-2	0	0	0	0	C1-5-2	0	1	0	1
A1-5-3	1	2	3	6	B1-5-3	0	0	2	2	C1-5-3	2	7	18	27
A1-5-4	0	1	2	3	B1-5-4	0	3	15	18	C1-5-4	0	1	2	3
A1-5-5	0	0	0	0	B1-5-5	0	4	12	16	C1-5-5	0	5	13	18
A1-6-1	0	0	0	0	B1-6-1	5	4	3	12	C1-6-1	1	1	4	6
A1-6-2	0	0	0	0	B1-6-2	1	2	2	5	C1-6-2	0	0	0	0
A1-6-3	0	1	0	1	B1-6-3	0	2	1	3	C1-6-3	1	3	6	10
A1-6-4	0	3	12	15	B1-6-4	1	4	12	17	C1-6-4	3	6	9	18
A1-6-5	0	7	24	31	B1-6-5	0	3	5	8	C1-6-5	2	7	18	27
A2-1-1	0	0	0	0	B2-1-1	2	3	5	10	C2-1-1	0	0	0	0
A2-1-2	0	0	0	0	B2-1-2	0	1	2	3	C2-1-2	0	4	9	13
A2-1-3	0	0	0	0	B2-1-3	1	0	2	3	C2-1-3	0	1	2	3
A2-1-4	0	0	0	0	B2-1-4	0	2	0	2	C2-1-4	0	0	0	0
A2-1-5	0	0	0	0	B2-1-5	0	0	0	0	C2-1-5	0	0	0	0
A2-2-1	0	0	0	0	B2-2-1	9	2	2	13	C2-2-1	4	5	2	11
A2-2-2	0	0	0	0	B2-2-2	2	0	0	2	C2-2-2	6	3	1	10

A2-2-3	0	0	0	0	B2-2-3	0	0	0	0	C2-2-3	7	0	2	9
A2-2-4	0	0	0	0	B2-2-4	0	1	3	4	C2-2-4	1	1	0	2
A2-2-5	0	0	0	0	B2-2-5	0	0	0	0	C2-2-5	0	0	1	1
A2-3-1	0	0	0	0	B2-3-1	0	0	0	0	C2-3-1	0	0	0	0
A2-3-2	0	0	0	0	B2-3-2	0	0	0	0	C2-3-2	0	0	0	0
A2-3-3	0	0	0	0	B2-3-3	0	0	0	0	C2-3-3	0	0	0	0
A2-3-4	0	0	0	0	B2-3-4	0	0	0	0	C2-3-4	0	0	0	0
A2-3-5	0	0	0	0	B2-3-5	0	0	0	0	C2-3-5	0	0	0	0
A2-4-1	0	0	0	0	B2-4-1	0	0	0	0	C2-4-1	0	0	0	0
A2-4-2	0	0	0	0	B2-4-2	0	0	0	0	C2-4-2	0	0	0	0
A2-4-3	0	0	0	0	B2-4-3	0	0	0	0	C2-4-3	0	0	0	0
A2-4-4	0	0	0	0	B2-4-4	0	0	0	0	C2-4-4	0	0	0	0
A2-4-5	0	0	0	0	B2-4-5	0	0	0	0	C2-4-5	0	0	0	0
A2-5-1	0	0	0	0	B2-5-1	0	0	0	0	C2-5-1	0	0	0	0
A2-5-2	0	0	0	0	B2-5-2	0	0	0	0	C2-5-2	0	0	0	0
A2-5-3	0	0	0	0	B2-5-3	0	0	0	0	C2-5-3	0	0	0	0
A2-5-4	0	0	0	0	B2-5-4	0	0	0	0	C2-5-4	0	0	0	0
A2-5-5	0	0	0	0	B2-5-5	0	0	0	0	C2-5-5	0	0	0	0
A2-6-1	0	0	0	0	B2-6-1	0	0	0	0	C2-6-1	1	3	4	8
A2-6-2	0	0	0	0	B2-6-2	0	0	0	0	C2-6-2	0	2	2	4
A2-6-3	0	0	0	0	B2-6-3	0	0	0	0	C2-6-3	0	1	2	3
A2-6-4	0	0	0	0	B2-6-4	0	0	0	0	C2-6-4	0	6	11	17
A2-6-5	0	0	0	0	B2-6-5	0	0	0	0	C2-6-5	0	2	4	6
A3-1-1	2	1	9	12	B3-1-1	4	2	6	12	C3-1-1	0	2	2	4
A3-1-2	0	0	2	2	B3-1-2	1	1	4	6	C3-1-2	0	3	5	8
A3-1-3	1	2	5	8	B3-1-3	0	1	0	1	C3-1-3	0	0	0	0
A3-1-4	0	5	14	19	B3-1-4	2	0	1	3	C3-1-4	1	1	5	7
A3-1-5	0	0	2	2	B3-1-5	2	1	1	4	C3-1-5	0	0	0	0
A3-2-1	2	2	3	7	B3-2-1	1	5	10	16	C3-2-1				0
A3-2-2	0	0	0	0	B3-2-2	0	2	4	6	C3-2-2				0
A3-2-3	0	3	7	10	B3-2-3	0	0	3	3	C3-2-3				0
A3-2-4	0	1	1	2	B3-2-4	0	0	0	0	C3-2-4				0
A3-2-5	1	0	3	4	B3-2-5	0	0	2	2	C3-2-5				0
A3-3-1	1	2	8	11	B3-3-1	3	1	0	4	C3-3-1	0	4	9	13
A3-3-2	0	2	5	7	B3-3-2	2	1	3	6	C3-3-2	7	3	7	17
A3-3-3	0	2	0	2	B3-3-3	0	2	1	3	C3-3-3	15	6	7	28
A3-3-4	1	0	2	3	B3-3-4	1	1	3	5	C3-3-4	4	0	0	4
A3-3-5	2	2	3	7	B3-3-5	0	0	1	1	C3-3-5	0	0	0	0
A3-4-1	3	2	11	16	B3-4-1	0	0	0	0	C3-4-1	3	5	12	20
A3-4-2	1	1	5	7	B3-4-2	0	0	0	0	C3-4-2	0	1	0	1
A3-4-3	1	0	1	2	B3-4-3	0	0	0	0	C3-4-3	0	2	3	5
A3-4-4	0	2	4	6	B3-4-4	0	0	0	0	C3-4-4	1	1	2	4
A3-4-5	0	0	0	0	B3-4-5	0	0	0	0	C3-4-5	2	0	0	2
A3-5-1	1	0	3	4	B3-5-1	0	0	0	0	C3-5-1	1	3	6	10
A3-5-2	0	0	1	1	B3-5-2	0	2	2	4	C3-5-2	2	2	0	4
A3-5-3	9	8	7	24	B3-5-3	0	0	2	2	C3-5-3	0	0	1	1

A3-5-4	0	0	0	0	B3-5-4	0	2	5	7	C3-5-4	0	0	2	2
A3-5-5	0	0	0	0	B3-5-5	0	1	0	1	C3-5-5	0	2	5	7
A3-6-1	1	2	4	7	B3-6-1	0	3	0	3	C3-6-1	1	3	10	14
A3-6-2	1	0	0	1	B3-6-2	0	0	0	0	C3-6-2	0	2	3	5
A3-6-3	1	3	8	12	B3-6-3	0	0	0	0	C3-6-3	1	0	1	2
A3-6-4	0	2	0	2	B3-6-4	1	1	0	2	C3-6-4	0	0	0	0
A3-6-5	0	0	0	0	B3-6-5	0	0	0	0	C3-6-5	1	2	2	5
A4-1-1	0	1	4	5	B4-1-1	0	2	6	8	C4-1-1	0	2	5	7
A4-1-2	1	2	9	12	B4-1-2	0	2	3	5	C4-1-2	0	4	14	18
A4-1-3	0	1	4	5	B4-1-3	0	0	0	0	C4-1-3	0	1	1	2
A4-1-4	0	2	5	7	B4-1-4	0	1	3	4	C4-1-4	0	0	2	2
A4-1-5	0	0	2	2	B4-1-5	0	0	0	0	C4-1-5	0	0	1	1
A4-2-1	0	1	8	9	B4-2-1	0	0	0	0	C4-2-1	1	1	4	6
A4-2-2	0	0	1	1	B4-2-2	0	2	3	5	C4-2-2	0	1	3	4
A4-2-3	1	9	21	31	B4-2-3	0	0	1	1	C4-2-3	0	0	0	0
A4-2-4	2	9	23	34	B4-2-4	0	1	3	4	C4-2-4	0	2	2	4
A4-2-5	0	0	0	0	B4-2-5	0	0	1	1	C4-2-5	0	2	5	7
A4-3-1	1	0	3	4	B4-3-1	0	0	0	0	C4-3-1	2	1	3	6
A4-3-2	1	0	2	3	B4-3-2	0	0	0	0	C4-3-2	1	2	2	5
A4-3-3	0	1	5	6	B4-3-3	0	3	9	12	C4-3-3	0	2	2	4
A4-3-4	0	4	9	13	B4-3-4	0	0	2	2	C4-3-4	0	0	2	2
A4-3-5	0	2	6	8	B4-3-5	0	4	10	14	C4-3-5	0	1	2	3
A4-4-1	0	3	8	11	B4-4-1	0	0	2	2	C4-4-1	6	2	3	11
A4-4-2	0	2	4	6	B4-4-2	0	0	1	1	C4-4-2	0	1	3	4
A4-4-3	0	1	5	6	B4-4-3	0	0	0	0	C4-4-3	0	0	0	0
A4-4-4	0	1	4	5	B4-4-4	0	0	4	4	C4-4-4	1	4	10	15
A4-4-5	0	2	6	8	B4-4-5	0	0	0	0	C4-4-5	0	0	0	0
A4-5-1	0	2	3	5	B4-5-1	3	0	1	4	C4-5-1	0	4	12	16
A4-5-2	1	0	2	3	B4-5-2	0	0	0	0	C4-5-2	0	2	5	7
A4-5-3	0	0	0	0	B4-5-3	0	1	2	3	C4-5-3	0	3	3	6
A4-5-4	0	1	4	5	B4-5-4	0	0	0	0	C4-5-4	0	0	3	3
A4-5-5	1	0	0	1	B4-5-5	3	1	0	4	C4-5-5	0	1	3	4
A4-6-1	2	0	2	4	B4-6-1	2	3	9	14	C4-6-1	0	1	2	3
A4-6-2	1	1	0	2	B4-6-2	0	0	1	1	C4-6-2	0	3	7	10
A4-6-3	0	0	0	0	B4-6-3	0	4	6	10	C4-6-3	0	4	10	14
A4-6-4	1	3	14	18	B4-6-4	0	2	5	7	C4-6-4	0	0	0	0
A4-6-5	0	0	0	0	B4-6-5	0	2	4	6	C4-6-5	0	4	11	15

B. Appendix

The biometric data are retrieved from measurements on a photograph done post spawning of every female that completed the egg production for five subsequent days. The following equations where used for calculations (Miller et al., 2000).

$$\text{Lipid-sac volume} = \frac{\pi \times \text{lipid-sac } area^2}{4 \times \text{body length}} \tag{B.1}$$

$$\text{Body volume} = \frac{\pi \times area\ body^2}{4 \times \text{body length}} \tag{B.2}$$

$$\text{Lipid-sac percent} = \left(\frac{\text{Lipid-sac volume}}{\text{Body volume}}\right) \times 100 \tag{B.3}$$

TABLE B.1: Raw data from the biometric measurements. The dark grey areas represent those females that did not spawn during the incubation period. LS= lipid-sac.L-S%= percent lipid-sac of the whole body.

CO2	Temp	Tank	L-S length	Body length	L-S volume	Body volume	L-S %
380	11	A	1.157	2.52	0.023	0.865	2.625
380	11	A	1.916	2.477	0.023	0.777	2.989
380	11	A	0	0	0	0	0
380	11	A	0.409	2.588	0.079	0.998	1.2
380	11	A	1.164	2.387	0.133	0.713	1.672
380	11	A	1.171	2.418	0.186	0.744	3.117
380	11	B	1.164	2.383	0.045	0.617	7.384
380	11	B	0	2.572	0	0.713	0
380	11	B	0	0	0	0	0
380	11	B	1.122	2.397	0.012	0.693	1.758
380	11	B	1.283	2.517	0.04	0.754	5.359
380	11	B	1.797	2.537	0.025	0.768	3.274
380	11	C	1.446	2.669	0.027	0.891	3.083
380	11	C	0	2.731	0	0.865	0
380	11	C	1.496	2.324	0.025	0.608	4.099
380	11	C	0.397	2.271	0.002	0.548	0.417
380	11	C	1.562	2.678	0.059	0.809	7.354
380	11	C	1.89	2.7	0.103	1.018	10.121
380	14	A	0	0	0	0	0
380	14	A	0	0	0	0	0
380	14	A	0	0	0	0	0
380	14	A	0	0	0	0	0
380	14	A	0	0	0	0	0
380	14	A	0	0	0	0	0
380	14	B	0	2.24	0	0.575	0

380	14	B	0.74	2.368	0.003	0.737	0.484
380	14	B	0	0	0	0	0
380	14	B	0	0	0	0	0
380	14	B	0	0	0	0	0
380	14	B	0	0	0	0	0
380	14	C	0.355	2.406	0.001	0.697	0.167
380	14	C	0	2.356	0	0.663	0
380	14	C	0	0	0	0	0
380	14	C	0	0	0	0	0
380	14	C	0	0	0	0	0
380	14	C	0	0	0	0	0
2080	14	A	0.433	2.499	0.002	0.807	0.26
2080	14	A	0.721	2.287	0.008	0.683	1.29
2080	14	A	0	2.417	0	0.822	0
2080	14	A	0	2.467	0	0.791	0
2080	14	A	0	2.378	0	0.664	0
2080	14	A	0	2.151	0	0.541	0
2080	14	B	0.853	2.396	0.01	0.656	2.02
2080	14	B	1.75	2.336	0.05	0.581	8.203
2080	14	B	0	2.328	0	0.659	0
2080	14	B	0	2.378	0	0.627	0
2080	14	B	1.097	2.508	0.02	0.924	2.186
2080	14	B	0	0	0	0	0
2080	14	C	0	2.302	0	0.609	0
2080	14	C	0	0	0	0	0
2080	14	C	0.837	2.403	0.005	0.621	0.761
2080	14	C	0.65	2.22	0.001	0.451	0.31
2080	14	C	1.13	2.396	0.017	0.74	2.343
2080	14	C	0	2.322	0	0.7	0
2080	11	A	0	2.144	0	0.523	0
2080	11	A	1.641	2.478	0.031	0.67	4.608
2080	11	A	0	2.341	0	0.534	0
2080	11	A	0	2.472	0	0.744	0
2080	11	A	2.153	2.43	0.058	0.609	9.532
2080	11	A	1.968	2.431	0.045	0.651	6.875
2080	11	B	0	2.454	0	0.612	0
2080	11	B	0	2.445	0	0.724	0
2080	11	B	0	2.57	0	0.763	0
2080	11	B	0	2.383	0	0.644	0
2080	11	B	1.094	2.533	0.019	0.911	2.091
2080	11	B	0	2.654	0	0.906	0
2080	11	C	1.66	2.322	0.071	0.658	10.932
2080	11	C	1.02	2.184	0.01	0.438	2.36
2080	11	C	1.375	2.163	0.027	0.48	5.705
2080	11	C	0.697	2.332	0.003	0.528	0.517
2080	11	C	1.41	2.5	0.017	0.682	2.471
2080	11	C	0.638	2.289	0.01	0.604	1.65

TABELL B.2: The mean results from the biometric data (mean±SD, except for the warming treatment (n=2), n=). LS= lipid-sac. Ambient (380 ppm, 11˚C), warming (380pm, 14˚C), acidified (2080ppm, 11˚C), warming*acidified

Treatment	LS length	Body length	LS volume	Body volume	LS%
ambient	1.1±0.60	2.5±0.14	0.05±0.05	0.8±0.13	3.40±2.79
warming	0.3±0.31	2.3±0.06	0.00±0.00	0.7±0.06	0.16±0.20
acidified	0.8±0.77	2.4±0.14	0.02±0.02	0.6±0.13	2.60±3.41
warming*acidified	0.5±0.54	2.4±0.09	0.01±0.01	0.7±0.11	1.09±2.02

C. Appendix

TABELL C.1: Results from tests of equal variance, Levene's tests, done on every data set to explore the variance within each set. Values $p > 0.05$ are considered as equal variance.

	d.f	F-value	*p*-value
EPR	3,16	1.492	0.255
HS	3,16	0.395	0.757
Sex ratio	3,8	0.781	0.537
Body volume	3,7	0.802	0.527
Lipid-sac volume	3,7	3.889	0.055
Percent volume lipid-sac	3,7	1.923	0.204