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Effects of predicted ocean acidification scenarios on the early developmental stages of *Calanus finmarchicus*

A multigenerational study

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ABSTRACT

In recent years, ocean acidification through absorption of anthropogenic CO_2 has been recognised as a threat to marine ecosystems. The effects of environmental hypercapnia have been investigated for a broad selection of marine invertebrate taxa, and tentative conclusions have been drawn about their relative sensitivity to ocean acidification. However, the main body of knowledge in this regard stems from short-term exposure studies, and few studies have included the full life cycle or multiple generations.

The marine calanoid copepod *Calanus finmarchicus* is a key species in the North Atlantic, where it constitutes an important link between primary producers and higher trophic levels. In the present study, two consecutive generations of *C. finmarchicus* were reared under four different exposure conditions. These CO_2 regimes represented the present status (380 ppm), two near-future scenarios based on published projections for the year 2100 and 2300 (1080 ppm and 2080 ppm respectively), as well as a positive control of 3080 ppm CO_2. This thesis work focused on the early developmental stages of the second generation, and examined the effects of elevated CO_2 levels on the rates of ontogenetic development as an indirect measure of hypercapnic stress.

A general increase was seen in both overall development time and stage specific development times for both of the highest exposure groups, but significant delays in development was only observed for the 3080 ppm treatment group, and here only for the stages NV, NVI and CI. The delays were in the order of approximately 2 - 4 days for these stages, and increased with each successive moult. Such developmental delays may cause *C. finmarchicus* to miss important ecological windows of opportunity to which their life cycle is finely tuned. As no significant delay was observed for the year 2100 and 2300 scenarios, *C. finmarchicus* may appear to be relatively robust regarding near-future ocean acidification. However, significant differences found in feed availability during the time of stages NV, NVI and CI indicate a potential for compensatory feeding in the year 2300 exposure group.

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1 INTRODUCTION

1.1 Elevated CO_2 levels and ocean acidification

Anthropogenic CO_2 is emitted into the atmosphere as a result of the combustion of fossil fuels, while more indirect anthropogenic sources of CO_2 include deforestation and altered land use. In the atmosphere, CO_2 acts as a greenhouse gas, whose insulating properties in combination with its increased concentration, is a probable cause of the rise in global mean temperatures seen today (IPCC, 2007).

During the past two and a half centuries, the level of atmospheric CO_2 has increased by around one third, from about 280 ppm (parts per million) in pre-industrial times to approximately 380 ppm at present (Feely et al., 2004; IPCC, 2007). The global ocean surface average was 392.6 ppm for the year 2012, according to data from NOAA/ESRL (Dlugokencky and Tans, 2013), and is expected to increase further unless significant reductions in emissions are achieved (Gattuso and Hansson, 2011). On a geological time scale, the rate of increase documented in the last century alone is 100 times higher than any change in atmospheric CO_2 observed in geological records from the past 650,000 years (Siegenthaler et al., 2005).

All soluble gases are exchanged across the air-sea interface. Any change in the concentration of a given soluble gas in the atmosphere, such as the rise in CO_2 levels observed today, will lead to a similar change in the concentration of that gas in the world oceans, as equilibrium is re-established between the two reservoirs (Williams and Follows, 2011). Thus, the increase in atmospheric CO_2 is not just causing global warming, but also a decrease in ocean pH, due to the properties of the carbonate system in seawater (Caldeira and Wickett, 2003; Feely et al., 2004; Caldeira and Wickett, 2005; Orr et al., 2005). This phenomenon, termed ocean acidification (Gattuso and Hansson, 2011), takes place on a time scale of decades or longer (Williams and Follows, 2011), and has only recently been included in the efforts to mitigate climate change (Turley and Boot, 2011).

1.1.1 Carbon dioxide capture and storage

The prospect of melting ice caps, elevated sea levels and significant environmental changes caused by climate change has led to political and societal calls to reduce atmospheric CO_2 concentrations (Hey, 2005). Propositions have centred on carbon dioxide capture and storage (CCS) strategies which mitigate climate change while giving science a chance to find more permanent solutions (IPCC, 2005). One of the alternatives evaluated has been deep-sea injection of CO_2 (Marchetti, 1977; IPCC, 2001), with the assumption that the liquefying effect of pressure will allow the gas to remain relatively stationary near the seafloor. However, due to the lack of knowledge about, and reliable models of, the retention time and the possible consequences of local elevation of CO_2 concentrations (e.g. Seibel and Walsh, 2003), the issue remains controversial (IPCC, 2005). Deep sea storage was not included in the amendment to the 1996 *London Protocol* for the regulation of marine pollution, by the International Maritime Organization (IMO, 2007), and is not a legal alternative in the North-East Atlantic (Haugan et al., 2006). Instead, efforts and consequently policy and legislation (e.g. IMO, 2007), have focused on geological storage, in which CO_2 is injected into a porous reservoir rock capped by a compact cap rock. Such geological storage reservoirs are being used both on land and under the seabed (IPCC, 2005), and the technological solutions involved derive from enhanced oil recovery (EOR) techniques (IPCC, 2005; Steeneveldt et al., 2006). The first full-scale implementation of sub-seabed CO_2 sequestration was established at the Sleipner field by Statoil in 1996, in the Norwegian sector of the North Sea (Holloway, 2005). As of September 2012, 13.5 Mt of CO_2 have been injected in this reservoir (Fjæran, 2012). In addition, Statoil sub-seabed CCS efforts include storage at the Snøhvit field in the Barents Sea, as well as the projects being developed at Mongstad and Kårstø (Gines, 2012).

1.1.2 Leakage from sub-seabed CO_2 storage sites

While large-scale capture and storage of atmospheric CO_2 has the potential to reduce the rates of both global warming and ocean acidification, it is a solution that involves continued storage on a very long time frame, potentially for millennia (Holloway, 2001; Turley et al., 2004). Even in suitable and well managed storage locations, where minimum everyday risk

is assumed, the prospect of monitoring and maintaining the site for millennia poses a challenge regarding risk management. According to (Turley et al., 2004), the estimated probability of a leakage event during a period of 1000 years is 34 %, and the average leak is expected to release 0.2 % of the stored CO_2. Simulations of leakage events, when compared to studies of the effects of seepage from natural CO_2 vents (e.g. Tunnicliffe et al., 2009), indicate that leaks of this magnitude will pose a serious threat to the environment surrounding the site, in which organisms are not adapted to high CO_2 levels (e.g. Seibel and Walsh, 2003; Widdicombe and Needham, 2007). Essentially, sub-seabed storage of CO_2 involves a trade-off between avoiding the imminent risk of climate change and ocean acidification in near-surface waters in the present, and the risk of significant negative impacts on the deep-sea marine environments near storage sites in the event of leaks in the future (Gattuso and Hansson, 2011).

1.2 Properties of carbon dioxide in water

As mentioned, a gas which is present in the atmosphere will dissolve in water until equilibrium is attained. Henry's law states that the solubility of a gas in a liquid is proportional to the pressure of the gas over the solution (Chang, 2003), according to Equation 1.

$$c = k \times P \tag{1}$$

Here, c is the molar concentration (moles/L) of the dissolved gas, P is the partial pressure (in atmospheres) of the gas over the solution, and k is the temperature-dependent solubility constant (in moles/L atm).

When dissolved in water, carbon dioxide acts as a weak acid. Aqueous carbon dioxide (CO_2 (aq)) reacts with water to form the transient product carbonic acid (H_2CO_3), which dissociates almost immediately into bicarbonate (HCO_3^-), carbonate (CO_3^{2-}) and hydrogen ions. As the concentration of carbonic acid is much lower (≤ 0.03 %) than that of aqueous carbon dioxide and the two compounds are chemically inseparable, the sum of these two

molecules is often denoted CO_2^* and the first two steps described above are condensed to one. The first and second dissociation constants for carbonic acid are denoted K_1 and K_2 respectively (Zeebe and Wolf-Gladrow, 2001; Williams and Follows, 2011). This equilibrium reaction is shown in Equation 2.

$$CO_2^* + H_2O \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons CO_3^{2-} + 2H^+ \quad (2)$$

In the ocean, the majority of this dissolved inorganic carbon is present in the form of bicarbonate ions (Zeebe and Wolf-Gladrow, 2001). Carbon dioxide has a relatively high solubility in water, approximately 30 times that of oxygen (at 1 atm and 15°C), precisely because of this equilibrium reaction, which removes free CO_2 from the water and thus causes more CO_2 to be absorbed from the atmosphere (Schmidt-Nielsen, 1997; Williams and Follows, 2011). In addition, the solubility of CO_2 is known to decrease with increasing salinity and temperature and increase with increasing hydrostatic pressure and partial pressure (Williams and Follows, 2011).

Ocean pH is a measure of ocean acidity, defined as pH = -log[H^+], and is reported on a scale from 1 to 14, where 7 corresponds to the neutral point at 25°C, at which the concentration of hydrogen ions equals that caused by the dissociation of water alone (Williams and Follows, 2011). Though several different pH scales exist, the total scale is the one recommended (Dickson et al., 2007a), in the interest of standardisation of results. The p*K* value is defined as the pH at which the concentration of an acid equals that of its conjugate base (Zeebe and Wolf-Gladrow, 2001). At a temperature of 10°C and a salinity of 35 ppt, the p*K* values of carbonic acid in surface seawater are given by pK_1 = 5.99 and pK_2 = 9.21 (Lueker et al., 2000; Zeebe and Wolf-Gladrow, 2001; Lavigne and Gattuso, 2012; R Core Team, 2012). Figure 1.1 shows the Bjerrum plot for the carbonate system under these conditions, pK_1 occurring at the intersection between the concentration curves of CO_2^* and HCO_3^- (solid and dashed lines respectively), and pK_2 where the curve for HCO_3^- intersects that of CO_3^{2-} (dotted line). A decrease in temperature (e.g. to 8°C) will cause both pK_1 and pK_2 to shift to slightly higher pH values, but the typical sea surface pH of 8.1 still falls well within the

portion of the pH scale in which bicarbonate ions are the dominant form of dissolved organic carbon present (Zeebe and Wolf-Gladrow, 2001; Williams and Follows, 2011).

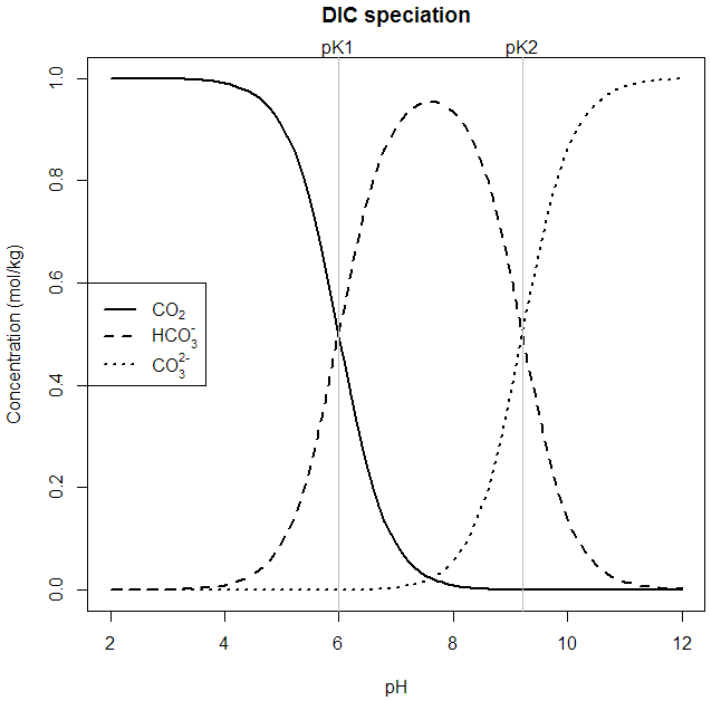

Figure 1.1: The *Bjerrum plot* for the carbonate system shows the relative concentrations of the various carbonate species as functions of pH. Dissolved inorganic carbon is distributed between CO_2^* (solid line), HCO_3^- (dashed line) and CO_3^{2-} (dotted line). For this plot, the carbonate system was solved at a fixed temperature (10°C) and salinity (35 ppt); pK_1= 5.99 and pK_2= 9.21. (Modified from (Zeebe and Wolf-Gladrow, 2001), with K_1 and K_2 from Lueker et al. (2000), using R (Lavigne and Gattuso, 2012).

Since the industrial revolution, the world oceans have absorbed approximately one-third of the anthropogenic carbon dioxide produced, and the oceans thus temper the rate of global warming (Sabine et al., 2004). The carbonate system is the main buffering system in the ocean (Williams and Follows, 2011). Buffers are compounds which act to minimize perturbations in pH, by reacting with exogenous H^+ ions (van Slyke, 1922; Roos and Boron, 1981). Addition of dissolved CO_2^*, as a result of an increase in the atmospheric concentration of CO_2, lowers the pH, which makes the partitioning of carbonate species shift. As is evident from the Bjerrum plot in Figure 1.1, a decrease in pH, which indicates an increase in [H+], will consume carbonate ions and favour the formation of bicarbonate and CO_2^*. This will lead to less favourable conditions for further uptake of CO_2 from the atmosphere (Zeebe and Wolf-Gladrow, 2001). Thus, ocean acidification indirectly contributes to a reduction in the tempering effect of the ocean on global warming. In addition, the same buffering process also contributes to a decrease in the concentration of carbonate ions, which are vital to calcifying organisms. This lowering of seawater carbonate saturation state, Ω, the

difference between the actual $[CO_3^{2-}]$ and the critical level, $[CO_3^{2-}]_{sat}$, below which calcium carbonate ($CaCO_3$) structures begin to dissolve (Williams and Follows, 2011), has been found to be detrimental to calcifying organisms from a broad spectrum of taxonomic groups. Calcifying organisms secrete $CaCO_3$ in two forms, calcite and the more soluble aragonite, and when $\Omega > 1.00$, calcification is favoured, whereas the reverse reaction, decalcification, is favoured when $\Omega < 1.00$ (Lalli and Parsons, 1997; Fabry et al., 2008). A reduction in calcification rates or an increase in dissolution rates have been documented for coccolithophores and other calcifying plankton (Riebesell et al., 2000; Orr et al., 2005), corals (Kleypas et al., 1999; Hoegh-Guldberg et al., 2007), echinoderms (Kurihara and Shirayama, 2004), gastropods (Bibby et al., 2007) and bivalves (Michaelidis et al., 2005).

1.3 Carbon dioxide as a marine pollutant

The problem of decreased calcification rates, or outright decalcification, is not caused by elevated levels of CO_2 *per se*, but rather by the consequent lowering of the saturation state for $CaCO_3$. There are, however, more direct physiological consequences of elevated levels of CO_2, i.e. hypercapnia, in marine environments.

1.3.1 Physiological effects and compensatory strategies

Both aqueous CO_2 and carbonic acid are uncharged, lipid-soluble molecules, and this permits their diffusion across biological membranes (Gutknecht et al., 1977), and allows them to equilibrate in both extra- and intracellular spaces. When the CO_2 levels increase in seawater, CO_2 dissociates in the internal body fluids of marine organisms, heightening the $[H^+]$ and lowering the internal pH (Roos and Boron, 1981; Pörtner et al., 2004; Fabry et al., 2008). The excess of hydrogen ions thus produced causes acidosis in tissues and body fluids (Pörtner et al., 1998; Michaelidis et al., 2005).

The presence of excess H^+ ions in body fluids poses a threat to enzymatic processes. The structural folding, and therefore functioning, of enzymes are dependent on interactions which are easily perturbed by the highly reactive hydrogen ions, as well as their hydrated counterparts (H_3O^+) (Berg et al., 2002). In order to counteract this internal acidification,

animals selected from a broad range of phyla have been found to employ a few common mechanisms originally evolved to eliminate the H^+ ions and CO_2 produced by metabolism (Fabry et al., 2008). These include the short-term measures of *metabolic suppression* and passive buffering, as well as the longer-term strategies of acid-base regulation through *ion transport* and *bicarbonate accumulation*. During metabolic depression (e.g. Langenbuch and Pörtner, 2004), in which energetically expensive processes, especially protein synthesis (Hand, 1991), are shut down, thus reducing growth and reproduction, the organism effectively hibernates while awaiting the end of transient acidification events (e.g. Busa and Nuccitelli, 1984). Passive buffering of intra- and extracellular fluids, a common strategy in species capable of anaerobically fuelled locomotion, such as epipelagic fish and cephalopods (Castellini and Somero, 1981; Seibel and Walsh, 2003) is achieved by the high activity of anaerobic metabolic enzymes which normally buffer pH changes caused by burst locomotion. More sedate species tend to have lower levels of these enzymes, and thus cannot compensate equally well by this mechanism (Fabry et al., 2008). Acid-base regulation through ion transport exploits the same machinery employed in handling the CO_2 produced during metabolism. Intracellular CO_2 is hydrated by the enzyme carbonic anhydrase, to form bicarbonate and hydrogen ions, and while the hydrogen ions are buffered passively or transported out of the cell by ion transport proteins in the cell membranes, in exchange for chloride ions, the bicarbonate ions may either be exchanged or remain as a buffer, depending on the pH being compensated. Species with low rates of metabolism generally also have lower concentrations of the Na^+/K^+ and H^+-ATPases responsible for ion transport, which may indicate lower capacity for acid-base compensation (Heisler, 1989; Gibbs and Somero, 1990). The ion transport involved in this strategy requires energy, and is thought to be unsustainable in time frames longer than hours to a few days (Fabry et al., 2008). Lastly, bicarbonate accumulation involves the active transport of additional buffering bicarbonate ions into the cells, (Pörtner and Reipschläger, 1996) a process in which stored $CaCO_3$, in the form of specific stores, exoskeletons or indeed calcified sensory organs are dissolved (e.g. Langenbuch and Pörtner, 2004; Michaelidis et al., 2005; Fabry et al., 2008). All these compensation strategies come at a cost; they require energy, which could otherwise have been used for growth, development and reproduction, and are thus likely to affect the rates of these processes (Fabry et al., 2008). The sensitivity to CO_2 exposure shows great variability across taxonomic phyla (Fabry et al., 2008), and even between closely related

species (Kurihara et al., 2004a). In addition, findings suggest that in marine invertebrates levels of tolerance will vary throughout the life cycle, depending on developmental stage (e.g. Melzner et al., 2009).

1.4 Calanus finmarchicus

As a group, copepods (Crustacea; Copepoda) are considered to be the most numerous multicellular organisms on Earth (Mauchline, 1998). The marine calanoid copepod *Calanus finmarchicus* (Gunnerus, 1770) seasonally dominates the North Atlantic zooplankton biomass, contributing to more than half of the total copepod biomass (Marshall and Orr, 1955; Conover, 1988; Planque and Batten, 2000), with an estimated annual production of approximately 74 million tonnes (wet weight) in the Nordic Seas alone (Aksnes and Blindheim, 1996). *C. finmarchicus* is considered a key species in the pelagic food web of the North Atlantic, including the Arctic Basin and the Barents Sea (Mauchline, 1998; Vadstein, 2009). By feeding on seasonal phytoplankton blooms, and being an important source of food for planktivorous fish and fish larvae, *C. finmarchicus* constitutes a link between primary producers and higher trophic levels (Runge, 1988; Planque and Batten, 2000). The larvae of several species, including economically important fisheries species such as Atlantic cod (*Gadus morhua*), herring (*Clupea harengus*), mackerel (*Scomber scombrus*) and haddock (*Melanogrammus aeglefinus*), are dependent on *C. finmarchicus* (Kane, 1984; Kaartvedt, 2000). In addition, the need for alternative marine lipid sources in fish feed in a growing aquaculture industry (FAO, 2010; 2012) has sparked an interest in the *n*-3 highly unsaturated fatty acids, mostly in the form of wax esters (Bergvik et al., 2012b), contained in the lipid stores of *C. finmarchicus*. *Calanus* oil is being evaluated as a component in feed for cultured species such as Atlantic salmon (*Salmo salar*) (Olsen et al., 2004; Bergvik et al., 2012a; Bergvik et al., 2012b).

Calanus finmarchicus is widely distributed in the North Atlantic (Conover, 1988; Planque and Batten, 2000), its southern limit of distribution corresponding to the position of the separation between the sub-Arctic gyre and the warmer waters of the temperate subtropical gyre, (Planque and Batten, 2000), where its dominance is replaced by that of *Calanus helgolandicus* (Fromentin and Planque, 1996). Similarly, *Calanus hyperboreus* (Krøyer, 1838) and *Calanus glacialis* (Jaschov, 1955) attain dominant roles in arctic waters

(Mauchline, 1998). In their analysis of pan-Atlantic plankton data spanning four decades, Planque and Batten (2000) identified four core regions of particularly high abundance of *C. finmarchicus*; the North Sea and the Norwegian Sea, as well as a region to the southwest of Iceland, on the eastern side of the North Atlantic, and the regions around the Newfoundland Bank and the Labrador Basin, as well as Nova Scotia and the Gulf of Maine on the western side. Both of the two main distribution centres on the eastern side are located within the North Atlantic sub-polar gyre (e.g. Aksnes and Blindheim, 1996). As a planktonic species, *Calanus finmarchicus* is subject to the horizontal movements of the water masses it inhabits, which create a temporal-spatial link; as a cohort grows and develops over time, it is subject to transport due to currents, and will encounter environmental variability on a level not easily modelled in laboratory studies (e.g. Mauchline, 1998).

1.4.1 Ontogenetic development and life strategies of *C. finmarchicus*

Copepods, like all crustaceans, employ moulting, the periodical shedding and re-formation of the integument, as a means to allow growth in spite of their rigid exoskeletons (Mauchline, 1998; Raven and Johnson, 2002). The life cycle of *C. finmarchicus*, which is illustrated in Figure 1.2, consists of six naupliar stages (denoted NI-NVI) and five copepodite stages (denoted CI-CV), before the sexually mature males and females emerge (Marshall and Orr, 1955; Mauchline, 1998). Between hatching and the adult stage, each successive developmental stage is followed by a moulting, a process in which most of the chitin from the old integument is recycled into the new one, but which is nonetheless energetically costly (Mauchline, 1998; Rey-Rassat et al., 2002). The NIII stage is the first stage in which feeding is documented (Hygum et al., 2000a; Våge, 2011); in stages prior to this one, *C. finmarchicus* subsists on the innate maternal lipid sac (Marshall and Orr, 1955; Mauchline, 1998).

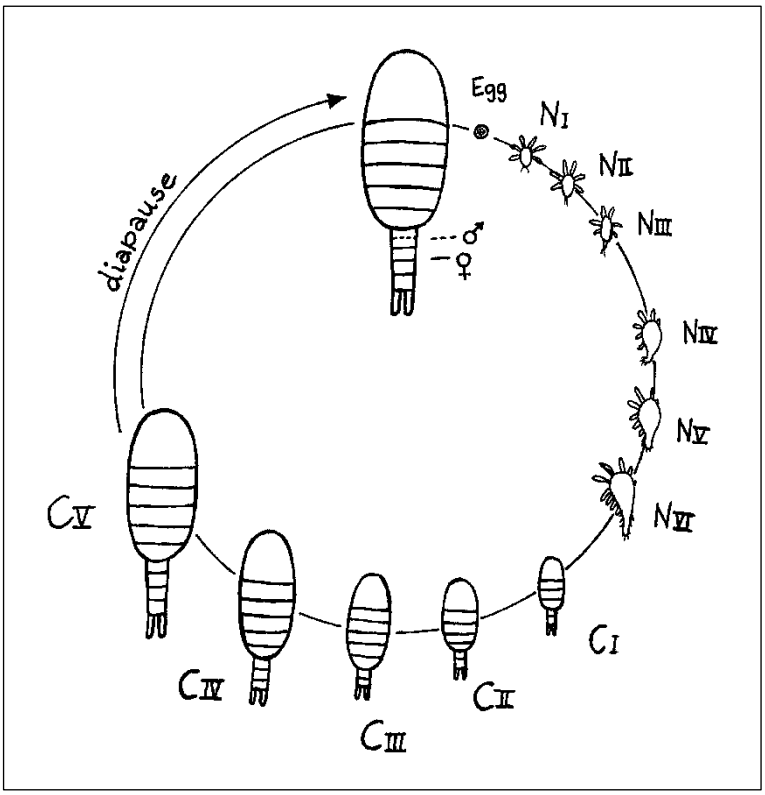

Figure 1.2: Simplified portrayal of the life cycle of *Calanus finmarchicus*, from egg, via six naupliar stages and five copepodite stages, to adult male (♂) or female (♀). The characteristics of the naupliar stages (NI – NVI) are based on the descriptions by Marshall and Orr (1972), while the depiction of the copepodite (CI – CV) and adult stages highlights the apparent segmentation of the metasome and urosome, as described by Mauchline (1998). The circular representation, including the prolonged stage NIII and the diapause, has been adapted from (Baumgartner, 2009).

While *C. finmarchicus* has traditionally been viewed as herbivorous (Marshall and Orr, 1955; Mauchline, 1998), it has been found to exhibit omnivory (Castellani et al., 2008; Vadstein, 2009), and even cannibalistic behaviour (Ohman and Hirche, 2001; Eiane et al., 2002; Bonnet et al., 2004). In feeding stages of the calanoid copepod life cycle, the initiation of each moult is linked to threshold levels in weight and lipid stores (Mauchline, 1998; Irigoien, 2004), and the time required to attain these will vary depending on environmental conditions such as temperature and food availability, as well as intrinsic factors causing variability between individuals (Mauchline, 1998).

The life strategies of *C. finmarchicus* include two general patterns of vertical migration in the water column. Their diel vertical migration (DVM) is a daily pattern in which they ascend to surface waters at sunset, feed on the phytoplankton in these waters throughout the night, and then return to deep waters as dawn approaches (Edvardsen et al., 2006). This behaviour, which is typical, albeit variable, among zooplankton (e.g. Dale and Kaartvedt, 2000; Baumgartner et al., 2011), is thought to be a response to the trade-off between the need for growth and the necessity of avoiding visual predators (Mauchline, 1998; Edvardsen et al., 2006).

In addition to their diel vertical migration, *C. finmarchicus* exhibits a seasonal, ontogenetic vertical migration, involving the ascent of CV (see Figure 1.3) and adult individuals from deep water towards the surface prior to the spring phytoplankton bloom, and a subsequent return to deep waters of late copepodite stages in the fall (Mauchline, 1998; Edvardsen et al., 2006). The number of generations produced per year varies depending on the region in question. Generally, only one generation completes its life cycle per year in the northern parts of the geographic distribution, whereas *C. finmarchicus* has been found to produce two, or even three, generations further south (Mauchline, 1998). Like most pelagic calanoids, *C. finmarchicus* is a broadcast spawner, in that the females release their individual fertilized eggs freely into the surrounding water masses (Mauchline, 1998; Kiørboe, 2008). Prior to egg release, however, males attach their spermatophore to the first somite of the urosome (Marshall and Orr, 1955; Mauchline, 1998), and fertilization takes place as the eggs pass through the genital pore. Hatching occurs within approximately 24 hours (Kiørboe and Sabatini, 1994; Mauchline, 1998), and the short stage duration of NI and NII (Mauchline, 1998; Hygum et al., 2000b; Campbell et al., 2001) ensures that first-feeding NIII individuals start emerging within the first week after spawning. The seasonal migratory behaviour has been found to be variable (e.g. Mauchline, 1998), but the timing of ascent, mating and egg release in nature is generally assumed to be linked to cues which precede the onset of the spring phytoplankton bloom, ensuring favourable conditions for the first-feeding stages of the life cycle (Mauchline, 1998; Irigoien, 2004; Kiørboe, 2008).

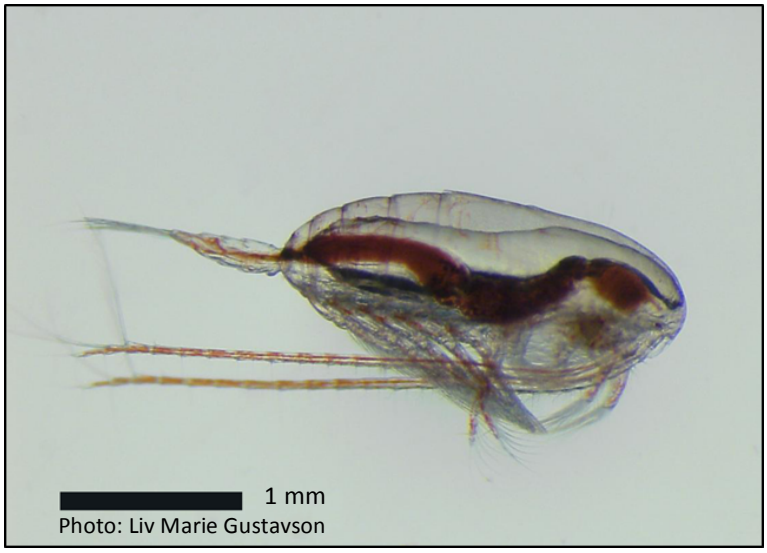

Figure 1.3: 2.0x magnification microscope photo of a live stage CV *Calanus finmarchicus*. The segmentation shown in Figure 1.2 is clearly visible. Note the large lipid sac extending for almost the entire length of the prosome and storing the energy to be utilised for the final moulting into the adult stage. The scale bar included is 1 mm.

1.4.2 Acid-base regulation in copepods

There is still much uncertainty regarding acid-base regulation, and consequently the mechanisms for pH compensation, in copepods. In fish and decapod crustaceans, such as shrimps, lobsters and crabs, the gills are known to be the primary location of such regulation (Wheatley and Henry, 1992). As decapod crustaceans have been found to have a similar selection of exchange proteins in their gills as those known to be responsible for proton exchange across the gills of fish, Wheatley and Henry (1992) propose a mechanism analogous to that found in fish, where the Na^+/H^+ ion exchange is driven by the sodium gradient set up by Na^+/K^+ ATPase. However, copepods lack gills (Wolvekamp and Waterman, 1960), and speculations as to the primary location of acid-base regulation have included the maxillary gland, the hindgut and the integument. The proposal involving the maxillary gland (e.g. Boxshall, 1982) is based on this being the main site of metabolic waste excretion in copepods (Le Borgne, 1986), and is an analogue to renal pH regulation observed in fish (Wood et al., 2002). The proposed role of the hindgut is based on inferences made from findings in terrestrial insects (e.g. Linser et al., 2009), and the involvement of the integument (e.g. Mauchline, 1998) has also been suggested, particularly in relation to the moulting process.

1.4.3 Effects of hypercapnia in *C. finmarchicus* and other copepods

Compared to other invertebrates, such as corals, molluscs and echinoderms, marine crustaceans appear to be relatively resilient when exposed to CO_2-induced hypercapnia (Byrne, 2011; Whiteley, 2011). Early studies, such as Marshall et al. (1935) and Grice et al. (1973) investigated the effect of acid-induced acidification on marine zooplankton, but as CO_2 is lipid soluble, such model systems are not realistic representations of CO_2-induced stress (Kikkawa et al., 2004). In her review of previous studies involving the exposure of crustaceans to elevated CO_2 concentrations, Whiteley (2011) found that the species investigated were generally tolerant to the acidification levels expected by the years 2100 (~700-1000 ppm CO_2) and 2300 (~2000 ppm CO_2) (Caldeira and Wickett, 2003; 2005; IPCC, 2007; Doney et al., 2009), though negative effects were observed for rates of calcification and growth, as well as embryonic development and moulting frequencies. Copepods have a

low degree of calcification, and a correspondingly higher chitin content in their exoskeletons, and are regarded as non-calcifiers (Fitzer et al., 2012). In their study involving CO_2 concentrations relevant for CCS leakage events (~8400 ppm), (Mayor et al., 2007) found no effect on adult growth in *Calanus finmarchicus*, but negative effects on egg production and hatching success. For acidification levels closer to those predicted for surface-driven ocean acidification, (Kurihara et al., 2004a; b) found a decrease in egg production for both *Acartia steueri* (Smirnov, 1936) (~2400 ppm) and *A. erythraea* (Giesbrecht, 1889) (~5400 ppm), as well as increased naupliar mortality in the latter species, while the former showed no effect on naupliar survival. In an exposure study lasting approximately one generation, and using four CO_2 levels spanning from normal to CCS-leakage relevant (390, 3300, 7300 and 9700 ppm CO_2 respectively), Pedersen et al. (2013) found negative effects on survival and growth for *Calanus finmarchicus* in the two highest treatments only, as well as indications of decreasing rates of ontogenetic development with increasing CO_2 levels. In a two-generation study of *Acartia tsuensis* (Ito, 1956) exposed to 2380 ppm CO_2, compared to present day level controls, Kurihara and Ishimatsu (2008) found no significant effects on survival, growth or development between exposure and control groups, and no inter-generational differences in egg production or hatching rates between the two exposed cohorts. Thus, these studies may appear to give little cause for concern within the stipulated year 2100 and 2300 ocean acidification scenarios (Caldeira and Wickett, 2003; 2005; IPCC, 2007; Doney et al., 2009), as significant adverse effects were only observed for exposure levels far exceeding those predicted for the near future. However, as pointed out by Whiteley (2011), generalisations based on short-term exposure should be approached with caution. With the exception of Kurihara and Ishimatsu (2008), all of the studies mentioned thus far in this section involved exposure in the relatively short term, spanning one generation at most, and none incorporated multiple stressors. A three-generational study by Fitzer et al. (2012) on the harpacticoid copepod *Tisbe battagliai* (Volkmann-Rocco, 1972), with CO_2 as the only stressor, showed negative effects on survival and hatching success at exposure levels below 1000 ppm CO_2. Vehmaa et al. (2012) conducted a two-stressor study on *Acartia bifilosa* (Giesbrecht, 1881), involving changes in temperature (17-20°C) and pH (ambient and year 2100, lowered by 0.4 units), in which both egg production and maternal effects were explored. Egg production increased with increasing temperature for all stressor combinations, but this effect was smaller when

combined with a lowering of the pH. In addition, hatching success was reduced upon transplantation of eggs from one stressor combination to another. The findings of Fitzer et al. (2012) and Vehmaa et al. (2012) thus support the call for multigenerational, multistressor studies put forth in recent reviews of ocean acidification research (e.g. Byrne, 2011; Whiteley, 2011).

1.5 The present study

In recent years, the increase in awareness and knowledge of the threat posed by ocean acidification, *"The Other CO_2 Problem"* (Turley, 2005; Henderson, 2006; Doney et al., 2009), as well as the potential effects of future leaks from sub-seabed CO_2 storage sites, has led to a growing concern about impacts on marine invertebrates. Knowledge of trends in the sensitivity and responses of marine species is essential for a more integrated understanding of the phenomenon on an ecosystem level (e.g. Byrne, 2011). The calls for ecosystem-based management of marine resources (McLeod and Leslie, 2009), both by scientists and policy-makers (UNEP/GPA, 2006), underline the need for studies of key species and their response to environmental hypercapnia. As a copepod, *Calanus finmarchicus* represents a key group of organisms, as well as being a key species in its own right (Mauchline, 1998; Vadstein, 2009). Knowledge of its sensitivity to ocean acidification will be important for a broader understanding of the future of the ecosystems in which it participates (e.g. Doney et al., 2009).

The objective of the present study is to investigate the effects of elevated CO_2 concentrations on the full life cycle of *C. finmarchicus* in the course of two consecutive generations, in terms of ontogenetic development. The main body of knowledge on the responses of marine invertebrates to ocean acidification stems from short-term exposure studies, and the need for longer-term studies including the entire life cycle, or even multiple generations has been pointed out (e.g. Byrne, 2011; Whiteley, 2011; Fitzer et al., 2012). To our knowledge, only three multigenerational studies on the effects of hypercapnia in marine copepods have been published to date, involving the species *Tisbe battagliai*

(Fitzer et al., 2012) and *Acartia tsuensis* (Kurihara and Ishimatsu, 2008), as well as *Acartia bifilosa* (Vehmaa et al., 2012). Previous studies have indicated that the early developmental stages of marine copepods are less tolerant of CO_2 exposure than adult individuals (Kurihara et al., 2004a; Melzner et al., 2009). As the juvenile stages have also been identified as a potential bottleneck for species persistence in a number of other marine invertebrates (e.g. Byrne, 2011), the present study will focus on the pre-adult stages of *C. finmarchicus*. Development rate, in terms of development time and stage duration, has been used as an indirect measure of the energetic cost of stress in other copepod studies (e.g. Campbell et al., 2001). Delayed development as an indicator of hypercapnic stress has also been documented in other crustaceans. For larvae of the shrimp *Pandalus borealis*, Bechmann et al. (2011) found delayed development in response to CO_2 concentrations well within the scenarios predicted for the year 2300. To the author's knowledge, this is the first study to investigate the effect of hypercapnia on the development rate of the full life cycle of *C. finmarchicus* in a multi-generational context.

2 MATERIALS AND METHODS

2.1 Experimental set-up for CO_2 exposure

The experiment was carried out at the NTNU Centre of Fisheries and Aquaculture (SeaLab), between November 2011 and March 2012. Two consecutive generations of *Calanus finmarchicus* were reared in mesocosm systems at four different CO_2 concentrations (denoted Control and Treatment 1, 2 and 3, respectively). For each CO_2 concentration, three replicates were included (denoted A, B and C). The adult *C. finmarchicus* used to initiate the first generation were obtained from a continuous culture maintained at the NTNU Centre for Fisheries and Aquaculture according to the description in Hansen et al. (2007); for details, see Appendix A. The seawater used in the present experiment was filtrated to remove particulate matter, matured for a minimum of 24 hours in aerobic conditions, treated to prevent supersaturation, carefully temperature controlled and filtrated to remove bacteria; for further details, see Appendix A.

A flow chart illustrating the experimental set-up is included in Figure 2.1. In essence, a gas mixer provided the four desired gas mixtures, which were used to equilibrate filtrated seawater in four equilibration columns. The equilibrated water was then directed to the appropriate replicate tanks, where a secondary, in-tank equilibration system allowed further equilibration of the water. A peristaltic pump delivered a continuous flow of algae to the exposure tanks, via separate lines, thus providing feed for *C. finmarchicus*.

The experiment took place in a climate room at a temperature of approximately 10°C, and in a 12:12 hour L:D cycle with low light conditions. The duration of the experiment was a total of 136 days, with the first and second generations spanning 79 and 57 days respectively. All developmental stages were sampled for determination of density and stage distribution, and this thesis work has focused on the second generation.

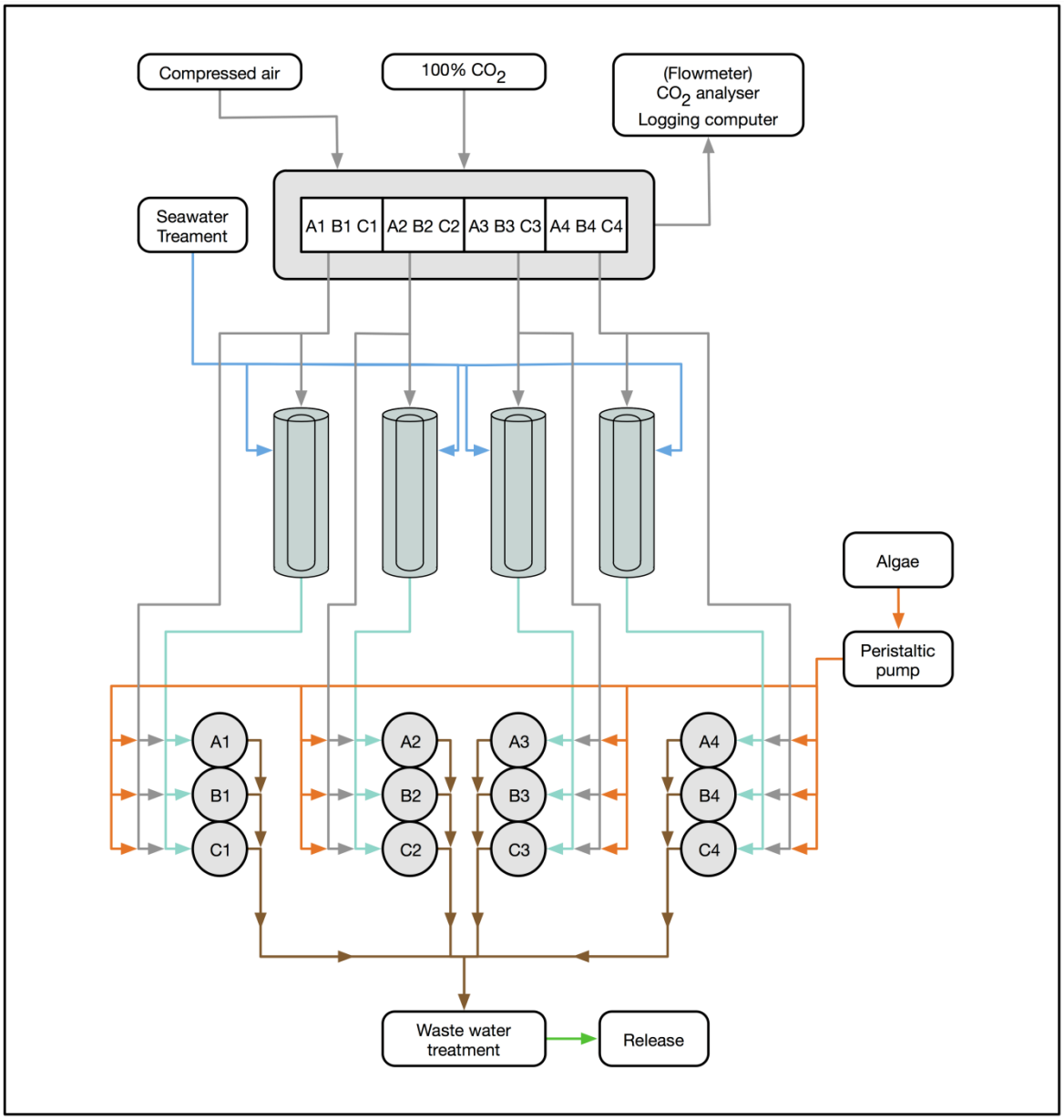

Figure 2.1: A flow chart illustrating the CO_2 exposure system. Compressed air and 100% CO_2 were mixed in a custom-built, adjustable gas mixer, producing four different CO_2 concentrations. Grey lines exiting the gas mixer represent the four gas mixtures, each being led off to two separate systems of gas-water equilibration. The primary system consisted of four double cylinders, one for each CO_2 concentration, in which a counter-current system facilitated the dissolution of CO_2 in seawater (incoming blue lines), and the resulting water (turquoise lines) filled the twelve 90 litre exposure tanks, three replicate tanks (A, B and C) per exposure group (1, 2, 3 and 4). The secondary equilibration system set up a counter-current in a column inside each exposure tank, in which gas from the appropriate gas mixture was dissolved in the water. A peristaltic pump continually distributed feed algae (orange lines) to all the tanks. Waste water (brown lines) was treated chemically before release.

2.1.1 Exposure levels and automated distribution

Four target CO_2 concentrations were selected, including the present day status (Control, 380 ppm) (IPCC, 2007), and near-future scenarios based on projections made in previous studies (Treatment 1, 1080 ppm; Treatment 2, 2080 ppm; and Treatment 3, 3080 ppm) (Caldeira and Wickett, 2003; IPCC, 2007; Våge, 2011). A custom-built automated gas mixing system (HTK Hamburg GmbH, Germany) was used to provide four different levels of CO_2 enriched air. The composition of the gas mixture output from each channel was dependent on the ratio between compressed air (central compressed air distribution at SeaLab) and 100% CO_2 gas (Mapcon® CO_2, YARA Praxair) mixed by the system. The gas flow to each tank was monitored and regulated through the use of separate gas flow rotameters connected to each output channel. These were adjusted manually as needed, in order to stabilize the pH in the tanks. The CO_2 outputs from the channels were 500, 2200, 4000 and 5800 ppm CO_2 respectively.

2.1.1 The equilibration columns

The four gas mixtures established in the gas mixer were fed to four separate equilibration columns, each of which consisted of two cylinders, one nested inside the other, as shown in Figure 2.2. A wooden air stone (Aqua Medic GmbH, Germany) near the bottom of the inner cylinder released the gas mixture into the filtrated seawater, while an aquarium pump (Micro-Jet MC450, Aquarium Systems) situated at the top of the column facilitated the movement of water from the outer column into the inner one. The resultant downward gravimetric flow of water combined with the simultaneous upward movement of rising gas bubbles, created a counter-current system in the inner cylinder which caused effective dissolution of CO_2 in the seawater. While the filtrated seawater was let into this equilibration system at the top of the column, lateral holes at the bottom of the inner cylinder allowed the gravimetric flow of equilibrated water to exit the equilibration column.

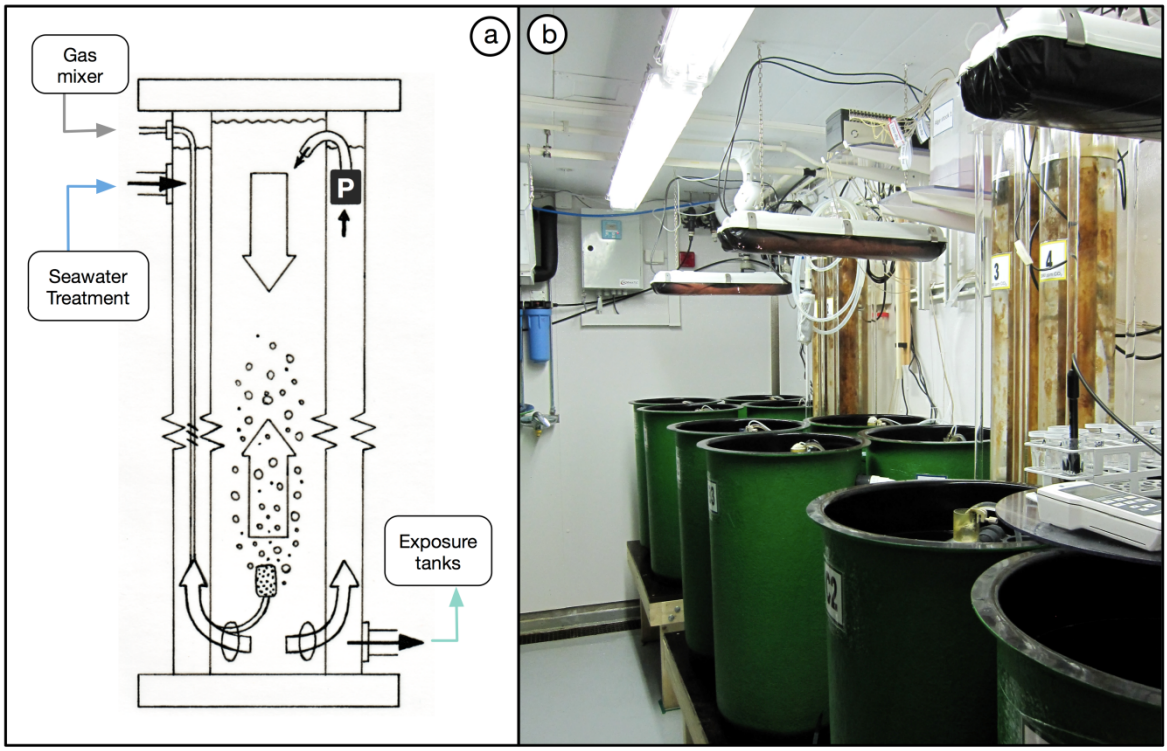

Figure 2.2: a) A schematic illustration of an equilibration column, consisting of two cylinders, one inside the other. The inner cylinder had lateral holes at the bottom. Seawater (blue line) entered at the top of the outer column, and a pump (P) raised the water level of the inner cylinder, thus forming a gravimetric flow. Gas from the gas mixer (grey line) was released at the bottom of the inner column, and the resultant counter-current system (indicated by the two vertical white arrows) allowed effective dissolution of CO_2. The gravimetric flow carried the equilibrated water out through lateral holes at the bottom of the inner column, and it was led off (turquoise line) to the exposure tanks. **b)** A photograph of the laboratory, showing the light system, equilibration columns, exposure tanks and feed distribution.

2.1.2 The exposure tanks

Twelve 90 litre polyester tanks were mounted on tables for stability, as shown in the photograph in Figure 2.2. Each tank was cylindrical, with a conical base, and gas-equilibrated seawater entered through two inlets, one at the base (30% of the total flow) and one near the top (70% of the total flow), thus providing a degree of vertical mixing, while waste water exited through an overflow outlet at the surface level. The water exchange rate of each tank was 90 L/day. A secondary gas equilibration column, covered with filtering mesh to prevent animals from entering, was mounted vertically inside the tank. At the base of this column, a

gas mixture identical to that used to equilibrate the water in the primary equilibration columns was released through a wooden air stone (Aqua Medic GmbH, Germany), and an aquarium pump (Micro-Jet MC450, Aquarium Systems) created a downward gravimetric movement of water inside the column, by pumping water from the tank through the filtering mesh and subsequently recycling it back into the column. Equilibrated water was released back into the tank at the bottom of the column, and this in-tank counter-current gas equilibration system also contributed to vertical mixing of the water. A cross section of an exposure tank, including the relevant components and processes described above is shown in Figure 2.3 part a).

The spatial arrangement of the tanks within the climate room was selected to minimise temperature gradients within the room which were discovered during preparations. The tanks were arranged in groups of four, according to replicate group, as shown in Figure 2.3 part b), rather than by CO_2 level, as depicted in the Figure 2.1 flow chart. This blocking arrangement (Wild and Seber, 2000), along with the addition of an electric ceiling fan, aimed to minimise the effect of the temperature gradient relating to the distance from the door of the room. Another more moderate temperature gradient, where close proximity to the wall meant lower temperatures, was not included in the design, as the size of the room did not allow this.

2.1.3 Distribution of feed

The *C. finmarchicus* reared in the present study were fed a monoculture of *Rhodomonas baltica* Karsten *sensu* Zimmerman 1925 (Chromopyta, Cryptophyceae) (Throndsen, 1997). The spherical diameter of 6-8 µm of the algae ensured their suitability as feed for the feeding stages of *C. finmarchicus* (Nejstgaard and Solberg, 1996; Throndsen, 1997), which have previously been shown to utilise these (Båmstedt et al., 1999). The algae were kept in suspension and added continuously at levels required to maintain a carbon level of approximately 200 µg C/L (for details, see Skogstad (2010)). Automated distribution of the algae from this container was accomplished through the use of a peristaltic pump, as shown in Figure 2.1, and the algae entered the tanks near the upper inlet for equilibrated seawater, as depicted in Figure 2.3 a).

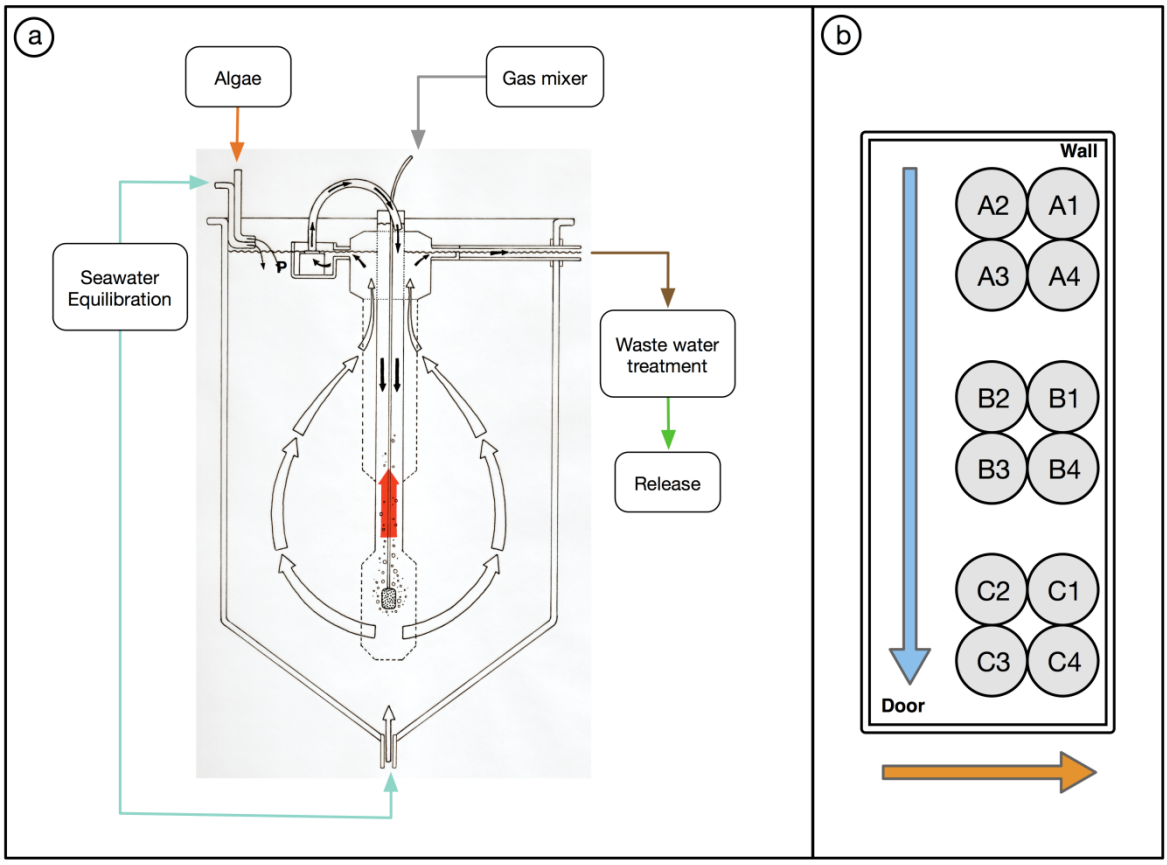

Figure 2.3: a) A diagram showing the cross-section of one of the exposure tanks. Water from the primary equilibration system (turquoise lines) entered through inlets at the top and bottom of the tank, providing a degree of circulation. Feed algae (orange line) also entered at the surface (wavy, horizontal line). A secondary equilibration column was mounted vertically inside the tank, and filtering mesh (dashed lines) prevented *C. finmarchicus* from entering this system. At the top, a pump (P) caused recycling and downward movement of water (black arrows), while gas from the appropriate gas mixture (grey line) was released at the bottom of the column (red arrow), the resulting counter-current facilitating dissolution of CO_2 in the water. The movement of equilibrated water from the bottom of the column, into the tank and then back into the recirculation loop is represented by the white arrows. Waste water (brown arrow) exited through an overflow outlet at the surface level, and was chemically treated (green arrow) before release. **b)** The twelve exposure tanks were spatially arranged in three replicate groups, each of which contained one tank from each of the exposure regimes. The positions of these groups in relation to the door and walls are also shown, as well as the lengthwise temperature gradient (thick blue arrow) and the wall-related temperature gradient (thick orange arrow).

2.2 Parameters describing the system

According to Dickson et al. (2007a), the measurement of two carbon parameters, along with temperature, pressure, salinity and the appropriate equilibrium constants, is generally regarded as sufficient to provide a complete description of the acid-base system in seawater. The carbon parameters determined in the present study were total pH (pH_{tot}) and total alkalinity (A_T), the average pressure was 10 dbar and the salinity was 35 ppt. The pH of all the experimental tanks was measured daily using a potentiometric pH-meter, and once a week using a spectrophotometer. In addition, temperature and algal feed concentrations were monitored on a daily basis and adjustments made so as to maintain the highest possible degree of stability throughout the experiment.

With the exception of the sampling of animals, and the temperature measurements involving the glass thermometer, all *in situ* measurements and sampling of water from the tanks took place inside a sieve cup with a mesh size of 64 µm. The use of this sieve cup served to include algae, while preventing animals and debris from affecting measurements or being included in samples, and also breaking the oily surface film. Thus, all the samples for the determination of water parameters were taken from the top 10 cm of the water column.

Between tanks, all equipment used for all types of sampling and measurements was flushed with seawater to dislodge any animals attached to the outer surfaces, then with fresh water to further minimize the likelihood of transfer of live animals, and finally in seawater in order to keep from salinity shocking the animals in the next tank.

2.2.1 Potentiometric pH determination

A potentiometric pH meter (pHenomenal® pH 1000 H, VWR International GmbH, Darmstadt, Germany) measuring pH_{tot} was used for daily pH determination. Prior to measuring, the pH meter was calibrated using a one-point calibration in Tris-buffer (Tris + Tris-HCl in synthetic seawater; Scripps Institution of Oceanography, La Jolla, CA, USA). This buffer, having the same ionic strength as seawater (approximately 0.7 mol/kg), defines pH on the total scale (Hansson, 1973; Nemzer and Dickson, 2005). The temperature readings

from the pH meter were recorded, but not used in further calculations. The measuring electrode was held vertically and moved gently around inside the filtering cup, until the pH value stabilized, before the value was recorded. Between measurements, the electrode was placed in a small beaker of seawater.

2.2.2 Spectrophotometric pH determination

In addition to daily potentiometric pH measurements, the pH_{tot} in the exposure tanks was measured spectrophotometrically once a week, using a method modified from Dickson et al. (2007b). Spectrophotometric pH determination exploits the pH-dependent colouration of an indicator dye, in this case *m*-cresol purple, a sulfonephthalein indicator, as it transitions between its monoanionic (HIn^-) and dianionic (In^{2-}) forms, and the resulting change in its absorption spectrum (Dickson et al., 2007b). Samples were collected from the near-surface water of the experimental tanks using 50 mL borosilicate glass bottles. Each bottle was rinsed twice in the water before being filled completely, resulting in a positive water meniscus, and sealed with a gas-tight glass stopper. The samples were then transferred to a thermostat water bath and equilibrated to (25°C). The use of stopper grease, though recommended by Dickson et al. (2007b), was not deemed necessary, as the samples were always analysed within an hour of collection. In order to prevent water from the water bath from contaminating the samples, the bottles were never fully submerged.

A 2 mM *m*-cresol purple solution was prepared, and its ionic strength adjusted through the use of sodium chloride (not seawater as described in Dickson et al. (2007b)). This stock solution was subsequently stored in the dark, and its bottle wrapped in aluminium foil.

Once the samples had attained a temperature of 25°C, they were analysed one by one, using a spectrophotometer (Cary 50 Bio UV-visible Spectrophotometer, Varian Inc., Mulgrave, Australia), with a temperature-controlling (25°C) single cell peltier unit (Cary Single cell peltier accessory, Varian Inc., Mulgrave, Australia). Sampled water was transferred into a 3.7 mL gas-tight special optical glass cuvette (corresponding to a light path of 1 cm, not 10 cm as described in Dickson et al. (2007b)), which, along with its cap, was rinsed in sample water three times before the actual measurement was taken. Once again, a positive meniscus and subsequent careful replacement of the Teflon® cap removed excess

water and minimized air bubbles. The outer surfaces of the cuvette were carefully cleaned, and the sample was then placed in the spectrophotometer. Using the Varian Cary® WinUV RNA/DNA Estimation Application, Varian Inc., Middelburg, the Netherlands), its absorbance was measured at three different wavelengths: 434 and 578 for the absorption maxima of In^{2-} and HIn^{-} respectively, and 730 nm, a non-absorbing wavelength for *m*-cresol purple, in order to correct for background absorbance. Next, the cuvette was emptied, and 50 µL of *m*-cresol was added, followed by water from the same sample. The cap was replaced, and the contents of the cuvette were mixed carefully. Once a uniform colour had been attained, the absorbance was measured at the same three wavelengths, and the pH of the sample was calculated from the absorbance values of the sample with and without the addition of *m*-cresol, according to Equations 2.1 and 2.2. Between samples, the cuvette was rinsed two times in distilled water.

$$pH = 8.0056 + log\left(\frac{\left({}^{A_1}/_{A_2} - 0.00691\right)}{\left(2.2220 - \left({}^{A_1}/_{A_2} \times 0.1331\right)\right)}\right) \quad (2.1)$$

where

$${}^{A_1}/_{A_2} = \frac{\left(A_{578cresol} - A_{578blank} - \left(A_{730cresol} - A_{730blank}\right)\right)}{\left(A_{434cresol} - A_{434blank} - \left(A_{730cresol} - A_{730blank}\right)\right)} \quad (2.2)$$

and 0.00691, 2.2220 and 0.1331 were the extinction coefficient ratios for *m*-cresol purple (λ_1 = 578 nm and λ_2 = 434 nm) as shown in Dickson et al. (2007b).

An increase in temperature causes a decrease in pH, due to the endothermic nature of the dissociation of water (Chang, 2003). As the pH determination took place at 25°C, the pH at the temperatures in the tanks was calculated using the free software package "CO_2-SYS" version 2.1 (Pierrot et al., 2006). Silicate and phosphate measurements for the seawater used in the experiments were not made, and these values were thus assumed to be zero by

the software package (Pierrot et al., 2006). No correction was made for the introduction of *m*-cresol purple into the acid-base system (Dickson et al., 2007a).

2.2.3 Determination of total alkalinity

The total alkalinity (A_T) of the water in one of the control tanks (A1, 380 ppm CO_2) was measured once a week, using an open-cell titration method modified from Xiaowan et al. (2009). Before sample analysis, this method was evaluated by measuring the A_T of Standard Seawater (Scripps Institution of Oceanography, La Jolla, CA, USA) with known pH, salinity and A_T. Samples were collected in sealable 50 mL plastic vials and equilibrated to 25°C prior to analysis as described for pH samples. A TitraLab TIM860 Titration Manager equipped with a PHC2001-8 pH electrode (Radiometer Analytical SAS; Villeurbanne, France) and a water-jacketed titration cell with a magnet stirrer and stir bar ensured that the titration process would take place at a stable temperature of 25°C (±0.2°C), as recommended by Xiaowan et al. (2009). An Erlenmeyer flask containing the titrant, a solution of 0.02 M HCl with 0.68 M sodium chloride (NaCl) was connected to the Titration Manager and placed inside the thermostat bath. Thus, NaCl was used to adjust the ionic strength of the titrant, not artificial seawater as indicated in Xiaowan et al. (2009). All air bubbles were removed from the dosing tube, as well as from all other tubing in the Titration Manager. The mass of approximately 25 ml of the sampled water was determined using a Precisa® 180A Precision Balance, (Precisa Gravimetrics AG, Aldingen, Germany) and entered into the method program on the Titration Manager. The A_T of the sample was then measured by titration as described by Xiaowan et al. (2009). The A_T concentration (M_{At}), given as µmol/kg, was calculated automatically by the Titration Manager, based on the equivalence point of the titration, according to Equation 2.3:

$$M_{At} = c_{HCl} \times \frac{v_{HCl}}{m_{sw}} \qquad (2.3)$$

where c_{HCl} = molarity of the HCl solution

v_{HCl} = ml of HCl solution consumed

m_{sw} = mass (g) of seawater sample

2.2.4 Determination of temperature

In addition to the temperature measurements from the potentiometric pH-meter, the temperature in the experimental tanks were measured and recorded daily, using a VWR® Precision Thermometer certified by VWR® as accurate within ± 0.3°C (VWR International GmbH, Darmstadt, Germany).

2.3 Regulation of feed concentrations

A filtering cup with a pore size of 64 µm was placed into the water of each experimental tank, and a 25 ml cell counter cup was filled with about 20 ml of water from inside this cup. Between tanks, the filtering cup was thoroughly rinsed. The water sample was placed on a stirring table (150 rpm) (Labotron 960742, Infors HT, Bottmingen), and gently shaken just prior to analysis, to prevent sedimentation of particles. The samples were then analysed using a particle-sizer system (Multisizer^{TM} 3 Coulter Counter®, and the Beckman Coulter Particle Characterization software package, version 3.51, both from Beckman Coulter, Inc.), which extracted and analysed three 1 mL sub-samples from each sample. The Coulter principle states that the amplitude of the voltage pulse generated from the passage of a particle through the aperture of the counter system will be directly proportional to the spherical volume of the particle in question (Coulter, 1956 as cited in Graham, 2003).

The three resulting curves of particle number as functions of particle size were averaged, if found to be reasonably consistent, and the area under this curve was calculated for particles ranging in size from 5.363 µm to 9.561 µm, which includes the peaks representing the size range of *Rhodomonas baltica* (Møller, 2005). Algal concentrations significantly above or below the target concentration of 200 µg C/L caused the feeding regime to be adjusted for the tank in question, thus keeping the concentrations as stable as possible.

2.4 Two generations of *Calanus finmarchicus*

Adult *C. finmarchicus* tend to consume eggs and young nauplii, and as a feeding strategy cannibalism has been found to contribute to the population dynamics of this species (Bonnet et al., 2004). To avoid grazing of eggs and nauplii by the later developmental stages, the two generations reared in the present experiment were kept separate, through isolation of adult females for egg-laying and subsequent separation of eggs from adult individuals.

2.4.1 Collection of females for egg-laying

2.4.1.1 The first generation

Prior to the start of the first generation, adult *C. finmarchicus* were transferred, through careful filtration, from their polyester culture tanks and distributed equally among six 50 L polyethylene egg-laying tanks. The animals were kept submerged at all times during this procedure, and care was taken to minimize the risk of animals being damaged. After the transfer, algal stock was added to the egg-laying tanks until an effective concentration of 600 µg C/L was attained. As food supply has been found to affect egg production rates in copepods (Marshall and Orr, 1955), this increase in algal concentration was used to stimulate egg production. Due to low egg production initially, the procedure for isolation of females was repeated, and visual inspection revealed broken antennae and other physical damage likely caused by this process.

2.4.1.2 The second generation

For the initiation of the second generation, adult C. finmarchicus were transferred in the same manner as for the first generation, except that this time fertilization and egg release took place under the appropriate CO_2 conditions. The white egg-laying tanks were filled with water from the same tank as the one each batch of females had come from, and the exposure regimes were continued all through this procedure. Thus, for each tank, the entire life cycle was subjected to the same acidification scenario, as recommended by Byrne (2011). Due to low egg-laying rates in some tanks, the egg-laying was allowed to continue

for 72 hours. Consequently, some eggs hatched during this time, and the average age of the cohort at their return to the 90L tanks was 36 hours (1.5 days).

2.4.2 Collection and incubation of eggs

2.4.2.1 The first generation

The first generation of eggs were collected 24 hours after the adults were placed in the six egg-laying tanks. All the adult individuals were removed by filtration and the eggs and nauplii were all pooled into one batch. The total volume was adjusted to 1000 mL, and after gentle mixing in order to evenly distribute the eggs and nauplii in the water column, 10 mL subsamples were collected and used to determine the density of eggs and nauplii, using a Leica MZ12.5 stereomicroscope (Leica Microsystems, Wetzlar, Germany). Based on this sample, the total number of eggs and nauplii in the 1000 mL batch was estimated to be 24600. The batch was evenly distributed amongst the twelve experimental tanks, giving an estimated 2050 eggs and nauplii per tank at the starting point of the experiment.

2.4.2.2 The second generation

After three days (72 hours) of incubation in the egg-laying tanks, the second generation of eggs and newly hatched nauplii (with an average age of 1.5 days) were separated from the adult individuals by filtration. The eggs and nauplii from each tank were diluted, using seawater with the appropriate pH, to a volume of 500 mL, from which representative subsamples of 10 mL were collected. The number of eggs and nauplii in these subsamples was determined by counting under a stereomicroscope as described above, and used to estimate the number of eggs and nauplii collected from each tank. The lowest number estimated in this way was from tank A4, which had 2000 eggs and nauplii, and this was consequently set as the target starting number for all the other tanks as well. Thus, the volume corresponding to 2000 eggs and nauplii for each 500 mL stock was calculated, and after gently mixing the contents of each stock this volume was measured out and added to the water in the appropriate experimental tank.

2.4.3 Sampling for determination of stage distribution

The various developmental stages of *C. finmarchicus* exhibit duration times ranging from mere hours to several days (e.g. Hygum et al., 2000a; Campbell et al., 2001). Therefore, in order to depict the development of each stage in sufficient detail, while reducing the likelihood of over-sampling the long-duration stages, the sampling frequency needed to be adjusted according to the expected duration time of the stages dominating in the cultures at a given point in time. The first naupliar stages were thus sampled three times a day, while the subsequent stages were sampled less frequently. In addition, the sampled volume was increased as the density in the tanks decreased, in order to ensure samples with a sufficient number of animals to provide a representative measure of the density and stage distribution in the tanks. Sampling for the second generation began eight hours after placement in the experimental tanks. The fixative used to preserve the samples was Fytofix, a Lugol-based fixation agent, which was prepared as described in Appendix A.

Each sample was taken using a measuring beaker (500 – 1000 mL) attached to a length of inflexible PVC tubing. In order to reduce any non-uniform distribution patterns for the animals in the tank, both within and between developmental stages, a stirring routine was carried out. The sampling beaker was first moved gently up and down in the entire water column, and then emptied carefully into the tank in order to minimize the degree of damage to the animals. This stirring procedure was repeated three times before the actual sample was collected. The collected sample was then filtered through a filter attached to the outlet of a funnel.

As the cohort progressed from nauplii to the much larger copepodite stages, the pore size of the filter was also increased (starting at 32 µm, then 64 µm and finally 120 µm) to prevent clogging. Any animals remaining in the empty beaker were flushed onto the filter using seawater, and the funnel above the filter was then flushed in the same manner. The sampled animals, along with any faecal pellets and other debris, were then reverse-flushed off the filter and into an 8 mL glass bottle containing 16 µl of Fytofix. The sample bottles were stored upright in the dark at 10°C for the duration of the experiment, while long-term storage was at 4°C.

2.4.4 Stage determination

The contents from each sample bottle were transferred to the well of a microscopy slide using a glass Pasteur pipette, which was carefully rinsed to prevent retention of animals. The sampled animals were then placed under a Leica M205 C stereomicroscope (Leica Microsystems, Wetzlar, Germany), and photographed using a Nikon DS-5M digital camera (Nikon Instruments, Inc., Düsseldorf, Germany). The sample bottles were inspected for remaining animals under the microscope. For each sample, the developmental stages of all individuals, including eggs, were determined according to Marshall and Orr (1972) and Mauchline (1998). Stages NI and NII were distinguished mainly on the basis of the spines on the posterior ends of their bodies, while the other naupliar stages (NIII – NVI) were identified by the number of appendages and the number of posterior spines. The copepodite stages (CI – CV, male and female) were identified by the apparent segmentation of the metasome and the urosome. Each sample was assigned a table, in which all the animals found in the sample were registered according to their developmental stage. In order to minimize counting mistakes, the registered individuals were grouped by stage along the edge of the well, and then re-counted. After counting, stage determination and photography, the animals were transferred back into their bottle of origin. Care was taken to minimize the loss of animals by rinsing the pipette with the remaining water in the well after transfer.

All the samples from the second generation were analysed by the same person, and in order to reduce the problem of misidentification whenever a new stage appeared in the samples, some samples were re-counted later around these transition points.

2.5 Statistical analysis

All data analyses and statistical tests were carried out using Microsoft Excel version 2010 (Microsoft, Redmond, WA) and SigmaPlot version 12.3 (Systat Software, Inc., San Jose, CA), the latter of which was also used for all the plots. The significance level was set at $p \leq 0.05$ for all tests.

2.5.1 Tests on parameters describing the system

For both generations of *C. finmarchicus*, observed gradients in temperature and feed availability (algal concentrations) were explored using one way ANOVA on the means and standard deviations found for the four different exposure groups. In both these cases, a Shapiro-Wilk test confirmed the normality of the data. In cases where a significant difference was found, a *post hoc* Dunnett's test (Dunnett, 1955; Zar, 1999) was performed.

2.5.2 Estimates of density

The total number of animals, including eggs, found in each sample was normalised by adjusting for differences in the sampled volume, in order to provide an estimate of the total density in each experimental tank at the time of each sampling. This data set has been included in Appendix C. Based on the density in the individual replicate tanks, the mean and standard error for each treatment group (n = 3 replicate tanks) was then calculated for each sampling point. An attempt was made at identifying and removing outliers, but no systematic solution could be identified. A one-way ANOVA was used to test for significant differences in mean density between the treatment groups.

2.5.3 Stage determination data – MDT

The stage determination data has been included in Appendix D, and was analysed using a procedure resembling that of Campbell et al. (2001). Cumulative proportions for each stage were calculated for each sample, by including all individuals which had progressed to, or

past, a given stage. For each experimental tank, these cumulative proportions were then plotted for each developmental stage, and the linear portion of the resulting sigmoidal curve was isolated for linear regression analysis. Due to the relatively low number of points comprising each curve, the selection of data for linear regression was carried out based on a $y \in \langle 0.05,\ 0.95 \rangle$ inclusion window removing the 'tails' of the sigmoidal cumulative proportion plots, not the 0.10-0.90 limits used by Campbell et al. (2001). To counter the potential for inclusion of 'tail' values caused by sampling error (Hu et al., 2007), an additional limit of two consecutive values of zero or one was chosen to terminate a linear trend. This last provision was similar to that made by Hu et al. (2007) in their calculations. For each stage in each tank, the linear portions thus isolated were then analysed by least squares linear regression (see Appendix B), and the linear equations fit to the data were used to determine the median development time (MDT), the time from the midpoint of egg-laying (1.5 days) to when 50% of the cohort has developed to, or past, the stage in question (Landry, 1975; Campbell et al., 2001).

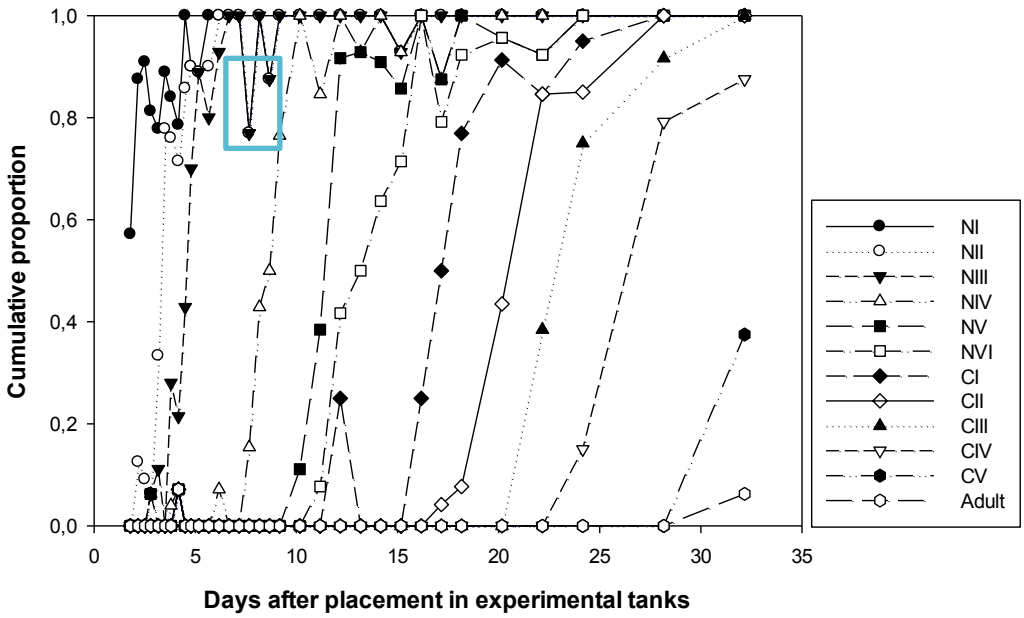

Figure 2.4: The plot shows an example of the cumulative proportions for each stage, as a function of time, for the stage frequency data from one of the experimental tanks. Typically, the curve for stage NI had progressed past the 50% point at the time of the first sampling, and stage C5 and the adult stage were not well represented. The blue rectangle marks the challenging tail values, which kept 'bouncing' both prior to and after the clear linear portion of the curve. Such tail values were removed by adding the rule of two consecutive values of 0 or 1 to terminate a linear trend.

2.5.4 Comparison of MDT

For comparison of median development times between exposure groups, the MDT value of a given stage from each of the three replicate tanks within each group was used as the basic unit. These were tested for normality using a Shapiro-Wilk test, and in instances where the assumptions of normality and equal variances were fulfilled, the MDT values were analysed using a one-way ANOVA. A significance level of $P<0.05$ was adopted in all analyses. For stages where a significant difference was detected by the one-way ANOVA, the *post hoc* Dunnett's test (Dunnett, 1955; Zar, 1999), which compares several treatment groups to a single control group, was carried out. This test was selected based on the experimental design, in which the control group formed the baseline in any comparison intended in the design. If a treatment did not produce a significant difference relative to its control, then it would not be expected to produce a significant difference compared to other treatments.

In one instance, the assumptions of normality and equal variances were violated, and as the transformation of the data failed to rectify this, the non-parametric Kruskal-Wallis one-way ANOVA on ranks procedure was used. As this test returned a non-significant result, no further non-parametric tests were carried out.

3 RESULTS

3.1 Parameters describing the system

3.1.1 Actual CO_2 concentrations

As the experimental set-up permitted a certain degree of gas exchange with the surroundings, the actual CO_2 concentrations in the four exposure groups differed slightly from the target of 380 ppm (Control), 1080 ppm (Treatment 1), 2080 ppm (Treatment 2) and 3080 ppm (Treatment 3). The measured values for the water parameters from the first and second generation are presented as mean values ± 1 standard deviation (SD) in Tables 3.1 and 3.2, respectively. During the rearing of the first generation, the actual CO_2 concentrations were 437 ± 17 ppm (Control), 1102 ± 77 ppm (Treatment 1), 2307 ± 303 ppm (Treatment 2), and 3502 ± 262 ppm (Treatment 3). During the rearing of the second generation, the CO_2 concentrations were 414 ± 17 ppm (Control), 1055 ± 93 ppm (Treatment 1), 2035 ± 139 ppm (Treatment 2), and 3644 ± 197 ppm (Treatment 3). The relatively large increase in CO_2 concentration in Treatment 3 (up 142 ppm) from the first to the second generation is worth noting, as is the even larger decrease in mean CO_2 concentration observed in Treatment 2 (down 273 ppm). The daily pH measurements using the potentiometric pH meter were not included in Tables 3.1 and 3.2, as the temperature sensor was not considered to be sufficiently accurate, but measurements were used for day-to-day monitoring, and are shown as a background scatter plot in Figure 3.1. The more reliable spectrophotometric pH measurements are superimposed as mean ± SD in the same plot. During the egg-laying period at the beginning of the second generation, some technical issues, as well as low egg-laying rates necessitated a prolonged stay in the non-flow-through conditions of the egg-laying tanks, which appears to have caused the pH to drop. (As the only measurements available form this period stem from the pH meter, this apparent decrease in pH may have been due to incorrect measurements.) Any eggs included in the second generation remained under these conditions for a maximum of 3 days, the average egg age at replacement in the flow-through tanks being 1.5 days.

Table 3.1: A summary of the measured concentration of CO_2 (ppm), pH (total scale) based on spectrophotometric measurements, temperature and total alkalinity in the four exposure groups during the **first generation**. All parameters were measured in water from the experimental tanks. Values are mean ± SD, N = 3 replicate tanks, and mean values are based on all measurements taken during the experiment. N = 35 per replicate tank for pH and [CO_2], N = 23 for temperature and N = 38 for total alkalinity. Total alkalinity (A_T) was measured for tank A1 only.

Exposure group	[CO_2] (ppm)	pH_T	Temp. (°C)	A_T (µmol/kg)
Control	437 ± 17	8.00 ± 0.01	9.75 ± 0.15	2269 ± 21.8
Treatment 1	1102 ± 77	7.64 ± 0.03	9.89 ± 0.15	-
Treatment 2	2307 ± 303	7.33 ± 0.06	9.87 ± 0.15	-
Treatment 3	3502 ± 262	7.15 ± 0.03	9.68 ± 0.12	-

Table 3.2: A summary of the measured concentration of CO_2 (ppm), pH (total scale) based on spectrophotometric measurements, temperature and total alkalinity in the four exposure groups during the **second generation**. All parameters were measured in water from the experimental tanks. Values are mean ± SD, N = 3 replicate tanks, and mean values are based on all measurements taken during the experiment. N = 7 per replicate tank for pH and [CO_2], N = 37 for temperature and N = 14 for total alkalinity. Total alkalinity (A_T) was measured for tank A1 only.

Exposure group	[CO_2] (ppm)	pH_T	Temp. (°C)	A_T (µmol/kg)
Control	414 ± 17	8.02 ± 0.02	9.80 ± 0.11	2245 ± 16.36
Treatment 1	1055 ± 93	7.65 ± 0.04	9.96 ± 0.11	-
Treatment 2	2035 ± 139	7.38 ± 0.03	9.96 ± 0.10	-
Treatment 3	3644 ± 197	7.13 ± 0.02	9.80 ± 0.09	-

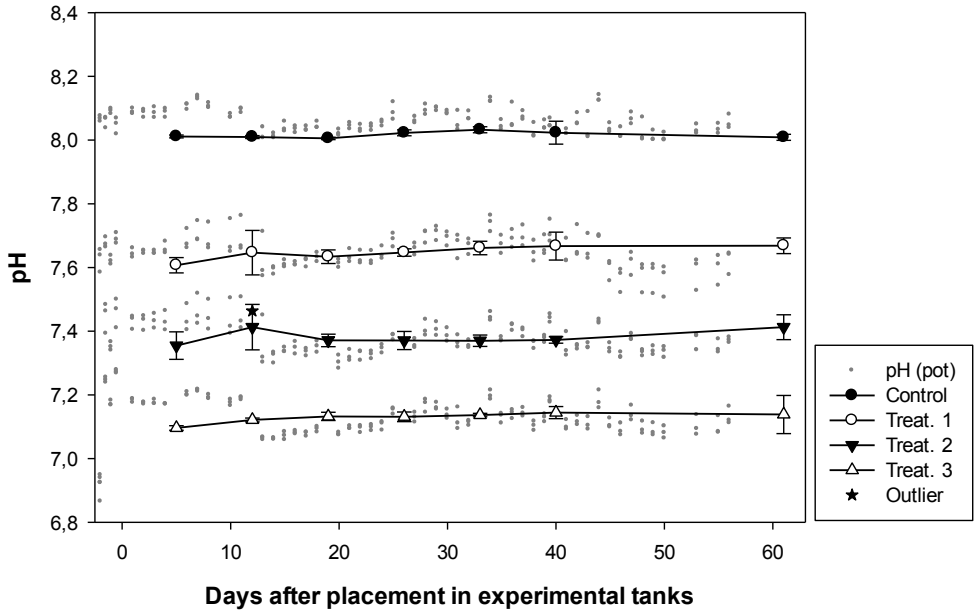

Figure 3.1: The plot shows the pH in each of the four exposure groups, based on spectrophotometric measurements, as mean ± SD (N = 3 replicate tanks) for the duration of the second generation. A scatter plot of the pH measurements from the potentiometric pH meter is included in the background, to illustrate the daily development in pH during the intervals between each spectrophotometric measurement.

3.1.2 Temperature gradients detected

The temperature in each experimental tank was relatively stable throughout the experiment, and the degree of variability was approximately the same for all tanks within the same time frame, as exemplified for Generation 2 in Figure 3.2. No statistically significant difference was detected in mean temperature between the four treatment groups in either generation (one way ANOVA, $F_{(3,8)} = 1.459$, $p = 0.297$ and $F_{(3,8)} = 2.421$, $p = 0.141$ for the first and second generation respectively). However, a non-significant, but nonetheless consistent pattern was observed. As shown in Table 3.1, during the first generation, the mean temperature was 9.75 ± 0.15°C for the Control group, 9.89 ± 0.15°C for Treatment 1, 9.87 ± 0.15°C for Treatment 2, and 9.68 ± 0.12°C for Treatment 3. Correspondingly, for the second generation the mean temperatures were 9.80 ± 0.11°C for the Control group, 9.96 ± 0.11°C for Treatment 9.96 ± 0.10°C for Treatment 2, and 9.80 ± 0.09°C for Treatment 3, as shown in Table 3.2.

A comparison of the mean temperatures for generation 1 and 2 reveals that for the Control tanks, there was a mean increase in temperature of about 0.05°C from the first to the second generation, while for the three other exposure groups this increase was approximately 0,1°C.

The temperature gradient which was detected in the room prior to the start of the experiment, which led to lower temperatures in the tanks situated close to the door of the room (see Figure 2.3 b, blue arrow), as well as the gradient causing lower temperatures in the tanks in close proximity to the longitudinal wall of the room (see Figure 2.3 b, orange arrow), are both evident in the temperature results. Both gradients are visible in the left plot of Figure 3.2, in which the tanks have been sorted by spatial arrangement. The tanks which were situated closest to the wall, namely the Control and Treatment 3 tanks, all exhibited lower mean temperatures than the Treatment 1 and Treatment 2 tanks. Also, the Control and Treatment 3 tank in spatial group C had lower temperatures than those of spatial groups A and B, which were further away from the door.

The right plot of Figure 3.2, in which the tanks have been sorted by exposure group, shows a pattern where two out of three replicate tanks in each treatment group exhibit approximately the same mean temperature, whereas the third, the one from spatial group C, has a lower mean temperature relative to the other two.

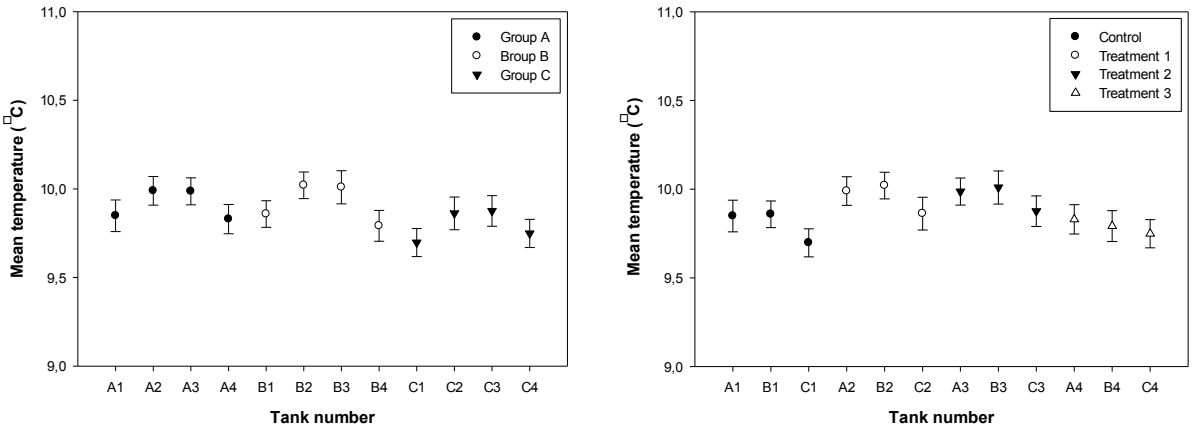

Figure 3.2: Right: The plot shows the temperature variation in the twelve tanks, as mean ± SD (N = 37), for the second generation. The tanks are sorted by spatial arrangement, in order to visualise the way proximity to the wall, as well as the door (see Figure 2.3 b), affected tank temperatures. **Left:** The plot depicts the temperature variation in the twelve experimental tanks over the duration of the second generation. Values are mean ± SD (N = 37), and the tanks are sorted by exposure group. Due to the wall-related gradient, which the spatial blocking design did not counter, the tanks from spatial group C have lower mean temperatures, compared to those from the other two spatial groups.

3.1.3 Feed concentrations

Despite efforts to manually adjust the algal concentrations in each exposure tank in response to daily measurements, feed availability varied throughout both generations. The mean algal concentrations (mean ± SD), expressed in micrograms of carbon per litre (µg C/L), for each of the twelve tanks during the first and second generation are presented in Table 3.3. During the first generation, the overall mean algal concentrations in each treatment group were 180.63 ± 75.08 µg C/L (Control), 184.90 ± 72.86 µg C/L (Treatment 1), 195.47 ± 69.31 µg C/L (Treatment 2), and 230.75 ± 58.00 µg C/L (Treatment 3). A one-way ANOVA comparing these means showed no statistically significant overall difference ($F_{(3,8)}$ = 0.325, p = 0.808) in feed availability. For the second generation, the mean algal concentrations were 194.50 ± 68.77 µg C/L (Control), 198.76 ± 65.08 µg C/L (Treatment 1), 208.99 ± 62.72 µg C/L (Treatment 2), and 226.01 ± 56.22 µg C/L (Treatment 3). Again, a one-way ANOVA comparing these means showed no statistically significant overall difference ($F_{(3,8)}$ = 0.147, p = 0.929) in feed availability between treatment group.

However, an overall non-significant pattern was observed, which can be visualised from Table 3.3. In the first generation, there was a slight, but systematic, increase in algal concentration with increasing CO_2 concentration. During the second generation, this gradient was reduced somewhat, but still discernible.

Table 3.3: Algal density, the feed concentrations available to *C. finmarchicus*, averaged within the twelve exposure tanks, for the duration of the first and second generations. Values are mean ± SD. Carbon per litre was calculated based on cell counts, using the results for *R. baltica* found by Skogstad (2010). The two highest exposure groups show slightly higher mean algal concentrations relative to the control and low exposure groups.

		GENERATION 1	GENERATION 2
EXPOSURE GROUP	Tank	Algal density (µg C/L)	Algal density (µg C/L)
Control	A1	168.43 ± 73.82	191.58 ± 67.17
	B1	168.15 ± 75.47	186.93 ± 66.82
	C1	205.30 ± 75.96	204.99 ± 72.33
Treatment 1	A2	166.42 ± 73.93	199.78 ± 54.34
	B2	199.99 ± 71.42	205.21 ± 74.74
	C2	188.28 ± 73.22	191.28 ± 66.15
Treatment 2	A3	207.55 ± 63.51	229.43 ± 75.12
	B3	196.58 ± 71.19	193.76 ± 61.33
	C3	182.28 ± 73.22	203.77 ± 51.70
Treatment 3	A4	217.89 ± 62.82	228.48 ± 61.77
	B4	226.21 ± 61.44	212.94 ± 52.95
	C4	248.16 ± 49.73	236.61 ± 53.94

Focusing on the second generation, the increase in algal concentration with increasing CO_2 level was especially clear between day 22 and day 35, as indicated by the two vertical dashed lines in the top plot of Figure 3.3. In addition, a sharp reduction in algal density occurred simultaneously in all of the exposure groups, from approximately day 2 to day 5 (see asterisk in Figure 3.3) after the placement of eggs in the flow-through tanks.

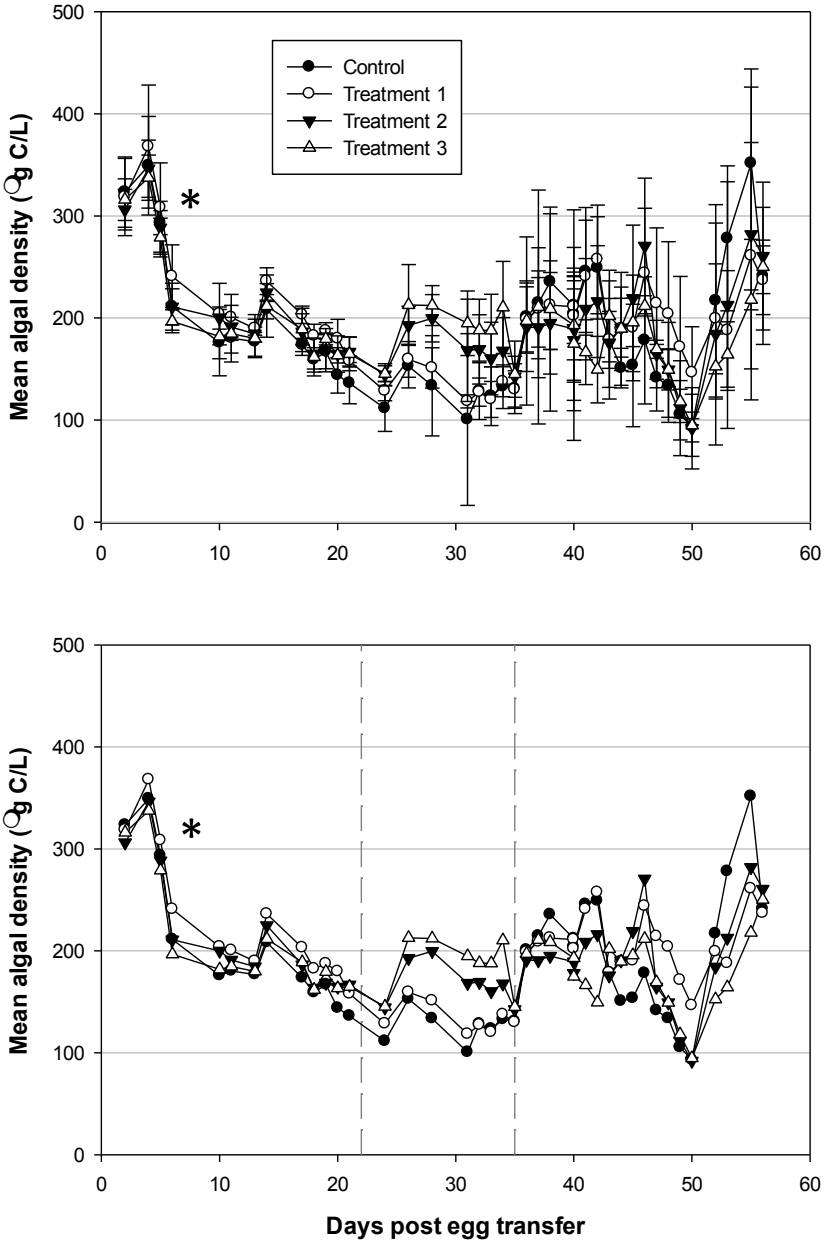

Figure 3.3: Top: Mean algal density (µg C/L) for each of the four exposure groups during the second generation. Between day 2 and day 5, the feed concentrations drop rapidly in all four treatment groups (marked by an asterisk), and this time interval corresponds to the appearance of the first-feeding stage NIII (see Figure 3.5). Values are mean ± SE (N = 3 replicate tanks). **Bottom:** Mean algal density (µg C/L) for each of the four exposure groups during the second generation, but with the standard errors left out for clarity.

During these few days, the concentration of algal carbon fell from approximately 350 µg C/L to about 200 µg C/L, and despite the increases made in algal input, mean concentrations continued to fluctuate. Between day 22 and day 35, the concentrations in the Control and Treatment 1 tanks sometimes fell as low as 100 µg C/L.

Isolating the time interval indicated in the bottom plot of Figure 3.3 yielded a clearer picture. A one way ANOVA comparing the mean values for each of the four exposure groups during the time span between day 22 and day 35 revealed a significant difference ($F_{(3,40)}$ = 13.262, $p < 0.001$) in algal concentrations. The subsequent *post hoc* Dunnett's test identified a significant increase in algal density in both Treatment 2 ($p = 0.002$) and Treatment 3 ($p < 0.001$), when compared to the Control group. In Figure 3.3, the sharp drop (asterisk) in algal concentration corresponding to the appearance of NIII has been marked by an asterisk, while the trend observed in Table 3.3 is visible between day 22 and day 35 (dashed vertical lines). Here, the food availability clearly increases with increasing CO_2 concentration.

3.2 Estimates of density for the copepod cultures

The density calculated for each sampling point varied greatly within each exposure group, as shown in Figure 3.4., which indicates that the sampling method did not provide representative samples regarding density. A one-way ANOVA comparing the means for the sampling points between day 23 and day 29, which was the only time interval to display any sign of a clear trend, detected no significant difference in mean density.

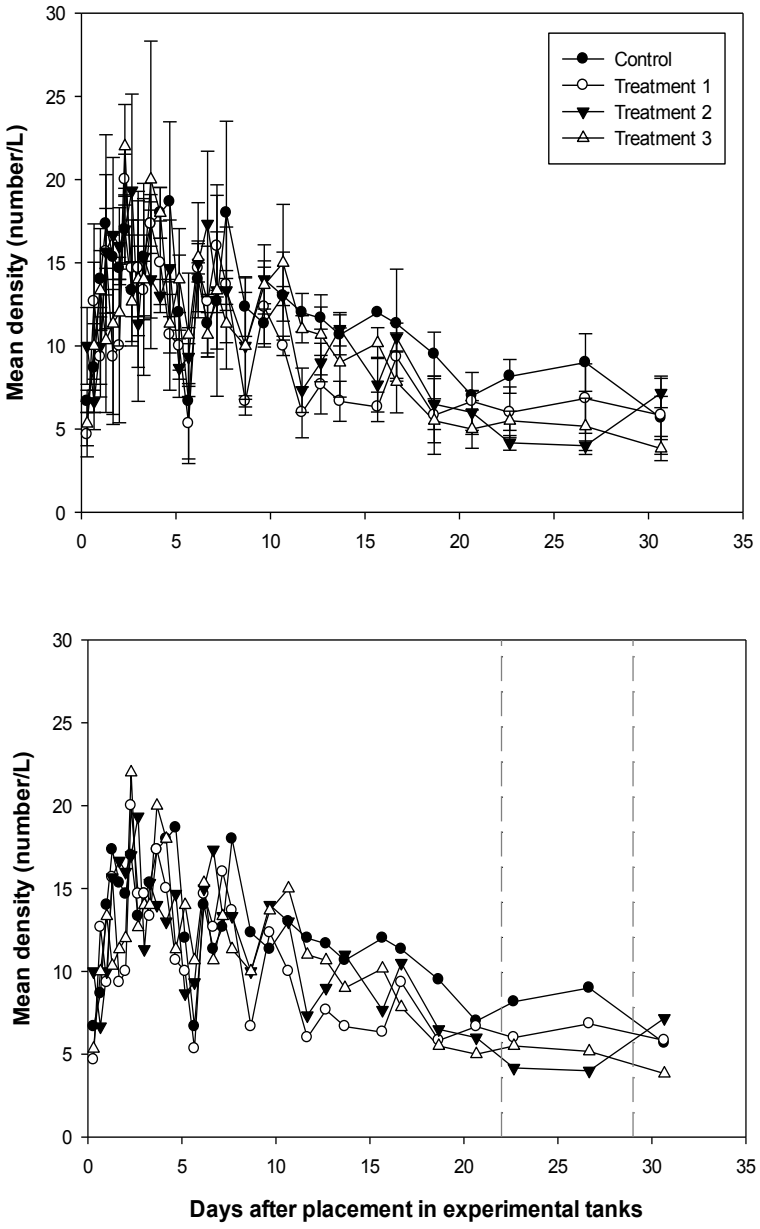

Figure 3.4: The plots show mean density in the four exposure groups during the second generation. **Top:** The values are mean ± SE (n = 3 replicate tanks). **Bottom:** The plot shows the same mean values as the top one, but here standard errors have been removed for clarity, and dashed lines isolate the time interval between day 23 and day 29.

3.3 Stage distribution

3.3.1 Median Development Time – MDT

The stage-wise mean MDT values for each of the four exposure groups have been plotted in Figure 3.5 for the naupliar stages and the early copepodite stages. A one-way ANOVA revealed a significant difference in mean MDT for the stages NV, NVI and CI ($p = 0.002$, 0.008 and 0.007 respectively). A *post hoc* Dunnett's test on these stages revealed that significant differences in MDT existed only between Treatment 3 and the Control group, with $p = 0.001$ for NV, $p < 0.05$ for NVI and $p = 0.005$ for CI. In all of these cases, the mean MDT increased with increasing CO_2 concentration, indicating a delay in development in the Treatment 3 group (see Figure 3.5). A non-significant continuation of this trend was also seen in stages NIV and CII. For the stages where a significant effect was found, the mean developmental delay was 2.03 days for NV, 4.13 days for NVI and 3.91 days for CI, when comparing Treatment 3 and the Control group.

3.4 Practical findings regarding the stage determination of early stages

Concerning the naupliar stages, the description in Marshall and Orr (1972) includes the number of dorsal setae on the distal segment of each antennule, which may be used to distinguish a late-stage NIV, for example, from an early NV. In the present experiment, while posing less of a threat to Health, Safety and Environment (HSE) than a formaldehyde-based fixative (Jaspers and Carstensen, 2009), the fixating agent Fytofix caused relatively drastic bending and shrinkage in post-NII individuals, and in most cases, the antennae were no longer positioned in a way that permitted the visual determination of such details. The degree of distortion caused by the fixating agent rendered the specimens unsuited for biometric measurements, but still enabled the determination of stage from visual characteristics such as the number of appendages and the shape and size of the body, as well as the positioning, size and number of spines on the posterior end of the body.

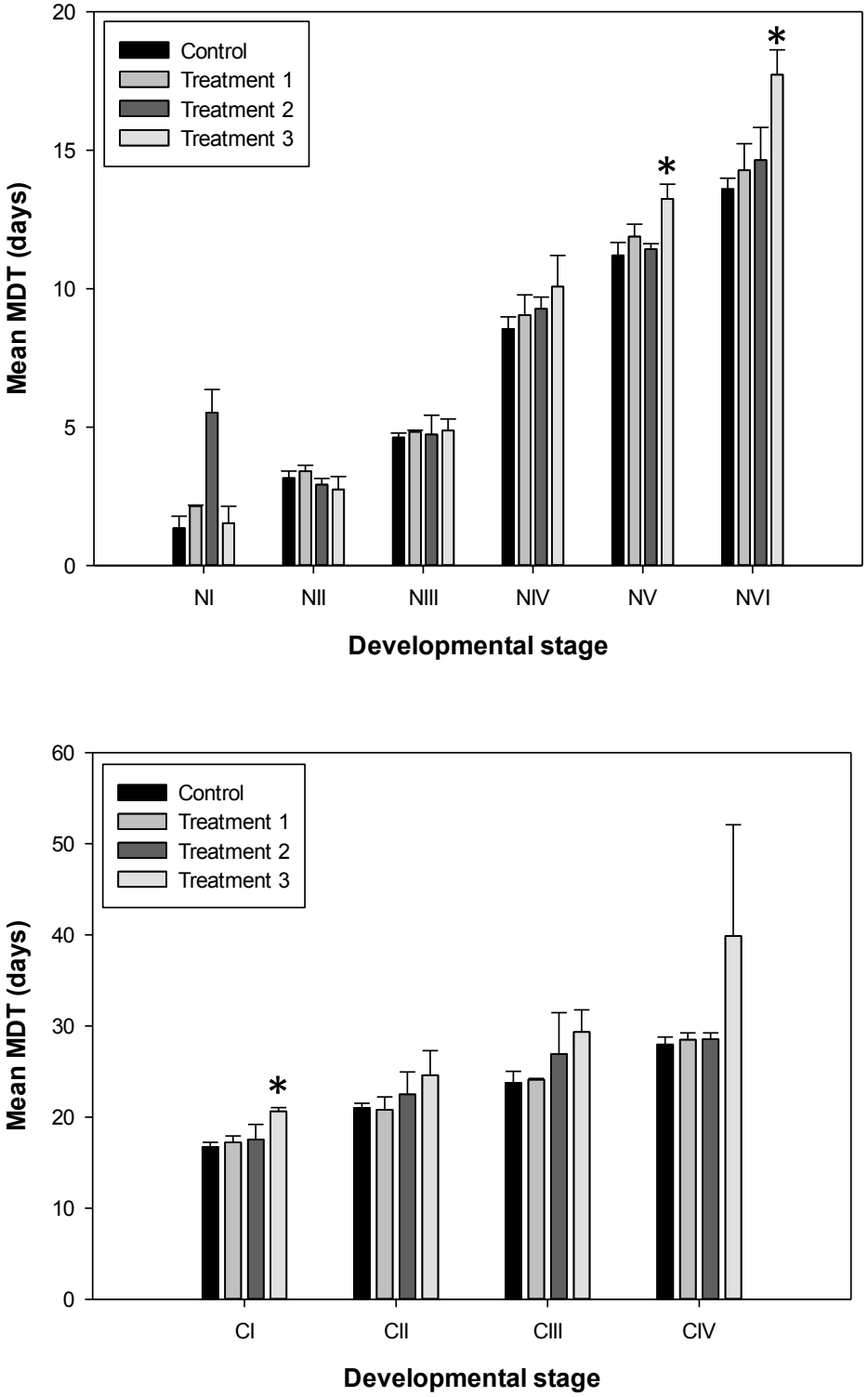

Figure 3.6: The mean values for Median Development Time (MDT) for each of the (**top**) six naupliar stages and (**bottom**) four youngest copepodite stages of *Calanus finmarchicus*, plotted for within-stage comparison between the four exposure groups. Values are mean ± SD (n = 3 replicate tanks). The asterisks mark the instances in which a *post hoc* Dunnett's test identified a significant difference between a treatment and the control.

4 DISCUSSION

4.1 Evaluation of exposure conditions

The variations observed in temperature, algal density and within-treatment pH were not part of the experimental design, and their various causes and possible effects on the ontogenetic development rates of *C. finmarchicus* are therefore discussed in the light of findings reported in previous studies.

4.1.1 Variation in CO_2 concentrations

The CO_2 concentrations employed in the four exposure regimes were relatively stable within each of the two generations. From the first to the second generation, the CO_2 levels decreased in the Control, Treatment 1 and Treatment 2 groups, while an increase was observed for Treatment 3. In addition, it is worth noting that during the egg-laying which initiated the second generation, the pH in the egg-laying tanks became quite low (see scatter plot in Figure 3.1). This was probably due to respiration and the excretion of wastes (Mauchline, 1998), in an environment which lacked water exchange. Thus the eggs and early nauplii were subjected to short-term exposure (1.5 days on average) to pH levels which differed markedly from those experienced by later stages.

Early developmental stages have been found to be more sensitive to CO_2 exposure than adult individuals, both for other marine copepods (Kurihara et al., 2004; Melzner et al., 2009) and for a number of other marine invertebrates (Byrne, 2011). The initial lowering of the pH may therefore have affected the eggs, as well as stage NI and NII, which were present at the end of the egg-laying period. However, as the decrease in pH during these first days was similar in all tanks ($\sim$ 0.1 pH units), a comparison between treatment groups is still considered to be justified.

4.1.2 Temperature gradients

The experimental design did not include temperature as a variable, and steps were taken to minimise and block the temperature gradients discovered, but the data from the daily

measurements nevertheless revealed systematic differences in temperature within and between treatment groups, as well as between generations. According to Campbell et al. (2001), the mean temperature increase of 0.05-0.1°C from one generation to the next should not be sufficient to produce significant effects on growth, as the temperature interval falls within that for which they found normal growth. However, it may still be biologically significant, especially in combination with elevated CO_2 levels (e.g. Byrne, 2011; Whiteley, 2011; Vehmaa et al., 2012). In addition, the differences in temperature within and between treatment groups may have been biologically significant on the scale of the entire life cycle. In his review of temperature effects on copepod development rates, Mauchline (1998) evaluates the Bělehrádek equations developed for *Calanus finmarchicus* in various studies, and shows that while such a small difference in temperature may not significantly influence the development rate of individual stages, the additive, stage-wise increase or decrease in development rate may still significantly affect the overall duration of the life cycle and timing of ontogenetic events.

4.1.3 Variation in feed availability

The simultaneous, and very rapid, decrease in algal concentrations observed in all tanks during days 2 to 5 (see Figure 3.3) coincides with the appearance of the first-feeding stage, NIII, followed by subsequent stages, as indicated in the MDT plot in Figure 3.5. Following this initial decrease, the algal concentrations remained low in the Control and Treatment 1 tanks, even falling below 100 µg C/L for some of the tanks, for the duration of the copepodite stages. The drop in algal concentration took place despite repeated increases in the input of algae. In comparison, the *C. finmarchicus* in the two highest exposure groups experienced algal concentrations closer to the target concentration of 200 µg C/L.

The trend observed with respect to inter-treatment differences in algal concentrations is particularly clear between day 22 and day 35, during which time the copepodite stages CII to CIV, and later stages (for which data was not sufficient), were shown to dominate the cohorts in these two exposure groups (see Figure 3.6). Here, a statistically significant difference in algal concentrations existed between the Control group and both Treatment 2

and Treatment 3, and the *C. finmarchicus* in the two highest exposure groups thus experienced significantly higher feed availability.

The non-significant decrease in copepod density with increasing CO_2 level mirrors the observed increase in algal concentration. Thus, the higher feed availability in the high exposure groups may have been caused by lower population density alone, possibly because of reduced hatching success or survival. Visual inspection of animal densities in the tanks during the experiment supports this view. As the method for sampling *C. finmarchicus* proved problematic with regard to providing representative samples for determination of density, this correlation could not be investigated directly in the present study. In a separate experiment, however, Tagliati (2012) found significantly reduced hatching success in eggs produced by individuals from the second generation, which provides additional support for this assumption.

An increase in food density generally makes feeding less energetically costly (Mauchline, 1998; Kiørboe, 2008). Mayor et al. (2006) demonstrated that the feeding rates of *C. finmarchicus* correlate with feed availability, and in their short- term exposure study of the effects of ocean acidification on the calanoid copepod *Centrophages tenuiremis* (Thompson and Scott, 1903; *nomen dubium*), Li and Gao (2012) found an increase in feeding rate at exposure levels of $\sim$ 1000 ppm CO_2. In addition, the latter found that this increase was correlated with an increasing rate of aerobic metabolism, and thus suggested that the increased feeding activity might be a means of compensating for the energetic cost of maintaining acid-base balance in a high CO_2 environment. In the present study, such compensatory feeding, if present, may thus have tempered the adverse effects of elevated CO_2 levels on the development rates of *C. finmarchicus* in the high exposure groups.

However, the results of Håkedal (2013) from the same study appear to indicate the absence of such compensation, as he observed a non-significant reduction in filtering and ingestion rates for the Treatment 2 and Treatment 3 exposure groups when compared to the Control group. As responses may differ between short-term exposure settings and chronic exposure in a multigenerational context (Byrne, 2011; Whiteley, 2011), this aspect may warrant further exploration. If high CO_2 exposure lowers the appetite, or feed acquisition ability, of

C. finmarchicus, then compensatory feeding may not be an option for supplying the extra energy needed for maintaining homeostasis in the context of ocean acidification.

4.2 Evaluation of experimental methods

4.2.1 Feeding regime issues

Much of the within-group variation observed in the algal density measurements may be due to technical issues such as clogging of feed distribution tubes, clogging of the sieve mesh in the flow-through tanks (see Figure 2.3). Copepods do not feed at a constant rate (Kiørboe and Sabatini, 1994; Mauchline, 1998), and adjusting the algal distribution rates in response to fluctuating feeding rates proved a challenge. The reduction in the overall difference between algal concentrations in the low and high exposure groups from the first to the second generation may have been caused by closer monitoring of the feeding system. The monitoring data indicate that food limitation may have occurred for short time intervals in some of the control tanks, but in general the carbon concentrations fell within the interval required for normal growth and development in *C. finmarchicus* (Hygum et al., 2000; Campbell et al., 2001). For the naupliar stages of *C. finmarchicus*, Hygum et al. (2000) found that nearly maximal developmental rates could be attained at feed levels as low as 50 µg C/L, while Campbell et al. (2001) report near maximum (90%) development rates at about 70 µg C/L for both nauplii and copepodites. Thus, outright food limitation does not appear to have been an issue in the present experiment, though the gradients observed for feed availability may nonetheless have resulted in differential opportunity for compensatory feeding.

4.2.2 Sampling and sampling method

The sampling of animals was carried out without replacement, and due to the low initial number of eggs used to start the generation, the removal of animals will have represented a significant form of mortality in the populations in the tanks (Wild and Seber, 2000). This effect is visible in the average densities registered at different sampling times. Arguably, all tanks were subject to approximately the same 'predation' due to sampling, but if population

densities were in fact lower in the high CO_2 treatment groups, then each individual removed through sampling will have represented a larger loss in the sparsely populated tanks. Thus, the sampling itself may have represented a source of bias which may have contributed to obscuring potential differences between treatments.

The sampling method, specifically the use of the beaker and the stirring routine, was not tested prior to the start of the experiment, and its ability to retrieve representative samples must be questioned. In addition, sampling was carried out by more than one person, and despite efforts to standardise the routine, subtle interpersonal differences may have affected the sampling. The density results clearly indicate that samples were not representative regarding this parameter, while the findings for the stage frequency analysis show that the method might have been better suited for giving a representative view of stage distribution. A more optimal approach might have been to sample the naupliar stages using a plankton net with a known opening area, pulled vertically through the water column in the tank, and subsequently calculate the volume of water passed through the net (Mauchline, 1998; ICES, 2000). For the copepodites, which have a more pronounced escape reaction, a 'core sampling' water sampler with a near-instantaneous closing mechanism would have been a better choice. This would have reduced the probability of escape, and thus lowered the risk of underestimating the density and stage dominance of these stages (Mauchline, 1998; ICES, 2000).

In addition, the use of filters with increasing mesh size as the cohort progressed, to prevent clogging and make the procedure less time consuming, may have resulted in the loss of data on the variation in development rates within a cohort, as both the particularly late hatching individuals and the early offspring of individuals with particularly high development rates may have been discarded through filters with large mesh sizes. Such intrinsic spread and variability within a cohort is known to become exacerbated as stages progress (Mauchline, 1998), and while this complicates data analysis, it is nonetheless information which might prove relevant in the context of how a species responds to environmental stressors (Mauchline, 1998; Kimmerer and Gould, 2010).

The sigmoidal curves of cumulative proportions for each developmental stage revealed a lower relative number of sampling points in the copepodite stages, especially in the later

ones, than in the early naupliar stages. Thus, despite the attempt at adapting the sampling frequency to the expected duration of each stage, some stages appear to have been over-sampled, while others have been under-sampled. In addition, the relatively late start of the sampling resulted in a lack of information about the development curves for hatching, as well as for the two earliest naupliar stages, NI and NII. Similarly, because sampling was terminated before the adult stage, and was infrequent for the late copepodite stages, it was not possible to obtain good estimates of MDT for these stages, or indeed an estimate of the generation time. A higher number of eggs used to initiate each generation, as well as more through monitoring of the stage distribution during the experiment might have resolved these issues.

4.2.3 Stage determination and choice of fixating agent

The two earliest naupliar stages, NI and NII, proved to be difficult to distinguish from one another, especially in instances where the posterior spines had been broken during handling and filtration. There were also difficulties in ascertaining the stages of other nauplii, particularly in cases where the individuals were close to a moult, and size could not always be used as a supporting criterion for stage determination. Literature describing the early developmental stages of *Calanus finmarchicus*, such as Marshall and Orr (1972) and Mauchline (1998), tends to focus on characteristics which may be distinguished with relative ease when studying live individuals immobilised by tranquilising agents such as Finquel® MS-222 (Argent Chemical Laboratories, Inc., Redmond, WA). However, these may be less useful as guidelines when a fixating agent has caused the individuals to shrink, bend or become distorted in other ways.

The choice of fixating agent for the stage distribution samples was influenced by several factors. Traditionally, the use of buffered formaldehyde-based fixatives has been the default method of choice for the preservation of zooplankton samples (e.g. Steedman, 1976; Gifford and Caron, 2000), and much is known about their effects on various planktonic organisms. However, during the last decades, emerging concerns about the health risks involved in the subsequent storage and processing of such samples (e.g. Black and Dodson, 2003; National Toxicology Program, 2011) has led to an increased awareness of the need to develop

methods for fixatives which comply with HSE standards. The use of Fytofix simplified the procedural steps necessary for safe storage and handling of samples, especially during counting and stage determination, thus reducing the potential for damage or loss of sampled individuals. In addition, the experiment by Våge (2011), which preceded this one, also used Fytofix on *C. finmarchicus*. Jaspers and Carstensen (2009), who investigated the Lugol-induced shrinkage of various zooplankton, found 17 % shrinkage for copepods, and that shrinkage remained stable between 36 hours and 3 months after fixation.

In the present study, an effort was made to photograph the various developmental stages in order to generate data on biometric characteristics such as length, circumference and volume. However, the degree of distortion was such that the specimens could not be properly positioned. In their study of the early stages of the harpacticoid copepod *Tisbe battagliai,* Fitzer et al. (2012) used the buffered formaldehyde-based McDowell and Trump's fixative, and their specimens did not exhibit the lengthwise distortion which complicated the present study. In an effort to further the use of non-carcinogenic fixatives, Black and Dodson (2003) suggest a method for fixation of *Daphnia* using ethanol. Their method was developed for freshwater zooplankton samples, and its hypothetical adaptation to marine species such as *C. finmarchicus* would involve the adjustment of the osmolality of the fixative to avoid alterations in the volume of fixated specimens (Altin, 2013, personal communication). Also, while the reduction of shrinkage would be advantageous for biometry, the use of ethanol would turn fixated specimens opaque (Goodsir et al., 2008), which might impede the evaluation of other anatomical characteristics. The findings regarding the stage determination of nauplii and copepodites preserved in Fytofix suggest that while this fixating agent has its merits concerning HSE and methodological simplicity, its use on *C. finmarchicus* is not suitable for samples intended for biometric measurements, while the distortion of fixated specimens did not significantly hamper the stage determination.

4.3 Density estimates

The eggs of *Calanus finmarchicus* sink (Mauchline, 1998), and could thus be expected to come to rest against the bottom of the tanks shortly after being placed into the water. Therefore, the density displayed by the sampling was expected to show an initial increase, due to hatching which would consequently introduce nauplii into the water column. Following this increase, the density was then expected to decrease in all the tanks, regardless of CO_2 level, as the number of eggs at the start of the second generation was relatively low and sampling took place repeatedly and without replacement. This can be viewed as a form of 'predation' imposed by the sampling itself (Wild and Seber, 2000). In addition, if the selected CO_2 levels had adverse effects on the hatching success or survival of *C. finmarchicus*, the density at any time of sampling would be expected to decrease with increasing CO_2 concentration. The initial increase followed by gradual decrease in density displayed high variability but was nonetheless discernible in the mean density data (see Figure 3.4), while the trend inter-treatment differences was more uncertain. The non-significant tendency discovered for the time interval between day 23 and day 29 may indicate lower densities in the high-exposure groups, compared to the control, but the trend was not consistent for other time intervals.

For animals retrieved from the second generation in the present experiment, Tagliati (2012) found a significant reduction in hatching success only in the highest exposure group, while a non-significant trend indicated a general decrease in this parameter with increasing CO_2 level. This, combined with visual evaluations of density in the various tanks during the experiment, may indicate that the density was in fact lower in the high CO_2 treatment groups. The fact that this tendency was not clearly revealed in the density data suggests that the sampling method did not result in representative samples with regard to density.

For the time interval between day 22 and 35, the feed availability was significantly higher in the Treatment 2 and Treatment 3 exposure groups, when compared to the Control. This provides further support for the non-significant trend observed for density. The assumption of equal input and exchange rates for the algae in all tanks leads to two hypotheses: If the density was in fact lower in the high-exposure tanks, then that explains the smaller decrease in algal concentration compared to the Control. Alternatively, if the density was not

significantly lower in the high-exposure tanks, then the *per capita* feeding rate must have been lower. As Håkedal (2013) found a non-significant but systematic decrease in ingestion rate in the Treatment 2 and Treatment 3 groups compared to the Control, while Tagliati (2012) found lower hatching success in the Treatment 3 group, the explanation may appear to be a combination of both hypotheses. However, no clear trend was discernible from the density data themselves, and this issue remains unclear.

4.4 MDT – Delayed development

A significant difference in median development time was detected only between the Control and Treatment 3 groups. As these two groups were very similar in terms of temperature (see Figure 3.2), and none of them were permanently food limited (Campbell et al., 2001), the observed difference in MDT was probably caused by the differing CO_2 regimes, and consequent difference in hypercapnic stress. Compared to the control, the average developmental delay in the Treatment 3 tanks was approximately 2 days for stage NV, 4 days for NVI and 3.9 days for CI. The reduction in ontogenetic development rates may reflect a depletion in energy, possibly due to increased acid-base compensation placing additional demands on the energy budgets, thus delaying the energy demanding process of moulting (Rey-Rassat et al., 2002). The visibly additive nature of the delay for each new stage fits the description given in Mauchline (1998) for other stressors. The slightly longer delay for stage NVI likely reflects the additional time required for attaining the energy stores required for completing the next moult into the anatomically very different CI stage (Mauchline, 1998). Similarly, Håkedal (2013) found significant developmental delays in stages NV, NVI and CIV for the high CO_2 treatments compared to the Control in the first generation of the present study.

In general, the limited number of eggs obtained at the beginning of each generation, as well as problems with the procedure for animal sampling, caused much of the uncertainty inherent within the resulting stage determination data. During the experiment, the sample size (n) became a trade-off between the need to sample enough individuals to permit statistical analysis and the need to prevent depletion of the populations in the tanks, and thus allow the initiation of subsequent generations. This multi-generational aspect was not

a concern in the relevant previous studies (Hygum et al., 2000; Campbell et al., 2001; Gentleman et al., 2008; Kimmerer and Gould, 2010). Upon visual inspection, the population density appeared lower in some tanks, and as all tanks were to be treated the same regarding sample volume, the lowest densities formed the basis of such decisions. The target minimum sample size was thus tentatively set at 10 individuals.

The stage determination data revealed the simultaneous presence of two to three stages throughout the development of the generation (see Appendix D), indicating that in order to provide representative information on the stage distribution in a tank at a given time, the samples would need to contain a minimum number of animals. Current literature on the statistical analysis of stage determination data from copepod cohorts suggests sample sizes (n) from 50 to 100 animals (Campbell et al., 2001; Gentleman et al., 2008), n = 40 (Hygum et al., 2000) and $n \geq 30$ (Kimmerer and Gould, 2010). Thus, the lower limit of inclusion for sample sizes (n_{min}) for stage distribution data should fall within the interval of 30 to 50 animals. However, when applied to the data from the present study, including outliers, even an $n_{min} \geq 30$ excluded 99.5% of the samples. Even the initial target sample size of 10 individuals was found to exclude too many sampling points, and thus all samples containing four or more animals were included.

The densities and sample sizes obtained in the present study did not fulfil the assumptions of the method of analysis involved in MDT determination (Hu et al., 2007). As such, the obtained estimates of development time should not be viewed as representative for the species, but rather as relative values suitable for detection of differences between the exposure groups. In addition to this uncertainty, the late onset of sampling meant that the two earliest naupliar stages, and especially NI, had progressed quite far before their presence was documented. This led to poor estimates of MDT for these early stages, and also impeded further calculations of other measures of development rate (Hygum et al., 2000; Campbell et al., 2001), as these are based on estimates for the first stage.

The chosen method for calculating MDT was modified from Campbell et al. (2001), and was carried out on untransformed data (e.g. Hygum et al., 2000). This method is subjective, does not make use of all the information available in the data set, and provides no estimates of error (Kimmerer and Gould, 2010). Other approaches to modelling copepod development

from stage frequency data, such as Bayesian models and stage-based models using differential equations (e.g. Gentleman et al., 2008; Kimmerer and Gould, 2010) were found to be beyond the scope of the current investigation based on the inherently high uncertainty in the data obtained. However, these methods should be considered before including the findings from the present study in any larger ecological models.

4.5 Viewing the main findings in a natural context

Care should always be taken when extending the conclusions from laboratory experiments to models of natural populations. The natural variability inherent in the environment experienced by a population of copepods is not readily modelled in laboratory mesocosm studies (Mauchline, 1998), and it is therefore necessary to view any such results in the natural context, and discuss the ways in which any differences between the experimental and natural conditions may affect the interpretation of the results.

The physical constraints of the experimental tanks prevented the diel- and ontogenetic vertical migrations, as well as the diapause, which characterise the life cycle of *Calanus finmarchicus*, and the absence of such energy requiring behaviour (Mauchline, 1998) will have affected the energy budget of the individuals used in the experiment. The energy saved through lack of migratory behaviour will have been available for other processes (Irigoien, 2004), such as acid-base compensation (Fabry et al., 2008), and may potentially have masked or tempered the adverse effects of environmental hypercapnia. In addition, natural variability in temperature, salinity and the type and availability of feed were not included as factors in the experimental design, and may prove to affect the way *C. finmarchicus* responds to ocean acidification (e.g. Fabry et al., 2008; Byrne, 2011; Vehmaa et al., 2012).

Another factor present in the natural environment is predation. In their review of studies on the effect of predation on natural populations of copepods, Mauchline (1998) report that mortality due to predation has been found to be a main factor in controlling population size for parts of the life cycle in several copepod species, and that the stage specific effects are generally highest in the early developmental stages. In the present study, the *C. finmarchicus* used will have been spared much of the energetic cost of predator avoidance,

except at sampling time, which may have contributed to more effective compensation for CO_2 exposure.

4.5.1 Comparison of main findings with those of previous studies

In their single generation study of the effects of CO_2 on *Calanus finmarchicus*, Pedersen et al. (2013) report no negative effects on survival and growth for the 3300 ppm treatment. While the data from the present study gave no clear density estimates, the combination of observations made in the tanks and data on hatching success (Tagliati, 2012) seems to indicate a reduction in density in the exposure group closest to 3000 ppm CO_2. Using near-future CO_2 levels, the three-generational study by Fitzer et al. (2012) and the two-stressor study by Vehmaa et al. (2012), the latter employing both increasing temperature and CO_2 concentrations, both showed a clear negative effect in post-hatching densities for much lower levels of ocean acidification. Thus, the results of the present study may give reason for concern about population size.

Regarding ontogenetic development, the observed delays support the expectation of heightened sensitivity to environmental hypercapnia when the entire life cycle is involved, compared to the short-term exposure of adult individuals (e.g. Mayor et al., 2007). The developmental delay is also in accordance with the results of Pedersen et al. (2013), though the present study shows significant differences at lower levels of exposure, which might be expected as the feeding regime used by Pedersen et al. (2013) (600 µg C/L) ensured far higher feed availability, and thus a higher possibility for compensatory feeding, than the present study.

Kurihara and Ishimatsu (2008), on the other hand, found no significant effect of exposure to 2380 ppm CO_2 on the survival, growth or development of *Acartia tsuensis*, so this trend clearly varies between copepod species. However, as *A. tsuensis* exhibits a much shorter generation time, compared to *C. finmarchicus*, the studies are not fully comparable, as time-dependent effects are more likely to manifest within a few generations in species with longer generation times (Mauchline, 1998).

In a multigenerational context, the observed developmental rates may also be used to make tentative inferences about the potential for adaptation to elevated CO_2 concentrations (e.g. Dupont and Thorndyke, 2009). In a two-generation experiment exposing the copepod *Acartia tonsa* to CO_2 levels similar to those used in the present study, Dupont and Thorndyke (2008) found that while the first generation exhibited developmental delay, such delays were absent in the subsequent generation. In the present experiment, a comparison of the findings from the two separate generations revealed no obvious differences in median development time from the first to the second generation (see Håkedal, 2013). Thus, no signs of adaptation were observed for *Calanus finmarchicus* after two generations.

4.5.2 Ecological implications and future prospects

The developmental delays observed for *Calanus finmarchicus* in the present study translates into more time spent in the more vulnerable, early stages of the life cycle, and in their natural environment this involves a higher risk of predation (Lopez, 1996). In addition, the delays, if applicable to natural populations, may have several implications for the life cycle, and, by extension, the role of *C. finmarchicus* in marine ecosystems. The added energetic cost of increased acid-base compensation (Fabry et al., 2008) may cause delayed development through the mechanism of prolonged periods needed to achieve the lipid stores required to for moulting, as hypothesised by Irigoien (2004). If this is the case, then a larger proportion of a given population of *C. finmarchicus* may miss important, lipid-related windows of opportunity to which their life cycle is finely tuned. Failure to attain a threshold level of lipid stores before descending to diapause depth, for example, has been suggested as an explanation for the presence of *C. finmarchicus* in surface waters during winter (Irigoien, 2004). In addition, lipid stores appear to have a function in post-diapause egg production. Hirche et al. (2001) conclude that lipid stores allow egg production to supersede the level expected based purely on the pre-bloom feed concentrations available to the adult female. Thus, in order to time the appearance of the first-feeding stage (NIII) with the peak of the spring phytoplankton bloom, females with sufficient energy stores are able to produce eggs prior to the bloom. If, however, developmental delays cause an increase in

failure to attain the energy stores necessary, then a larger proportion of the females may have to wait until ambient feed concentrations allow an increase in egg production, and their offspring may not have the opportunity to exploit the peak conditions (Irigoien, 2004). This scenario is in accordance with Cushing's match-mismatch hypothesis (Cushing, 1990), where the success or failure of an individual or group is understood in terms of their ability to time their own development and needs to relevant windows of opportunity in their environment. Developmental delays of 2 – 4 days will probably be significant in this context (Mauchline, 1998). The potential consequences of such an increased degree of mismatch within a population include lower reproductive capacity and educed juvenile survival, and in combination with the possible reduction in hatching success this may cause significant reductions in population size. As *C. finmarchicus* constitutes a key species in the pelagic food web of the North Atlantic (Mauchline, 1998; Vadstein, 2009), and serves as an important feed organism for the juvenile stages of several species of fish (Planque and Batten, 2000), such a response to ocean acidification may cause major changes in the structure and function of marine ecosystems.

Significant delays in development were detected for the Treatment 3 exposure group only, and though a non-significant but similar trend was also observed at lower CO_2 levels, *C. finmarchicus* appears to be relatively resilient to the levels of ocean acidification predicted for the coming 100 to 300 years (Caldeira and Wickett, 2003; 2005). However, the findings also hold relevance when it comes to leakages from sub-seabed CO_2 storage sites, due to their diapause in deep waters (Edvardsen et al., 2006).

4.6 Suggestions for future research

No significant effects, in terms of developmental delay, were found for the earliest naupliar stages in the present study. As there was a general lack of data for these stages, and previous studies have indicated that they may be particularly sensitive to ocean acidification (Kurihara et al., 2004; Melzner et al., 2009; Våge, 2011), further exploration of their responses is necessary. The case of NIII warrants particular interest, as this is the stage where exogenous feeding starts, and though feeding stages may be better acid-base regulators, due to their higher metabolic rates (Fabry et al., 2008), the handling of feed

particles and the need to compensate for weight loss in earlier stages have been pointed out as potential challenges facing this stage (Mauchline, 1998).

As the present study focused on the effects of CO_2 as a single stressor, further investigations are also required before conclusions can be drawn about the future prospects of natural populations of *Calanus finmarchicus*. The adverse effects of hypercapnia in combination with other stressors, such as an increase temperature, have been shown to supersede those caused by hypercapnia as a single stressor (Vehmaa et al., 2012). Several recent reviews of current knowledge on the consequences of ocean acidification have pointed out the need for multigenerational, multistressor studies (e.g. Fabry et al., 2008; Byrne, 2011; Whiteley, 2011). In addition, there is currently an increasing focus on the importance of incorporating natural variability in parameters such as temperature and the concentrations of CO_2 and O_2 (Gattuso and Hansson, 2011), in order to mimic the variation experienced by the organisms in their natural environment. Thus, the results from the present study might be used as a basis for more realistic scenarios, incorporating multiple stressors and less static conditions.

4.7 Conclusion

The present study confirms that the developmental rate of the key species *Calanus finmarchicus* is affected by levels of CO_2 relevant in realistic future ocean acidification scenarios. Significant developmental delays were only detected in the > 3000 ppm CO_2 treatment group, which is more relevant to localised deep water seeping from sub-seabed storage of CO_2, but a similar, if non-significant trend for the year 2300 projection was also seen. While *C. finmarchicus* may be among the more resilient copepod species in the context of near-future ocean acidification, even developmental delays in the order of a few days may cause significant changes in the dynamics of natural populations, whose life-cycles are finely tuned to environmental windows of opportunity. Such changes may in turn cause major structural and functional changes in the ecosystems in which *C. finmarchicus* plays a key role.

5 REFERENCES

AKSNES, D. L. and BLINDHEIM, J. (1996) Circulation patterns in the North Atlantic and possible impact on population dynamics of *Calanus finmarchicus*. *Ophelia,* **44,** 7-28.

BAUMGARTNER, M. F. (2009) *Calanus finmarchicus life cycle*. Woods Hole Oceanographic Institution. Accessed February 8th 2012. URL: http://www.gulfofmaine.org/gomt/?p=32.

BAUMGARTNER, M. F., LYSIAK, N. S. J., SCHUMAN, C., URBAN-RICH, J. and WENZEL, F. W. (2011) Diel vertical migration behavior of *Calanus finmarchicus* and its influence on right and sei whale occurrence. *Marine Ecology Progress Series,* **423,** 167-184.

BECHMANN, R. K., TABAN, I. C., WESTERLUND, S., FJONE GODAL, B., ARNBERG, M., VINGEN, S., INGVARSDOTTIR, A. and BAUSSANT, T. (2011) Effects of ocean acidification on early life stages of shrimp (*Pandalus borealis*) and mussel (*Mytilus edulis*). *Journal of Toxicology and Environmental Health, Part A,* **74,** 424-438.

BERG, J. M., TYMOCZKO, J. L. and STRYER, L. (2002) *Biochemistry*. 5th ed. W. H. Freeman and Company, New York, pp. 1-974.

BERGVIK, M., LEIKNES, Ø., ALTIN, D., DAHL, K. R. and OLSEN, Y. (2012a) Dynamics of the lipid content and biomass of *Calanus finmarchicus* (copepodite V) in a Norwegian fjord. *Lipids,* **47,** 881-895.

BERGVIK, M., OVERREIN, I., BANTLE, M., EVJEMO, J. O. and RUSTAD, T. (2012b) Properties of *Calanus finmarchicus* biomass during frozen storage after heat inactivation of autolytic enzymes. *Food Chemistry,* **132,** 209-215.

BIBBY, R., CLEALL-HARDING, P., RUNDLE, S., WIDDICOMBE, S. and SPICER, J. (2007) Ocean acidification disrupts induced defences in the intertidal gastropod *Littorina littorea*. *Biology Letters,* **3,** 699-701.

BONNET, D., TITELMAN, J. and HARRIS, R. (2004) *Calanus* the cannibal. *Journal of Plankton Research,* **26,** 937-948.

BOXSHALL, G. A. (1982) On the anatomy of the misophirioid copepods, with special reference to *Benthomisophria palliata* Sars. *Philosophical Transactions of the Royal Society,* **297,** 125-181.

BUSA, W. B. and NUCCITELLI, R. (1984) Metabolic regulation via intracellular pH. *American Journal of Physiology,* **246,** 409-438.

BYRNE, M. (2011) Impact of ocean warming and ocean acidification on marine invertebrate life history stages: Vulnerabilities and potential for persistence in a changing ocean. *Oceanography and Marine Biology: An Annual Review,* **49,** 1-42.

BÅMSTEDT, U., NEJSTGAARD, J. C. and SOLBERG, P. T. (1999) Utilisation of small-sized food algae by *Calanus finmarchicus* (Copepoda, Calanoida) and the significance of feeding history. *Sarsia,* **84,** 19-38.

CALDEIRA, K. and WICKETT, M. E. (2003) Anthropogenic carbon and ocean pH. *Nature,* **425,** 365.

CALDEIRA, K. and WICKETT, M. E. (2005) Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. *Journal of Geophysical Research,* **110** 1-12.

CAMPBELL, R. G., WAGNER, M. M., TEEGARDEN, G. J., BOUDREAU, C. A. and DURBIN, E. G. (2001) Growth and development rates of the copepod *Calanus finmarchicus* reared in the laboratory. *Marine Ecology Progress Series,* **221,** 161-183.

CASTELLANI, C., IRIGOIEN, X., MAYOR, D. J. and HARRIS, R. P. (2008) Feeding of *Calanus finmarchicus* and *Oithona similis* on the microplankton assemblage in the Irminger Sea, North Atlantic. *Journal of Plankton Research,* **30,** 1095-1116.

CASTELLINI, M. A. and SOMERO, G. N. (1981) Buffering capacity of vertebrate muscle: correlations with potentials for anaerobic function. *Journal of Comparative Physiology,* **143,** 191-198.

CHANG, R. (2003) *General Chemistry: The Essential Concepts*, 3rd ed., London, pp. 1-764.

CONOVER, R. J. (1988) Comparative life histories in the genera Calanus and Neocalanus in high latitudes of the northern hemisphere. *Hydrobiologia,* **167-168,** 127-142.

DALE, T. and KAARTVEDT, S. (2000) Diel patterns in stage-specific vertical migration of *Calanus finmarchicus* in habitats with midnight sun. *ICES Journal of Marine Science,* **57,** 1800-1818.

DICKSON, A. G., SABINE, C. L. and CHRISTIAN, J. R. E. (eds) (2007a) *Guide to Best Practices for Ocean CO_2 Measurements* Vol. 1. PICES Special Publication 3, 191 pp.

DICKSON, A. G., SABINE, C. L. and CHRISTIAN, J. R. E. (2007b) SOP 7 Determination of the pH of sea water using the indicator dye *m*-cresol purple. *In: Guide to best practices for ocean CO_2 measurements.* PICES Special Publication 3, 191 pp.

DLUGOKENCKY, E. and TANS, P. (2013) Globally averaged marine surface annual mean data. NOAA/ESRL Dataset, Accessed April 11th, 2012. URL: ftp://ftp.cmdl.noaa.gov/ccg/co2/trends/co2_annmean_gl.txt.

DONEY, S. C., FABRY, V. J., FEELY, R. A. and KLEYPAS, J. A. (2009) Ocean acidification: the other CO_2 problem. *Annual Review of Marine Science,* **1,** 169-192.

DUNNETT, C. W. A. (1955) A multiple comparison procedure for comparing several treatments with a control. *Journal of the American Statistical Association,* **50,** 1096-1121.

EDVARDSEN, A., PEDERSEN, J. M., SLAGSTAD, D., SEMENOVA, T. and TIMONIN, A. (2006) Distribution of overwintering *Calanus* in the North Norwegian Sea. *Ocean Science,* **2,** 87-96.

EIANE, K., AKSNES, D. L., OHMAN, M. D., WOOD, S. and MARTINUSSEN, M. B. (2002) Stage-specific mortality of *Calanus* spp. under different predation regimes. *Limnology and Oceanography,* **47,** 636-645.

FABRY, V. J., SEIBEL, B. A., FEELY, R. A. and ORR, J. C. (2008) Impacts of ocean acidification on marine fauna and ecosystem processes. *ICES Journal of Marine Science,* **65,** 414-432.

FAO (2010) *The State of the World Fisheries and Aquaculture 2010*. Food and Agriculture Organisation of the United Nations, Rome, pp. 1-197.

FAO (2012) *The State of the World Fisheries and Aquaculture 2012*. Food and Agriculture Organisation of the United Nations, Rome, pp. 1-209.

FEELY, R. A., SABINE, C. L., LEE, K., BERELSON, W., KLEYPAS, J. A., FABRY, V. J. and MILLERO, F. J. (2004) Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. *Science,* **281,** 237-240.

FITZER, S. C., CALDWELL, G. S., CLOSE, A. J., CLARE, A. S., UPSTILL-GODDARD, R. C. and BENTLEY, M. G. (2012) Ocean acidification induces multi-generational decline in copepod naupliar production with possible conflict for reproductive resource allocation. *Journal of Experimental Marine Biology and Ecology,* **418-419,** 30-36.

FJÆRAN, T. (2012) Mapping of geological CO_2 storage sites: an overview. Statoil, CCOP EPPM Seminar, September 12-14, 2012, Bali.

FROMENTIN, J.-M. and PLANQUE, B. (1996) *Calanus* and environment in the eastern North Atlantic. II. Influence of the North Atlantic Oscillation on *C. finmarchicus* and *C. helgolandicus*. *Marine Ecology Progress Series,* **134,** 111-118.

GATTUSO, J.-P. and HANSSON, L. (2011) *Ocean Acidification*. Oxford University Press, New York, pp. 1-326.

GIBBS, A. H. and SOMERO, G. N. (1990) Na^+-K^+ adenosine triphosphatase activities in gills of marine teleost fishes, changes with depth, size and locomotory activity level. *Marine Biology,* **106,** 315-321.

GINES, J. K. (2012) The role of international organizations. In: J. K. Gines (ed) *Climate Management Issues: Economics, Sociology, and Politics.* CRC Press, Boca Raton, Florida, pp. 213-231.

GRAHAM, M. D. (2003) The coulter principle: foundation of an industry. *Journal of the Association for Laboratory Automation,* **8,** 72-81.

GRICE, G. D., WIEBE, P. H. and HOAGLAND, E. (1973) Acid-iron waste as a factor affecting the distribution and abundance of zooplankton in the New York Bight: I. Laboratory studies on the effects of acid waste on copepods. *Estuarine and Coastal Marine Science,* **1,** 45-50.

GUTKNECHT, J., BISSON, M. A. and TOSTESON, F. C. (1977) Diffusion of carbon dioxide through lipid bilayer membranes: effects of carbonic anhydrase, bicarbonate and unstirred layers. *The Journal of General Physiology,* **69,** 779-794.

HAND, S. C. (1991) Metabolic dormancy in aquatic invertebrates. In: R. Gilles (ed) *Advances in Comparative and Environmental Physiology.* Vol. 8. Springer-Verlag, Heidelberg, pp. 1-50.

HANSEN, B. H., ALTIN, D., NORDTUG, T. and OLSEN, A. J. (2007) Suppression subtractive hybridization library prepared from the copepod *Calanus finmarchicus* exposed to a sublethal mixture of environmental stressors. *Comparative Biochemistry and Physiology,* **Part D 2,** 250-256.

HANSSON, I. (1973) A new set of pH-scales and standard buffers for sea water. *Deep-Sea Research,* **20,** 479-491.

HAUGAN, P. M., TURLEY, C. and PÖRTNER, H. O. (2006) *Effects on the Marine Environment of Ocean Acidification Resulting from Elevated Levels of CO_2 in the Atmosphere*. Norwegian Directorate for Nature Management, Oslo, pp. 1-32.

HEISLER, N. (1989) Interactions between gas exchange, metabolism and ion transport in animals: an overview. *Canadian Journal of Zoology,* **67,** 2923-2935.

HENDERSON, C. (2006) Ocean acidification: the other CO_2 problem. *New Scientist. Accessed May 3rd, 2013. URL: http://environment.newscientist.com/article/mg19125631.200.*

HEY, C. (2005) EU environmental policies: A short history of the policy strategies. In: S. Scheuer (ed) *EU Environmental Policy Handbook: A Critical Analysis of EU Environmental Legislation: Making it Accessible to Environmentalists and Policy Makers.* European Environmental Bureau, Brussels, pp. 18-30.

HOEGH-GULDBERG, O., MUMBY, P. J., HOOTEN, A. J., STENECK, R. S., GREENFIELD, P., GOMEZ, E., HARVELL, C. D., SALE, P. F., EDWARDS, A. J., CALDEIRA, K., KNOWLTON, N., EAKIN, C. M., IGLESIAS-PRIETO, R., MUTHIGA, N., BRADBURY, R. H., DUBI, A. and HATZIOLOS, M. E. (2007) Coral reefs under rapid climate change and ocean acidification. *Science,* **318,** 1737-1742.

HOLLOWAY, S. (2001) Storage of fossil fuel-derived carbon dioxide beneath the surface of the earth. *Annual Review of Energy and the Environment,* **26,** 145-166.

HOLLOWAY, S. (2005) Underground sequestration of carbon dioxide: a viable greenhouse gas mitigation option. *Energy,* **30,** 2318-2333.

HU, Q., PETRIK, C. M. and DAVIS, C. S. (2007) Normal versus gamma: stochastic models of copepod molting rate. *Journal of Plankton Research,* **29,** 985-997.

HYGUM, B. H., REY, C. and HANSEN, B. W. (2000a) Growth and development rates of *Calanus finmarchicus* nauplii during a diatom spring bloom. *Marine Biology,* **136,** 1075-1085.

HYGUM, B. H., REY, C., HANSEN, B. W. and TANDE, K. (2000b) Importance of food quantity to structural growth rate and neutral lipid reserves accumulated in *Calanus finmarchicus*. *Marine Biology,* **136,** 1057-1073.

IMO (2007) International Maritime Organization: New international rules to allow storage of CO_2 under the seabed. Accessed April 6th, 2013. URL: http://www.imo.org/blast/mainframe.asp?topic_id=1472&doc_id7772.

IPCC (2001) Climate Change 2001: The Scientific Basis. In: J. T. Houghton, Y. Ding, D. J. Griggs, M. Noguer, P. J. Van Der Linden, X. Dai, K. Maskell and C. A. Johnson (eds) *Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change.* The Intergovernmental Panel on Climate Change, Cambridge, pp. 1-896.

IPCC (2005) Summary for policy makers and technical summary. In: B. Metz, O. Davidson, H. C. De Coninck, M. Loos and L. A. Mayer (eds) *IPCC Special Report on Carbon Dioxide Capture and Storage.* Intergovernmental Panel on Climate Change, New York, pp. 1-49.

IPCC (2007) Climate change 2007: The physical science basis: Contribution of working group I to the fourth assessment report of the Intergovernmental Panel on Climate Change. In: S. Solomon, D. Quin, M. Manning, Z. Chen, M. Marquis, K. B. Averut, M. Tignor and H. L. Miller (eds) *IPCC*

Fourth Assessment Report: Climate Change 2007. Cambridge University Press, New York, 996 pp.

IRIGOIEN, X. (2004) Some ideas about the role of lipids in the life cycle of *Calanus finmarchicus*. *Journal of plankton research,* **26,** 259-263.

JASPERS, C. and CARSTENSEN, J. (2009) Effect of acid Lugol solutionas preservative on two representative chitinous and gelatinous zooplankton groups. *Limnology and Oceanography: Methods,* **7,** 430-435.

KAARTVEDT, S. (2000) Life history of *Calanus finmarchicus* in the Norwegian Sea in relation to planktivorous fish. *ICES Journal of Marine Science,* **57,** 1819-1824.

KANE, J. (1984) The feeding habits of co-occurring cod and haddock larvae from Georges Bank. *Marine Ecology Progress Series,* **16,** 9-20.

KIKKAWA, T., KITA, J. and ISHIMATSU, A. (2004) Comparison of the lethal effect of CO_2 and acidification on red sea bream (*Pagrus major*) during the early developmental stages. *Marine Pollution Bulletin,* **48,** 108-110.

KIØRBOE, T. (2008) *A Mechanistic Approach to Plankton Ecology*. Princeton University Press, Princeton, New Jersey, pp. 1-209.

KIØRBOE, T. and SABATINI, M. (1994) Reproductive and life cycle strategies in egg-carrying cyclopoid and free-spawning calanoid copepods. *Journal of Plankton Research,* **16,** 1353-1366.

KLEYPAS, J. A., BUDDEMEIER, R. W., ARCHER, D., GATTUSO, J.-P., LANGDON, C. and OPDYKE, B. N. (1999) Geochemical consequences of increased atmospheric carbon dioxide on coral reefs. *Science,* **284,** 118-120.

KURIHARA, H. and ISHIMATSU, A. (2008) Effects of high CO_2 seawater on the copepod (*Acartia tsuensis*) through all life stages and subsequent generations. *Marine Pollution Bulletin,* **56,** 1086-1090.

KURIHARA, H., SHIMODE, S. and SHIRAYAMA, Y. (2004a) Effects of raised CO_2 concentration on the egg production rate and early development of two marine copepods (*Acartia steueri* and *Acartia erythraea*). *Marine Pollution Bulletin,* **49,** 721-727.

KURIHARA, H., SHIMODE, S. and SHIRAYAMA, Y. (2004b) Sub-lethal effects of elevated concentration of CO_2 on planktonic copepods and sea urchins. *Journal of Oceanography,* **60,** 743-750.

KURIHARA, H. and SHIRAYAMA, Y. (2004) Effects of increased atmospheric CO_2 on sea urchin early development. *Marine Ecology Progress Series,* **274,** 161-169.

LALLI, C. M. and PARSONS, T. R. (1997) *Biological Oceanography: An Introduction*. 2nd ed. Elsevier Butterworth-Heinemann, Oxford.

LANDRY, M. R. (1975) The relationship between temperature and the development of life stages of the marine copepod *Acartia clausi* Giesbr. *Limnology and Oceanography,* **20,** 854-857.

LANGENBUCH, M. and PÖRTNER, H. O. (2004) High sensitivity to chronically elevated CO_2 levels in a eurybathic marine sipunculid. *Aquatic Toxicology,* **70,** 55-61.

LAVIGNE, H. and GATTUSO, J.-P. (2012) Seacarb: Seawater carbonate chemistry with R. R Package version 2.4.4, Accessed March 5th, 2013. URL: http://rgm3.lab.nig.ac.jp/RGM/r_function?p=seacarb&f=bjerrum.

LE BORGNE, R. (1986) The release of soluble end products of metabolism. In: E. D. S. Corner and S. C. M. O'hara (eds) *The Biological Chemistry of Marine Copepods.* Oxford University Press, Oxford, pp. 109-164.

LINSER, P. J., SMITH, K. E., SERON, T. J. and OVIEDO, M. N. (2009) Carbonic anhydrases and anion transport in mosquito midgut pH regulation. *The Journal of Experimental Biology,* **212,** 1662-1671.

LUEKER, T. J., DICKSON, A. G. and KEELING, C. D. (2000) Ocean pCO_2 calculated from dissolved inorganic carbon, alkalinity, and equations for K_1 and K_2: validation based on laboratory measurements of CO_2 in gas and seawater at equilibrium. *Marine Chemistry,* **70,** 105-119.

MARCHETTI, C. (1977) On geoengineering and the CO_2 problem. *Climatic Change,* **1,** 59-68.

MARSHALL, S. M., NICHOLLS, A. G. and ORR, A. P. (1935) On the biology of *Calanus finmarchicus*. Part VI. Oxygen consumption in relation to environmental conditions. *Journal of the Marine Biological Association of the United Kingdom,* **20,** 1-27.

MARSHALL, S. M. and ORR, A. P. (1955) *The Biology of a Marine Copepod*. Oliver and Boyd, Edinburgh, pp. 188.

MARSHALL, S. M. and ORR, A. P. (1972) *The Biology of a Marine Copepod*. Reprint. Springer Verlag, Berlin, pp. 1-195.

MAUCHLINE, J. (1998) *The Biology of Calanoid Copepods*. In: J. H. S. Blaxter, A. J. Southward and P. A. Tyler (eds) *Advances in Marine Biology.* Vol. 33. Academic Press, London, pp. 1-710.

MAYOR, D. J., MATTHEWS, C., COOK, K., ZUUR, A. F. and HAY, S. (2007) CO_2-induced acidification affects hatching success in *Calanus finmarchicus*. *Marine Ecology Progress Series,* **350,** 91-97.

MCLEOD, K. L. and LESLIE, H. M. (2009) *Ecosystem-based Management for the Ocean*. Island Press, Washington, DC, 390 pp.

MELZNER, F., GUTOWSKA, M. A., LANGENBUCH, M., DUPONT, S., LUCASSEN, M., THORNDYKE, M. C., BLEICH, M. and PÖRTNER, H.-O. (2009) Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences,* **6,** 2313-2331.

MICHAELIDIS, B., OUZOUNIS, C., PALERAS, A. and PÖRTNER, H. O. (2005) Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovincialis*. *Marine Ecology Progress Series,* **293,** 109-118.

MØLLER, E. F. (2005) Sloppy feeding in marine copepods: Prey-size-dependent production of dissolved organic carbon. *Journal of Plankton Research,* **27,** 27-35.

NEJSTGAARD, J. C. and SOLBERG, P. T. (1996) Repression of copepod feeding and fecundity by the toxic haptophyte *Prymnesium patelliferum*. *Sarsia,* **81,** 339-344.

NEMZER, B. V. and DICKSON, A. G. (2005) The stability and reproducibility of Tris buffers in synthetic seawater. *Marine Chemistry,* **96,** 237-242.

OHMAN, M. D. and HIRCHE, H. J. (2001) Density-dependent mortality in an oceanic copepod population. *Nature,* **412,** 638-641.

OLSEN, R. E., HENDERSON, R. J., SOUNTAMA, J., HEMRE, G., RINGO, E., MELLE, W. and TOCHER, D. R. (2004) Atlantic salmon, *Salmo salar*, utilizes wax ester-rich oil from *Calanus finmarchicus* effectively. *Aquaculture,* **240,** 433-449.

ORR, J. C., FABRY, V. J., AUMONT, O., BOPP, L., DONEY, S. C., FEELY, R. A., GNANADESIKAN, A., GRUBER, N., ISHIDA, A., JOOS, F., KEY, R. M., LINDSAY, K., MAIER-REIMER, E., MATEAR, R., MONFRAY, P., MOUCHET, A., NAJJAR, R. G., PLATTNER, G.-K., RODGERS, K. B., SABINE, C. L., SARMIENTO, J. L., SCHLITZER, R., SLATER, R. D., TOTTERDELL, I. J., WEIRIG, M.-F., YAMANAKA, Y. and YOOL, A. (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature,* **437,** 681-686.

PEDERSEN, S. A., HANSEN, B. H., ALTIN, D. and OLSEN, A. J. (2013) Chronic exposure of the North Atlantic copepod *Calanus finmarchicus* (Gunnerus, 1770) to CO_2-acidified seawater; effects on survival, growth and development. *Biogeosciences Discussions,* **10,** 5273-5300.

PIERROT, D., LEWIS, E. and WALLACE, D. W. R. (2006) MS Excel program developed for CO_2 system calculations. ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee.

PLANQUE, B. and BATTEN, S. D. (2000) *Calanus finmarchicus* in the North Atlantic: the year of *Calanus* in the context of interdecadal change. *ICES Journal of Marine Science,* **57,** 1528-1535.

PÖRTNER, H. O., LANGENBUCH, M. and REIPSCHLÄGER, A. (2004) Biological impacts of elevated ocean CO_2 concentrations: lessons from animal physiology and Earth history. *Journal of Oceanography,* **60,** 705-718.

PÖRTNER, H. O. and REIPSCHLÄGER, A. (1996) Ocean disposal of athropogenic CO_2: Physiological effects on tolerant and intolerant animals. In: B. Ormerod and M. Angel (eds) *Ocean Storage*

of CO_2: Environmental Impact. Massachusetts Institute of Technology and International Energy Agency, Greenhouse Gas R&D Programme, Cheltenham, Boston, pp. 57-81.

PÖRTNER, H. O., REIPSCHLÄGER, A. and HEISLER, N. (1998) Acid-base regulation, metabolism and energetics in *Sipunculus nudus* as a function of ambient carbon dioxide level. *The Journal of Experimental Biology,* **201,** 43-55.

R CORE TEAM (2012) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Accessed March 5th, 2013. URL: http://www.R-project.org/.

RAVEN, P. H. and JOHNSON, G. B. (2002) Arthropods. In: *Biology.* 6th ed. McGraw-Hill, London, pp. 924-925.

REY-RASSAT, C., IRIGOIEN, X., HARRIS, R. P. and CARLOTTI, F. (2002) Energetic cost of gonad development in *Calanus finmarchicus* and *C. helgolandicus*. *Marine Ecology Progress Series,* **238,** 301-306.

RIEBESELL, U., ZONDERVAN, I., ROST, B., TORTELL, P. D., ZEEBE, R. E. and MOREL, F. M. M. (2000) Reduced calcification of marine plankton in response to increased atmospheric CO_2. *Nature,* **407,** 364-367.

ROOS, A. and BORON, W. F. (1981) Intracellular pH. *Physiological Reviews,* **61,** 296-434.

RUNGE, J. A. (1988) Should we expect a relationship between primary production and fisheries? The role of copeod dynamics as a filter of trophic variability. *Hydrobiologia,* **167-167,** 61-71.

SABINE, C. L., FEELY, R. A., GRUBER, N., KEY, R. M., LEE, K., BULLISTER, J. L., WANNINKHOF, R., WONG, C. S., WALLACE, D. W. R., TILBROOK, B., MILLERO, F. J., PENG, T.-H., KOZYR, A., ONO, T. and RIOS, A. F. (2004) The oceanic sink for anthropogenic CO_2. *Science,* **305,** 367-371.

SCHMIDT-NIELSEN, K. (1997) *Animal Physiology: Adaptation and Environment*. 5th ed. Cambridge University Press, New York, pp. 5-612.

SEIBEL, B. A. and WALSH, P. J. (2003) Biological impacts of deep-sea carbon dioxide injection inferred from indices of physiological performance. *The Journal of Experimental Biology,* **206,** 641-650.

SIEGENTHALER, U., STOCKER, T. F., MONNIN, E., LUETHI, D., SCHWANDER, J., STAUFFER, B., RAYNAUD, D., BARNOLA, J. M., FISHER, H., MASSON-DELMOTTE, V. and JOUZEL, J. (2005) Stable carbon cycle - climate relationship during the late Pleistocene. *Science,* **310,** 1313-1317.

SKOGSTAD, M. E. (2010) *Effect of food concentration on growth, egg production and hatching success in Acartia tonsa (Copepoda: Calanoida) feeding on Rhodomonas baltica.* MSc thesis, Norwegian University of Science and Technology.

STEENEVELDT, R., BERGER, B. and TORP, T. A. (2006) CO_2 capture and storage: closing the knowing-doing gap. Trans IChemE, Part A. *Chemical Engineering Research and Design,* **84 (A9),** 739-763.

THRONDSEN, J. (1997) The planktonic marine flagellates. In: C. R. Tomas (ed) *Identifying Marine Phytoplankton.* Academic Press, San Diego, California, pp. 591-728.

TUNNICLIFFE, V., DAVIES, K. T. A., BUTTERFIELD, D. A., EMBLEY, R. W., ROSE, J. M. and CHADWICK, W. W. (2009) Survival of mussels in extremely acidic waters on a submarine volcano. *Nature Geoscience,* **2,** 344-348.

TURLEY, C. (2005) The other CO_2 problem. *openDemocracy.* Accessed May 3rd, 2013. URL: http://www.acamedia.info/sciences/sciliterature/globalw/reference/carol_turley.html.

TURLEY, C. and BOOT, K. (2011) The ocean acidification challenges facing science and society. In: J.-P. Gattuso and L. Hansson (eds) *Ocean Acidification.* Vol. 1. Oxford University Press, New York, pp. 249-271.

TURLEY, C., NIGHTINGALE, P., RILEY, N., WIDDICOMBE, S., JOINT, I., GALLIENNE, C., LOWE, D., GOLDSON, L., BEAUMONT, N., MARIOTTE, P., GROOM, S., SMERDON, G., REES, A., BLACKFORD, J., OWENS, N., WEST, J., LAND, P. and WOODASON, E. (2004) *Literature Review: Environmental Impacts of a Gradual or Catastrophic Release of CO_2 into the Marine*

Environment Following Carbon Dioxide Capture. The UK Department for Environment, Food and Rural Affairs, London.

UNEP/GPA (2006) *Ecosystem-based Management: Markers for Assessing Progress*. United Nations Environment Programme / Global Programme for Action for the Protection of the Marine Environment from Land-Based Activities, The Hague, Netherlands, pp 1-49.

VADSTEIN, O. (2009) Interactions in the planktonic food web. In: E. Sakshaug, G. Johnsen and K. Kovacs (eds) *Ecosystem Barents Sea.* Tapir Academic Press, Trondheim, pp. 251-266.

VAN SLYKE, D. D. (1922) On the measurement of buffer values and on the relationship of buffer value to the dissociation constant of the buffer and the concentration and reaction of the buffer solution. *Journal of Biological Chemistry,* **52,** 525-570.

VEHMAA, A., BRUTEMARK, A. and ENGSTRÖM-ÖST, J. (2012) Maternal effects may act as an adaptation mechanism for copepods facing pH and temperature changes. *Plos One,* **7,** Doi: 10.1371/journal.pone.0048538.

VÅGE, V. T. (2011) *Effects of high CO_2 concentrations on the early developmental stages of the marine copepod Calanus finmarchicus (Gunnerus, 1770).* MSc thesis, Norwegian University of Science and Technology.

WHEATLEY, M. G. and HENRY, R. P. (1992) Extracellular and intracellular acid-base regulation in crustaceans. *Journal of Experimental Zoology,* **263,** 127-142.

WHITELEY, N. M. (2011) Physiological and ecological responses of crustaceans to ocean acidification. *Marine Ecology Progress Series,* **430,** 257-271.

WIDDICOMBE, S. and NEEDHAM, H. R. (2007) Impact of CO_2-induced seawater acidification on the burrowing activity of *Nereis virens* and sediment nutrient flux. *Marine Ecology Progress Series,* **341,** 111-122.

WILD, C. J. and SEBER, G. A. (2000) *Chance Encounters: A First Course in Data Analysis and Inference*. Wiley and Sons, New York, pp. 1-611.

WILLIAMS, R. G. and FOLLOWS, M. J. (2011) *Ocean Dynamics and the Carbon Cycle: Principles and Mechanisms*. Cambridge University Press, Cambridge, 416 pp.

WOLVEKAMP, H. P. and WATERMAN, T. H. (1960) Respiration. In: T. H. Waterman (ed) *The Physiology of Crustacea.* Academic Press, New York, pp. 35-100.

WOOD, C. M., WILSON, P., BERGMAN, H. L., BERGMAN, A. N., LAURENT, P., OTIANG'A-OWITI, G. and WALSH, P. J. (2002) Obligatory urea production and the cost of living in the *Magadi tilapia* revealed by acclimation to reduced salinity and alkalinity. *Physiological and Biochemical Zoology,* **75,** 111-122.

XIAOWAN, T., YUNYAN, N., XIAOQIANG , P., CHUN, Y. and ANPING, H. (2009) Determination of total alkalinity and calcium concentration of seawater rapidly and automatically with small-amount samples. *The 3rd International Conference on Bioinformatics and Biomedical Engineering (ICBBE 2009).* Beijing, China, pp. 1-4.

ZAR, J. H. (1999) *Biostatistical Analysis*, 4th ed. Prentice-Hall, Upper Saddle River, New Jersey, pp. 718.

ZEEBE, R. E. and WOLF-GLADROW, D. A. (2001) *CO_2 in Seawater: Equilibrium, Kinetics, Isotopes.* Vol. 65, Elsevier Science, Amsterdam, pp. 346.

APPENDIX A - Details on seawater supply, *Calanus* cultures and sample fixation

Seawater supply and waste water treatment

The seawater used in the present experiment was taken from the Trondheim fjord, at a depth of approximately 70 metres. A set of two automated sand filters removed particulate matter exceeding 40 μm in size, and the water was allowed to mature in a 6 m^3 maturation tank for a minimum of 24 hours, to prevent sudden changes in its microbial community. No UV filtration was used; instead, removal of bacteria was accomplished using Krüger Kaldnes K3 biofilm carriers (1 m^3, 600 m^2) (Krüger Kaldnes, Sandefjord, Norway), to which the bacteria attached themselves, and which were later removed. Continuous aeration of the water, through vigorous bubbling, ensured that aerobic conditions were maintained.

In the maturation tank, a continuous flow circulation through a heat exchange system and a de-gasser heated the water to 11°C and removed excess gas, thus preventing supersaturation. Upon leaving the maturation tank, the water was subjected to another round of heat exchange and de-gassing and thus attained a temperature of 9°C, which was elevated to about 10°C by the time the water entered the experimental system. Before the water entered the experimental tanks, a Cuno Aqua-Pure® Water Filter System (AquaPure 3M, Neuss, Germany) was used to filter the water to 1 μm.

All the water used in the experiment was continually collected, and pathogens were rendered harmless by the addition of sodium hypochlorite (NaOCl) (3.5 mg/L, 30 min). The resultant redox reaction caused the formation of sodium chloride, and the water was released into the fjord.

Calanus cultures of origin

The adult C*alanus finmarchicus* used to initiate this experiment were obtained from a continuous culture maintained at the NTNU Centre for Fisheries and Aquaculture. These were established at the NTNU facilities at the Brattøra Research Centre in Trondheim, from

copepodites, primarily stage CV, collected from Trondheimsfjorden in the late autumn of 2004, using a Nansen zooplankton net (70 cm diameter, 180 μm mesh, Hydro-Bios, Kiel, Germany). The cultures were raised at +8.0 °C, and maintained at +8-10 °C in 280 L polyester containers in running natural seawater. Feed consisted of a mixture of the unicellular algae *Rhodomonas baltica*, *Isochrysis galbana* and *Dunaliella tertiolecta*, and the feed input was adjusted in order to maintain the level of algal carbon in excess of 150 μg C/L, which has been shown to be sufficient for normal growth and development in *C. finmarchicus* (Campbell et al., 2001). Each new generation was separated from the last by transfer of nauplii to new containers during the first week post-hatch (Hansen et al., 2007).

Preparation of the fixation agent Fytofix

Fytofix is a Lugol-based fixating agent, and was prepared as follows: 200 ml of distilled water was acidified with 20 ml glacial (anhydrous) acetic acid, and 20 g potassium iodide (KI) was then dissolved in the solution, followed by 10 g of iodine (I_2).

APPENDIX B – Linear regression tables

Table B.1: Stage-wise linear regressions for the cumulative stage proportions calculated for each of the Control tanks. The coefficients of the linear equation y = ax + b are included, and the R^2 value describes the fit. ND = No data, and occurs in instances where there was insufficient data.

CONTROL		Least squares linear regression		
Tank ID	**Stage**	**a**	**b**	**R^2**
A1	NI	0.4510	-0.3340	0.85
	NII	0.2440	-0.2230	0.655
	NIII	0.2750	-0.8210	0.849
	NIV	0.2140	-1.2380	0.936
	NV	0.3300	-3.0240	0.990
	NVI	0.2110	-2.3130	0.720
	CI	0.1530	-2.0540	0.912
	CII	0.0793	-1.1450	0.844
	CIII	0.0586	-0.8790	0.943
	CIV	0.1060	-2.5180	0.991
	CV	ND	ND	ND
B1	NI	0.2410	0.2340	0.466
	NII	0.3600	-0.7400	0.849
	NIII	0.3070	-0.9020	0.882
	NIV	0.3810	-2.7430	0.958
	NV	0.1520	-1.2390	0.659
	NVI	0.1490	-1.4990	0.909
	CI	0.1640	-2.3290	0.908
	CII	0.1370	-2.3380	0.899
	CIII	0.0820	-1.3520	0.847
	CIV	0.0906	-1.9470	0.835
	CV	ND	ND	ND
C1	NI	0.1620	0.3220	0.956
	NII	0.3730	-0.6430	0.639
	NIII	0.3000	-0.8520	0.887
	NIV	0.2790	-2.0100	0.904
	NV	0.1290	-0.9850	0.885
	NVI	0.1440	-1.5230	0.698
	CI	0.0979	-1.0900	0.732
	CII	0.0833	-1.2980	0.851
	CIII	0.1020	-2.0620	0.978
	CIV	0.0975	-2.2690	0.998
	CV	ND	ND	ND

Table B.2: Stage-wise linear regressions for the cumulative stage proportions calculated for each of the Treatment 1 tanks. The coefficients of the linear equation y = ax + b are included, and the R^2 value describes the fit. ND = No data, and occurs in instances where there was insufficient data.

TREATMENT 1		Least squares linear regression		
Tank ID	**Stage**	**a**	**b**	**R^2**
	NI	0.2380	-0.0169	0.746
	NII	0.2430	-0.3740	0.718
	NIII	0.1610	-0.2850	0.730
	NIV	0.1190	-0.4790	0.565
	NV	0.1070	-0.7770	0.848
A2	NVI	0.0943	-0.8550	0.894
	CI	ND	ND	ND
	CII	0.0914	-1.4610	0.822
	CIII	0.0903	-1.6630	0.998
	CIV	ND	ND	ND
	CV	ND	ND	ND
	NI	0.9900	-0.6550	0.989
	NII	0.3620	-0.6550	0.822
	NIII	0.3240	-1.0460	0.866
	NIV	0.1120	-0.5440	0.152
	NV	0.1810	-1.5650	0.895
B2	NVI	0.0950	-0.7610	0.646
	CI	0.0780	-0.8040	0.746
	CII	0.0550	-0.5560	0.768
	CIII	0.0963	-1.8350	0.933
	CIV	0.0743	-1.5780	0.890
	CV	ND	ND	ND
	NI	0.7140	-1.0130	0.878
	NII	0.2240	-0.2740	0.874
	NIII	0.3400	-1.1480	0.544
	NIV	0.1550	-0.9890	0.975
	NV	0.1110	-0.8660	0.913
C2	NVI	0.1400	-1.6270	0.890
	CI	0.0800	-0.9170	0.825
	CII	0.0681	-0.9830	0.709
	CIII	0.0554	-0.8350	0.810
	CIV	0.0708	-1.5550	0.984
	CV	ND	ND	ND

Table B.3: Stage-wise linear regressions for the cumulative stage proportions calculated for each of the Treatment 2 tanks. The coefficients of the linear equation y = ax + b are included, and the R^2 value describes the fit. ND = No data, and occurs in instances where there was insufficient data.

TREATMENT 2		Least squares linear regression		
Tank ID	**Stage**	**a**	**b**	**R^2**
	NI	0.1560	0.4540	0.816
	NII	0.3450	-0.4530	0.955
	NIII	0.3490	-1.0050	0.943
	NIV	0.2300	-1.5310	0.707
	NV	0.2020	-1.7880	0.942
A3	NVI	0.1050	-0.8970	0.835
	CI	0.1170	-1.4990	0.814
	CII	0.0891	-1.3470	0.792
	CIII	0.0485	-0.6280	0.722
	CIV	0.0564	-1.0820	0.947
	CV	ND	ND	ND
	NI	0.1770	0.3730	0.716
	NII	0.3990	-0.7690	0.684
	NIII	0.4740	-1.5620	0.956
	NIV	0.1310	-0.7670	0.751
	NV	0.0732	-0.3270	0.725
B3	NVI	0.0701	-0.5580	0.843
	CI	0.0776	-1.0030	0.828
	CII	0.0495	-0.7520	0.896
	CIII	0.0256	-0.3190	0.590
	CIV	ND	ND	ND
	CV	ND	ND	ND
	NI	0.0465	0.6460	0.138
	NII	0.3030	-0.3560	0.887
	NIII	0.2050	-0.6350	0.974
	NIV	0.2560	-1.8880	0.965
	NV	0.1750	-1.5400	0.927
C3	NVI	0.0964	-0.9980	0.782
	CI	0.0595	-0.4610	0.941
	CII	0.1330	-2.3540	0.759
	CIII	0.0733	-1.3680	0.893
	CIV	0.0444	-0.7890	0.855
	CV	ND	ND	ND

Table B.4: Stage-wise linear regressions for the cumulative stage proportions calculated for each of the Treatment 3 tanks. The coefficients of the linear equation y = ax + b are included, and the R^2 value describes the fit. ND = No data, and occurs in instances where there was insufficient data.

TREATMENT 3		Least squares linear regression		
Tank ID	**Stage**	**a**	**b**	**R^2**
	NI	0.8580	-1.0070	0.883
	NII	0.2160	-0.114	0.417
	NIII	0.1570	-0.3360	0.845
	NIV	0.1570	-0.8800	0.698
	NV	0.1820	-1.8080	0.886
A4	NVI	0.0399	0.0278	0.502
	CI	0.0913	-1.3430	0.963
	CII	0.0708	-1.3760	0.999
	CIII	0.0736	-1.5950	0.884
	CIV	ND	ND	ND
	CV	ND	ND	ND
	NI	0.5550	-0.580	0.996
	NII	0.6260	-1.4760	0.892
	NIII	0.4340	-1.5860	0.925
	NIV	0.1240	-0.8200	0.746
	NV	0.0979	-0.8450	0.770
B4	NVI	0.0626	-0.5700	0.849
	CI	0.0689	-0.9300	0.818
	CII	0.0421	-0.4550	0.795
	CIII	0.0337	-0.5810	0.588
	CIV	0.0148	-0.2180	0.442
	CV	ND	ND	ND
	NI	0.1930	0.2870	0.486
	NII	0.1620	0.1380	0.387
	NIII	0.2230	-0.5020	0.969
	NIV	0.0965	-0.5430	0.710
	NV	0.0430	-0.0725	0.501
C4	NVI	0.0592	-0.5870	0.820
	CI	0.0998	-1.5920	0.857
	CII	ND	ND	ND
	CIII	0.0901	-1.9740	0.944
	CIV	0.0685	-1.6400	0.869
	CV	ND	ND	ND

APPENDIX C – Density data

Table C.1: Density (number of individuals per litre) raw data for the second generation, based on the number of individuals found in each sample taken for stage determination. Eggs were not included in the density data. The values for day 16 have been removed due to uncertainty about the volume sampled. Due to unsuccessful fixation of the samples from the final two sampling points during this generation, the data only extends to day 32. (*based on the mid-point of egg-laying)

	EXPOSURE GROUP											
Time of sampling (days*)	**Control** (number/L)			**Treatment 1** (number/L)			**Treatment 2** (number/L)			**Treatment 3** (number/L)		
	A1	**B1**	**C1**	**A2**	**B2**	**C2**	**A3**	**B3**	**C3**	**A4**	**B4**	**C4**
1.79	14	14	10	12	14	12	8	20	14	10	10	8
2.17	8	16	6	22	10	12	8	8	10	22	6	4
2.50	14	22	12	10	8	12	16	8	20	12	22	10
2.79	49	16	14	21	11	18	17	25	10	11	19	8
3.17	18	18	16	12	16	2	24	20	8	8	28	4
3.50	16	18	14	10	18	4	22	16	12	16	10	12
3.79	18	25	15	25	19	23	13	21	24	19	28	21
4.17	12	28	10	16	20	12	10	22	32	18	16	8
4.50	24	14	8	10	22	16	10	4	20	16	22	4
4.79	16	20	14	20	10	14	10	24	16	16	26	4
5.17	22	18	22	20	18	18	20	16	12	4	34	26
5.67	21	20	16	20	18	10	14	0	14	16	20	20
6.17	12	28	16	6	14	14	14	12	20	14	12	8
6.67	18	12	6	8	8	14	8	12	8	10	22	12
7.17	4	16	10	10	4	2	6	12	10	8	20	6
7.67	18	13	14	17	15	14	15	13	17	10	20	17
8.17	8	14	12	16	8	16	12	28	14	12	12	8
8.67	12	16	12	24	14	12	12	22	8	26	8	8
9.17	29	17	9	14	20	8	11	15	15	6	15	13
10.17	15	9	13	7	6	7	18	8	4	9	5	16
11.17	12	13	9	11	17	9	10	17	15	14	16	11
12.17	14	12	13	11	10	9	8	14	17	11	22	12
13.17	10	14	12	4	9	5	6	10	6	12	12	10
14.17	13	11	11	7	11	5	7	9	11	12	14	6
15.17	10	14	9	5	9	7	10	13	10	9	10	5
17.17	12	12	12	5	8	6	4,5	10	8,5	12	9,5	12
18.17	16	5	13	9	12	7	12	11	9	6	6,5	5,5
20.17	7	12	10	3	11	4	4,5	9,5	5,5	5	7,5	3
22.17	7	6,5	7,5	3,5	7	9,5	6,5	8	3,5	4,5	7	3
24.17	8	10	6,5	5	9	4	5	4	3,5	4	5,5	6,5
28.17	6	12	9	4,5	11	5	3,5	4	4,5	5	8,5	3
32.17	5,5	8	3,5	3	11	4	8,5	5,5	7,5		2,5	4

APPENDIX D – Stage determination data

Table D.1: Stage determination raw data (not standardised per litre) for the second generation. The table continues on subsequent pages. The data for day 16 and all points after day 32 have been removed, due to uncertainty about volume, as well as unsuccessful fixation. The sampled volume has been included, as well as the conversion factor necessary for calculation of density. (*based on mid-point of egg-laying)

Sampling		Conversions		DEVELOPMENTAL STAGE													
Time (days*)	Tank (ID)	Volume (mL)	Factor (x)	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
1.79	A1	500	2	4	3												
2.17	A1	500	2	1	3												
2.50	A1	500	2	2	4	1											
2.79	A1	1000	1	3	21	7	18										
3.17	A1	500	2		2	7											
3.50	A1	500	2	1	1	5	1										
3.79	A1	1000	1	2	5	8	3										
4.17	A1	500	2	1		4	1										
4.50	A1	500	2	1	1	5	5										
4.79	A1	500	2	1		1	6										
5.17	A1	500	2	3	1		7										
5.67	A1	1000	1	2		1	18										
6.17	A1	500	2		1		5										
6.67	A1	500	2			1	8										
7.17	A1	500	2					2									
7.67	A1	1000	1				11	7									
8.17	A1	500	2				2	2									
8.67	A1	500	2				2	4									
9.17	A1	1000	1	1			8	19	1								
10.17	A1	1000	1					11	4								
11.17	A1	1000	1					4	8								
12.17	A1	1000	1						12	2							
13.17	A1	1000	1						3	6	1						
14.17	A1	1000	1				2	2	2	6	1						
15.17	A1	1000	1				1			6	2	1					
17.17	A1	2000	0,5						1	6	3						
18.17	A1	2000	0,5							4	23	5					
20.17	A1	2000	0,5							1	3	10					
22.17	A1	2000	0,5							2	2	5	5				
24.17	A1	2000	0,5						1	1		4	9	1			
28.17	A1	2000	0,5								1	2	4	5			
32.17	A1	2000	0,5										1	5	5		

Sampling		Conversions		DEVELOPMENTAL STAGE													
Time	Tank	Volume	Factor	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
(days*)	(ID)	(mL)	(x)														
1.79	B1	500	2	3	4												
2.17	B1	500	2	1	6	1											
2.50	B1	500	2	1	9	1											
2.79	B1	1000	1	3	12				1								
3.17	B1	500	2	2	4	2	1										
3.50	B1	500	2	1	1	7											
3.79	B1	1000	1	4	2	12	6	1									
4.17	B1	500	2	3	1	7	2									1	
4.50	B1	500	2		1	3	3										
4.79	B1	500	2	1		2	7										
5.17	B1	500	2	1			8										
5.67	B1	1000	1		2	2	16										
6.17	B1	500	2			1	12	1									
6.67	B1	500	2				6										
7.17	B1	500	2				8										
7.67	B1	1000	1	3			8	2									
8.17	B1	500	2				4	3									
8.67	B1	500	2	1			3	4									
9.17	B1	1000	1				4	13									
10.17	B1	1000	1					8	1								
11.17	B1	1000	1				2	6	4	1							
12.17	B1	1000	1					1	6	2	3						
13.17	B1	1000	1				1		6	7							
14.17	B1	1000	1					1	3	7							
15.17	B1	1000	1	1				1	2	10							
17.17	B1	2000	0,5							3	1						
18.17	B1	2000	0,5						1	2	9	1					
20.17	B1	2000	0,5					1		1	11	10					
22.17	B1	2000	0,5					1		1		6	5				
24.17	B1	2000	0,5							1	2	2	12	3			
28.17	B1	2000	0,5									2	3	19			
32.17	B1	2000	0,5										2	8	5		1

Sampling		Conversions		DEVELOPMENTAL STAGE													
Time	Tank	Volume	Factor	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
(days*)	(ID)	(mL)	(x)														
1.79	C1	500	2	2	3												
2.17	C1	500	2		2	1											
2.50	C1	500	2		5	1											
2.79	C1	1000	1	3	8	2			1								
3.17	C1	500	2	1	2	4	1										
3.50	C1	500	2	1	1	5											
3.79	C1	1000	1	1	4	9	1										
4.17	C1	500	2			5											
4.50	C1	500	2			2	2										
4.79	C1	500	2			2	5										
5.17	C1	500	2	1	1	2	7										
5.67	C1	1000	1	1	1		13				1						
6.17	C1	500	2				8										
6.67	C1	500	2				3										
7.17	C1	500	2				5										
7.67	C1	1000	1		1		12	1									
8.17	C1	500	2				4	2									
8.67	C1	500	2				4	2									
9.17	C1	1000	1			1	2	5			1						
10.17	C1	1000	1				3	10									
11.17	C1	1000	1					5	4								
12.17	C1	1000	1				1	2	6	4							
13.17	C1	1000	1				1	2	5	4							
14.17	C1	1000	1				1	1	5	4							
15.17	C1	1000	1					1	1	4	3						
17.17	C1	2000	0,5							1		2					
18.17	C1	2000	0,5						3	5	16	2					
20.17	C1	2000	0,5					1	1		7	10			1		
22.17	C1	2000	0,5							2	2	9	2				
24.17	C1	2000	0,5						2	1	1	4	4	1			
28.17	C1	2000	0,5				1					2	6	8	1		
32.17	C1	2000	0,5										1	6			

Sampling		Conversions		DEVELOPMENTAL STAGE													
Time	Tank	Volume	Factor	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
(days*)	(ID)	(mL)	(x)														
1.79	A2	500	2	3	2	1											
2.17	A2	500	2		10	1											
2.50	A2	500	2	2	3												
2.79	A2	1000	1	2	12	7											
3.17	A2	500	2	1	4	1											
3.50	A2	500	2			5											
3.79	A2	1000	1	2	5	14	4										
4.17	A2	500	2	1	2	2	3										
4.50	A2	500	2			2	3										
4.79	A2	500	2	2	1	3	4										
5.17	A2	500	2			3	7										
5.67	A2	1000	1	2	4		14										
6.17	A2	500	2	1	1	1											
6.67	A2	500	2			1	3										
7.17	A2	500	2		1		4										
7.67	A2	1000	1	2		2	10	3									
8.17	A2	500	2		1		7										
8.67	A2	500	2	1	1		1	9									
9.17	A2	1000	1	1	1	1	1	10									
10.17	A2	1000	1				1	4	1	1							
11.17	A2	1000	1				3	3	5								
12.17	A2	1000	1				1	4	4	2							
13.17	A2	1000	1				2		2								
14.17	A2	1000	1					1	2	4							
15.17	A2	1000	1				1		1	3							
17.17	A2	2000	0,5				2	1		5							
18.17	A2	2000	0,5						1	2	12	3					
20.17	A2	2000	0,5								3	2	1				
22.17	A2	2000	0,5					1		2	1	3					
24.17	A2	2000	0,5					1		1		3	5				
28.17	A2	2000	0,5									1	3	5			
32.17	A2	2000	0,5											3	3		

Sampling		Conversions		DEVELOPMENTAL STAGE													
Time	Tank	Volume	Factor	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
(days*)	(ID)	(mL)	(x)														
1.79	B2	500	2	4	3												
2.17	B2	500	2	1	3	1											
2.50	B2	500	2		3	1											
2.79	B2	1000	1		10		1										
3.17	B2	500	2		3	4	1										
3.50	B2	500	2			9											
3.79	B2	1000	1		2	15	2										
4.17	B2	500	2		2	6	2										
4.50	B2	500	2	1		7	3										
4.79	B2	500	2			2	3										
5.17	B2	500	2		2	1	6										
5.67	B2	1000	1	1	1	1	15										
6.17	B2	500	2				7										
6.67	B2	500	2				4										
7.17	B2	500	2				1	1									
7.67	B2	1000	1	1			13	2									
8.17	B2	500	2	1			2	1									
8.67	B2	500	2				5	2									
9.17	B2	1000	1		2		4	13	1								
10.17	B2	1000	1					5	1								
11.17	B2	1000	1					7	10								
12.17	B2	1000	1					2	8								
13.17	B2	1000	1				2		3	4							
14.17	B2	1000	1				1		3	6	1						
15.17	B2	1000	1						2	4	3						
17.17	B2	2000	0,5					1	1	1		2					
18.17	B2	2000	0,5					2	4	1	17						
20.17	B2	2000	0,5						1	1	9	9		1			
22.17	B2	2000	0,5				1		1		4	8					
24.17	B2	2000	0,5							1	1	5	10	1			
28.17	B2	2000	0,5						1		1	2	8	9	1		
32.17	B2	2000	0,5										2	13	6		

Sampling		Conversions		DEVELOPMENTAL STAGE													
Time	Tank	Volume	Factor	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
(days*)	(ID)	(mL)	(x)														
1.79	C2	500	2	5	1												
2.17	C2	500	2	2	4												
2.50	C2	500	2	1	5												
2.79	C2	1000	1	2	9	7											
3.17	C2	500	2		1												
3.50	C2	500	2	1	1												
3.79	C2	1000	1	5	3	13	2										
4.17	C2	500	2	1		3	2										
4.50	C2	500	2	1	1	5	1										
4.79	C2	500	2			1	6										
5.17	C2	500	2	2		2	5										
5.67	C2	1000	1		2	1	7										
6.17	C2	500	2				7										
6.67	C2	500	2		1		6										
7.17	C2	500	2				1										
7.67	C2	1000	1				14										
8.17	C2	500	2				6	2									
8.67	C2	500	2				4	2									
9.17	C2	1000	1				4	3	1								
10.17	C2	1000	1				3	4									
11.17	C2	1000	1					6	3								
12.17	C2	1000	1			1		3	4	1							
13.17	C2	1000	1						5								
14.17	C2	1000	1				1		3		1						
15.17	C2	1000	1	1				1	1	2		2					
17.17	C2	2000	0,5						1	1	1		1				
18.17	C2	2000	0,5						1	7	6						
20.17	C2	2000	0,5							2	4	2					
22.17	C2	2000	0,5								5	9	4		1		
24.17	C2	2000	0,5				1	1		1			4	1			
28.17	C2	2000	0,5								1	1	4	2	2		
32.17	C2	2000	0,5									1	1	5	1		

Sampling		Conversions		DEVELOPMENTAL STAGE														
Time (days*)	Tank (ID)	Volume (mL)	Factor (x)	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
1.79	A3	500	2	1	2										1		
2.17	A3	500	2	1	2	1											
2.50	A3	500	2	1	4	3											
2.79	A3	1000	1	2	7	7		1									
3.17	A3	500	2		4	7											
3.50	A3	500	2	1	2	6	1			1							
3.79	A3	1000	1		1	7	4	1									
4.17	A3	500	2		2	1	1			1							
4.50	A3	500	2			2	3										
4.79	A3	500	2			2	2	1									
5.17	A3	500	2		1		9										
5.67	A3	1000	1		1		12	1									
6.17	A3	500	2				6				1						
6.67	A3	500	2				4										
7.17	A3	500	2				3										
7.67	A3	1000	1		1		10	4									
8.17	A3	500	2				5	1									
8.67	A3	500	2				4	2									
9.17	A3	1000	1				1	10									
10.17	A3	1000	1				2	11	4				1				
11.17	A3	1000	1				1	5	4								
12.17	A3	1000	1					2	6								
13.17	A3	1000	1				1		2	2		1					
14.17	A3	1000	1						1	6							
15.17	A3	1000	1				1		2	5	2						
17.17	A3	2000	0,5				1		1	3	1			1			
18.17	A3	2000	0,5						1	4	18	1					
20.17	A3	2000	0,5							1	3	1	3		1		
22.17	A3	2000	0,5								3	6	3	1			
24.17	A3	2000	0,5							1	2	1	6				
28.17	A3	2000	0,5										3	4			
32.17	A3	2000	0,5			1		1		1			2	5	7		

Sampling		Conversions		DEVELOPMENTAL STAGE													
Time	Tank	Volume	Factor	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
(days*)	(ID)	(mL)	(x)														
1.79	B3	500	2	3	7												
2.17	B3	500	2	1	3												
2.50	B3	500	2	1	1	1			1								
2.79	B3	1000	1	1	18	5			1								
3.17	B3	500	2	1	4	5											
3.50	B3	500	2		1	6	1										
3.79	B3	1000	1	2	5	10	4										
4.17	B3	500	2	2		4	4	1									
4.50	B3	500	2			1	1										
4.79	B3	500	2	1		2	9										
5.17	B3	500	2				7	1									
5.67	B3	1000	1	1		1	10										
6.17	B3	500	2	1			5										
6.67	B3	500	2				6										
7.17	B3	500	2				6										
7.67	B3	1000	1				13										
8.17	B3	500	2	1			11	2									
8.67	B3	500	2	1	1		4	4				1					
9.17	B3	1000	1	1		1	8	4		1							
10.17	B3	1000	1				2	6									
11.17	B3	1000	1			1	2	8	6								
12.17	B3	1000	1				2	2	10								
13.17	B3	1000	1				2	2	4	2							
14.17	B3	1000	1				1	1	4	3							
15.17	B3	1000	1				1	2	4	5	1						
17.17	B3	2000	0,5				1		3	5		2					
18.17	B3	2000	0,5				1	2	3	2	12	1					
20.17	B3	2000	0,5					2	1	4	7	4	1				
22.17	B3	2000	0,5					1		3	6	6					
24.17	B3	2000	0,5					1	1		2	2	2				
28.17	B3	2000	0,5								2	2		4			
32.17	B3	2000	0,5							1	2	3	3	2			

Sampling		Conversions		DEVELOPMENTAL STAGE													
Time	Tank	Volume	Factor	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
(days*)	(ID)	(mL)	(x)														
1.79	C3	500	2	2	4	1											
2.17	C3	500	2	1	4												
2.50	C3	500	2	3	4	3											
2.79	C3	1000	1	2	7					1							
3.17	C3	500	2		1	3											
3.50	C3	500	2		1	5											
3.79	C3	1000	1		5	16	3										
4.17	C3	500	2	1	1	10	4										
4.50	C3	500	2		1	7	2										
4.79	C3	500	2	1	1	3	3										
5.17	C3	500	2	3			3										
5.67	C3	1000	1				14										
6.17	C3	500	2				10										
6.67	C3	500	2	1			3										
7.17	C3	500	2			1	4										
7.67	C3	1000	1			1	14	2									
8.17	C3	500	2				6	1									
8.67	C3	500	2				3	1									
9.17	C3	1000	1				7	8									
10.17	C3	1000	1				1	2	1								
11.17	C3	1000	1				1	9	5								
12.17	C3	1000	1				3	2	11	1							
13.17	C3	1000	1						6								
14.17	C3	1000	1					1	6	4							
15.17	C3	1000	1					2	3	5							
17.17	C3	2000	0,5														
18.17	C3	2000	0,5						2	4	11			1			
20.17	C3	2000	0,5				1		2		6	2					
22.17	C3	2000	0,5							1		5	1				
24.17	C3	2000	0,5								2	3	2				
28.17	C3	2000	0,5									1	5	3			
32.17	C3	2000	0,5									1	3	10	1		

Sampling		Conversions		DEVELOPMENTAL STAGE													
Time	Tank	Volume	Factor	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
(days*)	(ID)	(mL)	(x)														
1.79	A4	500	2	3	2												
2.17	A4	500	2	1	8	1			1								
2.50	A4	500	2		5	1											
2.79	A4	1000	1	5	4	1		1									
3.17	A4	500	2		1	3											
3.50	A4	500	2		2	5	1										
3.79	A4	1000	1		2	11	5	1									
4.17	A4	500	2	2		4	2			1							
4.50	A4	500	2		1	5	2										
4.79	A4	500	2	1	1	2	4										
5.17	A4	500	2				2										
5.67	A4	1000	1	1	1	3	11										
6.17	A4	500	2				7										
6.67	A4	500	2				4		1								
7.17	A4	500	2				4										
7.67	A4	1000	1	1	1		8										
8.17	A4	500	2				4	2									
8.67	A4	500	2		1		7	4									
9.17	A4	1000	1				1	5									
10.17	A4	1000	1				4	5									
11.17	A4	1000	1			1		10	2								
12.17	A4	1000	1				3	3	5								
13.17	A4	1000	1														
14.17	A4	1000	1					1	5	6							
15.17	A4	1000	1					2	5	5							
17.17	A4	2000	0,5														
18.17	A4	2000	0,5					5	3	8	7						
20.17	A4	2000	0,5				1	1	1	4	4		1				
22.17	A4	2000	0,5					1	2		6	1					
24.17	A4	2000	0,5							1	5	2	1				
28.17	A4	2000	0,5							1	2		4	1			
32.17	A4	2000	0,5						1			2	4	3			

Sampling		Conversions		DEVELOPMENTAL STAGE													
Time	Tank	Volume	Factor	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
(days*)	(ID)	(mL)	(x)														
1.79	B4	500	2	3	2												
2.17	B4	500	2		3												
2.50	B4	500	2	2	8	1											
2.79	B4	1000	1	1	15	3											
3.17	B4	500	2	3	2	8	1										
3.50	B4	500	2		1	4											
3.79	B4	1000	1	1	5	20	2										
4.17	B4	500	2			7		1									
4.50	B4	500	2			6	5										
4.79	B4	500	2	1		6	6										
5.17	B4	500	2	1	1	2	11	1		1							
5.67	B4	1000	1		2	2	13				2	1					
6.17	B4	500	2				6										
6.67	B4	500	2	1			10										
7.17	B4	500	2	1			9										
7.67	B4	1000	1				19				1						
8.17	B4	500	2				5	1									
8.67	B4	500	2				4										
9.17	B4	1000	1		1		12	2									
10.17	B4	1000	1				2	2			1						
11.17	B4	1000	1				6	8	2								
12.17	B4	1000	1				5	12	5								
13.17	B4	1000	1				4	3	5								
14.17	B4	1000	1				1	1	9	2			1				
15.17	B4	1000	1				1	2	3	3				1			
17.17	B4	2000	0,5														
18.17	B4	2000	0,5					1	2	5	5						
20.17	B4	2000	0,5				1	3	1	4	6						
22.17	B4	2000	0,5					1	2		5	5		1			
24.17	B4	2000	0,5						1	1	2	4	3				
28.17	B4	2000	0,5							2	3	3	4	3	2		
32.17	B4	2000	0,5								2	1	1	1			

Sampling		Conversions		DEVELOPMENTAL STAGE													
Time	Tank	Volume	Factor	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
(days*)	(ID)	(mL)	(x)														
1.79	C4	500	2		4												
2.17	C4	500	2		2												
2.50	C4	500	2		5												
2.79	C4	1000	1	2	1	4		1									
3.17	C4	500	2		1	1											
3.50	C4	500	2		1	3	2										
3.79	C4	1000	1	1	3	11	5	1									
4.17	C4	500	2		1	3											
4.50	C4	500	2			2											
4.79	C4	500	2				2										
5.17	C4	500	2	1		4	8										
5.67	C4	1000	1	1	1	2	13			1	1	1					
6.17	C4	500	2				3				1						
6.67	C4	500	2				5	1									
7.17	C4	500	2				3										
7.67	C4	1000	1			1	14	1				1					
8.17	C4	500	2				2	2									
8.67	C4	500	2	1			2	1									
9.17	C4	1000	1				10	3									
10.17	C4	1000	1				9	6	1								
11.17	C4	1000	1				5	5	1								
12.17	C4	1000	1				7	1	4								
13.17	C4	1000	1				1	2	7								
14.17	C4	1000	1				1	1	4								
15.17	C4	1000	1					1	4								
17.17	C4	2000	0,5				1	2									
18.17	C4	2000	0,5				1	3	1	2	4						
20.17	C4	2000	0,5						3	1	2						
22.17	C4	2000	0,5				1	1		1	3						
24.17	C4	2000	0,5					1			5	5	1	1			
28.17	C4	2000	0,5								2		3	1			
32.17	C4	2000	0,5									1	2	4	1		