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# COMBINED EFFECTS OF OCEAN ACIDIFICATION, OCEAN WARMING AND OIL SPILL ON ASPECTS OF DEVELOPMENT OF MARINE INVERTEBRATES

**by**

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## i. Abstract

For decades, humans have impacted marine ecosystems in a variety of ways including contamination by pollution, fishing, and physical destruction of habitats. Global change has, and will, lead to alterations in  in a number of abiotic factors of our ocean in particular reduced oxygen saturation, salinity changes, elevated temperature (ocean warming or OW) and elevated carbon dioxide (ocean acidification or OA). Now and in the future, OA and OW will operate together with local anthropogenic drivers such as oil pollution. And yet, at present, very little is known about their potential combined interactive effects on physiological performance and tolerance of marine organisms. Therefore, multiple driver experiments are required if we are to understand and predict future vulnerability of species, populations and ecosystems.

Early life stages of invertebrates are generally considered most vulnerable to environmental stress. However, few studies consider the combined effects OA and OW on survival and growth during early development of marine invertebrates, and to our knowledge, there is no information on the additional effects of oil pollution. Therefore, the aim of this thesis was to investigate the effects of combined exposure to OA, OW, and incorporating local drivers such as oil pollution on the development, morphology and physiology of three economically and ecologically important marine invertebrates. These are Northern shrimp *Pandalus borealis*, Northern krill *Meganyctiphanes norvegica*, and the green sea urchin *Strongylocentrotus droebachiensis*. All are cold-water species, assumed to have a narrower tolerance than more temperate species, and so could be particular sensitive to combined stressor affects.

Both Northern krill and to a lesser extent Northern shrimp larvae survived experimental conditions, mirroring those predicted under a future global change scenario (combined OA and OW exposure). Neither was hatching success affected. Both shrimp and krill larvae exhibited accelerated developmental rates and incurred greater maintenance costs as a result of exposure to these stressors. Shrimp larvae showed accelerated developmental rates (-9 days), increased metabolic rates (+20 %), and increased feeding rates (+20 %), but reduced growth (- 9 %) when exposed to OW compared with the control. OA increased development rate but only at the control temperature. Although juvenile mortality of krill was not affected by predicted OA/OW conditions, metabolic rate increased significantly (+ 36 %), as did larval developmental rate, while number of moults, feeding rate and growth (- 67 %) decreased significantly (- 67 %, -60 % and -8 % respectively). Accelerated development was accompanied by greater maintenance costs possibly due to experience a mismatch between energy supply and demand. Both species had an excess of food, and so growth reduction was more likely to be associated with higher metabolic demands in the future global change treatments. Food shortage *in situ*, due to variable food availability in the sea and/or mismatch with key prey species (algae and zooplankton) could result in more negative effects on growth and ultimately survival.

Green sea urchins were also able to survive OA exposure, without detectable effects on hatching success. However, at day 44 post-fertilization, larval body length in the OA treatment was 9 % lower compared to the control. Furthermore, there was a significant tendency of urchin larvae to increase swimming activity in the OA conditions that might indicate compensatory feeding.

Elevated maintenance and repair costs as a result of exposure to multi-stressors affected the energy budget of all the three species studied here resulting in reduced growth. Global drivers (OA and OW) resulted in trade-offs with more energy reallocated to swimming activity and metabolism, rather than growth. Exposure to oil reduced the acquisition of energy by reduced feeding which in turn resulted in less energy being available for growth. Both shrimp and sea

urchin larvae showed reduced activity and feeding when exposed to oil. It is possible that the reduced swimming activity observed may be due to a narcotic effect of the oil. Furthermore, early stage sea urchin larvae showed increased mortality when exposed to oil while the older larvae did not, indicating a stage specific toxicity to oil for sea urchin larvae.

The combination of global drivers and oil pollution acted additively on growth for both sea urchin and shrimp larvae. The impact of combined drivers on the size of shrimp larvae was equal the sum of the negative impacts observed for each driver: a 5 % reduction when exposed to OA and OW, a 9 % reduction when exposed to oil, and a cumulative 15 % reduction when exposed to all stressors. Similarly, the impact of combined drivers on the size of sea urchin larvae was equal to the sum of the negative impacts observed for each driver: a 14 % reduction when exposed to OA, a 9 % reduction when exposed to oil, and a 21 % reduction when exposed to all drivers. Therefore, the study demonstrated the additive physiological effects of OA, OW and a contaminant, and indicated that larval (sea urchin and shrimp) resilience to future changes (i.e. pollution) could be greatly reduced if larvae were already energy limited and severely stressed (reduced development) as a result of exposure to the global drivers. This study therefore shows the importance that the effective management of local drivers such as oil pollution could have against the backdrop of OA and OW, and emphasises that it is important to study impacts of toxicants, such as an oil pollution, in the context of predicted changes in the environment, as OW and OA are becoming major concerns. Finally, the fact that some local and global drivers seem to act additively should encourage local managers to act on local driver regulations, to obtain positive effects on local populations and environment and thereby rendering them more resilient to the negative impacts of future global drivers.

## ii. Contents

## iii Abbreviations

BLGR – Body growth length

C – Consumption

C2 – Calyptopis 2

C1 – Calyptopis 1

CL – Carapace length

DIC – Dissolved inorganic carbon

DM – Dry mass

dph – Days post hatch

dpf – Days post fertilization

ESD – Equivalent spherical diameter

F – Faeces

GLM – General linear model

MN – Metanauplius

MPMO – Multiple performance multiple optima

N1 – Nauplius 1

N2 – Nauplius 2

NBS – National Bureau of Standards

NR - Neutral red

NRRT – Neutral red retention time

OA – Ocean acidification

OAW - Combined ocean acidification and ocean warming condition

OCLITT – Oxygen and capacity-limited thermal tolerance

OW – Ocean warming

P – Production

PAH – Polycyclic aromatic hydrocarbons

PFA – Paraformaldehyde

POL – Posterolateral rod length

R - Respiration

RRGR – Respiration growth rate

SI – Symmetry index

SL – Stomach length

SST – Sea water temperature

SVGR – Stomach width growth rate

SW – Stomach width

TA – Total alkalinity

TL – Total length

## iv Figure legends

**Figure 1.1** Past and contemporary variability of marine pH. Future predictions are model-derived values based on the Intergovernmental Panel on Climate Change (IPCC 2014) mean scenarios. (Source: Turley *et al*, 2006, Cambridge University Press, 8, 65 – 70).

**Figure 1.2** Time series of annual average sea surface temperature anomaly (°C), referenced to the average temperature between 1970 and 2012, in the global ocean and in each of the European seas. Data sources: SST datasets from the Hadley Centre (HADISST1 (global)), MOON-ENEA (Mediterranean Sea), and Bundesamt für Seeschifffahrt und Hydrographie (Baltic and North Seas), and My Ocean. (Source: Sea surface temperature (CLIM 013) - Assessment published Mar 2014 by European Environment Agency).

**Figure 1.3** The light green-shaded area represents the ecological niche of a species. Stress arises when an environmental factor increases from point 1 to 2 such that the species is forced out of its ecological niche (red arrowed line). Various stress response reactions provide temporary survival under stress, and a return to the niche (blue arrowed line). If the boarders of the niche are extended through adaptation, what was stress is not stress any more (green arrowed line) (Source: Van Straalen 2003).

**Figure 1.4** Representation of the major competing physiological sinks in the energy budget of marine organisms redrawn from Clarke (1987).

**Figure 1.5** OCLTT (Oxygen and capacity-limited thermal tolerance) as a concept integrating multiple stressors and various processes and their indicators (simplified from Pörtner 2010). (A) Limited thermal windows are set by (aerobic) performance capacity as the first level of thermal limitation. Optimized oxygen supply to tissues between low and high *pejus* temperatures (top) combined with the kinetic stimulation of performance rates by warming supports temperature-dependent performance and a functional optimum (i.e. an optimum of aerobic scope) close to upper *pejus* temperature. Note that in sub-polar and polar climates, due

to falling oxygen demand and rising oxygen concentrations, the oxygen limitation of cold tolerance may not be seen, leaving a limitation through functional capacity (Wittmann et al. 2012). (Source: Pörtner 2012).

**Figure 1.6** The reduced pH exposure system reproduced from the aqua medic operation manual (http://www.aqua-medic.com/pH_computer.shtml). The pH of the header tanks was recorded every five minutes. The figure shows the variation in pH with time during 24 hours. Different volume header tanks and flow where used in different experiments depending on the species and exposure. (Drawing: courtesy of Renée K. Bechmann).

**Figure 1.7** Schematic drawing of the larval stages of P. borealis. Females beat their pleopods or swim freely in the water and the larvae (stage I) are released in the water column. The larvae go through a series of moults starting with stage I, stage II stage III, stage IV, stage V and a sixth transitional stage, the megalopa (M). Juveniles first mature as males, mate as males for one to several years and then change sex to spend the rest of their lives as mature females (with permission from Rasmussen and Aschan (2011)).

**Figure 1.8** Schematic life cycle of the *M. norvegica*. Adults release fertilized eggs and into the water column. Pelagic embryos develop and hatch, then goes through a series of moults starting with nauplius (2 stages), metanauplius (1 stage), calyptopis (3 stages), and furcilia (7 stages) larvae. Juveniles sexually mature after 9 - 12 months and become adults. (Source of drawings the marine Species identification portal after Lebour (1924), http://species-identification. org/species).

**Figure 1.9** Schematic life cycle of *S. droebachiensis*. Benthic adults release eggs and sperm in the water, where fertilization occurs. Planktonic embryos develop into pluteus larvae, which metamorphose, and transform into a benthic juvenile. Juveniles sexually mature after 3 years and become adults. (Adapted from Figure 8, in Dorey (2013)).

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**Figure 2.2** Feeding test of larva of *P. borealis,* 5 shrimp larvae and 150 Artemia nauplii were placed in 1 L seawater. After 6 hours the remaining *Artemia* were counted. (Drawing: Renée K. Bechmann).

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**Figure 2.5** The effect of decreased pH and elevated temperature on mean feeding rates (measured as mean number prey eaten $indiv^{-1}$ $h^{-1}$) for P. borealis larvae at stage II, III and IV. Control (pH 8.1 / 6.7 ºC, clear) (N = 33), decreased pH (pH 7.6 / 6.7 ºC, light grey) (N = 21), elevated temperature (pH 8.1 / 9.5 ºC, dark grey) (N = 27), combined (pH 7.6 / 9.5 ºC, black) (N = 24). Histograms represent means ± SE. Significantly different treatments ($P \leq 0.05$) are indicated by upper case letters and lower case letters indicate significant differences among the different stages according to the Estimate Marginal Mean test.

**Figure 2.6** The effect of decreased pH an elevated temperature on mean oxygen consumption (nmol $O_2$ $h^{-1}$ mg $^{-1}$ DM) for P. borealis larvae stage IV. Control (pH 8.1 / 6.7 ºC, clear) (N = 9), decreased pH (pH 7.6 / 6.7 ºC, light grey) (N = 7), elevated temperature (pH 8.1 / 9.5 ºC, dark grey) (N = 9), combined exposure (pH 7.6 / 9.5 ºC, black) (N = 7). Histograms represent means ± SE. Significantly different treatments ($p \leq 0.05$) are indicated by upper case letters according to Estimate Marginal Mean test.

**Figure 2.7** Relationships between oxygen consumption and feeding rates [$R^2$ = 0.1209, Y= 0.3927x + 2.502, df = 31, *P* = 0.0472] in stage IV larvae of *P. borealis* maintained under different temperature and pH conditions. Control (pH 8.1 / 6.7 ºC, clear), decreased pH (pH 7.6 / 6.7 ºC, light grey), elevated temperature (pH 8.1 / 9.5 ºC, dark grey), combined exposure (pH 7.6 / 9.5 ºC, black). The continuous line represents the line of best fit (linear regression) for all the data.

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**Figure 3.4** The effect of control and OAW conditions on the development of *M. norvegica* larvae (stage *nauplius* I – *calyptopis* III) exposed to a) control (pH 8.1, 7 ºC) and b) OAW (pH 7.6, 10 ºC) conditions. N1 = nauplius I, N2 = nauplius II, MN = metanauplius, C1 = calyptopis I, C2 = calyptopis II and C3 = calyptopis III (N = 3).

**Figure 3.6** The effect of control and OAW conditions on mean feeding rates (measured as mean number prey eaten indiv.$^{-1}$ h$^{-1}$) of juvenile *M. norvegica*. Control (pH 8.1, 7 ºC, clear) (N = 15), OAW (pH 7.6, 10 ºC, black) (N = 15). Histograms represent means ± SD. Significantly different treatments ($p \leq 0.05$) are indicated by large upper case letters.

**Figure 3.7** The effect of control and OAW conditions on mean oxygen consumption (µmol $O_2$ h$^{-1}$ mg $^{-1}$ DM$^{-1}$) of juvenile *M. norvegica*. Control (pH 8.1, 7 ºC, clear) (N = 16), OAW (pH 7.6, 10 ºC, black) (N = 18). Histograms represent means ± SD. Significantly different treatments ($p \leq 0.05$) are indicated by capital upper case letters.

**Figure 4.1** Adult *Pandalus borealis* with embryos (Photo: Renée K. Bechmann).

**Figure 4.2** The effect of control and OAW conditions and oil on mean Neutral Red Retention Time (NRRT) (measured as mean time for approximately 75 % of the lysosomal cells were destroyed) of P. borealis larvae. a) Control vs OAW treatment, b) Non-oil vs oil treatment. Histograms represent means ± SD. Significantly different treatments ($p \leq 0.05$) are indicated by upper case letters.

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**Figure 4.4 a-c** The effect of OAW conditions and oil and their interaction on mean feeding rates (measured as mean number prey eaten $indiv^{-1}$ $h^{-1}$) for P. borealis larvae at stage II, III and IV. a) Mean feeding rates for stages II, III and IV, b) mean feeding rates for oil exposed and non-exposed larvae, c) mean feeding rates for control and OAW exposed larvae. Histograms represent means ± SD. Significantly different treatments ($p \leq 0.05$) are indicated by upper case letters and upper case letters indicate significant differences among the different stages and treatments according to 2-way ANOVA.

**Figure 4.5 a-d** The effect of OAW conditions and oil and their interaction on the development of *P. borealis* larvae (stage II-IV) exposed to a) control (pH 8.0/6.7 ºC), b) control + oil (pH 8.0/6.7 ºC + oil), c) OAW (pH 7.6/9.5 ºC), d) OAW + oil (pH 7.6/9.5 ºC + oil). Percent stage is the % stage at the given time point.

**Figure 4.6** The effect of OAW conditions and oil on mean oxygen consumption (nmol $O_2$ $h^{-1}$ $mg^{-1}$ DM) for larval *P. borealis*. Control (pH 8.0/6.7 ºC, clear) (N = 6), control + oil (pH 8.0/6.7 ºC + oil, light grey) (N = 6), OAW (pH 7.6/9.5 ºC, dark grey) (N = 6), OAW + oil (pH 7.6/9.5 ºC + oil, black) (N = 6). Histograms represent means ± SD.

**Figure 4.7** a-b The effect of OAW conditions and oil on mean a) length, b) mass for stage IV *P. borealis* larvae. Control (pH 8.0/6.7 ºC, clear) (N = 6), control + oil (pH 8.0/6.7 ºC + oil, light grey) (N = 6), OAW (pH 7.6/9.5 ºC, dark grey) (N = 6), OAW + oil (pH 7.6/9.5 ºC + oil, black) (N = 6). Histograms represent means ± SD. Significantly different treatments ($p \leq 0.05$) are indicated by large upper case letters.

**Figure 4.8** The effect of OAW conditions and oil and their interaction on morphology of larval abdominal sixth segment on stage IV *P. borealis* larvae measured as mean percentage no abnormality (clear), unsymmetrical (light grey), missing endopodite (dark grey) and missing exopodite (black) for the different treatments. Significantly, different treatments ($p \leq 0.05$) are indicated by large upper case letters.

**Figure 4.9 a-b.** The effect of OAW conditions, oil, and their interaction on swimming behavior. a) Simple swimming test on stage I larvae of *P. borealis* (measured as mean percentage of larvae at the surface was calculated after 2 min) for control and control + oil treatment.

Significantly different treatments ($p \leq 0.05$) are indicated by big cap letters. b) Advanced swimming test for stage III larvae for the different treatments (measured as the mean number beam breaks per hour. Control (pH 8.0/6.7 ºC, clear) (N = 6), control + oil (pH 8.0/6.7 ºC + oil, light grey) (N = 6), OAW (pH 7.6/9.5 ºC, dark grey) (N = 6), OAW + oil (pH 7.6/9.5 ºC + oil, black) (N = 6). Histograms represent means ± SD. Significantly different treatments ($p \leq 0.05$) are indicated by large upper case letters.

**Figure 5.1** Larva of the green sea urchin *Strongylocentrotus droebachiensis*. (Photo: Maj Arnberg)

**Figure 5.2** Morphometric coordinates and morphology of *S. droebachiensis* 4 - arm pluteus larva. BL, body length; SW, stomach width; SL stomach length; A1, A2, posterolateral rod length (POL) (both arms (A1, A2)) A1.

**Figure 5.3** Experiment 1: Impact of OA and a 4 d oil exposure (8 - 12 dpf) on larval *S. droebachiensis* (a) mortality rate (% $d^{-1}$), (b) body length growth rate (BL GR, µm $ln(d)^{-1}$), (c) postero-lateral arm growth rate (POL GR, µm $µm^{-1}_{BL}$), (d) stomach volume growth rate (SV GR, $µm^3$ $µm^{-1}_{BL}$), (e) symmetry Index (SI) and, (f) feeding between days 12 to 15 (fluorescence $exp^{-1}$). Values are expressed as means ± SD.

**Figure 5.4** Experiment 2: Impact of OA and an oil exposure (23 - 27 dpf) on larval *S. droebachiensis* (a) mortality rate (% $d^{-1}$), (b) body length growth rate (BL GR, µm $ln(d)^{-1}$), (c) postero-lateral arm growth rate (POL GR, µm $µm^{-1}_{BL}$), (d) stomach volume growth rate (SV GR, $µm^3$ $µm^{-1}_{BL}$), (e) symmetry Index (SI), (f) respiration rate (RR, pmol $O_2$ $ind^{-1}$ $h^{-1}$ $µm^{-1}$ $_{BL}$), (g) percentage of active larvae (day 31) and (h) feeding between days 23 and 33 (fluorescence $exp^{-1}$). Values are expressed as means ± SD.

**Figure 5.5** Metamorphosed larvae (means ± SD) of larval *S. droebachiensis* in control, OA, control + 4 d oil spill (0.5 mg $L^{-1}$), OA + 4 d oil spill (0.5 mg $L^{-1}$) treatments

## v Table legends

**Table 3.1** Mean body lengths (mm) of early larval *M. norvegica* in control and OAW conditions. Number in brackets indicate the number of larva measured. (* indicate single measurements, which should be taken with caution).

## vi Appendix legends

**Appendix 2.1** Seawater physico-chemical parameters measured in the experimental system: oxygen concentration ([$O_2$]), pH (NBS Scale), temperature, salinity, total alkalinity. Other parameters (as indicated by an '*') were calculated using the program CO2SYS (Pierrot et al. 2006), using constants from Mehrbach et al. (1973) refitted to the NBS pH scale by Dickson and Millero (1987) and the $KSO_4$ dissociation constant from Dickson (1990). Mean pH level was calculated based on the actual [H+] concentration and back-calculated to pH. These parameters included: dissolved inorganic carbon (DIC), bicarbonate and carbonate ions concentrations ([$HCO_3^-$] and [$CO_3^{2-}$] respectively), saturation status for calcite and aragonite (Ωcalc and Ωara respectively). For all measured parameters (with the exception of total alkalinity) mean values (±SD) were provided, whilst for total alkalinity and all calculated parameters range of values were provided. Finally, mean pH level was calculated based on the actual [H+] concentration and back-calculated to pH.

**Appendix 3.1** Seawater physico-chemical parameters measured in the experimental system: oxygen concentration ([$O_2$]), pH (NBS Scale), temperature, salinity and total alkalinity. Other parameters were calculated using the program CO2SYS (Pierrot et al. 2006), using constants from Mehrbach et al. (1973) refitted to the NBS pH scale by Dickson and Millero (1987) and the KSO4 dissociation constant from Dickson (1990). Mean pH level was calculated based on the actual [H+] concentration and back calculated to pH. These parameters included: dissolved inorganic carbon (DIC), bicarbonate and carbonate ions concentrations ([$HCO_3^-$] and [$CO_3^{2-}$] respectively), saturation status for calcite and aragonite (Ωcalc and Ωara respectively). For all measured parameters mean values (± SD) were provided, whilst for total alkalinity and all calculated parameters range of values were provided.

**Appendix 3.2** Hatching success (%), unhatched eggs (%), number of living larvae and dead larvae for the Northern krill *M. norvegica* for the current and future OAW conditions at sampling days 7, 14, 22, and 29.

**Appendix 4.1** Seawater physico-chemical parameters measured in the experimental system: oxygen concentration ([$O_2$]), pH (NBS Scale), temperature, salinity and total alkalinity. Other parameters were calculated using the program CO2SYS (Pierrot et al. 2006), using constants from Mehrbach et al. (1973) refitted to the NBS pH scale by Dickson and Millero (1987) and the KSO4 dissociation constant from Dickson (1990). Mean pH level was calculated based on the actual [H+] concentration and back calculated to pH. These parameters included: dissolved inorganic carbon (DIC), bicarbonate and carbonate ions concentrations ([$HCO_3^-$] and [$CO_3^{2-}$] respectively), saturation status for calcite and aragonite (Ωcalc and Ωara respectively). For all measured parameters mean values (± SD) were provided, whilst for total alkalinity and all calculated parameters range of values were provided.

**Appendix 4.2** Mean concentration (± SD) of PAHs (µg $L^{-1}$) in water samples from the current and future oil exposed aquaria. The samples were taken during the experiment (N = 2) for each treatment.nd = not detected.

**Appendix 4.3** Feeding (adult), oxygen consumption (adult), hatching success, first hatching day, hatching period, feeding (larvae) and swimming behavior for *P. borealis* Krøyer 1838 adult females and larvae for the different treatments. N = number of samples investigated.

**Appendix 5.1** The decrease in concentration of algae in the flow-through aquaria without sea urchin larvae present. 150 µg carbon/L *Rhodomonas* sp. was added to the aquaria (N = 3 aquaria). The decrease in algal concentration in the aquaria with time was measured by coulter counter. Samples were taken directly from the aquaria (not from the outlets). Figure S1 show the gradual decrease in density with time. After five hours there was almost no algae left in the aquaria.

**Appendix 5.2** Mean concentration (± SD) of PAHs (µg $L^{-1}$) in water samples from the control and oil exposed tanks. Samples were taken during the experiment (N = 3) for each treatment.

**Appendix 5.3** Seawater physico-chemical parameters measured in the experimental system: oxygen concentration ([$O_2$]), pH (NBS Scale), temperature, salinity, total alkalinity. Other parameters (as indicated by an '*') were calculated using the program CO2SYS (Pierrot et al. 2006), using constants from Mehrbach et al. (1973) refitted to the NBS pH scale by Dickson and Millero (1987) and the $KSO_4$ dissociation constant from Dickson (1990). Mean pH level was calculated based on the actual [H+] concentration and back-calculated to pH. These parameters included: dissolved inorganic carbon (DIC), bicarbonate and carbonate ions concentrations ([$HCO_3^-$] and [$CO_3^{2-}$] respectively), saturation status for calcite and aragonite (Ωcalc and Ωara respectively). For all measured parameters mean values (±SD) were provided, whilst for total alkalinity and all calculated parameters range of values were provided. Finally, mean pH level was calculated based on the actual [H+] concentration and back-calculated to pH.

**Appendix 5.4** Examples of relationships used for calculation of the different rates (control, no oil, replicate 3): a. linear relationship between mortality (%) and time ($d^{-1}$), (b) logarithmic relationship between the body length (BL, µm) and time ($d^{-1}$), (c) linear relationship between the postero-lateral arm (POL, µm) and the body length (BL, µm), (d) linear relationship between stomach volume (SV, µm3) and body length (BL, µm), (e) no relationship between symmetry Index (SI) and body length (BL).

**Appendix 5.5** Larval mortality rate (MR in % $d^{-1}$) for each treatment was calculated as the coefficient of the significant linear relationship between mortality and dpf. Results of the regressions (Intercept, *p* - value, $R^2$, F - value and df: degree of freedom) are given for each culture replicate with corresponding treatment. Data in bold (*p* - value > 0.05) were removed from subsequent analyses.

**Appendix 5.6** Body length growth rate (BL GR in µm ln $(d)^{-1}$) for each treatment was calculated as the coefficient of the significant logarithmic relationship between BL and tpf. Results of the regressions (Intercept, *p* - value, $R^2$, F - value and df: degree of freedom) are given for each culture replicate with corresponding treatment.

**Appendix 5.7** Postero - lateral arm growth rate (POL GR in µm $\mu m^{-1}_{BL}$) for each treatment was calculated as the coefficient of the significant linear relationship between POL (µm) and BL (µm). Results of the regressions (Intercept, *p* - value, $R^2$, F - value and df: degree of freedom) are given for each culture replicate with corresponding treatment.

**Appendix 5.8** Stomach volume growth rate (SV GR in $\mu m^3 \mu m^{-1}_{BL}$) for each treatment was calculated as the coefficient of the significant linear relationship between SV ($\mu m^{3)}$ and BL (µm). Results of the regressions (Intercept, *p* - value, $R^2$, F - value and df: degree of freedom) are given for each culture replicate with corresponding treatment. Data in bold (*p* - value > 0.05) were removed from subsequent analyses.

**Appendix 5.9** Larval mortality rate (MR in % $d^{-1}$) for each treatment was calculated as the coefficient of the significant linear relationship between mortality and tpf. Results of the regressions (Intercept, *p* - value, $R^2$, F - value and df: degree of freedom) are given for each culture replicate with corresponding treatment. Data in bold (*p* - value > 0.05) were removed from subsequent analyses.

**Appendix 5.10** Body length growth rate (BL GR in µm ln $(d)^{-1}$) for each treatment was calculated as the coefficient of the significant logarithmic relationship between BL and tpf. Results of the regressions (Intercept, *p* - value, $R^2$, F - value and df: degree of freedom) are given for each culture replicate with corresponding treatment.

**Appendix 5.11** Postero - lateral arm growth rate (POL GR in µm $\mu m^{-1}$BL) for each treatment was calculated as the coefficient of the significant linear relationship between POL (µm) and BL (µm). Results of the regressions (Intercept, *p* - value, $R^2$, F - value and df: degree of freedom) are given for each culture replicate with corresponding treatment.

**Appendix 5.12** Stomach volume growth rate (SV GR, $\mu m^3 \mu m^{-1}$BL) for each treatment was calculated as the coefficient of the significant linear relationship between SV (µm3) and BL (µm). Results of the regressions (Intercept, *p* - value, $R^2$, F - value and df: degree of freedom)

are given for each culture replicate with corresponding treatment. Data in bold (*p* - value > 0.05) were removed from subsequent analyses.

**Appendix 6.1** Publication 1: Arnberg, M., P. Calosi, Spicer, J. I., Tandberg, A. H. S., Nilsen, M., Westerlund, S., Bechmann, R.K. 2013. Elevated temperature elicits greater effects than decreased pH on the development, feeding and metabolism of Northern shrimp (*Pandalus borealis*) larvae. Marine Biology. 2013. 160(8): p. 2037-2048. DOI 10.1007/s00227-012-2072-9

**Appendix 6.2** Publication 2: Samantha, L., Garrard, R. C., Hunter, A. Y., Frommel, A. C., Lane, J. C., Phillips, R., Cooper, R., Dineshram, U. Cardini, S. J. McCoy and M. Arnberg, et al. 2013. Biological impacts of ocean acidification: a postgraduate perspective on research priorities. Marine biology DOI: 10.1007/s00227-012-2033-3

**Appendix 6.3** Publication 3: Chan, K.K.K, Grünbaum, D., Arnberg, M., Thorndyke, M., Dupont, S., 2013. Ocean acidification induces budding in larval sea urchins. Marine Biology (8) 2129-2135

**Appendix 6.4** Publication 4: Chan, K.K.K, Grünbaum, D., Arnberg, M., Dupont. S., 2015. Impacts of ocean acidification on survival, growth, and swimming behaviors differ between larval urchins and brittlestars. ICES J. Mar. Sci. doi:10.1093/icesjms/fsv073

**Appendix 6.5** Publication 5: Arnberg, M., Calosi, P., Spicer, J.I., Westerlund, S., Invarsdottir, A., Bechmann R.K. Effects of combined exposure to reduced pH and elevated temperature on the early life stages of the Northern krill, *Meganyctiphanes norvegica*. Submitted to ICES 2015

**Appendix 6.6** Publication 6: Arnberg, M., Calosi, P., Spicer, J.I., Taban, I.C., Bamber, S., Westerlund, S., Vingen, S., Baussant, T., Bechmann, R.K., Dupont, S. Additive effects of oil and global environmental drivers on two keystone marine invertebrates. Submitted Sci. Rep. 2015.

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## viii AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other university award without prior agreement of the Graduate Committee.

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**Renée K. Bechmann** and **Ingrid C. Taban** supervised me in designing the experiments (Chapter 2, 3, 4, 5), and helped me in the lab, especially the experiment in Chapter 4. **Stig Westerlund** was responsible for designing the pH exposure system (chapter 2) and helped me designing the pH exposure systems in chapter 3, 4, 5. Furthermore, he supervised me in the pH / chemistry system, calculations in CO2SYS and larval respiration (Chapter 2, 4). **Dan Mayor** and **Kathryn Cook** at the University of Aberdeen performed the analysis of the DNA damage in shrimp embryos (Chapter 4). **Leif Anderson** (Gothenburg University) performed the chemistry analysis of all the alkalinity samples

**Piero Calosi** has been my main supervisor during the writing process, and he has supervised me in preforming the data analysis in Chapter 2, 3, 4. **Sam Dupont** supervised me and helped with the data analysis in Chapter 5. **John Spicer** has read manuscripts, commented on thesis and has given scientific advices during the thesis work**.** Finally, **Renée K. Bechmann** and **Sam Dupont** have commented on the last versions of the thesis.

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Publications:

- **Arnberg, M.**, Calosi, P., Spicer, J.I., Taban, I.C., Bamber, S., Westerlund, S., Vingen, S., Baussant, T., Bechmann, R.K., Dupont, S. Additive effects of oil and global environmental drivers on two keystone marine invertebrates. Submitted Sci. Rep. 2015.
- **Arnberg, M**., Calosi, P., Spicer, J.I., Westerlund, S., Invarsdottir, A., Bechmann R.K. Effects of combined exposure to reduced pH and elevated temperature on the early life stages of the Northern krill, *Meganyctiphanes norvegica*. Submitted to ICES 2015
- Chan, K.K.K, Grünbaum, D., **Arnberg, M**., Dupont. S., 2015. Impacts of ocean acidification on survival, growth, and swimming behaviors differ between larval urchins and brittlestars. ICES J. Mar. Sci. doi:10.1093/icesjms/fsv073
- Chan, K.K.k, Grünbaum, D., **Arnberg, M**., Thorndyke, M., Dupont, S., 2013. Ocean acidification induces budding in larval sea urchins. Marine Biology (8) 2129-2135
- Samantha, L., Garrard, R. C., Hunter, A. Y., Frommel, A. C., Lane, J. C., Phillips, R., Cooper, R., Dineshram, U. Cardini, S. J. McCoy and **M. Arnberg**, et al. 2013. Biological impacts of ocean acidification: a postgraduate perspective on research priorities. Marine Biology DOI: 10.1007/s00227-012-2033-3
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# Chapter 1 – General introduction

## 1.1 BACKGROUND

### 1.1.1 Ocean acidification, ocean warming and oil pollution – future oceans will suffer from environmental pressures from several anthropogenic drivers

Human-driven changes to ocean biogeochemistry affect multiple marine processes and species (Doney et al. 2012; Bopp et al. 2013; Duarte et al. 2013). Indeed, no marine systems remain unaffected by human impacts. The future oceans will suffer from environmental pressures from several anthropogenic activities. Many of these anthropogenic drivers (including ocean warming (OW), ocean acidification (OA), ocean circulation change, ocean deoxygenation, coastal hypoxia, overfishing and pollution) co-occur in time and space. Marine organisms are therefore increasingly being subjected to the combined impact of multiple drivers. Drivers in marine systems can be separated into two categories (Noone et al. 2012), those that act globally and those that act locally.

Global drivers are chronic (Ghedini et al. 2013), lasting for an extended period of time, with varying intensity. Global change will lead to shifts in a number of abiotic factors in the oceans such as carbonate chemistry and temperature, producing two global drivers, OA and OW respectively. Both are linked to changes in environmental carbon dioxide ($CO_2$). Since the beginning of the industrial revolution in the mid - $18^{th}$ century, the atmospheric concentration of $CO_2$ has risen by more than 100 ppm, as a consequence of human-caused $CO_2$ emissions, mostly caused by the burning of fossil fuel. Different models have predicted anthropogenic increases in atmospheric $CO_2$ to increase, reaching 500 - 1000 ppm by the end of this century and up to 2000 ppm by year 2300 (IPCC 2014).

Such an increase in atmospheric $CO_2$ is driving changes in ocean seawater chemistry, due to the uptake of $CO_2$ by the oceans (Caldeira and Wickett 2003; Orr et al. 2005). The oceans are responsible for uptake of 30 % of the anthropogenic $CO_2$ emissions produced since 18$^{th}$ century (Sabine et al. 2004). This has resulted in a shift in seawater carbonate chemistry, which includes a decrease in oceanic pH (i.e. – log [$H^+$]). This has resulted in a phenomenon referred to as OA. Due to a threefold increase in $H^+$ (see equilibrium reactions below), the pH has already decreased by 0.1 units since pre-industrial times.

(1) $CO_2\,(aq) + H_2O \leftrightarrow H_2CO_3$ (carbonic acid)

(2) $H_2CO_3 \leftrightarrow H^+ + HCO_3^-$ (bicarbonate ion)

(3) $H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$ (carbonate ion)

Average surface ocean pH is predicted to decrease by 0.3 to 0.5 units by 2100 due to the increased levels of atmospheric $CO_2$. Projections based on the emission scenarios of the IPCC special report on emission scenarios predict reductions in average global surface ocean pH of between 0.14 and 0.35 units over the 21$^{st}$ century, adding to the present decrease of 0.1 units since pre-industrial times (IPCC 2014). Present changes in ocean carbon chemistry are rapid, at least 100 times more rapid than any experienced over the past 100,000 years (Fig. 1.1) (Haugan et al. 2006).

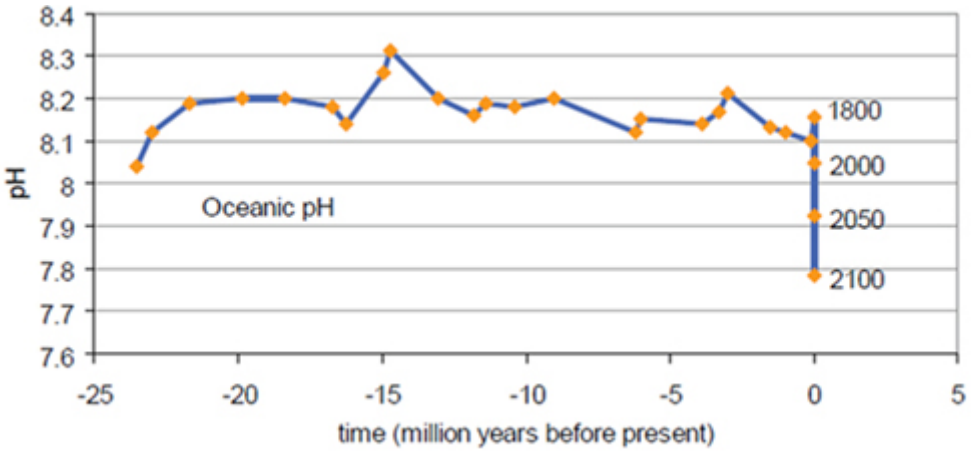


**Figure 1.1 Past and contemporary variability of marine pH. Future predictions are model-derived values based on IPCC mean scenarios. (Source: Turley *et al*, 2006, Cambridge University Press, 8, 65 – 70).**

OA is often been described as "global warming's evil twin" (quote attributed to R. Feely, NOAA) increasing temperature being the other twin**.** Global surface temperature has increased by approximately 0.76 ºC over the past 150 years. One side effect of heat absorption is increased OW, therefore the warming corresponds to an increase in mean ocean temperature of 0.31 °C during these 150 years (Levitus et al. 2000). Future elevated global temperatures due to anthropogenic $CO_2$ emissions will translate into further OW, and sea surface temperatures are predicted to increase by a further 3 - 5 ºC by the end of this century in the North Atlantic (Fig. 1.2) (Sokolov et al. 2009; IPCC 2014).

OA and OW are global phenomena and will persist for many generations; if OA and OW impair the recruitment success of key organisms to an ecosystem that will have serious consequences for that ecosystem. Because recovery of the key organisms is unlikely unless the larvae have the ability to show sufficient phenotypic plasticity or adapt to the rapid change in $CO_2$ and temperature (Eriksson et al. 2013).

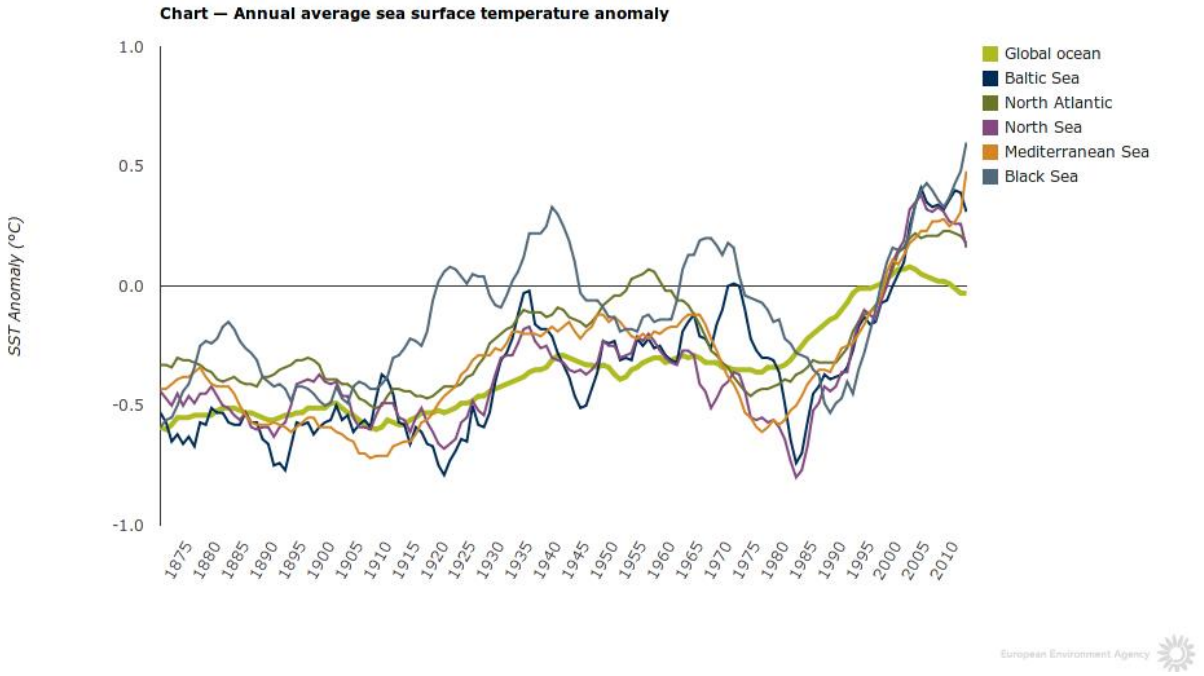


**Figure 1.2 Time series of annual average sea surface temperature anomaly (°C), referenced to the average temperature between 1970 and 2012, in the global ocean and in each of the European seas. Data sources: SST datasets from the Hadley Centre (HADISST1 (global)), MOON-ENEA (Mediterranean Sea), and Bundesamt für Seeschifffahrt und Hydrographie (Baltic and North Seas), and MyOcean. (Source: Sea surface temperature (CLIM 013) - Assessment published Mar 2014 by European Environment Agency).**

Other drivers can originate and act at local scales (Ghedini et al. 2013) and may be either chronic (e.g. nutrient input from rivers or overfishing) or transient and short lived (e.g. oil pollution). For example, reduced ice cover in the Arctic caused by on-going climate change, will lead to increased transport by ships, in response to the worldwide demand of more petroleum resources and search by the oil and gas industry, particularly into the European Arctic from the North Atlantic into Greenland, Northern Norway and Northwest Russia (AMAP 2007; Ermida 2014). This increases the geographical range, and number of ecosystems over which potential accidental oil pollution can occur. If a local stressor such as an oil pollution (in contrast

to global stressors) leads to high mortality of larvae, it is possible to argue that new larvae will come the following years, or that larvae will be transported from unaffected areas.

The species living in these areas will be exposed to multiple stressors like OA, OW, persistent organic pollutants and accidental discharges from the oil industry and from ships. Species ability to tolerate or adapt to future changes will determine persistence. Therefore, it is crucial to be able to make predictions of species survival. To do so one must take into consideration this complex array of both biotic and environmental changes. Interactions between, and among, global and local stressors created by human activities are poorly understood (Cooley 2012; Noone et al. 2012), despite the fact there are a number of reports on the actions of multiple stressors in specific locations. The possibilities for interactions between toxicant responses, OW and OA are many and complex. Unfortunately, the interactions between responses to toxicants and predicted environmental global change scenarios (e.g. temperature, pH and oxygen level) have been little studied to date (Nikinmaa 2013). Some interactions, mainly those between OA and OW, have been investigated (Byrne 2011; Todgham and Stillman 2013; Small et al. 2015) including their effects on invertebrate development (Przeslawski et al. 2015). With OW and OA becoming major public concerns more mechanistic studies of possible interactions between toxicants and predicted environmental conditions, particularly for different developmental stages, are urgently needed (Nikinmaa 2013), as those interactions, interact not with a static adult phase but with a developing individual.

### 1.1.2 Development of marine invertebrate larvae, stress, homeostasis and energy budget

The majority of benthic marine invertebrates have complex lifecycles (Thorson 1950; Strathmann and Strathmann 1982; Strathmann 1985), composed of different life stages (e.g. larvae, juvenile and adult). For benthic marine invertebrates, larval phase or development can be divided into two categories as outlined by Thorson (1950) and many others: benthic or

pelagic development. In both benthic and planktonic development, the offspring may have direct development to the adult. The planktonic (or pelagic) development can be divided into planktotrophic (i.e. larvae feed in the plankton) or lecithotrophic (larvae do not feed in the plankton) modes. Most lecithotrophic larvae are provided with some sort of nutritious source, usually with a yolk sac, a non-renewable food reserve; consequently, they have limited planktonic larval durations, and do not disperse over long distances (Hadfield and Strathmann 1996). Planktotrophic larvae on the other hand feed on phytoplankton and zooplankton in the water column, and spend longer time in the pelagic zone and have the potential to disperse over longer distances. Most benthic invertebrates have long-lived planktothropic development (Pechenik 1999) and this has guided the choice of the study species selected in this thesis. During the time in the water column, larvae feed, grow and go through further developmental stages before they seek the appropriate settlement substrate. These early developmental stages do not have the ability to move away from unfavourable environments, since they are embryos attached to their parents or they are planktonic larvae, drifting with currents. Therefore, they are subjected to the effects of drivers or external factors that characterise the water column, or area, where they are found.

Major environmental drivers to which these developmental stages could potentially be exposed could be OW, OA, salinity stress, low oxygen concentrations, ultraviolet irradiation and chemical pollution. Global change has led to a significant change in intensities of a number of these drives such as OA, OW, oxygen availability and salinity (IPCC 2014). It is often assumed that an organism is well adapted to live in its native environment. However, at the limit of their tolerance range for the intensity of a particular stressor or at the limit of their ecological niche, additional intensity will have strong impact on the survival of the species (Fig. 1.3) (Eriksson, Hernroth et al. 2013).

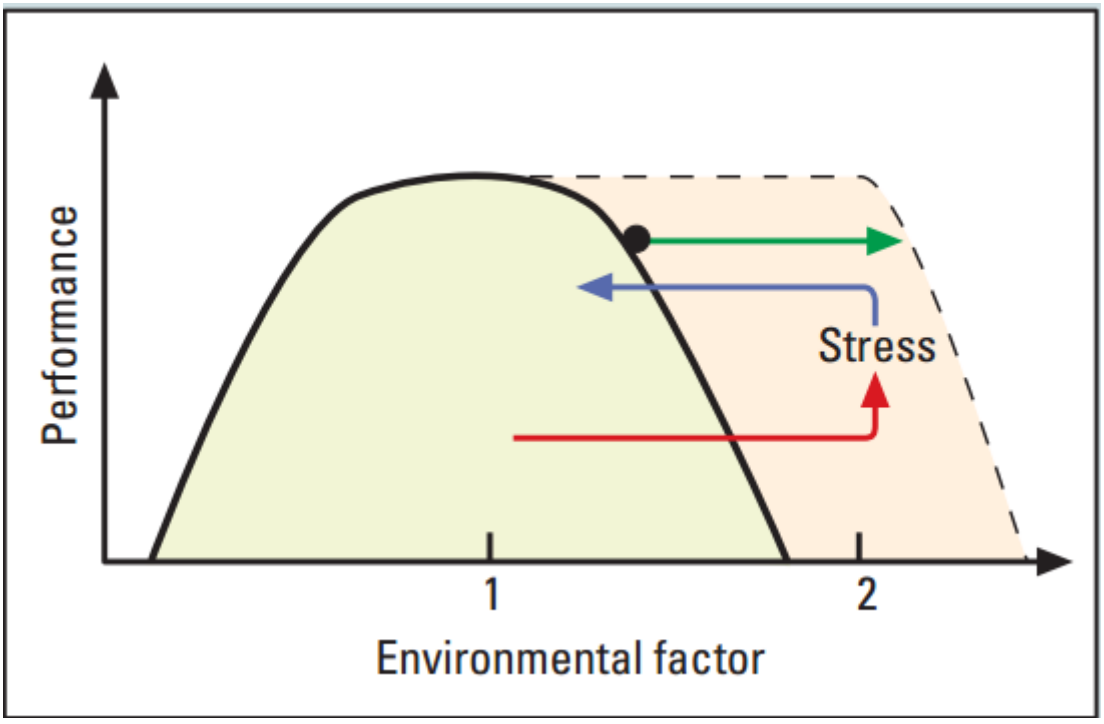


**Figure 1.3 The light green-shaded area represents the ecological niche of a species. Stress arises when an environmental factor increases from point 1 to 2 such that the species is forced out of its ecological niche (red arrowed line). Various stress response reactions provide temporary survival under stress, and a return to the niche (blue arrowed line). If the boarders of the niche are extended through adaptation, what was stress is not stress any more (green arrowed line) (Source: Van Straalen 2003).**

The ecological niche is the environmental conditions and resources necessary for an organism to maintain a viable population (Begon et al. 1996). Stress is when some environmental factor changes and organisms are no longer in the favoured ecological niche (Van Straalen 2003). Stress responses involve physiological mechanisms that counteract consequences of that stress *via* phenotypic plasticity. This provides short-term survival that can be relieved by either returning to the niche, or changing the boarders of the niche by acclimation, i.e. again *via* phenotypic plasticity, or adaptation. Responses to these changes (external factors) can challenge an organism's ability to maintain a stable internal environment (homeostasis). Homeostasis is energetically costly, and such stress compensation requires additional metabolic energy (Koop and Grieshaber 2000), consuming energy and resources in the process. Maintenance and repair

is the continuous effort organisms invest to keep metabolism in a viable state without association to (net) production (Kooijman 2013). Exposure to drivers can therefore trigger elevated energy demands and could lead to energy limitation and reallocation of resources, hence changing the energy budget within the animal. Organisms can be thought of as non-equilibrium, thermodynamically open systems relying on external energy sources and constant energy flow (Sokolova 2013). This notion of net energy balance or "energy budget" was first introduced by Winberg (1960) and its components have been standardized into an equation by the International Biological Program (IBP), in which C is consumption (energy acquired through food ingestion):

$$(1)\quad C = P + R + U + F$$

where P (production) is the energy incorporated into somatic and reproductive tissue; R (respiration) respiratory costs for somatic and reproductive production, basal maintenance metabolism and respiratory cost of locomotor activity; U is the energy execrated; F (faeces) is the energy of the ingested food that has not been absorbed. However, for most aquatic organisms the energy that is absorbed by consumption, is not simply directed into growth and reproduction, but could rather be redirected or distributed between four energy consuming groups or "sinks" growth, reproduction, maintenance and activity (Fig. 1.4) (Clarke 1987).

Metabolic adaptations to environmental stress can involve flexible allocation of energy resources between the four groups described above as well as a switch between different metabolic processes responsible for energy acquisition and conversion (Sokolova 2013).

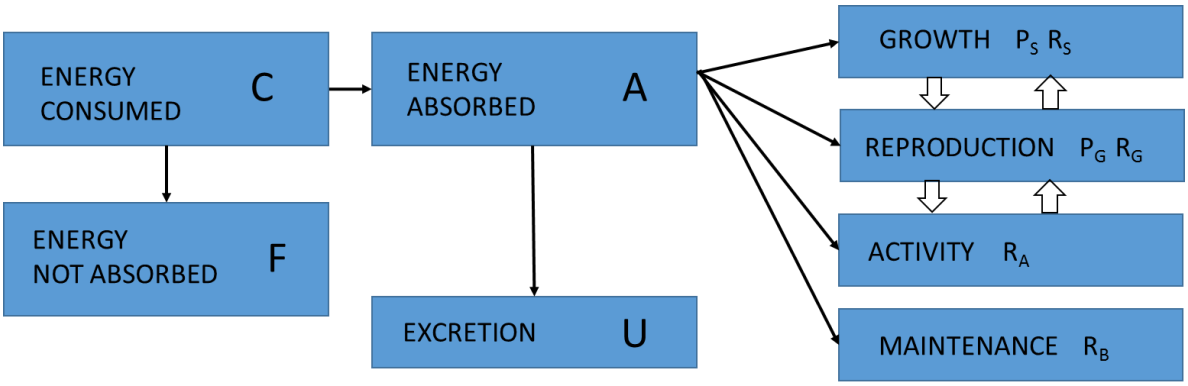


**Figure 1.4 Representation of the major competing physiological sinks in the energy budget of marine organisms redrawn from Clarke (1987).**

Examples of changes in energy budget and a response due to exposure to environmental drivers are energetic costs and energy allocation in the form of reduction in larval growth (Stumpp et al. 2011; Chapter 2; Kroeker et al. 2013; Stumpp et al. 2013). Reduction in growth or developmental rates could lead to larvae spending longer or shorter periods in the plankton before settlement (Moran and Grant 1993). Spending longer times in the plankton would have consequences for larval survival both directly, as natural mortality per day and indirectly, as this would increase risk of predation, and also have consequences for successful dispersal and recruitment (Lamare and Barker 1999; Dorey et al. 2013; Chan et al. 2015).

Changes in development and growth rates due to altered climate variables, e.g. changing temperature could also lead to shifts in phenology (Birchenough et al. 2015) resulting in a potential mismatch with processes timed according to constant cues (e.g. light) (Pörtner and Farrell 2008). The theory of how mismatch between a species and its food source can influence recruitment to a population has been developed by Cushing (1969; 1990) and is termed the match-mismatch hypothesis. Global change such as OW could potentially lead to mismatches between the reproductive cycles and development timing of larvae and its food (Koeller et al. 2009), this match being a key component for the survival and recruitment success of larvae (Ouellet et al. 2011). The mismatch is thought to be most significant during the planktotropic

larval phase and post-recruitment phase for benthic organisms, during which larvae are most dependent on phytoplankton food availability (Birchenough et al. 2015).

The larvae of marine invertebrates are small (high surface area-to-volume ratio) and go through several and complex moulting processes over a limited period of time, making them particularly vulnerable to environmental drivers (Pechenik 1999; Przeslawski et al. 2015). Consequently, particular developmental events (e.g. organogenesis) and particular developmental stages may cause a bottleneck for the success of the species in the future, given survival of a species is dependent on its ability to develop, grow and reproduce. Within a species, its physiological plasticity and ability to adapt will set the limits to how successful the animal will be to cope with stress factors (Eriksson et al. 2013). Insights into the sensitivities of larvae to particular environmental drivers can be provided by investigating the ontogeny of physiological functions throughout the larval development (Spicer and Gaston 1999; Walther et al. 2011; Small 2013; Schiffer et al. 2014; Small et al. 2015).

### 1.1.3 Impact of OA and OW on marine invertebrate development

As described above, the early life histories of marine invertebrates are particularly sensitive to environmental drivers such as OW and OA. Temperature is a major abiotic driver of larval development in marine invertebrates (Anger 2001). Changes in sea water temperature (SST) may result in increased mortality, change the time for spawning and alter embryonic or larval developmental rates. For example, benthic larval components of the zooplankton now peak 30 d earlier, compared to the 1960s, and have shifted forward their seasonality due to changes in SST (Edwards and Richardson 2004). Altered phenological processes or mortality of larvae due to OW has been reported, and this background information has helped inform the choice of the two species (Northern shrimp and Northern krill) in this thesis (Hirst et al. 2003; Koeller et al. 2009; Rindle et al. 2014).

In many marine invertebrates, developmental time decreases with increasing temperature (Weinberg 1982; Weiss, Heilmayer et al. 2009). However, maximum growth, faster developmental time and survival are all limited by the optimum temperature for the organism, or thermal optimum window, according to Pörtner (2012) as presented graphically in Figure 1.5.

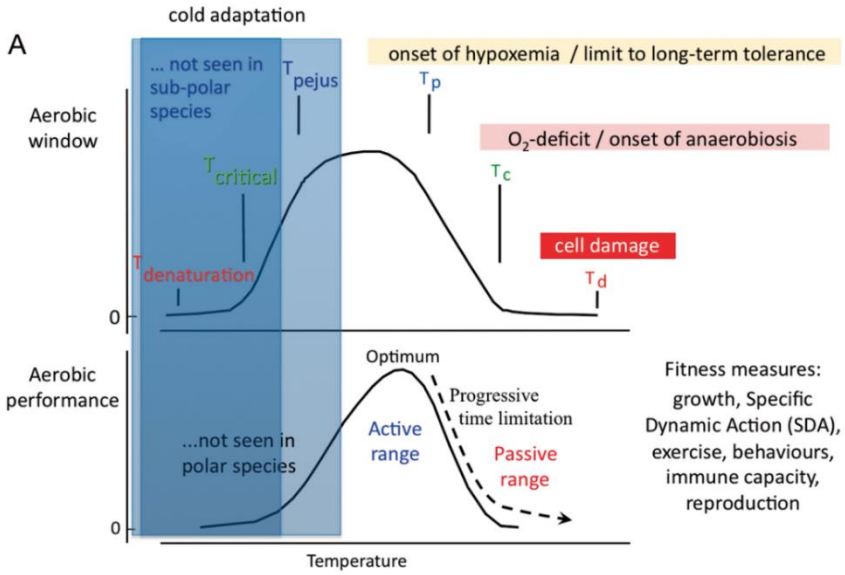


**Figure 1.5 OCLTT (Oxygen and capacity-limited thermal tolerance) as a concept integrating multiple stressors and various processes and their indicators (simplified from Pörtner 2010). (A) Limited thermal windows are set by (aerobic) performance capacity as the first level of thermal limitation. Optimized oxygen supply to tissues between low and high *pejus* temperatures (top) combined with the kinetic stimulation of performance rates by warming supports temperature-dependent performance and a functional optimum (i.e. an optimum of aerobic scope) close to upper *pejus* temperature. Note that in sub-polar and polar climates, due to falling oxygen demand and rising oxygen concentrations, the oxygen limitation of cold tolerance may not be seen, leaving a limitation through functional capacity (Wittmann et al. 2012). (Source: Pørtner 2012).**

For an organism to be able to invest in development, activity and growth, it must be able to sustain an aerobic scope, the fraction of energy flux and metabolic power available after basal maintenance costs are met (Sokolova 2013). The functional capacity of oxygen supply systems,

especially of the cardiovascular system, to fully match demand and sustain such aerobic scope is limited to a thermal window. This window is characterized by its species- and life stage-specific setting and width on the temperature scale (Pörtner 2012) which also has led to the theory of "oxygen and capacity-limited thermal tolerance" (OCLTT). Early life-history stages are proposed to have the narrowest aerobic thermal window, as opposed to juveniles and growing adults (Pörtner and Farrell 2008).

This thermal tolerance, or optimum, could also be narrowed by the combination of other stressors. Both according to the OCLTT concept by Pörtner (2012), and the "energy-limited concept of tolerance to stress" by Sokolova (2013), the ability to maintain positive aerobic scope is the key determinant in long term survival of an organism and their populations. If the aerobic scope is reduced under moderate stress (the pejus range), the aerobic scope is reduced but still remain positive and long term survival of the population is possible, but may come as a cost via reduced growth and reproduction (Sokolova 2013). Examples of such reduction in aerobic scope and consequent reduction of larval growth has been reported for crustacean larvae exposed to OW (Chapter 2 ; Small 2013). Futhermore, exposure to temperature in the upper pejus range can affect swimming performance and impair their ability to avoid predators or settle successfully as shown in the larval stages of the Chilean kelp crab, *Taliepus dentatus* (Storch et al. 2001). Similar stage specific sensitivity to OW and OA has also been found for the great spider crab, *Hyas araneus* (Walther et al. 2010) and European lobster, *Homarus gammarus* (Small 2013) where aerobic windows narrow at specific stages and individuals become more sensitive to OA especially under OW conditions.

Ern et al. (2015) have questioned the OCLTT therory by suggesting that there might be some fuctions that may be dictated by oxygen supply capacity, but others including those responsible for lethal tempeartures, dictated by nervous function. They have suggested an alternative hypothesis "Multiple performance – multiple optima" (MPMO) arguing that physiological

functions have different optimal temperatures and that their relative contribution to fitness varies between species. Furthermore, Clark et al (2013) and Gräns et al. (2014) have reported that the OCLTT theory concept does not apply generally across fish species. They suggest that other plausible factors can be responsible for governering temperature tolerance e.g. oxiditive stress cell damage, protein denaturation, disturbed neural function and effects on membrane fluidity.

Life cycle events and invertebrate development are also dependent on other climate related factors such as OA. The early developmental stages of marine invertebrates are generally negatively impacted by reduced pH associated with OA, both lethal and sub lethal effects have been observed (Dupont and Thorndyke 2009; Bechmann et al. 2011, Stumpp et al. 2011, Chapter 2 ; Small et al. 2015). For example, exposure to OA resulted in longer developmental time, higher mortality, and an increase in morphological abnormalities associated with calcification in bivalves and echinoderms (Kurihara 2008a; Parker et al. 2009; Gazeau et al. 2010; Stumpp et al. 2011; Dorey et al. 2013). Some invertebrate larvae are also able to cope with the effects of OA by energy reallocation (Findlay et al. 2010; Stumpp et al. 2011). Crustaceans are thought to be among the most robust groups to OA (Whiteley 2011; Kroeker et al. 2013; Przeslawski et al. 2015). Early life stages of several crustacean species seems to be resilient to OA (Kurihara and Ishimatsu 2008; Arnold et al. 2009; Egilsdottir et al. 2009; McDonald et al. 2009; Findlay et al. 2011; Pansch et al. 2012; Chapter 2 ; Styf 2014; Small et al. 2015). Nonetheless, it seems like there are species differences in the response to OA, and negative effects on larval development as result of exposure to reduced pH have been reported. Reduced hatching success has been observed for the copepod, *Calanus finmarchicus* and the barnacle, *Semibalanus balanoides* due to exposure of reduced pH (Mayor et al. 2007; Findlay et al. 2011). Furthermore, larval survival decreased in both king crab, *Paralithodes camtschaticus* and spider crab, *Hyas araneus*, for the latter increased developmental time.

Decreased growth rate and lipid content was also reported (Walther et al. 2011; Long et al. 2013).

According to a meta-analysis by Przeslawski et al. (2015) and other recent studies (Sheppard et al. 2010; Small 2013) OW in combination with OA, mitigated the negative effects of pH in most combined studies. This may be due to their faster growth rates and progression through developmental stages due to OW, thus reducing the time in their vulnerable planktonic life phase (Havenhand 1993). However, Gianguzza et al. (2014) reported that temperature appears to modulate the impact of decreasing pH on sea urchin, *Arbacia lixula*, larvae. Leading to a positive effect (faster growth rates compared to pH 8.2) of low pH at 20 °C, a neutral effect at 24 °C, and a negative effect (slower growth rates) at 26 °C, thus showing the response can be dependent upon initial temperature. Impacts of multiple drivers on marine invertebrate development are discussed below (Sect 1.1.5).

### 1.1.4 Impact of oil pollution on marine invertebrate development

Because of the great diversity of invertebrates in the marine environment, a wide range of biological responses to oil is reported. Oil and its degradation products have acute and chronic toxicity to marine invertebrates, and include death from direct oiling, as well as impaired feeding, growth and development (Suchanek 1993; Blackburn et al. 2014). For example, echinoderms can be particular sensitive to oil, and earlier oil spills have resulted in massive die offs (Nelson-Smith 1972), and early planktonic life stages have showed impaired embryogenesis and larval growth rates (Andersen 1991; Bellas et al. 2008; Rial et al. 2013).

Holoplankton (such as diatoms, radiolarians, dinoflagellates, foraminifera, amphipods, krill, copepods, salps), and temporary members (such as most larval forms of sea urchins, sea stars, crustaceans, marine worms, some marine snails, most fish), which are called meroplankton (Sakshaug et al. 2009) are exposed to both floating oil slicks, dissolved oil components and

oil droplets in the water column. The effects of an oil spill depend on where it happens, the amounts of oil, duration of the spill, oil type, wind and the state of the ocean with regards to mixture, temperature and currents (Grøsvik et al. 2014). Micron sized oil droplets can be formed naturally following an oil spill in the marine environment through the action of breaking waves (natural dispersion), this causes the oil to break and transports the oil droplets into the water column (Skadsheim et al. 2000; Hansen et al. 2012). Furthermore, sub-sea blowouts of oil at depths below 100 m will also have a size distribution of small oil droplets between 2 and 20 µm into the water column (Rye et al. 2000). For example, the polycyclic aromatic hydrocarbons (PAHs) in the water column after the blowout from The Ekofisk Bravo oil platform in 1977 was 8 µg $L^{-1}$ near the well and about 0.1 µg $L^{-1}$ at the edge of the area of floating emulsion (Wells et al. 1995). After the Exxon Valdez 1989, an oil tanker accident, it was found 12 µg $L^{-1}$ of PAHs 10 m under the slick, one week after the accident, however samples taken after the oil spill path during 1998 - 1999, 90 % of the samples contained less than 1 µg $L^{-1}$ total PAHs (Wells et al. 1995). Filter feeders such as some species of zooplankton filter particles in the same size range as small oil particles making them bioavailable to the organism (Gyllenberg 1981; Hansen et al. 2012). Furthermore, larvae are more sensitive to toxic fractions of oil and other stressors, due to their greater surface area to volume ratio, which produces a greater uptake rate of a contaminant, and the inability to move away from unfavourable environments. According to Suchanek (1993) larvae may be affected by oil by: 1) impaired abilities to avoid a predator; 2) reduced ability to locate suitable settlement sites; 3) limited ability to attach and/or maintain initial attachment to the preferred substrate; 4) lowered defense abilities against predators or competitors, and 5) inability to feed, grow or mature.

Generally, the toxicity of crude oil is attributed to the water-soluble monoaromatic hydrocarbons (MAHs) (i.e., benzene, toluene, ethylbenzene and xylene, commonly referred to

as BTEX) and PAHs (e.g. naphthalene's and a number of methylated derives). BTEX are fairly volatile compounds and are not expected to persist in the environment. PAHs are less volatile and the heavier PAHs (those with 4 or 5 rings) are known to persist and they can potentially have a long-term impact on the aquatic environment (Alford et al. 2015).

One mechanism that has been proposed to address the toxicity to hydrocarbon mixtures such as those found in crude oil, is non-polar narcosis, which results in the alteration of cell membrane function (Alford et al. 2015). PAHs and BTEX as lipophilic substances are easily taken up from the water phase and portioned into the cellular membranes of invertebrates (Douben, 2000). The presence of PAHs in the cellular membranes could subsequently lead to alterations in the membranes and can result in mild toxic effects or mortality depending upon the exposure via the non-polar narcosis (Klaasen 1996, Douben, 2000). Narcosis is a reversible state of arrested activity and can be caused by a wide variety of organic chemicals (Veith et al. 1983). For example, exposure of larvae of King crabs (Paralithodes) to water-soluble fractions of oil (WSF) of 0.5 mg $L^{-1}$ resulted in swimming cessation and ultimately death (Brodersen 1987). Furthermore, reduced feeding during oil exposures has been reported in several other studies most likely due to reduced activity (Berdugo et al. 1977; Hansen et al. 2012), and reduced growth (most likely due to reduced feeding) has been observed for sea urchins (Bellas et al. 2008). Furthermore, long term exposure to an oil concentration of 0.06 mg $L^{-1}$ to both embryo and larvae, resulted in significantly higher mortality of larvae, compared to the control (Taban et al. 2007; Bechmann et al 2010).

These negative impacts on early life stages of echinoderms and crustaceans show that they are more sensitive to pollution than adults are. This is important because their survival is critical for the survival of adult populations (Blackburn et al. 2014).

### 1.1.5 The impact of multiple drivers on marine invertebrate development

Marine invertebrates are rarely exposed to single drivers, but often to a mixture, or cocktail, of different drivers *in situ*. Although it is accepted that there is an urgent need for experiments investigating the effects of multiple drivers (Melatunan et al. 2011; Pistevos et al. 2011; Garrard et al. 2012; Byrne and Przeslawski 2013; Todgham and Stillman 2013) we are only beginning to understand their effects in combination. When multiple drivers act simultaneously, the effects could be additive (where a response can be predicted based on the effects of the single driver) synergistic, or antagonistic (where a response is greater or lesser than would be predicted from adding the independent effects of driver respectively) (Ghedini et al. 2013).

Many novel reviews on combined effects suggest that many drivers, combine in non-additive ways (i.e. synergistic or antagonistic) (Harvey et al. 2013; Boyd and Brown 2015; Przeslawski et al. 2015). However, other reviews suggest that synergies are not the norm, and that there is not sufficient evidence for synergies being the most common outcome of combined drivers (Kroeker et al. 2013; Ban et al. 2014). Furthermore, Brennan and Collins (2015) show that the number as well as the identities of drivers explained shifts in population growth rates in green algae: they demonstrated that the response to multiple environmental drivers depends on the response to the dominant driver, and the chance of a driver of large effect being present increases with the number of drivers. Furthermore, they demonstrated that interactions between drivers slightly counteracts the expected drop in growth and that population growth declined in a predictable way with the number of drivers.

Meta-analyses have been carried out to evaluate general patterns in marine embryos and larval responses to such multiple drivers (e.g. temperature, salinity and pH), and Przeslawski et al. (2015) find that interaction types varied among drivers, ontogenetic stages and biological responses, but were more consistent among phyla. Furthermore, they reported stage-specific effects, for example, that larvae were more vulnerable than embryos, and that early life stages

of echinoderms and mollusks were more vulnerable than the more robust arthropods and cnidarians to abiotic drivers. In addition to this Przeslawski et al. (2015) reported temperature to be the main driver and it mitigated negative effects of both pH and salinity, also reported by several other studies (Chapter 2; Small 2013; Styf 2014; Small, Calosi et al. 2015).

Several studies have also reported that embryonic life stages are less vulnerable than larvae (Chapter 2; Small 2013; Styf 2014). This could be because of their loading of protective stress proteins (Hamdoun and Epel 2007) and brooding species might be more resilient to abiotic drivers (Lucey et al. 2015), because brooding and encapsulation strategies are typically assumed to confer greater safety and protection to embryos.

Several researchers have highlighted the need for studies investigating the effects of global drivers combined with local anthropogenic drivers (Nikinmaa 2013; Przeslawski et al. 2015). The few recent studies looking at the potential for interactions between different global change drivers of toxicants are showing several different interaction patterns. These studies show that effects can be synergistic, antagonistic and additive (Pascal et al. 2010; Fitzer et al. 2013; Roberts et al. 2014; Styf 2014; Coelho et al. 2015). Regardless of the lack of consensus about how different drivers interact with each other, most researchers agree that it is challenging to identify interactive patterns across studies, and that there is a need for better designed experiments to be able to project the impact of multiple drivers on marine ecosystems to a certainty (Brown et al. 2013; Boyd and Brown 2015; Breitburg et al. 2015; Przeslawski et al. 2015).

## 1.2 THESIS AIMS AND OBJECTIVES

### 1.2.1 Background

Benthic marine invertebrates and their planktonic life stages face multiple environmental challenges in the ocean. There will be areas where combined or complex environmental challenges will occur with greater frequency and intensity than others, causing organisms to be exposed to multiple drivers including OA, OW, persistent organic pollutants and accidental discharges from the oil industry and from ships. Whilst we know much of the biological effects of each of these drivers individually, we are only beginning to understand their effects in combination. It has been highlighted that there is an urgent need for experiments investigating the effects of multiple drivers, which can provide a more realistic picture of what animal's experience in the wild (Melatunan et al. 2011; Pistevos et al. 2011; Todgham and Stillman 2013; Przeslawski et al. 2015). Nevertheless, until now the number of such studies has been limited by the logistic difficulties of carrying out experiments where the number of variables introduced quickly results in an unmanageable design (e.g. (Melatunan et al. 2011; Pistevos et al. 2011).

Therefore, the majority of ocean management strategies are not yet addressing multiple drivers in a coherent way; there is a lack of the combined perspective that addresses the impact of multiple drivers simultaneously (Noone et al. 2012). Multiple drivers have different interaction patterns see sect. 1.1.5 (Ghedini et al. 2013). Most importantly in ecological studies is that to understand the effect of a driver and the most effective way to manage them, is to identify whether there are interactive effects among drivers and of what type these are (Ghedini et al. 2013; Boyd and Brown 2015). Currently, the potential interactions between OA, OW and pollutants have not been well addressed (Nikinmaa 2013; Zeng et al. 2015).

### 1.2.2 Overall aim

**The overall aim of this thesis was to address some of the gaps in our knowledge of the single and combined interactive effects of multiple anthropogenic drivers, i.e. the effects of OA and OW (Chapters 1 and 2), and OA/OW with oil (Chapters 3 and 4) on the development, physiology and morphology of key-stone invertebrates, the Northern shrimp, *Pandalus borealis,* Green sea urchin, *Stongylocentrotus droebachiensis* and the Northern krill, *Meganyctiphanes norvegica*.**

The thesis focuses on early developmental stages, since larvae and juveniles are generally considered more vulnerable to environmental perturbations (as mentioned above), and their survival will largely determine population recruitment, and thus ultimately abundance, distribution and community structure (Kurihara and Ishimatsu 2008). The study species have been chosen since they are all cold-water key species (Sect. 1.4) in North Atlantic Ocean habitats, with broad distribution patterns including the Arctic Ocean, and with economic and ecological importance. Furthermore, the chosen species are cold-water species and could be particular sensitive to changes in temperature; tolerance windows are wider in tropical species than in polar species (Pörtner 2001). Thermal tolerance windows however are narrow in most species and thus sensitivities to combined stressor affects are likely to be higher in cold-adapted species rather than in species adapted to warmer seas (Jacob and Woodward 2012). Furthermore, the species live in the high latitudes where climate change is expected to be most pronounced (Kristiansen 1993). In addition to this, some of the species have distribution into the Arctic, and Arctic surface waters are predicted to become under saturated with aragonite by 2050 (Orr et al. 2005). Aragonite is an important element in the shells and tests of many marine planktonic invertebrate larvae. Hence, it is particularly important to study effect of global

change on organisms living in high latitude cold surface waters i.e. with planktonic and pelagic life stages such as the study species.

Invertebrates utilize broadcast spawning for propagation, and early life history stages occur in the water column where ocean change stressors – warming, acidification, hypercapnia and pollution are likely to have a deleterious impact on development (Byrne 2011). A high percentage of planktonic larvae die due to natural causes (predation, lack of food etc.). The species larvae are also planktotropic and dependent on feeding during the larval stages, thus the timing of the food source/quality are very important. Adding anthropogenic stressors like OA, OW and pollutants may increase the total mortality of early life stages and possibly cause reduced population size with time. Planktonic stages may be a population bottleneck in a changing ocean (Pechenik 1999).

Other life stages (adult and embryos for shrimp, juvenile for krill) were too included in the study because of the importance of investigating multiple life stages to provide a comprehensive assessment of the cumulative (potential) impacts of multiple drivers on a target species. This has already been suggested by other studies (Walther et al. 2009; Byrne and Przeslawski 2013; Kroeker et al. 2013; Sperfeld et al. 2014; Przeslawski et al. 2015; Small et al. 2015). Furthermore, stage-specific effects of both OA and OW have already been reported (Small 2013, Styf 2014). For example, the embryonic coating of shrimp eggs (Glas et al. 1997) may protect the shrimp embryos from exposure to the drivers, making them more resilient than other invertebrate larvae.

The choice of three species also makes one able to compare species sensitivity to the drivers, since species-specific effects of OA, OW and oil have been previously reported (Dupont and Thorndyke 2009). The difference in life history strategy may affect the tolerance of the early life stages. For example, shrimps hatch as zoea, a larger and more developed larval stage than

the krill nauplii, krill also go through even more developmental stages during the first period of their lives, therefore krill larvae may be more sensitive to the drivers than the shrimp larvae.

### 1.2.3 Aims and objectives of individual chapters

The aims and objectives of each chapter are as follow

Chapter 2

Aim: To characterize the physiological and developmental responses of larval stages of the Northern shrimp (*Pandalus borealis*) when exposed to reduced seawater pH and elevated temperature, both in isolation and in combination.

This will be achieved by rearing larvae under reduced pH (7.6) conditions predicted for the year 2100 (IPCC 2014)., and one of two water temperatures a control of 6.7 ºC, representing current seasonal average when the experiments were carried out, and 9.5 ºC representing a + 3 ºC associated with ocean warming (Solokov et al 2009). Individuals will be sampled at each developmental stage, and survival and hatching timing and success will be determined. Growth and developmental timing will be determined by staging and dry mass and length measurements. Oxygen consumption will be determined using closed respirometry techniques; in addition feeding rates for different stages will be determined preforming feeding tests. Feeding test are important since the larvae are planktotrophic. Use of proxies will allow the investigation of different larval stages sensitivities, together with changes in underlying physiology, which can link to altered life history traits as a result of these environmental drivers.

Chapter 3

Aim: To characterize the physiological and developmental responses of larval and juvenile Northern krill (*Meganyctiphanes norvegica*) when exposed to reduced pH at different temperatures.

This will be achieved by rearing larvae and juveniles under two different exposure treatments: (1) *current* (8.1 pH unit and 6.7 ºC water temperatures, conditions that the larva experience today) and (2) *future* (7.6 pH unit and 9.5 ºC forecasted pH and temperature for 2100). Juveniles were exposed for 14 d and larvae were exposed 30 d.

Larvae will be sampled at each developmental stage, and survival and hatching timing and success will be determined. Growth and developmental timing will be determined by staging and length measurements. Rates of feeding rates and oxygen consumption will be determined for larvae using feeding trials and closed respirometry respectively. Moulting success will also be determined as this is directly linked to favorable trophic and environmental conditions. Such proxies will allow the evaluation of different larval stages and juveniles sensitivities, along with changes in underlying physiology, which can link changes in life history traits to these environmental drivers.

Chapter 4

Aim: To investigate the effect of exposure to a realistic oil spill scenario on the development of embryos and larvae, and aspects of the physiological ecology of adults of the Northern shrimp *P. borealis* under "*current*" and a "*future"* ocean scenarios.

This will be done by rearing adults and larvae under two different exposure treatments : (1) *current* (8.1 pH unit and 6.7 ºC water temperatures, conditions that the larva experience today) and (2) *future* (7.6 pH unit and 9.5 ºC forecasted pH and temperature for 2100). The effect of both exposure treatments will be assessed with and without oil spill.

As female condition is important for the development of the shrimp embryos, feeding, respiration rate, and lysosomal membrane stability of adult females was investigated, as was hatching success, duration and time. Individual larvae will be sampled at each stage, and survival and hatching timing and success determined. Growth and developmental timing will be determined by staging and dry mass and length measurements. Oxygen consumption will be determined using closed respirometry. In addition feeding rates for different stages will be determined preforming feeding tests. Feeding test are important since they are feeding planktotrophic larvae. In addition, the swimming activity of the larva will be documented as oil may have narcotic effect on the larvae. Such proxies will allow the evaluation of different larval stages sensitivities, along with changes in underlying physiology, which may be linked to alterations in life history traits in response to these environmental drivers, oil spill, and their combination.

Chapter 5

Aims: To investigate (1) how echinoderms respond to an oil spill when cultured "current" and "future" conditions (2) interactions between a transient pollutant and *future* condition on aspects of echinoderm function.

This will be achieved by rearing larvae under two different exposure treatments: (1) *current* (8.1 pH unit, which are conditions that the larva experience today) and (2) *future* (7.6 pH forecasted pH for 2100) with and without oil spill. Individual larvae will be sampled at each day, and survival and hatching timing and success determined. Growth and developmental timing will be determined by morphological and length measurements. Oxygen consumption will be determined using closed respirometry; in addition feeding rates for will be determined preforming feeding tests during and after oil spill. Feeding tests are important since they are feeding planktotrophic larvae. In addition, activity of the larva as swimming response will be

examined as oil may have narcotic effect on the larvae. Such proxies will allow the evaluation of different larval stages sensitivities, along with changes in underlying physiology, which can link altered life history traits to these environmental drivers and oil spill and their combination.

## 1.3 TEST CONDITIONS/RESEARCH FACILITIES

The pH and temperature test conditions chosen for the experiments presented in this thesis were based on the "business as usual" emission scenarios for this century presented by IPCC (IPCC 2014) in addition to regional scenarios that were considered (Blackford and Gilbert 2007; Førland et al. 2009; Steinacher et al. 2009). European Project on Ocean Acidification (EPOCA) guidelines were followed (http://www.epoca-project.eu/index.php/guide-to-best-practices-for-ocean-acidification-research-and-data-reporting.html) in designing the experiments.

The oil concentration used in my experiments (0.5 mg $L^{-1}$ Arctic crude oil) simulates relevant oil concentrations in the water column following an oil spill. The concentration of total PAHs was 3.62 - 5.45 µg $L^{-1}$ (see section 1.1.4).

All experiments were performed at the marine research facility of International Research Institute of Stavanger (IRIS). IRIS research facility has built up an exposure system for continuous flow exposure to seawater with reduced pH (increased $pCO_2$) (Fig. 1.6). In addition, it was possible to regulate/set the temperature of the sea water and/or the room temperature. IRIS also has a continuous flow system for producing mechanically dispersed oil (Sanni, Oysaed et al. 1998).

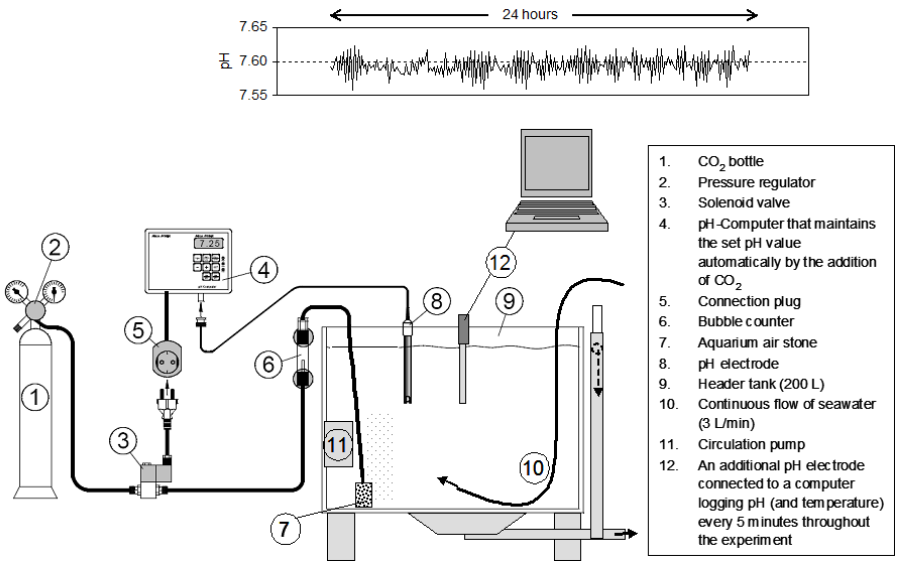


**Figure 1.6 The figure shows a reduced pH exposure system headertank, reproduced from the aqua medic operation manual (http://www.aqua-medic.com/pH_computer.shtml). The pH of the header tanks was recorded every five minutes. The figure shows the variation in pH with time during 24 hours. Different number, volume header tanks and flows where used in different experiments depending on the species and exposure (see Chapter's material and methods for exposure systems). (Drawing: courtesy of Renée K. Bechmann).**

## 1.4 STUDY SPECIES

### 1.4.1 Northern shrimp (*Pandalus borealis*)

Northern shrimp *Pandalus borealis* is an ecologically and commercially important species found in the colder regions of the North Atlantic and Pacific Oceans and represents a large proportion of the biomass of invertebrates in the Barents Sea and areas around Svalbard (Butler 1964; Garcia 2007). In 1992, the Northern shrimp from the Pacific Ocean was recognised as a separate Pacific species, and not a variety of *Pandalus eous.* However, the situation may still be unclear as it is reported that the two species may interbreed although some have reported they may be reproductively isolated.

Populations of Northern shrimp are found at the temperature range 1.6 - 11°C (Shumway et al. 1985). Northern shrimp in the North Atlantic breeds once a year, the female's eggs are usually extruded late summer to early autumn, and are carried on the pleopods (of the females) until spring (Bergstrom 2000). In the population examined here, eggs fertilization occurs in October-November, and hatching usually occurs in March (Bechmann et al. 2011). The bottom seawater temperatures in the collection area are fairly constant at 6 – 8 °C throughout the year (Arnberg and Bechmann pers. obs.). Subsurface seawater temperatures vary from year to year. However over the period 2001 - 2012, maximum seawater temperatures (10 – 15 °C) in the local system occurred in autumn, and the lowest water temperatures (4 – 8 °C) usually occurred in February-March, hence shrimp larvae hatch during the low-temperature period. The planktonic larvae develop through six different stages (I - VI) over 2 - 3 months after which they metamorphose to benthic post larvae (Fig. 1.7). The planktonic larvae live near the surface at 5 - 20 m depth for several months depending on temperature and feed on phytoplankton and small zooplankton (Koeller et al. 2009). Juveniles first mature as males, mate as males for one too several years, and then change sex to spend the rest of their lives as mature females (Bergstrom 2000).

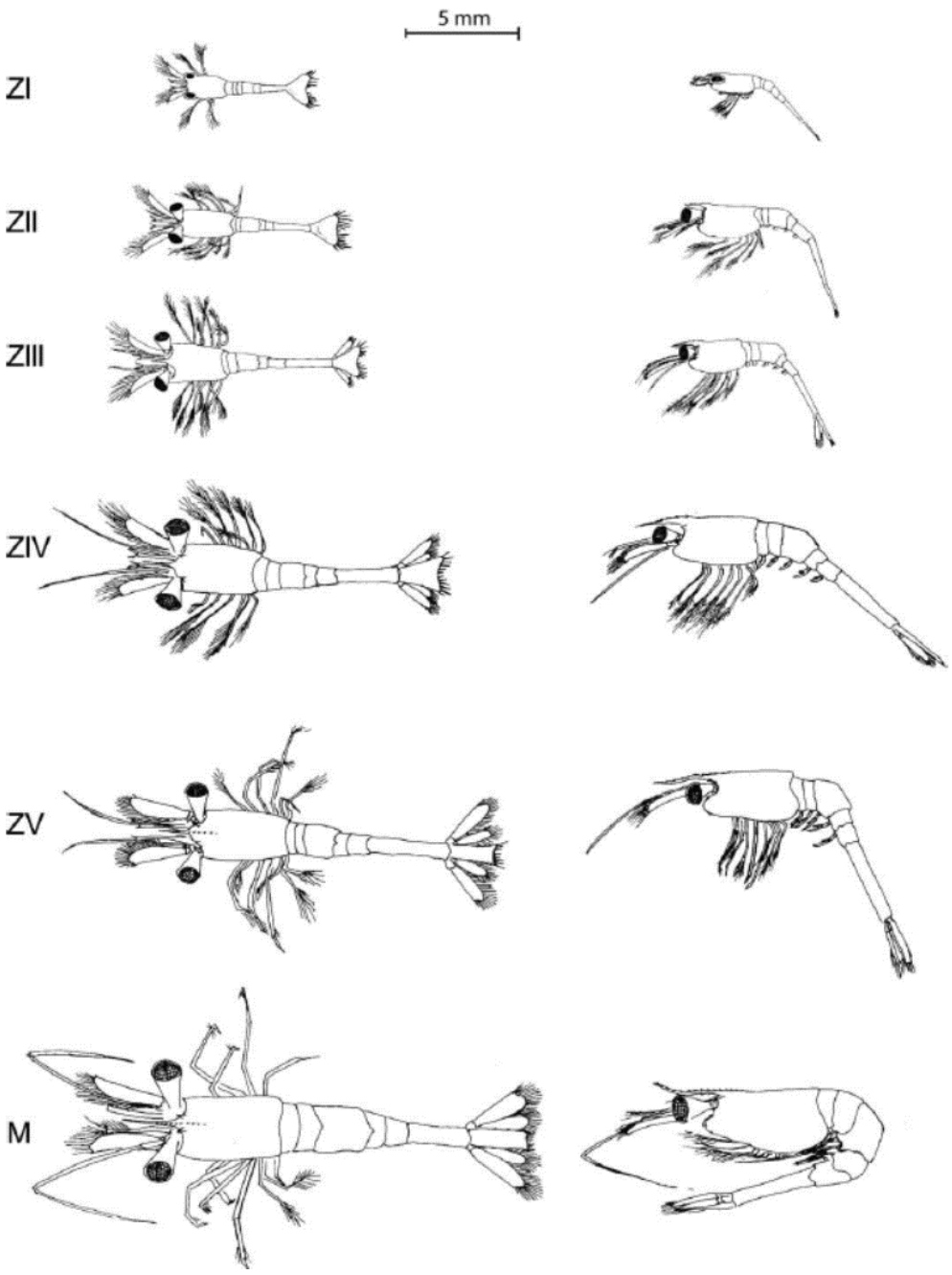


**Figure 1.7 Schematic drawing of the larval stages of *P. borealis*. Females beat their pleopods or swim freely in the water and the larvae (stage I) are released in the water column. The larvae go through a series of moults starting with stage I, stage II stage III, stage IV, stage V and a sixth transitional stage, the megalopa (M). Juveniles first mature as males, mate as males for one to several years and then change sex to spend the rest of their lives as mature females (with permission from Rasmussen and Aschan (2011)).**

### 1.4.2 Northern krill (*Meganyctiphanes norvegica*)

Northern krill *Meganyctiphanes norvegica* are found in the North Atlantic and the Mediterranean Sea, and are also abundant throughout the year in Norwegian fjords (Mauchline and Fisher 1967; Wiborg 1970). The populations are adapted to very different environments and trophic conditions, through the northern limits in Disco Bay (as cold as 2 °C), and along the European coast (5 – 10 °C) and down to the Lingurian Sea up to (18 °C).

Northern krill perform a diel vertical migration, which is limited to less than 100 m in shallow environments (e.g. Liljebladh and Thomasson (2001), but reaches more than 500 m in the Ligurian Sea (Tarling et al. 1999). Seasonal alterations in the distributions of the Northern krill has also been reported by Glover (1952). It moves toward coastal areas during the period January to May, and to deeper oceanic areas in June to December. The spring aggregations in coastal waters may be associated with mating and breeding, and once this is accomplished, dispersion takes place (Mauchline and Fisher 1967). This seasonal distribution pattern may be the case for the fjord population in the current study. Northern krill is abundant during spring, but few krill have been observed during trawling in the summer (Arnberg and Bechmann pers. obs.). Investigations of Northern krill in fjords of the west coast of Norway reported spawning to start in March-April or sometimes throughout June (Wiborg 1970). Hatching usually occurs in March (Arnberg and Ingvarsdottir *pers*. obs). The bottom seawater temperatures in the collection area are fairly constant at 6 - 8 °C throughout the year (Arnberg and Westerlund *pers*. obs.). Subsurface seawater temperatures vary from year to year. However over the period 2001 - 2012, maximum seawater temperatures (10 - 15 °C) in the local system occured in autumn and the lowest water temperatures (4 - 8 °C) usually occurred in February-March, hence krill larvae hatch during the low-temperature period.

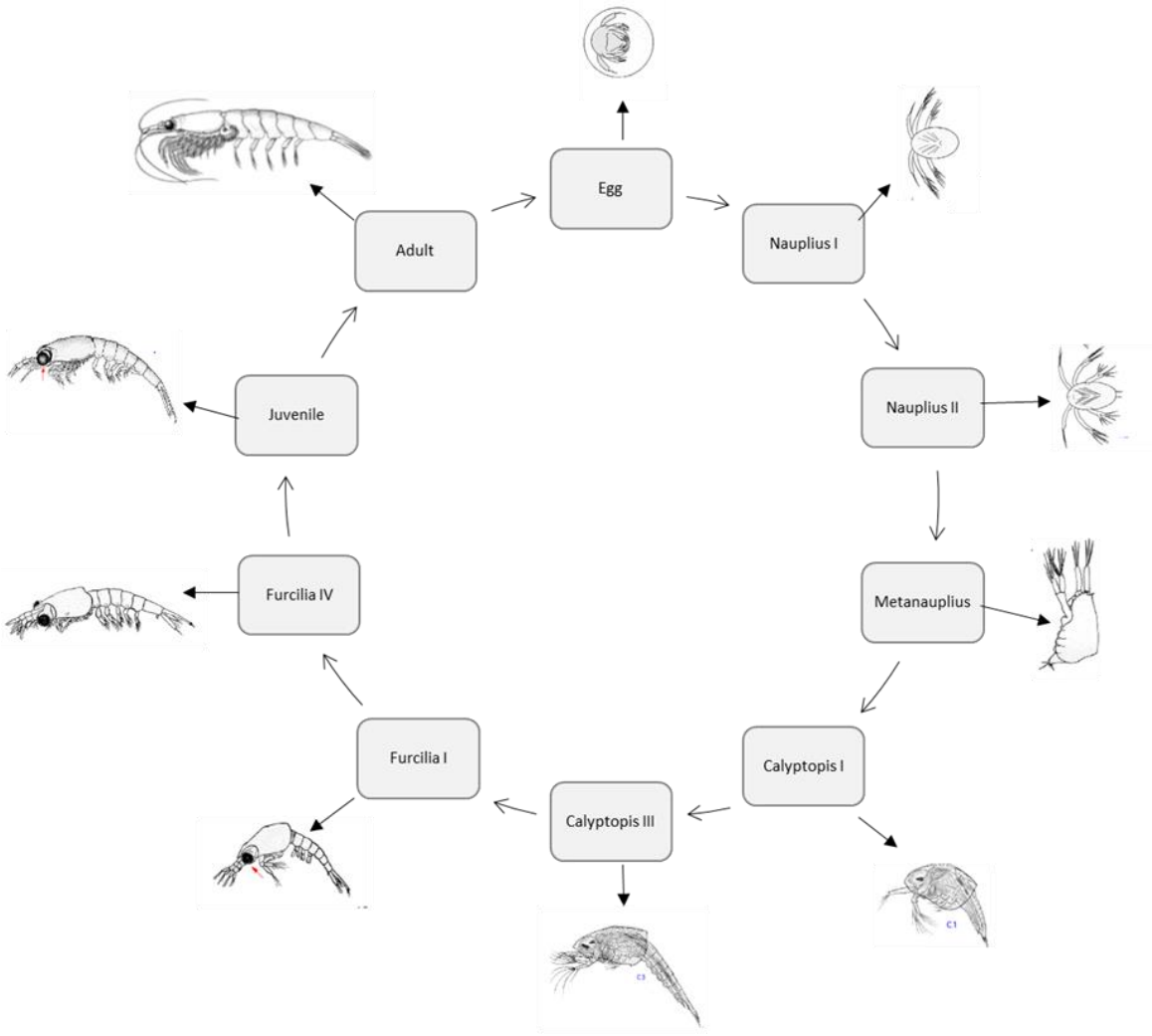


**Figure 1.8. Schematic life cycle of *M. norvegica*. Adults release fertilized eggs into the water column. Pelagic embryos develop and hatch, then go through a series of moults starting with nauplius (2 stages), metanauplius (1 stage), calyptopis (3 stages), and furcilia (7 stages) larvae. Juveniles become sexually mature after 9 - 12 months when they become adults. (Source of drawings the Marine Species identification portal after Lebour (1924), http://species-identification. org/species).**

The planktonic krill larvae go through a series of stages punctuated by moults (57 - 68 d dependent upon temperature), starting with the nauplius I and ending with the furcilla VII stage (Mauchline 1977) (Fig. 1.8). Both embryonic and larval development is known to be temperature and pH dependent (Hirst et al. 2003; Tarling and Cuzin-Roudy 2003; Yoshida et al. 2004). Northern krill is a member of a group of crustaceans called euphausiids, with a worldwide biomass in excess of 300 million tonnes; it is dominant in the pelagic communities

of the Southern Ocean, North Pacific and North Atlantic (Tarling et al. 2010). Consequently, Northern krill constitutes a major link between the primary productivity and secondary consumers and plays a significant role in the exchange of nutrients and material between the benthic and pelagic food webs.

### 1.3.3 Green sea urchin (*Strongylocentrotus droebachiensis*)

The green sea urchin *Strongylocentrotus droebachiensis*, is the most widely distributed member of the family Strongylocentrotidae, having a broad Arctic-boreal distribution ranging from temperate waters as far south as England and Puget Sound, to Arctic waters as far north as 81 degrees N (Scheibling and Hatcher 2001). The green sea urchin can tolerate a wide range of temperatures (range ≈ - 1 °C to 18 – 20 °C; optimum ≈ 9 °C to 13 °C), and compared to other sea urchins it can tolerate quite low salinities (down to S = 27) (Dorey 2013). The adult green sea urchin usually occurs on rocky substrates, and is most commonly found in the shallow subtidal from 0 - 50 m, but can also be found as deep as 300 m (Scheibling and Hatcher 2001)

It is a fast growing sea urchin, and develops gonads during the spring of their third year (Raymond and Scheibling 1987). Spawning usually occurs in early spring when the sea urchin release their eggs or sperm; directly into the water column where fertilization occurs. The sea urchin then goes through two major planktonic life stages in 4 - 21 weeks (blastula/gastrula and pluteus larvae), after which they metamorphose into benthic juveniles, and then to adults (Fig. 1.9). The metamorphosis from larva to a radially-symmetrical adult is complex and transitions from one developmental stage to another generally require major morphological and ecological changes (Dorey 2013)**.** Finally, green sea urchins are an ecologically important species as they play a key role in determining the distribution and abundance of macro algae, particularly kelps (Tegner and Dayton 2000). More recently, it has become commercially important because it is

fished and farmed for its roe (Hatcher and Hatcher 1997). The green sea urchin is therefore one of the most studied sea urchin species on the planet (Scheibling and Hatcher 2001).

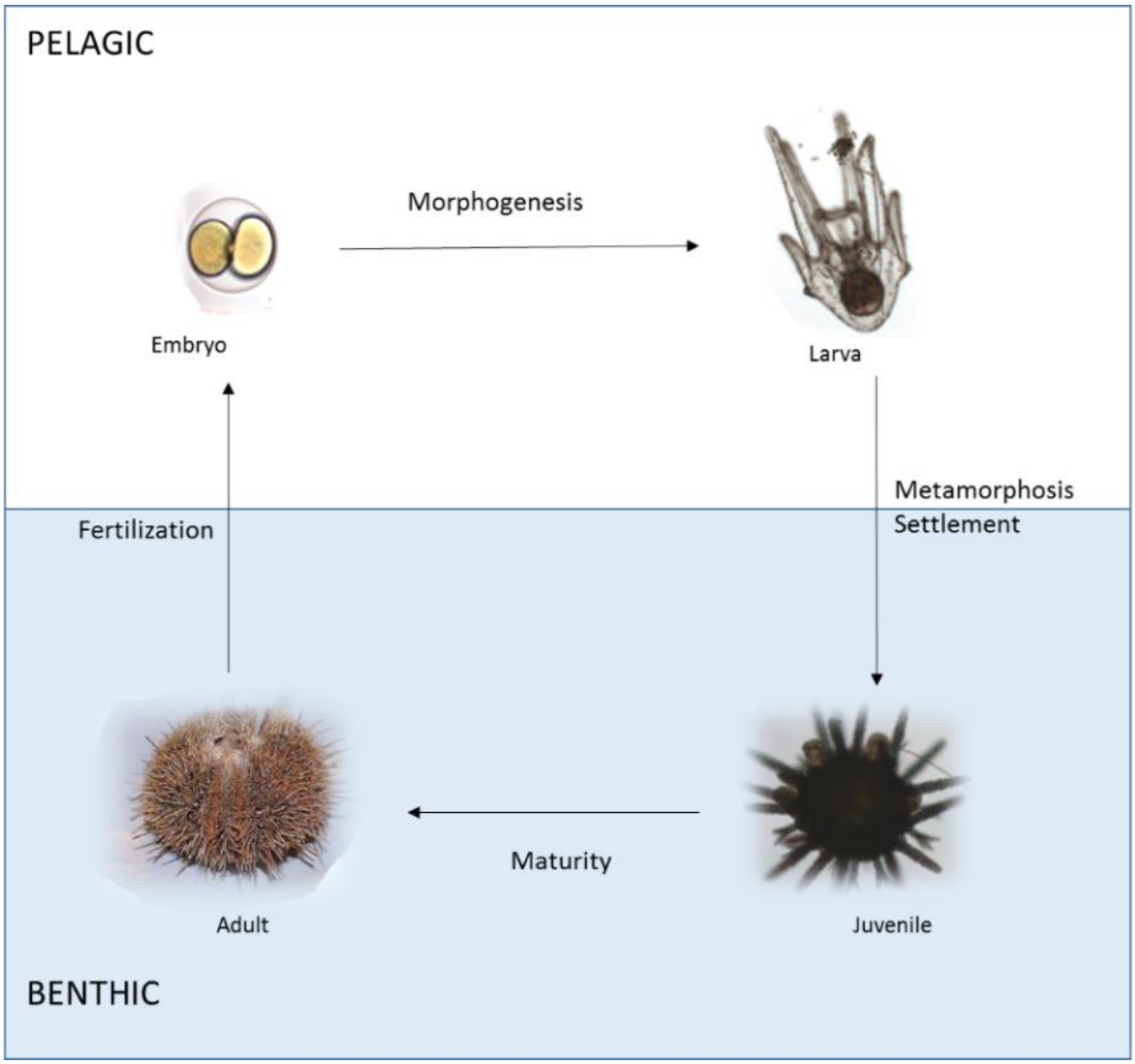


**Figure 1.9. Schematic life cycle of *S. droebachiensis.* Benthic adults release eggs and sperm in the water, where fertilization occurs. Pelagic embryos develop into pluteus larvae, which metamorphose, and transform into a benthic juvenile. Juveniles sexually mature after 3 years and become adults. (Adapted from Figure 8, in Dorey (2013)).**

## 1.5 COLLECTION SITES

Northern shrimps and Northern krill were collected *circa* 59° 04' 00'' N - 5° 45' 00' E in Hillefjord, north of Åmøy, Rogaland County, Norway. Shrimps were collected on two occasions: February 2010 and January 2012. Northern krill were collected February - March 2012 (Fig. 1.10). Sea urchins were collected by divers in Lysefjord at 15 m depth in February 2011 (Fig. 1.10).

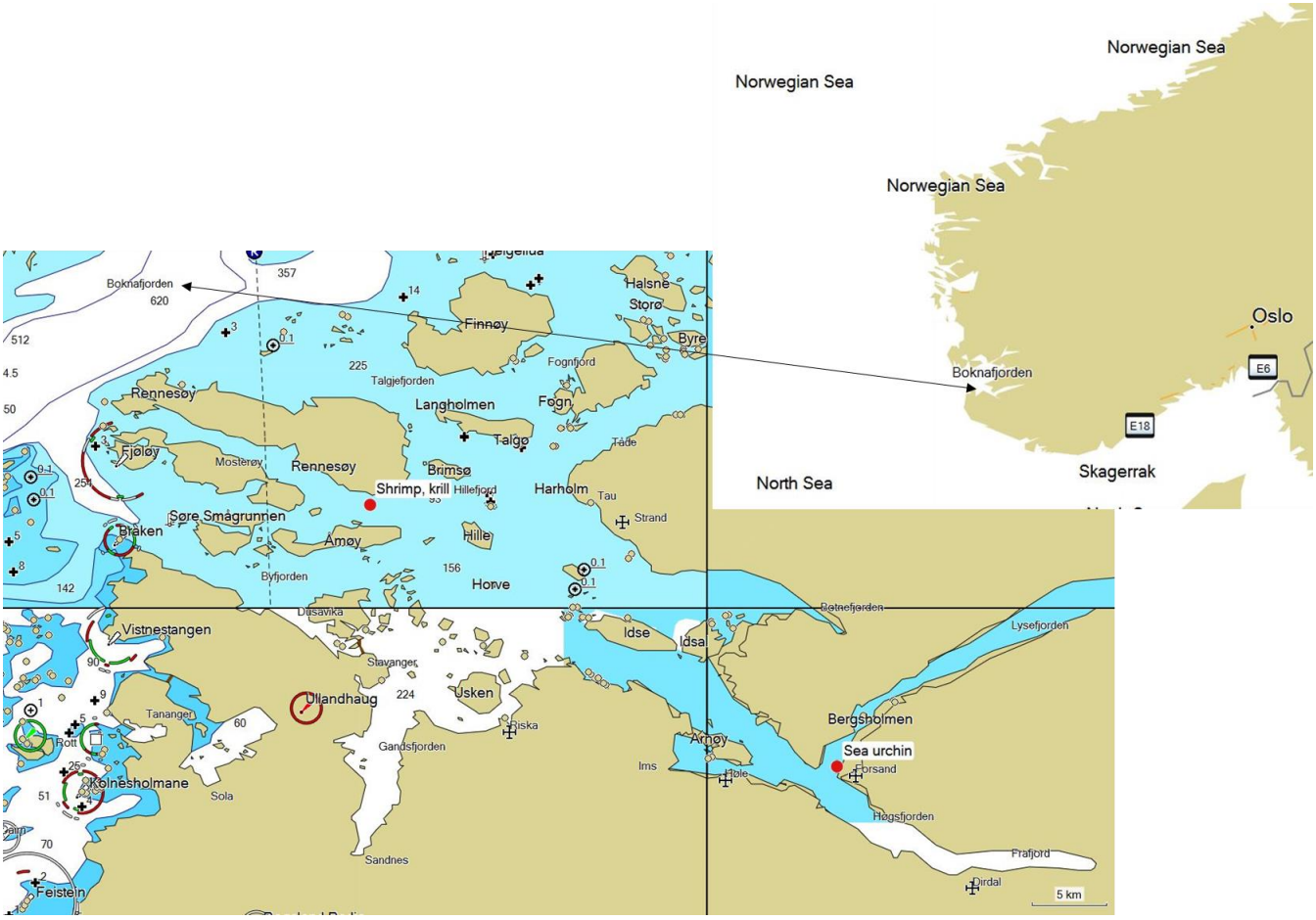


**Figure 1.10. Sampling locations for Northern shrimps, Northern krill and the Green sea urchins are indicated by the red circles on the detailed map, the expanded inset shows where detailed map are within Norway.**

**Chapter 2 – Combined effects of decreased pH and elevated temperature on development, feeding and metabolism of the Northern shrimp (*Pandalus borealis*) larvae.**

**Figure 2.1 Larva of Northern shrimp *Pandalus borealis* (Photo: IRIS).**

## 2.1 SUMMARY

Climate models predict that the average temperature in the North Sea could increase 3 - 5 °C and surface-waters pH decrease 0.3 - 0.5 pH units by the end of this century. Consequently, the combined effect of decreased pH (Control pH 8.1; decreased pH 7.6) and elevated temperature (Control 6.7 °C; elevated 9.5 °C) on the hatching timing and success, and the zoeal development, survival, feeding, respiration and growth (up to stage IV zoea) were investigated for the Northern shrimp *Pandalus borealis*. At elevated temperature, embryos hatched 3 d earlier, but experienced 2 - 4 % reduced survival. Larvae developed 9 d faster until stage IV Zoea under elevated temperature and exhibited an increase in metabolic rates (ca 20 %) and an increase in feeding rates (ca 15 - 20 %). Reduced pH increased development time but only at the low temperature. The conclusion is that warming will likely exert a greater effect on shrimp larval development than ocean acidification (OA) manifesting itself as accelerated developmental rates with greater maintenance costs, and decreased recruitment in terms of number and size.

## 2.2 INTRODUCTION

Ocean acidification (OA) has been shown to alter calcification, metabolism, the developmental trajectories and survival of marine invertebrates (Doney et al. 2009; Byrne 2011; Byrne 2012). As development, growth and metabolism are all largely temperature dependent; hence global warming is likely to also affect such functions (Cossin and Bowler 1987; Portner 2001; Koeller et al. 2009), ultimately modifying species distribution (Southward et al. 1995; Stillman 2003; Rosa and Seibel 2008; Jones et al. 2009). For example, Koeller et al. (2009) have shown that populations of the Northern shrimp; *Pandalus borealis* (Fig. 2.1) found along a thermal gradient in the North Atlantic have strongly adapted to local temperatures and correspond to algal bloom timing. This strategy is vulnerable to long-term climatic changes. It is therefore crucial to understand how concomitant temperature change can profoundly modify any effects of OA in a synergistic, antagonistic or additive manner (e.g. Rosa and Seibel 2008; Dissanayake and Ishimatsu 2011; Lischka et al. 2011; Melatunan et al. 2011; Wood et al. 2011; Mayor et al. 2012). Such effects also seem to differ in different life stages, populations, species and lineages (e.g. (Kurihara and Ishimatsu 2008; Dupont and Thorndyke 2009; Chen et al. 2011; Pistevos et al. 2011; Parker et al. 2012) but see (Findlay et al. 2011). Early developmental stages are presumed to be the most vulnerable part of a species life cycle, particularly when it comes to environmental changes (Thorson 1950; Spicer and Gaston 1999), and are therefore important to study if we are to accurately predict a species' response to OA.

Warming and OA will take place in concert, so understanding their combined effect, particularly on early developmental stages is vital to predict the impact of complex global change on marine taxa. There are relatively few investigations on the combined effects of OA and elevated temperature on crustaceans (see Whiteley (2011) and Byrne (2012) for a review), and this is particularly so for early developmental stages. To our knowledge the interactive

effects of elevated temperature and reduced pH on crustacean larval development has only been studied for four species. Larvae of two populations of the spider crab, *Hyas araneus* cultured at 710 ppm $CO_2$, showed delayed development, and reduced growth and fitness, with the northern-most populations (79 °N) being more sensitive to the negative impact of low pH at the zoeal stages, whilst the southern-most populations (54 °N) being more sensitive at the megalopa stages (Walther et al. 2010). Elevated temperature and $CO_2$ affected growth and shell development in the post larvae of two intertidal barnacles (*Semibalanus balanoides* and *Elminius modestus*), although elevated temperature appeared to be the overriding factor determining survival (Findlay et al. 2010). Nauplii and cyprids of the barnacle (*Amphibalanus improvisus*) exposed to different temperatures and reduced pH were more vulnerable to the negative effects of elevated temperature than to low pH (Pansch et al. 2012). Thus ignoring the existence of potential interactions between multiple environmental challenges, one may under- or overestimate the effects of either elevated temperature or OA in isolation.

The Northern shrimp, *Pandalus borealis* is an ecologically and economically important species (see Sect. 1.4.1 for more information about the species). Both embryonic and larval development are known to be both temperature and pH dependent (Weinberg 1982; Brillon et al. 2005; Bechmann et al. 2010). For example, Bechmann et al. (2010) showed a significant delay in zoeal progression (developmental time) at 5 °C for shrimp larvae exposed to pH 7.6 for five weeks.

Consequently, the aim of the present chapter was to investigate potential interactive effects of decreased pH and elevated temperature on aspects of development and developmental physiology of *P. borealis.* This was achieved by quantifying hatching timing and success, larval development rates, survival, growth, feeding and metabolic rates in *P. borealis.* In addition, the presence of fundamental relationships among key traits investigated was carried out in order to provide a more mechanistic understanding of the consequences of the exposure to elevated

temperature and decreased pH on the development of *P. borealis*. Ovigerous females and their larvae were exposed to one of two temperatures (6.7 and 9.5 ºC) and two pH levels (pH 8.1 and pH 7.6), the 'control' experimental temperature and pH treatments being within the typical thermal regime experienced by the local population of *P. borealis* examined here, and the 'future treatment' experimental conditions were based on those predicted for the year in the North Atlantic in 2100 (Caldeira and Wickett 2003; Caldeira and Wickett 2005; Sokolov et al. 2009).

The hypotheses were that: i) at the elevated temperature, time until first hatching, duration of hatching period, hatching success and larval development time will be reduced, whilst growth, feeding and metabolic rates will be increased; ii) decreased pH alone will cause an increase in time until first hatching, duration of hatching period and larval development time, and reduced hatching success, growth, feeding, and metabolic rates; and iii) increased temperature and reduced pH will have an interactive effect on *P. borealis* compared to one stressor alone (see Metzger et al. 2007; Pörtner and Farrell 2008).

## 2.3 MATERIALS AND METHODS

### 2.3.1 Adult animal collection and maintenance

Ovigerous females of *Pandalus borealis* (mean cephalothorax length 2.2 ± 0.2 cm) were collected using a bottom shrimp trawl from Hillefjord (North of Åmøy Rogaland County, Norway; 59° 04' 00" N - 5° 45' 00' E) during Feb 2010. Trawling lasted 15 - 20 min at 100 m depth. Instead of using a net, which may damage the shrimps, a barrel (1 m x 1 m) was secured, to the bottom (cod-end) of the trawl. The catch was a mixture of fish (haddock, cod, sole), krill and shrimp. Female shrimp were sorted by hand. Approximately 200 undamaged individuals were transferred to four 50 L large tanks each filled with fjord sea water to be transported to the laboratory within 2 h of capture. Upon arrival at the laboratory, shrimps were haphazardly

divided between three aquaria (vol. = 500 L, Temperature = 5 ºC, Salinity = 34, density approx. 66 indiv. *per* aquaria). Each aquarium was continuously supplied with sea water directly pumped from 75 m depth in the fjord close to the laboratory facilities. The sea water was sand-filtered prior to use in the experimental system. Shrimps were fed raw fish *ad libitium* three times a week during the acclimation period (23 d), and undigested material was removed the following day to avoid fouling of the water.

### 2.3.2 Experimental design and set-up

The effects of decreased pH, elevated temperature and their interaction on hatching success, larvae survival, development, feeding rate, and metabolic rate were assessed using a fully orthogonal experimental design including pH and temperature levels representing current conditions and conditions predicted for the year 2100. Ovigerous females and larvae (stage I - IV) of *P. borealis* were allocated to one of four treatments: 1) control ($pH_{NBS}$ 8.1, 6.7 ºC); 2) decreased pH ($pH_{NBS}$ 7.6, 6.7 ºC); 3) elevated temperature ($pH_{NBS}$ 8.1, 9.5 ºC) and 4) combined ($pH_{NBS}$ 7.6, 9.5 ºC). All individuals were kept in two separate continuous flow systems, consisting of two header tanks (vol. = 200 L, flow: 8.0 L $min^{-1}$) where temperature was tightly regulated at either 6.7 or 9.5 °C respectively, using heat exchangers. All sea water was double filtered, using a 115 and 40 µm plankton mesh (SEFAR PETEX® Heiden, Switzerland). From the header tanks sea water was pumped into 48 x 9 L aquaria, each containing one ovigerous shrimp (and later in the experiment a batch of 200 shrimp larvae). For each of the four treatments, threre were one header tank that supplied six aquarias. In the reduced pH treatment aquaria, the desired $CO_2$ equilibration was achieved, *via* manipulating pH using the method by Widdicombe and Needham (2007) employing pH-controllers (AB Aqua Medic GmbH pH computer, Bissendorf, Germany) set to maintain mean $pH_{NBS}$ at 7.6, *via* a solenoid valve, which allowed regulating the addition of $CO_2$ gas. Seawater $pH_{NBS}$ was recorded and logged every

five min using a pH probe (Orion Star Plus™ 3-Star and Ross® Electrodes, Thermo Fisher Scientific Inc, Beverly, USA) coupled to a calibrated pH meter (Orion Star Plus™, Thermo Fisher Scientific Inc, Beverly, USA and Consort and a multi-channel datalogger D-130, Consort, Turmhout, Belgium) using the Star Plus Navigator 21 Software (Thermo Fisher Scientific Inc., Beverly, USA.), and employing the NBS scale. In addition, water temperature and $pH_{NBS}$ were measured every second day, and oxygen levels three times over the duration of the experiment in all aquarias. Water temperature was measured using a calibrated glass thermometer (certified by Physikalisch-Technische Bundesanstalt (PTB), Braunschweig, Germany) and $pH_{NBS}$ using a hand held pH meter (Orion Star Plus™ 3-Star, Thermo Fisher Scientific Inc., Beverly, USA). Oxygen concentration was measured with an $O_2$ meter (Oxi 330i/SET, WTW, Weilheim, Germany). The salinity of the intake water was recorded every 5 minutes during the entire duration of the experiments using a CT-probe (Aqua TROLL 100®) with Win-Situ 5 data acquisition software (In-Situ Inc., Collins, USA). Values obtained from the literature on the study area (ranging from 1900 to 2300 µmol $kg^{-1}$ - (Schiettecatte et al. 2006; Bellerby et al. 2008; Findlay et al. 2008) were used to produce mean TA values used to calculate the carbonate system. The carbonate system parameters that were not directly measured (partial pressure of $CO_2$ ($pCO_2$), dissolved inorganic carbon (DIC), bicarbonate and carbonate ion concentration ([$HCO_3^-$] and [$CO_3^{2-}$] respectively), saturation status of calcite and aragonite ($\Omega_{calc}$ and $\Omega_{ara}$ respectively) were calculated from $pH_{NBS}$ and mean TA values using CO2SYS (Pierrot et al. 2006) with dissociation constants from Mehrbach et al. (1973) refitted by Dickson and Millero (1987).

### 2.3.3 Larval collection and maintenance

Individual ovigerous females (N = 94) were transferred to individual flow-through aquaria (vol. = 9 L; flow rate = 0.12 L $min^{-1}$). The total number of females was chosen to ensure that a large

number of shrimp larvae would hatch simultaneously, following an exposure period to experimental conditions (minimum two weeks before hatching). Each aquarium was checked every day in order to determine the time to first hatching. This was defined as the first day free swimming larvae were observed. The duration of the hatching period and percentage hatching success (percentage live larvae after hatching) was determined for six females from each treatment. Duration of the hatching period was measured as the number of days to hatch all the eggs. Batches of larvae (N = 200) all from the same female were transferred to the same type of individual glass aquaria as described above (vol. = 9 L). From each experimental treatment eight batches, each consisting of 200 larvae (< 24 h old), were exposed to the experimental treatments and monitored until they reached stage IV zoea.

### 2.3.4 Development of shrimp larvae

Post-hatch shrimp larvae were fed *ad libitium* on freshly hatched *Artemia salina* nauplii (*Artemia* length 450 - 550 µm, feeding density 1000 indiv $L^{-1}$) twice a day (morning and afternoon) for the entire duration of the experiment. In addition, for the first week, larvae were also fed with phytoplankton, *Thalassiosira weisslogi* 1200$^{TM}$ (Microalgae, Vigra, Norway), (2 x $10^4$ cells $L^{-1}$) once a day, to ensure feeding for stage I and II zoea (following indication by (Pedersen and Storm 2002; Ariza and Ouellet 2009). Dead *Artemia* were removed every three days to avoid seawater contamination. Mortality of shrimp larvae was calculated every third day and reported as % number of accumulated dead larvae at stage IV. Larval stage was determined using the descriptions of (Haynes 1979). The characters used to differentiate between stages were: 1) stalked eyes (stage II); 2) distinct exopodites on telson (stage III); and 3) stage IV larvae have larger endopodites than stage III larvae (Haynes 1979). The shrimp larvae aquaria were monitored daily visually, and the percentage of different larval stages was determined by counting the number of stage I - IV larvae in 3 L subsamples siphoned out from

each glass aquaria. When more than 40 % of the larvae in the control $pH_{NBS}$ aquaria had reached the next stage, all larvae were siphoned out and the developmental stage of each larva was determined. The larvae in the control aquaria and the larvae in the decreased pH aquaria were sampled the same day post hatch to detect possible effects of decreased pH. Since there was a decrease in development time for the elevated temperature aquaria, larvae for this treatment were sampled at a different time post hatch, than those from control temperature aquaria. After stage determination, larvae were returned to their aquaria.

### 2.3.5 Feeding experiment

To measure larvae feeding rates, a modification of the clearing rate methods of (Harvey and Morrier 2003) were used. Feeding trials were conducted in 1 L glass beakers containing filtered seawater from the treatment header tanks used in the experiment. Five larvae of the same stage of development were transferred into the experimental beakers with a Pasteur glass pipette with a cut end, placed in temperature controlled environments (6.7 or 9.5 °C) and starved for approx. 24 h prior to the experiments. Freshly hatched *Artemia* nauplii (initial prey conc. 150 indiv $L^{-1}$) was added to the water in the glass beakers containing the larvae. The beakers were then sealed with aluminium foil and incubated for 6 h at the respective temperature (Fig. 2.2). After the incubation period, shrimp larvae were carefully removed from the beakers and the remaining water, containing *Artemia* nauplii, was sieved using a 40 µm BD Falcon™ cell strainer (BD Biosciences, Franklin Lakes, USA), and individual nauplii counted. Amount of prey consumed was calculated as initial number of *Artemia* nauplii less their final number. Feeding rate was expressed as number of prey consumed *per* individual shrimp larvae *per* unit time (number of *Artemia* $indiv^{-1}\ h^{-1}$). A control sample from the reduced pH treatment without shrimp larvae was run through the experimental procedure, to test the efficiency of the sieving procedure and to see if $pH_{NBS}$ was maintained. All 150 *Artemia* sp. nauplii were recovered in

each control test. Over the incubation period, $pH_{NBS}$ had risen from $pH_{NBS}$ 7.6 to $pH_{NBS}$ 7.96 during the 30 h period.

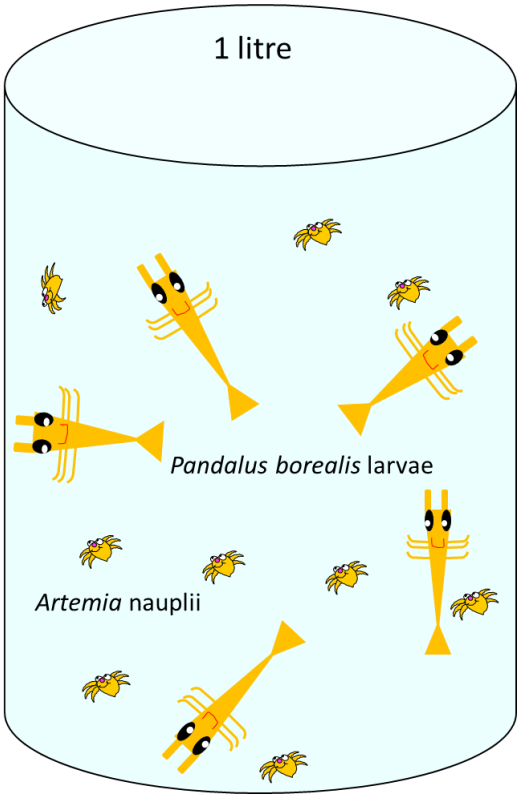


**Figure 2.2 Feeding test of larva of Northern shrimp *P. borealis*, 5 shrimp larvae and 150 *Artemia* nauplii were placed in 1 L seawater. After 6 hours the remaining *Artemia* were counted. (Drawing: Renée K. Bechmann).**

### 2.3.6 Determination of metabolic rates

To estimate stage IV larvae metabolic rates, their rates of oxygen consumption were measured following the methods of Taylor and Spicer (1989), using a custom-built closed glass-bottle respirometer (vol. = 300 mL), equipped with airtight stoppers in which $O_2$ electrodes were inserted to be in direct contact with the sea water inside. Blank low concentration $O_2$ water samples (N = 3) were used to verify that the respirometers were airtight. Shrimp larvae (N = 15 at each time) were placed in the incubation chambers with the appropriate treatment header tank water used in the experiment and sealed with the airtight stoppers, and then placed in flow through water baths to maintain a constant temperature. A total of 7 - 10 replicates were carried

out for each of the four $pH_{NBS}$/temperature combinations. Following preliminary trials, measurements of dissolved $O_2$ concentration in the respirometers were performed every 2 sec for the entire duration of the incubation (approx. 48 h) using $O_2$ electrodes (1302, Strathkelvin Instruments, Glasgow, UK) coupled to a multichannel oxygen meter (928, Strathkelvin Instruments, Glasgow, UK). Continuous measurements were conducted to demonstrate linearity of $pO_2$ decline in the respiration chambers during the incubation (N = 32). No changes in oxygen consumption rates were detected and $O_2$ concentration ranged from mean 278.1 ± 1.6 µmol $O_2$ $L^{-1}$ down to a minimum of 112.2 ± 1.6 µmol $O_2$ $L^{-1}$ at the beginning and end of the incubation period respectively. Larva free samples of sea water from the appropriate treatment header tank were used to assess background respiration rates caused by microorganisms in the system. Background respiration never exceeded 10 % of the total larval respiration (85.31 ± 25.0 µmol $O_2$ $h^{-1}$). These background rates were then used to correct the measured larval respiration values obtained. At the end of each trial, larvae were stored at – 80 °C in order, at a later date, to conduct morphological and dry mass determinations described by Ouellet and Chabot (2005). Total length (TL) was measured for a selection of larvae (6 - 13 indiv *per* trial, 7 - 12 trials per treatment). All the larvae from each trial were pooled to determine the total dry mass (DM). Oxygen consumption was expressed as nmol $O_2$ $h^{-1}mg^{-1}$ DM.

### 2.3.7 Statistical treatment

The effect of decreased pH, elevated temperature, and their interaction on the variables measured in this study was analysed using multiple ANOVA/ANCOVA tests, with ‘aquaria’ as a random factor nested within pH X temperature combinations. However, as aquaria had no significant effect on any of the parameters tested here (max. $F_{1, 106} = 1.339$, $p = 0.158$) this factor was removed from subsequent analyses. In addition, for feeding rates the term ‘stage’ were included, and for metabolic rates individual body mass as a covariate were included. All

data met assumption for normality of distribution (max. $F_{1,32} = 0.039$, $p = 0.845$), and variances were homogeneous for hatching success, hatching day, larval development, hatching period, feeding rate, and metabolic rates (max. $F_{3, 32} = 1.375$, $p = 0.278$). As our experimental design included four treatments and minimum six replicates *per* treatment *per* measurement, it was assumed that the ANCOVA design employed should be tolerant of deviation from the assumption of normality and heteroscedasticity (Sokal and Rolhf 1995). Pairwise comparisons were conducted using the Estimate Marginal Mean test with LSD correction. Finally, the data was analysed for the presence of fundamental relationships among the traits. This was done by investigating the presence of fundamental relationships among key physiological traits (i.e. metabolism, feeding) and developmental traits (i.e. first hatching day, body length and dry weight at stage IV) characterised in this study using the Pearson's correlation test. By so doing, the aim was to produce a more mechanistic understanding of the consequence of the exposure to elevated temperature and reduced pH. All analyses were conducted using v 19 SPSS® and v 5.1 Jump®.

## 2.4 RESULTS

### 2.4.1 Sea water chemistry

Mean ± SD for seawater physico-chemical parameters measured and calculated in the four treatments used in this study are reported in Appendix 2.1. Mean $pH_{NBS}$ for control treatments ranged between $pH_{NBS}$ 8.05 - 8.13 and in the decreased pH treatments between 7.56 - 7.68. Mean temperatures under control and elevated conditions were 6.7 ± 0.2 ºC and 9.5 ± 0.1 °C, respectively.

### 2.4.2 Hatching

Decreased pH and its interactions with elevated temperature had no significant effect on any of the parameters reported here (max. $F_{1, 23} = 3.065$, $p = 0.095$). Time to first hatching for *Pandalus borealis* females ranged between 16 and 18 d at 6.7 ºC and between 14 and 15 d at 9.5 ºC (see Fig. 2.3 a).

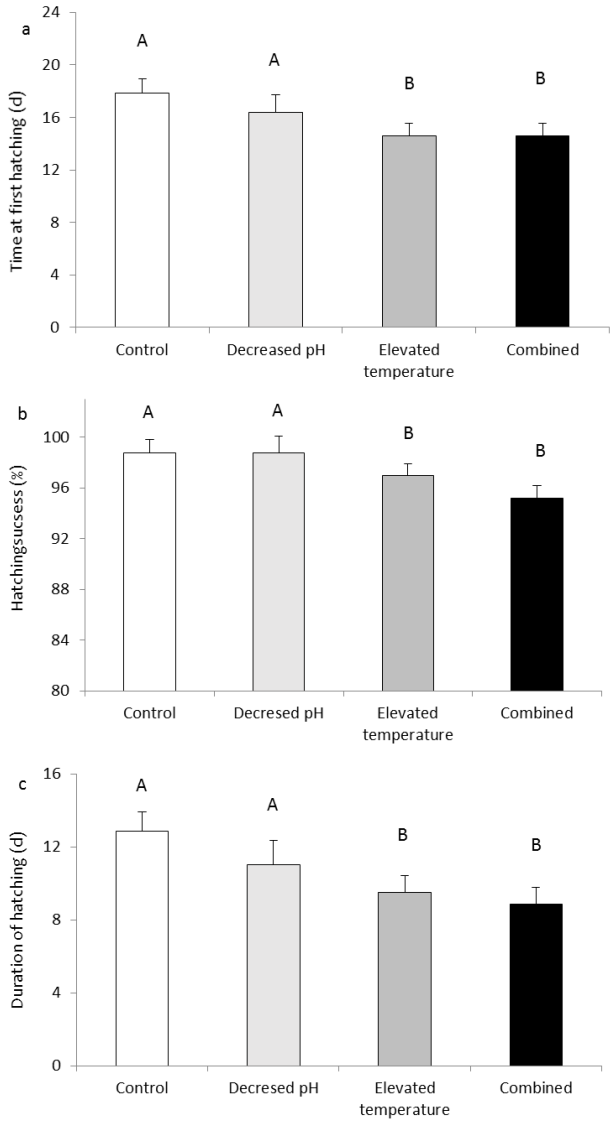


**Figure 2.3 Effect of decreased pH, elevated temperature and their interaction on mean, a) time to first hatching (N = 74), b) hatching success (N = 23), c) duration of hatching period in embryos (N = 23) of *P. borealis*. Control (pH 8.1 / 6.7 ºC, clear), decreased pH (pH 7.6 / 6.7 ºC, light grey), elevated temperature (pH 8.1 / 9.5 ºC, dark grey), combined (pH 7.6 / 9.5 ºC, black). Histograms represent means ± SE. Significantly different treatments ($p \leq 0.05$) are indicated by the difference in upper case letter according to the Estimate Marginal Mean test.**

Mean time to first hatching was being significantly lower at elevated temperature treatments compared with the control ($F_{1, 74} = 5.201$; $p = 0.001$). Mean percentage-hatching success for embryos of *P. borealis* were 98.7 % at control temperature conditions and 96.1 % at elevated temperature conditions (Fig. 2.3 b). Although the difference was only 1.6 % there was a significant effect of elevated temperature on this trait ($F_{1, 23} = 9.458$ $p = 0.006$). Finally, mean duration of the hatching period ranged between 11 and 13 d at control temperature conditions and 8 and 10 d at elevated temperature conditions (Fig. 2.3 c), being significantly lower at elevated temperature when compared to control temperature ($F_{1, 23} = 14.837$, $p = 0.001$).

### 2.4.3 Development of shrimp larvae

Detailed determination of the different stages was performed at different days for those from the two temperatures, and the data analyses consequently conducted separately for the two temperatures for all larvae in each aquarium (Fig. 2.4 a-b). For larvae at control temperature, the percentage of larvae at a given stage was determined for stage I and II 9 ± 0.45 d after hatching, for stage II - III 17 ± 0.32 d after hatching and for stage III - IV 28 ± 0.85 d after hatching. For elevated temperature treatments, the percentage of larvae at a given stage was determined for stage I and II larvae 7 ± 0.45 d after hatching, for stage II - III 13 ± 0.25 d after hatching and for stage III-IV 19 ± 0 d after hatching. Decreased pH was accompanied by a significant difference in percentage of stages of the shrimp larvae at control temperature conditions ($F_{1, 105} = 8.524$, $p = 0.0049$) (see Fig. 2.4), but had no significant effect of decreased pH under elevated temperature conditions ($F_{1, 105} = 1.91$, $p = 0.154$) (see Fig. 2.4). Mean (± SE) percentages of accumulated number of dead larvae at stage IV recorded at the end of the experiment (when the larvae had reached stage IV) were 11.6 ± 1.87 % at control conditions, 9.25 ± 2.19 % at decreased pH, 9.22 ± 2.56 % at elevated temperature, and 10.0 ± 4.54 % at combined treatment. However, there were no significant differences in mortality levels among

all treatments ($F_{1, 32} = 0.323$, $p = 0.809$). The mean (± SE) total length of larvae at stage IV was: control 9.50 ± 0.07 mm, decreased pH: 9.41 ± 0.08 mm, elevated temperature: 8.88 ± 0.07 mm and combined exposure: 9.21 ± 0.06. A significant difference in mean total length of larvae among treatments was detected; larvae of the stage IV being significantly smaller in individuals kept under elevated temperature treatment conditions ($F_{1, 34} = 105.137$, $p < 0.0001$).

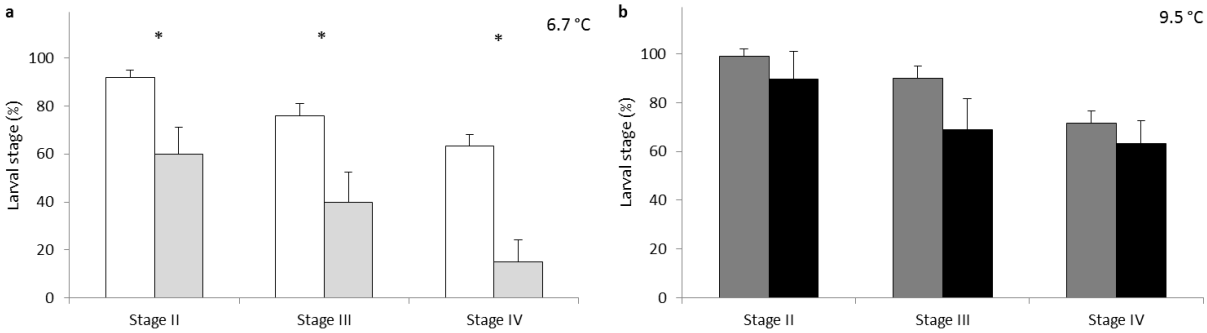


**Figure 2.4 Effect of exposure to decreased pH (pH 7.6, pH 8.1) on the development of *P. borealis* larvae (stage II-IV) exposed to, a) control (6.7 ºC) and, b) elevated temperature (9.5 ºC). Control (pH 8.1 / 6.7 ºC, clear (N = 33), decreased pH (pH 7.6 / 6.7 ºC, light grey) (N = 21), elevated temperature (pH 8.1 / 9.5 ºC, dark grey) (N = 27), combined exposure (pH 7.6 / 9.5 ºC, black) (N = 24). Histograms represent means ± SD. Significantly different treatments ($p \leq 0.05$) are indicated by asterix according to the Estimate Marginal Mean test.**

### 2.4.4 Feeding rates

Mean feeding rates of individual *P. borealis* larvae ranged between 2.6 and 3.1 prey eaten *per* hour for stage II, 2.9 and 3.6 prey *per* hour for stage III and 2.8 and 4.1 prey *per* hour for stage IV (Fig. 2.5). In general, feeding rates were higher at the elevated temperature (Fig. 2.5) ($F_{1, 105} = 27.648$, $p < 0.0001$); whilst decreased pH and the interaction between decreased pH and elevated temperature had no significant effect on feeding rates (max. $F_{1, 23} = 0.291$, $p = 0.591$). Different stages showed significantly different feeding rates (Fig. 2.5) ($F_{1\ 105} = 5.859$, $p <$

0.004). The mean value of feeding rate for stage II larvae (mean = 17.25) were significantly lower than those determined for stage IV (mean = 20.59) ($p$ = 0.002). No other comparisons showed significant differences ($p$ > 0.05).

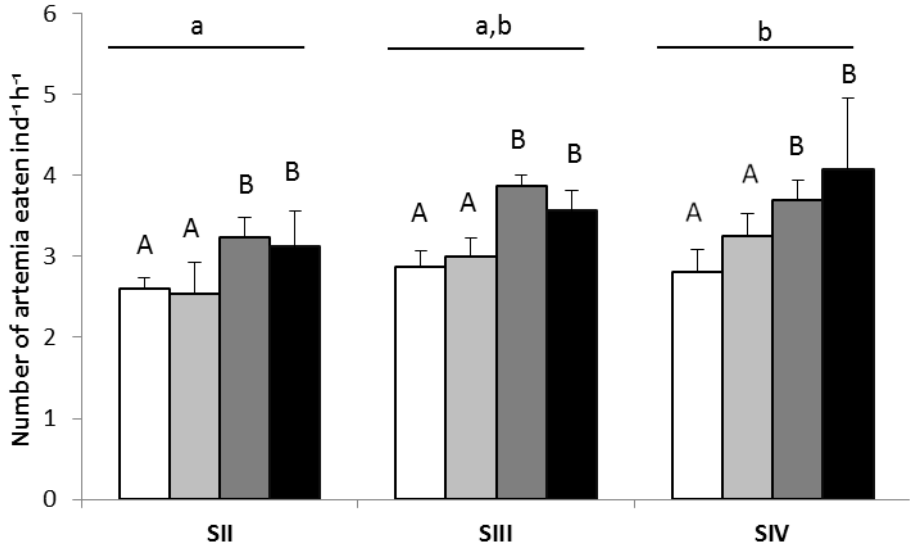


**Figure 2.5 The effect of decreased pH and elevated temperature on mean feeding rates (measured as mean number prey eaten indiv.$^{-1}$ h$^{-1}$) for *P. borealis* larvae at stage II, III and IV. Control (pH 8.1 / 6.7 ºC, clear) (N = 33), decreased pH (pH 7.6 / 6.7 ºC, light grey) (N = 21), elevated temperature (pH 8.1 / 9.5 ºC, dark grey) (N = 27), combined (pH 7.6 / 9.5 ºC, black) (N = 24). Histograms represent means ± SE. Significantly different treatments (P ≤ 0.05) are indicated by upper case letters and lower case letters indicate significant differences among the different stages according to the Estimate Marginal Mean test.**

### 2.4.5 Metabolic rates for shrimp larvae

Mean rates of $O_2$ consumption for *P. borealis* larvae stage IV ranged between 48.17 and 49.99 nmol $O_2$ ind$^{-1}$ h$^{-1}$ mg$^{-1}$ DM for control temperature conditions and 55.83 and 58.16 nmol $O_2$ ind$^{-1}$ h$^{-1}$ mg$^{-1}$ DM for the elevated temperature (Fig. 2.6). Rates of $O_2$ uptake were significantly greater at the elevated temperature ($F_{1, 31}$ = 8.014, $p$ = 0.009), whilst decreased pH and the interaction between decreased pH and elevated temperature had no significant effect on this parameter (max $F_{1,}$ = 0.129, $p$ = 0.722). The mean (± SE) DM (mg) per individual stage IV

larvae was: 0.99 ± 0.06 at control conditions, decreased pH: 0.97 (SE 0.19), elevated temperature: 0.79 (SE 0.07) and combined: 0.77 (SE 0.06).

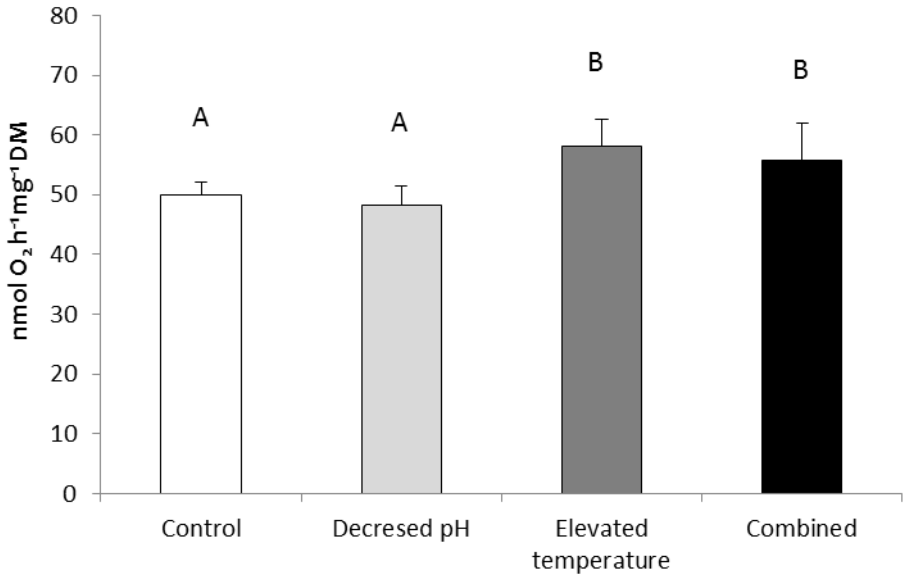


**Figure 2.6 The effect of decreased pH an elevated temperature on mean oxygen consumption (nmol $O_2$ $h^{-1}$ mg $^{-1}$ DM) for *P. borealis* larvae stage IV. Control (pH 8.1 / 6.7 ºC, clear) (N = 9), decreased pH (pH 7.6 / 6.7 ºC, light grey) (N = 7), elevated temperature (pH 8.1 / 9.5 ºC, dark grey) (N = 9), combined exposure (pH 7.6 / 9.5 ºC, black) (N = 7). Histograms represent means ± SE. Significantly different treatments ($p \leq 0.05$) are indicated by upper case letters according to Estimate Marginal Mean test.**

### 2.4.6 Relationships among traits

Feeding rates were found to be higher in stage IV larvae with higher levels of oxygen consumption, as indicated by the presence of a positive significant relationship between these two factors (Fig. 2.7) ($r_{37} = 0.377$, $p = 0.018$). However, no significant relationship was detected between time to first hatching and oxygen consumption or between time to first hatching and feeding rates (max $r_{29} = 0.291$, $p = 0.161$). Hatching success and time to first hatching displayed a significant positive relationship whilst oxygen consumption was inversely related to both DM and length at stage IV (minimum $r_{29} = 0{,}410 = p < 0.05$).

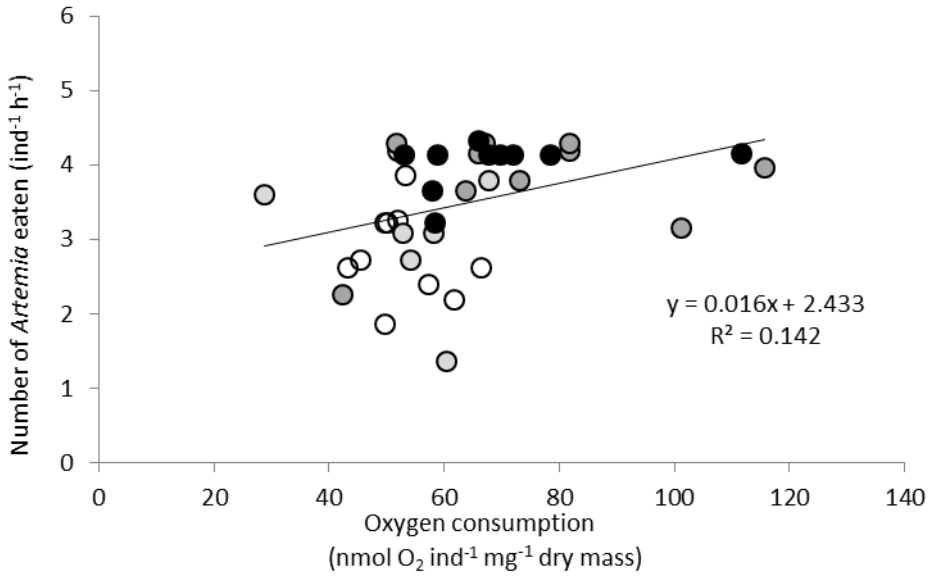


**Figure 2.7 Relationships between oxygen consumption and feeding rates [$R^2$ = 0.1209, Y= 0.3927x + 2.502, df = 31, *P* = 0.0472] in stage IV larvae of *P. borealis* maintained under different temperature and pH conditions. Control (pH 8.1 / 6.7 ºC, clear), decreased pH (pH 7.6 / 6.7 ºC, light grey), elevated temperature (pH 8.1 / 9.5 ºC, dark grey), combined exposure (pH 7.6 / 9.5 ºC, black). The continuous line represents the line of best fit (linear regression) for all the data.**

## 2.5 DISCUSSION

The present study shows that exposure to decreased pH is accompanied by a significant delay of larval development at control, but not at elevated temperature. Elevated temperature on its own had a marked effect on the embryonic development of the Northern shrimp, *Pandalus borealis*, with larvae hatching earlier, developing quicker but showing poorer survival. In addition, elevated temperature increases *P. borealis* larvae metabolic, feeding rates and decreases developmental rates but not survival in stage IV larvae. Consequently, it seems likely that ocean warming (OW) could exert a greater effect on Northern shrimp larvae development, than OA.

### 2.5.1 Hatching

Elevated temperature accelerated embryonic development, as it shortened time to first hatching (3 d) and the duration of the hatching period (3 d), although there was a reduction in hatching success (2 – 4 %). This indicates that under elevated temperature rates of embryonic development are faster thus matching our prediction. However, such acceleration in development is accompanied by a 2 - 4 % reduction in embryonic survival. Contrary to our predictions however, time to first hatching, hatching success and hatching period in *P. borealis* are not negatively affected by exposure to decreased pH.

Elevated temperature during the ovigerous period seems not only to have an effect on the duration of the embryonic development but also on hatching success. This is consistent with previous findings for this species (Shumway et al. 1985; Bergstrom 2000; Brillon et al. 2005). Nunes and Nishiyama (1984) found that developmental rates of *P. borealis* from Alaskan waters increased with increasing temperature within the interval 3 – 9 °C, and that the larvae hatched from eggs incubated at 3 °C had the greatest survival rates and significantly higher growth rate, when compared to larvae incubated at higher temperatures. Similarly Brillon et al. (2005) reported that hatching larvae from eggs incubated at 2 and 5 °C were larger and heavier than larvae hatched at 8 °C. Brillon et al. (2005) hypothesised that *P. borealis* embryos developing at higher temperatures may have hatched prematurely, and that this may explain the smaller size at hatching, the shorter incubation time and the higher proportion of abnormal larvae. This could potentially explain the observed reduced hatching success at elevated temperatures in correspondence with a faster embryonic development. In addition, larger larvae may be abler to survive post hatching because their greater swimming ability enabling them to capture prey more easily and also have access to larger (or a larger range of) prey organisms as food selection seemed to be size dependent (Pedersen and Storm 2002). Thus, elevated

temperature may lead to the development of smaller larvae, which could be more vulnerable to starvation and, in combination with lower hatching success, lead to lower recruitment levels.

### 2.5.2 Larval development

In addition to shortening embryonic development, OW seems to shorten shrimp larvae development by 9 d until stage IV. Furthermore, the dry mass of stage IV *P. borealis* larvae was 20 % lower at OW when compared to control conditions. Also in support of our predictions, larval development is delayed by exposure to decreased pH at control temperature, this effect being more pronounced for stage IV larvae. However, elevated temperature appears to override the effect of decreased pH on development, as no significant difference is found at the higher temperature level tested here. As for hatching, it appears that elevated temperature influences larval development in the Northern shrimp to a greater extent than decreased pH, with no indications of significant interactions between these two factors.

Our results are comparable to, and in agreement with, existing data on the importance of temperature in governing growth in the Northern shrimp larvae held under constant feeding conditions (Bergström 2000), and with larval developmental rates decreasing as culture temperature is increased (Weinberg 1982; Shumway et al. 1985; Ouellet and Chabot 2005). In addition, duration of larval stage in our study is comparable to those reported by Ouellet and Chabot (2005) and Weinberg (1982). Furthermore, our results indicating a delay of larval stages progression (but no effect on larvae survival rates) under decreased pH conditions at the control temperature partly confirm Bechmann et al. (2010) findings on the vulnerability of *P. borealis* larvae development to reduced pH conditions. Thus the Northern shrimps may be more sensitive to OA than the copepod, *Acartia tsuensis*, and the barnacle, *Amphibalanus amphitrite* (Kurihara and Ishimatsu 2008; McDonald et al. 2009) and may have comparable sensitivity to crustacean species such as European lobster, *Homarus gammarus,* the intertidal barnacles, *Semibalanus balanoides* and *Elminus modestus*, and the spider crab, *Hyas araneus* (Arnold et

al. 2009; Findlay et al. 2010; Walther et al. 2010). At the decreased pH treatments in our experiment, the sea water was under-saturated with respect to aragonite and the saturation for calcite was reduced to approximately 1. It has been predicted that calcifiers may be particularly sensitive to OA because of the reduced saturation states for $CaCO_3$ at lower pH (Orr et al. 2005; Kroeker et al. 2010). Crustacean exoskeleton consists of chitin, protein, high magnesium calcite and calcium phosphate (Mikkelsen et al. 1997). The reduction in developmental rate observed at OA conditions, coupled with low saturation states, may indicate a compromise in the ability of shrimp larvae to produce new exoskeletons, particularly at lower temperatures. Under such conditions larva have lower metabolic rates this dictating a lower scope for active calcification (Findlay et al. 2011; Melatunan et al. 2011). Slower development in shrimp larvae exposed to OA may, however, play an important role in 'colder' winters/springs in a future warmer ocean. Increased development time will presumably increase their time in the plankton and thus increase their chances of being predated upon. Thus, a small delay in development may potentially contribute greatly to an increase in mortality rates of planktonic larvae (Dupont et al. 2010). The mortality of early life stages of shrimp in nature is already high (Storm and Pedersen 2003). Thus additional mortality as an indirect consequence of OA could potentially affect recruitment to local populations.

### 2.5.3 Feeding

Feeding rates of Stage II-IV shrimp larvae show a significant increase in feeding with an increase in temperature, supporting our predictions and in agreement with the existing literature (Weinberg 1982; Shumway et al. 1985; Harvey and Morrier 2003). Paul and Nunes (1983) showed that larvae hatched at higher temperatures required more energy for metabolic requirements than if they hatched at lower temperatures. Contrary to the predictions, decreased pH has no effect on feeding.

Warming cues such as elevated temperature starts seasonal processes earlier (shifting phenology), causing potential mismatch with processes timed according to constant cues (e.g. light) (Portner and Farrell 2008). Global change such as elevated temperature could potentially lead to mismatches between the reproductive cycles of *P. borealis* and its planktonic food (Koeller et al. 2009), this match being a key component for the survival and recruitment success of Northern shrimp larvae (Ouellet et al. 2011). Ultimately, an increased demand of food, smaller sized shrimp larvae at elevated temperatures in combination with a mismatch with the biological production cycle, may lead to a decrease in the recruitment of *P. borealis* shrimp larvae (Ouellet et al. 2011). However, such a mismatch may not materialise if concurrent changes in the surface oceanography also result in earlier blooms (Koeller et al. 2009).

### 2.5.4 Metabolism

Stage IV shrimp larvae of *P. borealis* metabolic rates significantly increase with elevated temperature (20 % with a + 2.8 °C). Chabot and Ouellet (2005) described a similar temperature-dependent response of metabolic rates in *P. borealis* larvae stage IV and reported comparable levels of oxygen consumption to those reported in this study: 1.38 - 2.08 µg $O_2$ $h^{-1}$ for larvae reared at 7.5 - 9.0 ºC and 1.41 - 2.12 µg $O_2$ $h^{-1}$ for larvae reared between 6.7 - 9.5 ºC respectively. In addition, our study show that oxygen consumption positively relates to feeding, thus demonstrating the existence of a potential link between metabolic activity and the energy demand under decreased pH and elevated temperature conditions. Since the metabolic rates for *P. borealis* larvae significantly increases with elevated temperature, so must their energy demand. Whilst in our study stage IV larvae with higher metabolic rate show a higher food intake, they also show a reduction in length and mass, thus likely indicating a shift in energy intake efficiency and/or energy budget, as eventually energy is not available for growth. We might infer from the results of this study that there is a likely change in energy resource

allocation. Such a change has already been reported for organisms exposed to elevated temperature and decreased pH (Findlay, Kendall et al. 2010; Walther, Anger et al. 2010). Brillion et al. (2005) showed that an increase in temperature accelerates the duration of oogenesis and developmental time but reduces egg survival in *P. borealis*. Early hatch larvae in the present study were significantly smaller, and there was a positive significant relationship between time to first hatching and larval size. Brillon et al. (2005) observed that the higher temperature reduced the duration of the yolk reserves and the conversion efficiency of yolk into tissue growth, this leading to larvae with a lower size, mass and protein content. According to Clarke et al. (1991), the yolk in developing larvae has three potential energetic functions: (1) it enables the synthesis of new tissues; (2) it enables the maintenance and repair of existing tissues; and (3) it provides newly hatched larvae with an energy reserve before they can carry out exogenous feeding. Temperature affects the rate of yolk absorption and the efficiency that the yolk is converted into tissue, and in turn the larval size and growth during embryonic development (Heming 1982). Hence, larvae of *P. borealis* kept at elevated temperature may experience reduced duration of yolk reserves and conversion efficiency of yolk into tissue growth. Therefore, they may invest less energy into growth, as a consequence of the fact that they undergo a more accelerated development. This should ultimately result in the production of smaller larvae. As mentioned above, a combination of these two costs, an increased demand of food, and smaller sized shrimp larvae at elevated temperature could lead to a decrease in the recruitment of *P. borealis* shrimp larvae. Routine rates of oxygen consumption for stage I *P. borealis* larvae increased exponentially from 1.5 to 9 ºC and decreased from 9 – 12 ºC, indicating that the upper critical temperature for respiration is around 9 ºC for stage I larvae (Paul and Nunes 1983). Hence if we had employed higher experimental temperatures in our study presumably *P. borealis* larvae would eventually encounter their upper critical temperature for respiration and consequently survival could be affected.

### 2.5.5 Conclusions

In summary, this study suggests that recruitment success and population dynamics of *P. borealis* is most likely to be influenced by elevated temperature, rather than decreased pH, in predicted future global change scenario. Hatching success is in fact lower in eggs incubated under elevated temperature, and although the shrimp larvae were able to survive conditions predicted to occur under a future global change scenario, they still might experience a mismatch between energy supply and demand, together with smaller sized larvae, which could be more vulnerable to starvation, altogether resulting in lower recruitment. Finally, faster embryonic development and higher metabolic costs could make the shrimp larvae even more vulnerable to further environmental alternations such as coastal hypoxia and open-ocean deoxygenation, changes in salinity, rising nitrogen levels, and contaminants, among others (see Pörtner and Farrell 2008). However, recent studies highlight the carry-over effects of the exposure of OA from one life stage or generation to another (Dupont et al. 2012; Parker et al. 2012). Even if decreased pH effects were small during the larval stages of *P. borealis,* carry over effects on the post larvae, adults or the next generation may still exist and be important. Furthermore, abiotic variables such as temperature and pH vary spatially across *P. borealis* distributional range, and development in different populations and genetic strains might respond differently to OA and OW. This together with the species ability for rapid adaption to future climatic change scenarios (see (Collins and Bell 2004; Findlay et al. 2010; Pistevos et al. 2011; Sunday et al. 2011) should be considered warranting further study.

**Chapter 3 – The combined effects of ocean acidification and ocean warming on the development and metabolism on early life stages of Northern krill (*Meganyctiphanes norvegica*).**

**Figure 3.1. Adult *Meganyctiphanes Norvegica* (Photo: Renee Bechmann).**

## 3.1 SUMMARY

Climate models predict that the average temperature in the North Sea could increase 3 – 5 ºC and surface-water pH could decrease 0.3 – 0.5 pH units by the end of this century. Currently we know little of how ocean warming (OW) and ocean acidification (OA) will affect key components of temperate planktonic systems, such as the Northern krill, *Meganyctiphanes norvegica*. To improve our capability to predict the consequences of exposure to combined global stressors on marine ecosystems, a better understanding of key species' sensitivity to complex environmental challenges is needed. Consequently, aspects of the development and function of early life history stages of krill exposed to a combined OA and OW scenario (OAW: + 3.0 °C, - 0.5 pH) were investigated. Krill embryos and larvae were exposed for 30 d and juvenile krill were exposed for 14 d. While hatching success was not affected by exposure to predicted OAW conditions, the rate of larval development was increased. Although juveniles' mortality was not affected by predicted OAW conditions, metabolic rate increased significantly

(+ 36 %), and feeding rate (- 60 %) and number of moults (- 67 %) decreased significantly. The results indicate that predicted OAW conditions significantly affected early life stages of krill. Further research is needed to predict the potential implications for the temperate pelagic system they belong to.

## 3.2 INTRODUCTION

Global change is causing significant alterations in environmental conditions and will lead to shifts in a number of abiotic factors in our seas (Sect. 1.1.1). Both OA and OW will influence invertebrate development (Sect. 1.1.3). These drivers will act simultaneously on marine organisms, and it is therefore, important to study the impacts of these drivers in combination on key species living in global change hotspots. Changes to the biology and population dynamics of key species are likely to have consequences for whole ecosystems (Pimm 1991). The Northern krill, *Meganyctiphanes norvegica*, is one such key species (Fig. 3.1). It is pivotal in many pelagic systems (Sect. 1.4.2). More generally, krill constitute an important link in marine trophic chains (Pearcy et al. 1979), particularly between the primary producers and secondary producers, as they are a major food source for several species, including commercially important fish such as herring, *Clupea harengus*, cod, *Gadus morhua*, capelin, *Mallotus villosus* as well as whales (Gambaiani et al. 2009; Sakshaug et al. 2009; Simard and Harvey 2010).

Despite the importance of krill in marine ecosystems, there is surprisingly little information on the effect of OA and OW, in isolation on different krill species (Flores et al. 2012), and to date no study has investigated the combined effects of these stressors on krill. This may be because of the fragile nature of these organisms and the challenges of keeping them in good status under laboratory conditions (Kawaguchi et al. 2011; Sperfeld et al. 2014). Sub-adults of the Northern Atlantic krill, *Nyctiphanes couchii* were not affected by exposure to OA predicted to occur by

the end of the century ($pCO_2$ = 1,100 µatm), whereas feeding and excretion rates increased in Antarctic krill, *Euphausia superba* at moderately OA ($pCO_2$ = 672 ppm) (Saba et al. 2012; Sperfeld et al. 2014). Furthermore, rate of hatching of Antarctic krill was reduced by exposure to OA ($pCO_2$ = 1200 µatm), and embryonic development was slowed down at relatively low OA ($pCO_2$ = 2000 µatm) (Kawaguchi et al. 2011; Kawaguchi et al. 2013). Both embryonic and larval development are temperature and pH dependent in several krill species (Hirst et al. 2003; Tarling and Cuzin-Roudy 2003; Yoshida et al. 2004). Furthermore, early developmental stages are regarded as the most vulnerable part of a species life cycle, particularly in relation to environmental changes (Sect. 1.1.2). It is therefore important to study the response of early life stages if we are to predict a species' response to a combination of OA and OW.

Consequently, the aim of this study was to investigate the direct effects of exposure to simulated future combined OA and OW (OAW) conditions on key aspects of the development and function of the early life history stages of the Northern krill, *Meganyctiphanes norvegica*. Embryos/larvae and juveniles were collected from the North Atlantic surface waters outside Åmøy (Norway), and exposed to simulated current hereafter named control (pH 8.0, 7.0 °C), or future OAW (pH 7.6, 10 °C) conditions, based on IPCC "business as usual" scenario predictions for the year 2100 in the North Atlantic (Bindoff et al., 2013, IPCC 2014). In the first of two experiments, embryos and larvae were exposed for 30 d to OAW conditions, and a number of developmental parameters recorded namely: hatching success; developmental rate; mortality and length. In the second experiment, juveniles were exposed for 14 d to OAW conditions, and mortality, metabolic and feeding rates, and moulting frequency were determined.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Animal material collection and maintenance

In common with other Norwegian krill populations, spawning of *M. norvegica* may start in March in the North Atlantic (Wiborg 1970) (Arnberg and Bechmann *pers. obs*). For more information about the species, see sect. 1.4.2. In order to collect krill for investigating the effects of OAW on embryos and early larval stages the following protocol was followed. Ovigerous females were collected using a modified shrimp trawl fitted with a 10 m macro plankton net (22 mm mesh size) and a 100 L closed cod end. Instead of using a net that may damage the krill, a barrel (cod end) (1 m x 1 m) was secured, to the bottom end of the macro plankton net. Trawling (mean velocity = 9.26 km $h^{-1}$, 15 - 20 min) was carried out at 50 m depth in the Hillefjord (north of Åmøy Rogaland County, Norway; 59° 04' 00" N - 5° 45' 00' E) in March 2012. Sampling was carried out between 03:00 and 04:00 h local time, during hours of darkness, because krill are very photosensitive and their eyes can be permanently damaged by light (Gaten et al. 2010). Upon retrieval, krill were sorted by using large plastic spoons to avoid mechanical damage whilst handling them. Approximately 200 undamaged individual ovigerous females were transferred from the barrel to eight large aquaria (vol. = 50 L) each filled with local fjord sea water. These were transported within 2 h of capture to the laboratory, while being kept in the dark. Upon arrival at the laboratory, krill were transferred to 9 L aquaria, 5 indiv. in each, and kept at current conditions (T = 7 ºC, S = 33, $pH_{NBS}$ = 8.1) in a temperature-controlled room, and in the dark. Next day the sea water from the aquaria was siphoned out and allowed to drain through a sieve (mesh = 40 µm), submerged into the sea water. As Northern krill immediately release their eggs when captured, this operation enabled us to rapidly separate the eggs from the adults. The resulting filtrated sea water represented an egg suspension, from which we subsampled a given volume to determine egg density in each aquaria as follows. Briefly, ten samples (vol. = 10 mL each) of sea water were pipetted into individual Petri dishes (diam. = 9 cm, vol. = 100 mL), and eggs were counted under a stereomicroscope (Wild M28, Leica, Wetzlar, Germany) to determine egg density. These eggs were not used further in any

experiment, as the handling may have damaged them irreversibly. Approx. 90 - 100 untouched eggs were then transferred from the egg suspension into one of nine Plexiglas cylinders (diam. = 8 cm, height = 10 cm) each fitted with a bottom plankton mesh (40 µm). The cylinders were placed in one of twelve flow through aquaria (vol. = 9 L). The egg density *per* cylinder was 3 - 4 eggs *per* mL.

From day one post-hatch the larvae were fed once daily with the cryophyte algae *Rhodomonas* sp. 1 mL (with an original algal density approximately $6.7*10^{-6}$ cells $mL^{-1}$ and mean size 7.5 µm ± 0.8 µm). The algae were raised at T = 20 °C in filtered sea water containing growth media (Himedia, Mumbai, India).

To supply juveniles (mean carapace length 0.62 ± 0.06 mm) for the second experiment individuals were collected as described above for ovigerous females in February 2012. Upon retrieval juveniles were sorted by hand, and approximately 200 undamaged individuals transferred by plastic spoon from the barrel to eight large tanks (vol. = 50 L) each filled with local fjord sea water to be transported to the laboratory within 2 h of capture and kept in the dark. Upon arrival at the laboratory, juvenile krill were haphazardly divided between four aquaria (vol. = 500 L, T = 7 ºC, S = 33, density approx. 75 indiv. *per* aquaria). Each aquarium was continuously supplied with fresh sea water directly pumped from 75 m depth in the fjord close to the laboratory facilities. The sea water was sand-filtered prior to the experimental system, in order to preserve the piping system and remove large particles. The juveniles were fed *ad libitum* with freshly-hatched *Artemia salina* nauplii (*Artemia* length 450-550 µm, feeding density 1000 indiv $L^{-1}$), phytoplankton, *Thalassiosira weisslogi* 1200™ (Microalgae, Vigra, Norway), ($2 \times 10^4$ cells $L^{-1}$) as well as micro capsulated liquid larval diet (10 - 50 microns EZ larvae approx. 0.003 mL $L^{-1}$, Zeigler, Gardens, USA) twice a day. Undigested material was removed the following day to avoid fouling of the sea water.

### 3.3.2 Maintenance of pH and temperature

Embryos/early larvae (nauplius I – calyptopis III) in the first experiment, and juveniles in the second experiment, were allocated to aquaria belonging to one of two treatments: 1) control conditions ($pH_{NBS}$ 8.0, 7 °C), or 2) OAW conditions (IPCC 2014) ($pH_{NBS}$ 7.6, T = 10 °C). All individuals were kept in two separate continuous-flow systems, consisting of six header tanks (vol. = 12 L, flow rate = 1 L $min^{-1}$) where temperature was regulated to either 7 or 10 °C, using heat exchangers. From the header tanks sea water was pumped into a number of aquaria (12 x 9 L) each containing either (a) three cylindrical vessels containing embryo/larva for the first experiment or (b) five juveniles for the second experiment. In the aquaria with reduced pH treatment, the desired $pCO_2$ equilibration was achieved, *via* manipulating pH by employing pH-controllers (AB Aqua Medic GmbH pH computer, Aqua Medic, Bissendorf, Germany) set to maintain mean $pH_{NBS}$ at 7.6, *via* a solenoid valve, which allowed regulating the addition of pure $CO_2$ gas. In total, there were three headertanks per treatment each heardertank were suppling two aqaurias.

### 3.3.3 Sea water chemistry

Sea water $pH_{NBS}$ was recorded and logged every 5 min using a pH probe (Orion Star Plus™ 3-Star and Ross® Electrodes, Thermo Fisher Scientific Inc, Beverly, USA) coupled to a calibrated pH meter (Orion Star Plus™, Thermo Fisher Scientific Inc) and a multi-channel datalogger (D - 130, Consort, Turmhout, Belgium) using the Star Plus Navigator 21 Software (Thermo Fisher Scientific Inc.), and employing the NBS scale. In addition, water temperature and pH were measured every second day, and oxygen concentrations twice over the duration of the experiment in all aquarias. Water temperature was measured using a calibrated glass thermometer (certified by Physikalisch-Technische Bundesanstalt, Braunschweig, Germany) and pH using a hand held pH meter (Orion Star Plus™ 3-Star, Thermo Fisher Scientific Inc.).

Oxygen concentration was measured with an oxygen meter (Oxi 330i/SET, WTW, Weilheim, Germany). The salinity of the intake water was recorded every 5 min during the entire duration of the experiments using a CT-probe (Aqua TROLL 100®, In-Situ Inc., Collins, USA) with Win-Situ 5 data acquisition software (In-Situ Inc., Collins, USA). Sea water samples for alkalinity measurements were removed five times during the experiment at day 1, 7, 14, 20, and 30, total alkalinity (TA) of the sea water was analyzed in ten samples four times during the experiment from the two header tanks using high-precision potentiometric titration (Haraldsson et al., 1997). There was no significant difference in mean alkalinity (TA) ($F_{1,9}$ = 0.193, $p$ = 0.672) between the different treatments (mean alkalinity at $pH_{NBS}$ 8.0 was 2306.80 ± 10.53 SD µmoll $kg^{-1}$, at $pH_{NBS}$ 7.6 was 2303.30 ± 14.48 SD µmol $kg^{-1}$). Hence, the mean TA value for all samples was used as input to the program CO2SYS.EXE (Pierrot et al., 2006). There was no significant difference between replicates within the treatments for temperature ($F_{5, 59}$ = 0.468, $p$ = 0.799) or for $pH_{NBS}$ ($F_{5, 65}$ = 1.460, $p$ = 0.215). Neither was there a significant difference between the same treatment in the two experiments for temperature ($F_{1, 63}$ = 0.56, $p$ = 0.816) there was only a small difference in reduced $pH_{NBS}$ between the future treatments of the two experiments ($F_{1, 69}$ = 11.62, $p$ = 0.001), the mean $pH_{NBS}$ value for experiment 7.61 and 7.63, for experiment 1 and 2 respectively. The total mean alkalinity measured in the experiments was 2305 ± 12 µmol $kg^{-1}$. Therefore, the temperature, pH and alkalinity data from the two experiments were pooled, and entered into the program CO2SYS.EXE (Pierrot et al. 2006) to calculate the saturation state for aragonite and calcite ($\Omega_{arg}$ and $\Omega_{cal}$) and the other parameters in the carbonate system. The carbonate system parameters that were not directly measured were calculated using the program, employing constants from Mehrbach et al. (1973) refitted to the NBS pH scale by Dickson and Millero (1987) and the $KSO_4$ dissociation constant from Dickson (1990). The saturation states, dissolved inorganic carbon (DIC), concentrations of carbonate [$CO_3^{2-}$] and bicarbonate [$HCO_3^-$] were calculated using mean ± 1 SD of measured pH,

temperature and alkalinity thereby taking account of the variability in the measurements. The results from the CO2SYS calculations are presented in Appendix 3.1. Mean $pCO_2$ values in the treatments ranged from 470 to 537 µatm ($pH_{NBS}$ = 8.0) and up to 1349 to 1795 ($pH_{NBS}$ = 7.6). Sea water was under saturated with respect to aragonite when $pH_{NBS}$ was below 7.6 (Appendix 3.1).

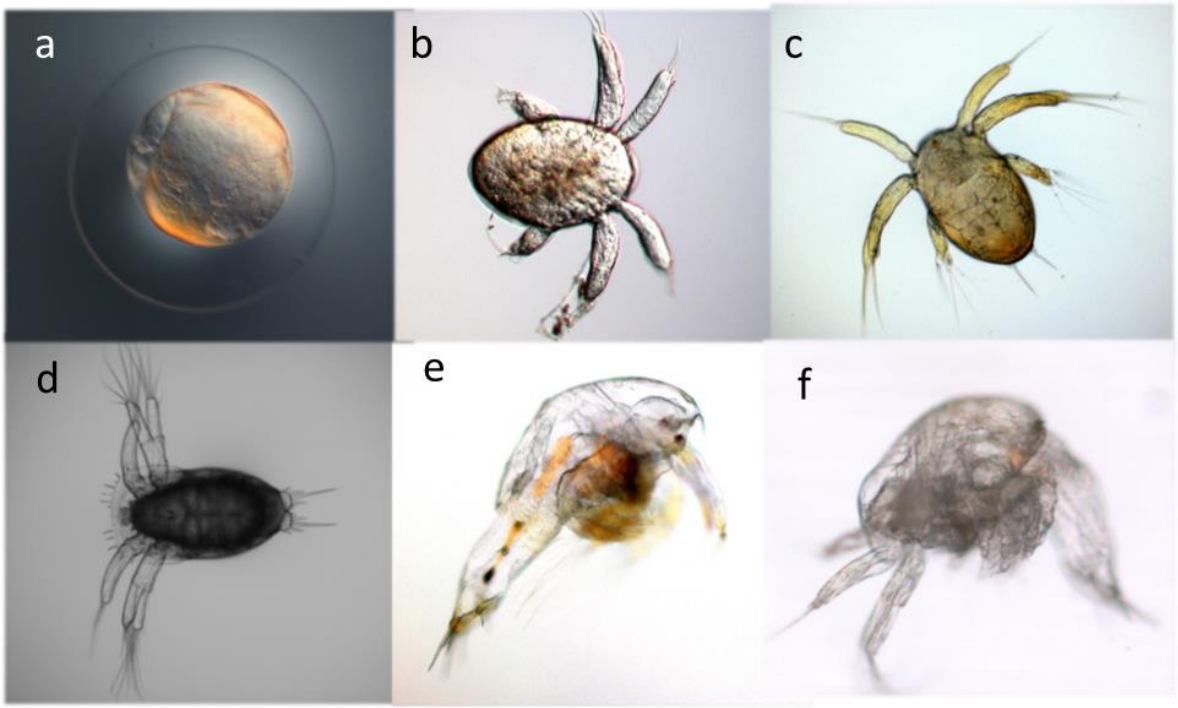


**Figure 3.2 Larval *M. norvegica* developmental stages a) developing egg, b) nauplius I, c) nauplius II, d) metanauplius, e) calyptopis I and f) calyptopis II (Photo appretiations to: Evgenia Dunaevskaya, Anna Ingvarsdottir and Maj Arnberg).**

3.3.4 Experimental procedures exp. 1

The cylinders containing newly hatched krill larvae were sampled at day 7, 14, 22 and 29 of the experiment. Three subsamples (initial embryo number 90) from each treatment were taken at each time interval. Unhatched eggs and larvae (both alive and dead) were siphoned out, counted under low power magnification (x 25) (Wild M28, Leica, Wetzlar, Germany), and stored in 4 % paraformaldehyde (PFA) for subsequent stage determination and length measurements.

Developmental stages (egg, nauplius I (N1), nauplius II (N2), metanauplius (MN), calyptopis I (C1), calyptopis II (C2), calyptopis III (C3)) were determined using the descriptions of Lebour and Sars 1898 (Lebour 1924) (see Fig. 3.2). Larvae were observed using a two imaging microscope (Axioplan, Zeiss, Wetzlar, Germany) fitted with a digital camera (MRc5, Axiocam, Wetzlar, Germany). Bifocal pictures were taken and length measurements of different larval stages were carried out. Hatching success was estimated at seven days post egg collection, and calculated as the percentage of living larvae over initial number of eggs. Mortality rates were defined as the number of living larvae over the initial number of eggs. Disappeared larvae or dissolved larvae were counted as dead.

### 3.3.5 Experimental procedures exp. 2

In the second experiment five juveniles of equivalent size (average total carapace length = 0.61 ± 0.06 cm) were allocated (after three weeks exposure under control conditions) to one of 12 aquaria (vol. = 9 L) each supplied with continuous flow-through sea water and assigned to one of the two treatments.

Juveniles were kept in the dark and fed twice daily (morning and afternoon) ad libitum on a mixture of, newly hatched *Artemia salina* nauplii (Artemia length 450-550 µm, feeding density 1000 indiv. $L^{-1}$), phytoplankton Thalassiosira weisslogi 1200TM (Microalgae, Vigra, Norway), (2 x 104 cells $L^{-1}$), and micro capsulated liquid juvenile diet (10-50 microns EZ larvae, 0.003 mL $L^{-1}$, (Zeigler, Gardens, USA). The number of dead krill per aquaria was determined every second day. Mortality was expressed as percentage dead juviniles in each treatment at the end of the experiment. Numbers of moults were recorded three times during the experiment at day 3, 7 and 11. Individuals were deemed to have moulted if an empty carapace (moult) was found in the aquaria. Moulting was expressed as mean moults per date per treatment.

Juvenile feeding rates were determined using a modification of the clearing rate methods by Harvey and Michel (2003). Feeding trials were conducted in glass beakers (vol. = 500 mL) each containing filtered sea water from the treatment header tanks used in the experiment. One juvenile was transferred into a beaker and placed at the respective temperature (7 or 10 °C). Freshly hatched *Artemia* nauplii (100 indiv.) were added to the water in 500 mL beakers containing the juveniles. The water in each beaker was then aerated using compressed air delivered through an air stone. The beaker was covered using aluminum foil to prevent evaporation and incubated for 20 h at the respective temperature. After the incubation period, juveniles were carefully removed from the beakers, with a small plastic spoon and the remaining sea water, containing *Artemia* nauplii, was sieved using a strainer (40 µm BD FalconTM cell, Biosciences, Franklin Lakes, USA), and individual nauplii counted. Amount of prey consumed was calculated as initial number of *Artemia* nauplii less their final number. Feeding rate was expressed as number of prey consumed per individual krill per unit time (number of *Artemia* indiv.$^{-1}$ h$^{-1}$). A control sample from the OAW treatment without krill was run through the experimental procedure, to test the efficiency of the sieving procedure. All 100 *Artemia* sp. nauplii were recovered in the control test.

The metabolic rate of juveniles was estimated 14 days after exposure, using rates of oxygen consumption as a proxy following the methods of Taylor and Spicer (1989). Individual juveniles were placed in a custom-built, closed glass-bottle respirometer (vol. = 300 mL), equipped with airtight stoppers into which $O_2$ electrodes were inserted and were in direct contact with the sea water within. Blank low concentration $O_2$ water samples (N = 3) were used to verify that the respirometers were airtight. Respirometers were supplied with sea water from the appropriate treatment header tank water used in the experiment and sealed with the airtight stoppers, and then placed in flow through water baths to maintain a constant temperature. A total of 20 replicates were measured for each of the two decreased pH/temperature

combinations. Following preliminary trials, measurements of dissolved $O_2$ concentration in the respirometers were performed every 2 sec for the entire duration of the incubation (approx. 6 h) using $O_2$ electrodes (1302, Strathkelvin Instruments, Glasgow, UK) coupled to a calibrated multichannel oxygen meter (928, Strathkelvin Instruments). Continuous measurements were conducted to demonstrate linearity of $pO_2$ decline in the respiration chambers during the incubation (N = 24).

Juvenile-free samples of sea water from the appropriate treatment header tank were used to estimate background respiration rates caused by microorganisms in the system. Background respiration never exceeded 10 % of the total juvenile respiration. Background rate respiration was used to correct the specimen's respiratory measurements. At the end of each trial, juveniles were removed from the respirometer, carefully blotted dry and then stored at – 80 °C for subsequent morphological and dry mass (DM) determinations, allowing us to present rates of oxygen consumption as µmol $O_2$ $h^{-1}$ $g^{-1}$ $DM^{-1}$. Total carapace length (TC) was measured for all juveniles. Individuals were dried to constant mass (approx. 24 h) on aluminum trays at 60 °C. They were then weighed on precision scales (Mettler-Toledo AT201, Oslo, Norway).

### 3.3.6 Statistical analysis

The effect of OAW conditions, for the biological parameters larval length, juvenile mortality, moulting, feeding and metabolic rates in this study was analyzed using General Linear Model (GLM) tests, one-way ANOVA. All these parameter data met assumption for normality of distribution, tested by the Kolmogorov-Smirnow test, and variances were homogeneous using the Levens – test®. A Wilcoxon Rank sum test for small sample sizes (Bhattacharyya and Johnson 1977) were used to analyze the effects of OAW conditions on the biological parameters larval hatching, mortality and developmental parameters. All analyses were conducted using v 21 SPSS®.

## 3.4 RESULTS

### 3.4.1 Experiment 1 - Larvae

#### 3.4.1.1 Hatching success, survival, developmental rate and length of krill larvae

The average hatching success was 42.2 ± 5.1 % and 36.3 ± 14.5 % for the control and OAW conditions respectively (Appendix 3.2). Hatching success was not significantly affected by OAW conditions ($p$ = 0.233). Mortality rates (Fig. 3.3) were high in both treatments, but mortality was significantly greater under OAW conditions, at all-time points sampled ($p_{max}$ = 0.05). Survival was high in both treatments during the metanauplius to calyptopis (MN - C1) stage but thereafter fell steeply during the C2 - C3 stage. In the control condition, this occurred from day 22 and in the OAW condition day 14. The results indicate that C2 larvae had similar mortality rates in both treatments (Fig. 3.3 and 3.4). At the end of the experiment (day 29), 22 % of the hatched larvae were still alive in the control conditions, but there were no survivors found in the OAW conditions (Fig. 3.3).

Furthermore, exposure to OAW conditions significantly decreased the development time for all the stages, with larvae reaching C3 more rapid compared to those raised under control conditions (Fig. 3.4) ($p_{max}$ = 0.025). In addition, larvae were significantly longer under OAW conditions at the MN stage (Table 3.1) ($F_{1, 15}$ = 17.980, $p$ = 0.003), but significantly reduced in length at the C2 stage (Table 3.1), ($F_{1, 41}$ = 10.100, $p$ = 0.003).

**Table 3.1 Mean body lengths (mm) of early larval *M. norvegica* in control and OAW conditions. Number in brackets indicate the number of larva measured. (* indicate single measurements which should be treated with caution).**

| Treatment | Nauplii | | Metanauplius | Calyptopsis | | |
|---|---|---|---|---|---|---|
| | NI | N2 | MN | CI | C2 | C3 |
| Control | 0.49 (4) | 0.49(25) | 0.52 (9) | 1.02(20) | 1.12(21) | 1.45 (2) |
| OAW | | 0.48 (7) | 0.57 (7) | | 1.04(22) | 1.39 * |
| Mauchline 1971 | 0.48 | 0.48 | 0.50 - 0.52 | 1.03* | 1.59* | 2.4* |

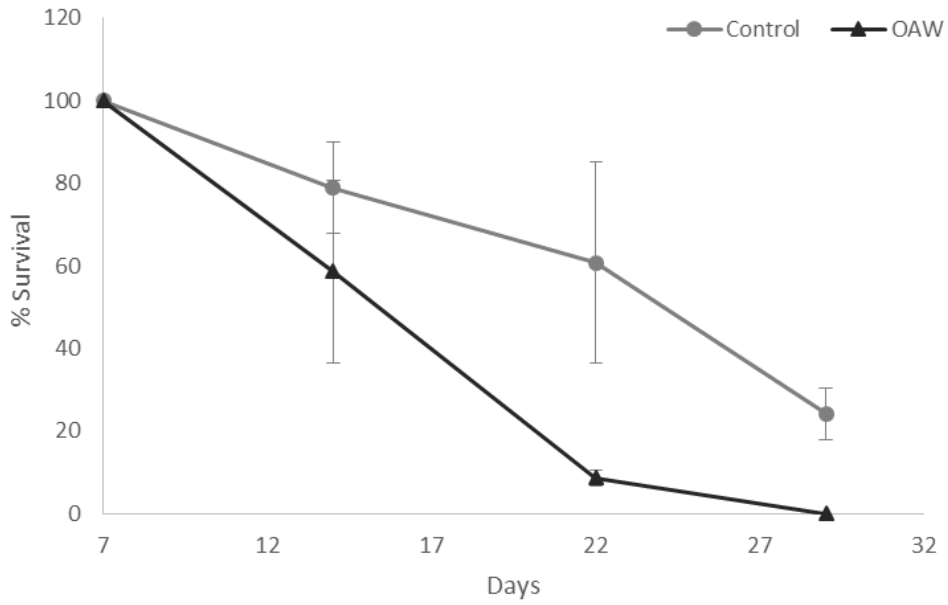


**Figure 3.3 The effect of control and OAW conditions on the mortality, % alive larvae of initial number hatched larvae, of larvae of *M. norvegica*. Control (pH 8.0, 7 ºC, light grey) (N = 3) and OAW (pH 7.6, 10 ºC, black) (N = 3).**

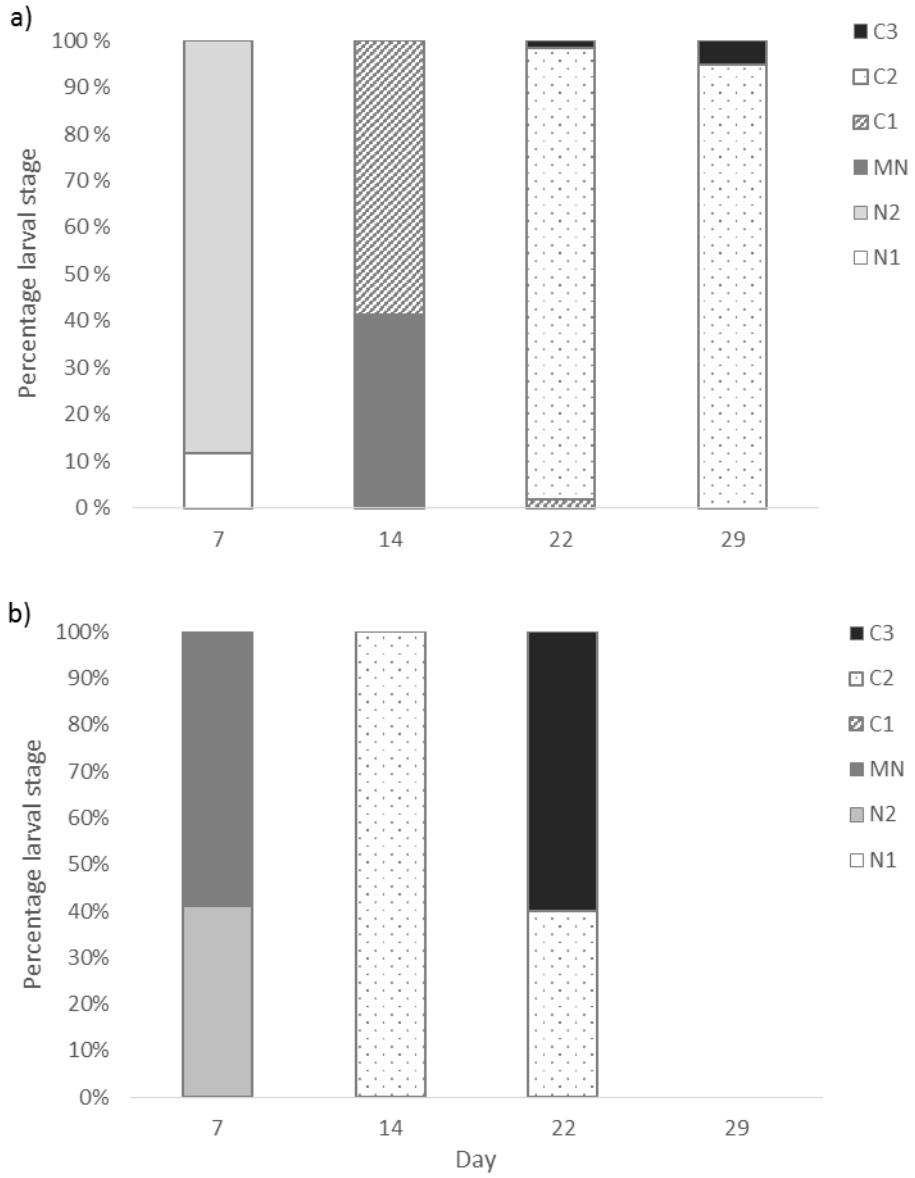


**Figure 3.4 The effect of control and OAW conditions on the development of *M. norvegica* larvae (stage *nauplius* I – *calyptopis* III) exposed to a) control (pH 8.0, 7 ºC) and b) OAW (pH 7.6, 10 ºC) conditions. N1 = nauplius I, N2 = nauplius II, MN = metanauplius, C1 = calyptopis I, C2 = calyptopis II and C3 = calyptopis III (N = 3).**

### 3.4.2 Experiment 2 - Juveniles

#### 3.4.2.1 Mortality and moulting

At the end of the exposure period (14 d) average mortality was 16.6 % and 20.0 % under control and OAW conditions respectively, but this difference was not significant ($F_{1, 88}$ = 1.010, $p$ =

0.428). However, exposure to the OAW conditions significantly reduced the number of moults by 67 % compared to control condition (Fig. 3.5) ($F_{1, 34}$ = 5.670, $p$ = 0.023). The number of moults was greater at day seven (Fig. 3.5), the term 'time' significantly influencing the number of moults ($F_{2, 33}$ = 4.5, $p$ = 0.019).

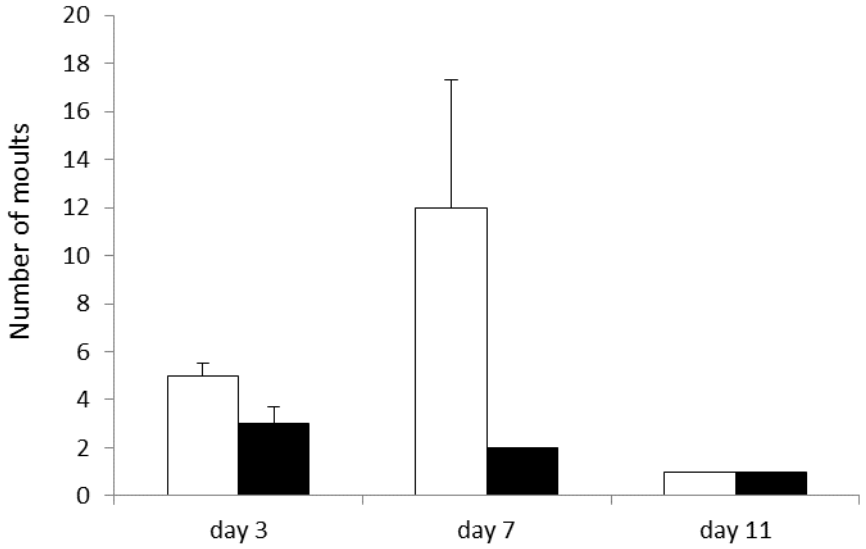


**Figure 3.5 The effect control and OAW conditions on mean moulting, number of moults *per* six aquaria, of juvenile *M. norvegica* for day 3, 7 and 11. Control (pH 8.0, 7 ºC, clear) (N = 6), OAW (pH 7.6, 10 ºC, black) (N = 6). Values are means ± SD.**

3.4.2.2 Feeding and metabolic rates

Exposure to OAW conditions significantly decreased juvenile feeding rates compared with the control conditions (Fig. 3.6) ($F_{1, 25}$ = 4.250, $p$ = 0.050). In addition, rates of $O_2$ uptake were found to be significantly greater (36 %) in individuals kept under OAW conditions (Fig. 3.7), with a mean value of 81.6 µmol $O_2$ $h^{-1}g^{-1}$ DM compared to the control conditions with a mean value of 52.2 µmol $O_2$ $h^{-1}g^{-1}$ DM ($F_{1, 32}$ = 13.53, $p$ = 0.01).

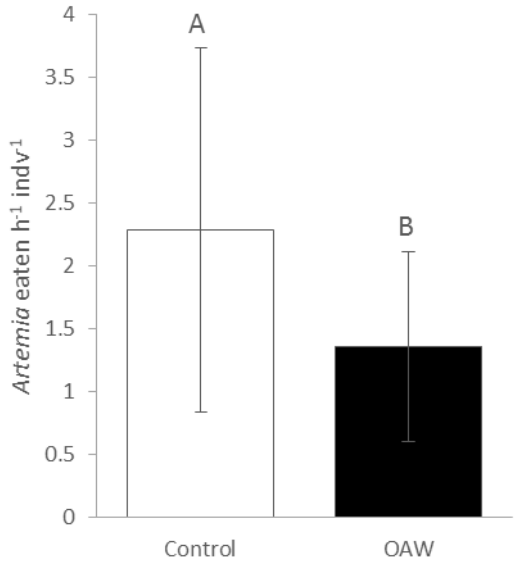


**Figure 3.6 The effect of control and OAW conditions on mean feeding rates (measured as mean number *Artemia* eaten indiv$^{-1}$ h$^{-1}$) of juvenile *M. norvegica*. Control (pH 8.0, 7 ºC, clear) (N = 15), OAW (pH 7.6, 10 ºC, black) (N = 15). Histograms represent means ± SD. Significantly different treatments ($p \leq 0.05$) are indicated by upper case letters.**

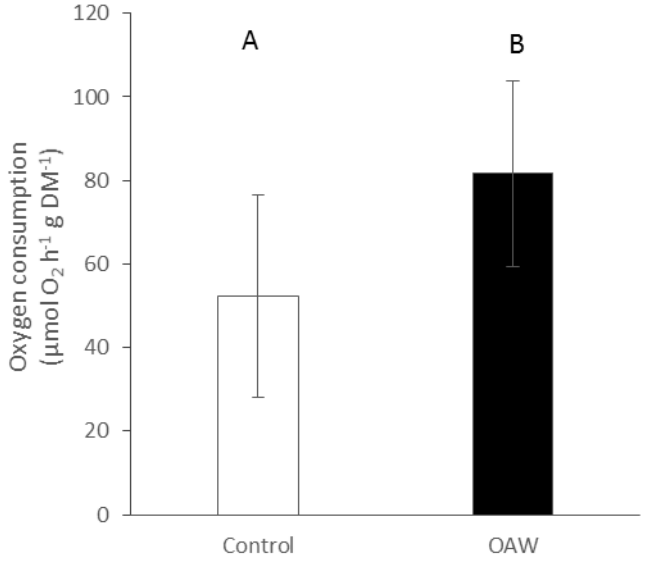


**Figure 3.7 The effect of control and OAW conditions on mean oxygen consumption (µmol $O_2$ h$^{-1}$ mg $^{-1}$ DM$^{-1}$) of juvenile *M. norvegica*. Control (pH 8.0, 7 ºC, clear) (N = 16), OAW (pH 7.6, 10 ºC, black) (N = 18). Histograms represent means ± SD. Significantly different treatments ($p \leq 0.05$) are indicated by upper case letters.**

## 3.5 DISCUSSION

These results are the first on the sensitivity of early life stages of a krill species to the combined exposure to OW and OA, OAW. Exposure to combined OAW conditions in the Northern krill *Meganyctiphanes norvegica* does not appear to affect embryo-hatching success but does lead to an increase in larval developmental rates. Furthermore, juvenile mortality does not change significantly following exposure to OAW conditions, whilst metabolic rate increased by 36 %, and feeding rates and number of moults decreased by 60 and 67 % respectively.

### 3.5.1 Impact of exposure of OAW conditions on embryos and larvae

Exposure of krill embryos/eggs to OAW had no effect on hatching success although it appeared to cause a reduction in larval developmental time. Ross and Quetin (1989), reported that embryos of the North Pacific krill, *Euphausia pacifica*, unlike adults and juveniles, were highly sensitive to handling, and that this may explain the variability in hatching rates reported in several studies of krill. Northern krill generally have relatively low survival in the laboratory (Buchholz 2003). In this study, the mean hatching success of embryos of Northern krill varied between 36 - 40 %, and was not affected by exposure to OAW conditions. The robustness of embryos to OAW has previously been reported for other crustacean species (e.g. Styf 2014). OW had no effect on hatching success in the Antarctic krill, *Euphausia superba* (Yoshida et al. 2004), whilst exposure to increased $pCO_2$ (1250 and 2000 μatm) reduced hatching success in the same species (Kawaguchi et al. 2011; Kawaguchi et al. 2013). This is the first study where the effects of OAW on krill hatching success has been investigated. The hatching success levels recorded for Northern krill embryos kept under control conditions in our experiment fall within the range reported for the Antarctic krill (Harrington and Ikeda 1986; Yoshida et al. 2004; Kawaguchi et al. 2011).

Survival rates of Antarctic krill are generally low during early development under laboratory conditions similar to our control (Hirano et al. 2003). This was also found for the early larval developmental stages of the Northern krill in this study, with only 22 % of larvae surviving under control conditions. Mortality of krill larvae was significantly greater under OAW conditions at all-time points. However, the stage specific mortality appeared to be similar because the larvae developed faster in OAW. In particular, the increased mortality observed at the transition between the C2 and C3 stages in both treatments indicates that this could be a critical developmental phase for the Northern krill. The stage-specific sensitivity that was observed in this study, suggests that further improvement of laboratory practice and husbandry techniques for the Northern krill larvae is required.

The accelerated development in the larvae raised in OAW, would lead to larvae spending shorter periods in the plankton. This would have beneficial consequences for larval survival both directly and indirectly via reducing developmental time and thus reduced predation risk. Alternatively, accelerated developmental rate can lead to higher mortality levels and often comes at the cost of increased energetic costs for maintenance and repair, which can lead to reduced larval size (e.g. Small et al. 2015).

Faster developmental rate has also been reported for krill species exposed to OW (Yoshida et al. 2004), and Northern krill seems similar in this regard, despite the fact that krill were tested for combined effects of OA and OW. Poulsen et al. (2011) showed that Antarctic krill exposed to a pollutant underwent an accelerated development from N1 to MN, the non-feeding larvae; they suggested that the accelerated development carried additional energetic costs, possibly preventing metamorphosis in following larval stages. In fact, the lipid reserves of the non-feeding larvae are being depleted when the larvae approach the feeding C1 stage (Ross and Quetin 1989), thus only a very narrow window remains to initiate feeding, to compensate for extra metabolic, maintenance and repair costs, when the larvae reach the C1 stage. If this

window is further reduced by the impact of OAW as it appears from our and other studies, survival of krill early life stages may be compromised (Poulsen et al. 2011).

Furthermore, this shifting in timing and shortening of feeding window may cause potential mismatch with processes timed according to constant cues (e.g. light). Global change such as OAW could therefore potentially lead to mismatches between the reproductive cycles of *M. norvegica* and its planktonic food, this match being a key component for the survival and recruitment success of Northern krill larvae, as documented for the Northern shrimp, *Pandalus borealis* (Koeller et al. 2009). However, such a mismatch may not materialise if concurrent changes in the surface oceanography also result in earlier blooms.

### 3.5.2 Impacts of exposure of future OAW conditions on juveniles.

Exposure to OAW conditions was not lethal to juvenile *M. norvegica* during the time course of our experiment. Juvenile mortality ranged between 16 and 20 % after 14 d exposure (total of four weeks in the laboratory), and fell within the mortality range of juvenile control groups of two other experiments with the same species (mortality after two weeks 15 and 21 %) performed at IRIS (Moodley and Invarsdottir *pers. comm.*). Another krill species widely distributed in the north Atlantic (*Nyctiphanes couchii*), had higher mortality rate (50 %) than in our experiment under comparable control conditions (Sperfeld et al. 2014). No lethal effects were detected for *N. couchii* following exposure to similar pH levels to those used in our study, although at lower pH (1700 µatm) most krill died within six days (Sperfelt et al. 2014). Despite the fact that no lethal effects of OAW conditions were found for juvenile Northern krill, a number of sub lethal effects were detected in the juveniles. Juveniles can compensate for acute temperature change through physiological plasticity (Saborowski et al. 2002; Strömberg and Spicer 2003). This ability to compensate for the negative direct effects of OW over a short period could explain the observed significant increase in metabolic rates (+ 36 %) in juvenile

krill exposed to future OAW conditions, in this study. On this basis, we predict that Northern krill will have greater maintenance and repair costs in future oceanic conditions. Increased energetic costs could be compensated *via* increased feeding effort or high food availability (Melzner et al. 2011). On the contrary, this study observed 60 % decreased feeding rates under OAW conditions. This result indicate that juvenile krill will be severely energetically challenged, possibly affecting survival and further development (Buchholz and Buchholz 2010). However, one must consider that abnormal swimming behaviour and associated reduced feeding activity have been observed for the Antarctic krill, *Euphausia superba* kept in small containers, where animals bumped into the walls of the containers more frequently (Price et al. 1988). The Northern krill in this experiment displayed higher metabolic rates due to the OAW conditions, and possible an associate greater level of activity (swimming) when compared to control conditions, which could explain the reduced feeding activity in under OAW conditions. Moreover, the reduction in feeding together with the increasing energy demand could possibly explain the decreased moulting observed in the OAW conditions; possibly the sign of a delay in development. A reduction in food may depress the hormonal trigger and delay moulting in Northern krill until the situation improves (Buchholz 2003). Krill can survive periods of high metabolic cost, induced by elevated temperature or starvation, by shrinking between moults even though food is abundant (Marinovic and Mangel 1999; Buchholz 2003). The moulting cycle of the krill in the Stavanger area has been estimated to be around 13.5 d at 7 °C (Ingvarsdóttir unpubl. data). The timing of the experiment and length could potentially influence the results and moulting rate data in shorter experiments such as ours, and therefore our results should be interpreted with some care. Nonetheless, as all krill were collected on the same date, and treated identically through the acclimation period, our data should to some degree be indicative of the effect on OAW conditions on moulting.

### 3.5.3 Conclusions

It has been shown that juvenile Northern krill can survive the OAW conditions tested here in this study. Nevertheless, there were noticeable sub-lethal effects, including increased metabolic costs, reduced feeding and possible interruption or delay of moulting. The larval stages on the other hand, appeared to be more sensitive to the OAW conditions displaying accelerated developmental rates. Even though the juvenile/adult krill may be able to adapt to OAW conditions (Sunday et al. 2011), due to the species ability to acclimatise to local temperature conditions (Saborowski et al. 2002; Strömberg and Spicer 2003) the larvae may not be able to acclimatize. The larvae have increased developmental rates, which may induce greater maintenance costs and narrowed feeding window in the future conditions. If the larvae are not able to meet their demand for energy to grow and develop due to possible mismatch with its food, mortality may follow in the further larval stages. This will represent an important challenge for the larvae especially during food-scares periods in the sea.

Although this was a relatively short-term experiment, the results highlight the need to understand more about the response of this keystone species to conditions predicted for the future of our oceans. Additionally, whilst long-term experiments are needed to understand longer-term effects on the species population biology, our results enable us to consider the potential implications for the temperate pelagic systems they belong too.

**Chapter 4. Combined effects of future climate conditions and oil spill on development, feeding and metabolism of the Northern shrimp (*Pandalus borealis*) larvae.**

**Figure 4.1 Adult *Pandalus borealis* with embryos (Photo: Renee Bechmann).**

## 4.1 SUMMARY

The future oceans will continue to experience increasing environmental pressure from several anthropogenic activities such as ocean warming (OW) and ocean acidification (OA). The effect of these global drivers is best studied in combination, and though there is increasing recognition that their effects may be exacerbated by the effects of other anthropogenic activities, such as offshore oil and gas industry, and ship transports. Nonetheless, our knowledge of combined global and local stressors effects on marine life has not kept pace. Consequently this study investigated the effects of a simulated seven day Arctic crude oil spill scenario (0.5 mg $L^{-1}$) on a number of key aspects of the biology of adults, embryos and larvae of the Northern shrimp, *Pandalus borealis* exposed to combined future conditions for OA and OW: OAW, + 3.0 °C and - 0.5 pH.

The exposure to OAW conditions together with an oil spill resulted in significantly lower neutral red retention time (NRRT) in gravid females, which is an indication of cellular stress. Embryos showed a reduction in mean time to first hatching when exposed to OAW, whilst neither factors had a significant effect on hatching success. Larvae exposed to oil under OAW conditions produced significantly smaller larvae (- 14 %) when compared to all other treatments, this being an additive effect of both OAW conditions (- 9 %) and oil spill (- 5 %). In addition, larvae developed nine days faster to stage IV under OAW conditions exhibited increased mortality (+ 30 %), swimming (80 %) and feeding rates (+ 15 - 20 %). In contrast, when exposed to a simulated oil spill in isolation, larvae showed reduced feeding (- 24 %) and swimming (- 57 %), these responses being independent from exposure to future OAW conditions. Furthermore, oil exposed larvae at stage IV showed a greater incident (+ 20 %) of deformities of the abdomen.

Shrimp larvae in the future ocean could experience accelerated developmental rates, accompanied by greater maintenance costs, which lead them to reach smaller size at stage IV. Furthermore, there were additive effects of combined exposure to OAW and an oil spill on the size of shrimp larvae, which suggest larval shrimp's resilience to local impacts (i.e. local oil spill) could be significantly reduced if they are exposed to future OAW, as they are already energetically limited and show a reduced development. Consequently, environmental policy management should consider the impacts of contaminants, such as such as oil spill, within the context of ongoing global change, if we are to preserve marine natural resources, biodiversity and ecosystems functions.

## 4.2 INTRODUCTION

Human-driven changes to ocean biogeochemistry affect multiple marine processes and systems. And future oceans will suffer from the impacts from several different drivers, drivers in marine

systems can be divided into two categories those that act globally and those that act locally (Sect. 1.1.1). Drivers such as OA and OW are predicted to have significant impacts on marine invertebrate species and ecosystems including changes in species dominance and local population extinction (Sect. 1.1.3).

Other drivers can originate and act at the local scale and may be either chronic (e.g. nutrient input from rivers) or transient, brief and short lived (e.g. oil spill) (Sect. 1.1.1). Previous work demonstrated that oil spills could have far-reaching biological consequences, including mass mortality (Conan et al. 1982; Peterson et al. 2003). Polycyclic aromatic hydrocarbons (PAHs) in oil in particular are known to be toxic pollutants for marine biota (Van brummelen et al. 1998; Skadsheim et al. 2000; Incardona et al. 2014; Incardona et al. 2015). Consequently, benthic marine invertebrates and their planktonic life stages face multiple environmental challenges with greater frequency and intensity in the future ocean: including OA, OW, persistent organic pollutants and accidental discharges from the oil industry and from ships. While we are starting to obtain a clearer picture of the biological effects of each of these drivers individually, we are only beginning to understand their effects in combination. There is indeed an urgent need for experiments investigating the effects of multiple drivers, which can provide a more realistic picture of what organisms will be experiencing in the wild (Sect. 1.1.5).

This is particularly important, as the majority of ocean management strategies are not yet addressing the issue of dealing with multiple drivers in a coherent way (Noone et al. 2012). When multiple drivers act simultaneously, there is a greater probability for additive, synergistic, or antagonistic effects occurring (Sect. 1.1.5). The first important step in ecophysiological studies is to understand whether there are interactive effects of multiple drivers and of what type these are to explore the most effective way to manage them (Ghedini et al. 2013; Nikinmaa 2013; Boyd and Brown 2015). Oil exposure may interact with exposure to OW and OA conditions, further complicating our ability to predict the true impact of an oil spill on marine

species, particularly on keystone species. Understanding the effects of environmental changes on key habitat species is an ecosystem conservation priority. In fact, changes or loss of key species is likely to have broad consequences for whole ecosystems (Pimm 1991). One of such species is the Northern shrimp, *Panadalus borealis* (Fig 4.1), which is a key organism in Arctic and subarctic benthic habitats (Anderson 2000; Olsen et al. 2012) and source of food and income for coastal fisheries, with ~16 000 t were landed in Norway in 2014 (NDF 2014).

Adult pandalid shrimps are known to be sensitive to oil contamination (Anderson et al. 1974; Vanderhorst et al. 1976; Sandborn and Malins 1980), with larvae being more sensitive than adults (Mecklenburg et al. 1977; Brodersen 1987). In addition, since larvae occur in surface waters they are also more likely to be exposed to higher concentrations of oil contamination from accidental oil spills. Altogether, *P. borealis* is therefore a relevant organism to use in future monitoring of regular discharges of oil and gas operations or following accidental discharges of petroleum compounds, since it can be found in the whole water column (Bechmann et al. 2010). In addition, Koeller et al. (2009) have shown that populations of the Northern shrimp, *P. borealis* found along a thermal gradient in the north Atlantic have strongly adapted to local temperatures and hatching time of the eggs correspond to timing of the algal bloom. This makes *P. borealis* vulnerable to long-term climatic changes. Consequently, with the current development of the oil industry and the ship transportation industry in areas where *P. borealis* occurs there is an increasing need to predict the likely consequences that an oil spill may have on northern ecosystems in a future warmer and more acidic ocean, particularly as these drivers supposedly have different mode of action.

OA and OW are believed to largely affect organisms' energy metabolism, and oil having both sub lethal effects, affecting larval activity, by narcotic or sluggish effects, leading to reduced feeding and growth, and lethal effects increasing mortality. The separate drivers therefore have a different mode of action on the larva. Both embryonic and larval development in *P. borealis*

is known to be temperature and pH dependent (Weinberg 1982; Brillon et al. 2005; Bechmann et al. 2010), with significant delay in zoeal development when exposed to low pH condition at 5 °C (Bechmann et al. 2010), but faster developmental time, increased metabolic rate, reduced survival and smaller size in larvae exposed to elevated temperature (Chapter 2).

Oil exposure of female shrimp carrying embryos for three months, caused increased mortality in the resultant larvae (Bechmann et al. 2010). Exposure of shrimp larvae to 0.015 mg $L^{-1}$ and 0.06 mg $L^{-1}$ of crude Arctic crude oil, caused increased mortality and developmental time with increasing concentration of oil, in addition to a 13 % decrease in growth of shrimp larvae exposed to the highest concentration of oil (Taban et al. 2007).

Planktonic larval stages are considered to be the most vulnerable life stage when a species is exposed to environmental (Sect. 1.1.2). Larvae are also expected to show more susceptibility to oil due to their higher surface area to volume ratio, and larvae could be exposed dispersion containing 10 µm oil particles (as for Bechmann et al. 2010) and dissolved oil components (Skadsheim et al. 2000). The oil droplets are approximately the same size range as phytoplankton and since stage I shrimp larvae are also known to ingest small particles such as phytoplankton (Pedersen and Storm 2002), this possibly representing a pathway of contamination for oil, together with adherence to and diffusion throughout the body.

No study has, to date, explored the combined effect of exposure to oil, OA and OW on marine organisms. Consequently, the aim of this chapter was to address this omission and investigate the possible interactive effects between a simulated seven days oil spill scenario of Arctic crude oil (0.5 mg $L^{-1}$) under OAW, for OA and OW predicted to occur in the North Sea for the year 2100, on the (1) physiology of adults, (2) the development of embryos, and (3) development and physiological ecology of larvae of the Northern shrimp, *P. borealis*. Our working hypothesis is that the combined exposure to an oil spill and OAW will lead to more severe effects on marine organisms' life history, developmental biology and ecophysiology, than the

single stressors. The results of the present study will be important not just to our understanding of multi-driver effects of marine organisms in general, but also to our ability to predict the future of an economically important resource in a warmer, more acid ocean, with the greater likelihood of encountering oil spills.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Adult collection and maintenance

Ovigerous females of *P. borealis* (mean ± SD cephalothorax length 2.4 cm ± 0.2) were collected using a bottom shrimp trawl from Hillefjord (north of Åmøy Rogaland County, Norway; 59° 04' 00" N - 5° 45' 00' E) during Jan 2012. Trawling lasted 15 - 20 min at 100 m depth at a speed of 9.26 km $h^{-1}$. Instead of using a net that may damage the shrimps, a barrel (1 m x 1 m) was secured, to the bottom (cod-end) of the trawl. The catch was a mixture of fish (haddock, cod, sole), krill and shrimp. Female shrimp were sorted by hand. Approximately 300 undamaged individuals were transferred to eight aquaria (vol. = 50 L, density approx. 38 indiv. *per* aquaria) each filled with fjord sea water and transported to the laboratory within 2 h from capture. Upon arrival at the laboratory, shrimps were haphazardly divided between five aquaria (vol. = 500 L, density approx. 60 indiv. *per* aquaria) and kept at laboratory conditions (T = 7 ºC, S = 33, $pH_{NBS}$ = 8.1). Each aquarium was continuously supplied with sea water directly pumped from 75 m depth in the fjord close to the laboratory facilities. The sea water was sand-filtered prior to use in the experimental system. Shrimps were fed raw fish *ad libitum* three times a week during the acclimation period (20 d), and undigested material was removed the following day to avoid fouling of the water.

4.3.2 Collection and maintenance of larval shrimps

Shrimp females were acclimated for 14 d to control and OAW conditions (see below for details) prior to exposure to oil. Ovigerous females (48 *per* treatment) were acclimated, and the total number of females was selected to ensure that a large number of shrimp larvae would hatch simultaneously (as in Chapter 2) following an exposure period to experimental conditions: minimum two weeks before hatching. Ovigerous females (N = 24 in total, six *per* treatment) were transferred to individual flow-through aquaria (vol. = 9 L; flow rate = 0.12 L $min^{-1}$), of the four different treatments. Shrimp larvae show photo-taxi and will immediately swim towards a light source; this means they were kept under a normal photoperiod of light and dark, they will swim towards the surface and thereby have the possibility to be trapped in the oil film. Therefore, and also to ensure similar conditions for all the treatments, females (experiment 2) and later larvae were kept in the dark with a light source positioned at the bottom of the aquaria. Each aquarium was checked every day in order to determine the time to first hatching (as described in experiment 1 below). Again hatching success was defined as the first day free-swimming larvae were observed. Batches of larvae (N = 200) all from the same female were kept in the same individual glass aquaria as described above (vol. = 9 L). From each experimental treatment six batches from six different mothers, were exposed and monitored until they reached stage IV zoea. The batches, consisting of 200 larvae (< 24 h old) from each mother, were kept separate and not mixed.

Post-hatch shrimp larvae were fed *ad libitum* on freshly hatched *Artemia salina* nauplii (*Artemia* length 450 - 550 µm, feeding density 1000 indiv $L^{-1}$) twice a day (morning and afternoon) for the entire duration of the experiment. In addition, for the first week, larvae were also fed with phytoplankton, *Thalassiosira weisslogi* 1200$^{TM}$ (Microalgae, Vigra, Norway, 2 x $10^4$ cells $L^{-1}$) once a day, to ensure feeding for stage I and II zoea following the protocols of Pedersen and Storm (2002) and Ariza and Ouellet (2009). Dead *Artemia* were removed every

day to avoid seawater contamination. Mortality of shrimp larvae was calculated every day and reported as percentage number of accumulated dead larvae.

4.3.3 Experimental design and set-up

The effects of OAW conditions, oil spill and their interaction on adult female shrimp feeding, respiration rate, lysosomal membrane stability, as well as hatching success, hatching time and duration of hatching (experiment 1, see below) and larvae survival, developmental time, feeding, swimming respiration and growth (experiment 2, see below) of *P. borealis* were investigated using an orthogonal experimental design including two climate scenarios (current (control) and future (OAW) conditions) and two 'oil spill conditions' (no oil spill and oil spill). Thus experimental treatments were as follows: [1] control ($pH_{NBS}$ = 8.1, T = 6.7 °C), [2], control + oil spill ($pH_{NBS}$ = 8.1, T = 6.7 °C, oil concentration = 0.5 mg $L^{-1}$), [3] OAW (IPCC 2014 scenarios + regional scenarios) ($pH_{NBS}$ 7.6, 9.5 °C), [4], OAW + oil spill ($pH_{NBS}$ = 7.6, T = 9.5 °C, oil concentration = 0.5 mg $L^{-1}$).

Ovigerous females and later larvae (stage I - IV) of *P. borealis* were allocated to one of these four treatments. Shrimps were kept in two separate continuous flow systems, consisting of six header tanks (vol. = 12 L, flow = 1 L $min^{-1}$) where temperature was tightly regulated at either 6.7 ± 0.08 or 9.5 ± 0.07 °C respectively, using heat exchangers. In the aquaria with reduced pH treatment, the desired $pCO_2$ equilibration was achieved, *via* manipulating pH using the method by Widdicombe and Needham (2007) employing pH-controllers (AB Aqua Medic GmbH pH computer, Bissendorf, Germany) set to maintain mean $pH_{NBS}$ at 7.6, *via* a solenoid valve, which allowed regulating the addition of $CO_2$ gas.

For experiment 1 the header tanks delivered sea water to 24 x 60 L aquaria using gravity. Each aquarium contained eight ovigerous females (N = 192 female shrimps in total). To ensure the female shrimps had a 14 d acclimation period to OAW conditions prior to be exposed to oil

exposure, adult females were exposed to oil (0.5 mg $L^{-1}$) between day 14 and 21. For each treatment 3 headertanks were used, each supplying to aquaria. For experiment 2, the 60 L aquaria were replaced by 24 x 9 L aquaria each containing a batch of 200 shrimp larvae. Larvae were exposed to oil from the day they hatched until 7 d post hatch (dph).

### 4.3.4 Characterization of the Carbonate System

Sea water $pH_{NBS}$ was recorded and logged every 5 min using a pH probe (Orion Star Plus™ 3-Star and Ross® Electrodes, Thermo Fisher Scientific Inc, Beverly, USA) coupled to a calibrated pH meter (Orion Star Plus™, Thermo Fisher Scientific Inc) and a multi-channel datalogger (D-130, Consort, Turmhout, Belgium) using the Star Plus Navigator 21 Software (Thermo Fisher Scientific Inc.), and employing the NBS scale. In addition, water temperature and $pH_{NBS}$ were measured every second day, and oxygen levels two times over the duration of the experiment in all aquarias. Water temperature was measured using a calibrated glass thermometer (certified by Physikalisch-Technische Bundesanstalt, Braunschweig, Germany) and $pH_{NBS}$ using a hand held $pH_{NBS}$ meter (Orion Star Plus™ 3-Star, Thermo Fisher Scientific Inc.). Oxygen concentration was measured with an oxygen meter (Oxi 330i/SET, WTW, Weilheim, Germany). The salinity of the intake water was recorded every 5 min during the entire duration of the experiments using a CT-probe (Aqua TROLL 100®, In-Situ Inc., Collins, USA) with Win-Situ 5 data acquisition software (In-Situ Inc., Collins, USA). Sea water samples for alkalinity measurements were taken five times during the experiment at day 1, 7, 14, 20, and 30, total alkalinity (TA) of the sea water was analyzed in ten samples four times during the experiment from the two header tanks using high-precision potentiometric titration (Haraldsson et al. 1997). There was no significant difference in mean measured alkalinity (TA) ($F_{1,9} = 0.193$, $p = 0.672$) between the different treatments [mean alkalinity at $pH_{NBS}$ 8.0 was 2306.80 ± 10.53 SD µmol $kg^{-1}$, at $pH_{NBS}$ 7.6 was 2303.3 ± 14.48 SD µmol $kg^{-1}$], hence the mean TA value for

all samples was used as input to the program CO2SYS.EXE (Pierrot, Lewis et al. 2006). The program CO2SYS.EXE (Pierrot et al. 2006) was used to calculate the saturation state for aragonite and calcite ($\Omega_{arg}$ and $\Omega_{cal}$) and the other parameters in the carbonate system: dissolved inorganic carbon (DIC) and concentrations of carbonate [$CO_3^{2-}$] and bicarbonate [$HCO_3^-$]. The input parameters were total alkalinity, $pH_{NBS}$, temperature and salinity in addition to the dissociation constants from Mehrbach, Cullberson et al. (1973) as refitted by Dickson and Millero (1987). The saturation states and dissolved inorganic carbon (DIC), concentrations of carbonate [$CO_3^{2-}$] and bicarbonate [$HCO_3^-$] (see Table 1) have been calculated using mean measured values minus standard deviation or mean values plus standard deviation for each parameter to take into consideration the variability in the measurements. The mean $pH_{NBS}$ level ± SD values, in addition to a range are presented in Appendix 4.1. Means were calculated on the actual [$H^+$] concentration and back calculated to $pH_{NBS}$. No significant difference in mean $pH_{NBS}$ between temperature treatments in the current and future OAW treatments were detected ($F_{1, 11} = 0.818$, $p = 0.559$) and mean $pH_{NBS}$ were 8.05 and 7.60 respectively. Mean temperatures were 6.7 °C in the current treatments and 9.5 °C in the future treatments with no significant difference between the replicates ($F_{1, 11} = 0.77$, $p = 0.787$). The total mean alkalinity measured in the treatments was 2305.01 ± 12.08. Mean $pCO_2$ values in the treatments ranged from 470 to 537 µatm ($pH_{NBS} = 8.1$) and up to 1349 to 1795 ($pH_{NBS} = 7.6$). Sea water was under saturated with respect to aragonite when $pH_{NBS}$ was below 7.6 (Appendix 4.1).

### 4.3.5 Oil exposure

A continuous flow system (CFS) was used to create a dispersion of crude Arctic crude oil in the sea water. A dispersion equivalent to a nominal oil concentration of 5 mg $L^{-1}$ was made by injecting oil into sea water under pressure (7 L $min^{-1}$ sea water and 0.042 µL $min^{-1}$ oil), to form small oil droplets (mean size 10 µm) (Sanni et al. 1998). To construct an exposure concentration

of 0.5 mg $L^{-1}$, the dispersion was first conducted into a 10 L glass-mixing flask, and thereafter pumped (6 mL $min^{-1}$) by peristaltic pumps (model 520, Watson and Marlow, Cornwall, UK) into the aquaria (flow = 120 mL $min^{-1}$). Water samples for the PAH analysis were collected three times during the exposure. PAH analyses of seawater were performed by Gas Chromatography (HP5890, Hewlett Packard, USA) and analyzed in ion mode (GC/MS – SIM) as described previously in Jonsson et al. (2004). Exposure concentrations were monitored from 26 different PAH compounds analyzed in the water samples based on a standard protocol (EPA 610) with modifications as previously described (Jonsson et al. 2004). Limit of quantification (LOQ) was set to approximately 0.005 µg $L^{-1}$ for each PAH component. The nominal oil concentration in the exposure set up was 0.5 mg $L^{-1}$ and the mean measured PAH concentration for each treatment was found to be 4.895 and 5.545 µg $L^{-1}$ for the current-oil treatment and the future-oil treatment respectively. Approximately 90 % of the PAHs in the exposure aquaria were C0-C3 naphthalene's, and the rest were 3-ring PAHs and dibenzothiophenes (DBTs). There was a significant difference in the mean concentration of naphthalene in the control and the oil treatments ($F = 38.30$, $p < 0.05$). A detailed overview of the different PAH compounds measured in the different exposure concentrations are listed in Appendix 4.2.

### 4.3.6 Experiment 1: Embryos and adult shrimp

#### 4.3.6.1 DNA damage in embryos

The viability of shrimp eggs was examined using a TUNEL staining kit (Invitrogen™ (C10246)), Thermo Fisher Scientific™, Waltham, USA) following the manufacturer's instructions and previously published methods (Mayor et al. 2012). Briefly, batches of formalin-fixed egg samples were rinsed with filtered sea water subjected to three freeze/thaw cycles using liquid nitrogen. They were subsequently incubated in a chitinase 1 mg $mL^{-1}$

solution for 50 min at room temperature to remove the chorion. Permeabilization of the egg membrane was achieved by treating the eggs with a Triton(R) X-100 (0.25 % TX) solution for 20 min at room temperature. The eggs were then incubated with terminal deoxynucleotidyl transferase buffer, enzymes and nucleotides 90 min 37 °C, and stained with Alexa Fluor(R) 594 dye in darkness using the Click-iT(R) reaction 30 min room temperature. This stain has fluorescence excitation and emission maxima at 590 and 615 nm, respectively. Positive controls were included to verify the staining procedure. Eggs were photographed using a microscope (Axiovert 25, Zeiss, Jena, Germany) fitted with an HBO mercury lamp and filter set 00. Egg images were analysed using Image J photo editing software (Schneider et al. 2012). The relative extent of apoptosis within the area of the photographed eggs was assessed using the mean brightness of the red histogram, with brighter values indicating a greater extent of apoptosis and *vice versa*.

4.3.6.2 Determination of hatching time, duration and success

To determine hatching time, duration of the hatching period for each female and the hatching success, three females were haphazardly selected from each treatment and placed in individual Plexiglas® cylinders, following an exposure period to experimental conditions. Each cylinder was checked every second day in order to determine the time to first hatching. This was defined as the first day free-swimming larvae were observed after the experiment started. The duration of the hatching period and percentage hatching success (% of living larvae after hatching) was determined for three females. Duration of the hatching period was measured as the number of days to hatch all the eggs.

4.3.6.3 Determination of feeding rate

To measure adult feeding rates, similar size slices of Saithe fish fillet (N = 8) were weighted and placed in aluminum brackets, one bracket *per* aquaria. Each aquarium contained eight female shrimps, which were allowed to feed for 24 h. After this time, the remains of the fillet were carefully removed and placed in pre-weighed aluminum brackets, and later dry mass determinations were performed. In order to back calculate to the wet mass, slices of fish fillet were dried and then weighed. Feeding experiments were performed three times during the experiment day 6, 14 and 18. Feeding rate was expressed as mg wet mass fish consumed *per* individual shrimp.

4.3.6.4. Measure of lysosomal membrane stability

In invertebrates, macrophage cells (granular haemocytes or coelomocytes) are important for the general defense system. In their cytoplasm these cells contain lysosomes, and a decrease in lysosomal membrane stability in the macrophage-like cells in aquatic invertebrates is often observed when animals are in a condition of stress for example the exposure to chemicals and pollutants (Lowe and Fossato 2000; Bechmann et al. 2010). Lysosomal membrane stability is measured by the means of so-called neutral red retention time (NRRT), and it is estimated based on the ability of the vesicles to retain the toxic neutral red (NR) dye before they lyse. This was measured in all the four different treatments. A total of 50 shrimps were measured. The adult shrimps were weighed and then immediately a 100 µL haemolymph sample was drawn using a 1 mL syringe (Becton and Dickinson and Company, NJ, USA) from right below the cephalothorax. The haemolymph was then diluted 1:1 in filtered sea water ($pH_{NBS}$ = 8.1), and 30 µL was immediately transferred on a microscopic slide. The slides were stored in an airtight moist box for 20 min, then 30 µL diluted NR solution were added to the cell slide. The NR samples were diluted as follows: 20 mg of NR in 1 mL DMSO, 5 µL of the stock solution were

diluted in 995 µL filtered sea water. The cell slide was checked under the microscope at 15, 30, 45, 60, 90, 120, 150, 180 and 240 min from the start of the assay. The time at which approx. 75 % of the cells were lysed was noted as NRRT in min. The cell slides were kept in the dark moist box during the whole procedure, with the exception for when they were controlled under the microscope.

4.3.6.5 Determination of metabolic rate

In order to determine adult metabolic rates, their rates of oxygen consumption as a proxy were measured following the closed-bottle respirometry method of Taylor et al. (1989). A custom-built closed glass-bottle respirometer (vol. = 1.28 mL) was fitted with airtight stoppers into which oxygen electrodes were inserted to be in direct contact with the sea water inside. Blank low concentration oxygen water samples (N = 3) were used to verify that the respirometers were gas tight, and to correct oxygen consumption of the shrimps for bacterial respiration. Adult shrimps were individually placed in the incubation chambers with the appropriate treatment sea water used in the experiment and sealed with the airtight stoppers, and then placed in the climate room to maintain constant temperature. A total of ten oxygen consumption replicates were carried out for each of the four treatments. Following preliminary trials, measurements of dissolved oxygen concentration in the respirometers were performed every 1 min for the entire duration of the incubation (approx. 4 h) using oxygen electrodes (DP-PSt3, PreSens, Regensburg, Germany) coupled to a four channel oxygen meter (OXY-4, PreSens, Regensburg, Germany). Continuous measurements were conducted to demonstrate linearity of oxygen partial pressure decline in the respiration chambers during the incubation (N = 8). No changes in oxygen consumption rates were detected. Blank trials with sea water from the appropriate treatment header tank (N = 4) were used to quantify background respiration rates caused by microorganisms in the system. Background respiration never exceeded 2 % of the total adult

respiration. These background rates were then used to correct the measured respiration values obtained. At the end of each trial, shrimps were weighed in water and later dry mass determinations were performed. Total length (TL) and carapace length (CL) were measured for all shrimps. Oxygen consumption was expressed as mg $O_2$ $h^{-1}$ $mg^{-1}$ DM.

### 4.3.7 Experiment 2: Shrimp Larvae

#### 4.3.7.1 Characterization of larval development

Larval stage was determined using the descriptions of Haynes (1979). The characters used to differentiate between stages were: 1) stalked eyes (stage II); 2) distinct exopodites on telson (stage III); and 3) stage IV larvae have larger endopodites than stage III larvae. The larvae kept under control conditions and the larvae kept under OAW conditions with and without oil spill were sampled the same day post-hatch (day 6, 9, 13, 19 and 27) to detect possible effects of OAW conditions and oil spill on development. The sampling time was based on the results of a previous study on the combined effects of OW and OA (see Chapter 2) on the developmental physiology of *P. borealis* (Chapter 2). Since there was a reduction in development time for the larva raised in the OAW conditions larvae were sampled on the same day to be able to determine the difference in development between the treatments. After stage determination, larvae were carefully returned to their respective aquaria.

#### 4.3.7.2 Determination of larval feeding rates

To quantify larvae feeding rates, a modification of the clearing rate methods by Harvey and Morrier (2003) were used. Feeding trials were conducted in 1 L glass bottles containing filtered sea water drawn from the treatment header tanks used in the experiment. Five larvae of the same stage of development were transferred into the experimental bottles, placed in temperature-controlled environments (T = 6.7 or 9.5 °C) and starved for approx. 24 h prior to

the experiments. Freshly hatched *Artemia* nauplii (initial prey conc. 150 indiv $L^{-1}$) were added to 1 L Schott bottles containing the larvae. The bottles were sealed with lids and incubated for 6 h at the respective temperature. After the incubation period, shrimp larvae were carefully removed from the bottles and the remaining water, containing *Artemia* nauplii, was sieved using a 40 µm BD Falcon™ cell strainer (BD Biosciences, Franklin Lakes, USA), and individual nauplii counted. Amount of prey consumed was calculated as initial number of *Artemia* nauplii minus their final number. Feeding rate was expressed as number of prey consumed *per* individual shrimp larvae *per* unit time (number of *Artemia* $indiv^{-1}\ h^{-1}$). A control sample from the control treatment without shrimp larvae was run through the experimental procedure, to test the efficiency of the sieving procedure. All 150 *Artemia* sp. nauplii were recovered in each control test.

4.3.7.3 Characterization of the swimming response

Swimming behavior in this study was defined as the intensity of the larval response to light. Newly hatched *P. borealis* are positively phototactic. Response to light is crucial in nature to put them in proximity to their food and therefore enhance their chances of successful growth and development. Two swimming response tests were performed hereafter-named simple swimming test and advanced swimming test. The simple swimming response test was that of Larsen (2004) modified as follows. Test were run in a dark control temperature room, 6.7 or 9.5 °C depending on the experimental treatment, and performed on stage I and II larvae during oil exposure. A total of six replicates from each exposure were used. Fifteen shrimp larvae were transferred to a 100 mL measuring cylinder with the right treatment water hanging in a burette holder. A flash light was positioned at the bottom of the cylinder, with the light beam pointing at the bottom, since the larvae are photo tactic, all larvae swam towards the bottom were the light source were positioned. This caused the larvae to settle there or swimming below the 20

mL line. When all the larvae were below level 1, the light was moved from the bottom position towards the top position and a stopwatch was started immediately. After 2 min, the numbers of larvae positioned in three different levels were recorded, level 1 (below the 20 mL line), level 2 (between the 20 and 80 mL line) and level 3 (above the 80 mL line). Based on data from these 6 tests the mean percentage of larvae at the surface was calculated after 2 min.

The advanced test of swimming behavior was measured using a combination of infrared and white light emitting diodes, together with a phototransistor. Test groups of between 10 and 15 larvae were placed into a glass beaker (vol. = 1 L, 9.2 cm diameter) using water from their specific treatment group. The infrared light emitting diode was aligned with the phototransistor on the opposite side of the glass beaker. Both components were encapsulated within 8 mm plastic tubes, which were supported in position by insertion into holes drilled into two wooden pillars. The width of the infrared light beam passing through the water, together with the surface area of the phototransistor open to this light, was controlled by the use of baffles pushed into the support pillars in front of the phototransistor and emitter. The baffles were drilled centrally to a diameter of 1.5 mm. As larvae swam through the light beam, breaking its path, the reduced amount of light falling onto the phototransistor caused a drop in the output voltage. In the absence of these baffles, the movement of the small larvae within the much broader beam would be more difficult to detect. The white light emitting diode was positioned directly above the phototransistor. It provided the light source towards which the larvae swam. Preliminary observations had shown that the larvae swam upwards into the path of light then ceased swimming, falling slowly back down within the vessel before recommencing swimming back towards the light. This behavior allows response to light to be measured as the intensity of repeated swimming activity recorded as the number beam breaks per hour. Voltage output from the phototransistor was logged at intervals of 0.2 sec throughout the one-hour test periods using a data logger (NI USB - 6009, National Instruments, Texas, USA). All tests were conducted in

an ambient low light environment. Stage I-II during oil exposure and stage III larvae after oil exposure was studied in this experiment.

4.3.7.4 Determination of metabolic rate and morphology

To estimate stage IV larvae metabolic rates, their rates of oxygen consumption were measured described above, the only difference being that the volume of the respirometers were smaller (vol. = 300 mL). Shrimp larvae (N = 15 at each time) were placed in the incubation chambers with the appropriate treatment header tank water used in the experiment and sealed with the airtight stoppers, and then placed in flow through water baths to maintain a constant temperature. A total of six replicates were carried out for each of the treatments. Following preliminary trials, measurements of dissolved oxygen concentration in the respirometers were performed every 1 min for the entire duration of the incubation (approx. 24 h) using oxygen electrodes (DP-PSt3, Presens) coupled to a four channel oxygen meter (OXY- 4, PreSens). Continuous measurements were conducted to demonstrate linearity of oxygen partial pressure decline in the respiration chambers during the incubation (N = 24). No changes in oxygen consumption rates were detected. Larval free samples of sea water from the appropriate treatment header tank were used to assess background respiration rates caused by microorganisms in the system. Background respiration never exceeded 2 % of the total larval respiration. These background rates were then used to correct the measured larval respiration values obtained. At the end of each trial, larvae were stored at – 80 °C in order, at a later date, to conduct morphological and dry mass determinations described by Ouellet and Chabot (2005). Total length (TL) was measured for a selection of larvae (6 - 13 indiv *per* trial, 7 - 12 trials *per* treatment). All the larvae from each trial were pooled to determine the total dry mass. Morphological classifications of abdominal sixth segment of the stage IV larvae were also performed, the larvae sixth element were classified as following no abnormality, missing

endopodite, missing exopodite and unsymmetrical, the classification of pigmented and not pigmented was also used. Resulting in the abnormality index (% of abnormal larvae). Oxygen consumption rate was expressed as nmol $O_2$ $h^{-1}mg^{-1}$ DM.

### 4.3.8 Statistical analysis

The effect of OAW conditions, oil spill, and their interaction on the variables measured in this study was analyzed using General Linear Model (GLM) tests, with 'aquaria' as a random factor nested within pH /temperature X oil combinations. As aquaria had no significant effect on any of the parameters tested here (max. $F_{1, 47} = 1.339$, $p = 0.158$) except one (abnormalities) this factor was removed from subsequent analyses. In those cases, where the factor 'aquaria' was significant, removing this factor did not change the patterns of significance for other factors, and thus, the aquaria effect was considered marginal here. In addition, for feeding rates, I also included the term 'stage' as a fixed factor, and for metabolic rates and NRRT I included individual body mass as a covariate. Mortality (percentage accumulated mortality) were tested for difference in treatments, for both for given time points (day 6, 9, 13, 17, 19 and 27) and for stage (stage II, III and IV).

All data met assumption for normality of distribution (max. $F_{1,32} = 0.039$, $p = 0.845$) except NRRT, and variances were homogeneous for hatching success, hatching day, larval development, hatching period, feeding rate, and metabolic rates (max. $F_{3, 32} = 1.375$, $p = 0.278$). As our experimental design included four treatments and minimum six replicates *per* treatment *per* measurement, I assumed that the orthogonal design employed should be tolerant of deviation from the assumption of normality and heteroscedasticity (Melatunan et al. 2009; Sokolov et al. 2009). Pairwise comparisons were conducted using the Estimate Marginal Mean test with LSD correction. All analyses were conducted using v 21 SPSS®.

## 4.4 RESULTS

### 4.4.1 Experiment 1: Adult Shrimp

#### 4.4.1.2 Effects on shrimp embryos

Mean percentage hatching success for embryos of *P. borealis* were high and ranged between 99.6 and 98.6 %. Mean time to first hatching was 10 d shorter in the OAW conditions when compared to control conditions (Appendix 4.3) ($F_{1, 11} = 6.35$, $p = 0.036$), whilst no significant effect of exposure to oil or the interaction between OAW condition and oil was found (max. $F_{1, 11} = 0.493$, $p = 0.502$).

DNA damage in embryos, hatching success, and duration of hatching was not significantly impacted by exposure to future OAW, oil and their combination (Appendix 4.3) (max. $F_{1, 257} = 2.289$, $p = 0.123$).

#### 4.4.1.3 Effects on female shrimp

Neutral red retention time (NRRT) was significantly lower in individuals exposed to OAW conditions (- 12 %) and oil (- 18 %) in isolation (Fig. 4.2) (min. $F_{1, 49} = 4.91$, $p = 0.032$), but not by their interaction (Fig. 4.2) ($F_{1, 49} = 1.75$, $p = 0.192$).

Adult female feeding (mg wet mass fish $ind^{-1}$) was not significantly impacted by exposure to OAW conditions, oil, or their interaction (Appendix 4.3) (max. $F_{1, 68} = 0.611$, $p = 0.437$), although there was a significant negative impact of time: adult females showed a 27 % reduction in feeding rate at the end of the experiment compared with the first sampling date (Appendix 4.3) ($F_{1, 68} = 4.590$, $p = 0.014$).

Rates of oxygen consumption (mg $O_2$ $h^{-1}$ $mg^{-1}$ DM) were not significantly impacted by exposure to OAW conditions, oil, or their interaction (Appendix 4.3) (max. $F_{1, 31} = 0.093$, $p = 0.749$),

although there was a significant effect of mass: with larger shrimps on average consuming more oxygen (Appendix 4.3) (max. $F_{1, 31} = 6.14$, $p = 0.024$).

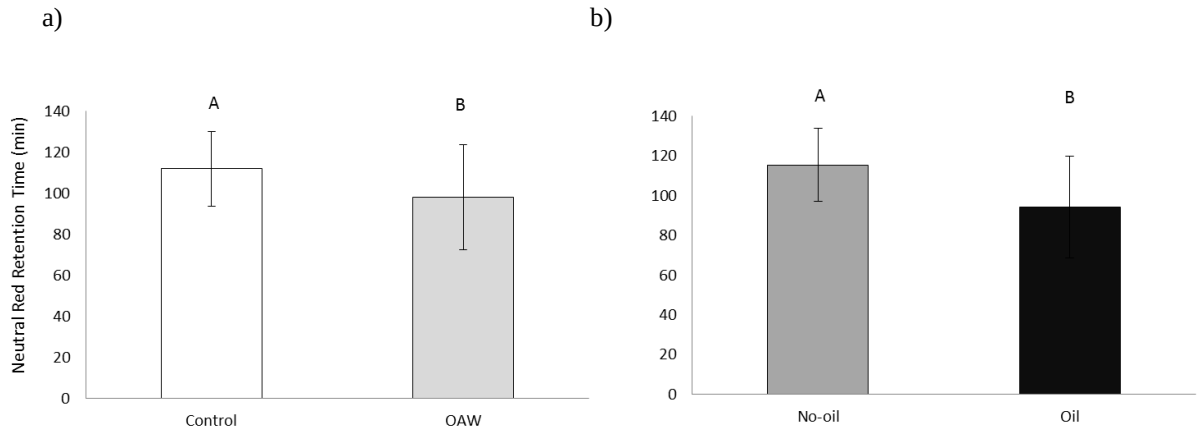


**Figure 4.2 The effect of control and OAW conditions and oil on mean Neutral Red Retention Time (NRRT) (measured as mean time for approximately 75 % of the lysosomal cells were destroyed) of *P. borealis* larvae. a) Control vs OAW treatment, b) Non-oil vs oil treatment. Histograms represent means ± SD. Significantly different treatments ($p \leq 0.05$) are indicated by upper case letters.**

### 4.4.2 Experiment 2. –Shrimp Larvae

#### 4.4.2.1 Mortality

The average mortality rate (MR, % $day^{-1}$) was 10 % higher for stage IV larvae when larvae were exposed to OAW conditions compared with control conditions (Fig. 4.3) ($F_{1, 19} = 7.45$, $p = 0.013$). Whilst no significant effect of exposure to oil or the interaction between OAW condition and oil was found (max $F_{1, 19} = 0.002$, $p = 0.964$).

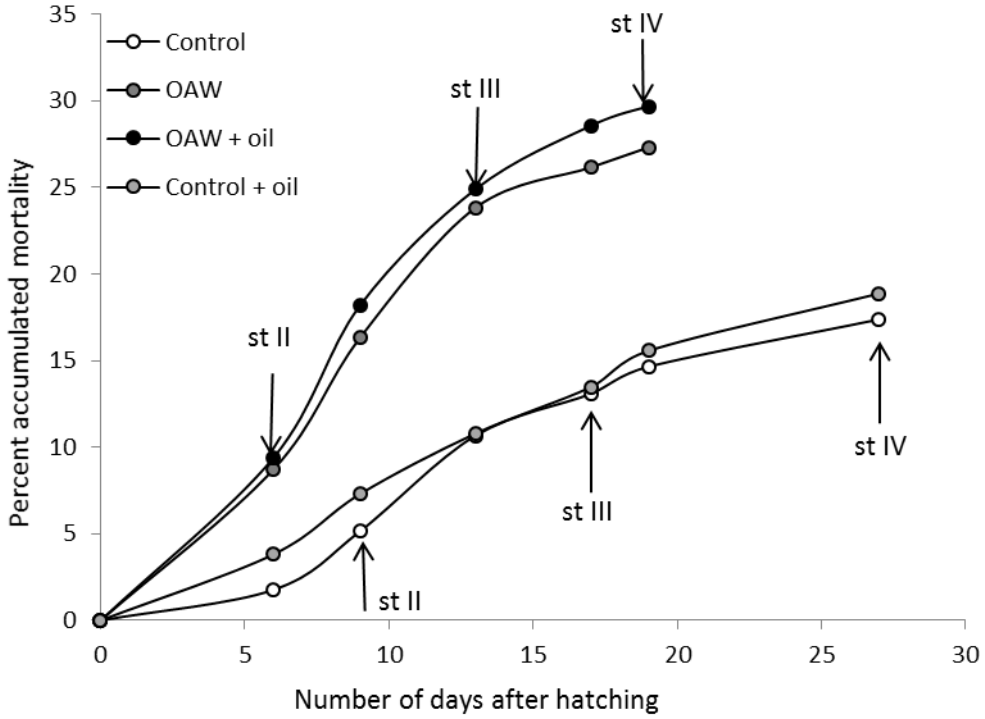


**Figure 4.3 The effect of control and OAW conditions, oil and time on mean accumulated mortality of *P. borealis* larvae. Control (pH 8.0/6.7 ºC, clear) (N = 6), control + Oil (pH 8.0/6.7 ºC + oil, light grey) (N = 6), OAW (pH 7.6/9.5 ºC, dark grey) (N = 6), OAW + oil (pH 7.6/9.5 ºC + oil, black) (N = 6). The different larval stages (stage II, stage III and stage IV) are indicated on the graph.**

4.4.2.2 Feeding rates

Feeding rates of individual *P. borealis* larvae ranged between 0.4 and 1.5 prey items for stage I-II larvae, 1.9 and 3.6 for stage III larvae, and 2.5 and 3.5 for stage IV larvae (Appendix 4.3). Stage I-II larvae fed on average 68 % less than stage III and IV larvae, this difference being significant (Fig. 4.4 a) ($F_{1, 19} = 30.069$, $p < 0.001$).

Feeding rates increased by 20 % in individuals on exposure to OAW conditions (Fig. 4.4 c) ($F_{1, 43} = 5.416$, $p = 0.023$), but decreased by 24 % in individuals exposed to oil spill (Fig. 4.4 b) (max $F_{1, 43} = 8.610$, $p = 0.006$) compared to control conditions. No significant effect of the interaction between OAW condition and oil was found ($F_{1, 43} = 0.559$, $p = 0.460$).

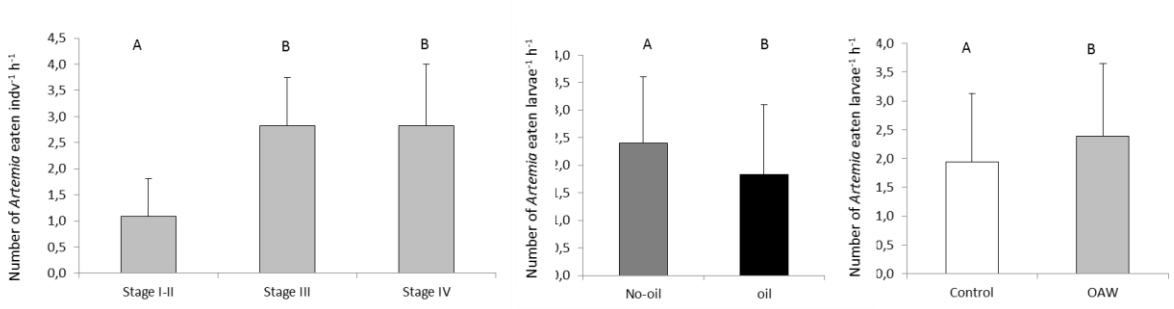


**Figure 4.4 a-c The effect of OAW conditions and oil and their interaction on mean feeding rates (measured as mean number prey eaten indiv$^{-1}$ h$^{-1}$) for *P. borealis* larvae at stage II, III and IV. a) Mean feeding rates for stages II, III and IV, b) mean feeding rates for oil exposed and non-exposed larvae, c) mean feeding rates for control and OAW exposed larvae. Histograms represent means ± SD. Significantly different treatments ($p \leq 0.05$) are indicated by upper case letters and upper case letters indicate significant differences among the different stages and treatments according to 2 way ANOVA.**

4.4.2.3 Development

Detailed determination of the different stages recorded at days 6, 9, 13, 17, 19 and 27 post-hatch for all of the treatments is presented in Fig. 4.5 a-d.

Development (% stages at given time points) was significantly impacted by OAW conditions, as larvae developed 9 d faster to stage IV in the OAW conditions compared to control conditions (min. $F_{1, 48} = 56.751$, $p < 0.001$). No significant effect of exposure to oil or the interaction between OAW condition and oil was found for stage I, II and IV (max. $F_{1, 48} = 0.273$, $p = 0.604$), with the exception of larva from stage III where an interaction between OAW condition and oil was found ($F_{1, 80} = 6.306$, $p = 0.015$).

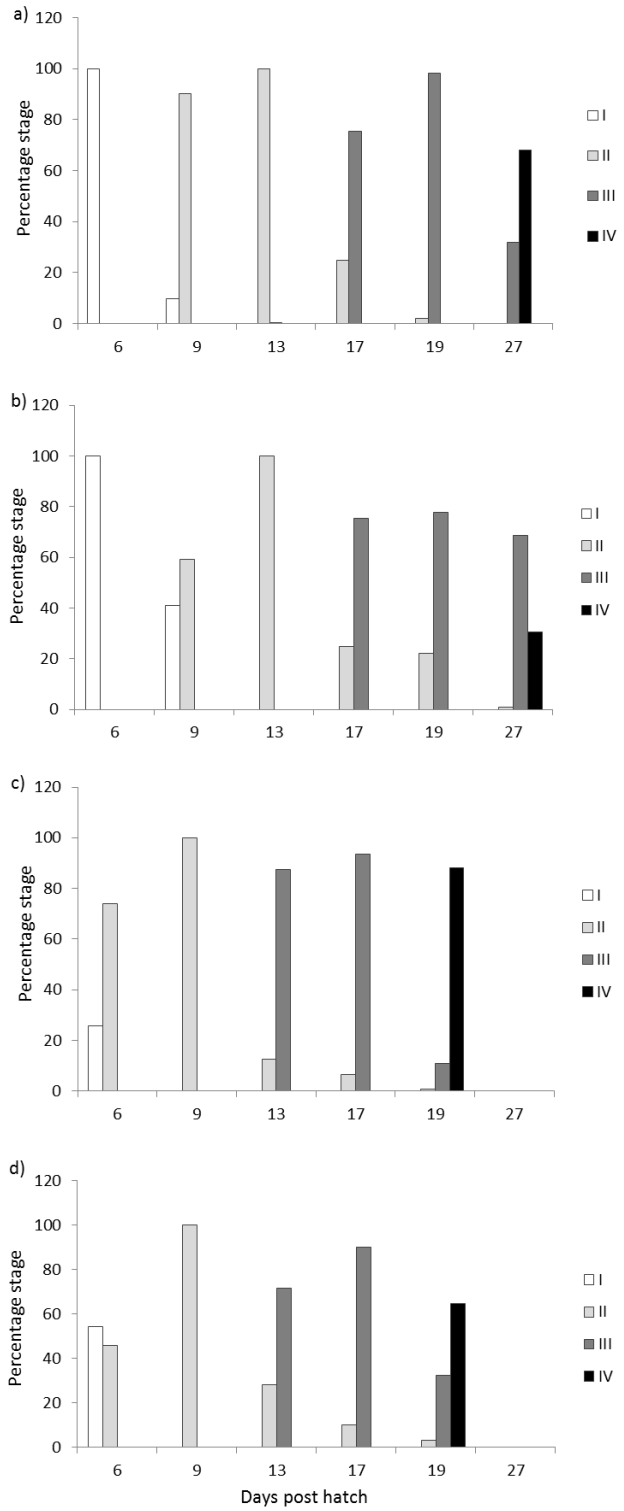


**Figure 4.5 a-d The effect of OAW conditions and oil and their interaction on the development of *P. borealis* larvae (stage II-IV) exposed to a) control (pH 8.0/6.7 ºC), b) control + oil (pH 8.0/6.7 ºC + oil), c) OAW (pH 7.6/9.5 ºC), d) OAW + oil (pH 7.6/9.5 ºC + oil). Percent stage is the % stage at the given time point.**

4.4.2.4 Metabolic rates for shrimp larvae

Rates of oxygen consumption for *P. borealis* larvae stage IV ranged between 51.90 and 62.76 nmol $O_2$ $h^{-1}$ $mg^{-1}$ DM for the control conditions, and 71.36 and 77.82 nmol $O_2$ $h^{-1}$ $mg^{-1}$ DM for OAW conditions (Fig. 4.6), however this parameter was not affected by exposure to OAW, oil or their interaction (Fig. 4.6) (max. $F_{3, 21} = 2.748$, $p = 0.075$). Finally, oxygen consumption rates significantly linearly increased with mass ($F_{3, 21} = 4.43$, $p = 0.050$).

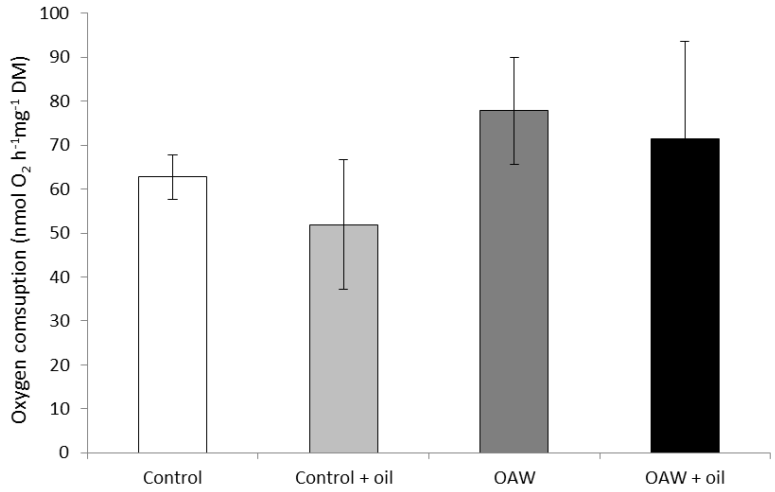


**Figure 4.6 The effect of OAW conditions and oil on mean oxygen consumption rates (nmol $O_2$ $h^{-1}$ $mg^{-1}$ DM) for larval *P. borealis* . Control (pH 8.0/6.7 ºC, clear) (N = 6), control + oil (pH 8.0/6.7 ºC + oil, light grey) (N = 6), OAW (pH 7.6/9.5 ºC, dark grey) (N = 6), OAW + oil (pH 7.6/9.5 ºC + oil, black) (N = 6). Histograms represent means ± SD.**

4.4.2.5 Length, mass and shrimp tail morphology

Mean values for body length and dry mass in stage IV larvae are represented in Fig. 4.7 a-b. Length (mm) of stage IV larvae was significantly reduced by exposure to OAW conditions and oil (max. $F_{1, 282} = 137{,}914$, $p < 0.0001$), with no significant interaction leading to an additive response to both drivers ($F_{1, 282} = 0.539$, $p = 0.463$).

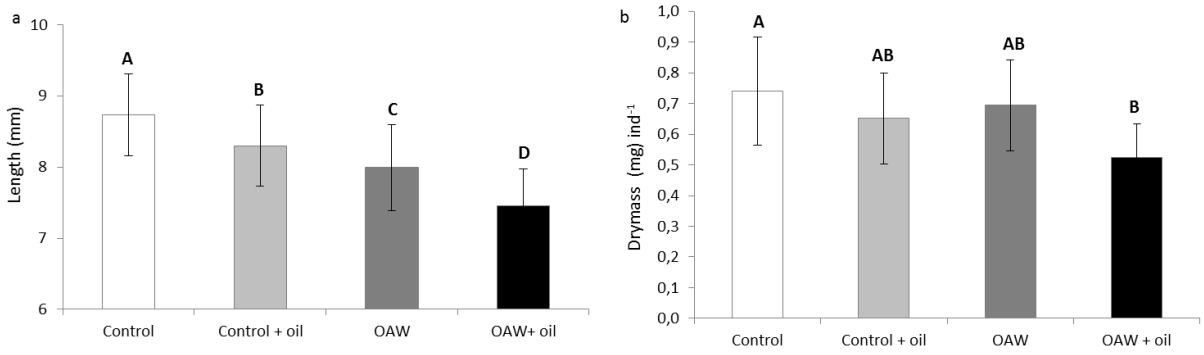


**Figure 4.7 a-b The effect of OAW conditions and oil on mean a) length, b) mass for stage IV *P. borealis* larvae. Control (pH 8.0/6.7 ºC, clear) (N = 6), control + oil (pH 8.0/6.7 ºC + oil, light grey) (N = 6), OAW (pH 7.6/9.5 ºC, dark grey) (N = 6), OAW + oil (pH 7.6/9.5 ºC + oil, black) (N = 6). Values expressed as means ± SD. Significantly different treatments ($p \leq 0.05$) are indicated by upper case letters.**

The mean dry mass *per* individual stage IV larvae kept under combined OAW and oil conditions was significantly lower than that of larvae kept under control conditions. ($F_{1, 10} = 6.25$, $p = 0.034$), whilst no other comparison was significant ($p > 0.05$). Abnormality during development was significantly impacted by oil exposure, with 20 % more larvae in the oil treatments presenting abnormalities compared to the non-oil treatments (Fig. 4.8) ($F_{3, 22} = 12.10$, $p = 0.002$). Whilst no significant effect of exposure to future OAW conditions ($F_{3, 22} = 0.92$, $p = 0.92$) or the interaction between future OAW condition and oil was found ($F_{3, 22} = 1.69$, $p = 0.21$).

There was a significant increase (35 %) in pigmented larvae when the shrimp larvae were raised at OAW conditions with and without oil ($F_{1, 3} = 31.80$, $p = 0.030$).

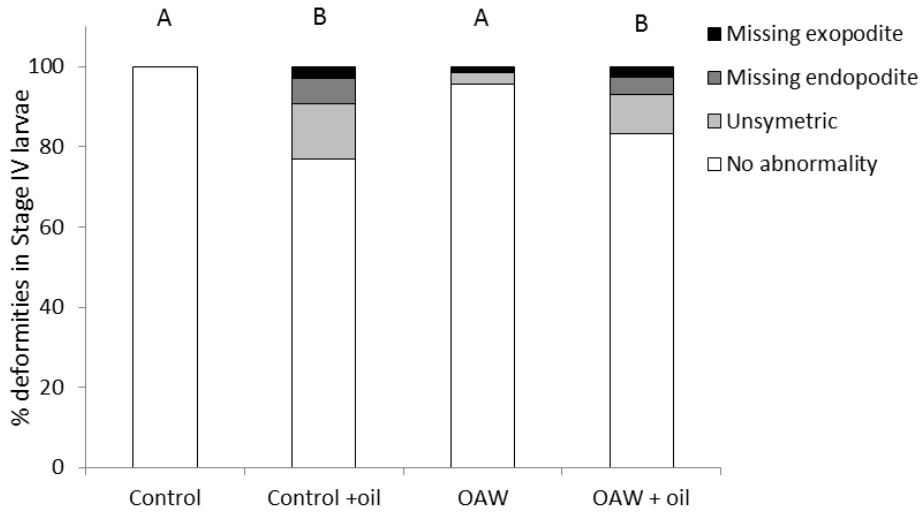


**Figure 4.8 The effect of OAW conditions and oil and their interaction on morphology of larval abdominal sixth segment on stage IV *P. borealis* larvae measured as mean percentage no abnormality (clear), unsymmetrical (light grey), Missing endopodite (dark grey) and missing exopodite (black) for the different treatments. Significantly different treatments ($p \leq 0.05$) are indicated by upper case letters.**

4.4.2.6 Swimming behavior

The swimming activity was variable between replicates of individuals from the same treatment/replicate aquaria. However, the simple swimming test showed that for stage I larvae exposure to oil under control conditions caused a significant reduction (- 57 %) in swimming activity, compared to the control treatment without oil (Fig. 4.9 a) ($F_{1, 26} = 14.97$, $p < 0.001$). The swimming index (% number of time beams broken) for stage III larvae was significantly impacted by OAW conditions, with increasing swimming by 80 % (Fig. 4.9 b) ($F_{1, 15} = 84{,}038$, $p < 0.001$) compared to control conditions. Whilst no significant effect of exposure to oil ($F_{1, 15} = 0.343$, $p = 0.567$) or the interaction between OAW condition and oil was found ($F_{1, 15} = 0.440$, $p = 0.517$).

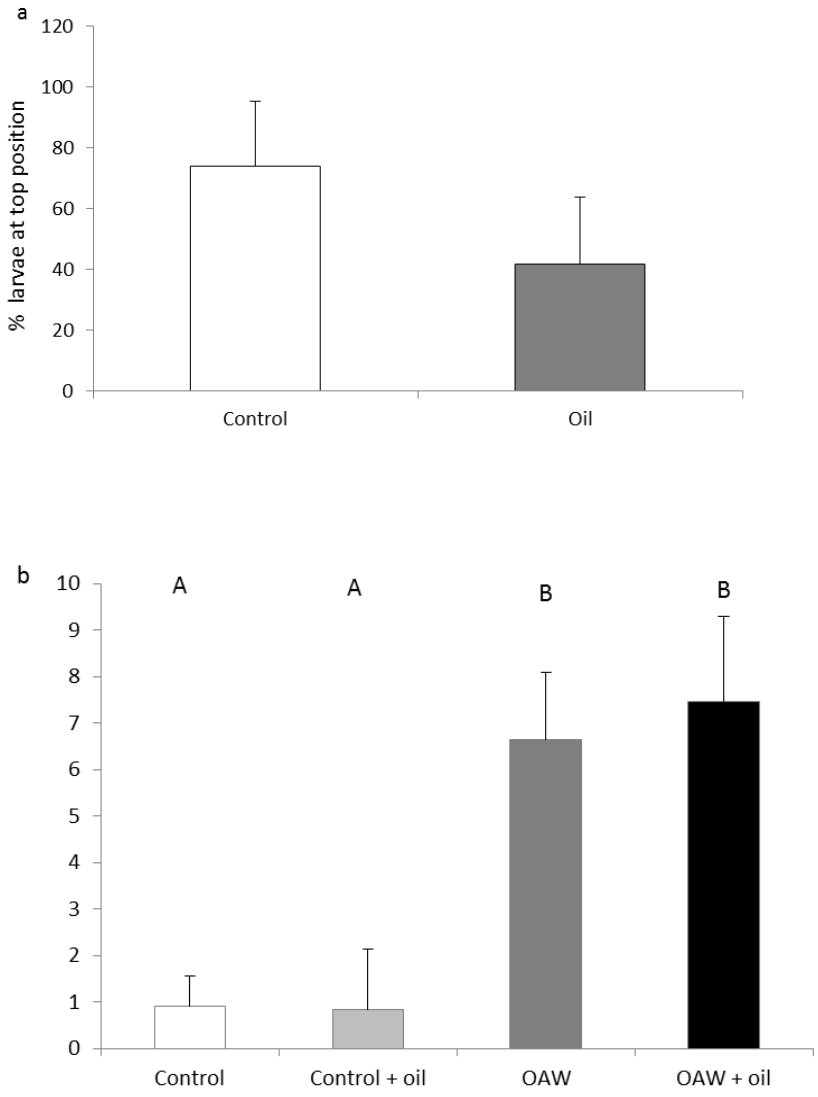


**Figure 4.9 a-b. The effect of OAW conditions and oil and their interaction on swimming behavior. a) Simple swimming test on stage I larvae of *P. borealis* (expressed as mean percentage of larvae at the surface after 2 min) for control and control + oil treatment. Significantly different treatments ($p \leq 0.05$) are indicated by upper case letters. b) Advanced swimming test for stage III larvae for the different treatments (as the mean number beam breaks per hour). Control (pH 8.0/6.7 ºC, clear), control + oil (pH 8.0/6.7 ºC + oil, light grey), OAW (pH 7.6/9.5 ºC, dark grey), OAW + oil (pH 7.6/9.5 ºC + oil, black) (N = 6 in each case). Values expressed as means ± SD. Significantly different treatments ($p \leq 0.05$) are indicated by upper case letters.**

## 4.5 DISCUSSION

This study provides the first experiments on the potential negative effects that the combined exposure to a local driver (i.e. oil spill) and multiple major global drivers OAW will exert on different life stages of an ecologically and economically important species with a complex life-history strategy, such as the Northern shrimp, *Pandalus borealis.*

Here, a number of important lethal and sub-lethal effects caused by the exposure to oil spill and future OAW conditions, in isolation and in combination are highlighted, on the larvae of *P. borealis*, indicating that the larval stages in this species are the most vulnerable life stages to the combined exposure to local and global changes. However, there was very little effects of a simulated oil spill and OAW conditions, in isolation and in combination, on embryonic development, and adult (female) physiology, with the exception of time to first hatching. Altogether, there were significant stage-specific responses to combined local and global change drivers, confirming the importance of investigating multiple life stages to provide a comprehensive assessment of the potential impacts of multiple drivers on a target marine invertebrate species with complex life history strategy (Section 1.1.5).

It is suggested that overall the combined exposure to future OAW and oil spills will result, broadly speaking, in a series of additive effects. In fact, it is shown that there were no significant interactive effects of the tested drivers, except for the timing of development at stage III, but there were negative additive impacts on several other parameters measured. Such additive effects were particularly evident for larvae. The findings are discussed within the context of how, based on our new evidence, we should manage oil spills, and in general local stressors, within the context of ongoing ocean change, as it is paramount that we acquire an understanding of life history bottle necks and mode of action of global and local drivers combined to best promote the conservation of natural resources of economic importance such as *P. borealis*, as well as general biodiversity levels in the ocean.

### 4.5.1 Impacts on embryos larvae and adults

The differences in the responses to different life stages to OAW and oil exposure as described below, highlights the importance to study significant stage-specific responses to local and global change drivers, to provide a comprehensive assessment of life story bottlenecks and the potential impacts of multiple drivers on the target species.

Adult (female) physiology was not impacted by the drivers tested, neither in isolation or combined, with the exception of the reduction in lysosomal stability following the exposure to oil. Reduced lysosomal stability due to oil exposure has already been reported in Northern shrimp *P. borealis* and the green shore crab, *Carcinus maenas* (Bechmann et al. 2010; Dissanayake and Bamber 2010). The energetic condition of females may play an important role in the development of shrimp embryos (Brillon et al. 2005). During normal embryonic development, females ventilate their eggs, through pleopod movements (Morritt and Spicer 1999; Baeza and Fernández 2002). The fact that the females were overall unaffected by the combined exposure to an oil spill and OAW conditions, might explain why there was very little effect of the tested stressors on embryonic development in our study. A similar level of embryonic tolerance to OAW conditions have already been reported for other crustacean species (Sect. 1.1.5) suggesting that early embryonic life stages are less vulnerable to OA and OW than later larval stages. However, Bechmann et al. (2010), reported no effect on hatching success of oil exposed embryos, but carry over effects of reduced larval survival. Although no effect on survival or hatching was observed for embryos, survival for larvae exposed to OAW conditions were reduced by 30 %. Furthermore, a number of abnormal larvae were found in the same treatment. This could partly be due to possible carry-over effects or early hatching in the OAW treatment. Fluctuations in temperature prior to larval hatching in combination with food availability, during and between larval seasons, are thought to have a profound effect on larval

survival (Nunes and Nishiyama 1984). In addition, Brillon et al. (2005) hypothesized that *P. borealis* embryos developing at higher temperatures may have hatched prematurely, this could explain smaller size at hatching and the higher proportion of abnormal larvae found in present study.

Oil exposure seven days from hatching in the present study did not affect mortality of shrimp larvae. However, Taban et al. (2007) showed that mortality increased in shrimp larvae following 18 d exposure to oil, even to lower oil concentrations than in the present study. For crustaceans, to be able to successfully moult and survive from one stage to the next, a minimum storage of energy reserves during each development stage is required (Ouellet et al. 1992). It is possible that some larvae from the Taban et al. (2007) study did not have enough energy to develop into stage III (day 18) and as a consequence died. However, in the present study, the oil exposure was shorter (one week) and it is therefore possible that the energy reserves were not depleted and the larvae survived and could develop further.

Furthermore, oil exposure led to a reduction in larvae swimming activity, this result being in agreement with other studies on shrimps and copepods exposed to oil (Brodersen 1987; Almeda et al. 2014). For example, exposure of larvae of King crabs (Paralithodes) to water soluble fractions of oil (WSF) of 0.5 mg $L^{-1}$ comparable to this study, resulted in swimming cessation and ultimately death (Brodersen 1987). However, comparison with other studies investigating the impact of oil spill simulations on planktonic invertebrates is difficult as the type of oil, the chemical composition, the exposure conditions, the fraction of oil tested, the analytical method and compounds analysed vary between studies (Bechmann et al. 2010; Ingvarsdóttir et al. 2012). Nonetheless, one can hypothesize that the reduced swimming activity during oil exposure may be due to a narcotic effect of the oil (Saiz et al. 2009; Almeda et al. 2014).

Reduced feeding rates were also observed and reduced growth for shrimp larvae exposed to oil for one week. This narcotic effect may have caused the observed reduction in feeding rates, and

reduced feeding will eventually lead to decreased growth due to energy limitation and increased mortality due to starvation (Hart and Strathmann 1994). Similar reduction of growth was observed for oil-exposed larvae of shrimp and herring (Taban et al. 2007; Ingvarsdóttir et al. 2012).

The feeding rate for oil exposed shrimp larvae returned to almost control levels after the oil exposure had ended. This reversible trend in feeding has also been observed for copepods first exposed to oil and then transferred to clean sea water (Berdugo et al. 1977). This reversible trend may explain why the early larvae of shrimps were less impacted by the oil exposure, as it seems like the larvae have enough energy reserves making them able to cope with a transient food shortage. Declining scope-for-growth is the net effect of hydrocarbons on several processes, but the primary cause of reduced scope-for-growth is reduced feeding rates (Rice et al. 1983).

Stage IV oil exposed larvae showed a high incident (20 %) of deformities of the abdomen in current study. Other studies have also reported abnormal embryo-larval development due to oil exposure (Baussant et al. 2011; Ingvarsdóttir et al. 2012).

Exposure to the simulated OAW conditions resulted in a decrease in development rate and in feeding of the *P. borealis* larvae in the present study. In a previous study Chapter 2, it was shown that OW elicits greater effects than OA on development, feeding and metabolism of Northern shrimp larvae. Impacts of OA was only accompanied by a significant delay of larval development but not under OW conditions (Chapter 2). The present study confirms the results from Chapter 2, exposure to OAW conditions compared to control conditions, shortens the development time with nine days until stage IV, shortens the larval length by 9 % and increased the feeding rate by 15 - 20 %. Although the present study confirms the results from Chapter 2, there are differences in absolute values. Metabolic rates for larvae in present study were 8 % higher and the body length 7 % smaller than in Chapter 2. Differences in year class of the

females and longer exposure time to OAW of the embryos, could explain the smaller larvae used in present study, as fluctuations in temperature prior to larval hatching in combination with food availability may have a profound effect on larval survival and growth (Nunes and Nishiyama 1984). In addition to this, Brillon et al. (2005) observed that higher temperatures reduced the duration of the yolk reserves and the conversion efficiency of yolk into tissue growth, resulting in smaller larvae. Furthermore, the higher metabolic rates in present study may be explained by the fact the larvae were smaller and had to swim faster over a longer distance to catch prey hence increasing the metabolic cost, this phenomenon has also been observed for larval cod (Herbing 2001).

The results are in agreement with existing data on the importance of temperature in governing growth in the Northern shrimp larvae, with developmental rates increasing as culture temperature is increased (Ouellet and Chabot 2005; Chapter 2). In our two studies, it was observed that *P. borealis* larvae with a higher metabolic rate associated with higher swimming activity and food intake, and a reduction in length and mass, indicating a shift in energy intake efficiency and/or energy budget, as energy is not available for growth. Similar reductions in developmental time and reduced growth were reported by Small (2013) for European lobster exposed to OW. Reduced larval size of marine invertebrates in future oceans will have a negative impact on feeding ability and make them more vulnerable to predators (Hart and Strathmann 1994). Global change such as OA and OW could also potentially lead to mismatches between the reproductive cycles of *P. borealis* and its planktonic food (Koeller et al. 2009) this being a key component for survival and recruitment success of Northern shrimp larvae (Ouellet et al. 2011). An increase in demand of food, smaller sized larvae at future conditions in combination with a mismatch with the biological production cycle, may lead to a decrease in the recruitment of *P. borealis* shrimp larvae (Ouellet et al. 2011). However, such a

mismatch may not materialize if concurrent changes in the surface oceanography also result in earlier algal blooms.

OAW conditions and oil affected shrimp larvae through reducing and shifting the energy budget through different mode of action (extra energy costs for future OAW conditions and the narcotic effect of oil) with no interaction. Consequently, they combine in an additive manner reducing growth, e.g. the size of the shrimp larvae is equal the sum of the reduction of each driver (future OAW) conditions (- 5 %), oil (- 9 %) and future OAW*oil (- 15 %).

### 4.5.2 Multiple drivers: future (OA and OW) and oil spill

Marine life faces multiple environmental challenges in the future ocean (Byrne and Przeslawski, 2013). In fact, there will be large areas of the ocean where marine organisms will be facing combined and complex environmental challenges, including OA, OW and accidental discharges from the oil industry and from ships, with a greater frequency and intensity seen to date. Reduced ice cover in the Arctic caused by on-going climate change, will lead to increased transport by ships, in response to the worldwide demand of more petroleum resources and search by the oil and gas industry, and therefore increased areas where oil pollution can be found (AMAP 2007; Ermida 2014). Many novel reviews on combined effects suggests that many stressors combine in non-additive ways (i.e. synergistic or antagonistic) (Harvey et al. 2013; Boyd and Brown 2015; Przeslawski et al. 2015). However, others suggest that synergies are not the norm, and that there is not sufficient evidence for synergies being the most common outcome of combined drivers (Kroeker et al. 2013; Ban et al. 2014). Recent studies investigating the potential for interactions between different global change drivers of toxicants show several different interaction patterns (Sect. 1.1.5).

Exposure to OAW, impacts larvae by reducing and shifting their energy budget through extra energy costs, and as a consequence, reducing growth producing smaller larvae. When larvae

are additionally challenged by an oil spill, reduced feeding and growth specifically due to OAW will likely make the larvae more vulnerable to the negative effects of exposure to oil. Both drivers were affecting growth for shrimps in a negative way, and in combination, the drivers were acting additively. It is suggested that the additive physiological effects of climate variables and a contaminant, and that larval resilience to future changes (i.e. pollution) could be greatly reduced if the larvae is already limited in energy and severely stressed (reduced development) from the OAW conditions.

Furthermore, the present study highlights the importance that the correct management of local drivers such as oil spills, but also other contaminants such as those from agricultural activities and pharmaceutical products and overfishing, will have within the context of OAW. Therefore the effective management of local drivers, such as oil pollution, could slow down the detrimental impact of future global environmental changes (Ghedini et al. 2013), increasing the resilience of entire ecosystems (Micheli et al. 2012). These findings could empower local managers, because they can show that reducing local impacts can reduce the effects of global stressors not under their governance (Connell et al. 2013).

However, the study focuses on a single life history stages and recent life cycle or multi-generational studies highlight the carry-over effects of the exposure of reduced pH from one life stage or generation to another (Dupont et al. 2012; Lohbeck et al. 2012; Parker et al. 2012). Even if future OAW effects were small during the larval stages of *P. borealis*, carry over effects on the post larvae, adults or the next generation may still exist and be important. Furthermore, abiotic variables such as temperature and pH vary spatially across *P. borealis* distributional range, and development in different populations and genetic strains might respond differently to OA/OW and oil spills. This together with the species ability for rapid adaption to future climatic change scenarios (see Collins and Bell 2004; Findlay et al. 2011; Pistevos et al. 2011; Sunday et al. 2011) should be considered and warrants further study.

# Chapter 5. Combined effects of low pH and oil spill on the development of green sea urchin (*Strongylocentrotus droebachiensis*).

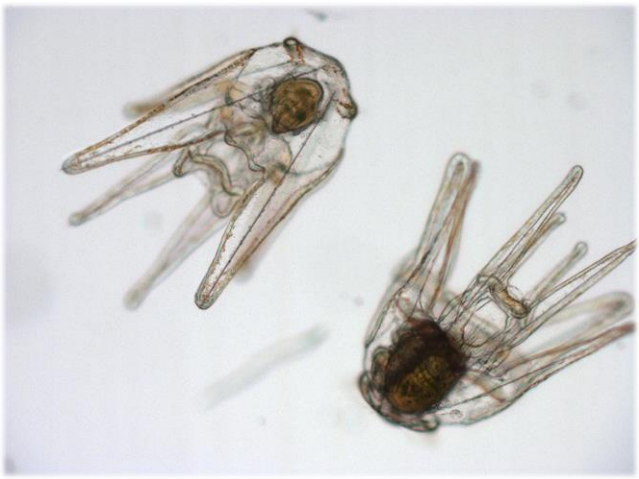

**Figure 5.1 Larva of *Strongylocentrotus droebachiensis*. (Photo: Maj Arnberg)**

## 5.1 SUMMARY

The ocean is under increasing environmental pressure from several anthropogenic activities. These include offshore oil and gas industry, increasing ship transport and global global change. The effects of global drivers such as ocean acidification (OA), resulting from the increasing $CO_2$ level in the atmosphere, or pollution, e.g. oil are mostly studied in isolation from other drivers. Exposure to OA or to increased concentrations of oil related components may alter an invertebrates' normal development and increase mortality, but the interaction between these two drivers has yet to be investigated.

Consequently, the impact of a simulated 4 - d Arctic crude oil spill scenario (0.5 mg $L^{-1}$), at two different water pHs (8.1 and 7.6) on aspects of the early development of the green sea urchin, *Strongylocentrotus droebachiensis* were tested, namely: hatching success, survival, growth, feeding, activity, swimming, respiration and settlement.

Early stage larvae (0 - 18 d post fertilization) showed increased mortality when exposed to oil while the older larvae (18 - 44) did not, indicating a stage specific toxicity to oil for sea urchin

larvae. OA did not influence the survival nor did the combined treatment (OA and oil condition).

Larvae showed reduced activity, feeding, growth and settlement when exposed to oil; OA alone reduced growth but did not affect the settlement. At day 44 post-fertilization, the body growth length of the larvae in the OA treatment was 9 % lower than under control condition without oil. Similarly, the oil treatment led to reduced body length (-14 % when compared to control). The combined treatment, OA and oil condition, led to a reduction in larvae body length (- 21 % when compared to control). These results indicate a reduction in growth for these treatments, and an additive effect of exposure to OA and oil spill. Larval sea urchin resilience to future changes (i.e. pollution) could be greatly reduced if the larvae are already limited in energy and stressed (reduced development) from exposure to OA. Therefore, environmental policy management should consider the role of contaminants, such as oil, within their assessment of changing environmental conditions.

## 5.2 INTRODUCTION

Human-driven changes to ocean biogeochemistry affect multiple marine processes and systems. Future oceans will suffer from the impacts from several different drivers, drivers in marine systems can be divided into two categories those that act globally and those that act locally (Sect. 1.1.1). Drivers such as OA are predicted to have significant impacts on marine invertebrate species and ecosystems including changes in species dominance and local population extinction (Sect. 1.1.3).

Other drivers can originate and act at the local scales and may be either chronic (e.g. nutrient input from rivers) or transient, brief and short lived (e.g. oil spill) (Sect. 1.1.1). The Arctic/sub-Artic is also one of the major areas in which increased oil and gas exploration are occurring, environmental pressures are highest in the North Sea, but there is an increasing high activity in

the Norwegian Sea and Barents Sea (Bakke et al. 2013). Crude oil contains numerous toxic compounds that are released into the water as it breaks down. The most toxic to marine species are Polycyclic Aromatic Hydrocarbons (PAHs) (Sect. 1.1.4). Species living in these areas may be exposed not just too local drivers but also to global drivers such as OA, hypoxia and warming (Doney, Ruckelshaus et al. 2012). When multiple drivers act simultaneously, there is a greater probability of additive effects, where a response can be predicted based on the effects of the single stressors, and synergistic or antagonistic (see section 1.1.5). Unfortunately, the majority of ocean management strategies proceed on a single issue-specific basis (Noone et al. 2012) and our understanding of the combined effects of multiple drivers lags far behind that of how each of the potential drivers acts in isolation. Therefore, there is a need to understand the interactive effects of multiple drivers on marine invertebrates and the ecosystem.

Marine life in general but some taxa in particular such as echinoderms, seems to be sensitive to the toxic effects of oil and negatively affected by OA (Sect. 1.1.3, 1.1.4). For example, sea stars (*Pisaster*) and sea urchins (*Strongylocentrotus*) were eliminated for several years following the 1957 wreck of the *Tampico Maru* off the coast of Baja California (Nelson-Smith A 1972). A global analysis of the literature revealed that sea urchins exposed to OA are generally negativly impacted but most effects are sub-lethal (Dupont and Thorndyke 2013).

Planktonic larval stages are widely considered to be the weakest link when a species is exposed to environmental changes (Kurihara 2008a; Dupont et al. 2010) and a population bottleneck (Pechenik 1999). Perhaps because larvae show more susceptibility to toxic fractions of oil and other stressors, because of their greater surface area to volume ratio which produces a greater uptake rate of a contaminant, and not having the ability to move away from unfavourable environments (Sect. 1.1.4). Furthermore; exposure to PAHs and other oil-related components alter normal invertebrate development and cause increased mortality (Vashchenko 1980; Veith et al. 1983; Suchanek 1993; Pillai et al. 2003; Bellas et al. 2008; Rial et al. 2013). Even at low

concentrations, oil seems to be often acutely narcotic (Klaassen 1996), although not lethal, to aquatic organisms over short exposure durations. Narcosis is a reversible state of arrested activity and can be caused by a wide variety of organic chemicals (Veith, Call et al. 1983).

The effects of OA on echinoderm larvae are generally negative but mostly sub-lethal. These include slower somatic growth and reflect a shift in energy budgets linked to additional costs of acid-base regulation (Dupont and Thorndyke 2013) and digestion (Stumpp et al. 2013) rather than a direct impact on calcification. A substantial amount of information regarding the effects of both oil and pH on invertebrate larvae is available; however how the effects of oil are modified by other drivers, for example OA, is largely unknown.

Consequently, the aim of the present chapter was to investigate possibilities for interactions between a transient pollutant (simulated 4 d Arctic crude oil spill scenario 0.5 mg $L^{-1}$) during early and later developmental stages, and OA (chronic exposure to reduced pH 7.6 average pH experienced by larvae by 2100 and extreme of the present natural variability; see Dorey et al. 2013, on aspects of the early development and settlement of the green sea urchin, *S. droebachiensis* (Fig. 5.1). Our working hypothesis is similar to that of the shrimp study (Chapter 4), in which it is predicted the combined exposure to an oil spill and future oceanic conditions will lead to additive effects on marine organisms' life history, developmental and ecophysiology.

## 5.3 MATERIALS AND METHODS

### 5.3.1 Adult collection and maintenance

Adult green sea urchins *S. droebachiensis* were collected by hand by divers in Lysefjorden, Norway at 15 m depth in mid-February, 2011. Urchins were transferred to the Sea Urchin farm at Finnøy and kept for two months in the laboratory before use in the experiments described below. During this time, they were kept in aquaria (1000 indv *per* 500 L aquaria) supplied with

natural flowing sea water (8 °C and $pH_{NBS}$ 8.0). Urchins were fed a diet of commercial 200 g dry-food pellets *ad libitum* each day (Skretting AS, Stavanger, Norway) and in addition brown algae, *Saccharina latissima*, until the beginning of the experiment.

### 5.3.2 Larval cultures

Spawning was induced on 11th April 2011 by intracoelomic injection of 0.5 M KCl in filtered (0.22 µm) sea water (FSW). For the experiment, eggs of eight females were collected in separate 250 mL beakers, washed, and fertilized by adding the dried sperm of three males (5 µl; to a final concentration of approximately ~1000 sperm $mL^{-1}$ , producing a fertilizations success > 95 %). Zygotes were allowed to divide once before they were pooled and were divided between a number of 13 L flow-through (flow rate = 100 mL $min^{-1}$) conical aquaria supplied with air stones, at a density of five embryos *per* mL, and at a temperature of 9.5 °C.

After five days, larvae were fed once a day at a concentration 150 µg Carbon $L^{-1}$ , with an initial algal density of approximately 6000 - 7000 cells $mL^{-1}$ and mean size 7.5 µm ± 0.8 µm on cryophyte algae, *Rhodomonas* sp.. *Rhodomonas* were raised in filtered sea water containing growth media (Himedia, Mumbai, India) maintained at T = 20 °C. Until day 18, the larvae were also fed with the algae, *Isocrysis.sp* (concentration 150 µg C $L^{-1,}$ with an initial algal density approximately 186000 cells $mL^{-1}$ and mean size 4.5 µm ± 0.5 µm). Carbon content of the algae was estimated based on bio- volume measurements as equivalent spherical diameter (ESD) with an electronic particle analyser (Elzone 5380 Micrometrics, Achen, Germany) and equations provided by Mullin et al. (1966). Algal cell density and size were measured using a coulter counter (Beckman coulter multisizer 3TM, Nerliens Meszansky AS, Oslo, Norway). To take account of the fact that the experimental system was based on a flow-through design, the retention time of the aquaria was tested to estimate how long food was available for the larvae after feeding. Water samples for retention time were taken after feeding with 30 min intervals

directly from the aquaria (not from the outlets). The reduction in algal concentration in the aquaria with time was measured using a coulter counter, Beckman coulter multisizer 3$^{TM}$, Nerliens Meszansky AS, Oslo, Norway. Appendix 5.1 show the gradual decrease in density with time (y = - 908.5 ln(x) – 1049). After five hours there was almost no algae left in the aquaria.

### 5.3.3 Experimental conditions/oil exposure

Sea urchin embryos and larvae were raised into two different pH treatments: control = $pH_{NBS}$ 8.0 and OA = $pH_{NBS}$ 7.6, average pH experienced by larvae at present and average pH experienced by larvae by 2100 and extreme of the present natural variability; see Dorey et al (2013). For each pH, six header tanks supplying 12 replicate chambers were used. In the reduced $pH_{NBS}$ treatment aquaria, the desired $CO_2$ equilibration was achieved, *via* manipulating $pH_{NBS}$ using pH-controllers (AB Aqua Medic GmbH pH computer, Bissendorf, Germany) set to maintain mean $pH_{NBS}$ at 7.6, *via* a solenoid valve, which allowed regulating the addition of $CO_2$ gas. Sea water $pH_{NBS}$ in the header tanks was recorded and logged every five min using a pH probe (Orion Star Plus™ 3-Star and Ross® Electrodes, Thermo Fisher Scientific Inc, Beverly, USA) coupled to a calibrated pH meter (Orion Star Plus™, Thermo Fisher Scientific Inc, Beverly, USA and Consort and a multi-channel datalogger D-130, Consort, Turmhout, Belgium) using the Star Plus Navigator 21 Software (Thermo Fisher Scientific Inc. Beverly, USA.). The pH probes were calibrated using NBS buffers (4 and 7 Merck, Damstadt, Germany). The impact of exposure to a 4 d oil spill was then tested in two different experiments. (i) In the first experiment, half of the replicates (N = 6 *per* tested $pH_{NBS}$) were exposed to oil between 8 - 12 days post fertilisation (dpf) and larval response was followed until day 17 dpf. (ii) At the end of this experiment, each culture that was not exposed to oil spill (N = 6 *per* tested $pH_{NBS}$) was divided into two replicated cultures (N = 12 replicates for each pH treatments) and half of

the replicates were exposed to oil at 23 - 27 dpf. Larval responses were monitored until day 44 dpf. A continuous flow system (CFS) was used to create a dispersion of crude Arctic crude oil in the sea water. A dispersion equivalent to a nominal oil concentration of 5 mg $L^{-1}$ was made by injecting oil into sea water under pressure (7 L $min^{-1}$ sea water and 0.042 µL $min^{-1}$ oil), to form small oil droplets (Sanni et al. 1998). To produce an exposure concentration of 0.5 mg $L^{-1}$, the dispersion was decanted into a 10 L glass mixing flask, and thereafter pumped (100 mL $min^{-1}$) by the mean of peristaltic pumps (Watson and Marlow model 520) into the header tanks (flow: 1 L $min^{-1}$) that supplied the aquaria.

### 5.3.4 Sea water chemistry

Water samples for the PAH analysis were collected 4 times during the first exposure and 3 times during the second exposure. Samples from the non-oil exposed aquaria were sampled as collective samples from all the aquaria in the treatment, to make sure none of the aquaria were contaminated. PAH analyses of seawater were preformed by Gas Chromatography (HP5890, Hewlett Packard, USA) and analyzed in ion mode (GC/MS – SIM) as described previously in Jonsson et al. (2004). Exposure concentration were monitored from 26 different PAH compounds analysed in the water samples based on a standard protocol (EPA 610) with modifications as previously described (Jonsson, Bechmann et al. 2004). Limit of quantification (LOQ) was set to approximately 0.005 µg $L^{-1}$for each PAH component.

Oil chemistry for treatments in experiment one and experiment two is summarized in Appendix 5.2. The nominal oil concentration in the exposure set-up was 0.5 mg $L^{-1}$ and the mean measured PAHs concentration for each treatment was 4.786 and 4.480 µg $L^{-1}$ for the oil and combined treatments, respectively. Approximately 90 % of the PAHs in the exposure tanks were C0 - C3 naphthalene's, and the remainder 3-ring PAHs and dibenzothiophenes (DBTs). There was a significant difference in the mean concentration of naphthalene between the control and the oil

treatments indicating that the control samples were not contaminated with oil ($F_{1,\ 10} = 38.30$, $p < 0.05$).

In addition, water temperature and pH were measured every second day, and oxygen concentration three times over the duration of the experiment in all aquarias. In the aquaria, water temperature was measured using a calibrated glass thermometer (certified by Physikalisch-Technische Bundesanstalt (PTB), Braunschweig, Germany) and $pH_{NBS}$ using a hand held pH meter (Orion Star Plus™ 3-Star, Thermo Fisher Scientific Inc., Beverly, USA). Oxygen concentration was measured with an $O_2$ meter and probe (Oxi 330i/SET, WTW, Weilheim, Germany). The salinity of the intake water was recorded every 5 min during the entire duration of the experiments using a CT-probe (Aqua TROLL 100®) with Win-Situ 5 data acquisition software (In-Situ Inc., Collins, USA).

The program CO2SYS.EXE (Pierrot et al 2006) was used to calculate the saturation state for aragonite and calcite ($\Omega_{arg}$ and $\Omega_{cal}$) and other parameters in the carbonate system: dissolved inorganic carbon (DIC) and concentrations of carbonate [$CO_3^{2-}$] and bicarbonate [$HCO_3^-$]. The input parameters were total alkalinity, pH, temperature and salinity in addition to the dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987). The saturation states and other parameters in Appendix 5.3 were calculated using mean measured values ± standard deviation (SD). The range is also given. Mean $pH_{NBS}$ level (and SD) in each aquaria was calculated on the actual [$H^+$] concentration and back calculated to $pH_{NBS}$.

Carbonate chemistry and temperature for treatments in experiment one and experiment two are summarized in Appendix 5.3. Although some significant differences were detected between aquaria for experiment one ($F_{23,\ 71} = 4.22$, $p < 0.0001$) the actual difference was very small. Temperature ranged from 9.4 - 9.5 °C. No significant differences were detected in temperature between aquaria for experiment two ($F_{23,\ 71} = 4.22$, $p < 0.0001$). No significant differences were

detected in the alkalinity of the OA treatments (TA = 2306.83 ± 13.73 µmol $kg^{-1}$) ($F_{1, 9}$ = 0.06, $p$ = 0.80), and only departed from the mean by 0.61 %. $pH_{NBS}$ fluctuated less than 1 % from the mean values. Mean $pCO_2$ values in the treatments ranged from 476 to 634 µatm ($pH_{NBS}$ = 8.0) and 1292 to 1569 ($pH_{NBS}$ = 7.6). Sea water was under saturated with respect to aragonite at $pH_{NBS}$ < 7.6 (Appendix 5.3).

### 5.3.5 Experiment 1, day 0 - 18

#### 5.3.5.1. Mortality and growth

Larval culture were monitored daily. Each day (day 0 - 18) 10 mL, was collected from each aquaria, counted and fixed in buffered 4 % paraformaldehyde (PFA) in FSW for later analysis. Percentage instantaneous mortality was calculated as $M_t$ = 1- $\frac{Nt}{Nt-1}$ *100 ($N_t$ density of larvae at time t) Eq 1. For each replicate, 10 - 15 individual larvae were photographed every other day with a digital camera mounted on a dissecting microscope (5x, 10x or 20x magnification, depending upon stage) using polarized light to visualize the skeleton. Five morphometric parameters (see Fig. 5.2) body length (BL), stomach width (SW), stomach length (SL) and posterolateral rod lengths (POL1, POL2) were measured for each larvae using Image J software (Fig. 5.2). A symmetry index (SI) was calculated for each larvae as follows SI = $\frac{Min(POL1,POL2)}{Max\ (POL1,POL2)}$ . Stomach volume (SV) was calculated using the equation:

(Eq. 2) $$SV = \frac{4}{3}\boldsymbol{\pi} \times \left[\frac{(SW+SL)}{4}\right]^3.$$


**Figure 5.2 Morphometric coordinates and morphology of *S. droebachiensis* 4 - arm pluteus larva. BL, body length; SW, stomach width; SL stomach length; A1, A2, posterolateral rod length (POL) (both arms (A1, A2)).**

5.3.5.2 Feeding

During the feeding test larvae were fed once each day with only 150 µg carbon $L^{-1}$ *Rhodomonas. sp*. Fluorescence microscopy (x 5 magnification) was used to assess the algal content in the stomachs of the sea urchin larvae at a given time point, to look for differences between the treatments, as indicative of differences in feeding rate. One hour after feeding, larvae were removed from each aquaria and pictures were taken. They were then photographed using a Zeiss Axioplan two imaging microscope fitted with an Axiocam MRc5 camera, bifocal (x5 magnification) with a fluorescent rhodamine filter. Semi-quantitative measurements of occurrence of algae in the digestive system of the sea urchin larvae was made by quantifying the saturation of red fluorescence in images collected with the rhodamine filter using Axiovision software. For each larva stomach fluorescence was measured. Values for stomach fluorescence were expressed as a function of exposure time, to obtain the differences in

exposure time (exp) and presented as red fluorescence $exp^{-1}$. Feeding was assessed in Experiment. 1 on days 12 - 15.

### 5.3.6 Experiment 2 day 18 – 44

#### 5.3.6.1. Mortality and feeding

Larval aquarias were monitored daily. Each day (day 18 - 44) two samples (10 mL), were collected from each aquaria, counted and fixed in buffered 4 % PFA in FSW for later analysis as described for experiment 1. Feeding was assessed day 23 – 33 as described for Experiment 1.

#### 5.3.6.2 Metabolic rates of larvae

Rates of oxygen ($O_2$) uptake were made on days 15, 23, 30 and 37, following the methods of Taylor and Spicer (1989). A custom-built closed, glass-bottle respirometer (vol. = 5 mL), was fitted with airtight stoppers into which $O_2$ electrodes had been inserted. A known amount of sea urchin larvae (N = 200) were placed in the incubation chambers filled with sea water at the appropriate pH and sealed with an airtight stopper. Chambers were submerged in flow-through water baths to maintain a constant temperature. A total of 6 replicates were carried out for each of the four decreased pH/oil combinations (one measurement per replicated aquaria). Measurements of dissolved $O_2$ concentration were performed every 2 sec for the entire duration of the incubation (approx. 48 h) using $O_2$ electrodes (1302, Strathkelvin Instruments, Glasgow, UK coupled to a multichannel $O_2$ meter (928, Strathkelvin Instruments, Glasgow, UK). $O_2$ electrodes were calibrated with air-saturated water (100 %) and deoxygenated water (0 %). Continuous measurements were conducted to demonstrate linearity of $pO_2$ decline in the

respiration chambers during the incubation. Seawater blanks (no larvae present) were run for each treatment to quantify background respiration rates caused by microorganisms in the system. Background respiration never exceeded 10 % of the total larval respiration (2.5 µmol $O_2$ $h^{-1}$). These background rates were then used to correct the measured larval respiration. At the end of each trial, viable larvae were counted (mean 0.8 ± 2.5 % were dead) and then fixed in buffered 4 % PFA in sea water for subsequent body size determination. Respiration (respiration refers to oxygen consumption) results were corrected by the number of living larvae per respirometer (individual $O_2$ consumption: pmol $O_2$ $ind^{-1}$ $h^{-1}$ $um^{-1}_{BL}$).

5.3.6.3 Swimming activity

Larva activity was assessed visually during day 23 of the oil exposure. For each aquaria, a sample of at least 20 larvae was placed in a Petri dish (diame. = 4 cm) and larvae were scored as active or passive (not swimming actively, only at the bottom of the Petri dish with no or little movement). A swimming activity test according to Yao et al. (2010) with modifications was performed on day 31. Six replicated aquarias in each treatment were tested. 15 larvae were transferred to 2 mL glass vials with the treatment sea water with control (pH 8.1) or OA ($pH_{NBS}$ 7.6), with (300 µg C $L^{-1}$) or without *Rhodomonas sp* algae. The syringe chamber was filled with 2 mL of test water with (300 µg C $L^{-1}$) algae and without algae, and was connected on to the glass vial with a silicon tube. After 24 h the number of larvae that had entered the syringe chamber was counted and classed as “active swimmers”.

5.3.6.4 Metamorphosis/larval settlement

At day 39, metamorphosis was induced in 8 - arm pluteus larvae with well-developed rudiments. For each replicated aquaria, 15 larvae were transferred in 24 wells (Falcon nunc plate) filled with 2 mL of sea water at the appropriate $pH_{NBS}$. Three different inducers were

compared (N = 24): 1) Plankton mesh with biofilm; 2) KCl (25 mmol $L^{-1}$) and plankton mesh with biofilm; 3) Coralline algae solution and plankton mesh with biofilm, and one control with only filtered seawater. The biofilm were prepared by allowing the plankton mesh to hang in flow through sea water for a month. The coralline solution was prepared by scraping off coralline algae from a rock and adding it to filtered seawater. The rate of larval metamorphosis after 24 h was expressed as the percentage of metamorphosed individuals.

### 5.3.7 Statistics

Larval mortality rates (MR in % $d^{-1}$) were calculated as the coefficient of the significant linear relationship between instant mortality $M_t$ (%) and tpf (day). Body length growth rates (BL GR in μm ln $(d)^{-1}$) were calculated as the coefficient of the significant logarithmic relationship between BL and tpf. Postero-lateral arm growth rates (POL GR in μm $\mu m^{-1}_{BL}$) were calculated as the coefficient of the significant linear relationship between POL (μm) and BL (μm). Stomach volume growth rates (SV GR, $\mu m^3$ $\mu m^{-1}_{BL}$) were calculated as the coefficient of the significant linear relationship between SV ($\mu m^3$) and BL (μm). Respiration rate growth rates (RR GR in pmol $O_2$ $ind^{-1}$ $h^{-1}$ $\mu m^{-1}_{BL}$) were calculated as the coefficient of the significant linear relationship between respiration rate (pmol $O_2$ $ind^{-1}$ $h^{-1}$) and BL (μm). Results of the regressions were used to test differences in the measured variables one -, 2-and 3-way ANOVA were performed with time, pH and oil as variables, followed by post-hoc Scheffe`s. Where there was no significant relationship ($p$ - value > 0.05), those data were removed from subsequent analyses.

The Kolmogorov-Smirnov test was used to test the data for normal distribution and the Levene test was used to test that variance was homogenous. All statistical analyses were performed using SAS software.

## 5.4 RESULTS

### 5.4.1 Experiment 1 – Impact of pH and oil exposure on green sea urchin 8 - 12 d post fertilization.

The average MR was 60 % higher for larvae when larvae were exposed to oil compared with controls (Fig. 5.3a) ($F_{3, 21} = 65.45$, $p < 0.0001$). Whilst no significant effect of exposure to OA or the interaction between OA and oil was found on mortality (max. $F_{3, 21} = 0.04$, $p = 0.93$). There were significant relationships between mortality (%) and time in all but three of the aquarias ($p < 0.05$ in each case, see Appendix 5.4 a, 5.5).

The BLGR was reduced by 11 % when larvae were exposed to oil, while exposure to OA decreased BLGR with 6 %, when larvae were exposed to both oil and OA, BLGR was decreased by 17 % compared with larvae raised in control conditions with no exposure to oil (Fig. 5.3b). Therefore both exposure to OA ($F_{3, 23} = 21.92$, $p < 0.0146$) and oil ($F_{3, 23} = 21.92$, $p < 0.0001$) had a significant effect on BLGR but there was no significant interaction ($F_{3, 23} = 0.77$, $p = 0.77$). There were significant relationship between BL (µm) and ln time (day) for all aquarias ($p < 0.05$, see Appendix 5.4 b, 5.6) allowing growth to be expressed as increased length per unit time (BL GR in µm ln $(d)^{-1}$).

Postero-lateral arm (POL) and stomach volume (SV), were not affected by oil, OA or their interaction (Fig. 5.3 c-d), (max. POL $F_{3, 22} = 0.87$, $p = 0.47$, SV $F_{3, 16} = 1.76$, $p = 0.20$). There was a significant relationship between POL length and time (µm; $p < 0.05$, see Appendix 5.4c and 5.7) allowing us to calculate another measure of growth (POL GR in µm $\mu m^{-1}_{BL}$). For all but two of the aquarias, there was a significant relationship between stomach volume (SV) and time ($p < 0.05$, see Appendix 5.4 d, 5.8) providing yet another measure of growth.

During and after exposure to oil, larvae were eating (feeding estimated as fluorescence exp) five times less food than the controls (Fig. 5.3 f) ($F_{3, 157} = 95.87$, $p < 0.0001$). No significant

effect of exposure to OA ($F_{3, 157} = 1.00$, $p = 0.32$) or the interaction between OA condition and oil ($F_{3, 157} = 0.15$, $p = 0.70$) was found.

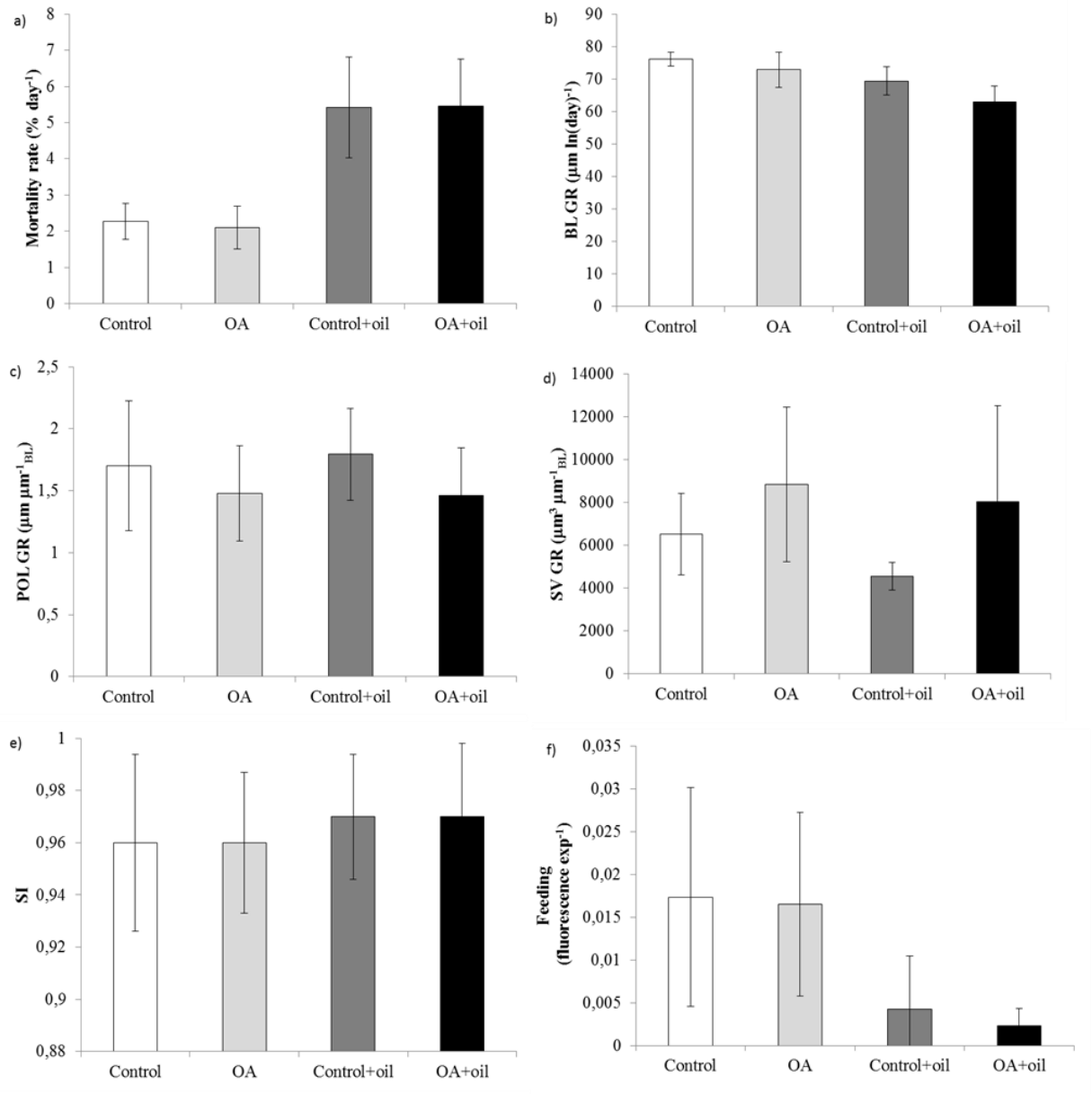


**Figure 5.3 Experiment 1: Impact of OA and a 4 d oil exposure (8 - 12 dpf) on *S. droebachiensis* larvae (a) mortality rate (% $d^{-1}$); (b) body length growth rate (BL GR, µmln(d)$^{-1}$); (c) postero-lateral arm growth rate (POL GR, µm µm$^{-1}_{BL}$); (d) stomach volume growth rate (SV GR, µm$^3$ µm$^{-1}_{BL}$); (e) symmetry Index (SI) and (f) feeding between d 12 to 15 (fluorescence exp$^{-1}$). Values are means ± SD.**

### 5.4.2 Experiment 2 – Impact of pH and oil exposure on green sea urchin 18 - 44 d post fertiliastion

MR ranged between 0.79 and 3.2 % d, and the average MR was 2.09 ± 0.67 % $d^{-1}$ with no significant difference between OA, oil or their interaction (Fig. 5.4a) ($F_{3, 18} = 0.02$, $p < 0.99$). Mortality (%) was significantly and linearly correlated with time in all aquarias except for two ($p < 0.05$, see Appendix 5.4 a, 5.9).

The BLGR decreased by 14 % when larvae were exposed to oil, while exposure to OA decreased BLGR by 9 %, when larvae were exposed to both oil and OA, BLGR was decreased by 21 % compared with larvae aquarias under control conditions with no exposure to oil (Fig. 5.4 b). Therefore both exposure to OA ($F_{3, 23} = 8.51$, $p = 0.0008$) and oil ($F_{3, 23} = 16.93$, $p = 0.0006$) had a significant effect on BLGR but there was no significant interaction ($F_{3, 23} = 1.85$, $p = 0.19$). There were significant relationships between BL (µm) and ln time (day) for all cultures ($p < 0.05$, see Appendix 5.4 b, 5.10) allowing growth to be expressed as increased length per unit time (BLGR in µm ln $(d)^{-1}$).

POL and SV were not affected by oil, OA or their interaction (Fig. 5.4 c-d) (max. POL $F_{3, 23} = 1.87$, $p = 0.17$, SV $F_{3, 19} = 2.06$, $p = 0.15$). There was a significant relationship between POL and time (µm; $p < 0.05$, see Appendix 5.4 c, 5.11) allowing us to calculate another measure of growth (POL GR in µm $\mu m^{-1}_{BL}$). For all but three of the aquarias there was a significant relationship between SV and time ($p < 0.05$, see Appendix 5.4 d, 5.12) providing yet another measure of growth. Symmetry Index was high in all aquarias (SI = 0.97 ± 0.03; Fig. 40 e) with no significant effects of oil, OA or replicate ($F_{23, 3524} = 0.64$, $p = 0.054$). Respiration rate (pmol $O_2$ $ind^{-1}$ $h^{-1}$) increased linearly with BL. However, no significant effect of OA ($F = 0.38$, $p = 0.54$), oil ($F = 4.30$, $p = 0.0543$) or their interaction ($F = 0.91$, $p = 34$) was detected (Fig. 5.4 f). There was significant swimming cessation (day 24 post fertilisation) in the oil exposed larvae with a mean of 34 % passive larvae in the oil exposure treatment compared to the mean of 4 %

passive larvae in the non-exposed larvae ($F_{1.23} = 50$, $p < 0.0001$). The presence of algae, alone or in interaction with other tested parameters, had no significant effect on the percentage of swimming larvae (SL, $F_{7, 45} = 4.64$, $p = 0.0008$; algae: $F = 2.55$, $p = 0.12$; all interactions $p > 0.05$). The percentage of active larvae was 2.5 times greater in the absence of oil and was higher at OA compared to controls (Fig. 5.4 g) (OA: $F = 4.50$, $p = 0.041$, oil: $F = 16.52$, $p = 0.0002$). Whilst no significant effect of exposure to the interaction between OA condition and oil ($F = 0.15$, $p = 0.70$) was found.

Scheffe's *post-hoc* test revealed that larvae raised at OA were eating 1.8 times less than those raised in control conditions. Moreover, exposure to oil resulted in an x 6.2 decrease in feeding compared to the control (Fig. 5.4 h). Larval feeding (estimated as fluorescence $exp^{-1}$) was therefore significantly affected by exposure to oil ($F_{2, 226} = 91.23$, $p < 0.0001$), OA ($F = 18.08$, $p < 0.001$) and their interaction ($F = 5.57$, $p = 0.02$).

Metamorphosis was induced using 8-arm pluteus larvae with well-developed rudiments. KCl (25 mmol $L^{-1}$) and plankton mesh with biofilm was most efficient inducer for larval metamorphosis with a mean of 43 % metamorphosed larvae. Plankton mesh with biofilm had a mean of 7 % metamorphosed larvae and 4 % metamorphosed in the coralline algae solution and plankton mesh with biofilm. No larvae metamorphosed in the control with FSW. Mean percentage metamorphosed larvae in the KCl (25 mmol $L^{-1}$) and plankton mesh with biofilm treatment were 63, 77, 6 and 27 % in the control, OA, control + oil and OA + oil, respectively (Fig. 5.5). Oil had significantly decreased metamorphosis in larvae ($F = 65.22$, $p \leq 0.001$). Whilst no significant effect of exposure to OA ($F = 1.44$, $p = 0.257$) or the interaction between OA condition and oil ($F = 0.25$, $p = 0.625$) was found.

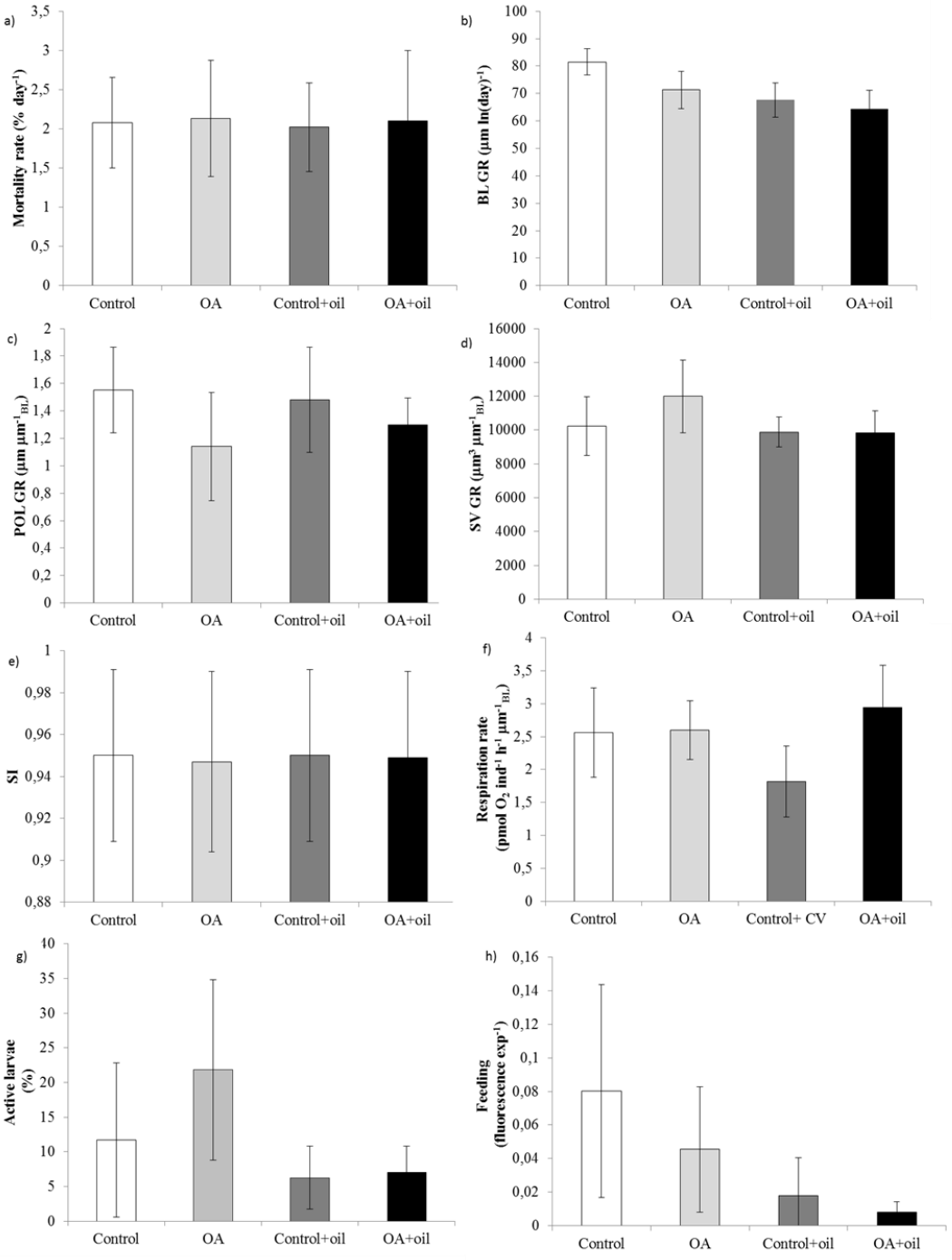


**Figure 5.4 Experiment 2: Impact of OA and an oil exposure (23 - 27 dpf) on *S. droebachiensis* larval (a) mortality rate (% $d^{-1}$), (b) body length growth rate (BL GR, µm $ln(d)^{-1}$), (c) postero-lateral arm growth rate (POL GR, µm $µm^{-1}_{BL}$), (d) stomach volume growth rate (SV GR, $µm^3$ $µm^{-1}_{BL}$), (e) symmetry Index (SI), (f) respiration rate (RR, pmol $O_2$ $ind^{-1}$ $h^{-1}$ $µm^{-1}_{BL}$), (g) percentage of active larvae (day 31) and (h) feeding between days 23 and 33 (fluorescence $exp^{-1}$). Values are expressed as means ± SD.**

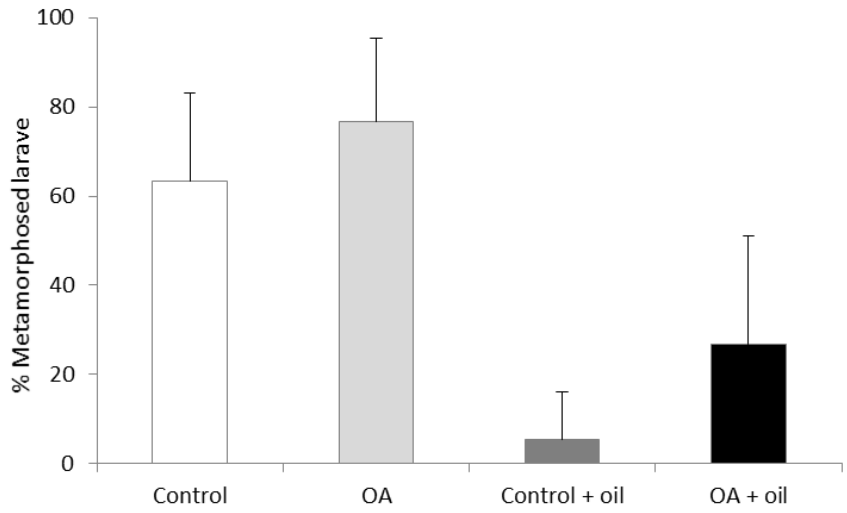


**Figure 5.5 Metamorphosed larvae (means ± SD) of larval *S. droebachiensis* in control, OA, control + 4 d oil spill (0.5 mg $L^{-1}$), OA + 4 d oil spill (0.5 mg $L^{-1}$) treatments.**

## 5.5 DISCUSSION

This study approaches the issue of the interactive effects caused by the combined exposure to a local driver (i.e. low concentration of oil) and exposure to a global driver (i.e. reduced pH) on the development on green sea urchin larvae. We hypothesized that the different drivers (OA and oil condition) would have different modes of action on the development of the larvae. Our results were consistent with this hypothesis. Exposure to oil resulted in a reduction in larval movement, feeding, and ultimately growth. Larvae raised in OA in this study showed delayed development most likely due to larger energy and metabolic costs. Growth rates of sea urchin larvae were 14 % lower in the oil treatment and 9 % lower in the reduced pH treatment, indicating a growth delay in these treatments compared to the control larvae. When combining the drivers, the size of sea urchin larvae was equal to the sum of the negative impacts observed for each driver: an additive effect and a reduction of 21 %.

Exposure to oil resulted in a reduction in larval feeding, movement and growth. Larvae were exposed to oil in water dispersion containing 10 µm oil particles (the same system and oil measured by Bechmann et al. 2010) and dissolved oil components (Skadsheim et al. 2000).

Filter feeders such as some species of copepods filter particles in the same size range as small oil particles making them bioavailable to the organism (Gyllenberg 1981; Hansen et al. 2012). Studies on copepods have shown that dissolved oil components will be taken up via diffusion over the general body surface, while oil droplets may be taken up through other routes, e.g. digestive system by ingested oil droplets and/or diffusion over body surface due to oil droplets adhering to external surfaces (Gyllenberg 1981; Berrojalbiz et al. 2009; Almeda et al. 2014). The same uptake route would also apply to sea urchin larvae, which are also filter feeders. Direct comparisons with other oil exposure studies is problematic, because the type of oil, the chemical composition, the exposure conditions, the fraction of oil tested, the analytical method and compounds analyzed vary a lot (Bechmann et al. 2010; Ingvarsdóttir et al. 2012). Even at low concentrations, PAHs seems to be often acutely narcotic (Klaassen 1996) but not lethal to aquatic organisms over short exposure durations. Narcosis is a reversible state of arrested activity and can be caused by a wide variety of organic chemicals (Veith et al. 1983). Reduced swimming activity during oil exposure may be due to a narcotic or sluggish effect of the the oil (Saiz et al. 2009; Almeda et al. 2013). For example, exposure of larvae of King crabs (*Paralithodes*) to water soluble fractions of oil (WSF) of 0.5 mg $L^{-1}$ comparable to our concentration resulted in swimming cessation and ultimately death (Brodersen 1987). Reduced activity and feeding was also observed during oil exposure. Reduced feeding during oil exposures has been reported in several other studies (Berdugo et al. 1977; Hansen et al. 2012), and reduced growth has been observed for sea urchin larvae (Bellas et al. 2008). This reduced activity and feeding could have contributed to the increased mortality and reduced growth in the oil exposed treatments. However, the oil effect was reversible. In fact, when the larvae were subjected to unpolluted water they started feeding again. This reversible trend was also observed for copepods after they were transferred to unpolluted water (Berdugo et al. 1977).

Another interesting find was the effects of oil on the larvae were stage specific. Exposure to the simulated oil spill resulted in high mortality for "young" larvae but not for "older" larvae; this suggests that the latter are more robust to oil pollution. This is of interest because sea urchin larvae are thought to be especially sensitive to xenobiotic toxins and were frequently used to determine the toxicity of oil products (Falk-Petersen 1978; Fernández et al. 2006; Bellas et al. 2008). Similar stage specific responses to oil have been reported for copepodites, that tend to be more sensitive to oil exposure than larger copepods, as it is for crustacean larvae when compared to adult forms more generally (Saiz et al. 2009; Jiang et al. 2012). Saiz et al. (2009) suggested that small copepods do not store large amounts of lipids in their body, and are therefore more sensitive to changes in food availability, due to reduced feeding, and hence also more sensitive to toxins.

Under food-limited conditions, sea urchin larvae develop smaller stomachs and rudiments (Strathmann and Strathmann 1994). Rudiments are united structures from the amniotic invagination and hydrocoel that that give rise to future adults (Hart and Strathmann 1994; Brey et al. 1995). The present study shows that fewer *S. droebachiensis* larvae undergo metamorphosis at day 44 when previously exposed to low concentrations of crude oil. Rinkevich and Loya (1977) reported that coral larvae exposed to concentrations 1.5 and 10 µg $L^{-1}$ of crude oil also significantly reduced their settlement. Kushmaro et al. (1997) exposed soft coral to different crude oil concentrations on the water surface the planulae of soft coral (*Heteroxenia fuscescens*) lost their ability to undergo metamorphosis with the increase in crude oil concentrations. These larvae were also significantly smaller than the ones not exposed to oil as a consequence of a delayed development. Meidel et al. (1999) showed that *S. droebachiensis* larvae that were fed a high of ration food were larger throughout most of the developmental period and reached competence to metamorphose 22 days earlier, than larvae fed a low ration

of food. The lower settlement success at day 44 in our study may be then partly explained by a delay in larval competency.

The delayed development of larvae observed in this study, are in agreement with other studies of negative effects of exposure to OA on growth, reduced size at a given time/or delayed development (Dupont and Thorndyke 2014). OA impacts metabolism, possibly in association with the organism's energy budget (food fueling soft and hard tissue growth, lipid storage as well as excretion and respiration) (Dorey et al. 2013). Two studies previously investigated and described increased metabolism in the sea urchin larvae under reduced pH conditions (Stumpp et al. 2011; Dorey et al. 2013). Stumpp et al. (2011) suggested that such an increase in metabolic cost could be responsible for slower development. No increased metabolic rates for the OA treatments were found in this study; however, there were large differences in the respiration between the replicates.

Furthermore; Stumpp et al. (2013) revealed that larvae exposed to OA experience a reduction in gastric pH, which directly translates into decreased digestive efficiencies, and triggers compensatory feeding. The study showed that the degradation rate of ingested *Rhodomonas* cells, measured as chlorophyll a-dependent fluorescence intensity, was significantly reduced (by 33 %, 8 - 16 d post fertilasation) under acidified conditions indicating a reduced digestive potential. A similar response in reduced digestive potential were found in the second experiment in this study were it was significantly reduced by 28 - 40 % (22 - 38 d post fertilization). Although compensatory feeding (as documented in Stumpp et al. (2013)) were not investigated/ documented in this study, there was a significant tendency of more active larvae (swimming) in the OA conditions that might indicate compensatory feeding.

A delay in growth, both seen in the larvae who were raised in OA and oil condition, and the additive effect on growth when the 2 factors were combined, would lead to larvae spending longer periods in the plankton (Moran and Grant 1993). This would have consequences for

larval survival both indirectly (physiological mortality 1.3 % larvae day$^{-1}$ from this study) and indirectly by predation (10 % larvae day$^{-1}$) (Lamare and Barker 1999; Dorey et al. 2013). Delayed metamorphosis even by a few days, can severely reduce the number of larvae reaching settlement, and lead to challenging constraints regarding recruitment and population maintenance.

It is suggested that there were additive physiological effects of OA and a contaminant. Therefore, larval sea urchin resilience to future changes (i.e. pollution) could be greatly reduced if the larvae are already limited in energy and severely stressed (reduced development) from OA. Consequently, environmental policy management should consider contaminants such as oil within the context of changing environmental conditions. This chapter focuses on a single life history stage, and recent studies highlighted the importance of carry-over and trans generational effects of the exposure to reduced pH (Dupont et al. 2012; Lohbeck et al. 2012; Parker et al. 2012; Thor and Dupont 2015). Furthermore, abiotic variables such as temperature and pH vary spatially across the distributional range of *S. droebachiensis*, and development in different populations and genetic strains might respond differently to reduced pH and oil. This together with the species ability for rapid adaption to future climatic change scenarios (see (Collins and Bell 2004; Collins and Bell 2006; Findlay et al. 2011; Pistevos et al. 2011; Sunday et al. 2011)) should be considered in further studies.

# Chapter 6 - General discussion

## 6.1 OVERVIEW

The main aim of this thesis was to increase our current understanding of the combined impacts of multiple anthropogenic drivers on the development, morphology and physiology of key marine invertebrates. The Northern shrimp (*Pandalus borealis*), the green sea urchin (*Stongylocentrotus droebachiensis*), and the Northern krill (*Meganyctiphanes norvegica*) were used as study species in Chapters 2 - 5. They were selected particularly for the important ecological role they play in the ecosystems and their economic importance (Sect. 1.4).

The first objective of the thesis (Chapter 2) was to assess whether physiological and developmental responses (hatching timing and success, development, survival, feeding, respiration and growth) of the Northern shrimp were affected by elevated temperature and reduced pH in line with predictions of future ocean warming (OW) and ocean acidification (OA) expected in 2100 (Blackford and Gilbert 2007; Førland et al. 2009; Steinacher et al. 2009). Another objective was to investigate possibilities for interactions between the two drivers.

The main conclusion from this experiment was that OW could possible exert a greater effect on shrimp larval development than OA manifesting itself as accelerated developmental rates with greater maintenance costs, and therefore decreased size of shrimp larvae.

The second objective of the thesis (Chapter 3), was to assess whether Northern krill (another important but different crustacean species) would respond in a similar way to the Northern shrimp, to elevated temperature and reduced pH in line with predictions of OW and OA predicted for 2100 (Blackford and Gilbert 2007; Førland et al. 2009; Steinacher et al. 2009). To investigate this the physiological and developmental (hatching success, mortality, development, length, moulting, feeding and respiration) responses of larval and juvenile Northern krill

exposed to a combination of OW and OA (OAW) in line with predictions for 2100, were assessed (see above).

Similar to the shrimp, the krill larvae showed increased rates of larval development when exposed to OAW. The experiment furthermore showed that juvenile Northern krill are likely to survive future OAW exposure, but nevertheless noticeable sub-lethal effects were observed, such as higher metabolic costs, reduction in feeding rates and possibly effects on moulting.

The third objective of the thesis (Chapter 4) was to investigate the effect of exposure of a realistic oil spill on the physiological ecology of the berried adults and the development of embryos and larvae of the Northern shrimp adult in a combined OAW scenario, predicted for 2100. An additional objective was to investigate possible interactions between the two drivers. To be able to describe physiological and developmental responses, adult female feeding, respiration rate, and lysosomal membrane stability were investigated, as well as hatching success, hatching time, duration of hatching, and larval survival, developmental time, feeding rates, swimming, oxygen consumption and growth. The results from the experiments described in Chapter 4 confirm the results from the experiment in Chapter 2 for the OAW treatment. Shrimp larvae exposed to future OAW experienced accelerated developmental rates, accompanied by greater maintenance costs, which resulted in smaller. Furthermore, the results indicate additive effects of combined exposure to OAW and an oil spill on the size of shrimp larvae. The results from these experiments suggest that the resilience of larval shrimps to local impacts (i.e. local oil spill) could possibly be significantly reduced when they are exposed to future OAW, likely due to the fact that they are already energetically limited and experiencing a reduced larval development rate by exposure to OAW.

The fourth and the last objective (Chapter 5), was to investigate if early life stages of green sea urchin would respond in a similar way to an oil spill as shrimp larvae, when larvae were energy limited due to higher maintenance costs, by global drivers (sea urchin aquarias at OA, shrimp

d at OAW). Therefore, physiological and developmental responses (hatching success, survival, growth, feeding, activity, swimming, respiration and settlement) were investigated.

As expected from previous literature, OA alone reduced growth in the green sea urchin due to increased maintenance costs, but did not affect settlement of larvae. In addition, and similar to the shrimp larvae, sea urchin larvae experienced reduced activity, feeding, growth and settlement behaviour when exposed to oil. Furthermore, similar to the shrimp larvae (Chapter 4), the study demonstrated additive effects of combined exposure to OAW and an oil spill on the size of sea urchin larvae. Therefore, also larval sea urchin resilience to future changes (i.e. pollution) can possible be greatly reduced if the larvae are already limited in energy and stressed (reduced development) from exposure to reduced pH.

Overall, for krill and shrimp larvae that were reared in OW conditions, temperature outside their normal thermal range for development, the duration of development was greatly decreased, while sea urchins reared in OA conditions increased their developmental rate. All larvae experienced increased maintenance costs. Metabolic rates, feeding and activity increased for larvae and juveniles when exposed to the global driver of OA. Activity was reduced due to narcotic effects of oil on the larvae when exposed to oil. No interactive effects of the combined drivers on activity, metabolic and feeding rates were detected. The increased rate of development and increased energy expenditure at future climate/OAW resulted in smaller krill and shrimp larvae, while greater energy expenditure from OA exposure resulted in smaller sea urchin larvae. Additive effects on size were observed for both sea urchin and shrimp when they were exposed to oil, OW and OA in combination. Each of the key findings of this thesis will be discussed.

## 6.2. STAGE-SPECIFIC RESPONSES TO MULTIPLE DRIVERS IN MARINE SPECIES

Larvae of marine invertebrates are small (large surface area-to-volume ratio) (compared to the adults) and go through several and complex moulting processes over a limited period of time. They have been recognised to be particularly vulnerable to environmental drivers (Pechenik 1999; Przeslawski et al. 2015). These early stages can be a bottleneck for the species success in the future, given survival of a species is dependent on its ability to develop, grow and reproduce. Several meta-analyses of available data on multiple stressors have revealed increased sensitivity of early life stages compared to adults (Harve et al. 2013; Kroeker et al. 2013; Przeslawski et al. 2015). The shrimp and the krill in this study seemed no different in this regard, as berried adults and juveniles seemed more robust than the larvae to exposure to OAW.

Furthermore, the analysis by Przeslawski et al. (2015) demonstrated that early embryonic life stages were less vulnerable to thermal and pH stress than larvae. For example, robustness of embryos of European lobster, *Homarus gammarus,* and Norway lobster, *Nephrops norvegicus,* exposed to thermal and pH stress has been previously reported (Small 2013; Styf 2014). The krill and shrimp embryos in this investigation appear to show a comparable level of embryonic robustness, to that reported for European and Norway lobster, e.g., there was no effect on hatching success of exposure to OA, OW and OAW conditions. There was, however one exception to this. Exposure of shrimp larvae to OW (Chapter 2) reduced their hatching success although admittedly, this was only by 1.6 %, and this was not confirmed by exposure to OAW in Chapter 4. The robustness of the embryos could possibly be explained by the fact that early cleavage embryos are already conditioned for environmental variations by having high levels of cellular defenses already present in the egg (Hamdoun and Epel 2007) and the fact that shrimps have protective embryonic coatings (Glas et al. 1997).

Different larval developmental stages may also have different sensitivities to different drivers. For example, Small (2015) showed that individual larval stages of *H. gammarus* show differing

sensitivities to elevated temperatures with regards to metabolism and survival, which can be translated as ontogenetic shifts in optimum temperature between stages. Furthermore, Saiz et al. (2009) reported that copepodites tend to be more sensitive to oil exposure than adult copepods. Similarly, early sea urchin stages seemed more sensitive with higher mortality due to impact of oil than later stages of sea urchin larvae. Sea urchin exposed to oil reduced their feeding rates, and it appeared that the later sea urchin developmental stages had more energy to cope with a transient food shortage because of reduced feeding rates.

**In conclusion, the larval stage seemed overall to be the most sensitive stages compared to the embryos, adult and juveniles. Early sea urchin stages seemed more sensitive to oil than later stages.**

## 6.3 SPECIES-SPECIFIC RESPONSES TO MULTIPLE DRIVERS IN MARINE SPECIES

Species, even when they are closely related, show considerable variation in their response to temperature and pH stress (Dupont and Thorndyke 2009), and furthermore to oil exposure (Suchanek 1993) and some species may be more resilient to certain drivers than others and become potential "winners" in future seas.

Previous meta-analyses showed that generally speaking crustaceans are a robust group of marine invertebrates when it comes to exposure to OA, whereas echinoderms tend to be more sensitive to the impacts of OA (Whiteley 2011; Byrne and Przeslawski 2013; Harvey et al. 2013; Kroeker et al. 2013; Przeslawski et al. 2015). The higher tolerance of crustaceans to OA have been attributed by Whiteley (2011) to their relatively higher metabolism and their ability to defend intracellular pH. Similar results were found in this study OA reduced the growth of sea urchin larvae and decreased the survival of early stage larvae. However, there were only very small effects of OA on larval shrimp, which has also been previously shown by other

studies (Bechmann et al. 2011; Chapter 2); suggesting shrimp larvae were more resilient than sea urchin larvae to OA exposure.

Furthermore, the krill larvae seemed more sensitive to OAW conditions than the shrimp larvae. After an exposure period of 30 d to these conditions, none of the exposed krill larvae were alive, whereas 70 % of the shrimp larvae survived these conditions. This may be explained by the fact that once shrimp larvae are hatched they start to feed immediately, while krill will go through three non-feeding stages before the C1 feeding stage. Krill energy reserves are then heavily depleted and immediate feeding is then imperative to ensure survival (Poulsen et al. 2011). However, OAW conditions might shorten this "feeding window" due to faster developmental rates of larvae and causing higher mortality rates.

Moreover, echinoderms can be particularly sensitive to acute exposure to oil (Suchanek 1993; Blackburn et al. 2014). This was confirmed here (Chapter 4), as after short-term oil exposure, almost all early stage sea urchin larvae died, but none of the early shrimp larvae died under similar conditions. This may be explained by the fact that whilst it was observed that reduced feeding occurred in both species during the oil exposure, shrimp larvae have more energy to cope with a transient food shortage or reduced feeding.

**In conclusion, sea urchins' larvae are more vulnerable to OA and short-term oil exposure than shrimps. Krill larvae were more vulnerable than shrimps to exposure of OAW conditions.**

## 6.4 ENERGY ALLOCATION SHIFTS AND REDUCED LARVAL SIZE CAUSED BY THE INVESTIGATED DRIVERS

Organisms can be described as non-equilibrium, thermodynamically open systems relying on external energy sources and constant energy flow (Sokolova 2013). For aquatic organisms the energy that is absorbed by consumption, is used by four energy consuming groups or "sinks", growth, reproduction, maintenance and activity (See Fig. 1.4).

The common energy currency in cellular metabolism is high-energy adenosine-triphosphate (ATP) molecules (Applebaum et al. 2014). Given that each organism is characterized by a maximal aerobic (and metabolic) capacity, available metabolic power (energy use per unit time) is limited and must be partitioned between different processes (Guderley and Pörtner 2010). Energy is required to maintain physiological homeostasis in response to environmental change, and therefore there will be an increase in demand of ATP for physiological maintenance. This could limit the allocation of ATP or "energy" to other energy consumer groups and lead to compromises among these functions such as the major fitness functions of growth, reproduction investment and locomotor activity (Guderley and Pörtner 2010).

In feeding developmental stages, the metabolic cost of growth is high, for instance for the sea urchin, *Lytechinus pictus* up to 75 % of the ATP pool is allocated to protein synthesis during growth (Pace and Manahan 2006). Studies of metabolic rate and ion regulation responses to environmental changes such as OA may result in increased metabolic costs to maintain homeostasis and thus decreases the scope for growth (Stumpp et al. 2011; Matson et al. 2012; Stumpp et al. 2012; Pedersen et al. 2014). For example, the sea urchin, *Strongylocentrotus purpuratus* larvae increased the metabolic rates of 100 % under elevated $pCO_2$, and spent 39 – 45 % of the available energy for somatic growth compared to the control larvae that allocated 78 - 80 %, resulting in a decreased scope for growth under hypercapnia (Stumpp et al. 2011). Although no change in metabolic rate for the sea urchin was found in this study, there were

more active larvae (swimming) in the OA treatment. This response to OA could point towards compensatory feeding, possibly to indicate higher metabolic costs. Therefore, reduced allocation of energy to growth could explain the 9 % reduction in growth for larvae in this treatment, similar to what was found by Stumpp et al. (2011).

Comparable increased metabolic costs to maintain homeostasis has also been reported for invertebrate larvae exposed to elevated temperature (Styf 2014). Furthermore, impairment of growth and smaller larvae as a result of increased metabolic cost due to warming has been found for larval European lobster, *Homarus gammarus* and sea stars, *Meridiastra calcar* (Nguyen et al. 2012; Small 2013). Many species already exhibit smaller sizes as a response to global change (Sheridan and Bickford 2011); following the functional ecological and metabolic rules, other species will also likely shrink in response to global change. Sheridan and Brickford (2011) suggest two ecological factors to be the most important in explaining the shrinking: nutrient limitation and changes in ectotherm metabolic rate. Furthermore, they suggest that a projected OW of about 1.1 - 6.4 ºC in 2100 (IPCC 2014) will result in a rise in metabolic rate for ectotherms by 10 – 75 %.

Shrimp larvae examined in the current study seem to respond to OW the same way as larvae of European lobster and sea stars with increased metabolic rates and increased activity at the cost of reduction in size. Activity, metabolic rate and feeding rate were not investigated for krill larvae in current study. However, at the end of the experiment, the length of the krill larvae was significantly smaller in the OAW treatment than in the control treatment for this species, suggesting that the same argument may apply for the krill larvae. Additional costs for acid-base regulation and /or thermal stress due to exposure for OA and OW, affects energy budget of the larvae in this study and as a consequence of this, increased costs for maintenance and homeostasis. The larvae could compensate for these increased costs, with higher food intake to meet the energy demand. Larvae in this study had excess of food and they increased their

feeding rates due to exposure to the global drivers. Nonetheless, it seemed that the increased feeding was not enough to meet their metabolic demand. Increases in metabolic demand due to OW or OA should be reflected in subsequent increases in trophic impacts of predators on prey. However, it seems like the metabolic demand was not met and therefore growth was decreased. The inability of some species to meet their metabolic demand at higher temperatures have also been reported by Twomey et al. (2012), and it seems like increases in feeding rates with OW and OA is generally weaker than those of respiration (Stumpp et al. 2011; Vucic-Pestic et al. 2011; Twomey et al. 2012).

If the larvae were to be limited with food shortage because of the mismatch with food or decreased feeding due to exposure of other stressors, it could have further implementations on growth and ultimately survival of the species. For example, Pansch et al. (2014) showed that energy availability could mediate the ability of barnacles, *Amphibalanus (Balanus) improvisus* to with stand moderate $pCO_2$. They showed that barnacles could tolerate $pCO_2$ when food was in excess, though under food-limitation barnacles showed reduced growth and development. Furthermore, a recent study showed that there is important additive to synergistic effects of food concentration and OW on developmental pace of larval sea star, *Acanthaster planci* (Uthicke et al. 2015).

Oil exposure of the larvae in this study affected activity, hence feeding, and therefore the assimilation of energy for the larvae. Less energy assimilated resulted in less energy to be used to maintain homeostasis, at the cost of growth for both shrimp and sea urchin larvae. Reduced feeding activity following exposure to dispersed oil has been shown previously for the copepod, *Calanus finmarchicus* and fish larvae, *Gadus morhua* (Nordtug et al. 2011; Hansen et al. 2012), and the reduction in energy status in shrimp, *Pandalus borealis* (Bechmann et al. 2010) may well be a direct effect of feeding activity.

**In conclusion, the studied drivers affected the energy budget for the larvae resulting in reduced growth. Global drivers (OA and OW) affected the use of energy with more energy reallocated to activity and metabolism resulting in reduced growth. Oil reduced the acquisition of energy by reduced feeding and also resulted in less energy for growth.**

Although the larvae were able to survive, grow and develop in the exposed treatments, increase in larval energy expenditure due to increased metabolic costs caused by the drivers, diverted energy away from growth and development. This could in turn affect larvae as juveniles after metamorphosis, as it has been shown that metamorphosis does not erase embryonic and larval history and it is not a "new beginning" (Pechenik 1999; Pechenik 2006). For example, fewer resources could be available for production of offspring, smaller organisms eat less, and hence reproduce less, and slower growth generally implies a delay in the start of reproduction (Jager 2015). Harmful long-term and transgenerational effects have already been demonstrated to occur in marine invertebrates due to exposure of current global change scenarios and oil exposure (Taban et al. 2007; Dupont et al. 2012; Incardona et al. 2015; Przeslawski et al. 2015; Vehmaa et al. 2015: Rodríguez-Romero et al. 2015). However, this mechanism could also be effective in buffering populations against negative effects of environmental change. For example, for a copepod when exposed to elevated $pCO_2$ beyond present day's natural variability, transgenerational effects set in so copepods were able to avoid further decreases in fecundity (Thor and Dupont 2015). Furthermore, it has also been shown that an organism's flexibility and plasticity is a key mechanism for survival. For instance, it has been reported that arrested or slower growth due to suboptimal environmental or trophic conditions, can be overcome by growth acceleration under more favourable conditions (Arendt 1997; Metcalfe and Monaghan 2001).

## 6.5 SHIFTING PHENOLOGY OR TIMING OF THE DEVELOPMENTAL CYCLES DUE TO EXPOSURE TO SELECTED DRIVERS

Phenology is defined as times of periodic plant and animal life cycle events and how these are influenced by seasonal and interannual variations in climate. In the upper water column of the oceans, seasonality sets much of the environmental variability experienced by organisms, and the range of the environmental conditions is typically greatest at mid and higher latitudes (Ji et al. 2010). To minimize exposure of sensitive life stages to stressful conditions, many organisms have evolved behavioural and life history strategies that exploit favourable periods of the year (those best matching optimal niche requirements) (Koeller et al. 2009; Ji et al. 2010; Varpe 2012). Such adaptations to seasonality have resulted in using particular periods of the year for reproduction, maintenance and growth often coupled with seasonal migrations, reduced activity and extensive energy storage (Varpe 2012).

It is now well established that organisms are responding to the unprecedented rate of global change, including the timing of biological activities and shifting of their phenology (Edwards and Richardson 2004; Burrows et al. 2011; Fly et al. 2015). If these changes are out of synch with the optimal conditions, for example if abundance of a species does not coincide with its food source, a mismatch situation can occur. There has already been reported an example of phenological decoupling due to OW, and these changes are faster than in many terrestrial ecosystems. In a further example, Asch (2015) showed that zooplankton did not change phenology synchronously with most fishes. Asch (2015) found a strong correlation between warmer ocean temperatures and changes in the timing of fish reproduction. Most of the fish species spawned earlier. Other species showed no long-term change in spawning cycles. A few species spawned later. Asch (2015) suggested that fishes that do not change their phenology synchronously with zooplankton might be subjected to mismatches with prey, potentially leading to reduced recruitment to fisheries. Furthermore, the seasonal peak timing of the

meroplankton has been shown to occur already 27 d earlier due to the North Atlantic warming over the last 45 years (Edwards and Richardson 2004). Alternatively, many long-term phytoplankton studies have observed that the phytoplankton bloom is constant; occurring at the same time each year under variable environmental conditions, suggesting that it is timed according to constant cues such as light (Sommer et al. 2012). OW leads to an increase in metabolic rates, and therefore an increase in energy demand, which will increase the food consumption by herbivores. Interestingly it has already been reported that there are stronger grazing pressures (Paul et al. 2015). Nonetheless, this does not translate into an increase in secondary production, and creates a mismatch with carnivores whose metabolic demand and foraging costs increase with OW and OA (Nagelkerken and Connell 2015).

Evidence suggests that food availability in the field is a critical factor in the development of larval crustaceans, occasionally important in mollusks, although rarely ever important in echinoderms (which may obtain a greater proportion of their nutrition from dissolved organic matter) (Olson and Olson 1989). Given the strong dependency of these larvae upon external food sources, and large seasonality shifts forward in seasonality shown by meroplankton (Edwards and Richardson 2004), it is likely that survival and recruitment of larval crustaceans are vulnerable to food limited situations linked to mismatches (Griffith 2013). Northern shrimp larvae may be particularly vulnerable since they are adapted to local temperatures and timing of the algal blooms, matching egg hatching and food availability under average conditions (Koeller et al. 2009).

Physiological plasticity appears to be key to krill, because juvenile and adult Northern krill are versatile in food choice that may make them less vulnerable to changes in the availability of different food items (Tarling et al. 1999). However, krill larvae could also be vulnerable to mistiming with its food due to shifting phenology. The lipid reserves of the non-feeding krill larvae are being depleted when the larvae approach the feeding C1 stage (Ross and Quetin,

1989). Only a very narrow window remains to initiate feeding, to compensate for the extra costs, when the larvae reaches the C1 stage. If this window is reduced, it could have serious consequences for krill survival (Poulsen et al. 2011). Ross and Quetin (1989) also reported that if initial delays in food avalibility exceeds a certain point (for larvae of Antarctic krill, *Euphausia superba* this was 10 - 14 d), starved larvae cannot recover when refed, and short delays in food affects the timing of development, and after long delays the larvae lost their ability to metamorphose.

For the green sea urchin mismatches with food, due to shifting phenology, does not seem to be a problem, because spawning is induced in response to a chemical released by phytoplanktonic blooming, and therefore the larvae will be present when food is available (Scheibling and Hatcher 2001). However, not only shortage of food but also insufficient food quality can be a limiting factor of invertebrate larval growth and reproduction (Sommer et al. 2012). Reduced light due to "global dimming" (Liepert 2002) and OW have been shown to reduce the cell size of phytoplankton (Sommer et al. 2012; Winder et al. 2012). So despite the fact that larvae can match their food in time and space, the recruitment success can be compromised if the food quality is poorer, or if the food is present only in limited amounts.

**In conclusion both krill and shrimp larvae were able to survive conditions predicted to occur under a future global change scenario, however there were accelerated developmental rates with greater maintenance costs meaning they could possible experience a mismatch between energy supply and demand. In the current study, the species had excess food, and growth was decreased due to higher metabolic demands in the climate exposure treatments. If the larvae were to be limited further with food shortage because of the mismatch with food, it could have further consequences for growth and ultimately survival of the species. Furthermore, smaller sized larvae could be**

**even more vulnerable to starvation, altogether resulting in lower recruitment of the species.**

**Finally, the smaller sized shrimp, krill and sea urchin larvae as secondary producers could possible create a mismatch with their predators whose metabolic demand and foraging costs may also increase with OW and OA.**

## 6.6 THE COMBINED EFFECTS OF MULTIPLE DRIVERS; THE IMPORTANCE OF UNDERSTANDING "THE MODE OF ACTION" OF THE DRIVERS AND MANAGING LOCAL IMPACTS IN THE FUTURE OCEAN

Predicting the impacts of multiple drivers on the ecosystems in the future oceans is a key challenge for global change biology today. The ability to make these predictions is dependent on knowledge on how drivers behave in combination, and the interaction of drivers is often characterised in three different ways: *synergistic, antagonistic* and *additive* as described in Sect. 1.1.5. Since it is difficult to test all possible combinations of drivers in the laboratory, it is important to understand how and why stressors interact (Løkke et al. 2013) and how drivers behave in combination, is strongly influenced by the drivers "mode of action".

Responding to several stressors simultaneously can result in considerable energy expenditures for an organism by involving multiple molecular and cellular signalling pathways (i.e. modes of action) (Breitburg et al. 2015). For example, the way hypoxia and hypercapnia can act on an organism is similar, with extra energy expenditure that is required to cope with these multiple stressors, meaning these can reduce the organisms' resilience to additional challenges (Melzner et al. 2013). Differently there are stressors that with differing modes of action that provide protective mechanisms ("cross-tolerance") to a range of stressors (Sinclair et al. 2013), and stressors that elicit similar response pathways without spending extra energy, that can result in responses less than additive or less severe (Breitburg et al. 2015).

Although the possibilities for interactions between toxicant responses, OW and OA are many, these interactions have been little studied (Nikinmaa 2013). The few studies that have investigated these drivers, have reported both synergistic, antagonistic and additive interactions (Sect. 1.5). In the two experiments described in Chapters 4 and 5 the possibilities for interactions between toxicant responses (short-term oil exposure), OW and OA were assessed. All the drivers had an effect on larvae by reducing and shifting the energy budget (as described in Sect. 6.3) through different modes of action, extra energy costs for global drivers and narcotic effect for oil. Both climate and oil were affecting growth for shrimps and sea urchins in a negative way, and in combination, the drivers were acting additively. The study demonstrated the additive physiological effects of climate variables and a contaminant, and that larval (sea urchin and shrimp) resilience to future changes (i.e. pollution) could be greatly reduced if the larvae is already limited in energy and severely stressed (reduced development) from the reduced climate variables. This shows the importance of an effective management of local drivers such as oil pollution, but also for example, other discharges of contaminants from agriculture, aquaculture and industry and also overfishing, will have within the context of OA and OW. The study also highlights the fact that it is important to study impacts of toxicants, such as an oil-pollution, in the context of predicted changes in the environment, as elevated OW and OA are becoming major concern. Furthermore, the fact that some local and global drivers seem to act additively could empower and encourage local managers to act on local driver regulations, to obtain positive effects on local populations and environment and make them more resilient to the negative impacts of future global drivers.

Drivers that affect organisms may also affect their ecological interactions, including predators and competitors for prey, and symbionts, and there is a wide range of interactive effects that act through ecosystems (Boyd and Brown 2015). The global drivers shifted the larval phenology of the studied species (Sect. 6.4); this could possible also, in turn influence larval trophic

interactions, and may possible lead to trophic cascades if consumers and producers' phenology's become decoupled.

## 6.7 CONCLUSIONS AND PERSPECTIVES

The aim of this thesis was to assess the impact of global and local drivers and their combination on early life stages in three marine invertebrates. A range of effects was observed depending on driver, species, stage of development and tested endpoints. This thesis therefore confirms the importance of investigating a number of different species and multiple successive early life stages before making predictions about the impacts of these drivers on the marine ecosystems. Despite the variability in response, no significant interaction was ever observed between the drivers examined with the exception OA and OW on neutral red retention time (NRRT) in adult shrimp (Sect. 4.4.1.2).

In summary, two major impacts on the larvae were observed when exposed to OA, OW and oil, one was shifts in energy allocation that lead to smaller larvae and the other shifting phenology or timing of the developmental cycles.

The greater energy demand due to exposure to the global drivers and hence the smaller size, could mean that the larvae would be even more vulnerable to potential mismatch with food, due to their shifting of phenology. Few studies on potential mismatches between benthic larval arrival and planktonic food source have been carried out so far, and temporal mismatches between invertebrate species and food source is difficult to test, due to the fact there is limited knowledge of the life cycles of many of the benthic species (Birchenough et al. 2015). However, to investigate mismatch potentials, targeted experiments looking at primary producers and invertebrate consumers with known nutrient loads and solar inputs in relation to global drivers such as OA and OW, will be needed to provide insights on how pelagic and benthic marine ecosystem will respond to the global change, as suggested by Birchenough et al. (2015).

Understanding the link between phenology and the trophic ecology of marine invertebrates is of utmost importance to understand the consequences of global change (Calado and Leal 2015). This because the organisms' response to global change may vary across functional groups and multiple trophic levels, and hence have impacts on food-web structure and ecosystem functioning (Edwards and Richardson 2004). Furthermore, standardized national and international long-time monitoring of both planktonic (phytoplankton and zooplankton) but also macro benthos, since many of the invertebrates have both pelagic and benthic life stages, including climatic data, would be useful to see how phenology of species is changing and if potential mismatches could occur, also suggested by Birchenough et al. (2015). Reports of possible cascading effects of higher tropic levels such as birds and fish resulting from of temporal mismatch, due to climate induced changes has already been reported (Burthe et al. 2012; Jochum et al. 2012) and mismatches in key species such as in this study, being at the at the basis of the food web could have cascading effects throughout the whole marine ecosystem. In addition to this fisheries and hence humans are also be likely to be affected by smaller organisms, the biomass may be reduced, because many people relay on fish and crustaceans as their main source of animal protein.

Furthermore, the thesis shows that global drivers and oil impacts on invertebrate marine larvae reduce the energy budget and shift its allocation through different mode of action (extra energy costs for global drivers and narcotic effect for oil) with no interaction. Consequently, they combine in an additive manner reducing growth. This suggest that larval resilience to local impacts (i.e. local oil pollution) could possibly be significantly reduced in combination with global drivers, probably due to the fact that they are already energetically limited and have increased developmental rate. The study therefore underlines the fact that it is important to study impacts of toxicants such as oil pollution in the context of predicted changes in the environment, and further studies are needed. Finally, the thesis highlights the importance of a

correct management of local drivers such as oil pollution, but also other contaminants such as those from agricultural activities and pharmaceutical product and overfishing, will have within the context of global change.

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**Appendix 2.1**

| Treatment | Control | OA | OW | OAW |
|---|---|---|---|---|
| **Measured** | | | | |
| $[O_2]$ (mg $L^{-1}$) | 9.5 ± 1 | 9.6 ± 1 | 9.4 ± 1 | 9.4 ± 0.9 |
| pH aquaria | 8.11 (8.08-8.13) | 7.65 (7.63-7.68) | 8.08 (8.05-8.11) | 7.60 (7.56-7.65) |
| Temperature (ºC) [tested in Aquaria] | 6.7 ± 0.2 | 6.7 ± 0.2 | 9.5 ± 0.1 | 9.5 ± 0.1 |
| Salinity | 34 ± 0.9 | | | |
| Total alkalinity(µmol $kg^{-1}$) | 1900-2300 | | | |
| **Calculated** | | | | |
| $pCO_2$ (µatm)* | 337-474 | 1038-1437 | 362-524 | 1147-1751 |
| DIC (µmol $kg^{-1}$)* | 1760-2173 | 1889-2313 | 1753-2169 | 1889-2324 |
| $[HCO_3]$ (µmol $kg^{-1}$)* | 1647-2049 | 1802-2204 | 1638-2043 | 1822-2210 |
| $[CO_3^{2-}]$ (µmol $kg^{-1}$)* | 82-116 | 31-45 | 84-121 | 30-46 |
| $\Omega_{calc}$* | 2.0-2.8 | 0.8-1.1 | 2.0-2.9 | 0.7-1.1 |
| $\Omega_{ara}$* | 1.3-1.8 | 0.5-0.7 | 1.3-1.8 | 0.5-0.7 |

**Seawater physico-chemical parameters measured in the experimental system: oxygen concentration ([$O_2$]), pH (NBS Scale), temperature, salinity, total alkalinity. Other parameters (as indicated by an '*') were calculated using the program CO2SYS (Pierrot et al. 2006), using constants from Mehrbach et al. (1973) refitted to the NBS pH scale by Dickson and Millero (1987) and the KSO4 dissociation constant from Dickson (1990). Mean pH level was calculated based on the actual [H+] concentration and back-calculated to pH. These parameters included: dissolved inorganic carbon (DIC), bicarbonate and carbonate ions concentrations ([$HCO_3^-$] and [$CO_3^{2-}$] respectively), saturation status for calcite and aragonite (Ωcalc and Ωara respectively). For all measured parameters (with the exception of total alkalinity) mean values (±SD) were provided, whilst for total alkalinity and all calculated parameters range of values were provided. Finally, mean pH level was calculated based on the actual [H+] concentration and back-calculated to pH.**

**Appendix 3.1**

| Treatment | Control | OAW |
|---|---|---|
| *Measured* $[O_2]$ (mg $L^{-1}$) | 8.7 ± 0.2 | 8.6 ± 0.1 |
| $pH_{NBS}$ (range pH) | 8.06 (8.03-8.08) | 7.62 (7.56-7.68) |
| ºC aquaria | 6.9 ± 0.27 | 9.7± 0.20 |
| Salinity | 33.13 ± 0.43 | |
| Total alkalinity (µmol $kg^{-1}$) | 2306.3 ± 10.5 | 2303.3 ± 14.5 |
| *Calculated* $pCO_2$(µatm) | 511 (470-537) | 1627 (1354-1795) |
| DIC (µmol $kg^{-1}$) | 2185 (2180-2189) | 2206 (2199-2209) |
| $[HCO_3]$(µmol $kg^{-1}$) | 2065 (2058-2070) | 2199 (2177-2206) |
| $[CO_3^{2-}]$(µmol $kg^{-1}$) | 95.6 (89.3-103) | 39.1 (34.8-46.9) |
| $\Omega_{calcite}$ | 2.30 (2.16-2.47) | 0.94 (0.84-1.13) |
| $\Omega_{aragonite}$ | 1.45 (1.36-1.56) | 0.60 (0.53-0.71) |

**Seawater physico-chemical parameters measured in the experimental system: oxygen concentration ($[O_2]$), pH (NBS Scale), temperature, salinity and total alkalinity. Other parameters were calculated using the program CO2SYS (Pierrot et al. 2006), using constants from Mehrbach et al. (1973) refitted to the NBS pH scale by Dickson and Millero (1987) and the KSO4 dissociation constant from Dickson (1990). Mean pH level was calculated based on the actual [H+] concentration and back calculated to pH. These parameters included: dissolved inorganic carbon (DIC), bicarbonate and carbonate ions concentrations ($[HCO_3^-]$ and $[CO_3^{2-}]$ respectively), saturation status for calcite and aragonite (Ωcalc and Ωara respectively). For all measured parameters mean values (± SD) were provided, whilst for total alkalinity and all calculated parameters range of values were provided.**

## Appendix 3.2

| | | Initial larvae number | Hatch rate | Unhatched living eggs | Living larvae | Dead larvae | Dissolved larvae/disappeared larvae | Number of samples |
|---|---|---|---|---|---|---|---|---|
| **Day 7** | | | | | | | | |
| | Control | 90 | 42.2 ± 5.1 | 48.3 ± 1.5 | 38 ± 4.6 | 0 | 4 | 3 |
| | OAW | 90 | 36.3 ± 14.5 | 24.3 ± 9.8 | 32.7 ± 13.1 | 1 | 32 | 3 |
| **Day 14** | | | | | | | | |
| | Control | 90 | | - | 29.7 ± 2.1 | 1.6 ± 1.1 | 55 | 3 |
| | OAW | 90 | | - | 18 ± 2 | 1.6 ± 0.6 | 38 | 3 |
| **Day 22** | | | | | | | | |
| | Control | 90 | | - | 25 ± 4 | 2.6 ± 2.3 | 4 | 3 |
| | OAW | 90 | | - | 3 ± 1 | 1.3 ± 0.6 | 15 | 3 |
| **Day 29** | | | | | | | | |
| | Control | 90 | | - | 8.3 ± 1.5 | 2 ± 1.7 | 17 | 3 |
| | OAW | 90 | | - | - | | | 3 |

**Hatching success (%), unhatched eggs (%), number of living larvae and dead larvae for the Northern krill *M. norvegica* for the current and future OAW conditions at sampling d 7, 14, 22, and 29.**

## Appendix 4.1

| Treatment | Control | OAW | Control + oil | OAW + oil |
|---|---|---|---|---|
| *Measured* $[O_2]$ (mg $L^{-1}$) | 8.5 ± 0.1 | 8.6 ± 0.1 | 8.6 ± 0.2 | 8.6 ± 0.1 |
| $pH_{NBS}$ (range pH) | 8.05 (8.03-8.08) | 7.59 (7.55-7.67) | 8.05 (8.03-8.08) | 7.60 (7.56-7.67) |
| ºC aquaria | 6.7± 0.08 | 9.5± 0.07 | 6.7 ± 0.07 | 9.5 ± 0.06 |
| Salinity | 33.13 ± 0.43 | | | |
| Total alkalinity (µmol $kg^{-1}$) | 2305 ± 12 | | | |
| *Calculated* $pCO_2$(µatm) | 511 (470-537) | 1627 (1354-1795) | 511 (470-537) | 1589 (1349-1753) |
| DIC (µmol $kg^{-1}$) | 2185 (2180-2189) | 2206 (2199-2209) | 2187 (2180-2189) | 2315 (2306-2318) |
| $[HCO_3]$(µmol $kg^{-1}$) | 2065 (2058-2070) | 2199 (2177-2206) | 2065 (2058-2070) | 2204 (2199-2207) |
| $[CO_3^{2-}]$(µmol $kg^{-1}$) | 95.6 (89.3-103) | 39.1 (34.8-46.9) | 95.6 (89.3-103) | 40.0 (35.6-46.4) |
| $\Omega_{calcite}$ | 2.30 (2.16-2.47) | 0.94 (0.84-1.13) | 2.30 (2.16-2.47) | 0.96 (0.86-1.12) |
| $\Omega_{aragonite}$ | 1.45 (1.36-1.56) | 0.60 (0.53-0.71) | 1.45 (1.36-1.56) | 0.61 (0.54-0.71) |

**Seawater physico-chemical parameters measured in the experimental system: oxygen concentration ([O2]), pH (NBS Scale), temperature, salinity and total alkalinity. Other parameters were calculated using the program CO2SYS (Pierrot et al. 2006), using constants from Mehrbach et al. (1973) refitted to the NBS pH scale by Dickson and Millero (1987) and the KSO4 dissociation constant from Dickson (1990). Mean pH level was calculated based on the actual [H+] concentration and back calculated to pH. These parameters included: dissolved inorganic carbon (DIC), bicarbonate and carbonate ions concentrations ([HCO3-] and [CO32-] respectively), saturation status for calcite and aragonite (Ωcalc and Ωara respectively). For all measured parameters mean values (± SD) were provided, whilst for total alkalinity and all calculated parameters range of values were provided.**

**Appendix 4.2**

| Chemical (µg $L^{-1}$) | Control | Oil | OAW\Oil |
|---|---|---|---|
| **Naphthalene** | nd | 0.299 ± 0.008 | 0.335 ± 0.025 |
| **C1-Naphthalene** | nd | 1.028 ± 0.015 | 1.143 ± 0.092 |
| **C2-Naphthalene** | nd | 1.785 ± 0.066 | 2.014 ± 0.143 |
| **C3-Naphthalene** | nd | 1.391 ± 0.041 | 1.597 ± 0.088 |
| **Sum 2 ring PAH** | - | 4.503 | 5.089 |
| **Fluorene** | nd | 0.025 ± 0.001 | 0.026 ± 0.001 |
| **Phenanthrene** | nd | 0.060 ± 0.004 | 0.068 ±0.007 |
| **C1-Phen/Anthr** | nd | 0.107 ± 0.004 | 0.122 ± 0.015 |
| **C2-Phen/Anthr** | nd | 0.123 ± 0.005 | 0.146 ± 0.009 |
| **Sum 3 ring PAH** | - | 0.315 | 0.362 |
| **Dibenzothiophene** | nd | 0.010 ± 0.001 | 0.011 ± 0.001 |
| **C1-Dibenzothiophene** | nd | 0.028 ± 0.002 | 0.035 ± 0.0053 |
| **C2-Dibenzothiophene** | nd | 0.029 ± 0.001 | 0.036 ± 0.004 |
| **Sum DBTs** | - | 0.067 | 0.080 |
| **Total PAHs** | - | 4.895 | 5.545 |

**Mean concentration (± SD) of PAHs (µg $L^{-1}$) in water samples from the current and future oil exposed aquaria. The samples were taken during the experiment (N = 2) for each treatment. PAH analyses of seawater were preformed by Gas Chromatography (HP5890, Hewlett Packard, USA) and analysed in ion mode (GC/MS – SIM) as described previously in Jonsson, Bechmann et al. (2004). nd = not detected.**

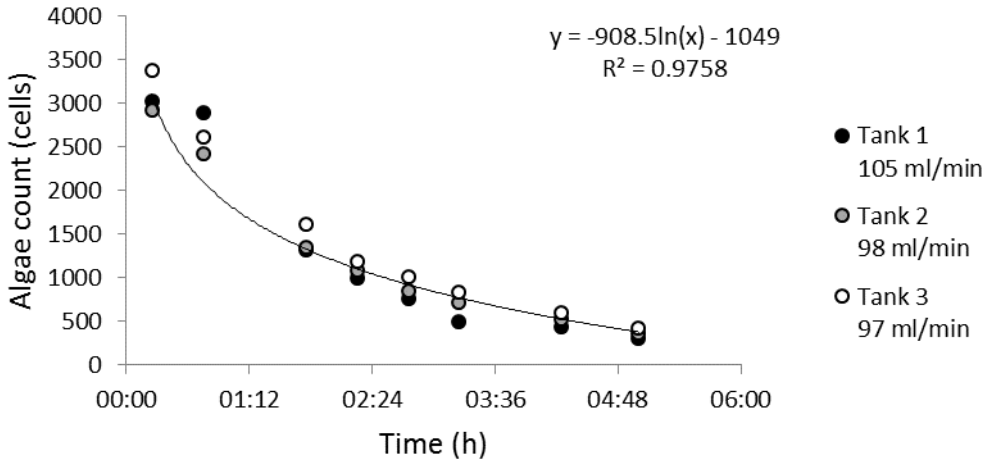
## Appendix 4.3

| Treatment | Current | Current + oil | Future | Future + oil |
|---|---|---|---|---|
| Feeding (Adult) | | | | |
| (mg wet mass fish $ind^{-1}$) | (N=8) | (N=3) | (N=8) | (N=3) |
| Day 6 | 382.95 ± 55.22 | 439.23 ± 16.81 | 358.59 ±169 | 452.03 ± 71.07 |
| Day 14 | 319.04±131.25 | 318.78 ± 33.45 | 316.24 ± 150.07 | 270.68 ± 43.09 |
| Day 18 | 313.17±129.17 | 290.57±254.60 | 245.50 ± 36.59 | 315.61±266.81 |
| Oxygen consumption | | | | |
| (mg $O_2$ $h^{-1}mg^{-1}$ dw) | (N=8) | (N=6) | (N=9) | (N=9) |
| | 0.096 ± 0.029 | 0.096 ± 0.031 | 0.098 ± 0.032 | 0.105 ±0.023 |
| DNA damage in embryo | | | | |
| (Florescence) | (N=61) | (N=63) | (N=71) | (N=66) |
| | 49.68 ± 44.94 | 57.37 ± 38.88 | 48.33 ± 39.68 | 43.21 ± 41.68 |
| | (N=3) | (N=3) | (N=3) | (N=3) |
| Hatching success (%) | 99.11 ± 0.86 | 99.58±0.36 | 98.83 ± 1.31 | 98.61 ± 0.82 |
| | (N=3) | (N=3) | (N=3) | (N=3) |
| First hatching day (d) | 44 ± 8.88 | 44 ± 8.74 | 37 ± 3.00 | 31 ± 3.22 |
| Duration of | (N=3) | (N=3) | (N=3) | (N=3) |
| hatching period (d) | 11.33 ± 2.30 | 11 ± 3.61 | 9 ± 3.46 | 8.33 ± 0.58 |
| Feeding (Larvae) | (N=6) | (N=6) | (N=6) | (N=6) |
| (Number of *Artemia* eaten $larvae^{-1}$ $h^{-1}$) | | | | |
| Stage I-II | 1.36 ± 0.93 | 0.42 ± 0.40 | 1.53 ± 0.19 | 0.81 ± 0.60 |
| Stage III | 2.83 ± 0.97 | 1.87 ± 1.29 | 3.63 ± 0.69 | 2.33 ± 0.98 |
| Stage IV | 2.85 ± 1.04 | 2.55 ± 1.36 | 3.48 ± 0.52 | 2.67 ± 0.56 |
| Swimming | | | | |
| Simple test (%) | | | | |
| Stage I | 74 ± 21.6 | 42 ± 22.0 | | |
| Stage II | | | 60 ± 19.0 | 60 ± 15.0 |
| Advanced test (%) | | | | |

| | | | | |
|---|---|---|---|---|
| Stage I | 14 ± 18.0 | 10 ± 14.0 | | |
| Stage II | | | 4 ± 6.0 | 6 ± 4.0 |

**Feeding (adult), oxygen consumption (adult), hatching success, first hatching day, hatching period, feeding (larvae) and swimming behavior for P. borealis Krøyer 1838 adult females and larvae for the different treatments. N= number of samples investigated.**

## Appendix 5.1




**The decrease in concentration of algae in the flow-through aquaria without sea urchin larvae present. 150 µg carbon/L *Rhodomonas* sp. was added to the aquaria (N = 3 aquaria). The decrease in algal concentration in the aquaria with time was measured by coulter counter. Samples were taken directly from the aquaria (not from the outlets). Figure S1 show the gradual decrease in density with time. After five hours there was almost no algae left in the aquaria.**

**Appendix 5.2**

| | | | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|---|---|
| **Chemical (µg $L^{-1}$)** | Control (N = 1) | OA (N=1) | Control + oil (N = 4) | OA + oil (N = 4) | Control + oil (N = 3) | OA + oil (N = 3) |
| **Naphthalene** | nd | nd | 0,435 ± 0,0295 | 0.450 ± 0.0566 | 0.445 ± 0.1595 | 0.338 ± 0.0347 |
| **C1-Naphthalene** | 0.0015 | 0.0021 | 0.493 ± 0.0361 | 0.539 ± 0.0682 | 0.523 ± 0.1869 | 0.3867 ± 0.0396 |
| **C2-Naphthalene** | nd | nd | 1.698 ± 0.1466 | 1.916 ± 0.2808 | 1.797 ± 0.6419 | 1.3212 ± 0.1224 |
| **C3-Naphthalene** | nd | nd | 1.4279 ± 0.1517 | 1.692 ± 0.4612 | 1.468 ± 0.5019 | 1.0642 ± 0.0969 |
| **Sum 2 ring PAH** | 0.0015 | 0.0021 | 4.054 | 4.616 | 4.233 | 3.111 |
| **Fluorene** | nd | nd | 0.039 ± 0.0040 | 0.040 ± 0.0068 | 0.046 ±0.0167 | 0.032 ± 0.003 |
| **Phenanthrene** | nd | nd | 0.090 ± 0.0086 | 0.095 ± 0.0070 | 0.103 ± 0.0388 | 0.074 ±0.004 |
| **C1-Phen/Anthr** | nd | nd | 0.139 ± 0.0143 | 0.167 ± 0.0429 | 0.166 ± 0.0580 | 0.118 ± 0.0068 |
| **C2-Phen/Anthr** | nd | nd | 0.164 ± 0.0201 | 0.234 ± 0.136 | 0.203 ± 0.006 | 0.150 ± 0.0138 |
| **Sum 3 ring PAH** | 0 | 0 | 0.463 | 0.582 | 0.561 | 0.406 |
| **Dibenzothiophene** | 0.0011 | 0.0004 | 0.016 ± 0.0014 | 0.017 ± 0.0023 | 0.018 ± 0.0077 | 0.014 ± 0.0006 |
| **C1-Dibenzothiophene** | nd | nd | 0.038 ± 0.007 | 0.058 ± 0.0286 | 0.047 ± 0.0164 | 0.035 ± 0.0053 |
| **C2-Dibenzothiophene** | nd | nd | 0.014 ± 0.0016 | 0.023 ± 0.0198 | 0.216 ± 0.0083 | 0.016 ± 0.0029 |
| **Sum DBTs** | 0.0011 | 0.0004 | 0.126 | 0.192 | 0.179 | 0.124 |
| **Total PAHs** | 0.0026 | 0.0025 | 4.628 | 5.340 | 4.944 | 3.620 |

**Mean concentration (± SD) of PAHs (µg $L^{-1}$) in water samples from the control and oil exposed tanks. Samples were taken during the experiment (N = 3) for each treatment. PAH analyses of seawater were preformed by Gas Chromatography (HP5890, Hewlett Packard, USA) and analysed in ion mode (GC/MS – SIM) as described previously in Jonsson, Bechmann et al. (2004).**

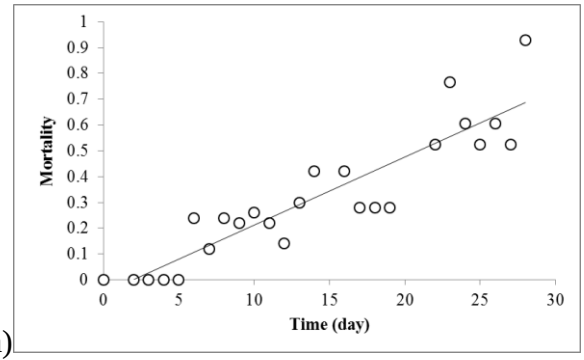
**Appendix 5.3**

| | Measured | | Calculated | | | |
|---|---|---|---|---|---|---|
| Treatment | $pH_{nbs}$ (range) | T(°C) | TA(µmol $kg^{-1}$) | $pCO_2$(µatm) | $\Omega_{calcite}$ | $\Omega_{aragonite}$ |
| Experiment 1 | | | | | | |
| Control | 8.03 (7.98 - 8.08) | 9.41 (± 0.03) | 2306 ± 14 | 555 (489 - 634) | 2.36 (2.07 -2.68) | 1.50 (1.31 – 1.70) |
| OA | 7.64 (7.59 - 7.68) | 9.41 (± 0.03) | 2306 ± 14 | 1455 (1323 – 1646) | 1.03 (0.89 -1.15) | 0.65 (0.56 – 0.73) |
| Control + oil | 8.06 (8.03 - 8.09) | 9.40 (± 0.11) | 2306 ± 14 | 515 (476 – 559) | 2.51 (2.30 – 2.74) | 1.59 (1.45 - 1.73) |
| OA + oil | 7.64 (7.61 - 7.68) | 9.52 (± 0.08) | 2306 ± 14 | 1455 (1323 -1569) | 1.03 (0.93 – 1.15) | 0.65 (0.59 – 0.73) |
| Experiment 2 | | | | | | |
| Control | 8.02 (7.98 - 8.07) | 9.45 ± 0.06 | 2306 ± 14 | 569 (502 - 634) | 2.32 (2.07 – 2.63) | 1.46 (1.31 – 1.67) |
| OA | 7.63 (7.59 – 7.68) | 9.43 ± 0.05 | 2306 ± 14 | 1485 (1318 – 1640) | 1.01 (0.89 – 1.15) | 0.64 (0.56 – 0.73) |
| Control + oil | 8.03 (7.99 - 8.07) | 9.45 ± 0.09 | 2306 ± 14 | 555 (501 – 618) | 2.36 (2.11 -2.63) | 1.50 (1.33 – 1.67) |
| OA + oil | 7.64 (7.59 – 7.69) | 9.49 ± 0.09 | 2306 ± 14 | 1455 (1292 – 1646) | 1.03 (0.89 – 1.18) | 0.65 (0.56 – 0.75) |

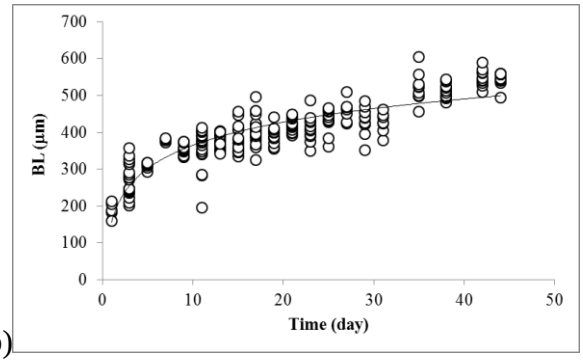
**Seawater physico-chemical parameters measured in the experimental system: oxygen concentration ([O2]), pH (NBS Scale), temperature, salinity, total alkalinity. Other parameters (as indicated by an '*') were calculated using the program CO2SYS (Pierrot et al. 2006), using constants from Mehrbach et al. (1973) refitted to the NBS pH scale by Dickson and Millero (1987) and the KSO4 dissociation constant from Dickson (1990). Mean pH level was calculated based on the actual [H+] concentration and back-calculated to pH. These parameters included: dissolved inorganic carbon (DIC), bicarbonate and carbonate ions concentrations ($[HCO_3^-]$ and $[CO_3^{2-}]$ respectively), saturation status for calcite and aragonite (Ωcalc and Ωara respectively). For all measured parameters mean values (±SD) were provided, whilst for total alkalinity and all calculated parameters range of values were provided. Finally, mean pH level was calculated based on the actual [H+] concentration and back-calculated to pH.**

## Appendix 5.4

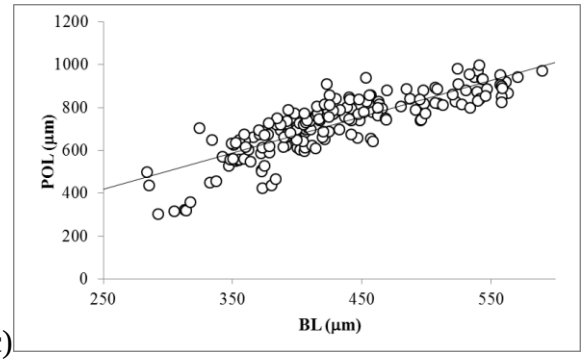
a)



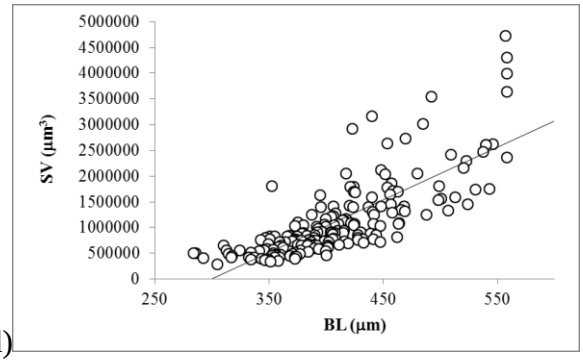

b)



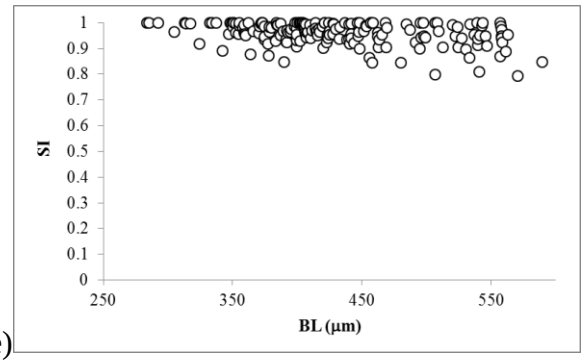

c)




d)




e)




**Examples of relationships used for calculation of the different rates (control, no oil, replicate 3):a. linear relationship between mortality (%) and time ($d^{-1}$), (b) logarithmic relationship between the body length (BL, µm) and time ($d^{-1}$), (c) linear relationship between the postero-lateral arm (POL, µm) and the body length (BL, µm), (d) linear relationship between stomach volume (SV, µm3) and body length (BL, µm), (e) no relationship between symmetry Index (SI) and body length (BL).**

**Appendix 5.5**

| Treat | Replicate | MR | Intercept | p-value | $R^2$ | F-value | df |
|---|---|---|---|---|---|---|---|
| Control | 1 | 1.53 | 0.25 | 0.0012 | 0.54 | 16.29 | 15 |
| | **2** | **0.95** | **0.05** | **0.23** | **0.10** | **1.60** | **15** |
| | 3 | 2.50 | -0.035 | <0.0001 | 0.75 | 41.09 | 15 |
| | 4 | 2.41 | 0.18 | 0.008 | 0.41 | 9.67 | 15 |
| | **5** | **0.98** | **0.074** | **0.16** | **0.14** | **2.19** | **15** |
| | 6 | 2.63 | -0.035 | <0.0001 | 0.75 | 43.12 | 15 |
| OA | 1 | 2.24 | 0.079 | 0.004 | 0.46 | 11.80 | 15 |
| | 2 | 2.96 | -0.0072 | <0.0001 | 0.67 | 28.86 | 15 |
| | 3 | 1.90 | -0.0080 | 0.0013 | 0.53 | 15.94 | 15 |
| | 4 | 2.11 | 0.013 | 0.0026 | 0.49 | 13.38 | 15 |
| | 5 | 1.32 | 0.11 | 0.021 | 0.33 | 6.77 | 15 |
| | **6** | **1.23** | **0.14** | **0.19** | **0.13** | **2.00** | **14** |
| Control + oil | 1 | 7.08 | -0.12 | <0.0001 | 0.96 | 304.73 | 15 |
| | 2 | 3.92 | -0.033 | <0.0001 | 0.67 | 27.88 | 15 |
| | 3 | 7.22 | -0.16 | <0.0001 | 0.92 | 157.46 | 15 |
| | 4 | 5.00 | -0.079 | <0.0001 | 0.84 | 73.67 | 15 |
| | 5 | 4.43 | -0.075 | <0.0001 | 0.75 | 41.54 | 15 |
| | 6 | 4.84 | 0.071 | 0.0072 | 0.41 | 9.88 | 15 |
| OA+ oil | 1 | 4.32 | -0.018 | <0.0001 | 0.83 | 68.34 | 15 |
| | 2 | 6.40 | -0.10 | <0.0001 | 0.87 | 90.79 | 15 |
| | 3 | 6.43 | 0.037 | <0.0001 | 0.87 | 96.99 | 15 |
| | 4 | 7.02 | -0.11 | <0.0001 | 0.87 | 96.01 | 15 |
| | 5 | 4.21 | -0.12 | 0.0002 | 0.65 | 25.88 | 15 |
| | 6 | 4.41 | 0.014 | 0.0002 | 0.66 | 25.26 | 14 |

**Larval mortality rate (MR in % $d^{-1}$) for each treatment was calculated as the coefficient of the significant linear relationship between mortality and tpf. Results of the regressions (Intercept, *p* - value, $R^2$, F - value and df: degree of freedom) are given for each aquaria replicate with corresponding treatment. Data in bold (*p* - value > 0.05) were removed from subsequent analyses.**

**Appendix 5.6**

| Treat | Replicate | BL GR | Intercept | p-value | $R^2$ | F-value | df |
|---|---|---|---|---|---|---|---|
| Control | 1 | 77.99 | 185.55 | <0.0001 | 79.56 | 463.21 | 120 |
| | 2 | 74.65 | 186.59 | <0.0001 | 81.71 | 558.53 | 126 |
| | 3 | 74.78 | 188.47 | <0.0001 | 69.91 | 297.40 | 129 |
| | 4 | 73.59 | 185.84 | <0.0001 | 79.22 | 381.27 | 101 |
| | 5 | 76.88 | 190.52 | <0.0001 | 73.03 | 362.85 | 135 |
| | 6 | 78.90 | 183.32 | <0.0001 | 76.19 | 393.56 | 124 |
| OA | 1 | 72.22 | 187.50 | <0.0001 | 55.63 | 151.70 | 122 |
| | 2 | 78.67 | 173.23 | <0.0001 | 74.68 | 377.54 | 129 |
| | 3 | 76.11 | 177.15 | <0.0001 | 78.27 | 360.12 | 101 |
| | 4 | 65.73 | 193.24 | <0.0001 | 76.56 | 382.24 | 118 |
| | 5 | 67.39 | 181.48 | <0.0001 | 84.58 | 685.55 | 126 |
| | 6 | 77.52 | 171.24 | <0.0001 | 80.35 | 363.89 | 90 |
| Control + oil | 1 | 66.49 | 188.23 | <0.0001 | 82.02 | 200.78 | 45 |
| | 2 | 66.86 | 197.32 | <0.0001 | 63.37 | 95.14 | 56 |
| | 3 | 71.93 | 178.05 | <0.0001 | 87.30 | 625.57 | 92 |
| | 4 | 67.95 | 181.72 | <0.0001 | 0.82 | 346.42 | 78 |
| | 5 | 65.89 | 205.69 | <0.0001 | 0.72 | 320.72 | 128 |
| | 6 | 77.07 | 167.58 | <0.0001 | 0.81 | 524.53 | 126 |
| OA + oil | 1 | 64.49 | 169.18 | <0.0001 | 66.57 | 161.28 | 82 |
| | 2 | 63.26 | 178.59 | <0.0001 | 76.67 | 207.02 | 64 |
| | 3 | 60.79 | 189.84 | <0.0001 | 65.00 | 183.87 | 100 |
| | 4 | 70.29 | 169.85 | <0.0001 | 88.25 | 202.78 | 28 |
| | 5 | 55.60 | 198.96 | <0.0001 | 65.33 | 131.88 | 71 |
| | 6 | 63.87 | 187.16 | <0.0001 | 70.71 | 255.96 | 107 |

**Body length growth rate (BL GR in µm ln $(d)^{-1}$) for each treatment was calculated as the coefficient of the significant logarithmic relationship between BL and tpf. Results of the regressions (Intercept, p - value, $R^2$, F - value and df: degree of freedom) are given for each aquaria replicate with corresponding treatment.**

**Appendix 5.7**

| Treat | Replicate | POL GR | Intercept | p-value | $R^2$ | F-value | df |
|---|---|---|---|---|---|---|---|
| Control | 1 | 2.08 | -248.98 | <0.0001 | 47.08 | 67.61 | 77 |
| | 2 | 1.82 | -146.75 | <0.0001 | 63.99 | 113.70 | 65 |
| | 3 | 1.76 | -71.06 | <0.0001 | 52.08 | 73.90 | 69 |
| | 4 | 0.69 | 280.98 | 0.0007 | 27.02 | 13.70 | 38 |
| | 5 | 2.13 | -206.69 | <0.0001 | 52.81 | 78.33 | 71 |
| | 6 | 1.73 | -71.40 | <0.0001 | 60.59 | 86.10 | 57 |
| OA | 1 | 1.75 | -89.19 | <0.0001 | 64.13 | 105.50 | 60 |
| | 2 | 1.46 | 30.70 | <0.0001 | 45.24 | 56.19 | 69 |
| | 3 | 1.42 | 51.49 | <0.0001 | 25.12 | 17.78 | 54 |
| | 4 | 0.81 | 289.18 | <0.0001 | 20.19 | 20.19 | 37 |
| | 5 | 1.50 | 20.84 | <0.0001 | 25.66 | 19.33 | 57 |
| | 6 | 1.94 | -196.58 | <0.0001 | 61.67 | 61.14 | 39 |
| Control + oil | 1 | 2.24 | -334.98 | <0.0001 | 81.98 | 100.06 | 23 |
| | 2 | 1.48 | -94.90 | <0.0001 | 57.12 | 29.31 | 23 |
| | 3 | 2.14 | -256.58 | <0.0001 | 67.94 | 97.48 | 47 |
| | 4 | 1.51 | -91.51 | 0.0003 | 33.16 | 16.38 | 34 |
| | 5 | 1.98 | -235.92 | <0.0001 | 46.89 | 72.41 | 83 |
| | 6 | 1.41 | -30.70 | <0.0001 | 65.11 | 119.45 | 65 |
| OA + oil | 1 | 1.06 | 108.78 | <0.0001 | 43.21 | 31.96 | 43 |
| | 2 | 1.65 | -103.18 | <0.0001 | 79.91 | 83.56 | 22 |
| | 3 | 1.72 | -155.32 | <0.0001 | 77.02 | 167.55 | 51 |
| | 4 | . | . | . | . | . | . |
| | 5 | 1.04 | 81.72 | 0.0026 | 25.66 | 10.70 | 32 |
| | 6 | 1.84 | -181.63 | <0.0001 | 51.21 | 65.08 | 63 |

**Postero - lateral arm growth rate (POL GR in $\mu m^3\ \mu m^{-1}_{BL}$) for each treatment was calculated as the coefficient of the significant linear relationship between POL (µm) and BL (µm). Results of the regressions (Intercept, p - value, $R^2$, F - value and df: degree of freedom) are given for each aquaria replicate with corresponding treatment.**

**Appendix 5.8**

| Treat | Replicate | SV GR | Intercept | p-value | $R^2$ | F-value | df |
|---|---|---|---|---|---|---|---|
| Control | 1 | 4454 | -860315 | <0.0001 | 26.57 | 35.82 | 100 |
| | 2 | 6122 | -1484280 | <0.0001 | 39.74 | 54.07 | 83 |
| | 3 | 6452 | -1652105 | <0.0001 | 47.60 | 83.56 | 93 |
| | 4 | 9064 | -2532556 | <0.0001 | 53.95 | 72.63 | 63 |
| | **5** | **5915** | **-1279603** | **0.059** | **3.47** | **3.64** | **102** |
| | 6 | . | . | . | . | . | . |
| OA | 1 | 10085 | -2791197 | <0.0001 | 60.64 | 129.44 | 85 |
| | 2 | 11992 | -3654478 | <0.0001 | 56.93 | 128.20 | 98 |
| | **3** | 13321 | -3832420 | 0.014 | 8.88 | 6.43 | 67 |
| | 4 | 3765 | -1657015 | <0.0001 | 38.13 | 44.99 | 74 |
| | 5 | 7650 | -1910608 | <0.0001 | 32.75 | 45.29 | 94 |
| | 6 | 6232 | -1436657 | <0.0001 | 41.13 | 46.82 | 68 |
| Control + oil | **1** | **2282** | **-251862** | **0.17** | **7.72** | **2.01** | **25** |
| | 2 | . | . | . | . | . | . |
| | 3 | 3650 | -724636 | 0.0002 | 17.57 | 15.34 | 73 |
| | 4 | 4580 | -1051041 | <0.0001 | 35.61 | 26.55 | 49 |
| | 5 | 4914 | -1144175 | <0.0001 | 32.19 | 51.27 | 109 |
| | 6 | 5114 | -1253188 | <0.0001 | 37.59 | 48.19 | 81 |
| OA + oil | 1 | . | . | . | . | . | . |
| | 2 | 13110 | -3915367 | <0.0001 | 69.61 | 96.20 | 43 |
| | 3 | 6423 | -1607604 | <0.0001 | 59.95 | 113.78 | 77 |
| | 4 | . | . | . | . | . | . |
| | 5 | . | . | . | . | . | . |
| | 6 | 4606 | -988761 | <0.0001 | 34.89 | 43.94 | 83 |

**Stomach volume growth rate (SV GR in $\mu m^3 \mu m^{-1}_{BL}$) for each treatment was calculated as the coefficient of the significant linear relationship between SV ($\mu m^{3)}$ and BL (µm). Results of the regressions (Intercept, p - value, $R^2$, F - value and df: degree of freedom) are given for each aquaria replicate with corresponding treatment. Data in bold (p - value > 0.05) were removed from subsequent analyses.**

**Appendix 5.9**

| Treat | Replicate | MR | Intercept | p-value | $R^2$ | F-value | df |
|---|---|---|---|---|---|---|---|
| Control | 1 | 1.1 | 0.05 | 0.0002 | 0.46 | 19.66 | 24 |
| | **2** | **0.16** | **0.10** | **0.61** | **0.012** | **0.27** | **24** |
| | 3 | 2.6 | -0.053 | <0.0001 | 0.82 | 108.22 | 24 |
| | 4 | 2.3 | 0.18 | <0.0001 | 0.64 | 40.33 | 24 |
| | 5 | 2.3 | -0.035 | <0.0001 | 0.63 | 38.72 | 24 |
| | 6 | 2.1 | 0.0026 | <0.0001 | 0.83 | 114.39 | 24 |
| OA | 1 | 2.6 | 0.051 | <0.0001 | 0.78 | 79.44 | 24 |
| | 2 | 2.9 | -0.002 | <0.0001 | 0.83 | 108.80 | 24 |
| | 3 | 2.4 | -0.058 | <0.0001 | 0.75 | 70.77 | 24 |
| | 4 | 1.9 | 0.033 | <0.0001 | 0.57 | 30.55 | 24 |
| | 5 | 0.79 | 0.15 | 0.0025 | 0.33 | 11.54 | 24 |
| | 6 | 2.2 | 0.076 | <0.0001 | 0.74 | 58.79 | 24 |
| Control + oil | 1 | 1.4 | 0.030 | <0.0001 | 0.71 | 55.00 | 24 |
| | **2** | **0.069** | **0.11** | **0.83** | **0.0020** | **0.05** | **24** |
| | 3 | 2.4 | -0.040 | <0.0001 | 0.80 | 93.17 | 24 |
| | 4 | 1.8 | 0.22 | <0.0001 | 0.51 | 23.78 | 24 |
| | 5 | 2.8 | -0.077 | <0.0001 | 0.66 | 44.40 | 24 |
| | 6 | 1.7 | 0.030 | <0.0001 | 0.68 | 48.85 | 24 |
| OA + oil | 1 | 2.8 | 0.028 | <0.0001 | 0.75 | 68.82 | 24 |
| | 2 | 2.6 | 0.020 | <0.0001 | 0.84 | 125.07 | 24 |
| | 3 | 3.2 | -0.12 | <0.0001 | 0.77 | 76.16 | 24 |
| | 4 | 1.5 | 0.062 | <0.0001 | 0.63 | 38.70 | 24 |
| | 5 | 0.89 | 0.14 | 0.0007 | 0.40 | 15.38 | 24 |
| | 6 | 1.6 | 0.11 | <0.0001 | 0.54 | 27.33 | 24 |

**Larval mortality rate (MR in % $d^{-1}$) for each treatment was calculated as the coefficient of the significant linear relationship between mortality and tpf. Results of the regressions (Intercept, *p* - value, $R^2$, F - value and df: degree of freedom) are given for each aquaria replicate with corresponding treatment. Data in bold (*p* - value > 0.05) were removed from subsequent analyses.**

**Appendix 5.10**

| Treat | Replicate | BL GR | Intercept | p-value | $R^2$ | F-value | df |
|---|---|---|---|---|---|---|---|
| Control | 1 | 77.28 | 182.87 | <0.0001 | 77.88 | 904.98 | 258 |
| | 2 | 79.51 | 176.84 | <0.0001 | 83.39 | 1254.82 | 251 |
| | 3 | 90.80 | 155.58 | <0.0001 | 78.07 | 879.31 | 248 |
| | 4 | 79.19 | 190.14 | <0.0001 | 77.93 | 713.60 | 203 |
| | 5 | 79.92 | 183.61 | <0.0001 | 75.44 | 823.41 | 269 |
| | 6 | 82.52 | 176.36 | <0.0001 | 78.92 | 943.58 | 253 |
| OA | 1 | 79.47 | 155.43 | <0.0001 | 71.03 | 625.30 | 256 |
| | 2 | 80.15 | 146.95 | <0.0001 | 71.91 | 708.96 | 278 |
| | 3 | 69.57 | 181.53 | <0.0001 | 73.27 | 644.11 | 236 |
| | 4 | 64.17 | 194.27 | <0.0001 | 78.08 | 887.16 | 250 |
| | 5 | 67.36 | 180.49 | <0.0001 | 78.24 | 945.93 | 264 |
| | 6 | 67.21 | 184.40 | <0.0001 | 74.51 | 646.16 | 222 |
| Control + oil | 1 | 73.18 | 189.78 | <0.0001 | 73.89 | 723.27 | 270 |
| | 2 | 59.62 | 207.01 | <0.0001 | 70.39 | 601.46 | 254 |
| | 3 | 68.99 | 196.34 | <0.0001 | 67.89 | 556.13 | 264 |
| | 4 | 62.75 | 199.48 | <0.0001 | 73.43 | 707.45 | 257 |
| | 5 | 75.96 | 187.64 | <0.0001 | 62.17 | 470.07 | 287 |
| | 6 | 65.20 | 203.00 | <0.0001 | 67.26 | 538.13 | 263 |
| OA + oil | 1 | 68.53 | 190.22 | <0.0001 | 59.19 | 387.17 | 268 |
| | 2 | 68.40 | 185.56 | <0.0001 | 68.39 | 527.86 | 245 |
| | 3 | 65.59 | 187.09 | <0.0001 | 71.27 | 602.68 | 244 |
| | 4 | 71.90 | 179.92 | <0.0001 | 71.56 | 646.76 | 258 |
| | 5 | 56.54 | 198.30 | <0.0001 | 75.19 | 799.93 | 265 |
| | 6 | 55.10 | 206.30 | <0.0001 | 56.79 | 286.54 | 219 |

**Body length growth rate (BL GR in µm ln $(d)^{-1}$) for each treatment was calculated as the coefficient of the significant logarithmic relationship between BL and tpf. Results of the regressions (Intercept, *p* - value, $R^2$, F - value and df: degree of freedom) are given for each aquaria replicate with corresponding treatment.**

**Appendix 5.11**

| Treat | Replicate | POL GR | Intercept | p-value | $R^2$ | F-value | df |
|---|---|---|---|---|---|---|---|
| Control | 1 | 1.86 | -125.09 | <0.0001 | 62.35 | 344.43 | 209 |
| | 2 | 1.74 | -82.74 | <0.0001 | 70.11 | 434.00 | 186 |
| | 3 | 1.69 | -5.31 | <0.0001 | 71.37 | 467.25 | 186 |
| | 4 | 1.01 | 189.75 | <0.0001 | 40.26 | 91.67 | 137 |
| | 5 | 1.64 | 17.72 | <0.0001 | 56.80 | 259.02 | 198 |
| | 6 | 1.36 | 101.01 | <0.0001 | 57.00 | 238.57 | 181 |
| OA | 1 | 1.63 | -7.54 | <0.0001 | 66.62 | 377.10 | 190 |
| | 2 | 1.51 | 49.42 | <0.0001 | 73.30 | 581.89 | 213 |
| | 3 | 0.84 | 297.00 | <0.0001 | 19.42 | 44.10 | 184 |
| | 4 | 0.67 | 358.88 | <0.0001 | 19.57 | 39.67 | 164 |
| | 5 | 1.31 | 107.31 | <0.0001 | 47.07 | 170.74 | 193 |
| | 6 | 0.88 | 212.13 | <0.0001 | 29.22 | 66.89 | 163 |
| Control + oil | 1 | 2.06 | -199.79 | <0.0001 | 69.25 | 506.79 | 226 |
| | 2 | 1.33 | 43.05 | <0.0001 | 51.58 | 202.36 | 191 |
| | 3 | 1.42 | 80.92 | <0.0001 | 57.50 | 271.96 | 202 |
| | 4 | 0.91 | 235.65 | <0.0001 | 31.97 | 90.68 | 194 |
| | 5 | 1.69 | -8.81 | <0.0001 | 69.96 | 517.04 | 223 |
| | 6 | 1.47 | 57.14 | <0.0001 | 55.19 | 237.69 | 194 |
| OA + oil | 1 | 1.50 | 31.91 | <0.0001 | 61.77 | 324.70 | 202 |
| | 2 | 1.58 | 11.09 | <0.0001 | 57.07 | 243.32 | 184 |
| | 3 | 1.25 | 144.50 | <0.0001 | 36.94 | 107.82 | 185 |
| | 4 | 1.14 | 186.35 | <0.0001 | 74.58 | 516.40 | 177 |
| | 5 | 1.10 | 179.31 | <0.0001 | 30.28 | 83.39 | 193 |
| | 6 | 1.21 | 112.82 | <0.0001 | 44.39 | 132.49 | 167 |

**Postero - lateral arm growth rate (POL GR in $\mu m^3\ \mu m^{-1}$BL) for each treatment was calculated as the coefficient of the significant linear relationship between POL (µm) and BL (µm). Results of the regressions (Intercept, p - value, $R^2$, F - value and df: degree of freedom) are given for each aquaria replicate with corresponding treatment.**

**Appendix 5.12**

| Treat | Replicate | SV GR | Intercept | p-value | $R^2$ | F-value | df |
|---|---|---|---|---|---|---|---|
| Control | 1 | 8342 | -2302844 | <0.0001 | 53.53 | 261.45 | 228 |
| | 2 | 9585 | -2729019 | <0.0001 | 61.87 | 326.11 | 202 |
| | 3 | 12774 | -3716178 | <0.0001 | 38.83 | 124.40 | 197 |
| | 4 | 11159 | -3289691 | <0.0001 | 53.03 | 180.66 | 161 |
| | 5 | 9275 | -2537793 | <0.0001 | 60.17 | 60.17 | 230 |
| | **6** | **28839** | **-9798347** | **0.12** | **1.20** | **2.47** | **205** |
| OA | **1** | **76770** | **-2789637** | **0.16** | **1.30** | **1.90** | **191** |
| | 2 | 15205 | -4864098 | <0.0001 | 65.25 | 396.16 | 212 |
| | **3** | 12883 | -3790401 | <0.0001 | 17.04 | 39.03 | 191 |
| | 4 | 11384 | -3329835 | <0.0001 | 50.66 | 207.40 | 203 |
| | 5 | 10867 | -3068109 | <0.0001 | 56.46 | 289.19 | 224 |
| | 6 | 9613 | -2608771 | <0.0001 | 46.81 | 168.95 | 193 |
| Control + oil | 1 | 9410 | -2722410 | <0.0001 | 56.59 | 319.41 | 246 |
| | 2 | 9135 | -2599021 | <0.0001 | 50.01 | 208.12 | 209 |
| | 3 | 10804 | -3246235 | <0.0001 | 57.90 | 308.05 | 225 |
| | 4 | 9200 | -2567345 | <0.0001 | 46.21 | 183.01 | 214 |
| | 5 | 10861 | -3163482 | <0.0001 | 28.03 | 94.27 | 243 |
| | **6** | **39023** | **-1366810** | **0.05** | **1.75** | **4.08** | **230** |
| OA + oil | **1** | **3935** | **-384983** | **0.05** | **1.47** | **3.84** | **219** |
| | 2 | 10408 | -3015780 | <0.0001 | 48.02 | 196.79 | 214 |
| | 3 | 9786 | -2695764 | <0.0001 | 11.07 | 24.40 | 197 |
| | 4 | 11257 | -3306177 | <0.0001 | 65.53 | 372.58 | 197 |
| | 5 | 9971 | -2733682 | <0.0001 | 42.50 | 171.47 | 233 |
| | 6 | 7809 | -1963488 | <0.0001 | 43.83 | 150.62 | 194 |

**Stomach volume growth rate (SV GR, $\mu m^3\ \mu m^{-1}$BL) for each treatment was calculated as the coefficient of the significant linear relationship between SV ($\mu m^3$) and BL (µm). Results of the regressions (Intercept, *p* - value, $R^2$, F - value and df: degree of freedom) are given for each aquaria replicate with corresponding treatment. Data in bold (*p* - value > 0.05) were removed from subsequent analyses.**

**Appendix 6.1 •Publication 1: Arnberg, M., P. Calosi, Spicer, J. I., Tandberg, A. H. S., Nilsen, M., Westerlund, S., Bechmann, R.K. 2013. Elevated temperature elicits greater effects than decreased pH on the development, feeding and metabolism of Northern shrimp (*Pandalus borealis*) larvae. Marine Biology. 2013. 160(8): p. 2037-2048. DOI 10.1007/s00227-012-2072-9**

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**Appendix 6.2 •Publication 2: Samantha, L., Garrard, R. C., Hunter, A. Y., Frommel, A. C., Lane, J. C., Phillips, R., Cooper, R., Dineshram, U. Cardini, S. J. McCoy and M. Arnberg, et al. 2013. Biological impacts of ocean acidification: a postgraduate perspective on research priorities. Marine biology DOI: 10.1007/s00227-012-2033-3**

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**Appendix 6.3 Publication 3: Chan, K.K.k, Grünbaum, D., Arnberg, M., Thorndyke, M., Dupont, S., 2013. Ocean acidification induces budding in larval sea urchins. Marine Biology (8) 2129-2135**

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**Appendix 6.4 Publication 4: Chan, K.K.K, Grünbaum, D., Arnberg, M., Dupont. S., 2015. Impacts of ocean acidification on survival, growth, and swimming behaviors differ between larval urchins and brittlestars. ICES J. Mar. Sci. doi:10.1093/icesjms/fsv073**

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**Appendix 6.5 Publication 5: Arnberg, M., Calosi, P., Spicer, J.I., Westerlund, S., Invarsdottir, A., Bechmann R.K. Effects of combined exposure to reduced pH and elevated temperature on the early life stages of the Northern krill, Meganyctiphanes norvegica. Submitted to ICES 2015**

# Effects of combined exposure to reduced pH and elevated temperature on the early life stages of the Northern krill, *Meganyctiphanes norvegica*.

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**ABSTRACT**

Climate models predict that the average temperature in the North Sea could increase 3 – 5 ºC and surface-water pH could decrease 0.3 – 0.5 pH units by the end of this century. Currently we know little of how ocean warming (OW) and ocean acidification (OA) will affect key components of temperate planktonic systems, such as the Northern krill *Meganyctiphanes norvegica*. To improve our capability to predict the consequences of exposure to combined global stressors on marine ecosystems, a better understanding of key species' sensitivity to complex environmental challenges is needed. Consequently, we investigated aspects of the development and function of early life history stages of krill exposed to a combined OA and OW scenario (OAW: + 3.0 °C, - 0.5 pH). Krill embryos and larvae were exposed for 30 d and juvenile krill were exposed for 14 d. While hatching success was not affected by exposure to predicted future OAW conditions, the rate of larval development was increased. Although juveniles' mortality was not affected by predicted future OAW conditions, metabolic rate increased significantly (+ 36 %), and feeding rate (- 60 %) and number of moults (- 67 %) decreased significantly. The results indicate that predicted future OAW conditions significantly affected early life stages of krill. Further research is needed to predict the potential implications for the temperate pelagic system they belong to.

**KEYWORDS:** Ocean acidification, ocean warming, moulting, development, global change hatching, body size, feeding, oxygen metabolic rate, Crustacea.

## INTRODUCTION

Global change is causing significant alterations in environmental conditions and will lead to shifts in a number of abiotic factors in our seas. Climate models predict that the average temperature in the North Sea could increase 3 – 5 ºC and surface-water pH could decrease as much as 0.3–0.5 pH units by the end of this century 2100 (Caldeira and Wickett, 2005; Sokolov et al., 2009). Since development, growth and metabolism are all largely temperature dependent, global ocean warming (OW) will likely affect such functions (Cossin and Bowler, 1987; Pörtner, 2001; Koeller et al., 2009), ultimately modifying species distribution and population dynamics (Southward et al., 1995; Stillman, 2003;Jones et al., 2009). Ocean acidification (OA) has been shown to significantly alter calcification, metabolism and the developmental trajectories and survival level of marine invertebrates (Doney et al., 2009; Przeslawski et al., 2015). However, these drivers will act simultaneously on marine organisms, and it is therefore, important to study the impacts of these drivers in combination on key species living in global change hotspots. Changes to the biology and population dynamics of key species are likely to have consequences for whole ecosystems (Pimm, 1991). The Northern krill *Meganyctiphanes norvegica*, is one such key species. It is pivotal in many pelagic systems in both the Mediterranean Sea and the higher latitudes of the North Atlantic, as well as occurring in large numbers in the deep lochs and fjords of north-west Europe (Tarling et al., 2010). More generally, krill constitute an important link in marine trophic chains (Pearcy et al., 1979), particularly between the primary producers and secondary producers, as they are a major food source for several species, including commercially important fish such as herring *Clupea harengus*, cod *Gadus morhua*, capelin *Mallotus villosus* as well as whales (Gambaiani et al., 2009; Sakshaug et al., 2009; Simard and Harvey, 2010). Despite the importance of krill in marine ecosystems, there is surprisingly little information on the effect of OA and OW, in isolation on different krill species (Flores et al., 2012), and to

date no study has investigated the combined effects of these stressors on krill. This may be because of the fragile nature of these organisms and the challenges of keeping them in good status under laboratory conditions (Kawaguchi et al., 2011; Sperfeld et al., 2014). Sub-adults of the Northern Atlantic krill *Nyctiphanes couchii* were not affected by exposure to reduced pH to occur by the end of the century ($pCO_2$ = 1,100 µatm), whereas feeding and excretion rates increased in Antarctic krill, *Euphausia superba* at moderately reduced pH levels ($pCO_2$ = 672 ppm) (Saba et al., 2012; Sperfeld et al., 2014). Furthermore, rate of hatching of Antarctic krill was reduced by exposure to reduced pH (1,200 uatm), and embryonic development was slowed down at relatively low pH ($pCO_2$ = 2,000 uatm) (Kawaguchi et al., 2011; Kawaguchi et al., 2013). Both embryonic and larval development are temperature and pH dependent in several krill species (Hirst et al., 2003; Tarling and Cuzin-Roudy, 2003; Yoshida et al., 2004). Furthermore, early developmental stages are regarded as the most vulnerable part of a species life cycle, particularly in relation to environmental changes (Thorson, 1950; Widdows 1991; Spicer and Gaston, 1999). It is therefore important to study the response of early life stages if we are to predict a species' response to OA and OW combined.

Consequently, the aim of this study was to investigate the direct effects of exposure to simulated future combined OA and OW (OAW) conditions on key aspects of the development and function of the early life history stages of the Northern krill *Meganyctiphanes norvegica.* We exposed embryos/larvae and juveniles, collected from the North Atlantic surface waters outside Åmøy (Norway), to simulated current (pH 8.0, 7.0 °C) or future OAW (pH 7.6, 10 °C) conditions, based on IPCC "business as usual" scenario predictions for the year 2100 in the North Atlantic (Bindoff et al., 2013, IPCC 2014). In the first of two experiments, embryos and larvae were exposed for 30 d to OAW conditions, and a number of developmental parameters recorded; namely: hatching success, developmental rate, mortality and length. In the second

experiment, juveniles were exposed for 14 d to OAW conditions, and mortality, metabolic and feeding rates, and moulting frequency were determined.

## MATERIALS AND METHODS

### *Animal material collection and maintenance*

In common with other Norwegian krill populations, spawning of *M. norvegica* may start in March in the North Atlantic (Wiborg, 1970) (Arnberg and Bechmann *pers. obs*). The bottom seawater temperature in the collection area is constant at T = 6 - 8 °C throughout the year (Arnberg and Bechmann *pers. obs.*), while subsurface seawater temperatures vary from year to year. This is important as *M. norvegica*, like most other krill species, undergo diel vertical migration which is limited to less than 100 m in shallow water habitats (e.g. (Liljebladh and Thomasson, 2001), but reaches more than 500 m in the Ligurian Sea (Tarling et al., 1999). Over an eleven-year period (2001-2012), maximum seawater temperatures (10 - 15 °C) in the fjord where krill were collected occurred in autumn, and the lowest water temperatures (4 - 8 °C) usually occurred in February-March. Consequently, krill larvae hatch during the low-temperature period. The planktonic krill larvae go through a series of developmental stages punctuated by moulting events (57 - 68 d dependent upon temperature), starting with the nauplius and ending with the furcilia VII stage (Mauchline, 1977). Juveniles are sexually mature after 9 - 12 months.

In order to collect krill for investigating the effects of OAW on embryos and early larval stages the following protocol was followed. Ovigerous females were collected using a modified shrimp trawl fitted with a 10 m macro plankton net (22 mm mesh size) and a 100 L closed cod end. Instead of using a net that may damage the krill, we secured a barrel (1 m x 1 m), to the bottom end of the macro plankton net. Trawling (mean. velocity = 9.26 km $h^{-}1$, 15 -

20 min) was carried out at 50 m depth in the Hillefjord (north of Åmøy Rogaland County, Norway; 59° 04' 00" N - 5° 45' 00' E) in March 2012. Sampling was carried out between 03:00 and 04:00 h local time, during hours of darkness, because krill are very photosensitive and their eyes can be permanently damaged by light (Gaten et al., 2010). Upon retrieval, krill were sorted by using large plastic spoons to avoid mechanical damages whilst manipulating them. Approximately 200 undamaged individuals were transferred from the barrel to eight large aquaria (vol. = 50 L) each filled with local fjord sea water. These were transported within 2 h of capture to the laboratory, while being kept in the dark. Upon arrival at the laboratory, krill were transferred to 9 L aquaria, 5 indiv. in each, and kept at current conditions (T = 7 ºC, S = 33, pH = 8.1), in a temperature-controlled room, and in the dark. Next day the sea water from the aquaria was siphoned out and allowed to drain through a sieve (mesh = 40 µm), submerged into the sea water. As Northern krill immediately release their eggs when captured, this operation enabled us to rapidly separate the eggs from the adults. The resulting filtrated sea water represented an egg suspension, from which we subsampled a given volume to determine egg density in each aquaria as follows. Briefly, ten samples (vol. = 10 mL each) of sea water were pipetted into individual Petri dishes (diam. = 9 cm, vol. = 100 mL), and eggs were counted under a stereomicroscope (Wild M28, Leica, Wetzlar, Germany) to determine egg density. These eggs were not used further in any experiment, as the handling may have compromised them irreversibly. Approx. 90 - 100 untouched eggs were then transferred from the egg suspension into one of nine Plexiglas cylinders (diam. = 8 cm, height = 10 cm) each fitted with a bottom plankton mesh (40 µm).The cylinders were placed in one of twelve flow through aquaria (vol. = 9 L). The egg density *per* cylinder was 3 - 4 eggs *per* mL.

One day post fertilisation the larvae were fed once daily with the cryophyte algae *Rhodomonas* sp. (concentration 150 µg C $L^{-1}$), with an original algal density approximately

670,000 cells $mL^{-1}$ and mean size 7.5 ± 0.8 µm. The algae were raised at T = 20 °C in filtered sea water containing growth media (Himedia, Mumbai, India).

To supply juveniles (mean carapace length 0.62 ± 0.06 mm) for the second experiment individuals were collected as described above for ovigerous females, February 2012. Upon retrieval juveniles were sorted by hand, and approximately 200 undamaged individuals transferred by plastic spoon from the barrel to eight large tanks (vol. = 50 L) each filled with local fjord sea water to be transported to the laboratory within 2 h of capture and kept dark. Upon arrival at the laboratory, krill were haphazardly divided between four aquaria (vol. = 500 L, T= 7 ºC, S = 33, density approx. 75 indiv. *per* aquaria). Each aquarium was continuously supplied with fresh sea water directly pumped from 75 m depth in the fjord close to the laboratory facilities. The sea water was sand-filtered prior to be used in the experimental system, in order to preserve the piping system and remove large particles. The juveniles were fed *ad libitum* with freshly-hatched *Artemia salina* nauplii (*Artemia* length 450-550 µm, feeding density 1,000 indiv. $L^{-1}$), phytoplankton *Thalassiosira weisslogi* 1200™ (Microalgae, Vigra, Norway), (2 x $10^4$ cells $L^{-1}$) as well as micro capsulated liquid larval diet (10-50 microns EZ larvae approx. 0.003 mL $L^{-1}$, Zeigler, Gardens, USA) twice a day. Undigested material was removed the following day to avoid fouling of the sea water.

*Maintenance of pH and elevated temperature*

Embryos/early larvae (nauplius I – calyptopis III) in the first experiment, and juveniles in the second experiment, were allocated to aquaria belonging to one of two treatments: 1) current conditions (pH 8.0, 7 °C) or 2) future OAW conditions (IPCC 2014) (pH 7.6, T = 10 °C). All individuals were kept in two separate continuous-flow systems, consisting of six header tanks (vol. = 12 L, flow rate = 1 L $min^{-1}$) where temperature was regulated to either 7 or 10 °C, using heat exchangers. From the header tanks sea water was pumped into a number of aquaria

(12 x 9 L) each containing either (a) three cylindrical vessels containing embryo/larva for the first experiment or (b) five juveniles for the second experiment. In the aquaria with reduced pH treatment, the desired $pCO_2$ equilibration was achieved, *via* manipulating pH by employing pH-controllers (AB Aqua Medic GmbH pH computer, Aqua Medic, Bissendorf, Germany) set to maintain mean pH at 7.6, *via* a solenoid valve, which allowed regulating the addition of pure $CO_2$ gas.

*Sea water chemistry*

Sea water pH was recorded and logged every 5 min using a pH probe (Orion Star Plus™ 3-Star and Ross® Electrodes, Thermo Fisher Scientific Inc, Beverly, USA) coupled to a calibrated pH meter (Orion Star Plus™, Thermo Fisher Scientific Inc) and a multi-channel datalogger (D-130, Consort, Turmhout, Belgium) using the Star Plus Navigator 21 Software (Thermo Fisher Scientific Inc.), and employing the NBS scale. In addition, water temperature and pH were measured every second day, and oxygen concentrations twice over the duration of the experiment in all aquarias. Water temperature was measured using a calibrated glass thermometer (certified by Physikalisch-Technische Bundesanstalt, Braunschweig, Germany) and pH using a hand held pH meter (Orion Star Plus™ 3-Star, Thermo Fisher Scientific Inc.). Oxygen concentration was measured with an oxygen meter (Oxi 330i/SET, WTW, Weilheim, Germany). The salinity of the intake water was recorded every 5 min during the entire duration of the experiments using a CT-probe (Aqua TROLL 100®, In-Situ Inc., Collins, USA) with Win-Situ 5 data acquisition software (In-Situ Inc., Collins, USA). Sea water samples for alkalinity measurements were removed five times during the experiment at day 1, 7, 14, 20, and 30, total alkalinity (TA) of the sea water was analyzed in ten samples four times during the experiment from the two header tanks using high-precision potentiometric titration

(Haraldsson et al., 1997). There was no significant difference in mean alkalinity (TA) ($F_{1,9} = 0.193$, $p = 0.672$) between the different treatments (mean alkalinity at $pH_{NBS}$ 8.0 was 2306.80 ± 10.53 SD µmol $kg^{-1}$, at $pH_{NBS}$ 7.6 was 2303.30 ± 14.48 SD µmol $kg^{-1}$). Hence, the mean TA value for all samples was used as input to the program CO2SYS.EXE (Pierrot et al., 2006).

There was no significant difference between replicates within the treatments for temperature ($F_{5, 59} = 0.468$, p = 0.799) or for pH ($F_{5, 65} = 1.460$, p = 0.215). Neither was there a significant difference between the same treatment in the two experiments for temperature ($F_{1, 63} = 0.56$, p = 0.816) there was only a small difference in reduced pH between the future treatments of the two experiments ($F_{1, 69} = 11.62$, p = 0.001), the mean pH value for experiment 7.61 and 7.63, for experiment 1 and 2 respectively. The total mean alkalinity measured in the experiments was 2,305 ± 12 µmol $kg^{-1}$. Therefore, the temperature, pH and alkalinity data from the two experiments were pooled, and entered into the program CO2SYS.EXE (Pierrot et al 2006) to calculate the saturation state for aragonite and calcite ($\Omega_{arg}$ and $\Omega_{cal}$) and the other parameters in the carbonate system. The carbonate system parameters that were not directly measured were calculated using the program, employing constants from Mehrbach et al. (1973) refitted to the NBS pH scale by Dickson and Millero (1987) and the $KSO_4$ dissociation constant from Dickson (1990). The saturation states, dissolved inorganic carbon (DIC), concentrations of carbonate [$CO_3^{2-}$] and bicarbonate [$HCO_3^-$] were calculated using mean ± 1 SD of measured pH, temperature and alkalinity thereby taking account of the variability in the measurements. The results from the CO2SYS calculations are presented in Table 1. Mean $pCO_2$ values in the treatments ranged from 470 to 537 µatm ($pH_{NBS}$ = 8.0) and up to 1,349 to 1,795 ($pH_{NBS}$ = 7.6). Sea water was undersaturated with respect to aragonite when $pH_{NBS}$ was below 7.6 (Table 1).

*Experimental procedures exp. 1*

The cylinders containing newly hatched krill larvae were sampled at day 7, 14, 22 and 29 of the experiment. Three subsamples (initial embryo number 90) from each treatment were taken at each time interval. Unhatched eggs and larvae (both alive and dead) were siphoned out, counted under low power magnification (x 25) (Wild M28, Leica, Wetzlar, Germany), and stored in 4 % paraformaldehyde (PFA) for subsequent stage determination and length measurements. Developmental stages (egg, nauplius I (N1), nauplius II (N2), metanauplius (MN), calyptopis I (C1), calyptopis II (C2), calyptopis III (C3)) were determined using the descriptions of Lebour and Sars 1898 (Lebour, 1924). Larvae were observed using a two imaging microscope (Axioplan, Zeiss, Wetzlar, Germany) fitted with a digital camera (MRc5, Axiocam, Wetzlar, Germany). Bifocal pictures were taken and length measurements of different larval stages were carried out. Hatching success was estimated at seven days post egg collection, and calculated as the percentage of living larvae over initial number of eggs. Mortality rates were defined as the number of living larvae over the initial number of eggs. Disappeared larvae or dissolved larvae were counted as dead.

*Experimental procedures exp. 2*

In the second experiment five juveniles of equivalent size (average total carapace length = 0.61 ± 0.06 mm) were allocated (after three weeks exposure under control conditions) to one of 12 aquaria (vol. = 9 L) each supplied with continuous flow-through sea water and assigned to one of the two treatments.

Juveniles were kept in the dark and fed twice daily (morning and afternoon) ad *libitum* on a mixture of, newly-hatched *Artemia salina* nauplii (*Artemia* length 450-550 µm, feeding density 1000 indiv. $L^{-1}$), phytoplankton *Thalassiosira weisslogi* 1200$^{TM}$ (Microalgae, Vigra,

Norway), (2 x $10^4$ cells $L^{-1}$), and micro capsulated liquid juvenile diet (10-50 microns EZ larvae, 0.003 mL $L^{-1}$, (Zeigler, Gardens, USA). The number of dead krill *per* aquaria was determined every second day. Mortality was expressed as percentage dead larvae in each treatment at the end of the experiment. Numbers of moults were recorded three times during the experiment day 3, 7 and 11. Individuals were deemed to have moulted if an empty carapace (moult) were found in the aquaria. Moulting was expressed as mean moults *per* date *per* treatment.

Juvenile feeding rates were determined using a modification of the clearing rate methods by Harvey and Michel (2003). Feeding trials were conducted in glass beakers (vol. = 500 mL) each containing filtered sea water from the treatment header tanks used in the experiment. One juvenile was transferred into a beaker and placed at the respective temperature (7 or 10 °C). Freshly hatched *Artemia* nauplii (100 indiv.) were added to the water in 500 mL beakers containing the juveniles. The water in each beaker was then aerated using compressed air delivered through an air stone. The beaker was covered using aluminium foil to prevent evaporation and incubated for 20 h at the respective temperature. After the incubation period, juveniles were carefully removed from the beakers, with a small plastic spoon and the remaining sea water, containing *Artemia* nauplii, was sieved using a strainer (40 µm BD Falcon™ cell , Biosciences, Franklin Lakes, USA), and individual nauplii counted. Amount of prey consumed was calculated as initial number of *Artemia* nauplii less their final number. Feeding rate was expressed as number of prey consumed *per* individual krill larvae *per* unit time (number of *Artemia* $indiv^{-1}h^{-1}$). A control sample from the OAW treatment without krill was run through the experimental procedure, to test the efficiency of the sieving procedure. All 100 *Artemia* sp. nauplii were recovered in the control test.

The metabolic rate of juveniles was estimated using rates of oxygen consumption as a proxy following the methods of Taylor et al. (1989). Individual juveniles were placed in a custom-

built, closed glass-bottle respirometer (vol. = 300 mL), equipped with airtight stoppers into which $O_2$ electrodes were inserted and were in direct contact with the sea water within. Blank low concentration $O_2$ water samples (N = 3) were used to verify that the respirometers were airtight. Respirometers were supplied with sea water from the appropriate treatment header tank water used in the experiment and sealed with the airtight stoppers, and then placed in flow through water baths to maintain a constant temperature. A total of 20 replicates were measured for each of the two decreased pH/temperature combinations. Following preliminary trials, measurements of dissolved $O_2$ concentration in the respirometers were performed every 2 sec for the entire duration of the incubation (approx. 6 h) using $O_2$ electrodes (1302, Strathkelvin Instruments, Glasgow, UK) coupled to a calibrated multichannel oxygen meter (928, Strathkelvin Instruments). Continuous measurements were conducted to demonstrate linearity of $pO_2$ decline in the respiration chambers during the incubation (N = 24). Larvae-free samples of sea water from the appropriate treatment header tank were used to estimate background respiration rates caused by microorganisms in the system. Background respiration never exceeded 10 % of the total larval respiration. Background rate respiration was used to correct the specimen's respiratory measurements. At the end of each trial, larvae were removed from the respirometer, carefully blotted dry and then stored at – 80 °C for subsequent morphological and dry mass (DM) determinations, allowing us to present rates of oxygen consumption as µmol $O_2$ $h^{-1}g^{-1}$ $DM^{-1}$. Total carapace length (TC) was measured for all juveniles. Individuals were dried to constant mass (approx. 24 h) on aluminium trays at 60 °C. They were then weighed on precision scales (Mettler-Toledo AT201, Oslo, Norway).

*Statistical analysis*

The effect of future OAW conditions, for the biological parameters larval length, juvenile mortality, moulting, feeding and metabolic rates in this study was analyzed using General

Linear Model (GLM) tests, one-way ANOVA. All these parameter data met assumption for normality of distribution, tested by the Kolmogorov-Smirnow test, and variances were homogeneous using the Levens – test. ®. A Wilcoxon Rank sum test for small sample sizes (Bhattacharyya and Johnson, 1977) were used to analyse the effects of OAW conditions on the biological parameters larval hatching, mortality and developmental parameters. All analyses were conducted using v 21 SPSS

## RESULTS

*Experiment 1 - Larvae*

*Hatching success, survival, developmental rate and length of krill larvae*

The average hatching success was 42.2 ± 5.1 % and 36.3 ± 14.5 % for the current and future OAW conditions respectively (Table 2). Hatching success was not significantly affected by future OAW conditions ($p = 0.233$). Mortality rates (Fig. 1) were high in both treatments, but mortality was significantly greater under future OAW conditions, at all-time points sampled ($p_{max} = 0.05$). Survival was high in both treatments during the metanauplius to calyptopis (MN-C1) stage but thereafter fell steeply during the C2-C3 stage. In the current condition, this occurred from day 22 and in the future OAW condition day 14. The results indicate that C2 larvae had similar mortality in both treatments (Fig. 1 and 2). At the end of the experiment (day 29), 22 % of the hatched larvae were still alive in the current conditions, but there with no survivors found in the future OAW conditions (Fig. 1). Furthermore, exposure to future OAW conditions significantly decreased the development time for all the stages, with larvae reaching C3 faster compared to those raised under current conditions (Fig. 2.) ($p_{max} = 0.025$).

In addition, larvae were significantly longer under future OAW conditions at the MN stage (Table 3) ($F_{1, 15}$ = 17.980, p = 0.003), but significantly reduced length at the C2 stage (Table 3), ($F_{1, 41}$ = 10.100, p = 0.003).

*Experiment 2 - Juveniles*

*Mortality and moulting*

At the end of the exposure period (14 d) average mortality was 16.6 % and 20.0 % under current and future OAW conditions respectively, but this difference was not significant ($F_{1, 88}$ = 1.010, p = 0.428). However, exposure to the future OAW conditions significantly reduced the number of moults by 67 % compared to current condition (Fig. 3) ($F_{1, 34}$ = 5.670, p = 0.023). The number of moults was greater at day seven (Fig. 3), the term 'time' significantly influencing the number of moults ($F_{2, 33}$ = 4.5, p = 0.019).

*Feeding and metabolic rates*

Exposure to future OAW conditions significantly decreased juvenile feeding rates compared with the current conditions (Fig. 4, $F_{1, 25}$ = 4.250, p = 0.050). In addition, rates of $O_2$ uptake were found to be significantly greater (36 %) in individuals kept under future OAW conditions (Fig. 5), with a mean value of 81.6 µmol $O_2$ $h^{-1}g^{-1}$ DM compared to the current conditions with a mean value of 52.2 µmol $O_2$ $h^{-1}g^{-1}$ DM ($F_{1, 32}$ = 13.53, p = 0.01).

**Discussion**

Our results are the first on the sensitivity of early life stages of a krill species to the combined exposure to ocean warming (OW) and ocean acidification (OA) (OAW). Exposure to predicted future combined OAW conditions in the Northern krill *Meganyctiphanes norvegica* does not appear to affect embryo hatching success but does lead to an increase in larval

developmental rates. Furthermore, juvenile mortality does not change significantly following exposure to OAW conditions, whilst metabolic rate increased by 36 %, and feeding rates and number of moults decreased by 60 and 67 % respectively.

*Impact of exposure of future OAW conditions on embryos and larvae*

Exposure of krill embryos/eggs to OAW had no effect on hatching success although it appeared to cause a reduction in larval developmental time. Ross and Quetin (1989), reported that embryos of the North Pacific krill *Euphausia pacifica*, unlike adults and juveniles, were highly sensitive to handling, and that this may explain the variability in hatching rates reported in several studies of krill. Northern krill generally have relatively low survival in the laboratory (Buchholz, 2003). In our study, the mean hatching success of embryos of Northern krill varied between 36 - 40 %, and was not affected by exposure to future OAW conditions. Embryos robustness to OAW has previously been reported for other crustacean species (e.g Styf, 2014). Elevated temperature had no effect on hatching success in the Antarctic krill, *Euphausia. superba* (Yoshida et al., 2004), whilst exposure to increased $pCO_2$ (1,250 and 2,000 μatm) reduced hatching success in the same species (Kawaguchi et al., 2011; Kawaguchi et al., 2013). This is the first study where the effects of OAW on krill hatching success has been investigated The hatching success levels recorded for Northern krill embryos kept under control conditions in our experiment fall within the range reported for the Antarctic krill (Harrington and Ikeda, 1986; Yoshida et al., 2004; Kawaguchi et al., 2011). Survival rates of Antarctic krill are generally low during early development under laboratory conditions similar to our control (Hirano et al., 2003). This was also found for the early larval developmental stages of the Northern krill in this study, with only 22 % of larvae surviving under control conditions. Mortality of krill larvae was significantly greater under future OAW conditions at all-time points. However, the stage specific mortality appeared to be similar

because the larvae developed faster in OAW. In particular, the increased mortality observed at the transition between the C2 and C3 stages in both treatments indicates that this could be a critical developmental phase for the Northern krill. We believe the stage-specific sensitivity we observed here, may require that we further improve laboratory practice and husbandry techniques for the Northern krill larvae.

The accelerated development in the larvae raised in OAW, would lead to larvae spending shorter periods in the plankton. This would have beneficial consequences for larval survival both directly and indirectly via reducing developmental time and thus reduced predation risk. Alternatively, accelerated developmental rate can lead to higher mortality levels (Flynn et al.2015) and often comes at the cost of increased energetic costs for maintenance and repair which can lead to reduced larval size (e.g. Small et al. 2015).

Faster developmental rate has also been reported for krill species exposed to elevated temperature (Yoshida et al., 2004), and Northern krill seems similar in this regard, despite the fact that we tested for combined effects of OA and OW. Poulsen et al. (2011) showed that Antarctic krill exposed to a pollutant underwent an accelerated development from N1 to MN, the non-feeding larvae; they suggested that the accelerated development carried additional energetic cost, possible preventing metamorphosis in following larval stages. In fact, the lipid reserves of the non-feeding larvae are being depleted when the larvae approaches the feeding C1 stage (Ross and Quetin, 1989), thus only a very narrow window remains to initiate feeding, to compensate for extra metabolic, maintenance and repair costs, when the larvae reaches the C1 stage. If this window is further reduced by the negative impact of OAW as it appears from our and other studies, survival of krill early life stages may be compromised (Poulsen et al. 2011).

Furthermore, this shifting in timing and shortening of feeding window may cause potential mismatch with processes timed according to constant cues (e.g. light). Climate change such as

OAW could therefore potentially lead to mismatches between the reproductive cycles of *M. norvegica* and its planktonic food, this match being a key component for the survival and recruitment success of northern krill larvae, as documented for the Northern shrimp *Pandalus borealis* (Koeller et al 2009). However, such a mismatch may not materialise if concurrent changes in the surface oceanography also result in earlier blooms.

*Impacts of exposure of future OAW conditions on juveniles*

Exposure to future OAW conditions was not lethal to juvenile *M. norvegica* during the time course of our experiment. Juvenile mortality ranged between 16 and 20 % after 14 d exposure (total of four weeks in the laboratory), and fell within the mortality range of juvenile control groups of two other experiments with the same species (mortality after two weeks 15 and 21 %) performed at IRIS (Moodley and Invarsdottir *pers. comm.*). Another krill species widely distributed in the north Atlantic (*Nyctiphanes Couchii*), had higher mortality rate (50 %) than in our experiment under comparable control conditions (Sperfeld et al., 2014). No lethal effects were detected for *N. couchii* following exposure to similar pH levels to those used in our study, although at lower pH (1,700 µatm) most krill died within six days (Sperfeld et al., 2014). Despite the fact that no lethal effects of future OAW conditions were found for juvenile Northern krill, a number of sublethal effects were detected in the juveniles.

Juveniles can compensate for acute temperature change through physiological plasticity (Saborowski et al., 2002; Strömberg and Spicer, 2000). This ability to compensate for the negative direct effects of elevated temperature over a short period could explain the observed significant increase in metabolic rates (+ 36 %) in juvenile krill exposed to future OAW conditions, in this study. On this basis, we predict that Northern krill will have greater maintenance and repair costs in future oceanic conditions. Increased energetic costs could be

compensated *via* increased feeding effort or high food availability (Melzner et al. 2011). On the contrary, we demonstrated 60% decreased feeding rates under future OAW conditions. This result indicate that juvenile krill will be severely energetically challenged, possibly affecting survival and further development (Buchholz and Buchholz 2010). However, we must consider that abnormal swimming behaviour and associated reduced feeding activity have been observed for the Antarctic krill *Euphausia superba* kept in small containers, where animals bumped into the walls of the containers more frequent (Price el al. 1988). The Northern krill in this experiment displayed higher metabolic rates due to the OAW conditions, and possible an associate greater level of activity (swimming) when compared to control conditions, which could explain the reduced feeding activity in under future OAW conditions. Moreover, the reduction in feeding together with the increasing energy demand could possible explain the decreased moulting observed in the future conditions; possibly the sign of a delay in development. A reduction in food may depress the hormonal trigger and delay moulting in Northern krill until the situation improves (Buchholz, 2003). Krill can survive periods of high metabolic cost, induced by elevated temperature or starvation, by shrinking between moults even though food is abundant (Marinovic and Mangel, 1999; Buchholz, 2003). The moulting cycle of the krill in the Stavanger area has been estimated to be around 13.5 d at 7 °C (Ingvarsdottir unpubl. data). Therefore, the timing of the experiment and length could potentially influence the results and moulting rate data in shorter experiments such as ours, and therefore our results should be interpreted with some care. Nonetheless, as all krill were collected on the same date, and treated identically through the acclimation period, our data should to some degree be indicative of the effect on future OAW conditions on moulting. Another possible influence on moulting data could be that krill eat their moults, as previously reported for lobster (Atema et al., 1979) as moults were not sampled each day. However, this has not been observed in our laboratory experiments with krill.

*Conclusions*

It has been shown that juvenile Northern krill can survive the future OAW conditions tested here in this study. Nevertheless, there were noticeable sub-lethal effects, including increased metabolic costs, reduced feeding and possible interruption or delay of moulting. The larval stages on the other hand, appeared to be more sensitive to the future OAW conditions displaying accelerated developmental rates. Even though the juvenile/adult krill may be able to adapt to future OAW conditions (Sunday et al 2014), due to the species ability to acclimatise to local temperature conditions (Saborowski et al., 2002; Strömberg and Spicer, 2000) the larvae may not be able to acclimatize. The larvae have increased developmental rates, which may induce greater maintenance costs and narrowed feeding window in the future conditions. If the larvae are not able to meet their demand for energy to grow and develop due to possible mismatch with its food, mortality may follow in the further larval stages. This will represent an important challenge for the larvae especially during food-scares periods in the sea.

Although this was a relatively short-term experiment, the results highlights the need to understand more about the response of this keystone species to conditions predicted for the future of our oceans. Additionally, whilst long-term experiments are needed to understand longer-term effects on the species population biology, our results enable us to consider the potential implications for the temperate pelagic systems they belong too.

**Acknowledgements**

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(200800/S40) lead by RKB .The experiments were performed at the marine research facility of IRIS. PC is supported by a Natural Sciences and Engineering Research Council of Canada Discovery Grant.

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Figure 1.

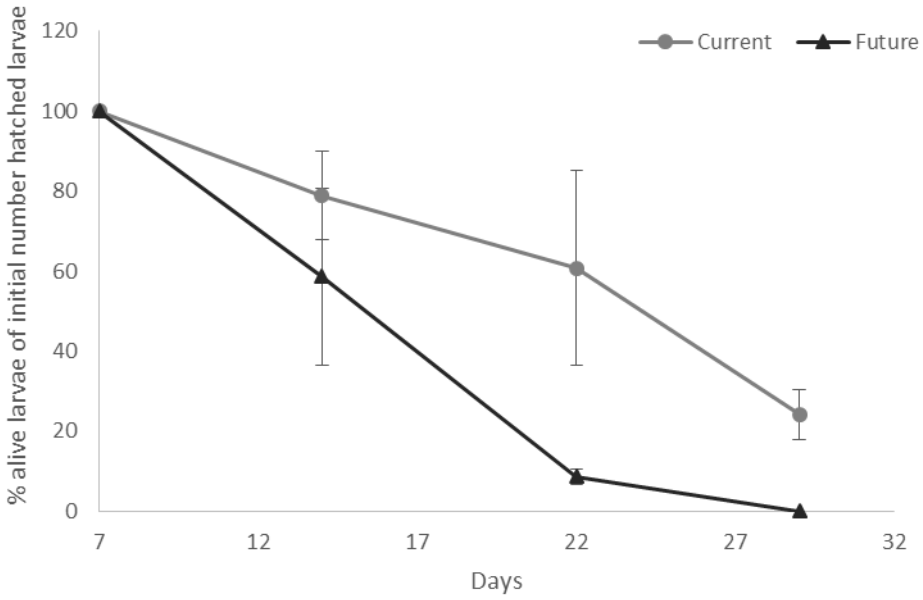


Figure 1.The effect of current and future elevated temperature and reduced pH (OAW) conditions on the mortality of larvae of *Meganyctiphanes norvegica.* Mean % surviving larvae of initial number hatched larvae, of larvae. Current (pH 8.0, 7 ºC, light grey) (N = 3) and future (pH 7.6, 10 ºC, black) (N = 3).

Figure 2.

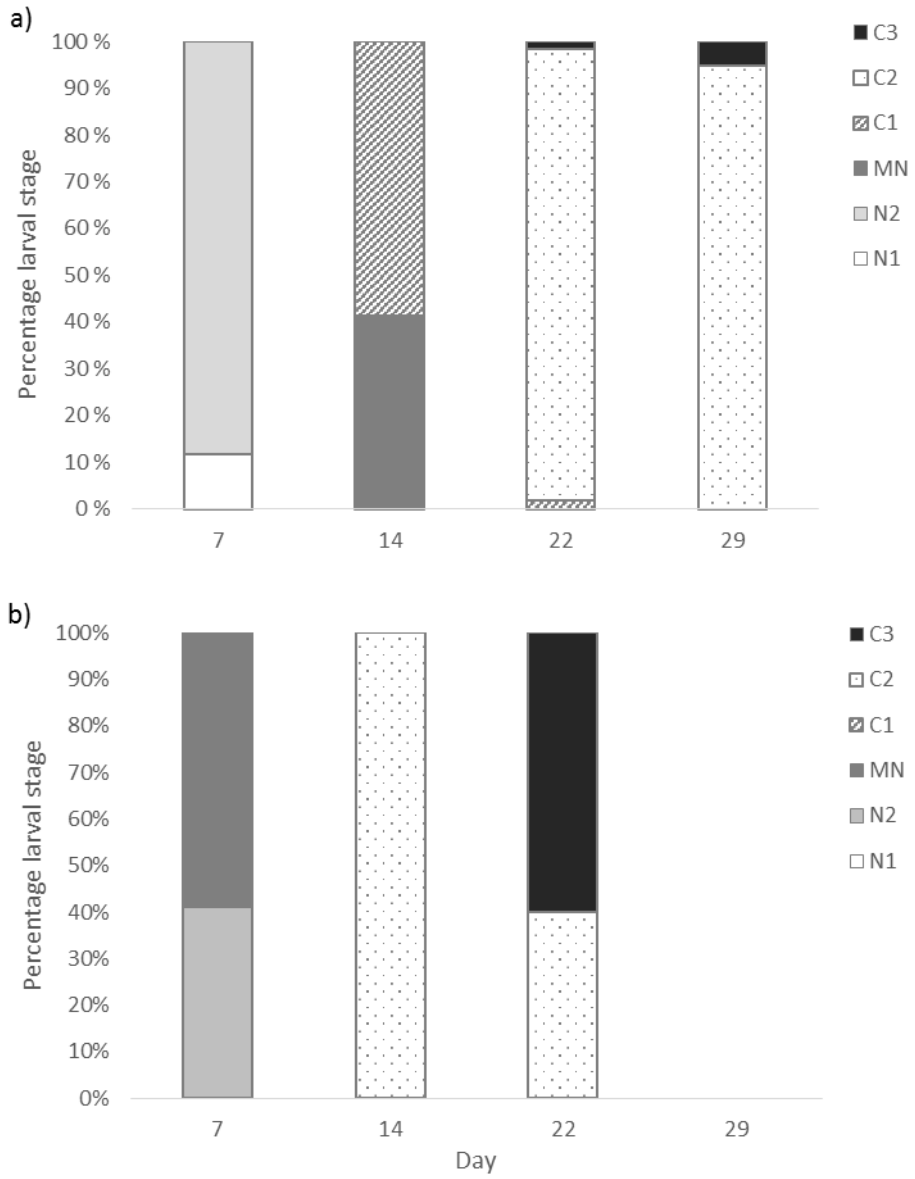


Figure 2. The effect of current and future OAW conditions on the development of *M. norvegica* larvae (stage *nauplius* I – *calyptopis* III) exposed to a) current (pH 8.0, 7 ºC) and b) future (pH 7.6, 10 ºC) conditions. Mean percentage of each larval stage 7, 12, 22 and 29 d after hatching. N1 = nauplius I, N2 = nauplius II, MN = metanauplius, C1 = calyptopis I, C2 = calyptopis II and C3 = calyptopis III (N = 3).

Figure 3

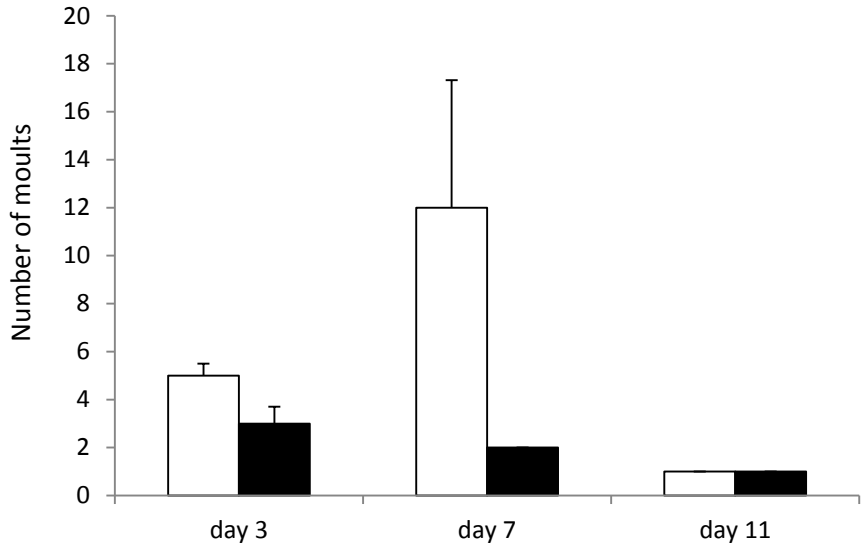


Figure 3. The effect of current and future OAW conditions on mean moulting of juvenile *M. norvegica*, number of moults *per* six aquaria, of juvenile for day 3, 7 and 11. Current (pH 8.0, 7 ºC, white) (N = 6), future (pH 7.6, 10 ºC, black) (N = 6). Histograms represent means ± SD.

Figure 4

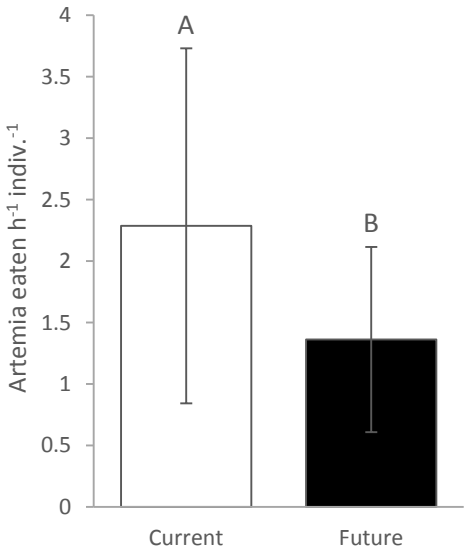


Figure 4. The effect of current and future OAW conditions on mean feeding rates of juvenile *M. norvegica* (measured as mean number prey eaten indiv.$^{-1}$ h$^{-1}$). Current (pH 8.0, 7 ºC, white) (N = 15), future (pH 7.6, 10 ºC, black) (N = 15). Histograms represent means ± SD. Significantly different treatments ($p \leq 0.05$) are indicated by different letters.

Figure 5

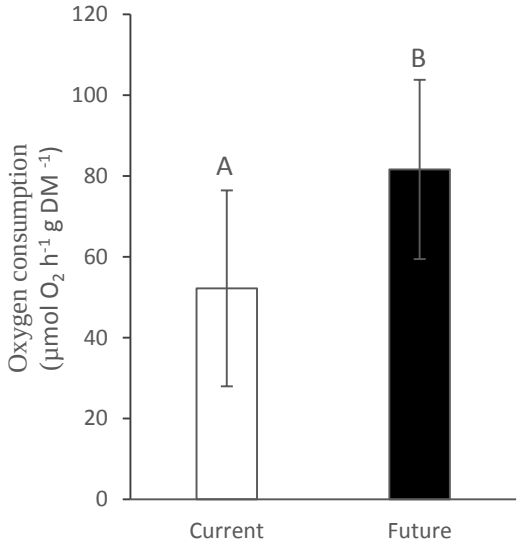


Figure 5. The effect of current and future OAW conditions on mean oxygen consumption (µmol $O_2$ $h^{-1}$ mg $^{-1}$ $DM^{-1}$) of juvenile *M. norvegica*. Current (pH 8.0, 7 ºC, white) (N = 16), future (pH 7.6, 10 ºC, black) (N = 18). Histograms represent means ± SD. Significantly different treatments ($p \leq 0.05$) are indicated by different letters.

Table 1.

| Treatment | Current | Future |
|---|---|---|
| *Measured* $[O_2]$ (mg $L^{-1}$) | 8.7 ± 0.2 | 8.6 ± 0.1 |
| $pH_{nbs}$ (range pH) | 8.06 (8.03-8.08) | 7.62 (7.56-7.68) |
| ºC aquaria | 6.9 ± 0.27 | 9.7± 0.20 |
| Salinity | 33.13 ± 0.43 | |
| Total alkalinity (µmol $kg^{-1}$) | 2306.3 ± 10.5 | 2303.3 ± 14.5 |
| *Calculated* $pCO_2$(µatm) | 511 (470-537) | 1627 (1354-1795) |
| DIC (µmol $kg^{-1}$) | 2185 (2180-2189) | 2206 (2199-2209) |
| $[HCO_3]$(µmol $kg^{-1}$) | 2065 (2058-2070) | 2199 (2177-2206) |
| $[CO_3^{2-}]$(µmol $kg^{-1}$) | 95.6 (89.3-103) | 39.1 (34.8-46.9) |
| $\Omega_{calcite}$ | 2.30 (2.16-2.47) | 0.94 (0.84-1.13) |
| $\Omega_{aragonite}$ | 1.45 (1.36-1.56) | 0.60 (0.53-0.71) |

Table 1. Seawater physico-chemical parameters measured in the experimental system: oxygen concentration ($[O_2]$), pH (NBS Scale), temperature, salinity and total alkalinity. Other parameters were calculated using the program CO2SYS (Pierrot et al. 2006), using constants from Mehrbach et al. (1973) refitted to the NBS pH scale by Dickson and Millero (1987) and the $KSO_4$ dissociation constant from Dickson (1990). Mean pH level was calculated based on the actual $[H^+]$ concentration and back calculated to pH. These parameters included: dissolved inorganic carbon (DIC), bicarbonate and carbonate ions concentrations ($[HCO_3^-]$ and $[CO_3^{2-}]$ respectively), saturation status for calcite and aragonite ($\Omega_{calc}$ and $\Omega_{ara}$ respectively). For all measured parameters

mean values (± SD) were provided, whilst for total alkalinity and all calculated parameters range of values were provided.

Table 2

| Day | Treatment | Initial larvae number | Hatch rate | Unhached eggs | Living larvae | Dead larvae | Dissolved larvae/dissapered larvae | Number of samples |
|---|---|---|---|---|---|---|---|---|
| **7** | | | | | | | | |
| | Current | 90 | 42.2 ± 5.1 | 48.3 ± 1.5 | 38 ± 4.6 | 0 | 4 | 3 |
| | Future | 90 | 36.3 ± 14.5 | 24.3 ± 9.8 | 32.7 ± 13.1 | 1 | 32 | 3 |
| **14** | | | | | | | | |
| | Current | 90 | | - | 29.7 ± 2.1 | 1.6 ± 1.1 | 55 | 3 |
| | Future | 90 | | - | 18 ± 2 | 1.6 ± 0.6 | 38 | 3 |
| **22** | | | | | | | | |
| | Current | 90 | | - | 25 ± 4 | 2.6 ± 2.3 | 4 | 3 |
| | Future | 90 | | - | 3 ± 1 | 1.3 ± 0.6 | 15 | 3 |
| **29** | | | | | | | | |
| | Current | 90 | | - | 8.3 ± 1.5 | 2 ± 1.7 | 17 | 3 |
| | Future | 90 | | - | - | | | 3 |

Table 2. Hatching success (%), unhatched eggs (%), number of living larvae and dead larvae for the Northern krill *M. norvegica* for the current and future OAW conditions at sampling days 7, 14, 22, and 29.

Table 3.

Table 3. Mean body lengths (mm) of early larval *M. norvegica* in current and future OAW conditions. Number in brackets indicate the number of larvae measured. (* indicate single measurements which should be taken with caution).

| **Treatment** | **Nauplii** | | **Metanauplius** | **Calyptopes** | | |
|---|---|---|---|---|---|---|
| | NI | N2 | MN | CI | C2 | C3 |
| Current | 0.49 (4) | 0.49 (25) | 0.52 (9) | 1.02 (20) | 1.12 (21) | 1.45 (2) |
| Future | | 0.48 (7) | 0.57 (7) | | 1.04 (22) | 1.39 * |
| Mauchline 1971 | 0.48 | 0.48 | 0.50 - 0.52 | 1.03* | 1.59* | 2.4* |

**Appendix 6.6 Publication 6: Arnberg, M., Calosi, P., Spicer, J.I., Taban, I.C., Bamber, S., Westerlund, S., Vingen, S., Baussant, T., Bechmann, R.K., Dupont, S. Additive effects of oil and global environmental drivers on two keystone marine invertebrates. Submitted Sci. Rep. 2015.**

# Additive effects of oil and global environmental drivers on two keystone marine invertebrates

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**Running title**: Impact of oil and global drivers on marine invertebrates

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**TYPE OF MANUSCRIPT**: Letter

**Abstract**

Projecting the impact of multiple drivers on marine ecosystems is challenging and relies on the existing knowledge of individual driver's mode of action. The mode of action can be acting on similar physiological processes, or on different processes. Drivers can also be non-interactive or interactive. For non-interactive stressor with dissimilar mode of action, theory predicts that their combined effect will be additive. We tested this hypothesis by investigating the effect of two independent drivers, oil exposure simulating an oil pollution as a local acute stressor and reduced pH and elevated temperature as combined chronic global drivers. This was tested on larvae of the Northern shrimp and the Green sea urchin. A range of effects was observed depending on driver, species, stage of development and tested endpoints. Despite this variability in response, no significant interaction was ever observed between the two drivers. As predicted, combined responses were always additive. This demonstrates the importance of a mechanistic understanding of individual drivers' mode of action and interactions to develop models that can predict the biological outcomes of exposure to cocktails of multiple drivers. This finding should empower local managers to act on local stressors to delay the negative impacts of future global drivers.

Human-driven changes to ocean biogeochemistry affect multiple marine processes and systems[3]. Drivers in marine systems can be divided into two categories[4]: those that act globally and those that act locally. Global drivers are chronic[5], lasting over an extended period of time. For example, average surface ocean pH is expected to decrease by 0.3 to 0.5 units by 2100 due to increased atmospheric carbon dioxide ($CO_2$), a phenomenon termed as ocean acidification (OA)[6,7]. I addition it is predicted that by 2100 sea surface temperatures will have increased by 2.0 – 4.5 °C[8]. Another result due to increasing $CO_2$ in the atmosphere is atmospheric warming which in turn results in increasing seawater temperatures, termed ocean warming (OW).

Other drivers can originate and act at the local scales[5] and may be either chronic (e.g. nutrient input from rivers) or transient, brief and short lived (e.g. oil pollution). For example, reduced ice cover in the Arctic caused by on-going climate change, will lead to increased transport by ships, as a response of the worldwide demand of more petroleum resources and search by the oil and gas industry, particularly into the European Arctic from the North Atlantic into Greenland, Northern Norway and Northwest Russia[9], increasing the geographical range, and ecosystems over which potential accidental oil pollution can occur.

Benthic marine invertebrates and their planktonic life stages will face multiple environmental challenges in the future ocean. In fact, there will be large areas of the ocean where marine organisms will be facing combined and complex environmental challenges, including OA, OW and accidental discharges from the petroleum industry and shipping with greater frequency and intensity seen to date. While we have some understanding of the biological effects of each of these drivers individually, we are only beginning to comprehend their effects in combination. The urge for experiments investigating the effects of multiple drivers, which can provide a more realistic picture of what animals experience in the wild, has been higlighted[10]. However, multi-drivers experiments are often challenging and lack the predictive power required for model projection. Another approach is to

develop a physiological understanding of single driver's effects and develop models that enable us to predict the effects of exposure to combined drivers. The simplest scenario is the combination of non-interactive drivers, i.e. drivers possessing different mode of action, thus simply leading to additive effects. The aim of our experiment was to experimentally test this idea.

We investigated the impact of combined effects of global drivers (OA: - 0.5 pH and OW: + 3 C°) and an Arctic crude oil spill (0.5 mg $L^{-1}$ nominal oil concentration), on larval stages of two keystone species, the northern shrimp (*Pandalus borealis*) and the green sea urchin (*Strongylocentrotus droebachiensis*). A range of endpoints was measured including growth, mortality, swimming, feeding and respiration. OA and OW affect energy, metabolism and ultimately growth in many marine organisms including sea urchin and shrimp larvae[11,12]. Differently, short-term oil exposure reduces larval activity leading to reduced feeding and ultimately mortality[13]. Therefore, our working hypothesis was that oil effects will be additive to OW and OA effects.

Chronic exposure to global drivers (OA and OW) increased the mortality of shrimp larvae by 30 %, but not acute exposure to oil or their interaction (Fig.1a, Table 1, S3). Exposure to global drivers (OA) did not affect mortality of sea urchin larvae, however a four days exposure to a simulated oil spill, resulted in high mortality for sea urchin larvae exposed early in their development (8-12 d post fertilisation (dpf)), but not for larvae exposed late in their development (23-27 dpf; Fig 1b, c, Table 1, S3) suggesting that later sea urchin larval stages were more robust to acute oil exposure. Oil exposed shrimp larvae showed a high incident (20 %) of abnormal larvae (Fig. 1g, Table 1, S3), while no change in symmetry index (SI) were observed among the sea urchin larvae (Fig. 1 h. i. Table 1, S3). Similar abnormal larval development as a result of oil exposure has previously been reported[14,15]. On the other hand, there was no significant effect of global drivers or interactions for these abnormality indexes.

The impact of combined drivers on the size of shrimp larvae was equal the sum of the negative impacts observed for each driver: a 5 % reduction when exposed to OA and OW, a 9 % reduction

when exposed to oil, and a cumulative 15 % reduction when exposed to all stressors (Fig. 1 d, Table 1, S3). Similarly the impact of combined drivers on the size of sea urchin larvae, was equal to the sum of the negative impacts observed for each driver: an 11 to 14 % reduction when exposed to OA, an 6 to 9 % reduction when exposed to oil, and an 17 to 21 % reduction when exposed to all drivers, respectively (Fig. 1 e- f, Table 1, S3). Additional costs for acid-base regulation and/or thermal stress can affect the energy budget and energy allocation of larvae. As a consequence of increased costs for maintenance and homeostasis, less energy is available for growth. Similar shifts in energy budget allocation due to exposure of reduced pH and elevated temperature, has been reported previously for these species[11,12].

All tested drivers (OA, OW and oil) affected larval feeding and activity: oil by reducing feeding and activity (Fig.S1 a. b. S2), and global drivers by increasing the activity of larvae in both species (Fig. S1 c-d, Table S3). Similar reductions in swimming and feeding were observed with in adult and nauplia copepods, and larval crabs exposed to oil[16,17]. However, comparison with other studies investigating the impact of oil polluton simulations on planktonic invertebrates is difficult as the type of oil, the chemical composition, the exposure conditions, the fraction of oil tested, the analytical method and compounds analysed vary between studies[15,18]. Nonetheless, we can hypothesize that the reduced swimming activity during oil exposure may be due to a narcotic effect of the oil, or the PAH in the oil,[16,17]. We observe reduced feeding rates and reduced growth for both species of larvae exposed to oil. This narcotic effect may be linked to observed reduction in feeding rates, and reduced feeding would eventually lead to decreased growth due to energy limitation and increased mortality due to starvation[19]. A similar reduction of growth was observed following oil exposed in larval shrimp[20] and herring[15]. The feeding rate for oil-exposed shrimp and sea urchin larvae returned almost to control levels after the oil exposure had ended. This reversible trend in feeding has also been observed for copepods first exposed to oil and then transferred to clean sea water[21]. This trend may explain why shrimps larvae and later stages sea urchin larvae were less impacted by the oil exposure

than the early sea urchin larval stages. Later developmental stages have more energy reserves and are then more able to cope with a transient food shortage[22]. This highlights the importance of considering several developmental stages and species when investigating the impacts of stressors to identify life history bottlenecks[23,24].

While a variety of responses were observed, dependent on species identity, stage and endpoint, no significant interaction was observed between global drivers and oil and the combined response was always additive. Different drivers may combine in synergistic, antagonistic and additive ways[25-31]. Moreover, similar drivers can interact in different ways depending on tested scenarios. One way to improve the predictive power of drivers interactions is to better understand their mode of action, i.e. the underlying biochemical and physiological mechanisms and pathways activated or deactivated, as a result of the exposure to a specific drivers: i.e. mode of actions. Our study demonstrates the potential of an alternative approach of making predictions based upon modes of action and interactions of drivers. Global drivers and oil impact larvae by reducing and shifting the energy budget through different mode of action, extra energy costs for global drivers and narcotic effect for oil, with no interaction. Consequently, they combine in an additive manner with negative effects for growth.

The present study highlights the importance that the correct management of local drivers such as oil pollution, but also for example contaminations from agricultural activities and pharmaceutical product and overfishing, will have within the context of OA and OW. In fact, an effective management of local drivers could slow down the detrimental impact of future global environmental change[32]. Just as Biodiversity at a regional level reflects Biodiversity at a global scale, adequate management actions at a local scale can have global consequences. This in turn would empower and encourage local decision makers to act on local driver regulations, and by doing so obtain positive effects on natural populations and environment more resilient to the negative impacts of future global drivers[33].

## Methods

**Global drivers in combination with oil-spill experiments**. Two experiments were performed to study the effect of a 4 d Arctic crude oil spill scenario (0.5 mg $L^{-1}$) and a chronic exposure to different pH (8.1 and 7.6) on the biology of larval stages of the green sea urchin (*Strongylocentrotus droebachiensis*) in April 2011 (duration exp.1: 18 d, exp. 2: 44 d). Adult green sea urchins *S. droebachiensis* were collected by scuba divers in Lysefjorden, Norway at 15 m depth in mid-February 2011. One experiment to study the effect of a 7 d Arctic crude oil spill scenario (0.5 mg $L^{-1}$) under high (T = 6.7 °C, pH = 8.1) and decreased pH scenarios (T= 9.5 °C, pH = 7.6) was carried out in February 2012 (duration 44 d). Adult females of the Northern shrimp (*Pandalus borealis*) with embryos were collected from Hillefjord (north of Åmøy Rogaland County, Norway; 59° 04' 00" N - 5° 45' 00' E) during January 2012. Six replicates for each four treatments were used for all three experiments. For a more detailed protocol, see supplementary information. All experiments were carried out at the IRIS (International Research Institute of Stavanger) environment research facilities (Stavanger, Norway). A continues flow system (CFS) was used to create a dispersion of crude Arctic oil in the sea water[34]. In the aquaria with reduced pH treatment, the targeted $pCO_2$ equilibration was achieved, by manipulating pH using the method by Widdicombe and Needham (2007)[35] and temperature was tightly regulated using heat exchangers. For seawater chemistry and oil measurements and results see supplementary information and Tables S1 and S2 for more information. Finally, for details on fertilization larval maintenance see supplementary information.

**Measurements of mortality.** Sea urchin larval cultures were monitored daily. Each day one subsample (exp. 1) or two subsamples (exp. 2) of 10 mL were collected from each culture, counted and fixed in buffered 4 % paraformaldehyde (PFA) in FSW for later analysis. Relative density (%) was significantly and linearly correlated with time in all 21 cultures except for three ($p < 0.05$, see Table S5 and S6). Shrimp larval cultures were monitored for dead larvae each day and relative density

(% of initial density) was significantly and linearly correlated with time in all cultures ($p < 0.01$; see Table S4). From these relationships, the mean mortality rate (MR in % $d^{-1}$) was calculated for each treatment.

**Morphometrics.** For the sea urchin experiment, 10-15 larvae were photographed every other day in each replicate with a digital camera mounted on a dissecting microscope (5x, 10x or 20x magnification, depending upon stage) using polarized light to visualize the skeleton. Three morphometric parameters (body length (BL) and posterolateral rod lengths (POL1, POL2)) were measured for each larvae using Image J software. Body length growth rates (BL GR in μm $ln(d)^{-1}$) were calculated from the slopes of the lines described by the significant, logarithmic relationship between BL (μm) and time (d). Symmetry index (SI) was calculated for each larvae as following $SI = \frac{Min(POL1,POL2)}{Max\ (POL1,POL2)}$. Total length (TL) was measured for a selection of shrimp larvae: 6 - 13 indiv. *per* aquaria, six aquaria *per* treatment. Morphological classifications (i.e. presence of abnormalities) of abdominal sixth segment of the stage IV shrimp larvae were also performed, the larvae sixth elements were classified as following: i. no abnormality, ii. missing endopodite, iii. missing exopodite and iv. unsymmetrical. All these classifications were pooled together and calculated as an abnormality index: i.e. % of abnormal larvae.

**Statistical analyses.** Statistical analyses were performed by using the SAS software. All endpoints (MR in % $day^{-1}$, BL GR in μm ln $(day)^{-1}$, SI, abnormality index and, shrimp larval length in mm) were analysed with a two-way analysis of variance (ANOVA) to test for significant differences between oil, global drivers and their interaction. Data was normally distributed according to the Kolmogorov-Smirnov test, and when not normally distributed $log_{10}$ transformed. Equal variance was tested using the Levene median test. The significance level α was set at 0.05.

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**Authors Contribution**

**M.A, R.K.B, ICT and SD** designed and conducted the experiments. **S.V.** and **S.W.** were responsible for designing the exposure systems and contributed to conducting the experiments. **T.B** contributed to feeding measurements of sea urchins. **M.A, R.K.B, P.C, S.D and J.S** analysed the data and wrote the manuscript with the help of all the co-authors. **S.B** contributed to shrimp swimming exercise

**Additional information**

Supplementary information

Competing financial interests

The author(s) declare no competing financial interests.

**Figure legends**

Figure 1. The effect of global drivers (pH and temperature) and Oil on larvae of the Norther shrimp *Pandalus borealis* and the green sea urchin *Strongylocentrotus droebachiensis*. a-c) mortality rate; d)

length; e-f) body length growth rate; g) abnormality; h-i) symmetry index. Control (pH 8.0, 6.7 ºC, white), Oi (pH 8.0, 6.7 ºC + Oil, light grey), OA/OW (pH 7.6, 9.5 ºC (for shrimp) and 6.7 ºC (for sea urchins), dark grey), OA/OW + Oil (pH 7.6, 9.5 ºC (for shrimp) and 6.7 ºC (for sea urchins) + Oil, black). Six replicates for each treatment. Histograms represent means values ± SD.

## Table legends

Table 1. The effects of global drivers (pH and temperature) and Oil on mortality, size/growth and abnormality/symmetry index. 0 no-significant effect, + significant positive effect and – significant negative effect.

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Figure 1.

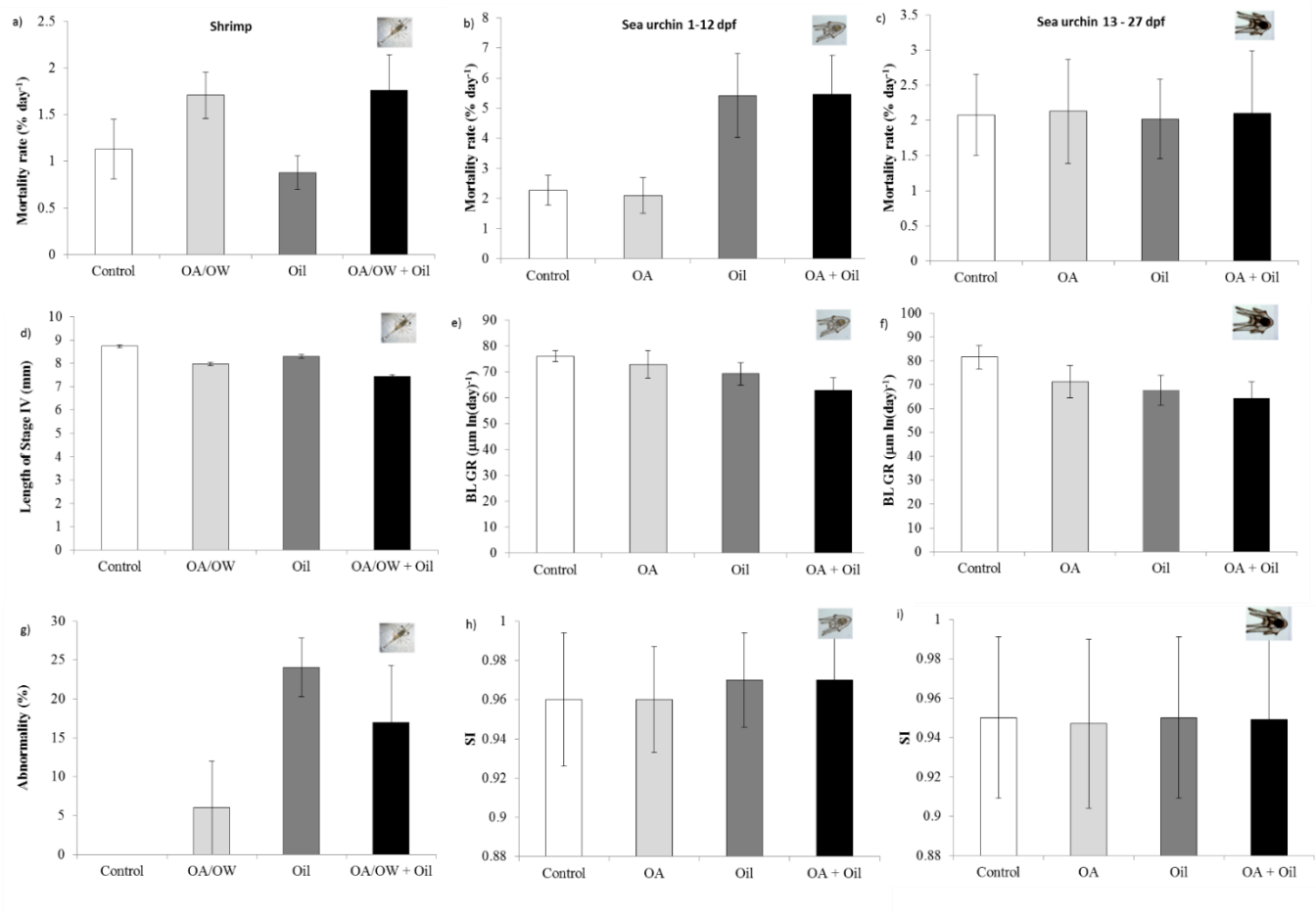
a)
Shrimp
Mortality rate (% day-1)
Control
OA/OW
Oil
OA/OW + Oil
b)
Sea urchin 1-12 dpf
Mortality rate (% day-1)
Control
OA
Oil
OA + Oil
c)
Sea urchin 13 - 27 dpf
Mortality rate (% day-1)
Control
OA
Oil
OA + Oil
d)
Length of Stage IV (mm)
Control
OA/OW
Oil
OA/OW + Oil
e)
BL GR (µm ln(day)-1)
Control
OA
Oil
OA + Oil
f)
BL GR (µm ln(day)-1)
Control
OA
Oil
OA + Oil
g)
Abnormality (%)
Control
OA/OW
Oil
OA/OW + Oil
h)
SI
Control
OA
Oil
OA + Oil
i)
SI
Control
OA
Oil
OA + Oil

Figure 2.

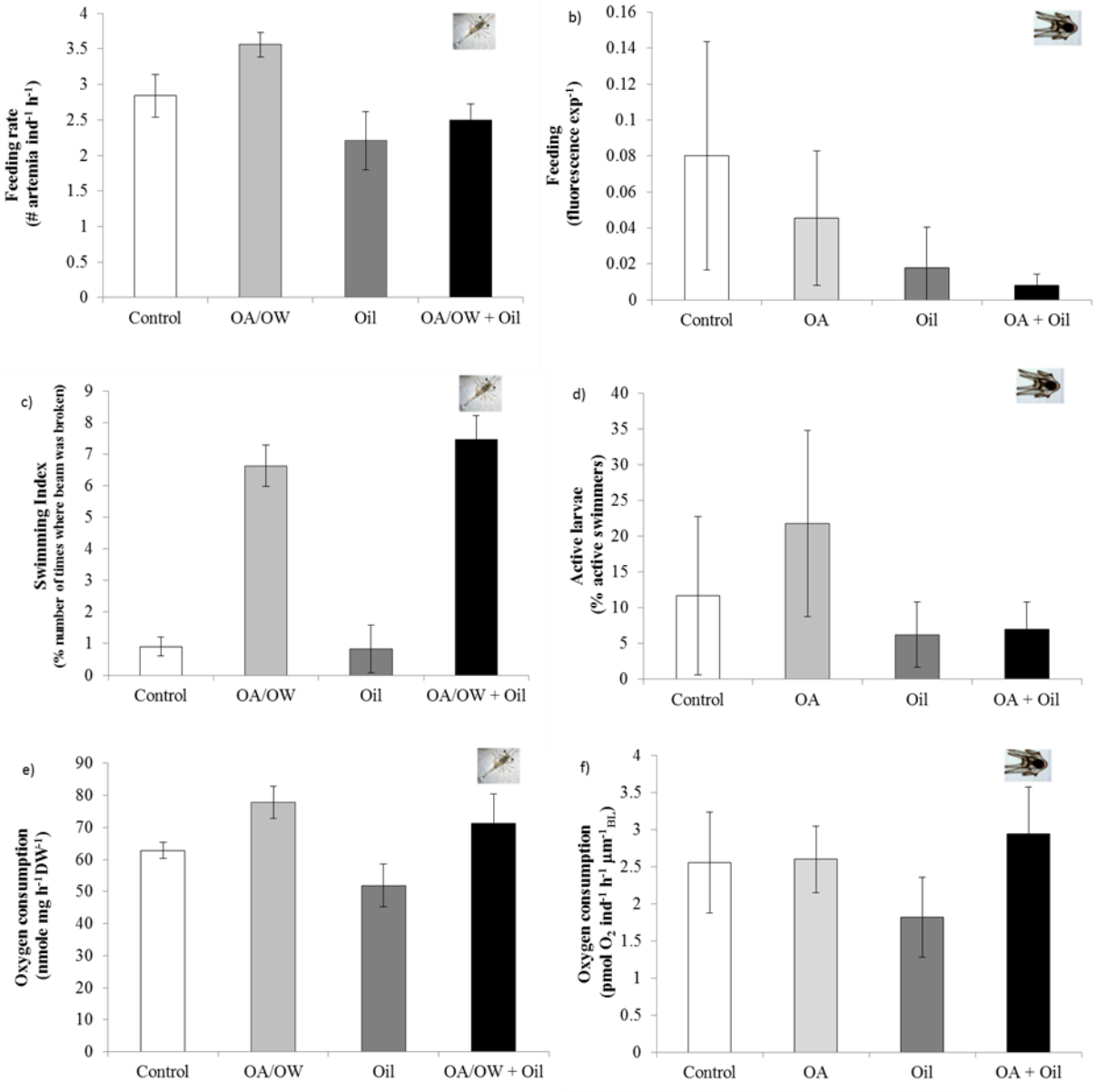
Feeding rate
(# artemia ind-1 h-1)
Control
OA/OW
Oil
OA/OW + Oil
b)
Feeding
(fluorescence exp-1)
Control
OA
Oil
OA + Oil
c)
Swimming Index
(% number of times where beam was broken)
Control
OA/OW
Oil
OA/OW + Oil
d)
Active larvae
(% active swimmers)
Control
OA
Oil
OA + Oil
e)
Oxygen consumption
(nmole mg h-1 DW-1)
Control
OA/OW
Oil
OA/OW + Oil
f)
Oxygen consumption
(pmol O2 ind-1 h-1 µm-1 BL)
Control
OA
Oil
OA + Oil

**Table 1**

| Species | Mortality | | Size / Growth | | Abnormality / SI | |
|---|---|---|---|---|---|---|
| **Shrimp** | Oil | 0 | Oil | - (9 %) | Oil | + (20 %) |
| | OA/OW | + (30 %) | OA/OW | - (5 %) | OA/OW | 0 |
| | OA/OW x Oil | 0 | OA/OW x Oil | - (15 %) | OA/OW x Oil | 0 |
| **Sea urchin (Early exposure to oil)** | Oil | + (250 %) | Oil | - (6 %) | Oil | 0 |
| | OA/OW | 0 | OA/OW | - (11 %) | OA/OW | 0 |
| | OA/OW x Oil | 0 | OA/OW x Oil | - (17 %) | OA/OW x Oil | 0 |
| **Sea urchin (Late exposure to oil)** | Oil | 0 | Oil | - (9 %) | Oil | 0 |
| | OA/OW | 0 | OA/OW | - (14 %) | OA/OW | 0 |
| | OA/OW x Oil | 0 | OA/OW x Oil | - (21%) | OA/OW x Oil | 0 |

Supplementary material

Results

**Seawater chemistry / Oil dispersal**

*Sea urchin*

Carbonate chemistry and temperature in Experiment 1 and 2 are summarized in Table S1. Although some significant differences in temperature were detected between aquaria for experiment one (One–way ANOVA, $F_{23, 71} = 4.22$, $p < 0.0001$), the actual difference was less than 0.1 °C. Temperature ranged from 9.4 to 9.5 °C. No significant differences were detected in temperature between aquaria for experiment two (One–way ANOVA, $F_{23, 161} = 1.20$, $p = 0.260$). No significant differences were detected in the alkalinity of the different pH treatments (TA = 2306 ± 14 µmol $kg^{-1}$, One-way ANOVA, $F_{1, 9} = 0.06$, $p = 0.803$), and only departed from the mean by 0.61 %. $pH_{NBS}$ fluctuated less than 1 % from the mean values. Mean $pCO_2$ values in the treatments ranged from 476 to 634 µatm at $pH_{NBS} = 8.0$ and 1292 to 1569 at $pH_{NBS} = 7.6$. Sea water was under saturated with respect to aragonite at $pH_{NBS} < 7.6$ (Table S1). Oxygen concentration in the treatments varied between 9.3 to 9.7 mg $L^{-1}$.

Oil chemistry for treatments in Experiment 1 and 2 is summarized in Table S2. The nominal oil concentration in the exposure set-up was 0.5 mg $L^{-1}$ and the mean measured PAHs concentration for each treatment was 4.786 and 4.480 µg $L^{-1}$ for the oil and combined treatments respectively. Approximately 90 % of the PAHs in the exposure tanks were C0-C3 naphthalene's, and the remainder 3-ring PAHs and dibenzothiophenes (DBTs). There was a significant difference in the mean concentration of naphthalene between the control and the oil treatments indicating that the control samples were not contaminated with oil (One-way ANOVA, $F_{1, 10} = 38.30$, $p < 0.050$).

*Shrimp*

No significant difference in mean $pH_{NBS}$ between temperature treatments in the current and future treatments were detected (One-way ANOVA, $F_{1, 11} = 0.81$, $p = 0.559$) and mean $pH_{NBS}$ were 8.05 and 7.60 respectively. Mean temperatures were 6.7 °C in the current treatments and 9.5 °C in the future treatments with no significant difference between the replicates (One-way ANOVA, $F_{1, 11} = 0.77$, $p = 0.787$). The total mean alkalinity measured in the treatments was 2305 ± 12 with no differences between the pH treatments (Mann-Whiteley U $p = 0.841$). Mean $pCO_2$ values in the treatments ranged from 470 to 537 µatm at $pH_{NBS} = 8.1$ and up to 1349 to 1795 at $pH_{NBS} = 7.6$. Sea water was under saturated with respect to aragonite when $pH_{NBS}$ was below 7.6 (Table S1).

The nominal oil concentration in the exposure set up was 0.5 mg $L^{-1}$ and the mean measured PAHs concentration for each treatment was found to be 4.895 and 5.545 µg $L^{-1}$ for the current-oil treatment

and the future-oil treatment respectively. Approximately 90 % of the PAHs in the exposure aquaria were C0-C3 naphthalene's, and the rest were 3-ring PAHs and dibenzothiophenes (DBTs). No naphthalene were detected in the control treatments. A detailed overview of the different PAH compounds measured in the different exposure concentrations are listed in table S2.

**Swimming, feeding, respiration**

*Sea urchin*

Experiment 1 – Impact of pH and oil exposure between 8-12 d post fertilisation (dpf).

Larval feeding (estimated as fluorescence $exp^{-1}$) was only affected significantly by the exposure to oil (ANOVA 2, model: $F_{3, 157} = 32.60$, $p < 0.0001$; oil: $F = 95.87$, $p < 0.0001$) but not by the OA ($F = 1.00$, $p = 0.32$) or their interaction ($F = 0.15$, $p = 0.70$). During and after exposure to oil, larvae were eating times less food than the controls (Figure S1b).

Experiment 2 – impact of pH and oil exposure between 23-27 dpf

Respiration rate (pmol $O_2$ $ind^{-1}$ $h^{-1}$) increased linearly with BL (Table S9). However, no significant effect of OA (ANCOVA, $F = 0.38$, $p = 0.540$), oil ($F = 4.30$, $p = 0.054$) or their interaction ($F = 0.91$, $p = 34$) was detected (Figure S1f).

During oil exposure (24 dpf), oil provoked significant swimming cessation in the oil exposed larvae with mean of 34 % passive larvae in the oil exposure treatment compared to the mean of 4 % passive larvae in the non-exposed larvae ($F_{1.23} = 50$, $p < 0.0001$). The presence of algae, alone or in interaction with other tested parameters, had no significant effect on the percentage of swimming larvae (SL; ANOVA 3, $F_{7, 45} = 4.64$, $p = 0.0008$; algae: $F = 2.55$, $p = 0.12$; all interactions $p > 0.05$). Both OA ($F = 4.50$, $p = 0.041$) and oil ($F = 16.52$, $p = 0.0002$) had significant effects on SL but there was no significant interaction ($F = 3.34$, $p = 0.09$). The percentage of active larvae was 2.5 times greater in absence of oil and was also higher at OA compared to control (Figure 1d).

Larval feeding (estimated as fluorescence $exp^{-1}$) was significantly affected by exposure to oil (ANOVA 2, model: $F_{2, 226} = 37.86$, $p < 0.0001$; oil: $F = 91.23$, $p < 0.0001$), the OA ($F = 18.08$, $p < 0.001$) and their interaction ($F = 5.57$, $p = 0.02$). Scheffe's post-hoc test revealed that larvae raised at OA were eating 1.8 times less than those raised at control. Moreover, exposure to oil resulted in a 6.2 times decrease in feeding compared to the control (Figure S1b).

*Shrimp*

For larvae stage 3 and 4, feeding rate (FR in artemia $ind^{-1}$ $h^{-1}$) developmental stages did not differ significantly among each other (ANOVA 3, stage F = 0.59, p = 0.45). Consequently, stages were pooled in future analyses. FR was significantly impacted by oil treatment (Figure S1a; ANOVA 2, $F_{3,43}$ = 4.49, p = 0.008; oil, F = 9.63, p = 0.004) but not OA/OW (F = 3.26, p = 0.08) or their interaction (F = 0.59, p = 0.45). The average FR was 1.2 times lower in larvae exposed to oil.

The swimming index (% number of time where laser beam was broken) was significantly increased by 8.1 in individuals exposed to OA/OW (Figure S2c; ANOVA 2, $F_{3, 18}$ = 29.74, p < 0.0001; OA/OW, F = 83.94, p < 0.0001) but not oil (F = 0.31, p = 0.58) or their interaction (F = 0.44, p = 0.52).

No significant impact (Figure S1e; ANOVA 2, $F_{3, 20}$=2.43, p=0.10) of oil and OA/OW could be detected for respiration (nmole $mg^{-1}$ dry weight $h^{-1}$).

Materials and methods

**Exposure systems**

*Sea urchin*

Sea urchin embryos and larvae were raised into two different pH treatments: control, $pH_{NBS}$ 8.0 (average $pH_{NBS}$ experienced by larvae at present) and OA, $pH_{NBS}$ 7.6, average pH experienced by larvae by 2100 and extreme of present natural variability; see Dorey et al 2013. For each pH, six flow through header tanks supplying 12 replicates chambers (vol. = 13 L, flow: 100 mL $min^{-1}$) conic aquaria bubbled with air stones. Seawater that supplied the aquaria were pumped directly from 75 m depth in the fjord close to the laboratory facilities, and filtered through two sand filters, two further filter stages (10 µm Claris filter and a 2 µm graded filter in Santoprene cartage holders) and then pumped into the header tanks where seawater was equilibrated at the target exposure conditions.

*Shrimp*

Shrimp larvae were raised into two different pH treatments: control, $pH_{NBS}$ 8.0, 6.7 °C, average $pH_{NBS}$ and temperature experienced by larvae at present, and OA/OW, $pH_{NBS}$ 7.6, 9.5 °C, average pH and temperature experienced by larvae by 2100; see IPCC 2013[1] scenarios and regional scenarios for relevant scenario[2-4]. Shrimp larvae were kept in two separate continuous flow systems, consisting of six header tanks (vol. = 12 L, flow = 1 L $min^{-1}$) where temperature was tightly regulated at either 6.7 ± 0.08 or 9.5 ± 0.07 °C respectively, using heat exchangers. Header tanks delivers seawater to 24 x 9 L aquaria each containing a batch of 200 shrimp larvae. Seawater that supplied the aquaria were pumped directly from 75 m depth in the fjord close to the laboratory facilities, filtered through two

sand filters, and then pumped into the header tanks where sea water was equilibrated at the target $pH_{NBS}$.

**pH manipulations**

In the aquaria with reduced pH treatment, the desired $pCO_2$ equilibration was achieved *via* manipulating pH using the pH-controllers (AB Aqua Medic GmbH pH computer, Bissendorf, Germany) set to maintain mean pH at 7.6, *via* a solenoid valve, which allowed regulating the addition of $CO_2$ gas. Seawater pH was recorded and logged every 5 min using a pH probe (Orion Star Plus™ 3-Star and Ross® Electrodes, Thermo Fisher Scientific Inc, Beverly, USA) coupled to a calibrated pH meter (Orion Star Plus™, Thermo Fisher Scientific Inc, Beverly, USA) and a multi-channel datalogger (D-130, Consort, Turmhout, Belgium) using the Star Plus Navigator 21 Software (Thermo Fisher Scientific Inc., Beverly, USA), and employing the NBS scale.

**Oil exposure**

A continues flow system (CFS) was used to create a dispersion of crude Arctic oil in sea water. A dispersion equivalent to a nominal oil concentration of 5 mg $L^{-1}$ was made by injecting oil into sea water under pressure (7 L $min^{-1}$ seawater and 0.042µL $min^{-1}$ oil), to form small oil droplets[5]. To make the exposure concentration of 5 µg $L^{-1}$, the dispersion was first conducted into a 10 L glass mixing flask for all three experiments.

*Sea Urchin*

To achieve the selected exposure concentration of 5 µg $L^{-1}$, the dispersion was first conducted into a 10 L glass mixing flask, and thereafter pumped (100 mL $min^{-1}$) by mean of peristaltic pumps (model 520,Watson and Marlow, Falmouth, UK) into the header tanks (flow: 1L $min^{-1}$) that supplied the aquaria. The impact of exposure to a 4 d oil spill was tested in two different experiments. (i) In the first experiment, half of the replicates (n = 6 *per* tested $pH_{NBS}$) were exposed to oil between 8 - 11 d dpf and larval response was followed till day 18 dpf. (ii) At the end of this experiment, each culture that was not exposed to oil spill (n = 6 for each $pH_{NBS}$ treatments) was split into two replicated cultures (n = 12 for each $pH_{NBS}$ treatments) and half of the replicates were exposed to oil at 23 - 27 dpf. Larval responses were monitored until day 44 dpf.

*Shrimp*

To construct an exposure concentration of 0.5 mg $L^{-1}$, the dispersion was first conducted into a 10 L glass-mixing flask, and thereafter pumped (6 mL $min^{-1}$) by peristaltic pumps (model 520, Watson and Marlow, Cornwall, UK) into the aquaria (flow = 120 mL $min^{-1}$). Shrimp larvae were exposed to oil from the day they hatched until 7 d post hatch.

**Larval fertilization, collection and maintenance**

*Sea urchin*

Spawning was induced in 11$^{th}$ of April 2011 by intracoelomic injection of 0.5 M KCl in filtered seawater (FSW). For the experiment, eggs of eight females were collected in separate 250 mL beakers, washed, and fertilized by adding dry sperm of three males to a final concentration of approximately ~1000 sperm $mL^{-1}$, allowing a fertilizations success > 95 %. Zygotes were allowed to divide once before they were pooled and were divided in the aquaria's. After 5 d and throughout the experiment, larvae were fed once a day (concentration 150 µg C $L^{-1}$ , with an initial algal density approximately 6,000-7,000 cells $mL^{-1}$ and mean size 7.5 µm ± 0.8 µm) with the cryophyte algae *Rhodomonas* sp., which were raised in filtered seawater containing growth media (Himedia, Mumbai, India) at 20 °C. Until day 18 the larvae were also fed with the algae *Isocrysis.sp* (concentration 150 µg C $L^{-1,}$ with an initial algal density approximately 186,000 cells $mL^{-1}$ and mean size 4.5µm ± 0.5µm). Carbon content of the algae was estimated based on bio volume measurements as equivalent spherical diameter (ESD) with an electronic particle analyser (Elzone 5380 Micrometrics, Achen, Germany) and equations provided by Mullin et al[6]. Algal cell density and size were measured using a coulter counter (Beckman coulter multisizer 3$^{TM}$, Nerliens Meszansky AS, Oslo, Norway).

*Shrimp*

Forty-eight ovigerous females were acclimated two weeks to laboratory control conditions (T = 7 °C, S = 33, pH = 8). The total number of females was selected to ensure that a large number of shrimp larvae would hatch simultaneously Arnberg et al.[7] following an exposure period to experimental conditions of a minimum of two weeks before hatching. Ovigerous females (N = 24 in total, six *per* treatment) were transferred to individual flow-through aquaria (vol. = 9 L, flow rate = 0.12 L $min^{-1}$). Each aquarium was checked every day in order to determine when hatching occurred. Batches of larvae (N = 200) all from the same female were kept in the same individual glass aquaria as described Arnberg et al.[7] (vol. = 9 L). From each experimental treatment six batches from six different mothers, were exposed and monitored until they reached stage IV zoea. The batches, consisting of 200 larvae (< 24 h old) from each mother, were kept separate and not mixed. Post-hatch shrimp larvae were fed *ad libitum* on freshly hatched, *Artemia salina* nauplii (*Artemia* length 450-550 µm, feeding density

1000 indiv $L^{-1}$) twice a day (morning and afternoon) for the entire duration of the experiment. In addition, for the first week, larvae were also fed with phytoplankton *Thalassiosira weisslogi* 1200™ (Microalgae, Vigra, Norway, 2 x $10^4$ cells $L^{-1}$) once a day, to ensure feeding for stage I and II zoea following the protocols of Pedersen and Storm[8] and Ariza and Ouellet[9]. Dead *Artemia* were removed every day to avoid seawater contamination.

**Feeding tests**

*Sea urchin*

During the feeding test larvae were feed once each day with only 150 µg carbon (C) $L^{-1}$ *Rhodomonas. sp*. Fluorescence microscopy was used to assess the algal content in the stomachs of the sea urchin larvae at a given time point, to look at the differences in the treatments. One hour after feeding, larvae were collected in each culture and pictures were taken. The picture were taken using a Zeiss Axioplan 2 imaging microscope fitted with an Axiocam MRc5 camera, bifocal (5X) with a fluorescent rhodamine filter. Semi-quantitative measurements of occurrence of algae in the digestive system of the sea urchin larvae was done by quantifying the saturation of red fluorescence in images collected with the rhodamine filter using Axiovision software. For each larva, body length (µm), area of larval stomach and stomach fluorescence were measured (Fig. S2 a-b). Stomach fluorescence was divided by the exposure time to obtain the differences in exposure time and presented as red fluorescence $ms^{-1}$. Feeding was assessed in exp. 1 12-15 dpf and in exp. 2 23-33 dpf.

*Shrimp*

To quantify larvae feeding rates, we used a modification of the clearing rate methods by[10]. Feeding trials were conducted in 1 L glass bottles containing filtered sea water drawn from the treatment header tanks used in the experiment. Five larvae of the same stage of development were transferred into the experimental bottles, placed in temperature-controlled environments (T = 6.7 or 9.5 °C) and starved for approx. 24 h prior to the experiments. Freshly hatched *Artemia* nauplii (initial prey conc. 150 indiv $L^{-1}$) was added to 1 L Schott bottles containing the larvae. The bottles were sealed with lids and incubated for 6 h at the respective temperature. After the incubation period, shrimp larvae were carefully removed from the bottles and the remaining water containing *Artemia* nauplii was sieved using a 40 µm BD Falcon™ cell strainer (BD Biosciences, Franklin Lakes, USA). Individual nauplii were counted. Amount of prey consumed was calculated as initial number of *Artemia* nauplii minus their final number. Feeding rate was expressed as number of prey consumed *per* individual shrimp larvae *per* unit time (number of *Artemia* $indiv^{-1}$ $h^{-1}$). A control sample from the current treatment

without shrimp larvae was run through the experimental procedure, to test the efficiency of the sieving procedure. All 150 *Artemia* sp. nauplii were recovered in each control test.

**Swimming/activity tests**

*Sea urchin*

A swimming activity test according to[11] with modifications was performed at 31 dpf. Six replicated cultures in each treatment were tested. 15 larvae were transferred to 2 mL glass vials with treatment sea water (pH 8.1 or 7.6), with 300 µg C $L^{-1}$ *Rhodomonas sp* algae or without algae. The syringe chamber was filled with 2 mL test water with 300 µg C $L^{-1}$ algae and without algae, and was connected on to the glass vial with a silicon tube. After 24 h the number of larvae that had entered the syringe chamber was counted as "active swimmers".

*Shrimp*

Swimming behavior was measured using a combination of infrared and white light emitting diodes, together with a phototransistor. Test groups of between 10 and 15 larvae were placed into 1 L glass beaker (9.2 cm diam.) using water from their specific treatment group. The Infrared light emitting diode was aligned with the phototransistor on opposite sides of the glass beaker. Both of these components were encapsulated within 8 mm plastic tubes, which were supported in position by insertion into holes drilled into two wooden pillars. The width of the infrared light beam passing through the water, together with the surface area of the phototransistor open to this light, was controlled by the use of baffles pushed into the support pillars in front of the phototransistor and emitter. The baffles were drilled centrally to a diam. of 1.5 mm. As larvae swam through the light beam, breaking its path, the reduced amount of light, falling onto the phototransistor caused a drop in the output voltage. In the absence of these baffles, the movement of the small larvae within the much broader beam would be more difficult to detect. The white light emitting diode was positioned directly above the phototransistor. It provided the light source towards which the larvae swam. Preliminary observations had shown that larvae swam upwards into the path of light then ceased swimming, falling slowly back down within the vessel before recommencing swimming back into the light. This behavior allows response to light to be measured as the intensity of repeated swimming activity recorded as the number beam breaks per hour. Voltage output from the phototransistor was logged at intervals of 0.2 sec. throughout the one-hour test periods using a data logger (NI USB - 6009, National Instruments, Texas, USA). All tests were conducted in an ambient low light environment. Stage III larvae after oil exposure was used in this experiment.

**Respiration**

*Sea urchin*

Respiration measurements were conducted at 15, 23, 30 and 37 dpf, following the methods of Taylor and Spicer (1989). A custom-built closed glass-bottle respirometer (vol. = 5 mL), equipped with airtight stoppers in which $O_2$ electrodes was used. Manually picked sea urchin larvae (N = 200) were placed in the incubation chambers filled with seawater at the appropriate pH and sealed with an airtight stopper. Chambers were submerged in flow through water baths to maintain a constant temperature. A total of six replicates were carried out for each of the four decreased pH/oil combinations (one measurement per replicated culture). Measurements of dissolved $O_2$ concentration were performed every 2 sec for the entire duration of the incubation (approx. 48 h) using $O_2$ electrodes (1302, Strathkelvin Instruments, Glasgow, UK) connected to a calibrated to a multichannel oxygen meter (928, Strathkelvin Instruments). $O_2$ electrodes were calibrated with 100% oxygenated water and 0% oxygenated water. Continuous measurements were conducted to demonstrate linearity of $pO_2$ decline in the respiration chambers during the incubation. Larva free seawater from the appropriate treatment was used to assess background respiration rates caused by microorganisms in the system. Background respiration never exceeded 10 % of the total larval respiration (2.5 µmol $O_2$ $h^{-1}$). These background rates were then used to correct the measured larval respiration. At the end of each trial, number of viable larvae were checked (mean 0.8 ± 2.5 % were dead) and then fixed in buffered 4 % PFA in seawater for later size determinations. Respiration results were corrected by the number of living larvae *per* respirometer (individual $O_2$ consumption: pmol $O_2$ $ind^{-1}$ $h^{-1}$ $um^{-1}$ $_{BL}$).

*Shrimp*

To estimate stage IV larvae metabolic rates, we measured their rates of oxygen consumption described above, the only difference that the volume of the respirometers were smaller (vol. = 300 mL). Shrimp larvae (N = 15 at each time) were placed in the incubation chambers with the appropriate treatment header tank water used in the experiment and sealed with the airtight stoppers, and then placed in flow through water baths to maintain a constant temperature. A total of six replicates were carried out for each of the treatments. Following preliminary trials, measurements of dissolved oxygen concentration in the respirometers were performed every 1 min for the entire duration of the incubation (approx. 24 h) using oxygen electrodes (DP-PSt3, PreSens, Regensburg, Germany) coupled to a calibrated four channel oxygen meter (OXY-4, PreSens). Continuous measurements were conducted to demonstrate linearity of oxygen tension decline in the respiration chambers during the incubation (N = 24). No changes in oxygen consumption rates were detected. Larva free samples

of sea water from the appropriate treatment header tank were used to assess background respiration rates caused by microorganisms in the system. Background respiration never exceeded 2 % of the total larval respiration. These background rates were then used to correct the measured larval respiration values we obtained. At the end of each trial, larvae were stored at – 80 °C in order, at a later date, to conduct morphological and dry mass determinations described by Arnberg et al.[7]

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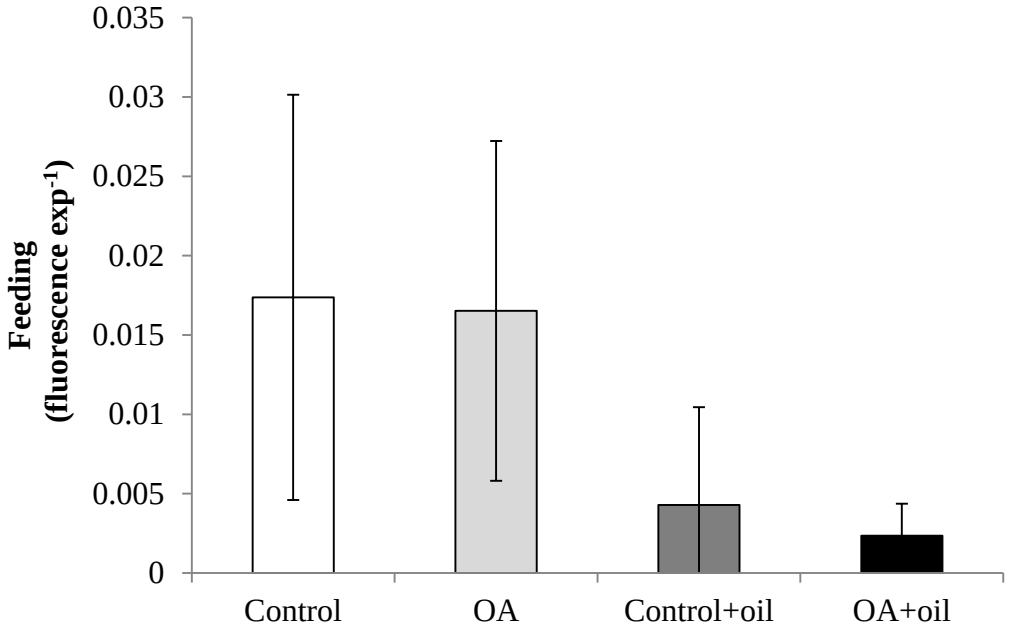


**Figure S1.** The effect of global drivers (pH and temperature) and Oil on early (day 8-12 post hatch) larval *Strongylocentrotus droebachiensis* on feeding rate. (pH 8.0, 6.7 ºC, white), Oil (pH 8.0, 6.7 ºC + Oil, light grey), OA/OW (pH 7.6, 9.5 ºC (for shrimp) and 6.7 ºC (for sea urchins), dark grey), OA/OW + Oil (pH 7.6, 9.5 ºC (for shrimp) and 6.7 ºC (for sea urchins) + Oil, black). Six replicates for each treatment. Histograms represent means values ± SD.

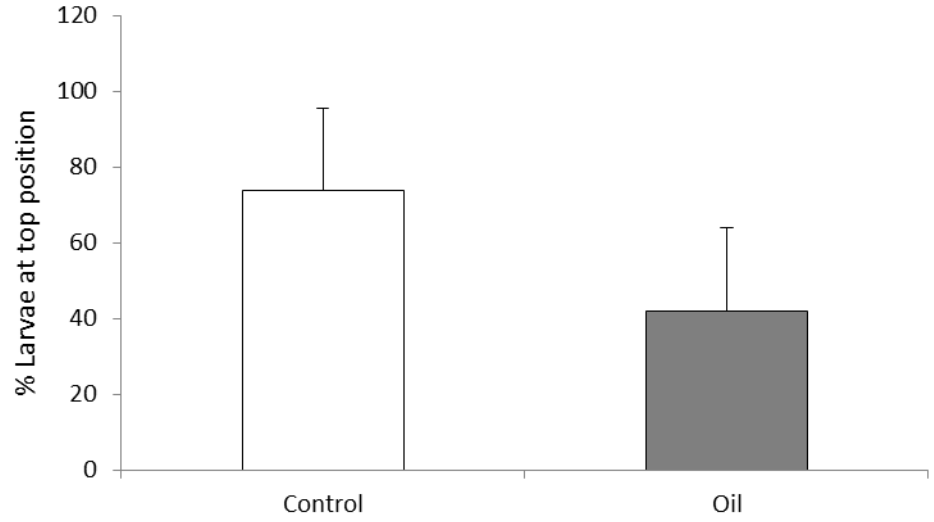


**Figure S1.** The effect of oil on larval *Pandalus borealis* swimming. (pH 8.0, 6.7 ºC, white), Oil (pH 8.0, 6.7 ºC + Oil, light grey) Six replicates for each treatment. Histograms represent means values ± SD.

**Table S1**. Seawater physiochemical conditions during the three experiments, (exp. 1.Sea urchin 18 d oil and OA exposure, exp. 2. Sea urchin 44 d oil and OA exposure, and exp. 3. Shrimp 30 d oil and OA/OW exposure). $pH_{NBS}$; pH NBS scale, $A_T$; total alkalinity, DIC ; total dissolved inorganic carbon, $pCO_2$; partial pressure of $CO_2$. For temperature mean values (± SD are provided), for all the calculated variables range of values are provided.

| Incubation group | Temperature (°C) | $pH_{NBS}$ | $CO_2$ (uatm) | DIC | $A_T$ |
|---|---|---|---|---|---|
| *Experiment 1* *(Sea urchin)* | | | | | |
| Control | 9.41 (± 0.03) | 8.03 (7.98 - 8.08) | 555 (489 - 634) | 2183 (2166-2199) | 2306 (± 14) |
| OA | 9.41 (± 0.03) | 7.64 (7.59 - 7.68) | 1455 (1323 – 1646) | 2307 (2283-2320) | 2306 (± 14) |
| Oil | 9.40 (± 0.11) | 8.06 (8.03 - 8.09) | 515 (476 – 559) | 2172 (2170-2176) | 2306 (± 14) |
| OA + Oil | 9.52 (± 0.08) | 7.64 (7.61 - 7.68) | 1455 (1323 -1569) | 2306 (2305-2307) | 2306 (± 14) |
| *Experiment 2* *(Sea urchin)* | | | | | |
| Control | 9.41 (± 0.03) | 8.02 (7.98 - 8.07) | 569 (502 - 634) | 2188 (2178-2193) | 2306 (± 14) |
| OA | 9.41 (± 0.03) | 7.63 (7.59 – 7.68) | 1485 (1318 – 1640) | 2307 (2283-2320) | 2306 (± 14) |
| Oil | 9.40 (± 0.11) | 8.03 (7.99 - 8.07) | 555 (501 – 618) | 2184 (2178-2190) | 2306 (± 14) |
| OA + Oil | 9.52 (± 0.08) | 7.64 (7.59 – 7.69) | 1455 (1292 – 1646) | 2307 (2291-2333) | 2306 (± 14) |
| *Experiment 3* *(Shrimp)* | | | | | |
| Control | 6.7 (± 0.08) | 8.05 (8.03-8.08) | 511 (470-537) | 2185 (2180-2189) | 2305 (± 12) |
| OA/OW | 9.5 ( ± 0.07) | 7.59 (7.55-7.67) | 1627 (1354-1795) | 2206 (2199-2209) | 2305 (± 12) |
| Oil | 6.7 ( ± 0.07) | 8.05 (8.03-8.08) | 511 (470-537) | 2187 (2180-2189) | 2305 (± 12) |
| OA/OW + Oil | 9.5 (± 0.06) | 7.60 (7.56-7.67) | 1589 (1349-1753) | 2315 (2306-2318) | 2305 (± 12) |

**Table S2.** Mean concentration (± SD) of PAHs (µg $L^{-1}$) in water samples from the control and OA/OW Oil exposed aquaria. The samples were taken during the experiment (n = 2) for each treatment.PAH analyses of seawater were preformed by Gas Chromatography (HP5890, Hewlett Packard, USA) and analysed in ion mode (GC/MS – SIM) as described previously in Jonsson, Bechmann et al. (2004). nd = not detected. SC = shrimp control, UC = sea urchin control, SOIL = shrimp Oil, SOA/OW + Oil = shrimp OA/OW + Oil, U1Oil = Sea urchin exp.1 Oil, U1OA + Oil = Sea urchin exp.1 OA + Oil, U2Oil = Sea urchin exp. 2 Oil and U2OA + Oil = Sea urchin exp.2 OA + Oil.

| Chemical (µg L-1) | SC | UC | S Oil | SOA/OW+Oil | U1 Oil | U1 OA+Oil | U2 Oil | U2 OA+Oil |
|---|---|---|---|---|---|---|---|---|
| Naphthalene | nd | nd | 0.299 ± 0.008 | 0.335 ± 0.025 | 0.435 ± 0.0295 | 0.450 ± 0.0566 | 0.445 ± 0.1595 | 0.338 ± 0.0347 |
| C1-Naphthalene | nd | 0.0018 | 1.028 ± 0.015 | 1.143 ± 0.092 | 0.493 ± 0.0361 | 0.539 ± 0.0682 | 0.523 ± 0.1869 | 0.3867 ± 0.0396 |
| C2-Naphthalene | nd | nd | 1.785 ± 0.066 | 2.014 ± 0.143 | 1.698 ± 0.1466 | 1.916 ± 0.2808 | 1.797 ± 0.6419 | 1.3212 ± 0.1224 |
| C3-Naphthalene | nd | nd | 1.391 ± 0.041 | 1.597 ± 0.088 | 1.4279 ± 0.1517 | 1.692 ± 0.4612 | 1.468 ± 0.5019 | 1.0642 ± 0.0969 |
| Sum 2 ring PAH | - | 0.0018 | 4.503 | 5.089 | 4.054 | 4.616 | 4.233 | 3.111 |
| Fluorene | nd | nd | 0.025 ± 0.001 | 0.026 ± 0.001 | 0.039 ± 0.0040 | 0.040 ± 0.0068 | 0.046 ±0.0167 | 0.032 ± 0.003 |
| Phenanthrene | nd | nd | 0.060 ± 0.004 | 0.068 ± 0.007 | 0.090 ± 0.0086 | 0.095 ± 0.0070 | 0.103 ± 0.0388 | 0.074 ±0.004 |
| C1-Phen/Anthr | nd | nd | 0.107 ± 0.004 | 0.122 ± 0.015 | 0.139 ± 0.0143 | 0.167 ± 0.0429 | 0.166 ± 0.0580 | 0.118 ± 0.0068 |
| C2-Phen/Anthr | nd | nd | 0.123 ± 0.005 | 0.146 ± 0.009 | 0.164 ± 0.0201 | 0.234 ± 0.136 | 0.203 ± 0.006 | 0.150 ± 0.0138 |
| Sum 3 ring PAH | - | - | 0.315 | 0.362 | 0.463 | 0.582 | 0.561 | 0.406 |
| Dibenzothiophene | nd | 0.0007 | 0.010 ± 0.001 | 0.011 ± 0.001 | 0.016 ± 0.0014 | 0.017 ± 0.0023 | 0.018 ± 0.0077 | 0.014 ± 0.0006 |
| C1-Dibenzothiophene | nd | nd | 0.028 ± 0.002 | 0.035 ± 0.0053 | 0.038 ± 0.007 | 0.058 ± 0.0286 | 0.047 ± 0.0164 | 0.035 ± 0.0053 |
| C2-Dibenzothiophene | nd | nd | 0.029 ± 0.001 | 0.036 ± 0.004 | 0.014 ± 0.0016 | 0.023 ± 0.0198 | 0.216 ± 0.0083 | 0.016 ± 0.0029 |
| Sum DBTs | - | 0.0007 | 0.067 | 0.08 | 0.126 | 0.192 | 0.179 | 0.124 |
| **Total PAHs** | **-** | **0.0025** | **4.895** | **5.545** | **4.628** | **5.34** | **4.944** | **3.62** |

**Table S3.** Results of multiple ANOVA testing for the effect of OA/OW and Oil, and their interaction on different parameters of the *Pandalus borealis* and *Strongylocentrotus droebachiensis* larvae. Degrees of freedom (df), mean of square (MS), F-ratio (F), probability level (*p*).

| **Parameter** | **Source** | **df** | **F** | **p** |
|---|---|---|---|---|
| *Shrimp larvae* | | | | |
| Mortality | Model | 3,23 | 2.98 | 0.05 |
| | OA/OW | 3 | 8.54 | 0.008 |
| | Oil | 3 | 0.25 | 0.2 |
| | OA/OW*Oil | 3 | 0.16 | 0.69 |
| Feeding rate | Model | 3,43 | 4.49 | 0.008 |
| | OA/OW | 3 | 3.26 | 0.08 |
| | Oil | 3 | 9.63 | 0.004 |
| | OA/OW*Oil | 3 | 0.59 | 0.45 |

| | | | | |
|---|---|---|---|---|
| Growth/size | Model | 3,282 | 66.79 | <0.0001 |
| | OA/OW | 3 | 137.91 | <0.0001 |
| | Oil | 3 | 62.25 | <0.0001 |
| | OA/OW*Oil | 3 | 0.54 | 0.46 |
| Swimming index | Model | 3,18 | 29.74 | <0.0001 |
| | OA/OW | 3 | 83.94 | <0.0001 |
| | Oil | 3 | 0.31 | 0.58 |
| | OA/OW*Oil | 3 | 0.44 | 0.52 |
| The abnormality index | Model | 3.22 | 4.75 | 0.012 |
| | OA/OW | 3 | 12.10 | 0.002 |
| | Oil | 3 | 0.92 | 0.92 |
| | OA/OW*Oil | 3 | 1.69 | 0.21 |
| Respiration | Model | 3,20 | 2.43 | 0.10 |
| *Sea Urchin larvae* | | | | |
| Experiment 1 | | | | |
| Mortality | Model | 3,21 | 21.83 | <0.0001 |
| | OA | 3 | 0.04 | 0.84 |
| | Oil | 3 | 65.45 | <0.0001 |
| | OA*Oil | 3 | 0.01 | 0.93 |
| Feeding rate | Model | 3,157 | 32.60 | <0.0001 |
| | OA | 3 | 1.00 | 0.32 |
| | Oil | 3 | 95.87 | <0.0001 |
| | OA*Oil | 3 | 0.15 | 0.70 |
| Growth/size | Model | 3.23 | 9.94 | 0.0003 |
| | OA | 3 | 21.92 | <0.0001 |
| | Oil | 3 | 21.92 | <0.0001 |
| | OA*Oil | 3 | 0.77 | 0.77 |
| Symmetry Index | Model | 23,560 | 1.53 | 0.054 |
| Experiment 2 | | | | |

| | | | | |
|---|---|---|---|---|
| Mortality | Model | 3,18 | 0.02 | p>0.99 |
| Feeding rate | Model | 2,226 | 37.86 | <0.0001 |
| | OA | 2 | 18.08 | <0.0001 |
| | Oil | 2 | 91.23 | <0.0001 |
| | OA*Oil | 2 | 5.57 | 0.02 |
| Growth/size | Model | 3,23 | 8.51 | 0.0008 |
| | OA | 3 | 6.97 | 0.0157 |
| | Oil | 3 | 16.73 | 0.0006 |
| | OA*Oil | 3 | 1.85 | 0.19 |
| Swimming index | Model | 7,45 | 4.64 | 0.0008 |
| | OA | | 4.50 | 0.041 |
| | Oil | | 16.52 | 0.0002 |
| | OA*Oil | | 3.34 | 0.09 |
| Symmetry index | Model | 23,3524 | 0.64 | 0.054 |

**Table S4** Results from regression analysis of shrimp mortality in *P. borealis* larvae raised in control (pH 8.1), OA/OW (7.6), Oil (pH 8.1 + Oil conc. 0.5 mg $L^{-1}$) and OA/OW + Oil (pH 7.6 + Oil conc. 0.5 mg $L^{-1}$).

| Treatment | Replicate | MR | df | F-value | Intercept | $R^2$ | p-value |
|---|---|---|---|---|---|---|---|
| Control | 1 | 2.61 | 24 | 132.48 | 113.03 | 0.85 | <0.0001 |
| | 2 | 1.27 | 24 | 382.75 | 103.78 | 0.95 | <0.0001 |
| | 3 | 0.45 | 24 | 513.13 | 101.12 | 0.96 | <0.0001 |
| | 4 | 0.67 | 24 | 277.48 | 101.66 | 0.94 | <0.0001 |
| | 5 | 0.60 | 24 | 403.92 | 101.51 | 0.95 | <0.0001 |
| | 6 | 1.17 | 24 | 571.51 | 100.85 | 0.96 | <0.0001 |
| OA/OW | 1 | 1.44 | 24 | 289.89 | 103.77 | 0.94 | <0.0001 |
| | 2 | 0.98 | 24 | 312.23 | 102.47 | 0.95 | <0.0001 |
| | 3 | 1.60 | 24 | 412.48 | 101.24 | 0.96 | <0.0001 |
| | 4 | 2.53 | 24 | 100.44 | 99.93 | 0.85 | <0.0001 |
| | 5 | 2.34 | 24 | 175.52 | 101.74 | 0.90 | <0.0001 |
| | 6 | 1.35 | 24 | 276.27 | 101.26 | 0.94 | <0.0001 |
| Oil | 1 | 1.53 | 24 | 637.42 | 104.21 | 0.97 | <0.0001 |
| | 2 | 0.30 | 24 | 516.64 | 99.72 | 0.96 | <0.0001 |
| | 3 | 0.98 | 24 | 443.23 | 97.78 | 0.95 | <0.0001 |
| | 4 | 0.61 | 24 | 241.36 | 101.71 | 0.93 | <0.0001 |
| | 5 | 1.18 | 24 | 523.96 | 102.49 | 0.96 | <0.0001 |
| | 6 | 0.68 | 24 | 290.24 | 100.65 | 0.94 | <0.0001 |
| OA/OW + Oil | 1 | 3.54 | 24 | 272.93 | 102.53 | 0.94 | <0.0001 |
| | 2 | 1.54 | 24 | 395.46 | 104.80 | 0.95 | <0.0001 |
| | 3 | 1.85 | 24 | 134.39 | 98.06 | 0.88 | <0.0001 |
| | 4 | 1.30 | 24 | 263.29 | 99.97 | 0.93 | <0.0001 |
| | 5 | 1.51 | 24 | 894.72 | 98.20 | 0.98 | <0.0001 |
| | 6 | 0.80 | 24 | 172.38 | 99.40 | 0.88 | <0.0001 |

**Table S5** Larval mortality rate (MR in % $day^{-1}$) in sea urchin exp.1 were calculated as the coefficient of the significant linear relationship between mortality and tpf. Results of the regressions (Intercept, p-value, $R^2$, F-value and df: degree of freedom) are given for each culture replicate with corresponding treatment. Data in bold ($p > 0.05$) were removed from subsequent analyses.

| Treatment | Replicate | MR | df | F-value | Intercept | $R^2$ | p-value |
|---|---|---|---|---|---|---|---|
| Control | 1 | 1.53 | 15 | 16.29 | 0.25 | 0.54 | 0.0012 |
| | **2** | **0.95** | **15** | **1.60** | **0.05** | **0.10** | **0.23** |
| | 3 | 2.50 | 15 | 41.09 | -0.035 | 0.75 | <0.0001 |
| | 4 | 2.41 | 15 | 9.67 | 0.18 | 0.41 | 0.008 |
| | **5** | **0.98** | **15** | **2.19** | **0.074** | **0.14** | **0.16** |
| | 6 | 2.63 | 15 | 43.12 | -0.035 | 0.75 | <0.0001 |
| OA/OW | 1 | 2.24 | 15 | 11.80 | 0.079 | 0.46 | 0.004 |
| | 2 | 2.96 | 15 | 28.86 | -0.0072 | 0.67 | <0.0001 |
| | 3 | 1.90 | 15 | 15.94 | -0.0080 | 0.53 | 0.0013 |
| | 4 | 2.11 | 15 | 13.38 | 0.013 | 0.49 | 0.0026 |
| | 5 | 1.32 | 15 | 6.77 | 0.11 | 0.33 | 0.021 |
| | **6** | **1.23** | **14** | **2.00** | **0.14** | **0.13** | **0.19** |
| Oil | 1 | 7.08 | 15 | 304.73 | -0.12 | 0.96 | <0.0001 |
| | 2 | 3.92 | 15 | 27.88 | -0.033 | 0.67 | <0.0001 |
| | 3 | 7.22 | 15 | 157.46 | -0.16 | 0.92 | <0.0001 |
| | 4 | 5.00 | 15 | 73.67 | -0.079 | 0.84 | <0.0001 |
| | 5 | 4.43 | 15 | 41.54 | -0.075 | 0.75 | <0.0001 |
| | 6 | 4.84 | 15 | 9.88 | 0.071 | 0.41 | 0.0072 |
| OA/OW + Oil | 1 | 4.32 | 15 | 68.34 | -0.018 | 0.83 | <0.0001 |
| | 2 | 6.40 | 15 | 90.79 | -0.10 | 0.87 | <0.0001 |
| | 3 | 6.43 | 15 | 96.99 | 0.037 | 0.87 | <0.0001 |
| | 4 | 7.02 | 15 | 96.01 | -0.11 | 0.87 | <0.0001 |
| | 5 | 4.21 | 15 | 25.88 | -0.12 | 0.65 | 0.0002 |
| | 6 | 4.41 | 14 | 25.26 | 0.014 | 0.66 | 0.0002 |

**Table S6** Larval mortality rate (MR in % $day^{-1}$) in sea urchin exp. 2 were calculated as the coefficient of the significant linear relationship between mortality and tpf. Results of the regressions (Intercept, p-value, $R^2$, F-value and df: degree of freedom) are given for each culture replicate with corresponding treatment. Data in bold ($p > 0.05$) were removed from subsequent analyses.

| Treatment | Replicate | MR | df | F-value | Intercept | $R^2$ | p-value |
|---|---|---|---|---|---|---|---|
| Control | 1 | 1.1 | 24 | 19.66 | 0.05 | 0.46 | 0.0002 |
| | **2** | **0.16** | **24** | **0.27** | **0.10** | **0.012** | **0.61** |
| | 3 | 2.6 | 24 | 108.22 | -0.053 | 0.82 | <0.0001 |
| | 4 | 2.3 | 24 | 40.33 | 0.18 | 0.64 | <0.0001 |
| | 5 | 2.3 | 24 | 38.72 | -0.035 | 0.63 | <0.0001 |
| | 6 | 2.1 | 24 | 114.39 | 0.0026 | 0.83 | <0.0001 |
| OA/OW | 1 | 2.6 | 24 | 79.44 | 0.051 | 0.78 | <0.0001 |
| | 2 | 2.9 | 24 | 108.80 | -0.002 | 0.83 | <0.0001 |
| | 3 | 2.4 | 24 | 70.77 | -0.058 | 0.75 | <0.0001 |
| | 4 | 1.9 | 24 | 30.55 | 0.033 | 0.57 | <0.0001 |
| | 5 | 0.79 | 24 | 11.54 | 0.15 | 0.33 | 0.0025 |
| | 6 | 2.2 | 24 | 58.79 | 0.076 | 0.74 | <0.0001 |
| Oil | 1 | 1.4 | 24 | 55.00 | 0.030 | 0.71 | <0.0001 |
| | **2** | **0.069** | **24** | **0.05** | **0.11** | **0.0020** | **0.83** |
| | 3 | 2.4 | 24 | 93.17 | -0.040 | 0.80 | <0.0001 |
| | 4 | 1.8 | 24 | 23.78 | 0.22 | 0.51 | <0.0001 |
| | 5 | 2.8 | 24 | 44.40 | -0.077 | 0.66 | <0.0001 |
| | 6 | 1.7 | 24 | 48.85 | 0.030 | 0.68 | <0.0001 |
| OA/OW + Oil | 1 | 2.8 | 24 | 68.82 | 0.028 | 0.75 | <0.0001 |
| | 2 | 2.6 | 24 | 125.07 | 0.020 | 0.84 | <0.0001 |
| | 3 | 3.2 | 24 | 76.16 | -0.12 | 0.77 | <0.0001 |
| | 4 | 1.5 | 24 | 38.70 | 0.062 | 0.63 | <0.0001 |
| | 5 | 0.89 | 24 | 15.38 | 0.14 | 0.40 | 0.0007 |
| | 6 | 1.6 | 24 | 27.33 | 0.11 | 0.54 | <0.0001 |

**Table S7** Body length growth rates (BL GR in µm ln(day)$^{-1}$) of sea urchin exp. 1 were calculated as the coefficient of the significant logarithmic relationship between BL and tpf. Results of the regressions (Intercept, p-value, $R^2$, F-value and df: degree of freedom) are given for each culture replicate with corresponding treatment.

| Treatment | Replicate | BL GR | df | F-value | Intercept | $R^2$ | p-value |
|---|---|---|---|---|---|---|---|
| Control | 1 | 77.99 | 120 | 463.21 | 185.55 | 79.56 | <0.0001 |
| | 2 | 74.65 | 126 | 558.53 | 186.59 | 81.71 | <0.0001 |
| | 3 | 74.78 | 129 | 297.40 | 188.47 | 69.91 | <0.0001 |
| | 4 | 73.59 | 101 | 381.27 | 185.84 | 79.22 | <0.0001 |
| | 5 | 76.88 | 135 | 362.85 | 190.52 | 73.03 | <0.0001 |
| | 6 | 78.90 | 124 | 393.56 | 183.32 | 76.19 | <0.0001 |
| OA/OW | 1 | 72.22 | 122 | 151.70 | 187.50 | 55.63 | <0.0001 |
| | 2 | 78.67 | 129 | 377.54 | 173.23 | 74.68 | <0.0001 |
| | 3 | 76.11 | 101 | 360.12 | 177.15 | 78.27 | <0.0001 |
| | 4 | 65.73 | 118 | 382.24 | 193.24 | 76.56 | <0.0001 |
| | 5 | 67.39 | 126 | 685.55 | 181.48 | 84.58 | <0.0001 |
| | 6 | 77.52 | 90 | 363.89 | 171.24 | 80.35 | <0.0001 |
| Oil | 1 | 66.49 | 45 | 200.78 | 188.23 | 82.02 | <0.0001 |
| | 2 | 66.86 | 56 | 95.14 | 197.32 | 63.37 | <0.0001 |
| | 3 | 71.93 | 92 | 625.57 | 178.05 | 87.30 | <0.0001 |
| | 4 | 67.95 | 78 | 346.42 | 181.72 | 0.82 | <0.0001 |
| | 5 | 65.89 | 128 | 320.72 | 205.69 | 0.72 | <0.0001 |
| | 6 | 77.07 | 126 | 524.53 | 167.58 | 0.81 | <0.0001 |
| OA/OW + Oil | 1 | 64.49 | 82 | 161.28 | 169.18 | 66.57 | <0.0001 |
| | 2 | 63.26 | 64 | 207.02 | 178.59 | 76.67 | <0.0001 |
| | 3 | 60.79 | 100 | 183.87 | 189.84 | 65.00 | <0.0001 |
| | 4 | 70.29 | 28 | 202.78 | 169.85 | 88.25 | <0.0001 |
| | 5 | 55.60 | 71 | 131.88 | 198.96 | 65.33 | <0.0001 |
| | 6 | 63.87 | 107 | 255.96 | 187.16 | 70.71 | <0.0001 |

**Table S8** Body length growth rates (BL GR in µm ln (day)$^{-1}$) of sea urchin exp. 2 were calculated as the coefficient of the significant logarithmic relationship between BL and tpf. Results of the regressions (Intercept, p-value, $R^2$, F-value and df: degree of freedom) are given for each culture replicate with corresponding treatment.

| Treatment | Replicate | BL GR | df | F-value | Intercept | $R^2$ | p-value |
|---|---|---|---|---|---|---|---|
| Control | 1 | 77.28 | 258 | 904.98 | 182.87 | 77.88 | <0.0001 |
| | 2 | 79.51 | 251 | 1254.82 | 176.84 | 83.39 | <0.0001 |
| | 3 | 90.80 | 248 | 879.31 | 155.58 | 78.07 | <0.0001 |
| | 4 | 79.19 | 203 | 713.60 | 190.14 | 77.93 | <0.0001 |
| | 5 | 79.92 | 269 | 823.41 | 183.61 | 75.44 | <0.0001 |
| | 6 | 82.52 | 253 | 943.58 | 176.36 | 78.92 | <0.0001 |
| OA/OW | 1 | 79.47 | 256 | 625.30 | 155.43 | 71.03 | <0.0001 |
| | 2 | 80.15 | 278 | 708.96 | 146.95 | 71.91 | <0.0001 |
| | 3 | 69.57 | 236 | 644.11 | 181.53 | 73.27 | <0.0001 |
| | 4 | 64.17 | 250 | 887.16 | 194.27 | 78.08 | <0.0001 |
| | 5 | 67.36 | 264 | 945.93 | 180.49 | 78.24 | <0.0001 |
| | 6 | 67.21 | 222 | 646.16 | 184.40 | 74.51 | <0.0001 |
| Oil | 1 | 73.18 | 270 | 723.27 | 189.78 | 73.89 | <0.0001 |
| | 2 | 59.62 | 254 | 601.46 | 207.01 | 70.39 | <0.0001 |
| | 3 | 68.99 | 264 | 556.13 | 196.34 | 67.89 | <0.0001 |
| | 4 | 62.75 | 257 | 707.45 | 199.48 | 73.43 | <0.0001 |
| | 5 | 75.96 | 287 | 470.07 | 187.64 | 62.17 | <0.0001 |
| | 6 | 65.20 | 263 | 538.13 | 203.00 | 67.26 | <0.0001 |
| OA/OW + Oil | 1 | 68.53 | 268 | 387.17 | 190.22 | 59.19 | <0.0001 |
| | 2 | 68.40 | 245 | 527.86 | 185.56 | 68.39 | <0.0001 |
| | 3 | 65.59 | 244 | 602.68 | 187.09 | 71.27 | <0.0001 |
| | 4 | 71.90 | 258 | 646.76 | 179.92 | 71.56 | <0.0001 |
| | 5 | 56.54 | 265 | 799.93 | 198.30 | 75.19 | <0.0001 |
| | 6 | 55.10 | 219 | 286.54 | 206.30 | 56.79 | <0.0001 |

**Table S9** Respiration rate growth rates (RR GR in pmol $O_2$ ind$^{-1}$ h$^{-1}$ µm$^{-1}{}_{BL}$) for sea urchins in exp. 2, were calculated as the coefficient of the significant linear relationship between respiration rate (pmol $O_2$ ind$^{-1}$ h$^{-1}$) and BL (µm). Results of the regressions (Intercept, p-value, $R^2$, F-value and df: degree of freedom) are given for each treatment.

| Treatment | RR GR | df | F-value | Intercept | $R^2$ | p-value |
|---|---|---|---|---|---|---|
| Control | 2.56 | 16 | 14.20 | -653.77 | 48.63 | 0.0019 |
| OA | 2.60 | 20 | 33.22 | -651.74 | 63.61 | <0.0001 |
| Oil | 1.82 | 20 | 11.16 | -470.82 | 37.00 | 0.0034 |
| OA + Oil | 2.94 | 16 | 21.05 | -762.18 | 58.39 | 0.0004 |