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Reduced pH increases mortality and genotoxicity in an Arctic coastal copepod, *Acartia longiremis*

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**SUMMARY**

This study investigates DNA damage and mortality in an Arctic marine copepod after long-term exposure to lowered pH. *Acartia longiremis* were collected from northern Norway and incubated in ambient pH 8.1, and reduced pH 7.6 and 7.2 over 3-4 weeks. Cumulative mortality was significantly elevated in the lowered pH treatments, decreasing after >14 days, and DNA damage was significantly higher in lowered pH conditions. This method is ideal for investigating oxidative stress and genotoxicity response to low pH in Arctic marine copepods exposed to future ocean acidification conditions.

1. Main

DNA damage can stunt development, reduce reproductive success, and inherited compromised DNA can adversely impact individual survival and population resilience (Depledge, 1998). In response to environmental stress, intracellular reactive oxygen species (ROS) may increase, leading to oxidative damage to DNA (Silva et al., 2016). Low seawater pH from rising atmospheric CO₂ emissions represents such an environmental stressor, especially in Arctic regions (Steinacher et al., 2009). Sea surface pH is projected to decrease by up to 0.5 pH units by 2100 (Ciais et al., 2019). Marine organisms have varying responses to reduced seawater pH (Kroeker et al., 2010), and copepods have demonstrated both inter- and intra-specific variability in acid-base regulation capacity (Zhang et al., 2011; McConvilje et al., 2013). Transcriptional changes in redox and DNA repair genes suggest a shift in oxidative balance and induction of DNA repair mechanisms induced by lowered pH in Arctic *Calanus glacialis* (Bailey et al., 2017). Negative sub-lethal effects may impact long-term fitness of copepod populations, and elevated mortality may affect species distribution and abundance patterns.

Here, mortality and DNA damage were analysed in the Arctic copepod *Acartia longiremis* during long-term exposure to ambient and reduced pH seawater (Table 1). The fast micromethod for analysis of DNA strand breaks and alkali-labile sites by fluorescence detection was used (Batel et al., 1999; Schröder et al., 2006; El-Bibany et al., 2014). It allows more rapid, precise, and equally sensitive genotoxicity analysis in comparison to the widely used comet assay (Bihari et al., 2002), and has been successfully applied to detect DNA damage in cells, whole larvae, tissues, and extracted DNA of several marine species (Reinardy and Bodnar, 2015; Reinardy et al., 2016), but not copepods. The method was modified to allow break-down of the chitinous crustacean exoskeleton by adding a homogenisation step, and the DNA denaturation pH was elevated to initiate alkaline unwinding of copepod DNA. This method facilitates genotoxicity analysis in non-model species and is well-suited to copepods preserved after experimentation.

*A. longiremis* were collected from Håkøybønn, Norway (69°40.19.6′N, 18°47.07′0.0′E). Seawater from an intake pipe at 60m depth was filtered (10µm) and pumped into a 1000L header-tank where CO₂ was injected by pH controller (Aquamedic). Untreated (pH 8.0-8.1) and low pH water was filled into twelve replicate 1L glass bottles (n=24); pH was measured before and after water replacement (3-4 days) using five (Exp1) or three (Exp2 and Exp3) electrodes (AquaMedic, total scale). Mean pH was calculated for each exposure treatment. *A. longiremis* (late copepodite stages CS and adults) were transferred into
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bottles and *Rhodomonas baltica* algal food (strain NIVA-5191) was added *ad libitum* and refreshed with the water changes. Bottles were placed on an immersed plankton wheel (0.26rpm) to maintain *in situ* temperature. Dead individuals were counted and discarded weekly. Relative and cumulative mortalities were determined from initial total and subsequent dead individual counts. Three bottles of each treatment were removed every seven days, live copepods were counted and preserved in RNA-later (Sigma Aldrich) for DNA damage analysis.

Three exposures to nominal pH 7.2 (Exp1) and 7.6 (Exp2 and Exp3) were conducted; measured mean pH fluctuated between water changes (Table 1). The mean pH difference in controls was -0.01±0.06 (Exp1), -0.01±0.04 (Exp2), and 0.00±0.01 (Exp3); the mean pH difference in the lowered pH treatments was 0.10±0.07 (Exp1), 0.07±0.07 (Exp2), and 0.07±0.02 (Exp3) (mean±s.d., n=6 Exp1, n=8 Exp2&3). Number of copepods per bottle was increased between exposures (Table 1) to yield enough survivors for DNA damage analysis. Exp1 was terminated after 21 days due to high mortality in the low pH treatment. Tissue samples from Exp1 were lost during transport and thus not available for DNA damage analysis.

For fast micromethod analysis, 16-21 individuals were pooled per bottle, and gently homogenised (4µl/individual, 20mM EDTA, 10% DMSO) with a hand-pestle to breakdown the chitinous exoskeleton. 20µl homogenate was loaded into triplicate or quadruplicate wells (equalling 5 individuals/well) of a 96-well black-walled plate. Lysis buffer (20µl 9M urea, 0.1% SDS, 0.2M EDTA, 2% Quant-iT™ PicoGreen®) was added for 40min lysis, in the dark and on ice. Homogenisation buffer and lysis solution were used for volume-matched blanks. Unwinding solution (200µl, 20mM EDTA, 1M NaOH, pH adjusted to 13.74±0.02 with NaOH or EDTA) was added to each well to initiate unwinding. Fluorescence was recorded immediately on initiation of unwinding and every 5min (gain 1500, excitation 485nm, emission 520nm POLARstar® Omega). Strand scission factor (SSF) was calculated after 20min: SSF = log(%dsDNAsample/%dsDNAcontrol); dsDNAsample is double-stranded (ds) DNA in lowered and ambient pH samples, and dsDNAcontrol is ambient pH samples only. Negative SSFs for DNA from lowered pH treatment wells indicated no DNA damage and were set to 0 (30% of lowered pH samples). Increasing fluorescence over the kinetic period of detection may indicate crosslinking of DNA (Schröder et al., 2006), and were excluded from the analysis, reducing technical replication from quadruplicate to triplicate or duplicate (5.6% of all wells).

Low pH treatments resulted in significantly higher cumulative mortality in all exposures (GLM, p < 0.05, Figure 1). Cumulative mortality fit significantly with a sigmoidal increase over time (logistic regression, p < 0.05, Figure 1). Relative mortality was below 20% at all time points in both Exp2 and Exp3 (not available for Exp1), generally higher in lowered pH treatments than controls, and significantly higher

| Exposure | Collection date | Start – end dates | Sampling (day) | Copepods per bottle | S (PSU) | T (°C) | Incubation pH | Incubation pCO₂ (µatm) | Incubation Control Low Control Low |
|----------|----------------|-------------------|----------------|--------------------|--------|-------|-------------|------------------------|---------------------|
| Exp1     | 11/07/2018     | 17/07/-07/08/2018 | 7, 14, 17, 21  | 20                 | 35     | 7.4 (±0.5) | 8.1 (±0.1) | 7.2 (±0.1) | 366 | 3319 |
| Exp2     | 20/06/2019     | 25/06/-23/07/2019 | 7, 14, 21, 28  | 70                 | 6.4 (±0.5) | 8.1 (±0.1) | 7.5 (±0.2) | 329 | 1437 |
| Exp3     | 19/07/2019     | 23/07/-20/08/2019 | 100            | 6.8 (±0.5) | 8.1 (±0.0) | 7.7 (±0.1) | 304 | 1002 |

Fig. 1. Cumulative mortality in percent in July 2018 (A), July 2019 (B), and August 2019 (C). Data are mean±s.e.m. *significant within-timepoint difference from control (one-way ANOVA or Kruskal-Wallis test, p < 0.05). Dotted line logistic regression, † significant fit (p < 0.05).
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in Exp3 than Exp2 (Figure 2A&B), despite similar pH regime (mean mortalities of 14.1±0.7% and 8.9±0.7%, respectively; one-way ANOVA, p<0.0001, n=120 measurements, Statgraphics 18×64). Mortality was highest in Exp2 on day 7, likely caused by low pH in the first four days. *Acartia* congeners show interspecific variability in susceptibility to lowered pH, with negative or negligible effects on survival at similar treatment levels. The cosmopolitan estuarine *A. tonsa* showed no effects on daily mortality at 1000µatm (pH 7.8) and 2000µatm (pH 7.6); only extreme conditions of 3000µatm (pH 7.4) and 6000µatm (pH 7.2) negatively affected survival (Cripps et al., 2014), similar to *A. spinicauda* (Zhang et al., 2011). *Acartia pacifica* and *Acartia erythrea* showed no increased mortality after exposure to 5000ppm (pH 7.0) and 10000ppm (pH 6.8) pCO2, while survival of *Acartia steueri* was not affected up to 10000ppm (pH 6.8) pCO2 (Zhang et al., 2011; Kurihara et al., 2004). The survival of the Arctic *A. longiremis* was affected at pH 7.6 and thus appears to be more sensitive to lowered pH than that of its temperate counterparts.

DNA damage was elevated in low pH treatments in Exp2 and Exp3 with a peak in low pH treatments on day 14 in both exposures (Figure 2C&D); DNA damage in Exp3 was significantly higher in the treatment than in the controls (GLM, p=0.01, n=24 bottles). Similar pH levels and exposure durations induced DNA damage in other marine organisms, including marine amphipods (Roberts et al., 2013), clams (Conradi et al., 2016), and lobster larvae (Iato et al., 2017). Additionally, increased oxidative stress was measured in copepods exposed to lowered pH (Zhang et al., 2012), suggesting that this could be the underlying mechanism behind genotoxicity. DNA damage reduced over time, possibly indicating upregulation of DNA repair. Our modified method for detection of DNA damage in crustacean tissue homogenates is well-suited to investigate the dynamic processes of oxidative stress, DNA damage, and induction of DNA repair.

Both mortality and DNA damage were higher in Exp3 compared to Exp2. Mean DNA damage in controls of Exp2 showed higher variation between replicate bottles and time points than controls in Exp3. Relative mortality also varied more between treatments and time points in Exp2, with significant difference on day 28 (one-way ANOVA, p=0.04, n=6). The observed differences between Exp2 (July) and Exp3 (August) were possibly due to seasonal differences in physiology (e.g. senescence).

In conclusion, *A. longiremis* mortality and sub-lethal DNA damage were elevated in lowered pH conditions of 7.7, 7.5, and 7.2 (mortality only). This may have important implications on vulnerability of Arctic copepod populations to changing environmental conditions such as...
ocean acidification. The fast micromethod is adaptable to pooled tissue homogenates of crustacean zooplankton and has potential for further modification to assess genotoxic susceptibility and DNA repair capacity in individual copepods. Our preliminary results provide data for further hypotheses testing, including clarifying the full oxidative stress and modification to assess genotoxic susceptibility and DNA repair capacity of Arctic marine organisms to lowered pH.

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CRediT authorship contribution statement

Claudia Halsband: Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Project administration, Funding acquisition. Mascha F. Dix: Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. Kristine Hopland Sperre: Data curation, Resources, Writing – review & editing. Helena C. Reinardy: Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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