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LETTER

Stage-specific vulnerability: Ocean acidification and hypoxia impair copepod recruitment through a naupliar bottleneck

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Scientific Significance Statement

Coastal marine ecosystems are increasingly exposed to concurrent acidification and deoxygenation, yet the combined effects of these stressors across zooplankton life stages remain poorly resolved. Using the egg-carrying copepod *Pseudodiaptomus annandalei*, we show that short-term adult survival was relatively insensitive to combined low pH and low oxygen, whereas naupliar development and reproductive output were strongly reduced. These results identify the naupliar stage as a critical bottleneck under combined acidification and hypoxia. This stage-specific vulnerability implies that adult survival alone can underestimate recruitment impacts of changing coastal carbonate and oxygen conditions.

Abstract

Progressive ocean acidification and deoxygenation are expected to threaten many marine zooplankton, but their combined effects across life stages remain poorly understood. We investigated stage-specific responses of the egg-carrying copepod *Pseudodiaptomus annandalei* to changes in pH_T (5.97–8.05) and dissolved oxygen (DO; 0.90–8.20 mg L^{-1}). Nauplii were the most sensitive stage in 24-h exposures, with survival dropping below 40% at pH_T 6.1 and declining to 55–70% when DO fell below 1 mg O_2 L^{-1}. Under combined stress, naupliar survival declined to about 65% in the most severe treatment (pH_T 7.11, DO 1.74 mg O_2 L^{-1}), whereas copepodites and adults showed no significant short-term survival response. In contrast, moderate combined stress (pH_T 7.62, DO 4.11 mg O_2 L^{-1}) produced sublethal effects, increasing development time by approximately 70%, reducing development success from 86% to 28%, and decreasing nauplii production by approximately one-third. Thus, nauplii form a critical bottleneck for recruitment under combined stress.

Ocean deoxygenation and acidification are major environmental challenges confronting marine ecosystems (Wang et al. 2017; Breitburg et al. 2018). Global warming increases seawater temperature and strengthens stratification, thereby reducing oxygen solubility and vertical oxygen transport, while anthropogenic CO_2 emissions increase CO_2 dissolution into seawater and lower seawater pH (Stendardo and Gruber 2012). In eutrophic coastal waters, phytoplankton blooms can enhance the subsequent microbial decomposition of organic matter, causing concurrent oxygen depletion and CO_2 accumulation; as a result, hypoxia and acidification often co-occur and jointly affect marine organisms (Steckbauer et al. 2011; Reed and Harrison 2016).

Zooplankton, particularly copepods, are key components of marine food webs and are sensitive to both acidification

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and low oxygen (Li and Gao 2012; Wishner et al. 2018). Low oxygen can affect copepods through both lethal and sublethal pathways. In *Acartia tonsa*, hypoxia reduced egg production and population growth even when adult mortality was limited (Marcus et al. 2004), and seasonal hypoxia reduced growth and produced smaller adults under thermally stressed conditions (Richmond et al. 2006). Low oxygen has also been linked to reduced egg hatching success and altered early-stage performance in calanoid copepods from coastal and upwelling systems (Ruz et al. 2018; Choi et al. 2023, 2024). Responses to acidification are likewise stage- and trait-dependent. Ocean acidification reduced naupliar production across generations in the harpacticoid copepod *Tisbe battagliai* (Fitzer et al. 2012) and challenged reproductive and developmental plasticity in *Acartia* spp. (Vehmaa et al. 2016). Multigenerational experiments with *A. tonsa* further showed rapid but incomplete fitness recovery under simultaneous warming and acidification and a thermal-tolerance cost of adaptation (Dam et al. 2021; deMayo et al. 2021).

Reduced O_2 availability and acidification may interact to affect copepod survival, development, reproduction, and behavior (Fabry et al. 2008; Breitburg et al. 2015). Hypoxia can alter vertical distribution and compress habitat space (Kaartvedt et al. 2021; Wyeth et al. 2022), whereas acidification may affect ion balance and energy metabolism (Lewis et al. 2013; Bailey et al. 2017). Hypoxia can reduce egg hatching and early-stage recruitment (Choi et al. 2023, 2024), whereas co-occurring hypoxia and acidification can alter locomotor behavior (Wyeth et al. 2024). However, copepod responses are not universally negative: some species may reduce exposure through behavioral avoidance or physiological acclimation, and life-history traits can alter exposure risk (Elliott et al. 2013; Graham and Barreto 2019; Sokolova et al. 2019; Tward et al. 2019).

Despite these advances, the combined effects of acidification and hypoxia across multiple copepod life stages remain insufficiently resolved, particularly for linking short-term survival to development and reproductive output. Here, we examined the stage-specific responses of the egg-carrying copepod *Pseudodiaptomus annandalei* to reduced levels of pH_T and dissolved oxygen (DO). We tested the following hypotheses: (1) reduced pH_T or DO would decrease survival more strongly in nauplii than in copepodites or adults; (2) combined acidification and hypoxia would prolong development and reduce the proportion of nauplii reaching later stages; and (3) adult short-term survival would not fully predict recruitment potential because naupliar development and reproductive output would be more sensitive endpoints.

Methods and materials

Preparation of seawater with different levels of pH and DO

Natural seawater (salinity 30 ppt) was collected from the coastal waters of Quanzhou (24.87°N, 118.68°E), China, filtered through 0.22-μm membranes, and autoclaved at 106°C for 20 min before use. Sterilized seawater was dispensed into 1-L polycarbonate bottles. Target pH and DO conditions were established using a CO_2-enrichment system (Model 0A100, Ruihua Instruments), which delivered controlled mixtures of CO_2/air or CO_2/air/N_2 through continuous bubbling until equilibrium was reached. Three treatment sets were prepared: (1) an acidification gradient (pH_T 5.97–8.05, DO 7.30 ± 0.08 mg L^{-1}); (2) a DO gradient (pH_T 7.98 ± 0.04, DO 0.90–8.20 mg L^{-1}); and (3) three combined treatments: ACAO (pH_T 8.02 ± 0.01, DO 6.73 ± 0.18 mg L^{-1}), MCMO (pH_T 7.63 ± 0.03, DO 4.06 ± 0.18 mg L^{-1}), and HCLO (pH_T 7.10 ± 0.02, DO 1.87 ± 0.31 mg L^{-1}), representing ambient to severe episodic conditions reported for eutrophic coastal waters of the East China Sea (C.-C. Chen et al. 2020; Miao et al. 2025). pH_{NBS}, DO, and temperature (25°C) were monitored during preparation. Conditioned seawater was transferred to airtight bottles without air bubbles to minimize gas exchange before use. Assay-specific measured pH_t and DO values are reported in the corresponding figure legends and Supporting Information (Supporting Information Tables S1–S5).

Determination of carbonate system parameters and DO

pH_{NBS} was measured with a calibrated pH meter (ORION STAR A211, Thermo Scientific), and converted to pH_T using CO2SYS. Total alkalinity was determined by titration (Edmond 1970). Other carbonate parameters (DIC, CO_3^{2-}, HCO_3^-, and pCO_2) were calculated using CO2SYS. Dissolved oxygen was measured with a PreSens system (Precision Sensing GmbH).

Copepod collection and maintenance

Pseudodiaptomus annandalei were collected from Wuyuan Bay (24.54°N, 118.18°E), Xiamen, China, in December 2020. Stock cultures were maintained in filtered seawater at 25°C and fed the microalga *Isochrysis galbana* every 48 h. *Isochrysis galbana* was cultured in f/2 medium at 25°C under a 12 h : 12 h light : dark cycle.

Determination of survival rate

Survival experiments were conducted with nauplius stage III, copepodite stage II, adult males, and females of *P. annandalei*. Individuals at each developmental stage were carefully selected under a stereomicroscope (SMZ745T, Nikon, Japan) and transferred into glass bottles (24.5 mL) containing treatment seawater. For each pH_T or DO level and each developmental stage, three replicate bottles were used. Each replicate contained 20 nauplius stage III or 10 copepodite stage II/adult individuals. During transfer, the amount of filtered seawater carried over with the organisms was minimized to less than 0.5 mL. Bottles were gently filled with treatment seawater without air bubbles, sealed, and incubated in the dark at 25°C for 4 or 24 h. Initial and final pH and DO were measured. Survival was assessed based on the response of individuals to gentle tactile stimulation under a stereomicroscope.

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Determination of development

For each treatment, 50 newly hatched nauplii were transferred into each of three replicate nominal 60-mL polycarbonate bottles (brimful capacity approximately 75 mL). Bottles were filled with approximately 75 mL of treatment seawater to eliminate headspace and sealed with Parafilm. To provide nutrition while limiting algal effects on seawater chemistry, the water in each bottle was renewed daily and supplemented with *I. galbana* at a final concentration of 3000 cells mL^{-1}. During each water change, the developmental stage of each individual was recorded under a stereomicroscope. Observations continued until all individuals had either reached sexual maturity or died. Median development time was defined as the time by which 50% of individuals reached the specified developmental endpoint (copepodite or adult) and was calculated using Probit analysis in SPSS 22.0. Model fit was evaluated using Pearson goodness-of-fit tests. Dissolved oxygen and pH were measured before and after daily water renewal; detailed variations in carbonate chemistry and DO are provided in Supporting Information Table S5.

Determination of reproduction

For each treatment, three polycarbonate mating bottles commercially designated and graduated as 250 mL were used. Because the bottle neck extended above the highest graduation, each bottle had a brimful capacity of approximately 300 mL. Each bottle was filled to capacity with approximately 300 mL of the corresponding treatment seawater to eliminate headspace and contained approximately 150 females and 100 males of *P. annandalei*. Adults were maintained under these conditions for approximately 24 h for short-term mating and collection of ovigerous females. This temporary high-density setup was used solely to obtain sufficient ovigerous females for individual incubation.

Ten ovigerous females were randomly selected from each mating bottle, yielding 30 females per treatment, and their clutch sizes were determined under a stereomicroscope. Each selected female was then transferred to a separate glass culture bottle filled to capacity with the corresponding treatment seawater and incubated individually. Each individually incubated female was used as an observational unit for clutch size, hatching, and nauplii production. The number of nauplii hatched in each bottle was recorded daily for three consecutive days. Nauplii production was expressed as the number of nauplii produced per female per day.

Data analysis

All data used in the analyses are available in W. Chen and Gao (2026). The effects of pH_T and DO on copepod survival were quantified by nonlinear regression analysis in Origin (version 2022, OriginLab Corporation). Model parameters were estimated using the Levenberg–Marquardt iterative algorithm, and the goodness of fit for each model was evaluated using the coefficient of determination (R^2) and the adjusted R^2.

For the pH_t–survival relationship, the 4- and 24-h exposure datasets were fitted independently using a single-phase exponential growth model:

$$\text{Survival}\,(\%) = A \times \{1 - \exp[-(\text{pH}_T - X_0)/t_1]\}$$

where A is the maximum asymptotic survival, X_0 is the fitted pH_T intercept at which predicted survival equals zero, and t_1 controls the curvature of the response.

For the DO–survival relationship, the 4- and 24-h exposure data were fitted using an exponential growth model:

$$\text{Survival}\,(\%) = y_0 + A_1 \times \exp(\text{DO}/t_1)$$

where y_0 is a baseline parameter, A_1 is the amplitude parameter, and t_1 is the scaling factor.

For survival, reproduction, and development data from the combined acidification–deoxygenation treatments, differences among ACAO, MCMO, and HCLO were assessed using one-way ANOVA in SPSS 22.0. When ANOVA indicated a significant treatment effect, Fisher's least significant difference post hoc test was used for pairwise comparisons. Normality and homogeneity of variance were evaluated using Shapiro–Wilk and Levene's tests, respectively.

Results and discussion

Stage-specific survival responses to acidification and hypoxia

Under single-stressor exposure, the 24-h survival rate of *P. annandalei* showed clear life-stage dependence. Under normoxic conditions (DO 7.30 ± 0.08 mg L^{-1}), survival declined sharply when pH_T fell below approximately 6.3 (Fig. 1E–H). Nauplii were the most sensitive stage to acidification, with 24-h survival dropping below 40% at pH_T 6.1, whereas copepodites, males, and females maintained survival rates of approximately 60–70% under the same pH conditions. Similarly, under ambient CO_2 conditions (pCO_2 483 μatm), hypoxia induced stage-specific responses. During 24-h exposure, naupliar survival declined to 55–70% when DO fell below 1 mg L^{-1}, whereas adults maintained survival rates of 70–80% under the same hypoxic conditions (Fig. 1A–D).

These results identify nauplii as the most sensitive acute-survival stage in *P. annandalei*. Similar stage-specific responses have been reported in other copepods exposed to low-oxygen or acidified conditions, although the magnitude of sensitivity varies among species, habitats, and exposure histories (Lewis et al. 2013; Cripps et al. 2014; Ruz et al. 2018). Therefore, our results should be interpreted as evidence for a sensitive naupliar window in this species under direct exposure, rather than as a universal response of all calanoid copepods.

Under combined stress conditions, the 24-h survival of nauplii exhibited a stress-intensity-dependent response. When conditions shifted from ambient (ACAO: pH_T 8.02, DO

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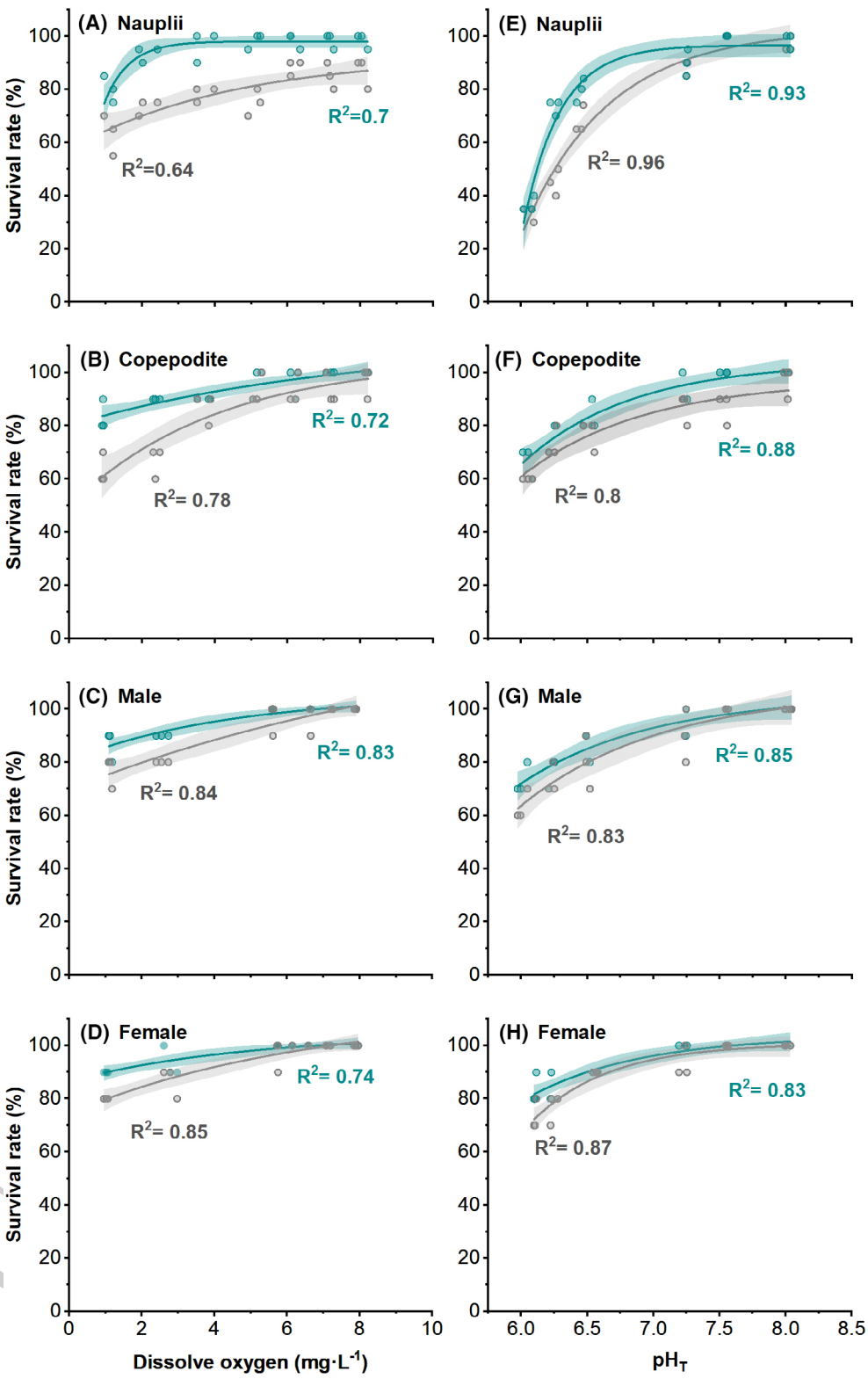


Fig. 1. Survival rates of *Pseudodiaptomus annandalei* exposed to different levels of pH_T and DO. Fitted survival curves (**A–D**) under ambient CO_2 conditions ($pCO_2 = 482.75 \pm 57.90$ μatm) across a range of DO levels (0.9–8.20 mg L^{-1}), and (**E–H**) under normoxia (7.29 ± 0.07 mg L^{-1}) across varied pH_T levels (5.97–8.04, induced by changed pCO_2). Data points represent nauplii (nauplius stage III), copepodites (copepodite stage II), adult males and females after 4 h (green line) and 24 h (gray line) exposures. Shaded areas indicate 95% confidence intervals.

6.60 mg L^{-1}) to moderate combined stress (MCMO: pH_T 7.65, DO 3.95 mg L^{-1}), mean naupliar survival decreased from 90% to 88.3% ($p = 0.81$), a non-significant change. However, when stress intensified to HCLO conditions (pH_T 7.11, DO 1.74 mg L^{-1}), 24-h survival significantly declined to 65% ($p = 0.01$, Fig. 2A). Comparison with the single-stressor data suggests that hypoxia was the dominant driver of reduced naupliar survival under HCLO, because survival at comparable DO levels was close to the HCLO response, whereas survival at comparable pH_T under normoxia remained higher.

In contrast to nauplii, copepodites and adults showed no significant differences in 24-h survival across all combined treatment groups (copepodites: $p = 0.32$; males: $p = 0.33$; females: $p = 0.13$, Fig. 2A,B). Additional time-course survival data are provided in Supporting Information Fig. S1 and Supporting Information Table S6.

The apparent tolerance of later stages should not be interpreted as complete physiological resistance. Rather, it indicates that short-term survival was less sensitive than development and reproduction for detecting the effects of combined acidification and hypoxia. Previous studies have similarly shown that low oxygen can reduce copepod egg production, hatching success, or population growth even when adult mortality is limited (Marcus et al. 2004; Sedlacek and Marcus 2005; Richmond et al. 2006). Field interpretation also depends on how copepods encounter hypoxia. In many stratified coastal systems, hypoxia is concentrated in bottom waters below the pycnocline, while surface waters remain more oxygenated. Mobile copepodites and adults may therefore reduce exposure by occupying better oxygenated layers (Pierson et al. 2017). Broadcast-spawning copepods may still be vulnerable because their eggs can sink into hypoxic bottom waters, where eggs or newly hatched nauplii may suffer mortality (Uye 1994; Roman et al. 2019). In contrast, egg-carrying species such as *P. annandalei* may experience different exposure pathways because embryos remain attached to females until hatching. Such exposure differences may favor egg-carrying taxa in systems with persistent bottom-water hypoxia. This life-history distinction cautions against directly extrapolating short-term laboratory exposure to all natural hypoxic systems.

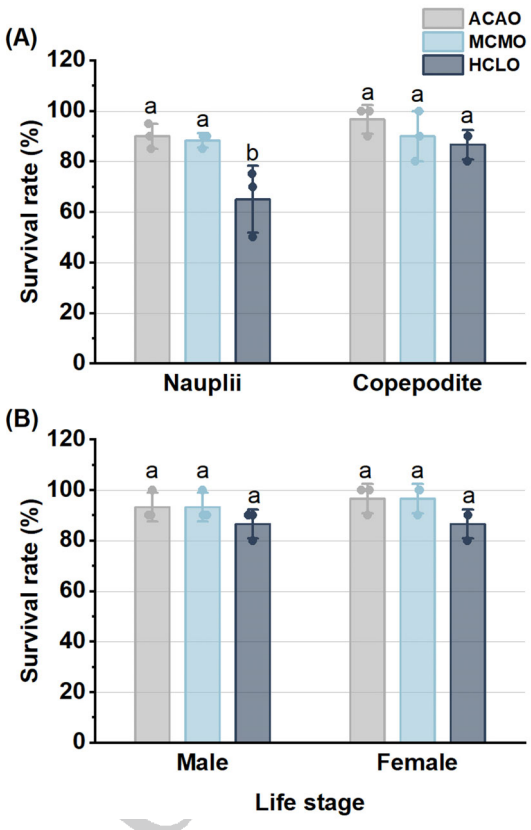


Fig. 2. Survival response of *Pseudodiaptomus annandalei* to combined acidification and deoxygenation during a 24-h exposure. Treatments were ACAO (pH_T 8.02 ± 0.01, DO 6.60 ± 0.20 mg L^{-1}), MCMO (pH_T 7.65 ± 0.04, DO 3.95 ± 0.20 mg L^{-1}), and HCLO (pH_T 7.11 ± 0.02, DO 1.74 ± 0.22 mg L^{-1}). Panels show (**A**) nauplii (nauplius stage III) and copepodites (copepodite stage II), and (**B**) adult males and females. Data are presented as means ± SD. Different lowercase letters indicate significant differences among treatments (one-way ANOVA, Fisher's least significant difference test, $p < 0.05$).

Developmental delay and reduced nauplii production constrain recruitment potential

Although acute survival under MCMO did not differ significantly from that under ACAO, development and recruitment-related endpoints were strongly affected by combined acidification and hypoxia (Fig. 3). In the HCLO treatment, no nauplii successfully developed to the copepodite stage, resulting in a development success rate of 0% (Fig. 3B). Under MCMO, development from nauplius to adult was significantly prolonged by nearly 70%, increasing from 11.5 d in ACAO to 19.7 d ($p < 0.0001$; Fig. 3A). Development success also declined sharply, from 86% in ACAO to 28% in MCMO ($p < 0.0001$; Fig. 3B). Thus, even when moderate combined stress did not cause acute naupliar mortality, it substantially reduced the probability that nauplii completed development. This decoupling between short-term survival and successful development indicates that survival assays alone may underestimate recruitment vulnerability. Prolonged development may also extend the period during which nauplii remain vulnerable to predation, although predation was not tested here.

A plausible explanation is that low oxygen constrained aerobic ATP production, whereas elevated CO_2 increased the energetic costs of ion transport and acid–base regulation (H. O. Pörtner and Farrell 2008; H.-O. Pörtner 2010; Thor et al. 2016). Together, these stressors may reduce the energy available for growth, cuticle formation, and repeated molting. This interpretation is consistent with a bioenergetic constraint (Sokolova 2013), but direct physiological measurements would be required to confirm it in *P. annandalei*. In addition, nauplii may have lower energy reserves and exhibit more continuous swimming behavior than later stages (Titelman and

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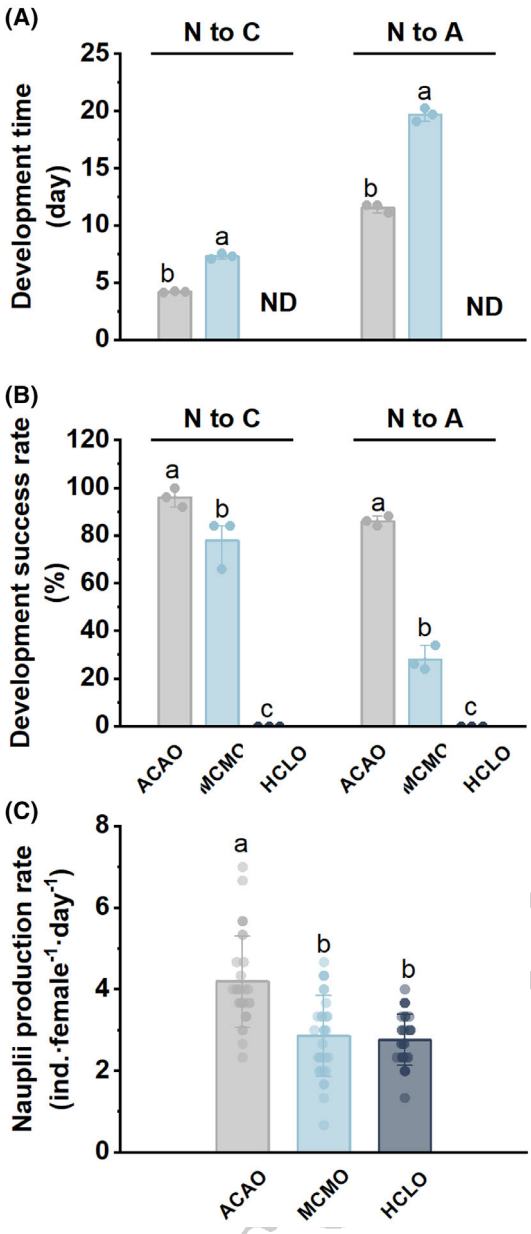


Fig. 3. Reproductive and developmental performance of *Pseudodiaptomus annandalei* under combined acidification and deoxygenation. Treatments were ACAO (pH_T 8.02 ± 0.01, DO 6.81 ± 0.11 mg L^{-1}), MCMO (pH_T 7.62 ± 0.02, DO 4.11 ± 0.15 mg L^{-1}), and HCLO (pH_T 7.09 ± 0.02, DO 2.06 ± 0.31 mg L^{-1}). Panels show (**A**) development time span, (**B**) development success rate, and (**C**) nauplii production. N-C represents development from nauplii to copepodite, and N-A represents development from nauplii to adult. Data are presented as means ± SD. "ND" indicates failed development. Different lowercase letters indicate significant differences among treatments (one-way ANOVA, Fisher's least significant difference test, $p < 0.05$).

Kiørboe 2003); therefore, survival assays in filtered seawater may have exaggerated early-stage vulnerability relative to food-replete field conditions (Cook et al. 2007).

The combined treatments also significantly reduced nauplii production by adult females. Compared with the ACAO group, which had a mean daily nauplii production of 4.19 ± 1.12 nauplii female^{-1} d^{-1}, nauplii production in the MCMO and HCLO groups decreased by 31.7% and 34.1%, respectively ($p < 0.0001$ for both comparisons; Fig. 3C). Nauplii production did not differ significantly between MCMO and HCLO ($p = 0.68$), suggesting that reproductive output declined once conditions reached the moderate combined-stress level and did not decrease further under the more severe treatment.

This reduction is consistent with earlier studies showing that hypoxia and acidification can impair copepod reproduction and early development. In *Acartia tonsa*, hypoxia reduced egg production and population growth rate (Marcus et al. 2004), and reproductive responses under low oxygen were further influenced by food availability and temperature (Sedlacek and Marcus 2005; Richmond et al. 2006). Acidification has also been shown to reduce naupliar production or challenge reproductive plasticity in copepods (Fitzer et al. 2012; Vehmaa et al. 2016; Dam et al. 2021). Collectively, these findings point to a recruitment bottleneck: combined stress not only reduces the number of nauplii produced but also lowers the likelihood that those nauplii reach adulthood. This bottleneck is inherently species- and environment-specific and is most relevant where nauplii are directly exposed to low pH and low O_2; therefore, it should not be generalized to all stratified systems or reproductive modes. Other co-occurring stressors, including warming and contaminants, may further affect recruitment but were not examined here.

Overall, these findings support the use of developmental and reproductive endpoints, rather than adult survival alone, when assessing copepod responses to co-occurring acidification and hypoxia.

Conclusion

Our findings show that combined acidification and hypoxia affected *P. annandalei* in a stage-specific manner. Copepodites and adults showed no significant short-term survival response, whereas naupliar development and nauplii production by adult females were substantially reduced. Combined acidification and hypoxia delayed development and lowered developmental success even without significant acute naupliar mortality, indicating that survival alone may underestimate recruitment vulnerability. However, the ecological consequences of such responses will depend on exposure duration, water-column structure, food availability, behavioral avoidance, predation, and reproductive mode. Future studies should integrate life-cycle experiments with field exposure scenarios to better evaluate population-level consequences.

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Author Contributions

Weijia Chen: investigation, writing original draft, visualization. Kunshan Gao: conceptualization, methodology, resources, supervision, writing – review and editing, funding acquisition.

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Conflicts of Interest

None declared.

Data Availability Statement

The data and metadata supporting the findings of this study are publicly available in the Figshare repository at https://doi.org/10.6084/m9.figshare.31869250.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

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