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Parental CO_2-vent history does not improve larval performance of the sea urchin *Arbacia lixula* under copper and acidification

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Abstract

CO_2-driven ocean acidification (OA) threatens marine calcifiers, while coastal ecosystems face increasing trace-metal contamination; their combined effects, and the modulating role of parental environmental history, remain poorly understood. We used the sea urchin *Arbacia lixula* from a naturally acidified CO_2 vent and a nearby ambient site at Ischia (Tyrrhenian Sea) to test how chronic adult exposure to low pH shapes larval responses to copper and low pH. Offspring from ambient (pH ~ 8.1) and vent (pH ~ 7.7) adults were reared for 48 h in a fully crossed design (two pH levels × three copper concentrations: 0, 5, 20 µg L^{-1}). At 24 hpf, development was dominated by parental history: ambient-derived larvae advanced rapidly, whereas vent-derived larvae showed higher arrest and slower progression, especially under low pH and high copper. By 48 hpf these effects diminished and pH and its interaction with copper became dominant, with both origins converging toward the echinopluteus stage. Nonetheless, malformations rose sharply under low pH regardless of copper, and skeletal-rod morphometrics revealed additional copper-related effects invisible to binary abnormality classifications. Chronic parental exposure did not enhance larval tolerance and sometimes increased sensitivity, underscoring the need to integrate multiple stressors and parental legacy.

1. Introduction

Ocean acidification (OA) is widely recognized as a major threat to marine biodiversity [1–3]. It arises from the ongoing uptake of anthropogenic CO_2 by surface waters, which drives a long-term decline in seawater pH and alters the marine carbonate system [4,5]. Projections indicate a decrease in the average surface open ocean pH of about 0.2–0.4 units by the end of the 21st century [6,7]. Syntheses across calcifying taxa indicate that OA commonly affects survival, growth, calcification, metabolic regulation, and early development, with echinoderm larvae frequently exhibiting increased mortality, delayed development and skeletal alterations under elevated CO_2 [8–13]. Despite the well-documented sensitivity of larval sea urchins to OA, several key aspects still need clarification, including the modulating roles of parental environmental history and multiple stressors.

Species and even population-specific responses to OA have been well documented. Local adaptation to the present range of natural variability, and the parental environmental history has emerged as key determinants of sensitivity to OA [14–17]. Work on sea urchins and other invertebrates has demonstrated that parental exposure to low pH during gametogenesis can partly buffer [18–20] or amplify [21] the negative effect of low pH on fecundity and offspring, altering egg size, growth and survival.

Coastal ecosystems are affected by multiple local (e.g. toxicants) and global changes (e.g. OA), including trace metals such as copper, one of the most widespread contaminants due to antifouling paints [22], industrial effluents [23,24], and urban runoff [25,26]. Copper is essential at low concentrations but becomes toxic when bioavailable levels increase, impairing fertilization, embryogenesis, and skeletogenesis in echinoderms and other invertebrates [27]. Studies have explored the combined effect of

OA with organic pollutants and metals, often revealing complex effects on physiology and survival [28–34]. For example, a polychaete population chronically exposed to low pH in the field showed enhanced resistance to low pH but modified the response to chemical stress, suggesting that selection or acclimation for OA tolerance may modify contaminant sensitivity [35].

The aim of this study is to evaluate how parental environmental history modulates the response of the offspring to exposure to copper and low pH, alone and in combination. We used the sea urchin *Arbacia lixula* (Linnaeus, 1758) as a model species. They were collected at two sites with contrasting natural pH levels. The shallow volcanic CO_2 vents of Castello Aragonese in Ischia (southern Italy) provide a natural laboratory to explore parental environmental history. At the Castello Aragonese site, intense CO_2 emissions generate sharp pH gradients over scales of tens to hundreds of metres, with vent areas characterized by mean pH values around 7.7 compared with nearby reference zones at about 8.1–8.2 [36–39]. The interplay between OA and copper contamination is particularly relevant for *A. lixula* along the Ischia coasts, where volcanic CO_2 emissions co-occur with anthropogenic inputs of metals from underground and thermal water [40,41] as well as the input of intense agriculture for wine production purposes [42–45]. Although copper concentrations were not directly measured at our sampling sites, dissolved trace metals in Mediterranean surface waters are generally higher than open-ocean baselines, reflecting the semi-enclosed nature of the basin and multiple anthropogenic inputs [46]. At the regional scale, sediment surveys from the nearby Port of Naples report highly variable and locally elevated copper concentrations typical of impacted harbor environments [47], while biomonitoring along Tyrrhenian coasts documents copper accumulation in algae and molluscs [48], indicating the presence of bioavailable copper in nearshore systems. Although these data do not imply metal enrichment at the vent system itself, they support the ecological plausibility of copper as a co-occurring coastal stressor in this region.

A. lixula were sampled from the two nearby sites at Ischia (naturally acidified vent area (pH ~ 7.7) and the ambient pH control site San Pietro (pH ~ 8.1)). Adults were induced to spawn, and their embryos were reared using a fully crossed design combining two pH levels (8.1 and 7.7) with three copper regimes (no copper, low and high sub-lethal concentrations). Larval development was followed at 24 and 48 h post-fertilization, scoring the proportion of embryos reaching successive developmental stages and quantifying the occurrence of skeletal abnormalities and plasticity.

2. Materials and methods

2.1. Organism collection

Adults of the sea urchin *A. lixula* were collected by SCUBA divers from subtidal rocky reefs on the northern coast of Ischia Island (Tyrrhenian Sea, Italy) at a maximum depth of 5 m during their main spawning season (July 2021). Adult sea urchins were visually selected in the field to ensure comparable body size, with test diameters ranging between approximately 4 and 5 cm across both vent and ambient populations, corresponding to sexually mature individuals [49]. Sea urchins were sampled at two sites that

differ in their long-term pH regime but are located within the same coastal sector. Populations from this species are often highly connected, as suggested by genetic studies across its range [49,50]. Although population connectivity has not been specifically assessed around the sampling site, this general pattern supports the assumption that nearby sites such as the CO_2 vents and the San Pietro control area, which are separated by only 1.5 km, may belong to the same regional population.

The ambient-pH site (hereafter “ambient site”) was located at San Pietro (40°44'47.6" N, 13°56'40.4" E), where the mean pH on the total scale (pH_T) is approximately pHT 8.14–8.18 with diel fluctuations [51,52]. The low-pH site (hereafter “vent site”) was located around the Castello Aragonese of Ischia (40°43'57.9" N, 13°57'51.8" E, stations N2 and S2 in Kroeker et al., 2011; pHT 7.8 ± 0.2 and 7.8 ± 0.3, respectively), at a naturally acidified CO_2 vent area characterized by shallow volcanic seeps that generate a persistent pH gradient along rocky reefs and *Posidonia oceanica* (Linnaeus) Delile, 1813 meadows [37]. Immediately after collection, sea urchins were transported in insulated containers filled with ambient seawater to the facilities of the Ischia Marine Centre of the Stazione Zoologica Anton Dohrn, where they were maintained in flow-through tanks supplied with filtered ambient seawater for approximately 24 hours prior to spawning induction and experimental exposures. This short holding period was applied to standardize pre-spawning conditions across treatments and minimize handling stress, while limiting potential acclimation effects relative to the long-term environmental history experienced by individuals *in situ*.

A. lixula is not a protected or endangered species, and all procedures complied with European legislation on the use of animals for scientific purposes (Directive 2010/63/EU). Both sampling sites fall within zone C of the Marine Protected Area “Regno di Nettuno”, and collections were carried out under authorization from the local management authority.

2.2. Experimental procedure

A. lixula adults were injected in the coelomic cavity with 1–2 mL of 0.5 M KCl to induce spawning [53]. Females were placed upside down in 300 mL beakers filled with 0.22 µm-filtered seawater (FSW) to collect the eggs, whereas sperm were collected dry with a micropipette and kept on ice until use to avoid premature activation. Gamete quality was checked under an optical microscope following established protocol [54], and fertilization was carried out using a sperm:egg ratio of 1250:1 [27]. Fertilization success was verified after 30 min by the elevation of the fertilization membrane, and only batches showing ≥ 95% fertilization success were used for the subsequent exposure. After fertilization, embryos from each parental origin were gently transferred into 25 mL polypropylene vials filled with FSW and tightly closed, to minimize gas exchange with the atmosphere and maintain stable pH conditions, as described by previous authors [20,55]. Embryo density was adjusted to approximately 1250 embryos per vial (*i.e.*, ~ 50 embryos mL^{-1}). Two different sets of vials were prepared for observation to be done at 24 hpf and at 48 hpf. All vials were maintained in a temperature-controlled room at constant temperature (22°C), within the thermal tolerance window for *A. lixula* larvae.

Natural seawater was 0.22 µm-filtered (38 PSU) and adjusted to two pH levels selected on the basis of the natural pH regimes experienced by *A. lixula* at the two sampling sites. The control treatment (pHT ≈ 8.1) corresponds to the stable mean pH characterising the ambient San Pietro site and the reference stations around the Castello Aragonese. The low-pH treatment (pHT ≈ 7.7) approximates the mean pH documented at intermediate-acidification zones of the Castello Aragonese vent system and reflects the conditions chronically experienced by the vent-associated *A. lixula* population from which adults were collected. This value lies well below the natural range of variability of the ambient site and therefore represents an ecologically relevant, site-matched acidification scenario rather than an open-ocean climate projection. We note that end-of-century open-ocean pH projections under high-emission scenarios (e.g., RCP 8.5: −0.3 to − 0.4 pH units by 2081–2100) [56] provide only a partial reference for coastal systems, where natural pH variability is typically much larger and future trajectories are modulated by local biogeochemical drivers [57,58]. Mediterranean-specific high-resolution projections [59,60] similarly indicate basin-wide declines of comparable magnitude under RCP 8.5, although coastal-scale projections remain limited.

For carbonate chemistry analyses, total alkalinity (TA) was determined on water sampled from the pre-equilibrated exposure media (without larvae) and maintained under identical experimental conditions. Samples for TA were collected in borosilicate bottles following standard operating procedures, fixed with saturated mercuric chloride, sealed gas-tight, and stored in the dark at 4°C pending analysis. An aliquot of 25 mL was titrated by potentiometric titration using an automated titrator (Mettler Toledo G20S). The HCl titrant (0.1 eq L^{-1}) was calibrated against certified reference materials distributed by A.G. Dickson (CRM, Batches #210 and #215). Seawater pH_T (total scale) was measured using a calibrated glass electrode using TRIS buffer. Measured pH_T and TA values were used to calculate the remaining carbonate system parameters using the CO2SYS Excel program [61], applying the dissociation constants [62,63]. Analytical precision based on CRM measurements was < 2 µmol kg^{-1}. To confirm pH stability over the course of the experiment, pH_T was measured at the beginning and end of each exposure period in representative replicate vials containing embryos/larvae. Copper additions (0, 5, 20 µg L^{-1}) did not measurably alter carbonate chemistry parameters (Table 1).

Table 1

Seawater carbonate chemistry parameters for each replicate at the start (T0) and end (T1) of the 48 h exposure period. AT was measured on pre-equilibrated exposure media without larvae. DIC, pCO_2, Ωa and Ωc were calculated from pHT and AT using CO2SYS [61], applying dissociation constants from Dickson and Millero (1987) and Dickson (1990); T = 22°C, S = 38, P = 0 dbar.

Condition	Cu (µg L^{-1})	Replicate	Timepoint	pHT	AT (µmol kg^{-1})	DIC (µmol kg^{-1})	pCO2 (µatm)	Ωa	Ωc
Ambient pH	0	1	T0	8.06	2602.70	2268.5	426.2	3.67	5.60
Ambient pH	0	1	T1	8.04	2602.70	2281.6	450.9	3.54	5.41
Ambient pH	5	2	T0	8.13	2680.35	2290.1	359.9	4.28	6.53
Ambient pH	5	2	T1	8.06	2680.35	2339.1	439.5	3.79	5.78
Ambient pH	20	3	T0	8.14	2646.28	2252.4	344.9	4.30	6.56
Ambient pH	20	3	T1	8.10	2646.28	2266.8	365.5	4.15	6.33
Vent pH	0	1	T0	7.67	2694.56	2565.6	1251.2	1.79	2.73
Vent pH	0	1	T1	7.68	2694.56	2561.2	1219.7	1.83	2.79
Vent pH	5	2	T0	7.73	2669.34	2514.2	1062.8	2.00	3.06
Vent pH	5	2	T1	7.66	2669.34	2545.5	1271.2	1.74	2.65
Vent pH	20	3	T0	7.74	2751.77	2588.7	1068.4	2.11	3.22
Vent pH	20	3	T1	7.70	2751.77	2607.5	1184.1	1.94	2.97

For each target pH, embryos were exposed to three copper treatments: a no-copper control and two nominal copper concentrations of 5 and 20 µg L^{-1} (hereafter Cu5 and Cu20), prepared by diluting a stock solution of copper sulfate (cupric sulfate, $CuSO_4$; Sigma-Aldrich) in filtered seawater. These levels are consistent with concentrations previously used to assess copper toxicity in sea urchins, alone or in combination with other stressors [64]. Copper concentrations in the experimental media were not analytically verified during the exposure; the values reported therefore correspond to nominal concentrations based on gravimetric preparation of stock solutions and subsequent dilution into FSW. Potential deviations from nominal values due to adsorption to vial surfaces, complexation with dissolved organic matter, or pH-dependent changes in copper speciation cannot be excluded, and actual bioavailable copper concentrations may differ from the nominal values reported here. This resulted in a fully crossed design (two pH levels × three copper treatments) for each parental origin for a total of 12

different conditions. For each condition, three replicate vials were used. Samples were collected after 24 and 48 h post-fertilization. At each time, the water was gently removed from the vials, and larvae were fixed using the cold-bath method [65], for subsequent assessment of developmental stage and the occurrence of skeletal abnormalities.

The treatment with ambient pH (8.1) and no added copper (Cu 0) was used as the negative control. Prior to the start of the exposure, early cleavage was visually verified to further confirm assay quality. At 48 hpf, control cultures showed proportions of normally developed larvae and inter-replicate variability consistent with standard quality criteria for sea urchin embryotoxicity assays [66,67], confirming overall biological quality of the assay. No replicate met criteria for exclusion (< 95% fertilization success).

2.3. Larval development and malformation assessment

Embryonic and larval development were evaluated under an optical microscope (Leica Z16 APO) equipped with a Leica DFC 300FX camera connected to a computer running Leica LAS software (Leica Application Suite, version 4.5). For each replicate vial, at least 100 embryos/larvae were photographed at 24 and 48 hpf. Developmental stage was assigned to each individual following the scheme used by previous authors [20]. Embryos and larvae were classified into five categories: (1) arrested embryos, unfertilized eggs and blastulae; (2) gastrulae, identified by the onset of archenteron invagination; (3) prisms, with the first calcareous rods visible and a cuboid shape; (4) early plutei, characterized by the initial formation of the four skeletal arms; and (5) echinoplutei, with fully developed postoral and somatic rods. For each replicate and time (24 and 48 hpf), the proportion of individuals in each category was calculated.

Skeletal abnormalities were assessed at 48 hpf in larvae that had reached the echinopluteus stage. Larvae were classified as morphologically normal when they displayed a complete and symmetrically organized larval skeleton, and as abnormal when they showed clear skeletal defects such as missing, bent, resorbed, thickened, or strongly asymmetric rods, or an evidently disorganized skeletal frame [67,68]. We acknowledge that this binary classification includes an element of operator judgement, especially for borderline phenotypes in which modest departures from the reference morphology may reflect graded stress responses rather than overt developmental failure. To reduce observer bias, all larvae were scored by a single trained operator using the same criteria across all treatments.

To complement this categorical assessment with a continuous descriptor of larval skeletal phenotype, morphometric measurements were also performed on digital images in those treatments where echinoplutei represented more than 20% of counted larvae. Specifically, the lengths of the postoral rods and of the somatic rods were measured using image analysis software (IMAQ™ Vision, version 6.0), and their ratio was subsequently calculated. These variables were used as descriptors of skeletal morphology and proportional allocation among larval skeletal elements, rather than as direct standalone proxies of overall larval growth or net calcification.

2.4. Statistical analyses

Statistical analyses were conducted in R (version 4.5.2) [69] using the packages dplyr, tidyr, ggplot2, car, emmeans, and vegan. Each replicate vial was treated as an independent experimental unit.

Developmental stage composition was analysed separately at 24 and 48 h post-fertilization (hpf). As an exploratory univariate approach, one-way ANOVAs were performed on arcsine square-root transformed stage proportions using treatment as a fixed factor, where treatment represented each unique combination of parental origin (ambient vs vent), pH (8.1 vs 7.7), and copper concentration (0, 5, 20 µg L^{-1}), yielding 12 levels in total. Tukey HSD tests were used for post hoc comparisons.

To assess overall multivariate differences in developmental structure among treatments, permutational multivariate analysis of variance (PERMANOVA) [70] was applied to Euclidean distance matrices calculated from square-root transformed stage frequencies. Separate PERMANOVAs were conducted at 24 and 48 hpf using parental origin, pH, copper treatment, and their interactions as fixed factors. When significant effects were detected, pairwise post hoc tests were performed, and Monte Carlo p-values were used when the number of permutations was limited. Non-metric multidimensional scaling (NMDS) ordinations were used to visualize multivariate patterns in developmental stage composition. Ordinations were based on Euclidean distances of transformed stage frequencies and computed using the metaMDS function in the vegan package.

To investigate specific developmental transitions underlying the observed stage distributions, binomial generalized linear models (GLMs) with a logit link function were fitted to three endpoints: (i) developmental arrest at 24 hpf, modelled as arrested vs non-arrested embryos; (ii) developmental progression at 24 hpf, expressed as the proportion of gastrulae among non-arrested individuals; and (iii) developmental outcome at 48 hpf, expressed as the proportion of echinopluteus-stage larvae among surviving individuals. In all models, parental origin, pH, copper treatment, and their interactions were included as fixed factors.

At 48 hpf, larval morphological quality was further analysed in two complementary ways. First, the proportion of abnormal larvae among echinoplutei was analysed using binomial GLMs, modelling abnormal vs normal individuals per replicate as a function of parental origin, pH, copper treatment, and their interactions. Second, skeletal morphometric variables (somatic rod length, postoral rod length, and somatic-to-postoral ratio) were analysed using linear models (lm) including parental origin, pH, copper treatment, and their interactions as fixed factors. Residual normality and homogeneity of variance were assessed using Shapiro–Wilk and Levene's tests, respectively. When significant effects were detected, estimated marginal means were computed and pairwise comparisons were performed with Tukey adjustment. Statistical significance was set at $\alpha = 0.05$.

During preparation of this manuscript, the authors used the generative AI tool Claude (Anthropic; accessed via claude.ai) for language editing and polishing only. It was not used to generate or analyse data or to produce scientific content; all data interpretation and conclusions are the authors' own, and the authors take full responsibility for the integrity of the work.

3. Results

3.1. Developmental stages at 24–48 hpf

At 24 hpf, developmental stage composition showed a strong divergence between progeny from ambient- and vent-site adults (Figs. 1A, 2A). Vent-derived progeny included a larger fraction of embryos in the arrested category, particularly under low pH and elevated copper, whereas ambient-derived progeny were more frequently represented by prism and early pluteus stages. Parental origin emerged as the main driver of developmental stage composition in the PERMANOVA ($R^2 \approx 0.56$, $p = 0.001$), with significant Parental × pH and Parental × Cu interactions indicating that acidification and copper modulated this effect. Consistently, the proportion of advanced larvae (prisms + plutei) differed significantly among the 12 treatment combinations ($F_{11,24} = 33.85$, $p < 0.001$).

Transition-specific models clarified the processes underlying this pattern (Fig. 3). At 24 hpf, vent-derived larvae showed consistently higher probabilities of developmental arrest than ambient-derived larvae, especially under low pH, where arrest further increased with copper concentration. Among non-arrested larvae, vent-derived individuals also showed a higher proportion of gastrulae, indicating slower progression toward prism and early pluteus stages. The early divergence between parental origins therefore reflected both higher developmental arrest and slower progression among non-arrested larvae.

By 48 hpf, differences among treatments were substantially reduced (Figs. 1B, 2B). Echinoplutei dominated most conditions, although their percentage still differed significantly among treatments ($F_{11,24} = 5.12$, $p = 0.0004$). Parental origin explained negligible and non-significant variance in the PERMANOVA ($R^2 \approx 0.02$), whereas pH emerged as the dominant factor ($R^2 \approx 0.23$, $p = 0.001$), with a significant pH × Cu interaction. In the NMDS ordination, ambient- and vent-derived progeny largely overlapped. Among non-arrested individuals, echinopluteus proportions were generally high across treatments, indicating only an apparent convergence at the level of stage classification, although vent-derived larvae showed greater variability under some low-pH and high-copper conditions.

Overall, these analyses reveal a sequential structure: at 24 hpf, parental origin governed both the probability of developmental arrest and the rate of progression among non-arrested larvae, whereas by 48 hpf these parental effects were largely superseded by pH and its interaction with copper.

3.2. Developmental abnormalities at 48 hpf

At 48 hpf, developmental abnormalities were widespread across all treatments (Fig. 4). Binomial GLM analysis revealed a significant effect of pH on the proportion of abnormal larvae ($p < 0.001$; SM-2). Parental origin, copper concentration, replicate, and all interaction terms were not significant ($p \geq 0.24$). Under pH 8.1, abnormal larvae accounted for approximately one third to one half of the population, whereas at pH 7.7 abnormality rates rose to approximately two thirds to more than three quarters of the larvae.

3.3. Skeletal rods morphometrics at 48 hpf

Parental origin and pH showed significant effects on the length of the somatic rod ($F_{1,2535}$ = 12.57, p = 3.9 × 10^{-4}; $F_{1,2535}$ = 19.88, p = 9.0 × 10^{-6}), whereas no significant effect was observed for copper ($F_{2,2535}$ = 2.57, p = 0.076). However, several interaction terms were significant, including Parental × pH, Parental × Cu, pH × Cu, and the three-way interaction Parental × pH × Cu (all $p < 0.05$). Overall, somatic rods were longer at pH 8.1 than at pH 7.7 and in larvae from parents originating from the ambient site (San Pietro) compared with those from the vent site. The effect of copper was more subtle and led to an increase in length with increasing concentration in some combinations of pH and parental origin (Fig. 5A).

For postoral rods, pH and copper significantly affected length ($F_{1,2535}$ = 139.80; $F_{2,2535}$ = 12.34; both $p < 0.001$). Postoral rods were consistently longer at pH 8.1 than at pH 7.7, and progressively shorter with increasing copper concentration across both pH levels. Although parental origin did not significantly affect length, significant Parental × pH, Parental × Cu, and Parental × pH × Cu interactions were observed (all $p \leq 8.1 \times 10^{-4}$), indicating subtle but complex differences among treatments (Fig. 5A).

The somatic-to-postoral ratio also varied significantly with Parental, pH, and Cu ($F_{1,2535}$ = 4.88, p = 0.027; $F_{1,2535}$ = 89.02, $p < 0.001$; $F_{2,2535}$ = 9.81, p = 5.7 × 10^{-5}) and exhibited a significant three-way interaction ($F_{2,2535}$ = 8.92, p = 1.4 × 10^{-4}). Across treatments, the ratio was generally higher at pH 7.7 than at pH 8.1, indicating a shift in the relative proportions of the two measured skeletal elements under acidified conditions. The ratio also tended to decrease with increasing copper, particularly at pH 8.1. Post hoc contrasts indicated that vent-derived larvae often showed slightly higher somatic-to-postoral ratios than ambient larvae under comparable pH–Cu combinations, although these differences were not consistent across all treatments (Fig. 5B).

4. Discussion

4.1. Parental legacy and early developmental trajectories

This study addressed two related questions: whether chronic parental exposure to low pH at CO_2 vents modifies larval responses to acidification and copper, and how these stressors jointly affect early development. Contrary to the expectation that chronic parental exposure would buffer offspring against acidification, vent-derived larvae showed poorer early developmental performance than those from the ambient site.

This pattern is consistent with previous work showing that parental exposure can modify egg provisioning, sperm performance, maternal mRNA composition, and other determinants of offspring quality, with consequences for larval development and stress sensitivity [19,20,54,71,72]. In some cases, transgenerational effects improve offspring performance under matching environmental conditions, whereas in others they fail to confer protection or may even increase sensitivity under multi-stressor exposure, consistent with the pattern observed here.

A complementary explanation is the possibility of some degree of site-associated differentiation. However, this should be interpreted cautiously, because connectivity among populations around Ischia is likely to be high and the dispersal potential of planktonic larvae remains substantial. The observed pattern is therefore more parsimoniously interpreted as a parental or environmental legacy effect than as clear evidence of local adaptation.

Our findings also contrast with results obtained in other taxa from the Ischia vent system, where chronic exposure to low pH has sometimes been associated with enhanced tolerance to acidification, albeit with possible costs under additional chemical stress. For example, polychaetes from the same system showed increased resistance to OA but altered sensitivity to pollutants [35], suggesting that physiological adjustments that help maintain acid–base balance may constrain other stress-response pathways.

More broadly, these findings reinforce the idea that offspring responses to ocean acidification depend not only on the direct effects of carbonate chemistry, but also on parental condition and on the interaction between global and local stressors.

4.2. Larval morphology and skeletal responses under OA and copper

Beyond the progression of developmental stages, a more informative signal of stress came from larval morphology, since early-stage abnormalities integrate multiple stress pathways and can reveal sublethal impacts not captured by survival or stage distribution alone [73]. At 48 hpf, the main treatment effect on morphological integrity was pH-driven, with abnormality rates markedly higher under low pH regardless of parental origin or copper exposure. This pattern aligns with previous evidence that near-future acidification increases malformation frequencies in echinoid larvae [74–76], although it should be noted that several of these studies did not explicitly correct for developmental delay, and apparent increases in malformation or reduced rod length can partly reflect earlier developmental stages rather than direct morphogenetic damage [77]. Nevertheless, the pattern observed here is consistent with the energetic cost of acid–base regulation documented in pluteus larvae exposed to low pH, which diverts resources from growth and calcification [78].

Contrary to our expectations, copper did not amplify the abnormality response, a result that contrasts with studies reporting synergistic OA × metal effects on developmental endpoints [79]. The most parsimonious explanation is that, under the specific copper concentrations and exposure duration tested here, pH dominated as the primary morphological driver and masked the contribution of moderate copper toxicity.

Skeletal rod measurements revealed more nuanced responses, with shorter postoral rods under low pH and high copper, and a three-way Parental × pH × Cu interaction on somatic rods. These patterns are difficult to interpret in isolation, since rod length also depends on developmental progression [77]; allometric corrections and longer-term exposures will be needed to disentangle direct stressor effects

from developmental delay before any functional or adaptive interpretation can be drawn. Together, these results reveal a sequential pattern in which parental legacy dominated early ontogeny while pH constrained larval quality at later stages, a pattern with direct implications for how CO_2 vents should be interpreted as proxies for future ocean conditions.

4.3. Integrating ecological and evolutionary implications

The design adopted here does not allow us to fully resolve how these parental and environmental effects interact across development; longer exposures and cross-generational designs would be required to reveal potential trade-offs between adult tolerance and offspring performance.

A relevant implication of our findings concerns the use of naturally acidified vents as predictive models for future OA. While adult persistence at the vent demonstrates tolerance at the organismal level, our results indicate that such tolerance does not necessarily extend to offspring, particularly when additional stressors are present. This caution against interpreting vent-associated phenotypes as uniformly pre-adapted to near-future ocean conditions, especially in coastal systems where acidification co-occurs with contaminant exposure [72,80].

Projecting the fate of *A. lixula* under future OA therefore requires a transgenerational, multi-stressor framework that explicitly integrates parental environmental history, early life-stage filters, and trait-level responses, rather than relying solely on survival or stage-based endpoints.

4.4. Ecotoxicological relevance and methodological considerations

From an ecotoxicological viewpoint, our results show that classical developmental endpoints and fine-scale morphometric traits capture different layers of stress response and should therefore be interpreted jointly. Standard sea urchin bioassays and regulatory protocols still rely largely on simple endpoints such as fertilization success or the percentage of morphologically "normal" plutei after 24–48 hpf [66,67,81,82]. Recent efforts to standardize and automate larval culture systems in aquatic model organisms further highlight the importance of refining experimental control and endpoint resolution in early life-stage assays [83–85]. Binary classifications effectively captured the dominant role of pH, but systematically underestimated sub-lethal and functionally relevant effects that emerged only from skeletal morphometrics, consistent with recent work proposing these metrics as sensitive indicators of multiple stressors [73,86,87].

Several limitations of our approach should be acknowledged. Exposures were relatively short and focused on early development, whereas longer-term experiments following larvae through settlement and juvenile growth would provide a more complete assessment of fitness consequences and allow direct quantification of early life bottlenecks [18,88]; longer designs would also help resolve direct stressor effects from developmental delay [77]. Copper speciation was not explicitly quantified, so we can only infer changes in bioavailability under different pH regimes based on general carbonate chemistry,

despite evidence that OA modulates metal bioavailability [89,90]; nonetheless, the nominal copper range applied here is comparable to that used in sea urchin studies with analytically verified concentrations [64], supporting the general interpretability of our results.

A further caveat concerns the use of CO_2 vents as a source of chronically exposed adults. Vent environments are inherently variable in space and time, with pH fluctuations driven by tidal cycles, local hydrodynamics and degassing intensity, so that adults classified as "vent-derived" experienced a fluctuating rather than a constantly reduced pH regime [37,38,51]. Moreover, as *A. lixula* is a mobile species in an open system, individuals collected at the vent site may have spent part of their life in waters of ambient pH, introducing unquantified heterogeneity in the duration and intensity of parental exposure. These limitations temper the interpretation of "parental history" as a well-defined experimental factor and suggest that controlled acclimation experiments, complemented by movement or isotopic tracking of adults, would help disentangle environmental legacy from short-term acclimation. Finally, we studied only two nearby sites, which may not capture the full range of environmental histories, genetic backgrounds and adaptive potentials for *A. lixula* across its distribution, as highlighted by multi-population climate studies [91,92].

5. Conclusions

In *Arbacia lixula* larvae, the effects of ocean acidification and copper unfolded sequentially across the first 48 hours of development and were shaped by parental environmental history. During the first day, developmental progression was dominated by parental origin, with vent-derived larvae showing higher rates of developmental arrest and slower progression than those from the ambient site. By 48 hpf, this parental imprint weakened and current pH emerged as the main driver of larval morphology, with abnormality rates markedly higher under low pH regardless of parental origin or copper exposure. Contrary to the expectation that chronic parental exposure to low pH at CO_2 vents would buffer offspring against acidification, vent-derived larvae showed poorer early developmental performance under the most stressful laboratory conditions, indicating that adult persistence at naturally acidified sites does not necessarily translate into offspring tolerance when additional stressors are present. These findings caution against interpreting vent-associated phenotypes as uniformly pre-adapted to future coastal conditions and support a transgenerational, multi-stressor framework that integrates parental history with early life-stage sensitivity when projecting the response of coastal calcifiers to concurrent global and local stressors.

Declarations

Conflict of interest statement

The authors declare no competing interests.

Ethics statement

This study involved work on the sea urchin *Arbacia lixula*, a marine invertebrate species that is not included among protected animals under current European Directive 2010/63/EU and corresponding Italian legislation on animal experimentation. Ethical review and approval were therefore not required. Adult specimens were collected at Ischia (Tyrrhenian Sea) under the general sampling authorizations of the Stazione Zoologica Anton Dohrn, and all procedures followed institutional guidelines for the handling and maintenance of marine invertebrates.

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Author Contribution

FGB: Investigation; Data curation; Visualization; Writing – review & editing.

AC: Investigation; Data curation; Formal analysis; Writing – review & editing.

ID: Investigation; Data curation; Formal analysis; Writing – review & editing.

CDT: Methodology; Resources; Supervision; Writing – review & editing.

MDV: Investigation; Data curation; Writing – review & editing.

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MaM: Conceptualization; Formal analysis; Supervision; Project administration; Funding acquisition; Writing – original draft; Writing – review & editing.

Data Availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher upon reasonable request. Requests to access the datasets should be directed to the corresponding author.

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Figures

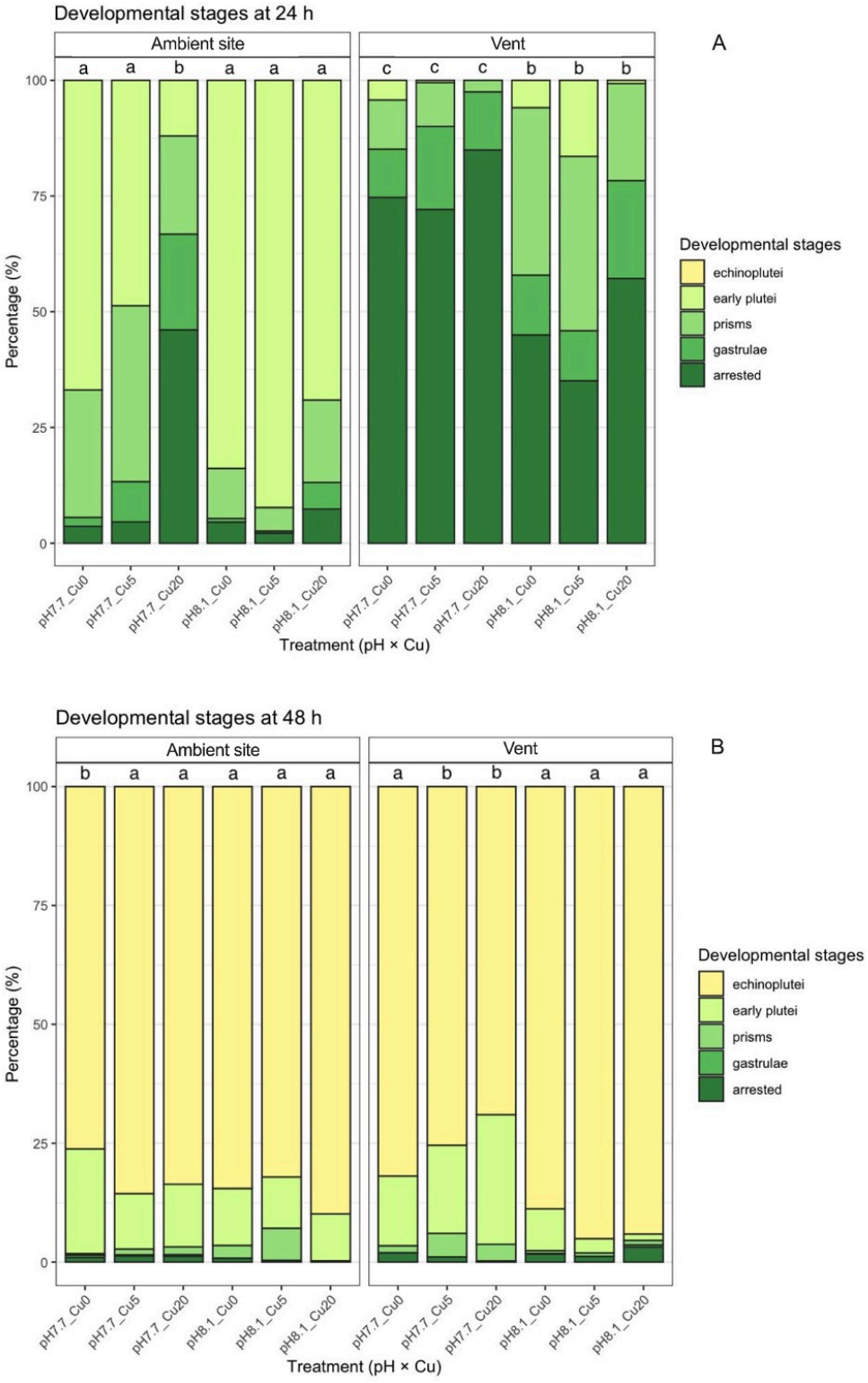


Figure 1

A-B. Percentage of developmental stages at 24 hpf (A) and 48 hpf (B) for offspring originating from ambient site (left panels) and vent site (right panels). On the X-axis, larval exposure conditions are shown as combinations of pH_T 7.7 or 8.1 and copper concentrations (Cu 0, 5, 20). Bars represent the mean proportion of arrested, gastrula, prism, early pluteus, and echinopluteus stages, in percentage.

When present, different letters above the bars indicate significant differences among treatments (Tukey HSD post hoc test, $p < 0.05$).

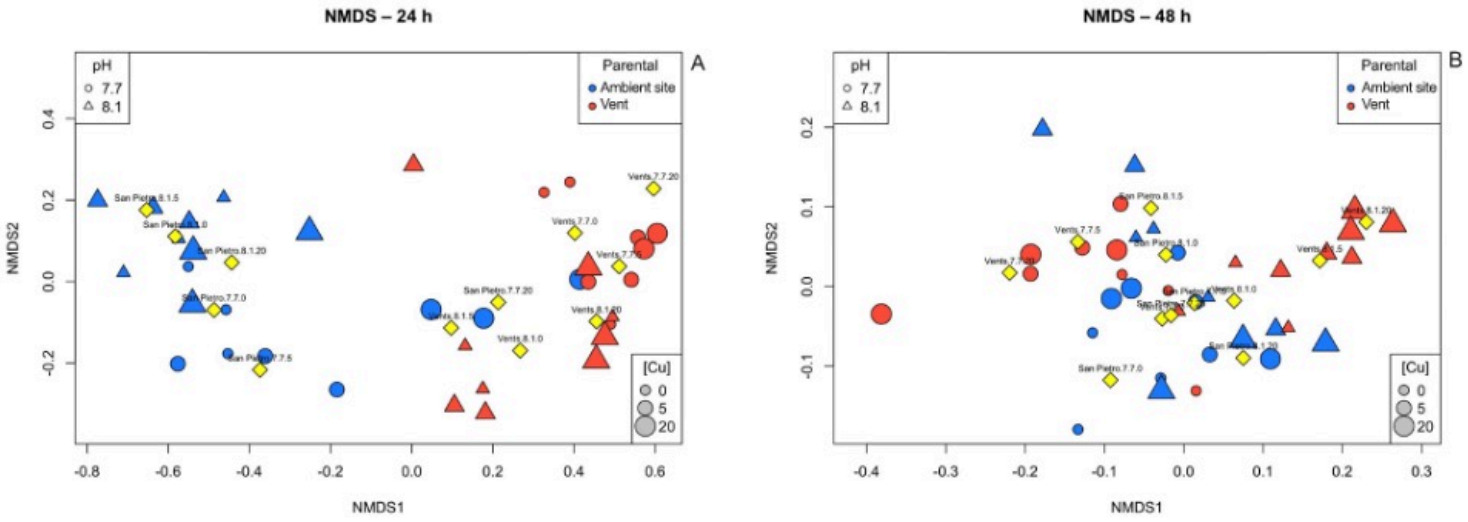


Figure 2

A–B. Non-metric multidimensional scaling (NMDS) ordination of developmental stage composition of A. lixula at 24 hpf (A) and 48 hpf (B). Ordinations are based on Euclidean distances of arcsine square-root transformed proportions of arrested, gastrula, prism, early pluteus and echinopluteus stages. Each point represents one replicate, coloured by parental origin (blue ambient site, red vent site), with point shape indicating pH (circles pH 7.7, triangles pH 8.1) and point size indicating copper concentration (small 0 µg L^{-1}, medium 5 µg L^{-1}, large 20 µg L^{-1}). Yellow diamonds show multivariate centroids for each Parental × pH × Cu combination, labelled with treatment codes. Axis values have no quantitative meaning and are interpreted solely in terms of relative distances among samples. At 24 hpf (A) ambient site and vent progeny form two well-separated clusters, whereas at 48 hpf (B) ambient and vent progeny largely share the same ordination space and the relative positions of samples mainly reflect differences among pH and copper treatments. Stress values were < 0.2, indicating an acceptable representation of the multivariate structure

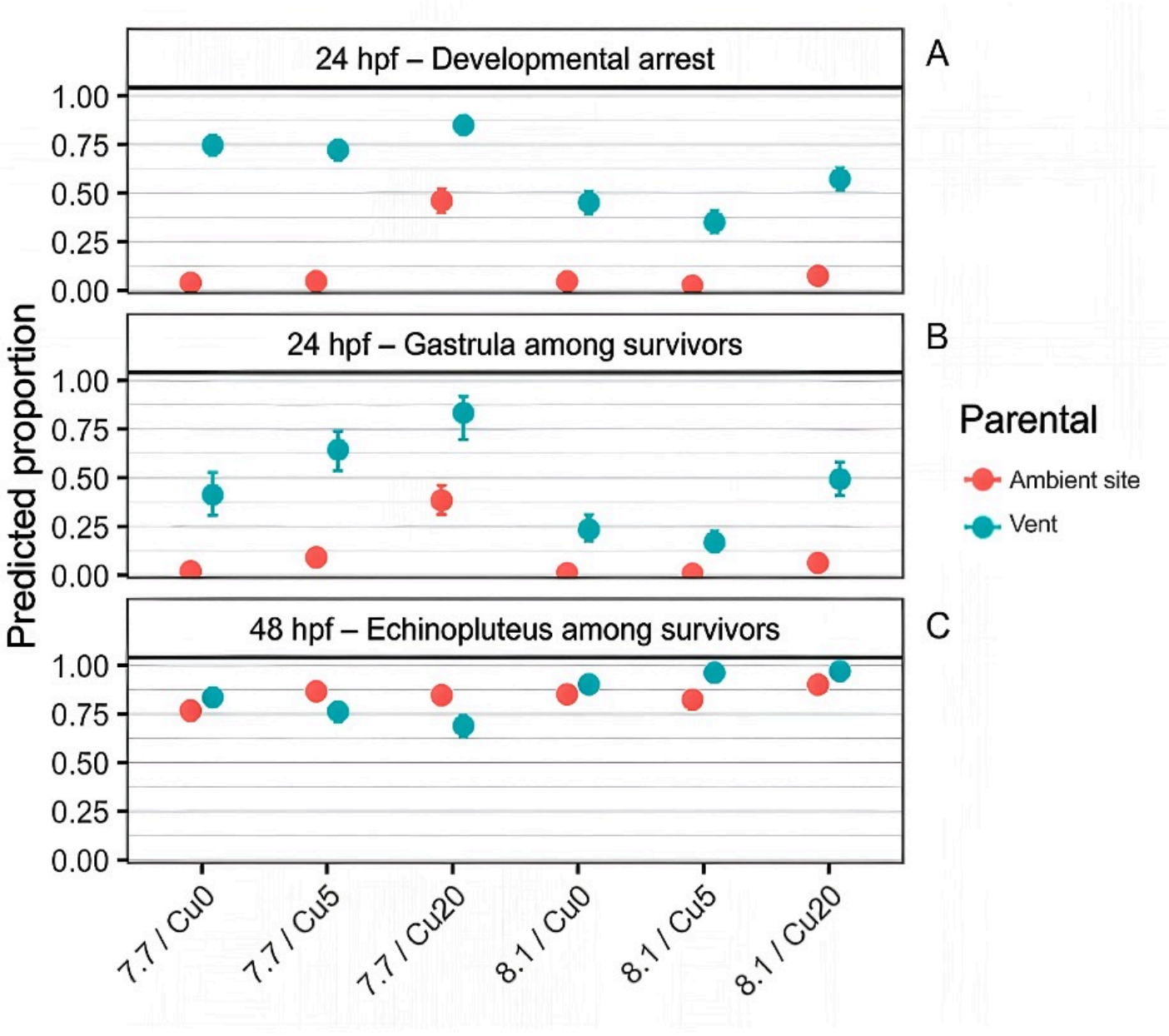


Figure 3

Model-based predictions of key developmental transitions in *Arbacia lixula* larvae under combined pH and copper treatments.

Predicted probabilities (± SE) derived from binomial generalized linear models are shown for (A) developmental arrest at 24 hpf, (B) proportion of gastrulae among surviving larvae at 24 hpf, and (C) proportion of echinopluteus-stage larvae among surviving individuals at 48 hpf. Colours indicate parental origin (San Pietro: ambient site; Vents: CO_2 vent site). The x-axis shows combinations of pH (7.7 and 8.1) and copper concentrations (Cu0, Cu5, Cu20). These panels represent sequential developmental filters, from early arrest to progression and final developmental outcome.

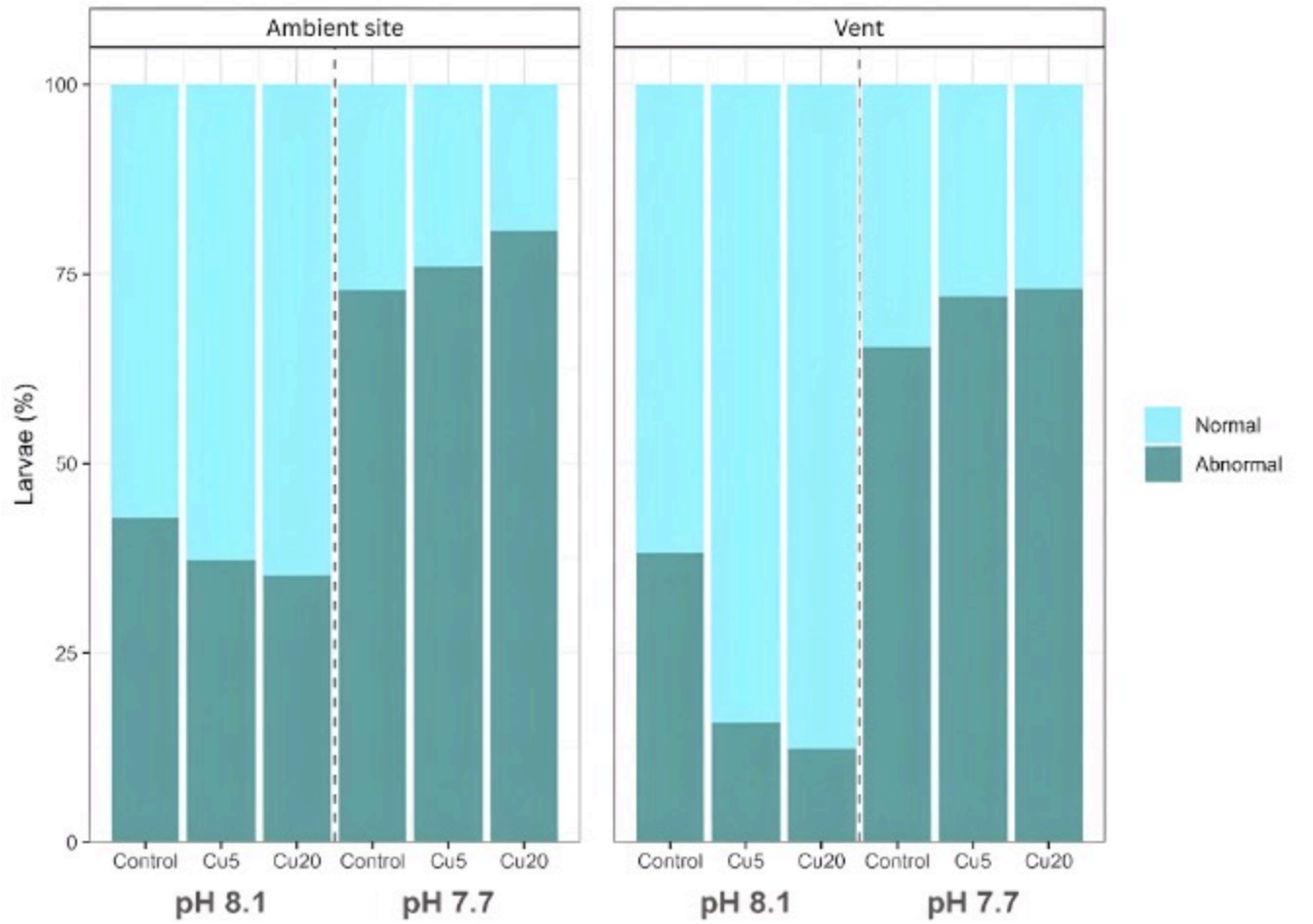


Figure 4

Proportion of normal and abnormal larvae at 48 hpf in offspring from parents collected at ambient and vent sites and raised in combinations of pH (8.1 or 7.7) and copper (Control, Cu5, Cu20).

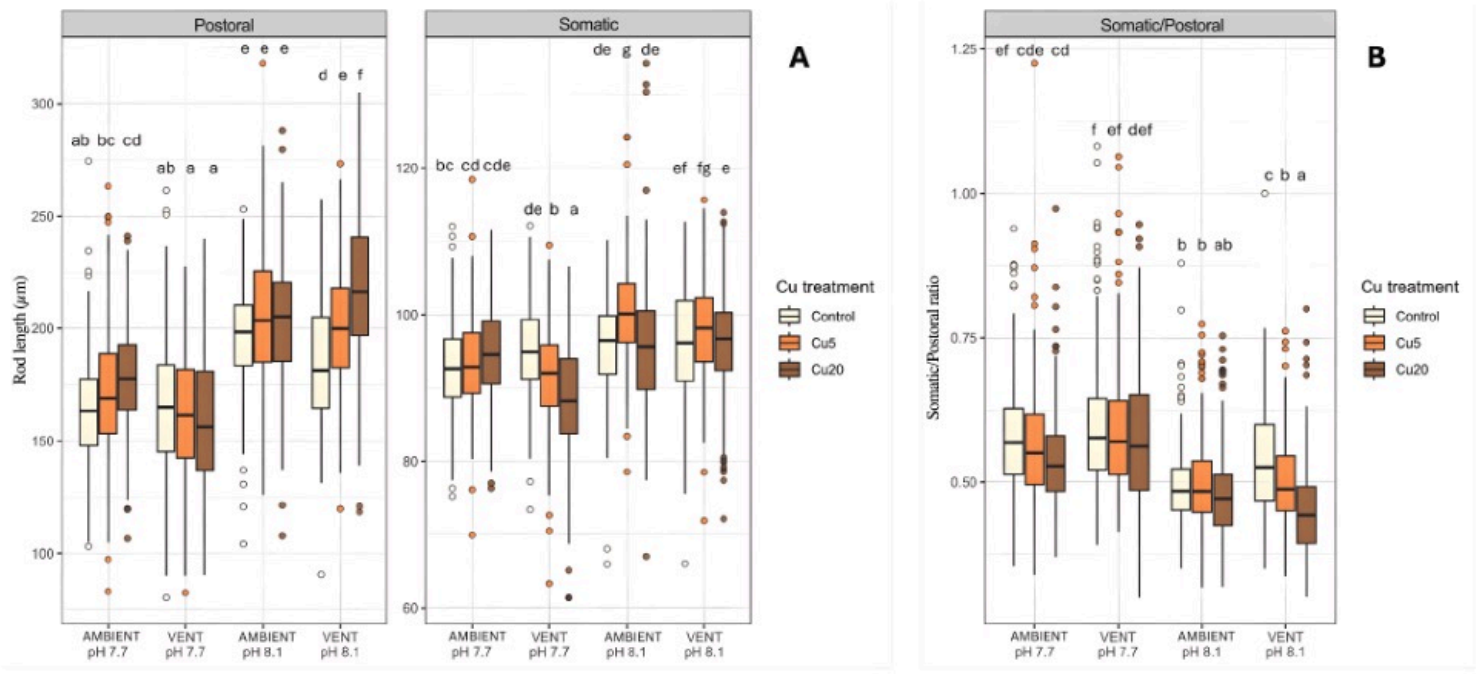


Figure 5

Boxplots of postoral rod length and somatic rod length (A), and the somatic-to-postoral ratio (B) in echinoplutei of *A. lixula* exposed to different pH conditions (8.1, 7.7) and copper treatments (control, 5 µg Cu L^{-1}, 20 µg Cu L^{-1}), and derived from parents collected at the two sites (ambient site, SP; vent site, VENT). Boxes indicate the interquartile range, horizontal lines indicate medians, whiskers extend to 1.5 × IQR, and points represent individual larvae. Different letters indicate significant pairwise differences among treatment combinations (Tukey HSD post hoc test following significant linear model effects, $p < 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.XLSX.xlsx
- Table2.XLSX.xlsx
- Table3.XLSX.xlsx