Doors are closing on early development in corals facing climate change

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Marine invertebrates are particularly vulnerable to climatic anomalies in early life history stages because of the time spent in the water column. Studies have focused on the effect of seawater temperature on fertilization, development, and larval stages in corals; however, none of them show comparative results along an environmental gradient. In this study, we show that temperatures in the range of 15–33 °C have strong effects on fertilization rates and embryonic stages of two coral species, Acropora munita in the subtropical environment and Acropora hyacinthus in subtropical and temperate environments. Deformations after the first cleavage stages were observed at low (15 °C) and high (33 °C) temperatures. Development was delayed by 6–7 h in the slightly non-optimal temperature of 20 °C. We found significant differences in fertilization rates and responses of embryos from different latitudes, with temperate corals being more sensitive to extremely hot temperatures and vice versa. We hypothesize that the coral development is restricted to a narrow temperature range and deviation outside this window could inhibit a species’ continuance and ecological success. Thus, it would have significant negative effects on adult populations and communities, playing a role in future of coral reef survival.

A biotic factors such as seawater temperature fluctuations, acidification, salinity, typhoons, changing weather patterns, and habitat characteristics have been considered primary factors affecting the survival/physiological performance, fitness, and distribution of marine organisms1–6. Broadcast-spawning marine invertebrates fertilize their gametes externally and have motile larval stages that spend days to months in the water column, making them vulnerable to various environmental perturbations4,7. Thus, it is essential to consider multiple life stages when assessing a species’ ability to tolerate stress. It is important for them to succeed at every stage of their early life cycle, including the fertilization of gametes, morula stage (also called the prawn-chip stage due to its irregular concave shape), gastrulation (the so-called donut shape), motile larval stage, settlement, and growth8,9,10. These are critical life history phases for many organisms, especially when exposed to anthropogenic stressors. The importance of successful early stages is critical as it determines the long-term viability of local populations8. In case of corals, this in turn can have community level consequences because they act as ecosystem engineers, providing habitat for myriad associated organisms while often excluding competitors for space8. Novel strategies or behaviors that increase overall reproductive success might be responsible for ensuring population survival9.

Studies conducted on scleractinian corals have shown conflicting results on fertilization rates when seawater temperature is a stress factor. While some studies observed no differences in fertilization rates with increasing temperatures10,11, an increase in cleavage rates were found in the corals Favites abdita, F. chinensis, and Mycedium elephantotus12, and A. millepora showed reduced fertilization13. Studies on other non-coral marine invertebrates have shown that when seawater temperature is used as a stress factor, fertilization is not affected but subsequent life stages are vulnerable8.
populations and communities’. Therefore, documenting the effects of temperature on early life stages is fundamental for understanding the potential for long-term resistance in corals to changing environmental conditions. Numerous studies (laboratory-based aquarium tank experiments and field-based sampling and observations) have been conducted to observe the responses of corals to seawater temperature stress in adults24–36 as well as larvae and pre-motility (fertilization and embryonic stages) stages37–40 (see Table 1 and references cited therein). However, there is a lack of information on how seawater temperatures that parent colonies are exposed to over the long term affect fertilization and development and whether the effects are the same at different latitudes. Furthermore, only a single study41 used gametes from known parent colonies and thus considered the genotypic effect on the response to temperature stress.

Studies have reported that the ideal temperature for fertilization and early development is 25–28°C42, whereas in cases of adult corals bleaching is observed at sea water temperatures around 31°C (1–2°C higher than the average summer seawater temperature)43–46 in tropical areas. However, the range of temperatures in which adult coral can thrive is between ~15°C (temperate) to ~35°C (in Persian Gulf)47. This suggests early developmental stages might be particularly sensitive to temperature stress. Hence, it is important to understand the effects of temperature on pre-settlement in order to understand how recruitment is influenced by external factors.

Here we report the effects of various seawater temperatures (at 15, 20, 25, 28, and 33°C) on the fertilization success, survival ratio, and development in two coral species: *Acropora hyacinthus*, at Penghu Marine Biology Research Center (PMBRC), Penghu, Taiwan (subtropical) and at the Biological Institute of Kuroshio (BIK), Kochi, Japan (temperate); and *Acropora muricata* at PMBRC (subtropical) (Fig. 1). We performed three different trials, one for *A. muricata* and two for *A. hyacinthus*. In each trial we exposed unfertilized gametes and subsequent embryo stages to five temperature treatments (15, 20, 25, 28, and 33°C). We crossed the gametes from three different colonies (for a total of six crosses, since we separated the maternal and paternal gametes from each hermaphrodite colony), with three replications for each cross, for a total of 18 repetitions per treatment (Fig. 2). Furthermore, we included three replicates for self-fertilization and controls (eggs cultivated in sperm-free sea water) (Fig. 2). Furthermore, we included three replicates for self-fertilization and controls (eggs cultivated in sperm-free sea water) (Fig. 2). Furthermore, we included three replicates for self-fertilization and controls (eggs cultivated in sperm-free sea water) (Fig. 2).

We hypothesized that there are inter- and intraspecies differences in their responses to seawater temperature stress and that the variations between the two locations were due to local adaptations to latitudinal differences. In addition, we tested for variability between colony crosses due to the genotypic effect on survival rate.

**Results**

**Seawater temperatures at two study locations.** Data loggers deposited at Penghu Kochi recorded hourly seawater temperature for a period of 14 months (Fig. 3). The minimum, maximum, and mean winter (January) temperatures were 14.5°C, 28.0°C, and 17.2 ± 3.5°C, respectively, in Penghu and 12.2°C, 18.7°C, and 16.9 ± 1.0°C, respectively, in Kochi. Minimum, maximum, and mean summer (August) temperatures were 25.8°C, 29.1°C, and 27.1 ± 0.6°C, respectively, in Penghu and 25.1°C, 29.6°C, and 28.0 ± 0.6°C, respectively, in Kochi. The experiment in Kochi was conducted in August 2012. In Penghu, the experiment was conducted in May 2012 when minimum and maximum seawater temperatures were 23.8°C and 30.8°C and the mean temperature was 25.8 ± 0.9°C. The transition in seawater temperatures from winter to summer was sharper in Penghu than Kochi (Fig. 3).

**Fertilization and development time.** Development in both *A. muricata* and *A. hyacinthus* (Fig. 4) was consistent with previous observations for other congeneric species48–52 until 24 h when they reached the ‘donut stage’. Although not completely overlapping, a similar pattern was observed in our study in the time required to achieve motility and develop into spindled planular larvae. *A. muricata* became motile after 41 h and *A. hyacinthus* after 38 h in both localities.

**Effect of temperature on fertilization and development.** There were differences in fertilization and development rates between temperature treatments in all three trials (Fig. 5). In all experiments, optimum fertilization and development were observed to occur at 25°C (Fig. 5), although the control temperature (ambient seawater temperature at the time of sampling and experimentation) was around 28°C at both locations. Each stage of the embryonic cycle (prawn chip, donut, and pear) took the same amount of time for completion under 25 and 28°C conditions (Fig. 6, 7, 8). At 20°C there was a delay in developmental time, and at 15°C fertilization was delayed by 6–7 h (Fig. 6, 7, 8). At the last observed stage (motile larvae), no survivors were observed at the more extreme temperatures (15°C and 33°C), the exceptions being in both species at PMBRC at 33°C with few survivors. Healthy larvae were observed to have high survival percentages at optimal temperatures (25°C and 28°C) (Figs. 5, 6, 7, 8). Treatments at 15, 20, and 33°C showed large variations (5–100%) in the number of eggs fertilized and those that underwent development. Although high mortality was observed at 20°C, a small percentage of the survivors reached the last stage (motile larvae) successfully (Figs. 5, 6, 7, 8). Responses to the 20°C treatment showed high variability (Fig. 5).

**Inter- and intra-location variation in fertilization and development of *A. hyacinthus* at PMBRC and BIK.** Fertilization success rates showed differences between the two localities at 33°C (Fig. 5, central and right columns). Although PMBRC (subtropical) fertilization success rates were highly variable at 15 and 20°C, and 100% at 33°C (Fig. 5), at BIK (temperate) we observed the opposite, with almost 100% fertilization at 15 and 20°C and a sharp drop to <30% at 33°C (Fig. 5). In the PMBRC trial, no survival was observed after fertilization at 15°C, while at 33°C an average of 60 and 40% of individuals reached the prawn-chip and donut stages, respectively. Conversely, no survivorship was observed in post-fertilization stages at 15 and 33°C in the BIK trial (Figs. 5, 7, 8). Development only rarely reached the motile phase at lower and higher temperatures, with only a few individuals still alive and functional at 33°C at PMBRC (Fig. 5). At the sub-optimal temperature of 20°C, better performance was observed in BIK with respect to PMBRC, with a higher average percentage of survival at every stage (Figs. 5, 7, 8). Multiple comparison results showed significant differences (p = 0.0001) in fertilization between locations at 33°C, with *A. hyacinthus* in BIK having lower rates compared to PMBRC (Fig. 5, Table 2). Similarly, there was a significant difference (p = 0.001) in prawn chip stage development at 33°C, with *A. hyacinthus* at BIK being more sensitive compared to PMBRC. There were no significant differences between the species in all other stages and temperature treatments (Fig. 5, Table 2).

**Inter- and Intraspecies variation in fertilization and development of gametes at PMBRC.** The general trends were similar for *A. muricata* and *A. hyacinthus* at PMBRC (Fig 5, left and central columns). Difference was found between the two species only in fertilization at 15°C, and the slight differences generally observed can be explained by inter-colony variation (see below). There were no differences in the effects of temperature stress on developmental stages between the two species. However, in the 33°C treatments, an average of 30% of *A. muricata* did reach the motile stage (Figs. 5, 6) while a few individuals of *A. hyacinthus* survived later than the donut stage (Figs. 5, 7). Treatments at 15, 20, and 33°C showed a large variation (5–100%) in the number of eggs fertilized and those that underwent development. There was more variation within *A. muricata*, which showed significant differences between temperature treatments (p ≤
### Table 1: Summary of available information on experiments conducted to observe the effects of different stresses on early development stages in corals. **Fertilization *Pre larval stages**

| Coral Species | Development stage observed | Type of stress | Main Results | Reference |
|---------------|----------------------------|----------------|--------------|-----------|
| **Acropora digitifera** | Fertilization, embryo development, larval survivorship and settlement | Sediment | Decreased fertilization, larval survivorship and settlement. Embryo development not affected | Gilmour, 1999 (74) |
| Porites astreoides | Larvae survivorships and metamorphosis | Temperature | Elevated temperature increases mortality and metamorphosis | Edmunds et al, 2001 (18) |
| **Diploria strigosa** | Fertilization, development, and larval survivorship | Temperature | No significant differences in fertilization at 30, 31, 32°C; aberration in development at 31°C and 32°C | Bassim et al, 2002 (10) |
| Diploria strigosa | Effect on larvae, larval survivorship, and metamorphosis | Combined effects of ammonium concentration and temperature | Ammonium and increased temperature cause a decrease in motility and rate of settlement | Bassim et al, 2003 (20) |
| Acropora palmata, Montastrea annularis, M. franksi | Larval survivorship | UV radiation | Larvae from deep water parents have lower survivorship than conspecifics from shallow water parents | Wellington and Fitt, 2003 (75) |
| Acropora muricata | Larval survivorship | Temperature, light condition, and presence of zooxanthellae | Presence of zooxanthellae did not affect survivorship; at 36°C all larvae die within 40 hours | Baird et al, 2006 (21) |
| **Favites abdita, F. chinensis, Mycedium elephantotus, Acropora millepora.** | Fertilization and early embryo development | Temperature | Reduced fertilization and more embryonic abnormalities with increasing temperatures in A. millepora; high level of fertilization in other corals | Negri et al, 2007 (12) |
| Acropora solitariaensis and Favites chinesis | Larval settlement and post-settlement survivorship | Temperature | Larval settlement is increased at higher temperatures, while post-settlement mortality increases with long exposure | Nozawa and Harrison, 2007 (22) |
| *Acropora palmata* | Development, survivorship, and settlement | Temperature | Development is accelerated at higher temperatures and rate of mortality and presence of abnormalities are increased | Randall and Szmant, 2009 (23) |
| *Fungiella repanda, Acropora millepora, A. spathulata, Symphyllia recta* | Larval pre-competency | Temperature | Higher % of larval metamorphosis in higher temperatures | Heyward and Negri, 2010 (38) |
| Porites panamensis | Larval survivorship and settlement, and growth into primary polyps | Combined effect of temperature and CO₂ | Survival and settlement unaffected by increasing CO₂ and 1°C; polyp growth reduced by the combined effect | Anlauf et al, 2011 (39) |
| *Montastraea faveolata* | Early embryo stages sensitivity | UV radiation | Low sensitivity during early development; susceptibility in the motile planula stage | Aranda et al, 2011 (76) |
| *Pocillopora damicornis*, *Seriatopora hystrix*, and *Stylophora pistillata* | Larval respiration | Temperature | Respiration rate was parabolic in relation to temperature, peaking at 28°C | Edmunds et al, 2011 (37) |
| *Acropora millepora, A. tenuis* | Larval metamorphosis | Combined effects of copper contamination and temperature | Synergic interactions: reducing Cu concentration prevents negative effect of 2–3°C increase | Negri and Hoogenboom, 2011 (40) |
| Goniatrea retiformis and Leptastrea cf transversa | Larval metamorphosis and settlement | CO₂ | No direct effects of acidification | Chua et al, 2012 (77) |
| **Acropora palmata** | Fertilization and development | Temperature and effect of different genotypes | Genotypic diversity affects the response of fertilization and developmental success | Baums et al, 2013 (42) |
| **Acropora tenuis, Acropora millepora** | Fertilization, development, survivorship, and settlement | Combined effects of CO₂ and temperature | No effect of CO₂; no effect of 2°C difference on fertilization, survivorship, and metamorphosis, increases rate of development | Chua, 2013 (11) |
| *Goniastrea favulus, Acropora spathulata* | Embryo and larval survivorship | Temperature | Slower development at 20°C. Temperature above ambient lower survival. A. spathulata is more affected that G. favulus | Woolsey et al, 2013 (17) |
0.001 for the fertilization, donut, and pear stages, and $p \leq 0.01$ for the prawn chip stage (Table 4). However, there were no significant differences among crosses at higher temperatures (25, 28, and 33°C). In the case of A. hyacinthus from PMBRC (Fig. 9 B), the difference was less significant ($p = 0.02$) between the cross at 15 and 20°C for fertilization but showed greater significance in other stages ($p = 0.001$) (Table 4). Unlike A. muricata, a significant difference ($p = 0.001$) was seen between crosses at 33°C in the donut stage (Table 4). A. hyacinthus from BIK (Fig. 9 C) showed significant differences ($p = 0.001$) between crosses only for fertilization, prawn chip and donut stages at 20°C (Table 4).

**Aberrant development of the embryos.** Aberrations in embryo development were detected at low (15°C) and high (33°C) temperatures. At 15°C, developmental deformations were observed immediately after fertilization, which was delayed by 2–3 h. Irregular and disproportional cell divisions were observed at 9 h after fusion of fertilized eggs. For this reason, embryos never reached the prawn-chip stage, instead continuing to fuse and degrade until the end of the experiment. This pattern was consistent for all three trials (two at PMBRC and one at BIK).

At 33°C, fertilization was faster than optimal conditions (25°C and 28°C treatment) by ~1 hour and was often followed by rapid and irregular cell division. At ~9 h, cell division had stopped and aberrant embryos started to degrade. Although all fertilized A. hyacinthus embryos at BIK underwent degradation, some fertilized embryos in both PMBRC trials were non-aberrant and survived till the end of the experiment.
experiment, and attained motility. The percentages of prawn chip, donut, and pear stages reflect only visually healthy and non-aberrant embryos (Fig. 5).

The above-described aberrations were not observed in control and self-fertilization treatments where non-fertilized eggs exhibited the round shape that gradually dissolves.

**Discussion**

This study is the first to address the effects of seawater temperature on the fertilization and early development in identical coral species living at two latitudes (subtropical and temperate) and during the same spawning year (2012) by conducting separate parental trials. The optimal temperature for survival after fertilization and before motility was 25°C irrespective of latitudinal differences in the seawater temperatures that corals are exposed to during gametogenesis and spawning. Although survival was highly affected by temperature of 15 and 33°C, this effect was different at the two latitudes. We posit that a narrow range of suitable seawater temperatures for early development in corals will create bottlenecks when seawater temperatures rise 2.0–3.0°C by 20504,53. This study is first to show in detail the effects of temperature on different stages of coral development as a function of latitude and species.

![Schematic representation of the experimental design.](image)

**Figure 2** | Schematic representation of the experimental design.

![Seawater temperature plot at Penghu (subtropical) and Kochi (temperate).](image)

**Figure 3** | Seawater temperature plot at Penghu (subtropical) and Kochi (temperate). Values represent hourly seawater temperatures plotted from December 2011–January 2013. The inset box plot represents seawater temperatures for those months in which the experiments were carried out at two locations.
The timing of developmental stages was similar for *A. muricata* and *A. hyacinthus* at both localities (Fig. 4) and congruent with the developmental cycles of other Acropora species49–52 for the first 24 h. Although not completely overlapping with the previous study51 a similar pattern was observed in the time required to achieve motility and develop into spindle planular larvae. *A. muricata* became motile after 41 h in this study, whereas it was previously observed52 to attain motility after 47 h in the same location (PMBRC). Similarly, *A. hyacinthus* became motile after 38 h in both localities, whereas50 found that it gained motility after only 36 h in concomitance with three other *Acropora* species. These slight differences may be due to different observation times during the experiments. Major differences were observed in this study between species and localities and with respect to previous studies in the time it takes to develop into spindle planular larvae. This last stage can be influenced by external conditions55.

Our latitudinal comparison showed that gametes from temperate Japan were susceptible to the high temperature of 33°C, but gametes of the same species from sub-tropical Taiwan were less sensitive (Fig. 5). In a similar study54, it was observed that the effect of temperature on the development of crown-of-thorns starfish depends on the geographic source of its larvae and the recent history of adult temperature exposures. Above or below the predicted temperature range (25–28°C), development was either delayed with a high frequency of subsequent death (20°C) or deformations in developmental stages occurred (15 and 33°C) (Figs. 6–8). Aberrant development was observed at lower and higher temperatures after the first cell division (Figs. 6–8). Aberrant embryos showed irregular cell division and fusion, always followed by degradation. Similarly, *A. millepora* showed deformed development at 32°C after the first cleavage stage56, while abnormalities in *Diploria strigosa* were observed later in their development57. However, gametes were able to fertilize at all temperatures they were exposed to, but at a significantly lower rate in the 33°C treatment for *A. hyacinthus* at BIK (Fig. 5). This might be because all ontogenic stages of a life cycle are exposed to environmental conditions, so population persistence depends on the performance of adults and offspring. However, if seawater temperatures become non-conducive for coral developmental stages, corals would have to shift their spawning away from summer by either advancing or delaying gametogenesis to cooler months58. Less affected fertilization rates can be attributed to stress resistant traits passed on directly to gametes from parents and genes for stress resistance in fertilized gametes not being expressed until after fertilization59. However, during the late stages of embryonic development, the differential expression of genes affects their ability to respond to particular stresses.

Studies on the fertilization of other marine invertebrates such as echinoids, polychaetes, mollusks, and echinoderms also indicate that gametes have the ability to fertilize over a wide temperature range (4–15°C) above local ambient fertilization temperatures60. Similarly, found61 no significant differences in fertilization success rates at 30, 31, and 32°C in *Diploria strigosa*, but developmental aberrations were observed at 31 and 32°C. At elevated temperatures (+5°C, 26–32°C), high levels of abnormalities and reduced fertilization rates occur in *Acropora millepora* and increased cleavage rates occur in *Favites chiensis*, *F. abdita*, and *Mycedium elephantotus*62. The developmental sequence in *Acropora palmata* was faster at 30 and 31.5°C than 28°C, but at higher temperatures greater rates of abnormal embryo development occur63. A similar response was observed in other marine invertebrates (e.g., oyster, sea urchin) tested for fertilization and development success at high temperatures64,65.

Our observations lead us to believe that the present increase in seawater temperatures will affect embryonic development and might
influence fertilization rates under more extreme conditions. This outcome might influence the timing of gametogenesis and spawning. In fact, water temperature affects the timing of reproduction for many invertebrates, including corals. It will also result in a decrease in the survival of corals at later life stages, thus determining the long-term viability of local populations. Our results show that temperatures deviating by 5 °C from the optimal development temperatures of 25–28 °C affected gamete development. This implies that a temperature fluctuation of ±5 °C will severely affect coral developmental stages in spite of their gametes being fertile. Corals will face greater seawater temperature fluctuations in the ocean change scenario when tropical corals are hypothesized to migrate to higher latitudes. In this event, it might result in the early life stages of corals being more impacted than adult corals since the latter are known to thrive in seawater temperatures above 35 °C in places like the Persian Gulf or also tolerate daily fluctuations in seawater temperature (up to 10 °C) as a result of internal waves and upwelling.

Our study also showed how parental lineage genotypes had a certain degree of influence on organism survival, especially genotype under high selective pressure at sub-optimal conditions (such as 20 °C, see figs. 5, 9 A, B, C, Table 4). We performed separate trials in this study for each parent cross (which was previously done only by), applying a series of different seawater temperatures to coral embryo stages to determine the role of genotype in reproductive success. We observed differences between crosses at different stages.
of development, particularly at lower temperatures (15 and 20 °C; Fig. 9 A, B, C, Table 4). Such variability across different crosses might also be due to phenotype. For example, there were higher success rates in certain parent crosses when fertilizing Acropora palmata under stressful conditions42. Future studies on this aspect will give important insight as to how genotypic and phenotypic characters influence the tolerance of corals to varying temperatures during their early life stages.

Due to their broad latitudinal distribution and associated temperature ranges, some reef communities may have a built-in adaptive capacity to accommodate temperature increases8. The water temperature increases slowly in Kochi from winter to summer compared to Penghu (Fig. 3). Although the summer average seawater temperature is similar (26–28 °C) at both locations, it might be that since adults are more accustomed to longer periods of low seawater temperatures, early coral life stages tolerate colder temperatures much better in temperate areas like Kochi. At 15 °C, A. hyacinthus at BIK had a 100% fertilization rate (Fig. 5, Fig. 9 C), and at 20 °C the variation between crosses was lower in comparison to A. hyacinthus from PMBRC (Fig. 5, Fig. 9 B). Upon exposure to high temperature (33 °C), early stages of A. hyacinthus from PMBRC fared better than those from BIK (Fig. 5, Fig. 9 A, B). The physiological and life history traits62 and exposure of adult corals to historical temperature63,64 could play a role in determining the thermal limits of early life stages2,4,9. Nevertheless, organisms will often be concurrently exposed to multiple stresses in addition to fluctuations in seawater temperature, including anthropogenic stresses8. This will add to the challenges that corals must endure in their early stages of development, and only time will tell if they can overcome this challenge through adaptive and acclimative mechanisms such as changes in larval dispersal and recruitment success, shifts in community structure, and range extensions through migration8.

Figure 6 | Time series photos showing the effect of temperature on embryonic development in A. muricata at PMBRC, Penghu, Taiwan. Columns represent the different times that elapsed from fertilization (0 h) to motility. Rows represent temperature treatments of 15, 20, 25, 28, and 33 °C. Scale bar = 500 µm.

Figure 7 | Time series photos showing the effects of temperature on embryonic development in A. hyacinthus at PMBRC, Penghu, Taiwan. Columns represent the different times elapsing from fertilization (0 h) to motility. Rows represent temperature treatments of 15, 20, 25, 28, and 33 °C. Scale bar = 500 µm.
Our study clearly shows that corals, like other marine invertebrates, do have a wide temperature tolerance range (15–33 °C in the present study) for successful gamete fertilization. Also, there is a latitudinal difference in response to temperature, so local adaptations to prevailing seawater temperatures might play an important role. Recent studies on climate change-induced ocean warming have indicated seasonal abnormal seawater temperature fluctuations leading to episodic high or low seawater temperatures in summer and winter. It is believed that stressors like ocean warming will have carryover effects from one life history stage to another, creating bottlenecks for populations that have sub-lethal or lethal consequences. Recent studies have shown that temperature stress can also influence circadian rhythms during early developmental stages in corals. Stresses like spawning, transported to BIK, and placed in individual 100 L containers with continuous flows of seawater and aeration. Seawater flow in the tanks was stopped daily after sunset (ca. 1830 hrs) throughout the spawning period. If no spawning was observed on any particular day, seawater flow was restored after 2330 hrs. At BIK, spawning was observed in situ by SCUBA diving every night from 1900 hrs during predicted spawning dates. On the peak spawning day, colonies with maximum egg-sperm bundles (as seen visually underwater) were collected 1 h before spawning, transported to BIK, and placed in individual 100 L containers with aeration. A. murex spawning was observed and their bundles collected at PMBRC on 11 May, five days after the full moon, and on 13 May for A. hyacinthus, seven days after the full moon. Collections at BIK for A. hyacinthus occurred on 7 August, five

| Table 2 | Inter-location comparison of the responses of A. hyacinthus to seawater temperature stress at PMBRC and BIK. Hy_P = A. hyacinthus from PMBRC, Hy_B = A. hyacinthus from BIK |
|----------|----------------------------------|
|          | 15 °C   | 20 °C   | 25 °C   | 28 °C   | 33 °C   |
| Fertilization |        |        |        |        |        |
| Hy_P vs. Hy_B | ns      | ns      | ns      | ns      | 0.0001  |
| Prawn chip    | Hy_P vs. Hy_B | ns      | ns      | ns      | ns      | 0.001   |
| Donut         | Hy_P vs. Hy_B | ns      | ns      | ns      | ns      | ns      |
| Pear          | Hy_P vs. Hy_B | ns      | ns      | ns      | ns      | ns      |

| Table 3 | Intra-location comparison of the responses by A. murex and A. hyacinthus to seawater temperature stress at PMBRC. Mu_P = A. murex from PMBRC, Hy_P = A. hyacinthus from PMBRC |
|----------|----------------------------------|
|          | 15 °C   | 20 °C   | 25 °C   | 28 °C   | 33 °C   |
| Fertilization |        |        |        |        |        |
| Mu_P vs. Hy_P |        |        |        |        | 0.01    |
| Prawn chip    | Mu_P vs. Hy_P |        |        |        |        | ns      |
| Donut         | Mu_P vs. Hy_P |        |        |        |        | ns      |
| Pear          | Mu_P vs. Hy_P |        |        |        |        | ns      |

Figure 8 | Time series photos showing the effects of temperature on embryonic development in A. hyacinthus at BIK, Nishidomari, Otsuki, Japan. Columns represent the different times elapsing from fertilization (0 h) to motility. Rows represent temperature treatments of 15, 20, 25, 28, and 33 °C. Scale bar = 500 μm.
treatments (15, 20, 25, 28, and 33°C columns are depicted in different colors). The crosses are: 1X2 = cross between colonies 1 and 2, 1X3 = cross between colonies 1 and 3 and 2X3 = cross between colonies 2 and 3. The box indicates the 25th and 75th percentiles, and the line within the box marks the median. Whiskers above and below the box indicate the 10th and 90th percentiles.

days after the full moon. *A. muricata* spawning was not observed during the survey period at BIK. Gamete bundles released from three colonies of each species onto the surface of the water in the buckets/containers were separately scooped up using tagged plastic cups and brought back to the laboratory for crossing experiments.

It was not possible to perform the same type of trial for *A. muricata* in both localities due to its overlap in spawning times in 2012. We tried to carry out the same experiment for *A. muricata* in 2013, but the experiment was unsuccessful due to gamete fertilization failure during two successive trials in June of that year.

**Experimental design and crossing experiments.** Crossing experiments for the three trials were performed by the same individuals (1st and 2nd authors) at both locations using the same experimental setup by shipping it to BIK after experiments were completed at PMBRC. Three colonies from each species were chosen for crossing and temperature stress experiment (Fig. 2). Bundles were filtered through a 120 μm plankton mesh to separate eggs and sperm, and aliquots of eggs and sperm were collected for density counts. A 9 μl aliquot of sperm was fixed with 1% formalin and the sperm counted in a haemocytometer using an Olympus microscope.

*Table 4 | Inter-cross comparison of the responses between A. muricata and A. hyacinthus to seawater temperature stress at PMBRC and BIK. Values in black = A. muricata (PMBRC), brown = A. hyacinthus (PMBRC), green = A. hyacinthus (BIK)*

|                | 15°C | 20°C | 25°C | 28°C | 33°C |
|----------------|------|------|------|------|------|
| Cross 1X2 vs. Cross 1X3 | 0.001 | 0.002 | ns | ns | ns |
| Cross 1X2 vs. Cross 2X3 | 0.001 | 0.02 | ns | ns | ns |
| Cross 1X3 vs. Cross 2X3 | ns | 0.001 | ns | ns | 0.04 |
| Cross 1X2 vs. Cross 1X3 | ns | 0.005 | ns | ns | ns |
| Cross 1X2 vs. Cross 2X3 | ns | 0.001 | ns | ns | ns |
| Cross 1X3 vs. Cross 2X3 | ns | 0.01 | ns | ns | ns |
| Cross 1X2 vs. Cross 1X3 | ns | 0.05 | ns | ns | ns |
| Cross 1X2 vs. Cross 2X3 | ns | 0.005 | ns | ns | ns |
| Cross 1X3 vs. Cross 2X3 | ns | 0.01 | ns | ns | ns |
| Cross 1X2 vs. Cross 1X3 | ns | 0.001 | ns | ns | ns |
| Cross 1X2 vs. Cross 2X3 | ns | 0.001 | ns | ns | 0.001 |
| Cross 1X3 vs. Cross 2X3 | ns | 0.001 | ns | ns | 0.001 |
| Cross 1X2 vs. Cross 1X3 | ns | 0.001 | ns | ns | 0.001 |
| Cross 1X2 vs. Cross 2X3 | ns | 0.001 | ns | ns | 0.001 |
| Cross 1X3 vs. Cross 2X3 | ns | 0.001 | ns | ns | 0.003 |

*Figure 9 | Quantitative data for inter-cross fertilization for the three trials separately: (A) *A. muricata* in PMBRC, (B) *A. hyacinthus* in PMBRC, and (C) *A. hyacinthus* in BIK at different temperature*
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