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Larval thermal windows in native and hybrid *Pseudoboletia* progeny (Echinoidea) as potential drivers of the hybridization zone

M. Lamare[1,*], J. Harianto[2], S. Uthicke[3], A. Agüera[4], S. Karelitz[1], D. Pecorino[5], J. Chin[1], M. Byrne[2,6]

[1]Department of Marine Science, University of Otago, Dunedin 9016, New Zealand
[2]School of Medical Sciences, University of Sydney, Sydney, NSW 2006, Australia
[3]Australian Institute of Marine Science, Townsville, QLD 4810, Australia
[4]Danish Shellfish Center, DTU-aqua, Danish Technical University, Øroddevej 80, 7900, Nykøbing Mors, Denmark
[5]Mesa Srl, University Campus - Palazzina Locatelli - Via Magliotto, 2 17100 Savona, Italy
[6]School of Life and Environmental Sciences, University of Sydney, Sydney, NSW 2006, Australia

ABSTRACT: For marine species that hybridize, the maintenance of separate lineages requires natural barriers that limit hybridization zones or species distributions in which hybrid progeny cannot survive across the entire range of the parent species. We examined this potential in the sea urchin species *Pseudoboletia maculata* and *P. indiana*, which have overlapping distributions in the Pacific and readily hybridize, yet have maintained separate lineages. We examined the role of developmental thermal windows in native and hybrid progeny reared across a temperature gradient (8 to 37°C) to determine if post-zygotic processes restrict the environmental isotherm hybridization zone along the eastern Australian coastline. Native and hybrid progeny of *Pseudoboletia* from Sydney Harbour were reared to late pluteus larvae and scored for development at 3 time points (10, 24 and 48 h post fertilization) to determine the thermal limits for normal early development. While hybrid progeny developed equally well within their thermal windows and at ambient temperature (22°C), they had thermal windows up to 10°C narrower than those of the maternal lineage. The geographic ranges known for the benthic adults coincide with the thermal windows of their progeny. Hybrid progeny were less tolerant of the warmer conditions experienced by the *Pseudoboletia* species. This indicates that offspring fitness may limit hybridization in the tropical regions. Given the potential for the emergence of new hybridization zones as the oceans warm, our observations highlight the need for a greater understanding of the thermal biology of hybrid offspring when predicting future distributions and the potential for expansion of hybridization zones.

KEY WORDS: Hybridization zones · Introgression · Larval development · Distribution · Ocean warming · Climate change · Echinoids

INTRODUCTION

Climate change-driven expansions of hybridization zones in terrestrial and marine ecosystems, and the resulting loss of biodiversity, are of increasing concern (Muhlfeld et al. 2014). Predicting the importance of this process requires a greater understanding of how hybridization influences species performance and distributions, and how it impacts native lineages in areas of sympatry. For marine species that naturally hybridize, the maintenance of separate lineages requires natural barriers that limit reproduc-

*Corresponding author: miles.lamare@otago.ac.nz

tive exchange (Gardner 1997) or distributions where hybrid progeny cannot survive across the entire range of the parent species (Pfennig et al. 2016).

Hybridization barriers in marine species are well studied in sea urchins (Lessios 2007) and are attributed to reproductive isolation that includes gamete incompatibility (McClary & Sewell 2003, Lessios 2007, Kosman & Levitan 2014), differences in reproductive periodicity (Muthiga 2003) and physical isolation through habitat separation (Lessios 2007). For some species, however, the mechanisms controlling the distributions of native species and their hybrids and the maintenance of separate lineages are unclear. Zigler et al. (2012), for example, examined hybridization in 2 sea urchin species, *Pseudoboletia maculata* and *P. indiana,* which have broadly similar temperate to tropical distributions but have no apparent barriers to hybridization (i.e. high gamete compatibility, overlapping reproductive cycles, sympatric and overlapping ecologies). Hybrids of the 2 species occur naturally, as do hybrids of several sympatric congeneric pairs of sea urchins and sea stars that have dispersive larvae (e.g. Byrne & Anderson 1994, Harper et al. 2007).

Dispersive larval stages can play an important role in hybridization and species introgression in the sea (Harper et al. 2007), and Gardner (1997) emphasized the need for a multi-disciplinary approach to understand hybridization in marine species, including an examination of reproductive ecology. This is particularly important as marine species around the globe are exhibiting changes in reproductive phenology and poleward migration through larval dispersal in response to warming climate and changes in ocean circulation (Pecl et al. 2017). Most (≈80 to 90%) marine species have a biphasic life cycle, with the thermal biology (i.e. their thermal windows) of their planktonic stages exerting a strong influence on the biogeographic distribution of the adults (O'Connor et al. 2007, Ling et al. 2009, Lamare et al. 2014, Byrne et al. 2016, 2017). There is growing interest in the role of larval thermal windows in driving (e.g. *Centrostephanus rodgersii*), or potentially driving (e.g. *Asterias amurensis*), the poleward range extensions of marine species as the climate warms (Ling et al. 2009, Pecorino et al. 2013, Hardy et al. 2014, Byrne et al. 2016, 2017). Thus far, however, no published studies have quantified the fitness of hybrid larval progeny to environmental gradients.

Pseudoboletia presents an excellent model system for the investigation of hybridization, as the process is readily detected in the genus due to the presence of intermediate adult phenotypes confirmed from genotyping (Zigler et al. 2012). There are 4 species of *Pseudoboletia*, including the sympatric species *P. maculata* Troschel, 1869 and *P. indiana* Michelin, 1862, that have very broad and often overlapping distributions. *P. indiana* has a broad Indo-Pacific distribution (Africa to Hawaii), while *P. maculata* is largely restricted in the Pacific (Australia to Japan) (Miskelly 2002). Although these species have maintained separate lineages for at least 2 million years and are taxonomically distinct, they have no apparent barriers to hybridization, including the gamete-binding protein system that ensures other congeneric urchins do not hybridize (Zigler et al. 2005). Inter- and intraspecific crosses give rise to equally high fertilization rates (Zigler et al. 2012) and normal embryonic and early larval development. Hybrids constitute up to 15% of one population (Zigler et al. 2012), and although the *Pseudoboletia* hybrid distributions are not well defined, hybridization zones are known to occur at the northern and southern range edges of the 2 species in the Pacific (Shigei 1986, Zigler et al. 2012).

To better understand processes controlling the distribution of marine hybridization zones and the potential that hybridization zones may be altered by climate warming (i.e. Harper & Hart 2007, Tsang et al. 2008, Chunco 2014), we determined the thermal window for development in native and hybrid progeny with respect to the occurrence of *Pseudoboletia* hybrids at their northern and southern Pacific (cooler) range edges (Shigei 1986, Zigler et al. 2012). We investigated the thermotolerance of the progeny of *P. maculata* and *P. indiana* and their hybrids from sympatric populations in southeastern Australia. This region is one of the fastest-warming marine environments, with ocean warming exacerbated by intensified poleward flow of the warm East Australian Current (Hobday & Lough 2011, Centina-Heredia et al. 2014). Sea surface temperatures (SSTs) in the region have increased ca. 1°C since 1960 and are forecast to increase by between 2 and 4°C by 2100 (Hobday & Lough 2011, Centina-Heredia et al. 2014). To test the 2 opposing hypotheses of heterosis (increased stress tolerance) and out-breeding depression (reduced stress tolerance) in hybrid progeny in response to temperature, intra- and interspecific crosses were generated. The thermal windows and fitnesses of *Pseudoboletia* progeny were determined using a 12-step thermal gradient (8.6 to 36.4°C), which provides a test of developmental responses not only at the site of study populations (Sydney Harbour) but also across the temperate range that the animal would

encounter within its Australian range, as well as temperatures beyond its thermal limits. Across this temperature gradient, we examined the pace of development and the percentage of normal progeny through the well-developed larval stage. Together with habitat maps of SST during the spawning season of the 2 *Pseudoboletia* species in eastern Australia (Zigler et al. 2012), we estimate the geographic range where native and hybrid larvae may develop at the present time and discuss implications for the projected near-future warming for the region.

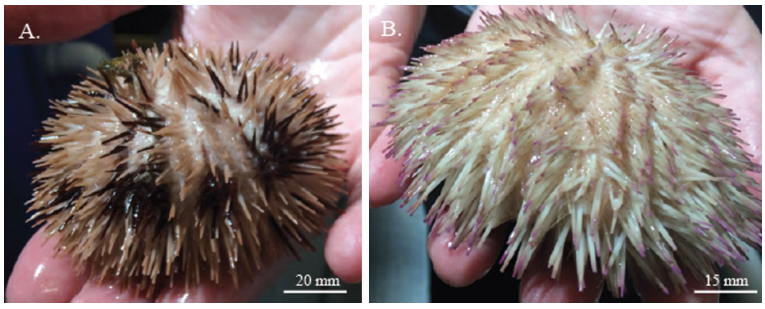

Fig. 1. (A) *Pseudoboletia maculata* and (B) *P. indiana* showing morphological differences between the two species. Distinguishing features include pigmentation of the test and spines and density of spine canopy

We tested the hypothesis that the thermal windows of native and hybrid offspring across an environmentally relevant temperature gradient would differ and thereby contribute to the limited distributions of hybrids and the maintenance of native species lineages. Specifically, we examined whether (1) the larval thermal windows are consistent with the distributions of native *Pseudoboletia*, (2) out-breeding depression under present day and future thermal conditions will reinforce distinct sympatric species, or (3) heterosis under future climate change scenarios predicted for Australian populations may drive loss of species differentiation through introgression and generation of evolutionary novel breeding lines.

MATERIALS AND METHODS

Water temperatures in Sydney Harbour

To assess the thermal environment encountered by the *Pseudoboletia* species at the site of collection, we used sea surface water temperatures recorded weekly between 2001 and 2007 at Neilsen Park (adjacent to our Camp Cove sea urchin population) (New South Wales Office of Environment and Heritage). We calculated the monthly average, maximum, minimum and 95% range of temperatures over the 7 yr period. Water temperature at the time of collection (February 2014) was 22.5°C.

Animal collection and spawning

Sympatric *Pseudoboletia maculata* and *P. indiana* (Echinoidea: Toxopneustidae) were collected from Camp Cove, Sydney Harbour (33° 50′ 27.3″ S, 151° 16′ 34.4″ E) at a depth of 2 to 3 m. Collections were made on 24 February 2014; animals were transported to the Sydney Institute of Marine Science (Chowder Bay, Sydney Harbour), where they were maintained in flow-through aquaria. The 2 species are easily recognized by differences in their pigmentation (Fig. 1), a technique shown by Zigler et al. (2012) to be valid for identifying non-hybrid individuals of both species, with pigment patches being diagnostic for *P. maculata* and pale tests with pink-tipped spines being diagnostic for *P. indiana*. Females of each species were also confirmed from known differences in egg size (Table S1 in Supplement 2 at www.int-res.com/articles/suppl/m598p099_supp.pdf). While the potential for fertile F_1 hybrid backcrosses with parental lineages appears low (<3% recorded by Zigler et al. 2012), ideally, parental identity would be confirmed by mtDNA analysis. However, morphological recognition of the 2 species and their hybrids is highly reliable, with Zigler et al. (2012) noting that only 1 of 53 individuals identified by parental morphologies displayed a mtDNA haplotype and bindin genotype of an F_2 or later generation hybrid.

The sea urchins were spawned by an intracoelomic injection of 0.5 M potassium chloride, with the volume injected (1 to 2 ml) depending on the size of each individual. As spawned *Pseudoboletia* can produce mucus that fouls gametes, eggs were collected dry from the aboral surface of females using a Pasteur pipette and then transferred to 500 ml glass beakers containing freshly filtered seawater (FSW, 0.45 µm). The eggs were washed in three 80% water changes. Sperm was collected dry from the aboral surface of males, stored in Eppendorf tubes and kept chilled until needed (for a maximum 1 h). For each experiment, to minimize any differences in gamete quality among individuals, pooled eggs and sperm from 3 females and 2 males were used. Fertilization was achieved by the addition of activated sperm to the eggs at a final sperm concentration of $2–3 \times 10^5$ sperm ml^{-1} as determined using a haemocytometer. Fertilization and

subsequent examination of thermal windows of successful development were undertaken in the following 4 crosses: (1) *P. maculata* ♀ × *P. maculata* ♂, (2) *P. indiana* ♀ × *P. indiana* ♂, (3) *P. maculata* ♀ × *P. indiana* ♂, and (4) *P. indiana* ♀ × *P. maculata* ♂. Experiments were run in 3 consecutive experiments over a 6 d period; the first experiment examined responses in *P. indiana* ♀ × *P. indiana* ♂ progeny, the second examined *P. maculata* ♀ × *P. maculata* ♂ and *P. maculata* ♀ × *P. indiana* ♂ progeny (using the same pooled eggs from *P. maculata* in both crosses), and the third examined *P. indiana* ♀ × *P. maculata* ♂ and *P. maculata* ♀ × *P. maculata* ♂ progeny. Shortly after insemination, eggs were examined for the presence of a fertilization envelope, and the proportion fertilized was determined to be >95% across all trials and species crosses.

Thermal window experimentation

Embryos and larvae were reared in aluminium thermal blocks (length = 784 mm, height = 60 mm, width = 170 mm) bearing 48 holes (diameter = 31 mm, depth = 54 mm) arranged in 4 columns × 12 rows (Fig. S1 in Supplement 1). To achieve a thermal gradient in the aluminium blocks from ≈8 to 37°C, heated or cooled water from temperature-controlled water baths was pumped through the opposite ends of the blocks. In total, there were 12 temperature treatments (8.6, 11.9, 14.4, 16.8, 19.4, 21.8, 24.2, 26.6, 29.0, 31.4, 33.7 and 36.4°C, Table S2 in Supplement 2), with each replicated 3 (interspecific crosses) to 6 (intraspecific crosses) times. Each hole in the block held a 40 ml glass vial with lid containing 30 ml of the embryo/larval suspension. For each experiment, 3 columns in each thermal block were used as temperature treatments, while the vials in the remaining columns were used either as a reservoir of FSW at the appropriate experimental temperature for partial water changes during the experiments or to record the temperatures every 15 min (Fig. S2 in Supplement 1) using thermistor probes connected to a Vernier LabQuest data logging system (Vernier Software). Water was replaced in each vial after each sampling time with seawater at the appropriate experimental temperature. Oxygen (O_2) concentrations and temperatures were measured independently and remained >96.4% O_2 saturation across all temperatures (Table S3 in Supplement 2).

At the start of the experiment, fertilized eggs were added to the vials to give a final concentration of 30 embryos ml^{-1}. A reference embryo sample was taken at the start of the experiment and subsequent 1.5 ml samples taken at 10, 24 and 48 h post-fertilization. Samples were fixed in 5% buffered formalin in seawater and stored for later classification of developmental stage. Embryos and larvae were initially classified across 8 developmental stages (Fig. 2) or identified as abnormal in appearance (i.e. irregular cell division, severe asymmetry, malformation). To statistically compare the response to temperature among progeny from the different type of cross, we pooled progeny into 4 developmental categories: (1) embryonic stages (pooling individuals from fertilized eggs to 16^{+}-cell stages), (2) unhatched blastula to gastrula stages (referred to as gastrulation stages), (3) pluteus stages (pooling prism and pluteus stages), and (4) abnormal development.

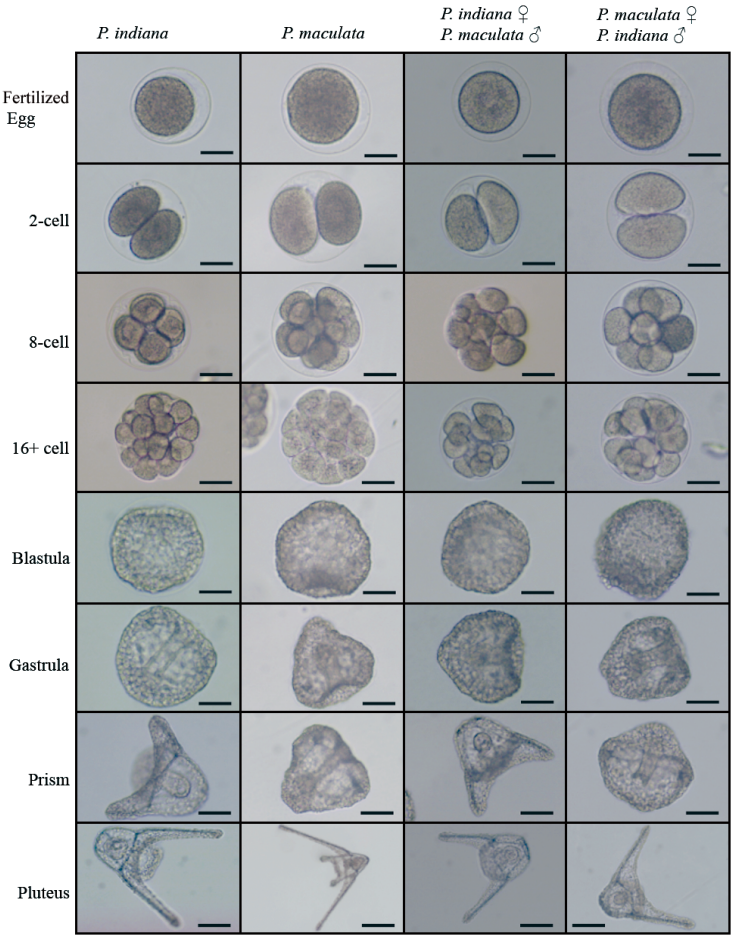

Fig. 2. Eight developmental stages seen up to 48 h post fertilization for *Pseudoboletia indiana* and *P. maculata* progeny and for hybrid *P. maculata* ♀ × *P. indiana* ♂ and *P. indiana* ♀ × *P. maculata* ♂ progeny. Scale bar: fertilized egg to prism = 50 µm; pluteus = 100 µm

Statistical comparisons of development among progeny

Development and temperature

The effect of temperature on development among the progeny of the 4 crosses was assessed qualitatively through direct observation of the distribution of developmental stages across temperature and time and quantitatively using generalized linear mixed models (GLMMs) fitted to the observations. For modelling of the distribution of developmental stages, the effect of temperature, time and their interactions for each of the progeny was quantified on either (1) the proportion of larvae reaching each developmental stage (embryo, gastrula, pluteus) at time or (2) the proportion of normal development at time. We subsequently focussed on the modelling results of the proportion of pluteus larvae at 24 and 48 h post fertilization, which integrates the pace of development across early embryonic and early pluteus development (a key component of potential larval survival in nature), while modelling the proportion of normal development at 48 h represents the potential to develop properly without considering the pace of development (i.e. the temperature limits for normal embryonic and larval development).

The temperature response was unimodal; therefore, orthogonal polynomials of temperature were used (up to level 3). Time also had a non-linear effect, with development stages appearing over time and disappearing as they progress to the next stage; therefore, orthogonal polynomials of time were used (up to level 2) for initial models. Subsequent model selection was done by comparison of the Akaike's information criterion (AIC), selecting the model with the lowest AIC. Models were fit using the function GLMM from the package lme4 v.1.1-12 (Bates et al. 2016), using a binomial distribution in which observations were treated as binary (for example, for normality: 1 = larvae with normal development, 0 = larvae with abnormal development). Observations resulted from counts in the same vials at 10, 24 and 48 h; therefore, the identification of these vials was set as a random variable that allowed observations from the same vial to be grouped together. Simulations were used to illustrate the development of the 4 species at each temperature and time to describe the change from one developmental stage to the next (Bates et al. 2016) and were performed using the package merTools v.0.3.0 (Knowles & Frederick 2016) in the software R v.3.3.0 (R Core Team 2014).

Using the generalized linear model (GLM) approach for pluteus development, we calculated the minimum and maximum temperature for this developmental stage (i.e. T_{50}^{min}, T_{50}^{max}, T_{90}^{min} and T_{90}^{max}) to assess overall developmental performance thresholds. The GLMM assessed the effect of temperature on the probability of normal pluteus development with time among the 4 progeny categories. For this, we performed a bootstrap procedure consisting of 1000 simulations of the model considering fixed (temperate, progeny, time) and random (vial) effects to obtain the maximum and minimum temperatures that yield 50 and 90% of normal pluteus larvae at 10, 24 and 48 h. From the simulations, differences among progeny in pluteus development at 48 h were determined from the 95% confidence intervals (Supplement 3).

A further set of GLMMs was used to assess the effect of temperature on the probability of normal development across all developmental stages at 10, 24 and 48 h post fertilization for the 4 progeny categories. We parameterized the GLMM for each set of progeny, considering time, temperature and their interactions. Again, temperature had a non-linear effect on abnormality probability, with the response data exhibiting a unimodal shape that was modelled considering orthogonal polynomials of temperature (up to degree 3). The GLMMs were used to calculate the minimum and maximum temperature limits of normal development (i.e. T_{50}^{min}, T_{50}^{max}, T_{90}^{min} and T_{90}^{max}) to assess performance thresholds based on the proportion of larvae developing normally. As before, we performed a bootstrapping procedure with the model to obtain the maximum and minimum temperatures that yield 50 and 90% of normal larvae at 10, 24 and 48 h and differences among progeny determined from the 95% confidence intervals. Model fit was selected based on the lowest AIC (Supplement 4).

SST and larval thermal window maps

April to June (austral autumn) is the major period of spawning of Sydney *Pseudoboletia* (Zigler et al. 2012). Therefore, the average SST measurements during May over a 13 yr time frame (2003 to 2016) were collected from the SST probe of the satellite Terra MODIS (http://oceancolor.gsfc.nasa.gov/) from southern Papua New Guinea to just south of Tasmania (latitude from 7° 55′ 00″ to 51° 39′ 60″ S, longitude from 138° 45′ 00″ to 163° 45′ 00″ E) at a resolution of 4 km. The area chosen encompasses the present geographical range of *Pseudoboletia* along the eastern Australian coast. By integrating the SST distributions

with the thermal windows for normal development of native and hybrid *Pseudoboletia* larvae, we determined the potential geographic area in which the larvae could develop normally in the water column. We overlaid on the thermal maps the documented occurrences of both *Pseudoboletia* species as reported in the Atlas of Living Australia (https:bie.ala.org.au/search?q=Pseudoboletia). To account for temporal and latitudinal variation in spawning period, in addition to the patterns reported for the reported peak spawning period (May), we also modelled distributions of *Pseudoboletia* assuming a wider autumn spawning period (i.e. April to June) using SST maps for the 3 mo (Fig. S3 in Supplement 1). SST maps were realized with the software R v.3.3.0 by using the functions image and contour of the base package.

RESULTS

Sea temperatures

Sea temperatures recorded weekly between 2000 and 2007 at Neilsen Park (Fig. 3A) ranged from 12.8°C (August 2004) to 25.0°C (February 2001), with an average temperature over the 7 yr period of 19.1°C. Mean monthly temperatures over the 7 yr period ranged from 15.49°C (July) to 22.57°C (February), with average temperatures during the spawning and larval development period (April to June) ranging from 22.57 to 16.62°C (Fig. 3B).

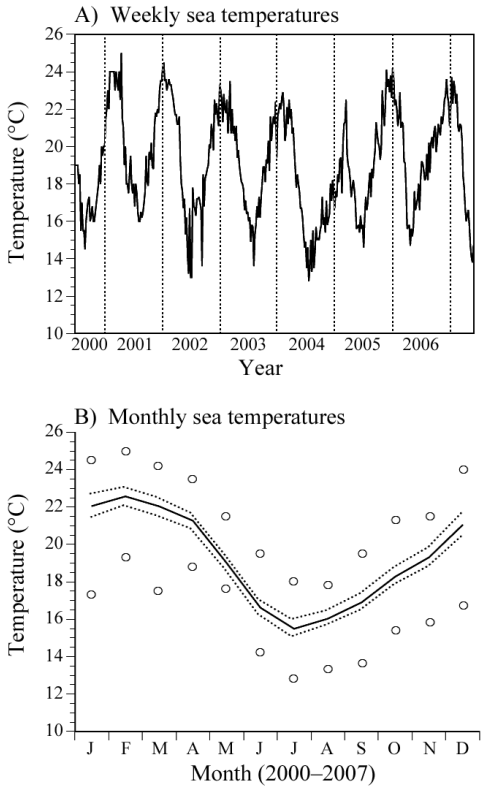

Fig. 3. Sea surface temperature at Neilsen Park, Sydney Harbour: (A) recorded approximately weekly between April 2000 and July 2007; (B) monthly average (—), with 95% confidence limits (·····); maximum and minimum values (°C) recorded over the 7 yr period

Embryonic and larval development

Pseudoboletia maculata females spawned larger eggs (mean 103.9 ± 1.09 µm) than *P. indiana* (mean 84.4 ± 1.12 µm, Table S1 in Supplement 2. Comparing development at the ambient temperature for Sydney Harbour at spawning (21.8°C), *P. maculata* showed a faster progression through developmental stages than *P. indiana*, at all times sampled (Table 1). For example, by 24 and 48 h, the majority of *P. maculata* progeny were in the prism and pluteus stages, respectively, while in *P. indiana*, most progeny were in the early blastula and gastrula stages, respectively (Table 1). In turn, at 21.8°C, hybrid progeny from both reciprocal

Table 1. Proportion of individuals (% ± SE) from 4 *Pseudoboletia* progeny at 9 developmental stages and abnormal stages reared at ambient temperatures (21.8°C). Proportions are given for 10, 24 and 48 h post fertilization

Stage	*P. indiana*	*P. maculata*	*P. indiana* ♀ × *P. maculata* ♂	*P. maculata* ♀ × *P. indiana* ♂
10 h post fertilization				
8-cell	0.9 ± 0.95	0	0	0
16+-cell	73.9 ± 4.97	0	0	0
Morula	22.1 ± 4.67	0	0	0
Unhatched blastula	0	0	0	0
Early blastula	0	99.5 ± 0.48	4.4 ± 1.79	0
Late blastula	0	0	83.1 ± 1.88	100
Abnormal	3.1 ± 1.20	0.5 ± 0.48	12.5 ± 2.80	0
24 h post fertilization				
Early blastula	62.1 ± 6.05	11.2 ± 1.65	0	0
Late blastula	37.9 ± 6.05	0	0	0
Gastrula	0	20.9 ± 2.53	0	0
Prism	0	67.9 ± 2.85	37.3 ± 6.1	39.0 ± 0.91
Pluteus	0	0	62.7 ± 6.1	59.1 ± 2.01
Abnormal	0	0	0	1.9 ± 1.85
48 hr post fertilization				
Late blastula	13.3 ± 7.31	0	0	0
Gastrula	86.7 ± 7.31	0	0	0
Prism	0	0	0	0
Pluteus	0	100	98.1 ± 1.0	100
Abnormal	0	0	1.9 ± 1.44	0

crosses developed fastest, with the majority of hybrid offspring in the pluteus stage by 24 h (Table 1). At ambient temperature, abnormality rates were low (<4%) across all 4 progeny types at each sample time, with the exception of *P. indiana* ♀ × *P. maculata* ♂, which recorded 12.5% abnormality at 10 h.

Developmental thermal windows

In terms of developmental progression, direct observations indicated *P. maculata* had the broadest optimal thermal window and a greater capacity to respond to warmer temperatures. This is seen in the faster development and a relatively larger number of advanced larvae at any given sampling time (Fig. 4). For example, at 10 h, the pace of development of *P. maculata* and its hybrids was only decreased below ca. 17°C, with no embryos found above that temperature. For *P. indiana,* however, embryonic stages were still present at temperature ≤24.2°C at 10 h and still occurred at 24 h. By 48 h, ~100% of the progeny of *P. maculata* and *P. indiana* were plutei at ≥24.2°C. However, the thermal window for *P. indiana* was narrower (19.3 to 29°C) than that of *P. maculata* (16.7 to 31.3°C). The hybrid progeny had the narrowest and cooler optimal thermal windows, with *P. indiana* ♀ × *P. maculata* ♂ plutei present at temperatures between 14.3 and 21.8°C, while *P. maculata* ♀ × *P. indiana* ♂ plutei were present at temperatures between 16.7 and 26.5°C (Fig. 4).

For statistical comparisons of pluteus thermal windows (Fig. 5), GLMMs considering the level 3 orthogonal polynomials of temperature and their interactions with level 2 orthogonal polynomials of time attained the best fit for the distribution of pluteus stages across the thermal gradient (Supplement 3). Model simulations showed different patterns in the timing and thermal windows of this stage among all 4 progeny types. At 48 h, the T_{50}^{min} for plutei was not significantly different ($p > 0.05$) between the hybrids and *P. maculata* (16.69 to 18.05°C) but was signifi-

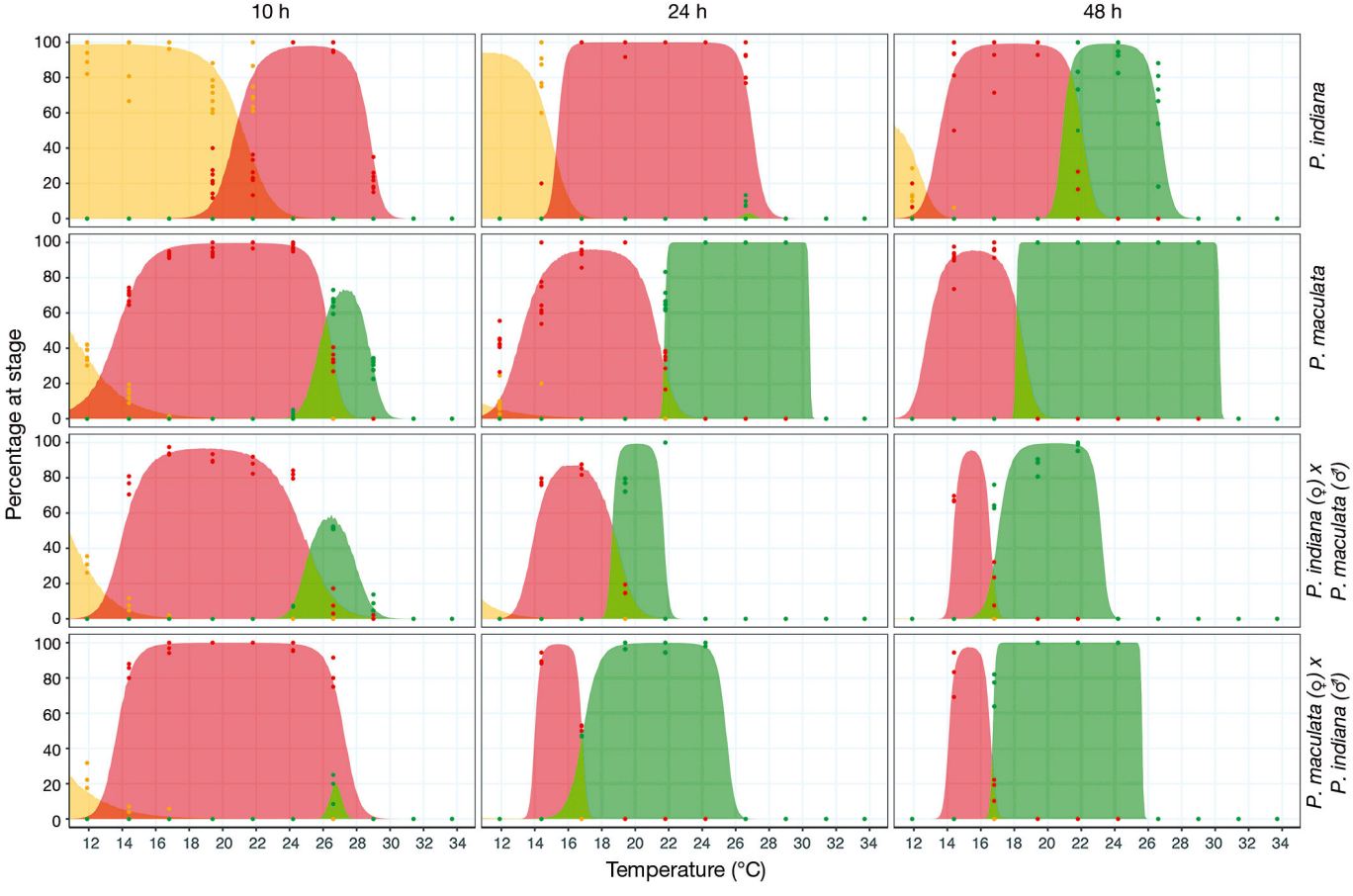

Fig. 4. Generalized linear mixed models of the average proportion of conspecific and hybrid *Pseudoboletia* progeny in 3 developmental stages (embryo, gastrula, pluteus) across a thermal gradient (8.5 to 35.8°C) at 10, 24 and 48 h post-fertilization. The dots indicate the measured proportions in each development stage. Embryos are presented in orange, gastrula in red, and larvae in green

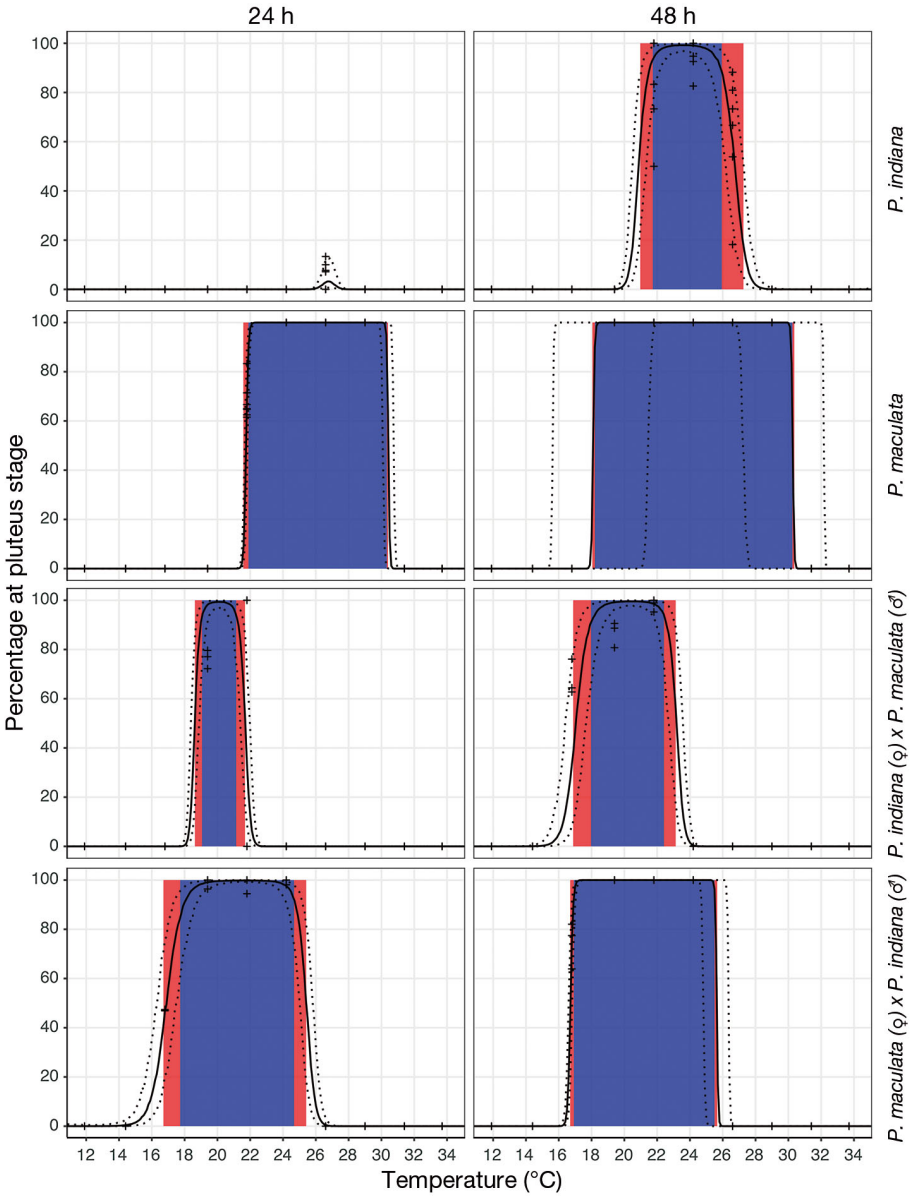

Fig. 5. Generalized linear mixed models of the proportion of normal conspecific and hybrid *Pseudoboletia* progeny pluteus larvae across a thermal gradient (8.5 to 35.8°C) at 24 and 48 h post-fertilization. The solid line indicates the model fit to the measured proportions (indicated by '+'), with the dotted line indicating 95% confidence intervals. Blue shading indicates the temperature range where >90% of the larvae developed normally. Red shading indicates temperatures where the proportion of larvae developing normally is between 50 and 90%

cantly different among all other comparisons (Supplement 3). The T_{90}^{min} of *P. maculata* and the hybrid plutei was not significantly different (16.92 to 18.21°C) but was significantly colder than the T_{90}^{min} of 21.73°C for *P. indiana* (Table 2, Fig. 5). Higher temperature limits show a different pattern: *P. maculata* had the significantly ($p < 0.05$) warmest T_{50}^{max} and T_{90}^{max} (30.36 and 30.24°C, respectively), followed by *P. indiana* (27.26 and 25.94°C, respectively). The hybrids exhibit lower T_{50}^{max} and T_{90}^{max} than the non-hybrids (all less than 25.49°C), with *P. indiana* ♀ × *P. maculata* ♂ hybrid plutei having the coldest T_{50}^{max} and T_{90}^{max} ($p < 0.05$) (Table 2, Fig. 5).

Thermal windows of normal development

We modelled the proportion of normal development across the temperature gradient to define the absolute thermal windows of each progeny line (Fig. 6). The GLMM, considering the level 3 orthogonal polynomials of temperature and their interactions with

Table 2. Temperature limits for plutei development after 48 h of treatment for 4 *Pseudoboletia* progeny. Limits are expressed as lowest temperatures (T_{50}^{min} and T_{90}^{min}) and warmest temperatures (T_{50}^{max} and T_{90}^{max}) where 50 and 90% of individuals have reached the plutei. The mean and 80% confidence interval (in parentheses) are reported

Progeny	T_{50}^{min}	T_{50}^{max}	T_{90}^{min}	T_{90}^{max}
P. indiana	20.98 (20.53–21.50)	27.26 (26.20–27.40)	21.73 (21.10–22.40)	25.94 (24.80–26.40)
P. maculata	18.05 (17.07–19.03)	30.36 (29.90–30.80)	18.21 (10.10–21.69)	30.24 (30.00–30.70)
P. indiana ♀ × *P. maculata* ♂	16.88 (16.40–17.70)	23.12 (22.70–23.60)	17.97 (17.30–18.90)	22.41 (21.70–23.10)
P. maculata ♀ × *P. indiana* ♂	16.69 (15.78–17.50)	25.66 (24.94–25.80)	16.92 (16.20–17.95)	25.49 (24.10–26.15)

time, attained the best fit for both conspecific progeny and the *P. indiana* ♀ × *P. maculata* ♂ hybrid (Supplement 4). The *P. maculata* ♀ × *P. indiana* ♂ hybrid thermal windows did not change significantly over time, and the model does not include that variable. Although the proportion of normally developing larvae changed significantly over time for both conspecific progeny and *P. indiana* ♀ × *P. maculata* ♂ hybrids ($p < 0.01$, Supplement 4), the thermal limits (T_{50} and T_{90}) did not change significantly between sampling times ($p > 0.05$, Supplement 4). Overall, we observed the thermal window of normally developed *P. maculata* (18.05 to 30.36°C) is the widest (Table 3, Fig. 6), with a T_{50}^{max} and T_{90}^{max} significantly higher ($p < 0.05$) than for the other progeny. The *P. indiana* ♀ × *P. maculata* ♀ hybrid (16.88 to 23.21°C) exhibited the narrowest thermal window (Table 3, Fig. 6), with the T_{50}^{max} and T_{90}^{max} significantly lower than for the remaining progeny ($p < 0.05$). *P. indiana* and *P. maculata* ♀ × *P. indiana* ♂ hybrids exhibit similar thermal windows (Table 3, Fig. 6), with no significant difference between these 2 in their upper thermal limits T_{50}^{max} and T_{90}^{max} ($p > 0.05$). There were no significant differences in the lower thermal limits between the 2 species and their hybrids (Table 3, Fig. 6).

SST and larval thermal window maps

The results of assuming a peak spawning in May and applying the 50 and 90% thermal windows for normal development in *Pseudoboletia* (Fig. 6) to

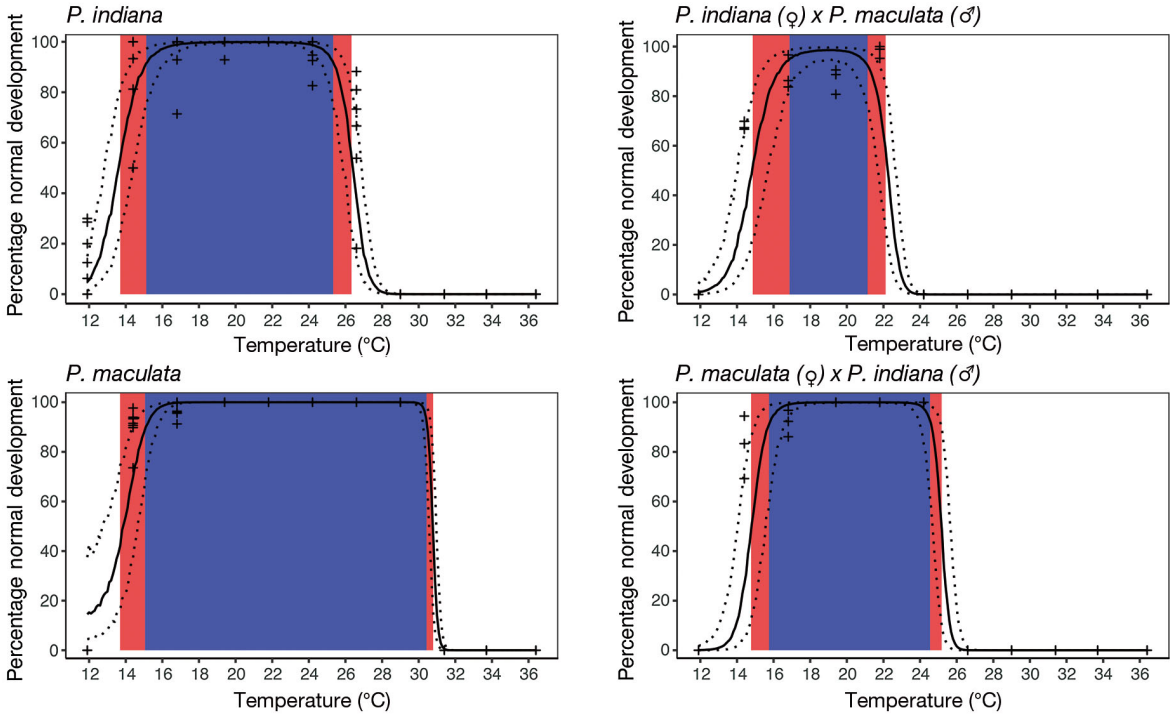

Fig. 6. Generalized linear mixed models of the proportion of normally developed conspecific and hybrid *Pseudoboletia* progeny across a thermal gradient (8.5 to 35.8°C) at 48 h post-fertilization. The solid line indicates the model fit to the measured proportions (indicated by '+'), with the dotted line indicating 95% confidence intervals. Blue shading indicates the temperature range where >90% of the larvae developed normally. Red shading indicates temperatures where the proportion of larvae developing normally is between 50 and 90%

Table 3. Temperature limits to normal development after 48 h of treatment in 4 *Pseudoboletia* progeny. Limits are expressed as lowest temperatures (T_{50}^{min} and T_{90}^{min}) and warmest temperatures (T_{50}^{max} and T_{90}^{max}) where 50 and 90 % of individuals have undergone normal development. The mean and 80 % confidence interval (in parentheses) are reported

Progeny	T_{50}^{min}	T_{50}^{max}	T_{90}^{min}	T_{90}^{max}
P. indiana	13.55 (12.85–14.45)	26.45 (25.85–26.85)	14.95 (14.15–15.95)	25.45 (24.75–26.05)
P. maculata	13.95 (12.85–14.45)	30.75 (30.55–30.95)	15.15 (14.45–15.75)	30.45 (30.25–30.65)
P. indiana ♀ × *P. maculata* ♂	14.75 (13.95–15.75)	22.25 (21.75–22.65)	16.25 (15.35–17.55)	21.25 (20.35–21.85)
P. maculata ♀ × *P. indiana* ♂	14.75 (13.85–15.55)	25.25 (24.65–25.85	15.65 (14.85–16.55)	24.65 (23.95–25.15)

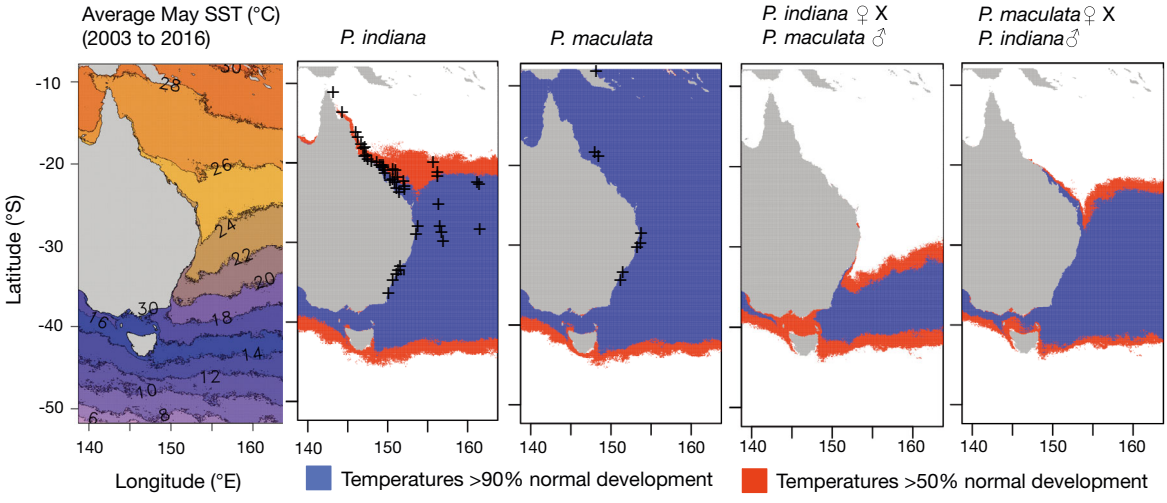

Fig. 7. Average sea surface temperatures (SSTs) for May between 2003 and 2016 along eastern Australia and the distribution of sea temperatures in May that correspond to the thermal windows for 50 and 90 % normal development in native and hybrid *Pseudoboletia* progeny. Overlaid on the *P. indiana* and *P. maculata* maps are the locations of recorded populations of each species (indicated by **+**)

the average May SST off eastern Australia (Fig. 7) indicate the potential differences among the progeny in the geographic range suitable for larval development (Fig. 7). *P. maculata* had the broadest potential range of 45° S through to the equator, while *P. indiana* had a potential range that did not extend north of 15° S latitude. Although information on the 2 species distributions in the Australian region is limited, there are no reports of populations outside our estimated larval thermal windows. In addition, the broader larval thermal window seen in *P. maculata,* which includes equatorial sea temperatures, is consistent with the broader latitudinal range seen in *P. maculata* (33° 50′ 27.3″ to 3° 48′ 23.83″ S) compared with *P. indiana* (36° 24′ 39.2″ to 10° 46′ 33.04″ S). The narrower larval thermal windows seen in the hybrid progeny correspond to a potentially narrower geographic range for normal development along eastern Australia. In this respect, while the hybrids have similar modelled southern limits to native progeny (ca. 45° S), the modelled northern distributions are narrower, especially for *P. indiana* ♀ × *P. maculata* ♂ (i.e. ca. 35° S) but also for *P. maculata* ♀ × *P. indiana* ♂ (i.e. ca. 25° S). We found a very similar pattern in distributions of *Pseudoboletia* progeny assuming a wider autumn spawning period (i.e. April, May and June) using SST maps for the 3 mo, with hybrids showing the same restricted southern range (Fig. S3 in Supplement 1).

DISCUSSION

To determine the outcomes of hybridization for *Pseudoboletia,* as a marine model system where the phenotype of adult and larval hybrids can be detected, we characterized the developmental fitness of native and hybrid progeny along a temperature gradient spanning from tropical to temperate isotherms and reflecting the broad latitudinal distribution of the 2 species. This allowed us to examine how temperature may determine the present and future success and distribution of native and hybrid *Pseudoboletia indiana* and *P. maculata* throughout their sympatric range in the eastern Pacific and to identify potential hybridization zones.

By all measures, *P. indiana* and *P. maculata* readily hybridize (Zigler et al. 2012), and in the present study, we found a >95% fertilization success in all possible crosses. We also found that during the first 48 h post-fertilization, the hybrid progeny developed to the pluteus stage equally well (if not slightly faster) in ambient temperatures for the Sydney Harbour populations. Hybridization appears common, and in the present study population in Sydney Harbour, Zigler et al. (2012) estimated that at least 15% of individuals had intermediate morphologies consistent with hybrids, including fertile hybrid females.

In contrast, for a number of sea urchin species, hybridization barriers and reproductive isolation include gamete incompatibility (McClary & Sewell 2003, Lessios 2007, Kosman & Levitan 2014), differences in reproductive periodicity (Muthiga 2003), and physical isolation through habitat separation (Lessios 2007). Differences in the gamete recognition proteins are especially well studied as a mechanism for maintaining species integrity in sea urchins (Zigler et al. 2005) and marine invertebrates in general (Vacquier & Swanson 2011). However, other key life history processes, such as the fitness of developmental stages, are less often examined as barriers to hybridization, despite their key role in determining reproductive success (Lessios 2007) and potentially controlling distributions and abundances of hybrid progeny across the species ranges. For example, Rahman & Uehara (2004) observed lower settlement success in *Echinometra* hybrid progeny, suggesting the fitness of the developmental stages may influence hybridization, although given that the settlement success rate was >60%, it may only be a relatively weak barrier to hybridization (Lessios 2007). Rahman et al. (2004) suggested that prezygotic barriers were likely more important in restricting hybridization between sympatric *Echinometra* species given that larval survival, metamorphosis and F_1 growth, survival and fertility were comparable among native and hybrid progeny.

There were differences among *Pseudoboletia* progeny in their response to temperature, with hybrid inferiority in terms of a narrower thermal window and a lower tolerance to warmer temperatures than native progeny. Specifically, the width of the thermal windows (T_{90}^{min} to T_{90}^{max}) were greater in *P. maculata* (15.3°C) and *P. indiana* (10.5°C) than in the hybrids, *P. maculata* ♀ × *P. indiana* ♂ (9.0°C) and *P. indiana* ♀ × *P. maculata* ♂ (5.0°C). Our findings are consistent with those seen by Beaumont et al. (2004) for *Mytilus edulis* and *Mytilus galloprovincialis* hybridization trials, where the hybrid veliger larvae performed more poorly under warmer temperature treatments compared with pure larvae. The underlying genetic basis for hybrid inferiority is attributed to genetic incompatibility and the effects of deleterious mutations and recombinant genotypes that have not been subjected to selection (Burke & Arnold 2001). In addition to the inherent differences between the 2 species in their thermal biology, thermal tolerance may also be due in part to levels of maternal provisioning associated with differences in egg size. The thermal windows of hybrid progeny derived from the larger *P. maculata* eggs (103.9 µm average diameter) were broader than those derived from *P. indiana* eggs (84.4 µm average diameter), which is consistent with the trend seen in the native progeny thermal windows. In this respect, the thermal window of *P. maculata* is ca. 5°C wider than that of *P. indiana*, as measured by T_{90} minimum and maximum temperatures, and is mainly in the ca. 5°C warmer temperatures. Egg size is related to composition and energy content in sea urchins (McAlister & Moran 2012), and maternal provisioning is important for tolerance to environmental stress (i.e. levels of heat shock proteins) (Hamdoun & Epel 2007) and antioxidant enzymes (Lister et al. 2016, 2017) during development.

The differences in the thermal windows among progeny may influence hybrid success and the extent of the hybridization zone of *Pseudoboletia*. For instance, the geographic distribution of adult *Pseudoboletia* along eastern Australia broadly reflects the thermal windows of the larvae; *P. maculata* has a broad geographical distribution, with individuals found from 35° S through to the equator, which encompasses the larval thermal window, while *P. indiana* appears to have a distribution that only extends north to ca. 10° S and which reflects the narrower, relatively cooler thermal window of the species. If this pattern holds true for the hybrid progeny, then the *P. indiana* ♀ × *P. maculata* ♂ hybrids will have the smallest geographic distribution, restricted to areas south of ca. 30° S, while *P. maculata* ♀ × *P. indiana* ♂ hybrids will be restricted to latitudes south of ca. 20° S along eastern Australia. Although there is presently not enough distributional data to test the relationship between thermal windows and hybrid distributions, previous studies on echinoderms show a clear link between the response of developmental stages to temperature (i.e. their thermal limits) and adult distribution patterns (Ling et al. 2009, Pecorino et al. 2013, Hardy et al. 2014, Lamare et al. 2014). This relationship also suggests that the hybridization zone of the species is most likely restricted to the southern limits of the species range in Australia

and provides a mechanism for the maintenance of *Pseudoboletia* lineages across its range.

The relationship between SST, larval thermal windows and potential distributions of native and hybrid populations will depend on the timing of annual spawning. Only 1 study has examined the annual reproductive cycle of *Pseudoboletia* near the southern end of the species range (Zigler et al. 2012), and it is possible that there are temporal and latitudinal differences in the reproductive timing. To account for this, we mapped the potential distributions of native and hybrid *Pseudoboletia* populations assuming an autumn spawning period but with some temporal variation (April to June). We found a similar pattern to that based on May spawning (Fig. S3 in Supplement 1), and which was consistent with our hypothesis that the hybridization zone of the species is most likely restricted to the southern limits of the species range in Australia.

The thermal windows of the *Pseudoboletia* progeny suggest that the hybrids have lower overall larval fitness across the temperature range investigated. There is, however, some suggestion of hybrid vigour especially at the ambient sea temperature used here (21.8°C). Hybrid vigour in sea urchins has previously been observed in *Echinometra* crosses in terms of faster growth in hybrid juveniles (Rahman et al. 2004a), and in the present study, hybrid *Pseudoboletia* larvae performed at least equally well in terms of development rate. For example, hybrids always showed a greater proportion of more advanced development stages at the 3 time points measured up to 48 h (Table 1). While we did not follow larval development rate through to settlement, at least in these key early stages, post-zygotic development rates in hybrids would not appear a barrier in the hybridization within the more temperate edges of the species ranges such as Sydney Harbour (i.e. 18 to 24°C).

Hybrid vigour at higher temperatures may also be an important mechanism whereby taxa can persist in an ecosystem during warming. Alternatively, hybrid or out-breeding depression may occur, where progeny are less well adapted to a range of environmental temperatures compared with their parents, who have accumulated adaptive differences that increase their fitness in the present environment (Charlesworth & Willis 2009). *Pseudoboletia* hybridization did not result in progeny with a broader temperature tolerance, and we found no evidence that hybridization could act as a mechanism for responding to warming or to expand their distributions into warmer or colder waters. In fact, hybrids may limit the expansion of the species ranges through the potential introgression of less tolerant alleles that depresses the overall fitness of peripheral populations (Bridle & Vines 2007). In this case, hybrid zones may act as sinks rather than sources of dispersal (Pfennig et al. 2016).

This study raised several interesting questions around the role of larval fitness in sea urchin hybridization. First, to better understand why hybrids appear to be characteristic of range edge populations, it will be important to characterize the thermal windows for development in central and more northern populations of *Pseudoboletia*, as parental thermal history is likely to influence thermotolerance of progeny (Byrne et al. 2011, Pecorino et al. 2013, Hardy et al. 2014). *Pseudoboletia* larvae derived from lower latitudes, including their hybrid offspring, would be expected to have a warmer thermal tolerance. Second, it will be important to understand the thermal tolerance in hybrid and native progeny beyond the 2 d larval stage, as thermal windows may change with further development. Hardy et al. (2014) and Lamare et al. (2014) both showed a slight narrowing of thermal windows in echinoid larvae during later development. Last, it is possible that introgression through the back-crossing of hybrids may result in F_2 hybrid progeny that have a thermal window closer to or exceeding the native progeny and, hence, a hybridization zone that is broader than the area we predicted. Introgression requires the production of fertile F_1 hybrids, and while this process does not always occur (i.e. in some *Mytilus* hybridization zones, Brannock et al. 2009), the presence of fertile *Pseudoboletia* hybrids in the Sydney populations (Zigler et al. 2012) indicates that introgression occurs. In addition, Zigler et al. (2012) reported at least 1 F_2 hybrid among 53 individuals studied. Introgression in echinoderms also occurs through dispersal of larvae (Harper & Hart 2007, Harper et al. 2007), and the dispersal of hybrid larvae may promote expansion of the hybridization zones through introgression into native populations, especially if F_2 larvae have thermal windows similar to or broader than those of their parents.

The relationship between species ranges and hybrid fitness has been widely discussed, although generally in terms of hybridization as a facilitator of range expansion (Pfennig et al. 2016), and much of the focus now centres on the effects of ocean warming and invasive species on the expansion of hybridization zones (Kelly et al. 2010). Examples exist on the effects of range expansion and hybridization in marine species among natural populations and from invasive species (Gardner 1997, Huxel

1999, Brannock et al. 2009). Changes in sea temperature and ocean currents are another important driver of hybridization in marine invertebrates. Harper & Hart (2007), for example, quantified hybridization and introgression between the sea stars *Asterias rubens* and *A. forbesi* as result of historic (post-glacial) sea temperature warming and secondary contact following the trans-Arctic interchange, while Tsang et al. (2008) documented hybridization in 2 subspecies of the barnacle *Tetraclita japonica*, where they attributed genetic patterns to the recent mixing of the 2 subspecies as a result of warming and poleward migration. Vertebrate examples also exist; Potts et al. (2014) documented evidence for hybridization in the coastal fish genus *Argyrosomus* with range expansions associated with warming in the Angola-Beguela frontal zone.

By examining the thermal windows of *Pseudoboletia* progeny, we were able to show that hybrid offspring have a narrower thermal window compared with their maternal lineage. Based on the observations that species ranges in native *Pseudoboletia* are tied to the thermal window of their developmental stages, we propose that progeny fitness may act to limit the hybridization zone in the *Pseudoboletia* genus through the lower capacity of hybrid progeny to complete development in the warmer ranges of the 2 species. Given the effects of ocean warming on species distributions and hybridization zones (Chunco 2014), our observations highlight the need for a greater understanding of the thermal biology of hybrid offspring when predicting future hybridization zones.

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