

Climate change implications for reproductive success: temperature effect on penis development in the barnacle *Semibalanus balanoides*

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ABSTRACT: Climate may influence biogeography through effects on adult survival and reproductive success. The acorn barnacle *Semibalanus balanoides* is the most widespread intertidal barnacle in the Northern Hemisphere. At its southernmost European distribution limit in northwestern Iberia (Galicia, northwestern Spain), winter temperatures are close to the thermal limit for penis development, and it is expected that a temperature increase would acutely reduce reproductive success. We studied the effect of temperature on penis development in the laboratory and related the results to field data to estimate fertilization probabilities at different temperatures and population densities. Exposure to colder water temperatures (14 and 17°C) allowed full penis development, but this was inhibited at warmer temperatures (20 and 23°C). Penis annulations and penis length were greater in barnacles exposed to colder water treatments than in those held at warmer temperatures. In the laboratory experiments, fertilization probability was higher at the lowest temperature (~100%). In the field, fertilization probability was higher in the localities with colder temperature as well as those with higher barnacle density. The results support the prediction that rising temperatures will likely reduce reproductive success of *S. balanoides* at its southernmost distribution limit through failure in fertilization. If similar mechanisms apply in other boreal species, climate change may shrink distribution ranges through large-scale reproductive failures at the southern limits.

KEY WORDS: Climate change · Temperature · Reproduction · Penis development · Fertilization success · Barnacles · *Semibalanus balanoides*

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1. INTRODUCTION

Ecological responses to climate change are already evident in most ecosystems. Ocean warming effects include biogeographic changes as species ranges expand and contract (Southward et al. 1995) as well as shifts in phenology (Walther et al. 2002, Parmesan & Yohe 2003), causing changes in composition and structure of intertidal communities (Hawkins et al. 2008). Rapid changes are occurring in the NE Atlantic Ocean in relation to warming trends (Stocker

et al. 2013). Marine ecosystems have responded to warming; long-term studies in the NE Atlantic have shown that the abundance and distribution of warmer- and colder-water species have varied with temperature over the past years (Southward et al. 1995, Beaugrand et al. 2002, Hawkins et al. 2008). On the Atlantic coast of the Iberian Peninsula, sea surface temperature increased by about 0.2°C decade⁻¹ during the period 1975–2007 (Gómez-Gesteira et al. 2008, deCastro et al. 2009), and although the warming is not geographically or seasonally uniform (Kout-

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sikopoulos et al. 1998, Lima & Wetthey 2012), it seems to be consistent with the large-scale trends of rising seawater temperatures.

Intertidal rocky habitats from the NE Atlantic region have shown clear patterns of ecological change in response to climate fluctuations. Such fluctuations have been documented in several warmer- and colder-water taxa, including algae (Martínez et al. 2012), gastropods (Mieszkowska et al. 2007), limpets (Southward et al. 1995, Lima et al. 2006) and barnacles (Mieszkowska et al. 2014). Barnacles have been extensively used as sentinel organisms for climate change responses (Southward et al. 1995, Wetthey & Woodin 2008, Wetthey et al. 2011b). In particular, the acorn barnacle *Semibalanus balanoides* (Linnaeus, 1767) has been long used as a model species in biogeographic studies; being widely distributed in boreo-arctic communities, easily manipulated and tracked in the field and sensitive to changes in temperature (Hutchins 1947).

The mechanisms of biogeographic response to climate include both intraspecific and interspecific processes. Hutchins (1947) hypothesized that the southern geographic limits of species may be most strongly influenced either by the effects of high summer temperatures on adult survival or by the effects of low winter temperatures on reproduction. *S. balanoides* is an excellent model for testing these alternative biogeographic hypotheses. Many studies have related the abundance of *S. balanoides* populations and their performance to changes in temperature. As early as 1954, Southward & Crisp (1954) pointed out that the decline in *S. balanoides* populations in the British Isles could be due to a temperature rise. Recent long-term studies have confirmed these trends by documenting a contraction in the distribution ranges of *S. balanoides* as well as a decrease in the abundance of southern populations (Wetthey & Woodin 2008). Likewise, the reproductive success of *S. balanoides* is highly mediated by temperature, as gonad development requires water temperatures below 15°C (Barnes 1963, Barnes & Stone 1972) and the onset of breeding requires water temperatures below 10°C (Crisp & Patel 1969). High recruitment rates are also strongly correlated with low temperatures (Kendall et al. 1985, Southward 1991, Drévès 2001).

There is an extensive literature on *S. balanoides* physiology (Crisp & Patel 1960, Barnes 1963, Barnes & Barnes 1968) and spatial distribution (Southward 1991, Southward et al. 1995, Hawkins et al. 2008). This species has a continuous distribution in the NE Atlantic from Svalbard Island to the middle of the French coast on the Bay of Biscay (Crisp & Fischer-

Piette 1959, Feyling-Hanssen 1963, Stubbings 1975), and there is a southern isolated disjunct population in Galicia, NW Iberian Peninsula (Fischer-Piette & Prenant 1956, Fischer-Piette 1963, Macho et al. 2010, Wetthey et al. 2011b). However, there are no studies on the species performance at its southernmost European limit, where seawater and air temperatures are higher than the values previously proposed for reproductive success. For example, seawater temperature in Galicia rarely drops below 10–12°C, which is a threshold that has been considered crucial for fertilization success in more northern populations (Barnes 1963, Crisp & Patel 1969, Crickenberger & Wetthey 2018). Wetthey & Woodin (2008) examined changes in the European southern limit of the species in relation to climate change and suggested that the southern limit of *S. balanoides* is mediated by intolerance of winter body temperatures above 10°C, leading to reproductive failure. Their results also indicate that the species has contracted from reproductive populations throughout the NW Iberian Peninsula in the 1950s and 1960s (Ardré et al. 1958, Fischer-Piette & Seoane-Camba 1963, Barnes & Barnes 1966) to a single reproductive population in the Ría de Arousa (Galicia, Spain) (Wetthey et al. 2011b), which can be understood in relation to the fine-scale temperature distribution, since coastal upwelling is a major driving factor for ecological processes in the region (Fraga 1981).

S. balanoides is an obligate cross-fertilizing hermaphrodite species, and breeding occurs synchronously once a year (Barnes 1963). Changes in phenology may increase the likelihood of reproductive failure for single-brooding colder-water species (Lewis 1986, Moore et al. 2011). *S. balanoides* gonads begin to develop during spring, although they are not conspicuous until summer when the penis develops and enlarges, reaching its maximum size just before the onset of fertilization in the late fall (Moore 1935, Barnes 1958, Crisp 1964). After copulation, the penis is lost with the molt and the embryos are brooded until late winter. Reproductive success is mediated by all these phases, and each one of them might be negatively influenced by a warmer climate scenario. Warmer temperatures not only may inhibit gonad development, prevent or reduce fertilization or delay its onset (Crickenberger & Wetthey 2018), affect the embryo brooding process (Rognstad & Hilbish 2014), alter pelagic larval development (Lucas et al. 1979) and influence settlement and recruitment success (Drévès 2001), but also may alter competitive ability (Wetthey 2002, Poloczanska et al. 2008, Mieszkowska et al. 2014).

In this paper, we use a combination of laboratory experiments and field observations to test the hypothesis that high temperatures inhibit *S. balanoides* penis development, becoming a limiting factor for fertilization and, therefore, for reproductive success at its southernmost distribution limit. By determining the reproductive threshold in Galicia, it will be possible to forecast the fate of this ecologically dominant species (Connell 1961, Poloczanska et al. 2008) at its southern distribution limit as influenced by a changing climate. This also provides a general test of the hypothesis of winter cold limitation of reproduction at the southern distribution limit of boreal species, testing whether the same mechanism is responsible for limits in both northern European populations and the isolated disjunct population in southern Europe.

2. MATERIALS AND METHODS

2.1. Study site

The study took place in the Rías Baixas (Galicia) in the NW Iberian Peninsula (Fig. 1). The Rías Baixas are located between Cape Finisterre and Portugal and are characterized by the presence of 4 rias with similar characteristics, since they are all V-shaped basin systems that progressively widen from the innermost part towards the mouth. The Rías Baixas are part of the NW Iberian Upwelling System, which is the northernmost limit of the Eastern North Atlantic

Upwelling System (Wooster et al. 1976, Alvarez et al. 2008). Upwelling in this region is mainly a spring–summer process, characterized by favorable northerly winds along the coast (Fraga 1981), although it has been documented in winter under some special conditions (Alvarez et al. 2003). The surface water that is displaced offshore is replaced by colder and nutrient-rich deeper water known as Eastern North Atlantic Central Water, characterized by high salinity values ranging from 35.67 to 35.83 psu and low temperatures ranging from 11.8 to 13.5°C (Alvarez et al. 2003). The highest upwelling intensity is found precisely in the Rías Baixas, between 42° and 43° N (Fraga 1981, deCastro et al. 2008), and there is a decreasing latitudinal gradient both northward and southward from this point (Alvarez et al. 2005b).

Two rias (Ría de Arousa and Ría de Vigo) were chosen based on their different population densities of *Semibalanus balanoides*. The Ría de Arousa is the only place in the Iberian Peninsula where high population density (≈ 100 ind. 100 cm^{-2}) and coverage (5–25% in the middle intertidal) of *S. balanoides* can be found (Macho 2006, Wetthey & Woodin 2008, Wetthey et al. 2011b). The southernmost distribution limit of *S. balanoides* is located in the Bay of Baiona in the southern margin of the Ría de Vigo (Wetthey & Woodin 2008), where its presence is scarce (less than 10 ind. 100 cm^{-2}). On rare occasions, a few individuals have been found in Viana do Castelo, Portugal, in exceptionally cold years (Fischer-Piette & Prenant 1957, Wetthey et al. 2011b).

Two localities were chosen in the middle part of each ria: A1 (42.57° N, 8.96° W) and A2 (42.61° N, 8.89° W) in the Ría de Arousa and V1 (42.11° N, 8.84° W) and V2 (42.12° N, 8.83° W) in the Ría de Vigo. The 2 rias are 45 km apart and within the dispersal range of *S. balanoides* larvae.

Both rias have a similar NE–SW orientation (Alvarez et al. 2005a) and also share similar hydrographic conditions, since they have a semidiurnal and mesotidal regime which contributes to water renovation within the rias (Alvarez et al. 2005a), and they are subjected to factors such as upwelling events (Rosón et al. 1995, Nogueira et al. 1997, Alvarez et al. 2005a), wind regimes within the rias (Chase 1975, Rosón et al. 1995) and freshwater input from rivers (Otto 1975, Ríos et al. 1992, Taboada et al. 1998). Temperature in

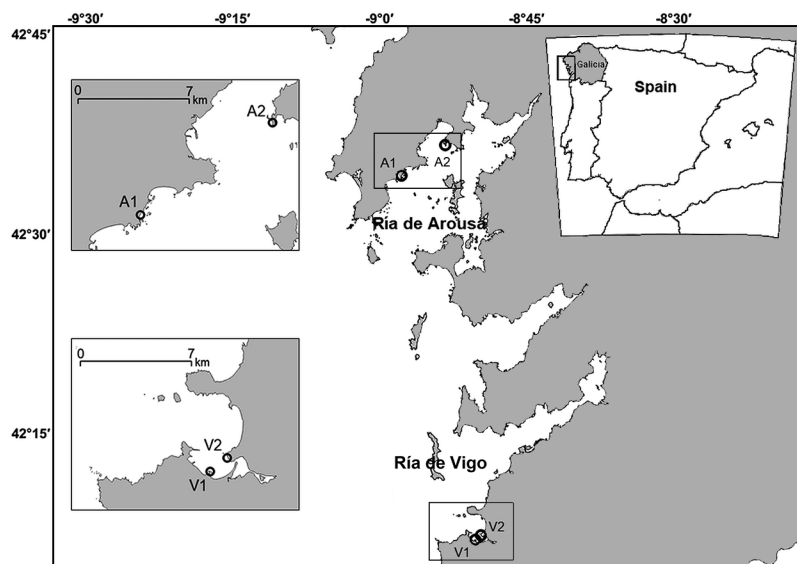


Fig. 1. The Rías Baixas region (Galicia, Spain), showing study locations in the Ría de Arousa (A1 and A2) and the Ría de Vigo (V1 and V2). Open circles indicate sampling locations

the rias varies between 11 and 20°C, and salinity varies between 32.0 and around 36.0 psu (Alvarez et al. 2005a). However, the Ría de Arousa is 1 to 2°C colder than the Ría de Vigo (Alvarez et al. 2005a, MeteoGalicia 2018) (Fig. 2).

2.2. Laboratory experimental design

Adult *S. balanoides* were collected from A1 in early September 2015, before the onset of penis development. Small rocks with aggregations of adults at intermediate densities (mean of 50 adult and 20 young of the year ind. 100 cm⁻²) were transported to the laboratory. These small rocks were haphazardly distributed in 33 l containers until there were approximately 100 individuals in each tank. Tanks were kept in an open circuit with 50 µm filtered seawater in an isothermal room where the air temperature and light cycle were adjusted every week to match those in the field. In addition, a 6 h morning low tide was simulated daily (low tide timing was not adjusted to match field tides). Barnacles were fed throughout the experiment (Sep 12 to Nov 4, 2015) for 3 h every 2 d with a mixture of *Chaetoceros* sp., *Phaeodactylum* sp., *Tetraselmis* sp. and *T-Isochrysis* at a final concentration of 10⁸ cells l⁻¹, which was confirmed by Coulter counter to be a sufficient feeding regime to avoid any food limitation. The experimental temperature treatments (see below) were in the range where cirral beat rate in *S. balanoides* is within 80 % of its maximum (Southward 1955), so any temperature limitation of feeding was unlikely.

Four different temperature treatments were used, 14, 17, 20 and 23°C, with 2 replicates per treatment (8 containers in total). The temperatures were selected to include a relevant range below and above the tem-

peratures observed in the field (Fig. 2). The lowest (14°C) and the highest (23°C) temperatures were included for comparison of ecological performance with populations in the center of the geographic range in northern Europe and with plausible conditions in the future under a warmer climate scenario, respectively. The differences between treatments were large enough to reliably maintain them without overlapping in our open-circuit seawater system. In all treatments, there was a head tank connected to a water chiller or heater used to maintain the target temperature. Head tanks distributed the water to insulated containers, and every container had a temperature data logger (Maxim iButton®) recording every 10 min.

A basal sample of 30 individuals was taken on the day the animals were collected. *S. balanoides* were sampled 3 times (Days 27, 41 and 53) during the 53 d experiment. Fifteen individuals with operculum size between 4 and 6 mm were randomly chosen from each replicate and carefully removed from the rock. Basal diameter at the widest point, operculum length at the longest point and height at the tallest point were measured for each individual. To determine the penis developmental stage, individuals were dissected and the number of annulations and length of the penis were measured under an optical microscope with image analysis software (Nis-Elements BR version 4.0, Nikon Instruments). Somatic tissue was separated, dried at 60°C for 48 h and then weighed with a microbalance (Sartorius LE225D, precision 0.1 mg). To determine the presence or absence of spermatozoa inside the penis, 10 individuals per replicate collected in each sampling were dissected, and the penis was processed for histology (fixed in Davidson formaldehyde for 24 h, dehydrated in an ethanol series, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin).

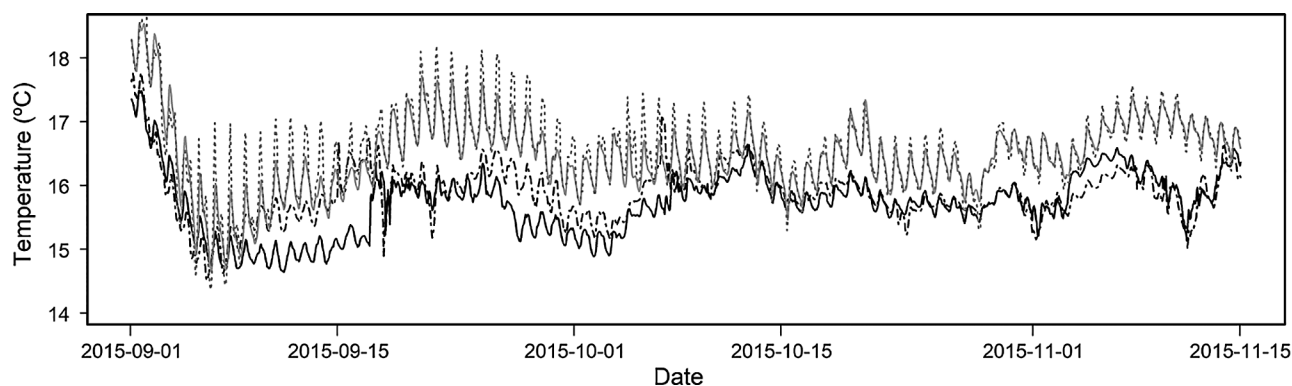


Fig. 2. Sea surface temperatures in study locations during the experiment (September 12 to November 4, 2015) from the MeteoGalicia Ocean Model (MOHID Water Modelling System). A1 (solid black line) and A2 (dot-dashed black line) in the Ría de Arousa and V1 (solid grey line) and V2 (dotted grey line) in the Ría de Vigo

2.3. Field sampling

To compare with the laboratory results and to integrate them into a population context, penis development, population density and temperature data from the field were also recorded. In late October 2015, coinciding with the end of penis development in the field and the end of the laboratory experiment, 20 barnacles were collected from each location at the Ría de Arousa, dissected in the laboratory, and penis developmental stage determined. The number of annulations and length of the penis were obtained as described above, and dry weights of somatic tissue were measured. No barnacles were sampled from the Ría de Vigo locations since population densities were too low to remove individuals without affecting the population. Thus, estimations of penis length from the Ría de Vigo were indirectly obtained as described in Sections 2.4 and 2.5.

Population density was recorded using photographs of 15 quadrats (10 × 10 cm) at each location where *S. balanoides* was present. All individuals in each quadrat were counted.

Hourly surface water temperatures at each sampling location, obtained from the MeteoGalicia ocean model (Fig. 2, MOHID Water Modelling System, www.mohid.com, MeteoGalicia 2018), were validated with iButton records from the intertidal for the duration of the laboratory experiment. Temperature was recorded on these iButtons every 45 min, and the ocean model temperatures were available at hourly intervals.

2.4. Estimation of penis length multiplier

To obtain the distribution of penis length, all laboratory measurements of the length of the relaxed penis were corrected by a penis multiplier, since during copulation the penis may stretch to 3 or 4 times its relaxed length (Klepal et al. 2010). The penis multiplier accounts for the difference in length from live animals in the field (extended penis) vs. dead animals analyzed in the laboratory (relaxed penis). This correction was made to avoid underestimates in length of the relaxed penis due to the previous freezing of the samples and to obtain an estimation of the extended length of the penis.

For this purpose, a field survey was carried out in December 2017 in A1 (colder) and V1 (warmer) locations after the end of the fertilization season. The sampling was designed to assess the distance between neighboring individuals at which fertilization fails. Fertilization in *S. balanoides* fails beyond the

penis length because this species is an obligate out-crosser (e.g. Crisp 1954). So, if the nearest neighbor is farther away than the penis length, fertilization should fail. Individuals were chosen whose nearest neighbors ranged from 0 to 5 cm distance (a range far beyond the maximum penis length, 2.5 cm) in 0.5 cm increments. Ten animals per distance increment were sampled at each location. Individuals were categorized as fertilized or not. Natural variation in the spacing among individuals was used for this purpose, so, for example, to determine whether individuals that were 4 cm apart from all potential mates were able to fertilize, only individuals with no potential mates closer than that distance were selected. From these fertilization distances obtained in the field (Fig. 3), we calculated the frequency of fertilization at different distances for both rias (Fig. 4).

We obtained the relaxed penis length distribution for the Ría de Arousa (colder ria) using samples collected from A1 and A2. The Gaussian distribution of relaxed penis length from the Ría de Arousa was converted to the cumulative exceedance distribution (1 – cumulative length distribution) to get the fraction of individuals with penis length exceeding a particular distance. As expected (Klepal et al. 2010), the distances derived from relaxed penis length were much smaller than the observed fertilization distances. The cumulative exceedance distribution of relaxed penis length from the Ría de Arousa was compared to the frequency of fertilization at different distances. Since the 60th and 30th percentiles of the relaxed penis length exceedance distribution were 0.71 and 0.52 cm, respectively, and the 60th and 30th percentiles of the fer-

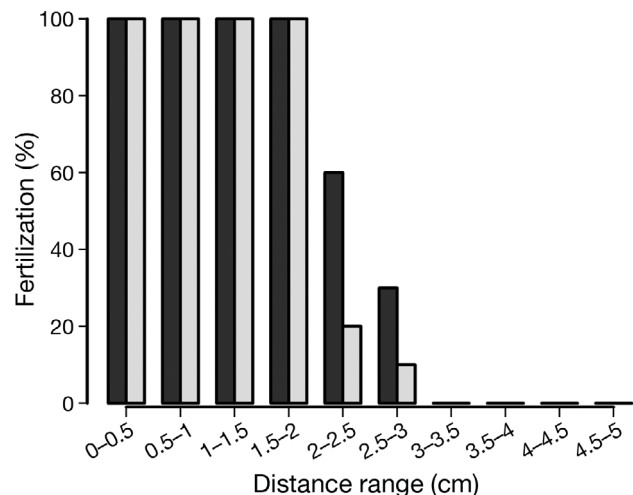


Fig. 3. Percentage of fertilized individuals in the field population at 10 different ranges of nearest neighbor distance. Dark grey bars: A1 (Ría de Arousa); light grey bars: V1 (Ría de Vigo)

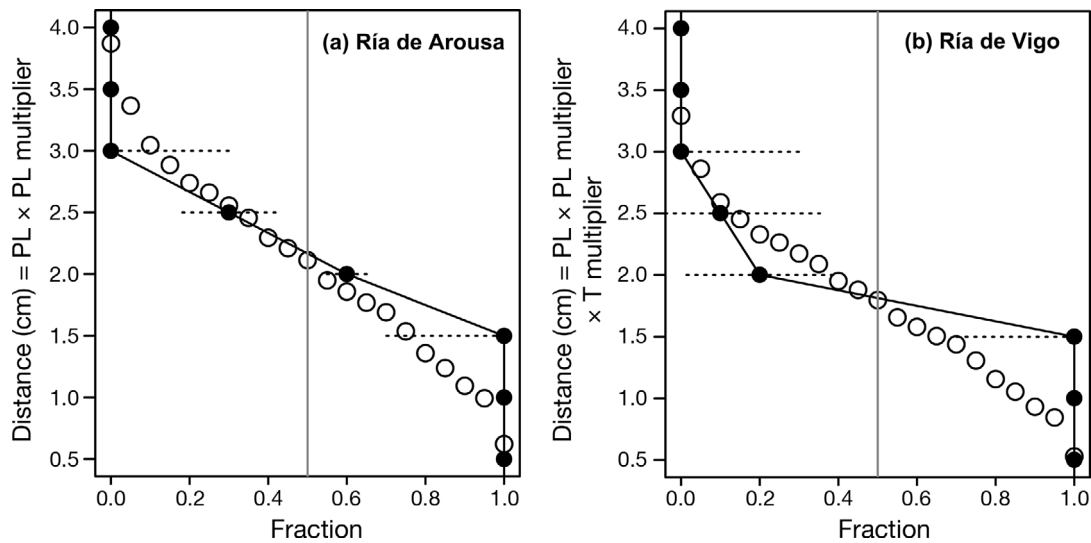


Fig. 4. Proportion of individuals fertilized in the field (black solid lines and circles) and proportion of individuals in the field with penis length exceeding each distance (open circles) on y-axis (1 – cumulative distribution of penis length), in (a) Ría de Arousa and (b) Ría de Vigo. Grey vertical line is the 50 % fertilization point. PL = penis length; PL multiplier = 3.6; temperature (T) multiplier = 0.85

tilization distance distribution were 2.5 and 2.0 cm (Fig. 3), respectively, we estimated 3.6 as the correct penis length multiplier (Fig. 4a). Multiplying by 3.6 allowed us to convert the relaxed penis lengths measured in dead individuals to the actual distances that penises can reach during copulation (extended length). The extended penis lengths estimated by this method (Fig. 4) all lie within the 95% confidence limits of the observed fertilization distance distribution (Fig. 4).

2.5. Estimation of temperature multiplier

The relationship between penis length and the average of aerial and submerged temperature in the laboratory was used to obtain an estimate of penis length in the Ría de Vigo. The mean of aerial and submerged temperatures was used because it is a better predictor of reproductive performance in *S. balanoides* than either aerial or submerged temperature alone (Rognstad & Hilbish 2014). Since there is a linear decrease in penis length as temperature increases, we calculated the linear regression between the penis lengths measured at the end of the 53 d experiment among the 4 treatment temperatures (14, 17, 20 and

23°C) to calculate the predicted penis length for temperatures in the warmer ria (Ría de Vigo) (Fig. 5).

To estimate the average of aerial and submerged temperature in the Ría de Vigo field locations during the same period at which the laboratory experiment was carried out, we used the NOAH intertidal temperature model (Wetthey et al. 2011a), which has been extensively validated (Mislan & Wetthey 2011, Kish et al. 2016). The substratum in the model was rock, the shore level was set to the levels of our photographic quadrats, albedo was set to barnacle re-

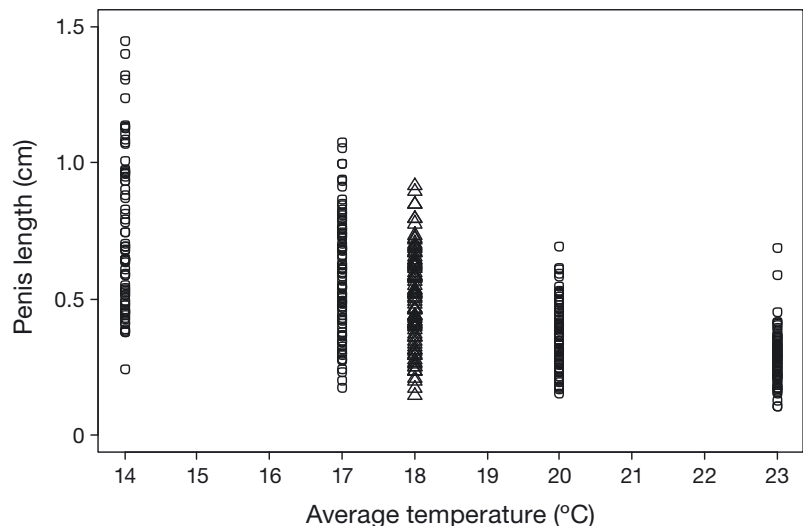


Fig. 5. Laboratory experiment Day 53 penis length data as a function of temperature (circles) and the estimated values for 18°C in the Ría de Vigo (triangles), assuming linear change between 17 and 20°C

flectivity and the surface was oriented directly south (azimuth 180°) with a 45° slope; the model was run using as forcing variables the MeteoGalia 1 km grid scale WRF meteorological forecasts for hourly solar and long-wave radiation, wind speed, air temperature, relative humidity, rainfall rate and atmospheric pressure and the MeteoGalia 250 m scale MOHID ocean forecasts for hourly tide level and water temperature. Model validation was carried out by comparing predictions from the Ría de Arousa to iButton data from the field. Model parameters were tuned to minimize the root mean square difference between observations and predictions.

The NOAH model hindcast of the average of aerial and submerged temperatures for barnacles in the Ría de Vigo locations was 18°C during the period when the laboratory experiment was carried out. Obtaining the average temperature at which barnacles were in the intertidal in the Ría de Vigo allowed us to estimate the effect of that temperature on penis length, given the temperature response observed in the laboratory experiment (Fig. 5). Assuming a linear effect of temperature between 17° and 20°C, at 18°C, penis length was determined to be 85% of the value at 17°C. Applying a temperature multiplier of 0.85 to the October 2015 field measurements of relaxed penis lengths from the Ría de Arousa allowed us to obtain the predicted cumulative exceedance distribution of extended penis length for the Ría de Vigo for the same date, which was plotted for comparison with the frequency of fertilization at different distances (Fig. 4b).

The NOAH intertidal temperature model (Wetthey et al. 2011a) was also run for the Ría de Arousa region using forcing data from 6 different general circulation models (UK Met Office Hadley Centre HadGEM-ES, Norwegian Climate Center NorESM1, Centre National de Recherches Météorologiques CNRM-CM5A, Irish Centre for High-End Computing EC-EARTH, Institut Pierre Simon Laplace CM5A-MR, Max Planck Institute for Meteorology ESM-LR), all dynamically downscaled to the CORDEX EUR-11 (11 km grid) (www.euro-cordex.net) region by the Swedish Meteorological and Hydrological Institute with the SMHI-RCA4_v1 model to assess the regional change in temperature conditions for the study region between the decades 1970–1979, 2006–2015 and 2090–2099. The NOAH simulations from CORDEX historical runs indicated a warming of 0.4 to 1.6°C between 1970–1979 and 2006–2015, consistent with observed ~0.6°C warming between those decades across Iberia (Gómez-Gesteira et al. 2008, deCastro et al. 2009) and serving as a validation of the NOAH CORDEX models.

2.6. Statistical analyses

All statistical analyses were performed with R v.3.3.1 (R Core Team 2016). The effect of temperature on penis length and number of penis annulations was assessed by ANCOVA (using the R package *car* v.2.1-3, Fox & Weisberg 2011) using the elapsed time in the experiment as the covariate and temperature as the treatment effect. This allowed us to measure the temperature effect at the mean elapsed time. The number of penis annulations was linearly regressed against relaxed penis length for each temperature treatment. The relationship between the number of annulations and relaxed penis length for all temperature treatments together was estimated by fitting a logarithmic model to the data. Both relaxed penis length and number of annulations at all temperatures were assessed in relation to body volume (covariate) to confirm that there were no effects of animal size on these 2 variables (Barnes 1992). Basal diameter (BD), operculum length (OL) and shell height (h), measured from each individual, were used to calculate body volume (V) as a truncated cone [$V = 1/3 \pi \times h \times (BD^2 + BD \times OL + OL^2)$]. The effect of temperature on body dry weight during the experiment was assessed by ANCOVA using elapsed time as the covariate and temperature as the treatment effect.

Fertilization probability was modeled for the 4 different locations using the cumulative distribution of nearest neighbor distances (Clark & Evans 1954) multiplied by the cumulative exceedance distribution of extended penis length from the field. The R *spatstat* package v.1.46-1 (Baddeley et al. 2015) was used for this purpose. Spatial coordinates of individuals, obtained using Image J software (Schindelin et al. 2012) on the photographs of field quadrats set at the sampling locations, were used to determine nearest neighbor distances among individuals (R *spatstat* package). Field fertilization probability for each location (A1, A2, V1 and V2) was calculated as the average of the fertilization probabilities in each of the 15 replicate photo quadrats.

In the same way, an idealized fertilization probability was obtained for each location using the mean of cumulative nearest neighbor distance from each location multiplied by the cumulative exceedance distribution of extended penis length at the end of the laboratory experiment from each of the temperature treatments (14, 17, 20 and 23°C). Fertilization probability was also assessed as a function of mean plus 1 standard deviation temperature in the laboratory treatments and the field. Mean temperature was

obtained taking into account submerged and aerial temperature observations for both the laboratory experiment and the field.

3. RESULTS

A strong temperature effect on number of annulations ($F_{3,116} = 51.6$, $p < 0.0001$) and on relaxed penis length ($F_{3,116} = 144.8$, $p < 0.0001$) was found for *Semibalanus balanoides*. Size (measured as body volume) of the individuals used (operculum size between 4 and 6 mm) did not affect penis development: (1) there was no significant relationship between body volume and number of annulations (ANCOVA, $F_{1,112} = 1.42$, $p = 0.23$) and no differences in the slopes among temperatures ($F_{3,112} = 0.60$, $p = 0.61$), and similarly, (2) there was no significant relationship between body volume and relaxed penis length (ANCOVA, $F_{1,112} = 0.0005$, $p = 0.98$) and no differences in the slopes among temperatures ($F_{3,112} = 0.53$, $p = 0.66$). The lack of a relationship between penis development and volume of the experimental individuals, as was previously stated by Barnes (1992), allowed us to confirm that our results concerning size and number of annulations in the penis were due to the effect of temperature.

Relaxed penises were longer in lower-temperature treatments (ANCOVA, $F_{3,441} = 88.2$, $p < 0.001$; all slopes differ from each other [$t < -3.8$, $p < 0.001$]) (Table 1). Barnacles exposed to lower temperatures (14 and 17°C) showed a significant average increase in relaxed penis length throughout the experiment (300 and 200 % at 14 and 17°C, respectively), whereas at 20°C, relaxed penis length increase was much smaller (60 %) and at 23°C no increment was observed (Figs. 6 & 7).

Similarly, the number of annulations significantly varied with temperature during the experiment (ANCOVA, $F_{3,441} = 33.7$, $p < 0.001$). Animals at the 2 lower temperatures (14 and 17°C) had the same num-

Table 1. Number of annulations and penis length (mean \pm SD) at the end of the laboratory experiment at all temperature treatments and in the field (A1 and A2, Ría de Arousa). Absolute values obtained from measuring penis length are shown i.e. not corrected for the penis length multiplier of 3.6

Temperature/ field location	No. of annulations	Length (cm)
14°C	213 \pm 11	1.01 \pm 0.19
17°C	219 \pm 16	0.78 \pm 0.16
20°C	185 \pm 27	0.44 \pm 0.11
23°C	149 \pm 35	0.28 \pm 0.10
A1	183 \pm 33	0.41 \pm 0.12
A2	192 \pm 19	0.41 \pm 0.06

ber of annulations ($t = 1.01$, $p = 0.32$), which was higher than the number of annulations at warmer-temperature treatments (Table 1). A difference in the number of annulations was also observed between the highest temperatures (20 and 23°C) ($t < -3.9$, $p < 0.001$) as well as a moderate increase over time in the number of annulations at 20°C during the experiment. In addition, although not statistically significant ($p = 0.058$), a slight decrease could graphically be observed in the number of annulations in the 23°C treatment (Fig. 8).

The slope of the relationship between the number of annulations and relaxed penis length increased with temperature (Fig. 9). There was no relationship between annulations and length in the 14°C treatment ($p = 0.42$, $R^2 = -0.01$, $df = 1, 28$), since the number of annulations was equally high for all the lengths from 5 to 15 mm. At 17°C, even though the relationship was significant, the slope was low (slope = 42, $p = 0.015$, $R^2 = 0.16$, $df = 1, 28$). However, the slope of the relationship between annulations and length was much larger at 20°C (slope = 200, $p < 0.0001$, $R^2 = 0.66$, $df = 1, 28$) and 23°C (slope = 316, $p < 0.001$, $R^2 = 0.72$, $df = 1, 28$). The relationship between the number of annulations and penis length for all temperature treatments together showed a good fit using a

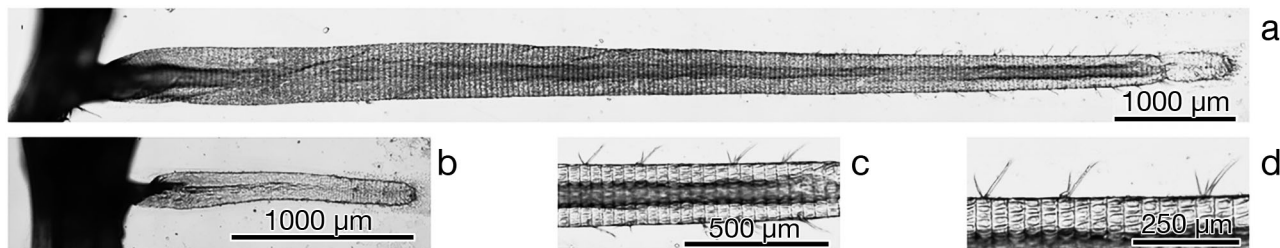


Fig. 6. Relaxed penis of *Semibalanus balanoides* individuals kept in the laboratory at (a) 14°C and (b) 23°C. Detail from the animal kept at 14°C of the (c) penis annulations and (d) setae under an optical microscope

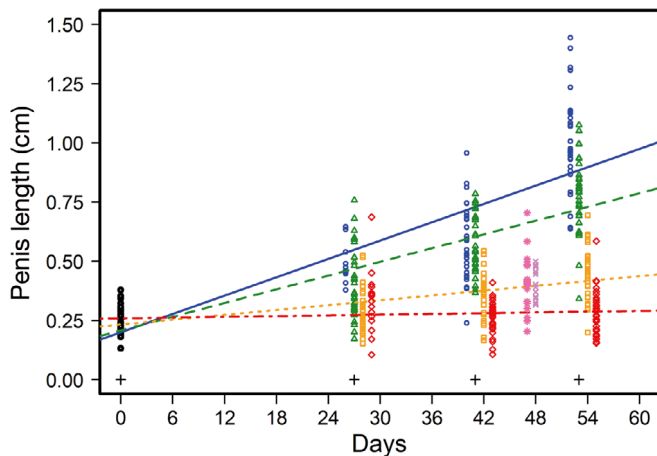


Fig. 7. Penis length versus elapsed time in the laboratory experiment. Symbols are offset for clarity; + symbols at bottom indicate sample dates. Black circles: initial data; blue circles and solid line: 14°C; green triangles and dashed line: 17°C; orange squares and dotted line: 20°C; red diamonds and dot-dashed line: 23°C; pink stars: A1 location data; violet x: A2 location data. Lines are linear regressions

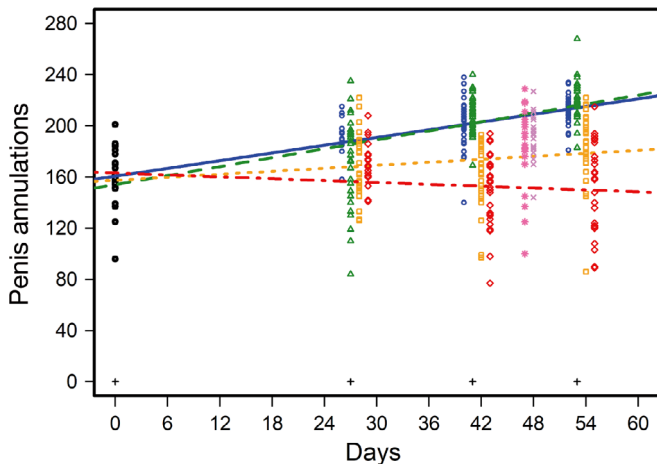


Fig. 8. Number of penis annulations versus elapsed time in laboratory experiment. Colors and symbols as in Fig. 7. Lines are linear regressions

logarithmic model ($R^2 = 0.9332$, $y = 46.268 \ln(x) - 206.53$).

Differences in penis development among treatments were also noticeable in 14 and 17°C treatments by the presence of abundant and long setae, which are involved in sensory responses (Klepal et al. 1972). Setae were absent in animals from the 20 and 23°C treatments (Fig. 6). Likewise, at the end of the laboratory experiment, all the individuals collected from the 14 and 17°C treatments had sperm inside the penis (Fig. 10), while only 15% of the animals from the 20°C treatment and none from the 23°C treatment had sperm inside. Annulations in penises of

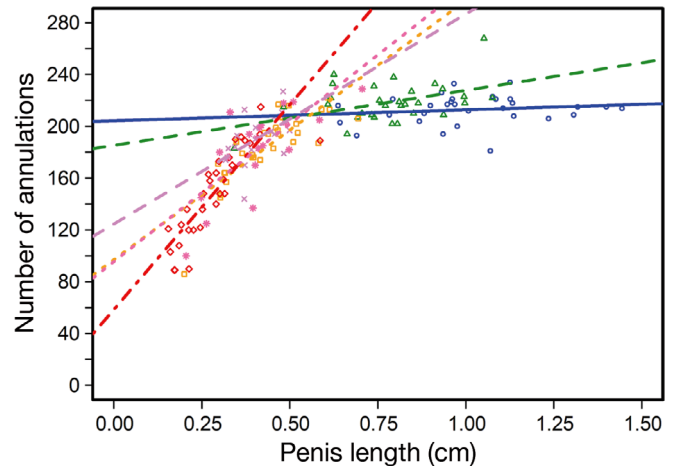


Fig. 9. Relationship between number of annulations and penis length at the end of the experiment. Colors and symbols as in Fig. 7. Lines are linear regressions

animals exposed to warmer temperatures (20 and 23°C) were often poorly defined, more towards the distal part and gradually less visible towards the base; in addition, when setae were present, they were noticeably few and shorter. Sperm packets were visible to the naked eye only in the 14 and 17°C treatments.

There were significant temperature effects on body dry weight when adjusting for the elapsed time of the laboratory experiment (ANCOVA, $F_{3,476} = 23.4$, $p < 0.001$). Barnacles held at 14 and 17°C treatments gained body dry weight and slope did not differ between them ($t < 0.26$, $p = 0.80$), whereas body weight decreased in animals held at 20 and 23°C ($t < -5.1$, $p < 0.001$).

The highest fertilization probability was found in the Ría de Arousa population and was higher at A1 ($\approx 95\%$) than at A2 ($\approx 82\%$) (Fig. 11, Table 2). In the Ría de Vigo population, fertilization probabilities were estimated to be lower than the Ría de Arousa values ($\approx 44\%$ for V2 and $\approx 39\%$ for V1) (Fig. 11, Table 2). Likewise, idealized fertilization probability was greater the lower the temperature was (Table 2). Fertilization probability also decreased when the mean temperature (plus SD) increased (Fig. 12).

In the field, the percentage of fertilized individuals in the population abruptly decreased between 2 and 3 cm of nearest neighbor distance (Fig. 3), which coincides with the approximate maximum distance the extended penis can reach (≈ 2.5 cm). Fertilization percentage in the colder ria (A1, Arousa) was double the value in the warmer ria (V1, Vigo) over the neighbor distance range from 2 to 3 cm (Fig. 3). Fertilization occurs at longer distances in the Ría de Arousa

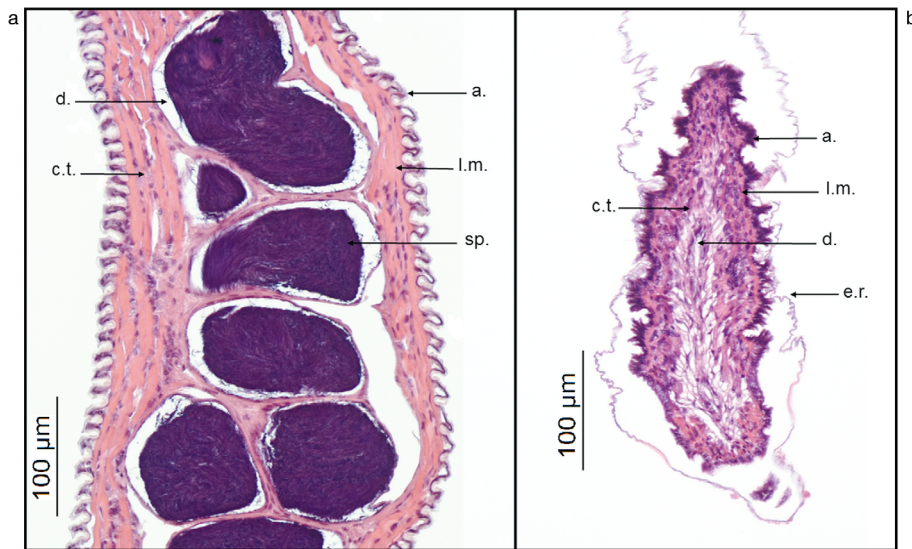


Fig. 10. Longitudinal section of *Semi-balanus balanoides* penis from (a) cold-water (20×) and (b) warm-water (20×) treatments. sp.: Spermatozoa; a.: annulations; l.m.: longitudinal muscles; d.: ductus; c.t.: connective tissue; e.r.: exoskeletal ridges. Sperm packets in the colder-treatment sample are evident in dark violet, but structures are hardly recognizable in the warmer treatment, where the exoskeletal ridges are detached from the annular ridges. See Klepal et al. (1972) for histology details

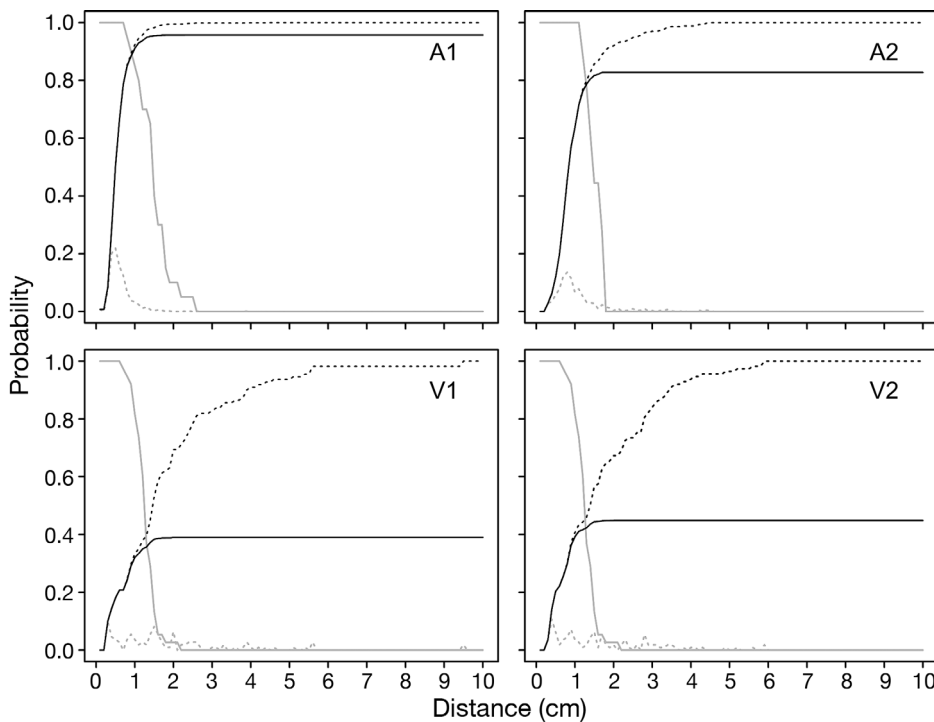


Fig. 11. Cumulative distribution of nearest neighbor distance (dotted black line), inverse cumulative distribution of penis length (solid grey line) (exceedance of penis length), field nearest neighbor distance frequency (dotted grey line) and fertilization probability (cumulative sum of products of the first 2, solid black line) for the 4 field locations (Ría de Arousa: A1 and A2; Ría de Vigo: V1 and V2)

consistent with the predictions from our laboratory results on the effect of temperature on penis length (Fig. 3). When temperature difference between the colder and the warmer ria is taken into account, the 50% fertilization point for the Ría de Vigo (warmer ria) fits the cumulative exceedance distribution of extended penis length with the penis length multiplier of 3.6 and the laboratory-derived temperature effect of 0.85 (Fig. 4b). Furthermore, all the values in the cumulative exceedance distribution of extended

penis length lie within the confidence limits of the fertilization fractions observed at different nearest neighbor distances in the field in the Ría de Vigo (Fig. 4b).

The NOAA CORDEX RPC 8.5 forecasts of the Ría de Arousa region predicted warming of 1.87 to 4.55°C between 2005–2015 and 2090–2099 (Table 3), which makes our highest temperature treatment of 23°C a reasonable representation of future conditions since it lies within this same range.

Table 2. Estimates for fertilization probability (FP) and idealized fertilization probability (IFP) at all locations. Penis length multiplier of 3.6 was applied for calculations. Mean \pm SD are shown for each location and temperature for laboratory predictions. A1, A2: Ría de Arousa locations; V1, V2: Ría de Vigo locations

	A1	A2	V1	V2
FP	0.95 \pm 0.09	0.82 \pm 0.23	0.39 \pm 0.23	0.44 \pm 0.21
IFP 14°C	0.98 \pm 0.06	0.95 \pm 0.10	0.78 \pm 0.33	0.83 \pm 0.25
IFP 17°C	0.97 \pm 0.10	0.89 \pm 0.16	0.68 \pm 0.30	0.73 \pm 0.23
IFP 20°C	0.92 \pm 0.14	0.67 \pm 0.25	0.36 \pm 0.22	0.43 \pm 0.15
IFP 23°C	0.74 \pm 0.16	0.42 \pm 0.19	0.21 \pm 0.16	0.27 \pm 0.12

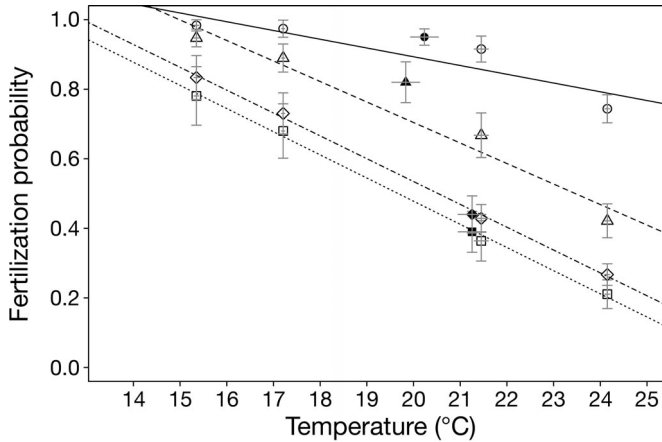


Fig. 12. Fertilization probability as a function of mean plus 1 SD temperature (submerged and exposed to air), based on laboratory penis lengths (open symbols) and field nearest neighbor distances and on field penis lengths (solid symbols), iButton field temperatures (Ría de Arousa: A1, A2), model hindcasts of field temperatures (Ría de Vigo: V1, V2) and field nearest neighbor distances. A1: solid black line; A2: dashed black line; V1: dotted black line; V2: dot-dashed black line. Open symbols are recorded laboratory penis lengths—circles: 14°C; triangles: 17°C; squares: 20°C; diamonds: 23°C. Vertical and horizontal grey bars are SE

4. DISCUSSION

The mechanisms responsible for setting the geographic limits of species are of great interest in a warming world. Hutchins (1947) argued that the most sensitive stages in life histories are most likely to limit geographic distribution, and he used the example of *Semibalanus balanoides* to propose the hypothesis that an equatorward geographic limit could be set by the effect of winter conditions on reproduction rather than by the more obvious effect of extreme summer conditions on adult survival. Here, we examined one of the rate-limiting steps in reproduction and its dependence on temperature at the equatorward geographic limit of the species. We

Table 3. NOAH model forecasts and hindcasts of warming in the Ría de Arousa region between the 1970s, early 2000s and 2090s. Model developers and model versions are listed in the model column (MOHC: Met Office Hadley Centre; NCC: Norwegian Climate Center; CNRM: Centre National de Recherches Météorologiques; ICHEC: Irish Centre for High-End Computing; IPSL: Institut Pierre Simon Laplace; MPI: Max Planck Institute for Meteorology). Global models were dynamically downscaled to the CORDEX EUR-11 (11 km) grid by the Swedish Meteorological and Hydrological Institute using the RCA_v1 regional model. ΔT is the mean temperature change between decades

Global model	2006–2015 vs. 1970–1979 ΔT (°C)	2090–2099 vs. 2006–2015 ΔT (°C)
MOHC HadGEM2-ES	1.61	4.55
NCC NorESM1	0.42	2.98
CNRM CNRM-CM5A	0.93	2.74
ICHEC EC-Earth	0.68	2.39
IPSL CM5A-MR	0.73	2.20
MPI ESM-LR	0.54	1.87

base our analysis on the observations (e.g. Barnes & Barnes 1968, Southward & Crisp 1954, Crickenberger & Wetthey 2018) that large-scale geographic patterns in physiology and distribution overwhelm local physiological acclimation effects.

High temperatures inhibit penis development in *S. balanoides*, as determined in our laboratory experiments (Figs. 5 & 7), and therefore diminish fertilization probabilities of the species, as confirmed in the field (Fig. 3). Penis development was completed in barnacles held at temperatures of 17°C or lower. The mean length of the fully developed penises (relaxed) was far greater at 14 and 17°C (10.1 and 7.8 mm, respectively) than at 20 and 23°C (4.4 and 2.8 mm, respectively) (Table 1). Klepal et al. (2010) observed that the relaxed penis length of mature *S. balanoides* from Scotland is 8 to 13 mm long, similar to our 14–17°C laboratory treatments and far longer than our field observations (4.1 mm in the Ría de Arousa). Barnacles maintained at 14 and 17°C showed a number of annulations corresponding to field observations in the UK of fully developed penises immediately before copulation (Barnes & Stone 1972, Barnes 1992), while the animals from warmer-temperature treatments (20 and 23°C) in the laboratory and from the Ría de Arousa showed a lower number of annulations (Fig. 8). These results suggest that the *S. balanoides* population in the Ría de Arousa does not show a fully developed penis in terms of length and number of annulations, as observed in other populations in the middle of its geographic range. Higher temperatures in Galicia, compared to northern popu-

lations, seem to be the driving factor for the inhibition of penis development in the *S. balanoides* southernmost population.

Although other authors found that absolute penis length is not an accurate measurement for assessing developmental stage because the penis is a very extensible organ (Barnes & Stone 1972), we obtained reliable measurements with our methodology, with low SDs and a high logarithmic fit between the number of annulations and penis length (Fig. 9). Moreover, our results accurately fit into the models and estimations of fertilization success both in the laboratory and in the field, allowing us to affirm that temperature is a determining factor in penis development. The fact that there is a linear relationship between the number of annulations and length in penises smaller than 5 mm may indicate that there is a size from which the penis is functional enough to develop the annulations that allow it to lengthen and shrink, since annulations provide a greatly increased surface area that contribute to the increase in length under turgor pressure (Klepal et al. 1972). It is possible that the penises must have at least this resting length to be functional, since above this length they all show the same number of annulations.

As expected, fertilization probability was higher in Ría de Arousa (up to approx. 95%), where temperatures are 1 to 2°C lower than in the Ría de Vigo and where fertilization probabilities do not exceed 44%. The higher fertilization probability at A1 in Ría de Arousa is explained by the population density, which is higher than at A2. As nearest neighbor distance increased, the proportion of fertilized individuals decreased (Fig. 3) (see also Hoch 2008). Barnacles in uncrowded groups have to reach greater distances to copulate with their potential partners; therefore, shorter penises would be a disadvantage. In contrast, barnacles in high-density aggregations have many potential partners for copulation within a short distance. Although we did not directly measure penis length in animals from the warmer ria, the relationship between fertilization percentage and nearest neighbor distance in Fig. 3 can mostly be explained by a difference in the length of the penises in the warmer ria population, which must be shorter than penises in the coldest ria population (Fig. 2). The estimation of penis length for Ría de Vigo (warmer ria) provided a test of our model of the effects of temperature and population density on fertilization, since we correctly predicted the 50% fertilization point (Fig. 4b) using the penis length multiplier of 3.6 and a temperature effect of 0.85, and the fraction of the population with penis length exceeding each dis-

tance fits within the confidence limits of the fertilization fractions.

According to these results, fertilization in the warmer ria will be negatively affected not only by low population densities, as was expected, but also by shorter penis lengths caused by exposure to higher temperatures. In a warmer geographic region, like Ría de Vigo, the length of the penis can condition the reproductive success of a population to the point of considerably reducing its chances of fertilization compared to a population in a colder region as in Ría de Arousa. Even in Ría de Arousa, if temperatures continue rising in a warming scenario, reproductive success can be diminished up to approx. 42%, as seen in the idealized fertilization probability at 23°C (A2, Table 2). This is a plausible future temperature scenario since NOAA/CORDEX RCP 8.5 forecasts of the penis development season in Ría de Arousa in 2090–2099 lie within this range. A likely consequence of reduced reproductive success would be an increasing dependence of recruitment on larval dispersal from colder rias to warmer rias near the southern geographic limit of the species. For example, it is likely that the episodic occurrence of *S. balanoides* in Viana do Castelo, Portugal (Fischer Piette & Prenant 1957, Wetthey & Woodin 2008, Wetthey et al. 2011b), in extremely cold years derived from dispersal from more northern populations in Galicia. In light of these results, it is also very likely that natural selection would favor longer penises with the upward trend in temperatures because of the effects of climate change. The fertilization values obtained from the field support this idea, suggesting that rising temperatures will likely affect fertilization success through the capacity of the penis to reach distant mating partners. Crickenberger & Wetthey (2018) made predictions for the southern range limit in Galicia using biogeographic models and stated that fertilization probability was between 14 and 89%, depending on temperature and population density, and suggested that fertilization is an important driver at the southern limit of the species. Our results seem to match the same variability they observed regarding the probabilities for fertilization. It is interesting to highlight that fertilization probability is best correlated with the mean of the submerged and aerial exposure temperatures, suggesting that penis development and the reproductive success related to it are likely influenced by both aerial and water temperature. These results support what other authors have observed (e.g. Wetthey & Woodin 2008, Rognstad & Hilbish 2014) about the importance of incorporating both seawater and aerial temperature when attempt-

ing to understand the influence of temperature on the physiology and ecology of intertidal organisms.

The penis is a very extensible organ and can be extended 3 to 4 times its resting length (Klepal et al. 1972, 2010). As sessile animals, barnacles are limited by the distance they are from conspecifics in their ability to carry out fertilization. Short penises caused by high temperatures would not be able to reach certain distances for mating. However, several studies (Hoch 2008, Neufeld & Palmer 2008) have shown that the penis is a structure with high phenotypic plasticity. Hoch (2008) found that barnacles in sparse and uncrowded aggregations had longer penises than those from densely crowded groups, suggesting a response to increase the possibility of reaching distant mating partners which, if applied in this case, could somehow counteract the negative effect of temperature on penis length, assuming such plasticity was not lost in the development process under warmer conditions. Our field observations suggest that higher temperatures override this phenomenon (Figs. 3, 4, 12).

Our study suggests that reproductive thresholds observed in the middle of the geographic range (France and further north) under the winter cold limitation of the reproduction hypothesis are exceeded in the Galician populations at the southernmost distribution limit on the European coast. In Galicia, the maximum temperature for reproductive success appears to be 14–17°C (Figs. 7–9, 12), whereas further north the threshold is closer to 10–12°C (Crisp & Patel 1969, Rognstad & Hilbush 2014, Crickenberger & Wetthey 2018). This shows the adaptability of the species to local conditions and points out that Galicia may serve as a refuge for *S. balanoides* and other boreal species in southern European waters. However, the results also point out that there is a critical threshold of temperature at which penis development fails and therefore *S. balanoides* reproduction fails. Reproductive success in barnacles, which have such a complex and dynamic life cycle, can be limited and affected by several factors at many levels and scales (e.g. Crickenberger & Wetthey 2018). As for many animals, in *S. balanoides*, temperature is one of the most important factors setting the distribution limit of the species; however, the importance of limits on other life stages are still unknown, and further studies are needed to clarify these patterns. In addition, interactions with other species in the region such as *Chthamalus montagui* and *Austrominius* (formerly *Elminius*) *modestus*, which have a higher tolerance to warmer temperatures, might add restrictions to the southern limits of *S. balanoides* (Barnes & Barnes 1966, Poloczanska et al. 2008, Mieszkowska et al. 2014) due to competition effect.

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