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Enhancing oocyte maturation and fertilisation in the black-foot limpet *Patella candei* d'Orbigny, 1840 (Patellidae, Mollusca)

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ABSTRACT

Limpet species are subjected to pressures of high exploitation. In the Madeira Archipelago (Portugal), the limpes are an economic and traditional fishing resource whose exploitation is regulated, to reduce the impact over the native populations. The effectiveness of the NaOH-alkalinized seawater baths to enhance the artificial maturation of *Patella candei* (D'orbigny, 1840) oocytes and fertilisation success in laboratory has been studied. First, the optimal combinations of pH and activation time to obtain the active oocytes were tested. Then, the viability of those oocytes was studied in a three factorial assay (pH x activation time x fertilisation time). Finally, the results obtained in the previous assays were cross tested to find a relationship between both. The viability of the oocytes over time were tested for methodology improvement. Results showed that the optimal conditions for the production of larvae was a pH of 9.5, activation time between 2 and 3 h and fertilisation time of 24:00 h, obtained a ratio of development between 0.65 and 0.75. The refrigeration could help to maintain the oocyte viability over time, but further research is required. This study establishes a foundational step towards the study the larval biology of *Patella candei* in laboratory, which is required to understand the ecological requirements for the early life stages of this species and promote aquaculture production.

1. Introduction

The genera *Patella* is one of the seven of the family Patellidae (Rafinesque 1815), a group of specialized marine gastropods known as "limpets". The limpets are key nodes in coastal and intertidal ecosystems, regulating microbial, algal and faunal populations (Plaganyi and Branch, 2000; Kaufmann et al., 2008; Riveira-Ingraham et al., 2011). The overexploitation of the limpets as a fishery resource is affecting the diversity of their populations (H.R. Martins et al., 1987; Martins et al., 2008; Henriques et al., 2017; Sousa et al., 2019b), and several limpet species are considered endangered, like the Mediterranean species *Patella ferruginea* Gmelin, 1791 and *Cymbula safiana* Lamarck, 1819 (Riveira-Ingraham et al., 2011); the Eastern-Pacific *Scutellastra mexicana* Broderip & Sowerby, 1829 (Carballo et al., 2020); and the Atlantic species *Patella candei* D'Orbigny, 1840 in the Canary Islands (Núñez et al., 2003; Tuya et al., 2006; González-Lorenzo et al., 2015) and *Patella aspera* Röding, 1798 in the Azores Archipelago (Thompson, 1979; Serrão Santos et al., 2010).

The black foot limpet, *Patella candei* D'Orbigny, 1840 is a native species of the intertidal ecosystems from the Webbnessia biogeographical area, which is composedof the archipelagos of Madeira, Selvagens and Canary Islands (Freitas et al., 2019; Weber and Hawkins, 2002). This species plays an important role as a gastronomic and economic resource for the islands. In Madeira, the fishery of limpets reported a total catch of 111 tonnes in 2017, worth 0.4 M€ (Sousa et al., 2019b). The regional governments developed legislative measures with the objective of maintaining and restoring the populations of native limpet species, such as the creation of marine reserves in the Canary Islands (Núñez et al., 2003; Ramírez et al., 2009) and capture restrictions in Madeira (Sousa et al., 2019a).

The aquaculture of limpets could be an alternative to the exploitation of the natural stocks. Limpets aquaculture is a relatively new and

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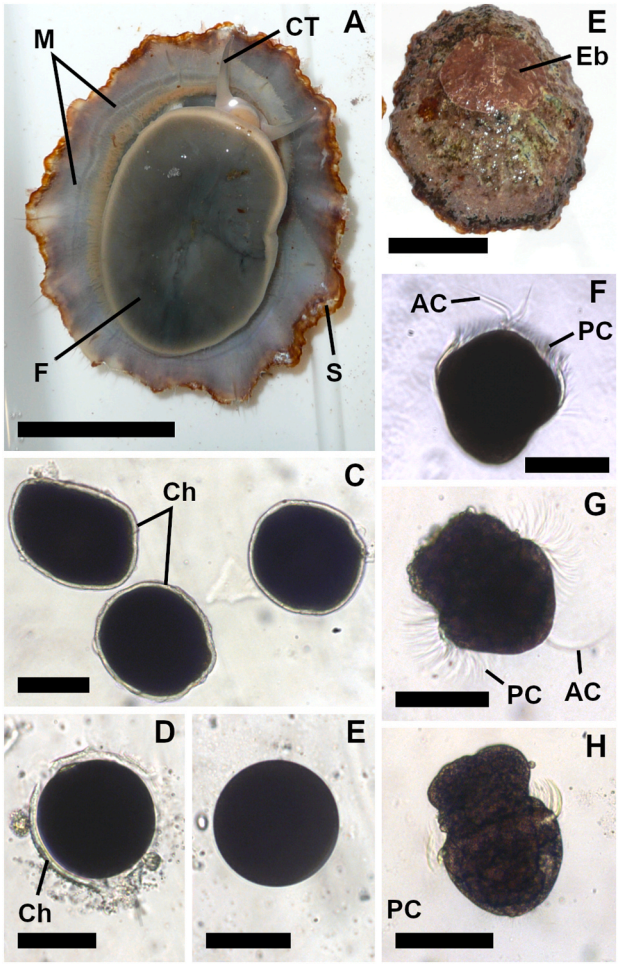

Fig. 1. *P. candei*. General view of the adult, oocytes, and trochophora larvae. A. Adult specimen, ventral view (scale bar = 20 mm). B. Adult specimen, dorsal view (scale bar = 20 mm). Activation process of the oocytes (scale bar = 100 μm) (C-E): C. Non-active oocytes showing a variable shape (from spherical to non-spherical) and chorion intact. D. Active oocyte showing spherical shape and chorion partially removed. E. Active oocyte showing spherical shape and chorion totally removed. Trochophora larvae (scale bar = 100 μm) (F-H): F-G. Trochophora larvae, normal morphology. H. Trochophora larvae, deformed morphology. Abbreviations: AC, apical cilia; Ch, chorion; CT, cephalic tentacles; Eb; epibionts; F, foot; M, mantle; PC, prototrochal cilia; S, shell.

expanding field led by studies on *Patella caerulea* (Ferranti et al., 2018) and *Patella ferruginea* (Espinosa et al., 2010; Guallart, 2010; Guallart et al., 2020) in the Mediterranean; and *Cellana sandwicensis* in Hawaii (Nhan and Ako, 2019). Major efforts were focused on adult feeding and management (Nhan and Ako, 2019; Guallart et al., 2020), natural spawning (Ferranti et al., 2018; Nakano et al., 2020), and laboratory-scale fertilisation trials and larval culture (Espinosa et al., 2006; Pérez et al., 2016). The aquaculture of limpetsis challenging as: (1) adults could be damaged during the capture and management, thus increasing mortality (Mau and Jha, 2018); (2) adequate aquafeeds had not been developed (Mau and Jha, 2018; Guallart et al., 2020); (3) there is no sexual dimorphism or effective spawning induction methods, thus biopsies and dissection are necessary to identify the animal sex and to obtain the gametes (Castejón et al., 2020); and (4) the larvae survival and settlement success has been limited thus far (Mau et al., 2018; Guallart et al., 2020).

The alternative to the spawning induction methods for the production of limpets larvae is the use of gametes obtained by dissection for fertilisation using in vitro procedures. The fertilisation's success is enhanced through the activation, a process in which the oocytes obtained by dissection are matured artificially using alkalinized seawater baths during a given time (Aquino De Souza et al., 2009; Castejón et al., 2020). Thus far, different studies used two alkalinizing agents, NaOH (Aquino De Souza et al., 2009; Guallart et al., 2020; Castejón et al., 2020), NH_4OH (Wanninger et al., 1999; Gould et al., 2001; Hodgson et al., 2007), or both (Pérez et al., 2016). The efficiency of the activation depends on the pH and the duration of the alkalinized seawater baths, the oocytes being considered viable when shape was spherical and the chorion removed (Castejón et al., 2020). In patellid limpets, the management of the sperm is comparatively easier, being diluted in seawater during a given time to achieve competence (Hodgson et al., 2007; Pérez et al., 2016; Castejón et al., 2020).

The objective of this study was to develop a reliable and replicable methodology for the production of *P. candei* larvae, using oocytes actived by NaOH alkalinized baths. The first step was to determine the optimal combinations of pH x activation time, to obtain the active oocytes. As a second step, the viability of those active oocytes was studied using different fertilisation times in a three factorial assay (pH x activation time x fertilisation time). Thirdly, the results obtained in the second assay were verified using a wider range of activation times. Finally, the viability of the limpet's gametes over time was tested for protocol optimization.The potential of this methodology for obtaining larvae of limpets for laboratory-scale experiments and aquaculture production is discussed.

2. Material and methods

The adult specimens of *P. candei* (152 in total; Fig. 1A) were obtained from the SW coast of Madeira Island through different samplings realized during the spawning season (from December 2019 to February 2020), to ensure higher proportion of specimens with mature gonads (Henriques et al., 2012). The animals were collected by local fishermen employing the special knife known as "lapeira" using a traditional method of manual capture. Immediately upon arrival to the Centro de Maricultura da Calheta (Madeira, Portugal), the animals were measured (carapace length, CL; Digital Stainless Calliper, precision = 0.01 mm), weighed (total weight, TW; Precision Balance RADWAG WTC 600.1, range = 600 g, precision = 0.01 g, Poland) and labelled (handmade

Table 1
P. candei. General information about the assays realized: date, male and female morphometry, temperature and oocyte density during the activation, temperature and oocyte density during the incubation.

Assay	date	males			females			activation conditions		incubation conditions	
		n	SL (mm)	TW (g)	n	SL (mm)	TW (g)	temp °C	OD oocyte ml^{-1}	temp °C	OD oocyte ml^{-1}
Assay 1: Production of active oocytes	December 2019	N.A.	N.A.	N.A.	4	57 ± 4	16 ± 4	22 ± 1	124 ± 20	N.A.	N.A.
Assay 2: Viability of the active oocytes	February 2020	3	55 ± 6	17 ± 7	4	48 ± 4	10 ± 2	20 ± 1	145 ± 17	20 ± 1	70 ± 8
Assays 3: RAO and oocyte viability	February 2020	3	47 ± 7	11 ± 3	5	46 ± 5	9 ± 3	20 ± 1	122 ± 8	19 ± 1	45 ± 3
Assay 4: Viability of the gametes over time	February 2020	3	47 ± 7	11 ± 3	5	46 ± 5	9 ± 3	20 ± 1	122 ± 8	19 ± 1	45 ± 3

Values are showed as average ± SD. Abbreviations: n, total number of specimens pooled; OD, oocyte density; temp, temperature; TW, total weight (fresh); SL, shell length.

vinyl tags sheet attached with cyanoacrylate), see Table 1 for details. The specimens were maintained in 200 l culture tanks. The animals were maintained in an open system (1–2 l min^{-1}, 50 μm filtered seawater) with natural temperature and salinity (20 ± 1 °C and 37 ± 1; Oxygen Probe, Handy Polaris v.3.11., OxyGuard®, Farum, Denmark; and Hand-Held Refractometer A.S.D. 0–100, ATAGO, Japan). The assays were realized between two and eight days after the arrival of the specimens. The details of the assays are showed in Table 1.

Extraction of the gametes. The sex of the limpets was determined by biopsy of the gonads using one ml syringes. The left side of the mantle was cut using a scalpel to expose the gonad. The gonad of each female was immersed in 100 ml of filtered seawater (FSW) and gametes were released using a plastic Pasteur's pipette. The oocytes were washed employing a 200 μm filter to collect solid debris and a 100 μm filter was used to collect and wash the oocytes carefully. The washed oocytes were then resuspended in 100 ml of FSW. Regarding the males, the sperm was pipetted from the fresh gonad and suspended in 100 ml of FSW. The suspension was filtered using a 50 or 100 μm mesh, discarding the retained phase and the sperm solution was collected.

Assay 1: Production of active oocytes. Different combinations of pH and activation time (AT) were tested in this two factorial assay (pH x AT), to find the combination with a higher ratio of active oocytes and shorter AT. For this purpose, four pH treatments (8.5, 9.0, 9.5 and 10.0 ± 0.1) plus control (8.2 ± 0.1) were combined with seven ATs (0:30 h, 1:00 h, 1:30 h, 2:00 h, 3:00 h, 4:00 h, and 5:00 h), giving a total of 35 treatments, and three replicates per treatment were used. The FSW was alkalinized using NaOH and pH was measured using a pH metre (pH Meter GRD 2, CRISON, Barcelona, Spain). The activation was realized in 100 ml plastic cups filled with 20 ± 1 ml of pH treatment. Each plastic cup corresponded to a single replicate.

The plastic cups were placed randomly in a grid marked with coordinates to facilitate the sampling when required. Activation started at the same time on all the treatments. At the end of each AT, a group of randomly selected plastic cups from each pH treatment were sampled to characterize their oocytes using an optical microscope (Zeiss Axioskop 2 Plus, Carl Zeiss Microscopy LLC, NY, United States). The characterization of the oocytes was based on their morphology (spherical or non-spherical) and the chorion integrity (intact, partially removed, totally removed) (Fig. 1B-D). Between 50 and 80 oocytes were characterized *per* replicate. The oocytes were considered "active oocytes" when the shape was spherical, and the chorion was partially or totally removed (Fig. 1C-D). Two observers were involved in the characterization of the oocytes. The assignation of the replicates for each observer, and the order followed to characterize those replicates were realized randomly. The sampled plastic cups were removed from the assay. The ratio of active oocytes (RAO = number active oocytes x total $oocytes^{-1}$) and the ratio of non-spherical oocytes (RNO = number non-spherical oocytes x total $oocytes^{-1}$) were calculated for each replicate.

Assay 2: Viability of the active oocytes. This assay tested the viability of the oocytes treated with the optimal pH x AT combinations from the previous assay. Three pHs were tested: 8.3 ± 0.1 as control, and 9.0 and 9.5 ± 0.1 as those that showed the higher RAO. Three ATs were tested: 3:00 h and 4:00 h as the shorter timing with higher RAO, and 1:30 h as a precaution following recommendations from previous publications (Aquino De Souza et al., 2009; Hodgson et al., 2007). Finally, three Fertilisation Times (FT) were tested: 1:30 h, 3:00 h and 24:00 h, to determine the viability of the sperm during the first hours and to determine if polyspermyoccurs with prolongued FT. Then, this assay combined three factors (pH x AT x FT), giving a total of 27 combined treatments, with four replicates *per* combined treatment.

The activation of the oocytes was realized in nine separated crystal beakers filled with 400 ml of pH solution, each one corresponding to one pH x AT combination. The activation was realized at room temperature. At the end of each AT, the corresponding oocytes were carefully washed in a 100 μm filter and kept in 100 ml of FSW until the start of the fertilisation. A sample of oocytes was taken and characterized from each pH x AT combination, then the RAO was calculated following the previous assay 1. The sperm pool solution was prepared at the end of the activation to maximize sperm freshness. The fertilisation began at the same time in all the combined treatments, adding the sperms for a final density of 2 × 106 sperm cells ml^{-1}. The treatments FT = 1:30 and 3:00 h were washed with FSW at the end of the FT to remove the sperm and 100 μm filters were used to gather the oocytes. Fertilisation and incubation were realized in 100 ml plastic cups filled with 40 ml FSW. Incubation was static and lasted 24:00 h at room temperature.

The sampling was realized at the end of the incubation period adding 2 ml of absolute ethanol to induce the sinking of the larvae and collecting as many specimens as possible from the bottom. The samples were fixed in 5.6% formaldehyde. The specimens collected in each replicate were characterized as: normal trochophora larvae (larvae with normal morphology; Fig. 1E-F), abnormal larvae (e.g. polyspermatic, deformed, cell clusters; Fig. 1G-H), and oocytes (non-fertilized oocytes; Fig. 1B-D). The ratio of development (RD = number normal larvae x total $specimens^{-1}$), the ratio of abnormality (RA = number abnormal larvae x total $specimens^{-1}$), and the ratio of fertilisation (RF = RD + RA) were calculated in each of the replicate.

Assay 3: Ratio of active oocytes and oocyte viability. This assay was realized to verify the potential use of the ratio of active oocytes (RAO) as indicator for the oocyte viability, measured as the ratio of development (RD) for normal larvae. This assay was based on the results obtained in the assay 2. For this purpose was employed a two factorial assay (pH x AT), combining two pH (9.0 and 9.5 ± 0.1) with five AT (1:00 h, 1:30 h, 2:00 h, 2:30 h and 3:00 h), resulting in 10 total combined treatments with four replicates *per* combined treatment. A control treatment with natural pH (8.2 ± 0.1) and four replicates was also realized. A sample of oocytes from each pH x AT combined treatment were characterized to calculate the RAO. Fertilisation and incubation took place in 100 ml plastic cups filled with 40 ml FSW. Incubation was static, employed 2 × 106 sperm cells ml^{-1} and lasted 24:00 h at room temperature. Then, the specimens were anesthetized with ethanol and fixed with formaldehyde. Following the assay 2, the specimens from each pH x AT replicate were characterized to calculate the RD, RA, and RF.

Assay 4: Viability of the gametes over time. This assay was conducted to study the viability of the gametes over time after their extraction and activation. For this purpose, gametes were used from the same pool as assay 3 (see Table 1). The oocytes employed in this study were activated at pH 9.5 during 3 h and kept an additional two hours in FSW, while preparing the sperm pool. Each pool of gametes (O = oocytes; S = sperm) was subdivided into two differentiated temperature treatments, room temperature (N = 20 ± 1 °C) and refrigeration temperature (R = 5 ± 2 °C), resulting in the following pools: oocytes at room temperature (ON), oocytes at refrigeration temperature (OR), sperm at room temperature (SN), and sperm at refrigeration temperature (SR). The pools were cross-fertilized as: ON x SN, OR x SN, ON x SR, and OR x SR. The Factor Time was established as the time passed after the control fertilisation test (Time = 0 h). Then, the cross-fertilisations were carried out at four different Time treatments (Time = 4:00 h, 8:00 h, 12:00 h and 24:00 h), resulting in a total of 16 cross-fertilisation x Time combined treatments. Four replicates *per* combined treatment were used. The incubation proceeded as: static, 2 × 106 sperm cells ml^{-1}, 24:00 h duration, and at room temperature. At continuation, the specimens were anesthetized and fixed to calculate the RD for each replicate.

Statistical analyses. The statistical software R version 3.6.3 (R Core Team, 2020) was used for all the statistical analyses. Square root transformations (RAO, RNO, RD, RA, and RF) were applied to comply ANOVA's requisites of normality of the residuals and homoscedasticity (Shapiro-Wilk test and Levene's test, respectively;package "car 3.0–7"; (Fox and Weisberg, 2019)). The statistical significance for both tests was established with a critical level (α) of 0.05 to reject the null hypothesis.

In assay 1, the RAO and RNO were analyzed with a two-way ANOVA (type III) (package "car 3.0–7"; (Fox and Weisberg, 2019)) using pH and AT as factors. Post hoc *t*-tests with Bonferroni correction were applied,

Table 2
P. candei. Assay 1. Average ratio of active oocytes RAO (mean ± SD) obtained at each combination of pH x Activation Time.

	0:30 h	1:00 h	1:30 h	2:00 h	3:00 h	4:00 h	5:00 h
pH 8.0	0.04 ± 0.07^{ab}	0.09 ± 0.07^{abc}	0.13 ± 0.02	0.15 ± 0.02	0.07 ± 0.05^{a}	0.06 ± 0.02^{a}	0.06 ± 0.05^{a}
pH 8.5	0.09 ± 0.08^{a}	0.21 ± 0.11^{a}	0.13 ± 0.10	0.15 ± 0.02	0.14 ± 0.02^{ab}	0.08 ± 0.01^{a}	0.08 ± 0.02^{ab}
pH 9.0	0.00 ± 0.00^{b}	0.18 ± 0.15^{ab}	0.20 ± 0.06	0.27 ± 0.05	0.35 ± 0.07^{bc}	0.50 ± 0.02^{b}	0.14 ± 0.07^{abc}
pH 9.5	0.00 ± 0.00^{b}	0.05 ± 0.05^{bc}	0.23 ± 0.13	0.31 ± 0.11	0.38 ± 0.10^{c}	0.51 ± 0.11^{b}	0.33 ± 0.04^{c}
pH 10.0	0.00 ± 0.00^{b}	0.01 ± 0.01^{c}	0.07 ± 0.04	0.16 ± 0.04	0.35 ± 0.07^{bc}	0.41 ± 0.19^{b}	0.25 ± 0.12^{bc}

Different letters indicate significant differences ($P < 0.05$) among treatments within the same activation time (column).

Table 3
P. candei. Assay 1. Average ratio of non-spherical oocytes RNO (mean ± SD) obtained at each combination of pH x Activation Time.

	0:30 h	1:00 h	1:30 h	2:00 h	3:00 h	4:00 h	5:00 h
pH 8.0	0.86 ± 0.07	0.76 ± 0.11^{a}	0.72 ± 0.10^{a}	0.72 ± 0.14^{a}	0.87 ± 0.01^{a}	0.83 ± 0.08^{a}	0.92 ± 0.05^{a}
pH 8.5	0.75 ± 0.03	0.63 ± 0.06^{a}	0.73 ± 0.15^{a}	0.68 ± 0.01^{ab}	0.75 ± 0.04^{a}	0.74 ± 0.08^{ab}	0.92 ± 0.02^{a}
pH 9.0	0.75 ± 0.05	0.34 ± 0.24^{b}	0.44 ± 0.04^{b}	0.40 ± 0.03^{c}	0.46 ± 0.11^{b}	0.35 ± 0.05^{c}	0.81 ± 0.04^{ab}
pH 9.5	0.69 ± 0.17	0.60 ± 0.07^{ab}	0.38 ± 0.24^{b}	0.43 ± 0.14^{bc}	0.42 ± 0.12^{b}	0.37 ± 0.10^{c}	0.61 ± 0.08^{b}
pH 10.0	0.70 ± 0.15	0.66 ± 0.09^{a}	0.47 ± 0.15^{ab}	0.64 ± 0.15^{abc}	0.37 ± 0.06^{b}	0.49 ± 0.26^{bc}	0.68 ± 0.11^{b}

Different letters indicate significant differences ($P < 0.05$) among treatments within the same activation time (column).

package "lsmeans 2.30–0"; (Lenth and Lenth, 2018)). The statistical significance was established with a critical level (α) of 0.05 to reject the null hypothesis.

In assay 2 the RD, RA, and RF from assay 2 were analyzed using a three-way ANOVA (type III) in which pH, AT, and FT were the factors. The differences among the combined pH x AT treatments were analyzed, subdividing the datasets into FT subsets (FT = 1:30 h, 3:00 h and 24:00 h) and applying post hoc *t*-tests with Bonferroni correction. In assay 3, the RD, RA, and RF were analyzed using a two-way ANOVA (type III) in which pH and AT were the factors. The differences among the combined pH x AT treatments were analyzed applying a post hoc *t*-tests with Bonferroni correction. In both assay 2 and 3 the relationship between RD and RAO was studied applying the standard linear regression model using the R software [lm(RD~RAO, DATASET)] to each FT subset from the assay 2 and the entire dataset from the assay 3. The statistical significance was established with a critical level (α) of 0.05 to reject the null hypothesis.

In assay 4 the RD was analyzed using a robust ANOVA as non-parametric method of analysis, applying the "t2way" function (package "WRS2 1.1–0″; Mair and Wilcox, 2018, 2019) for a two factorial design (cross-fertilisation x Time). At continuation, the cross-fertilisation treatments were analyzed in each Time subset (Time = 4 h, 8 h, and 12 h) using the post hoc *t*-tests with Bonferroni correction. The critical level (α) to reject the null hypothesis was 0.05.

3. Results

*Assay 1: Production of active oocytes.*The highest number of active oocytes (RAO) was obtained at a pH of 9 and combined with AT = 4:00 h ($p < 0.05$, Table 2), while lower RAO was generally observed at control treatment (pH 8.0) and AT = 0:30 h ($p < 0.05$, Table 2). On the contrary, the ratio of non-spherical oocytes (RNO) was higher at control and pH of 8.5 treatments ($p < 0.05$, Table 3). The influence of the interaction of pH x AT was significant for both RAO ($F_{4,70} = 5.79$, $p < 0.001$) and RNO ($F_{4,70} = 1.97$, $p < 0.01$).

Assay 2: Viability of the active oocytes. The higher ratio of development (RD) occurred generally at a pH of 9.5, except when combined with AT = 4:00 h ($p < 0.05$, Fig. 2A), while the lower RD was observed in control ($p < 0.05$, Fig. 2A). The three-way ANOVA showed pH x AT as the single significant interaction ($F_{4,81} = 4.00$, $p < 0.001$). The RD increased with the pH, being higher at AT = 3:00 h, in comparison to other AT treatments, but the influence of the FT was not clear ($p > 0.05$, Fig. 2A). The RD increased significantly with the RAO following a standard linear regression model in the three FT subsets (Fig. 3B): FT = 1:30 h (F-stat = 69.95, $p < 0.05$, $R^2 = 0.869$), FT = 3:00 h (F-stat = 49.11, $p < 0.05$, $R^2 = 0.857$), and FT = 4:00 h (F-stat = 79.11, $p < 0.05$, $R^2 = 0.907$).

Generally, the ratio of abnormality (RA) was low, with highest value occurring in the combined treatments of pH 9.0 and 9.5 x FT = 1:30 h ($p < 0.05$, Fig. 2B). The RA was lower at control pH independently of the AT or the FT ($p < 0.05$, Fig. 2B). Three-way ANOVA showed significant influence of the interactions pH x AT ($F_{4,81} = 5.90$, $p < 0.001$) and pH x FT ($F_{4,81} = 3.78$, $p < 0.01$). RA increased with the pH, but AT and FT did not show a clear influence on the RA ($p > 0.05$). The ratio of fertilisation (RF) was lower at control ($p < 0.05$,). The three-way ANOVA showed pH x AT as a significant interaction ($F_{4,81} = 19.87$, $p < 0.001$). RF increased with higher pH, while AT and FT did not showed a clear influence (Fig. 3A).

Assay 3: Ratio of active oocytes and viability. The control treatment showed a low RD (mean ± SD = 0.06 ± 0.02). Regarding the pH x AT combinations, the highest RD occurred at pH 9.5 x AT = 2:00 h (0.75 ± 0.07; $p < 0.05$; Fig. 4A), while the lowest RD occurred at pH 9.5 x AT = 3:00 h (0.22 ± 0.03; $p < 0.05$; Fig. 4A). The interaction pH x AT was significant ($F_{4,40} = 12.50$, $p < 0.05$). The RD apparently increased with the RAO, but the linear regression model was not significant (F-stat = 3.80, $p > 0.05$, $R^2 = 0.30$) (Fig. 4D).

The highest RA occurred at pH 9.5 x AT = 1:30 h, but it was similar among the remaining treatments ($p < 0.05$; Fig. 4B). Two-way ANOVA showed significance in the interaction between pH x AT ($F_{4,30} = 7.15$, $p < 0.01$). The RF was generally higher at pH 9.5 than at pH 9.0, but decreased when AT = 3:00 h (Fig. 4C). The interaction pH x AT was significant ($F_{4,30} = 22.34$, $p < 0.001$).

Assay 4: Viability of the gametes over time. The RD was higher at Time = 0 h, decreasing gradually with a longer Time. No larvae was obtained in any cross-fertilisation test at Time = 24:00 h (Table 4). Robust ANOVA showed significant influence in the interaction cross-fertilisation x Time ($F_{16,51} = 4.48$, $p < 0.001$). One-way ANOVAs showed significant differences at T = 8 h ($F_{3,12} = 7.29$, $p < 0.01$) and T = 12 h ($F_{3,12} = 22.24$, $p < 0.01$), in which the RD was higher in those treatments with refrigerated oocytes, i.e. OR:SN and OR:SR (Table 4).

4. Discussion

This study shows that the artificial maturation of the oocytes of *P. candei* using alkalinized seawater baths enhances larval production in the laboratory. The higher ratio of development was obtained using the following conditions: pH 9.5, activation time between 2:00 and 3:00 h, and fecundation time of 24:00 h, and maintaining a temperature of

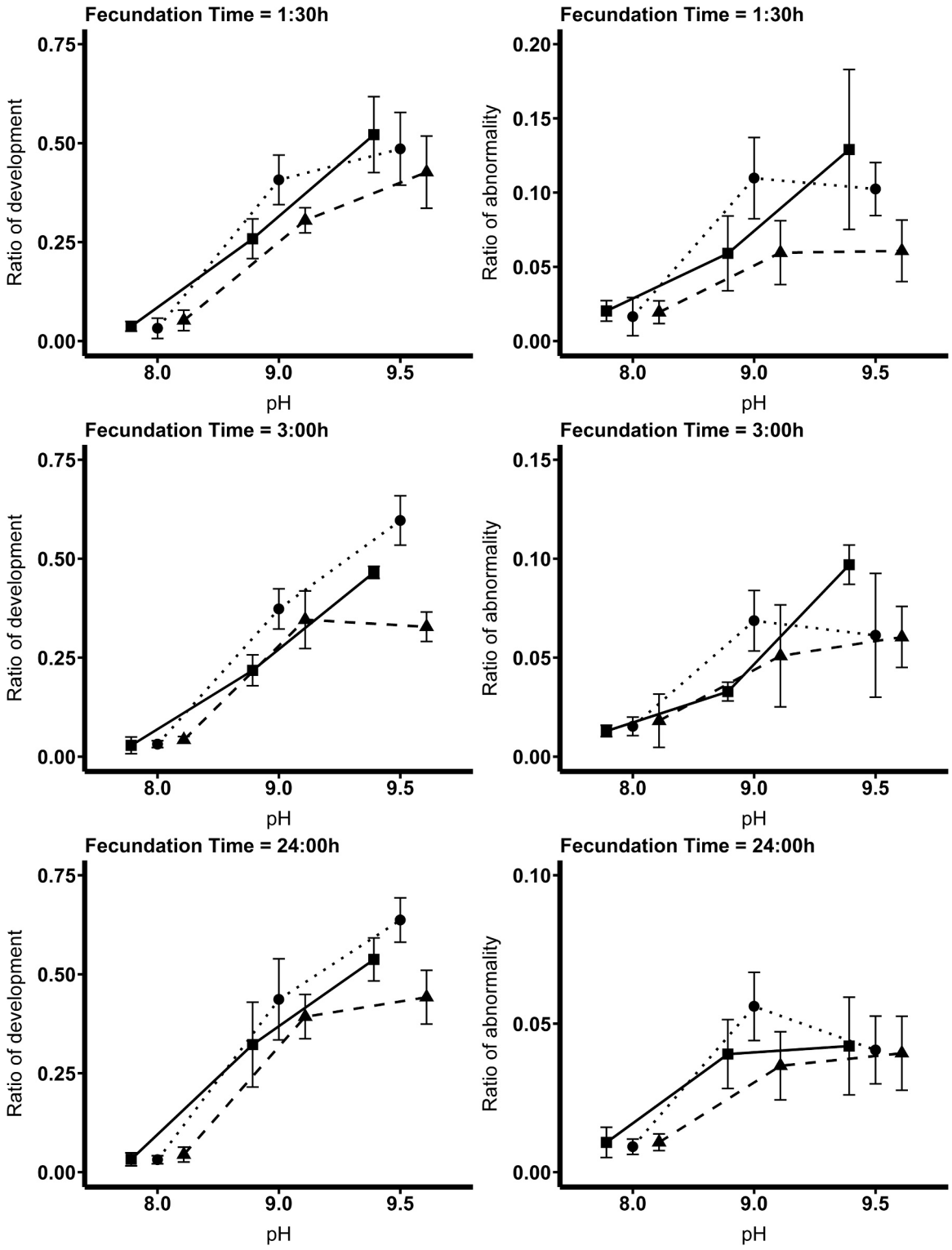

Fig. 2. *P. candei. Assay 2*: Influence of different combinations of factors on the larval production. A. Ratio of (normal) development at different pH x activation x fecundation times combinations. B. Ratio of abnormality at different pH x activation x fecundation times combinations. Different letters indicate significant differences ($p < 0.05$) among treatments.

20 ± 1 °C during this process. NaOH was an effective alkaline agent for the activation of *P. candei* oocytes. The NaOH was also effective to enhance the fertilisation success in other limpet species, i.e. *Patella aspera* (Castejón et al., 2020), *P. vulgata* and *P. depressa* (Aquino De Souza et al., 2009), *P. ferruginea* (Guallart et al., 2020), *Cellana nigro lineata* (Catalan and Yamamoto, 1993) and *Lottia gigantea* (Gould et al., 2001).

The assay 1 showed a peak on the ratio of active oocytes at pH 9.0 and 9.5 when activation time (AT) was between 3:00 and 4:00 h. Similar observation was done on *P. aspera*, still in this limpet species the ratio of active oocytes was high at AT = 5:00 h (Castejón et al., 2020). In other limpet species, the activation of the oocytes required between 2:00 and

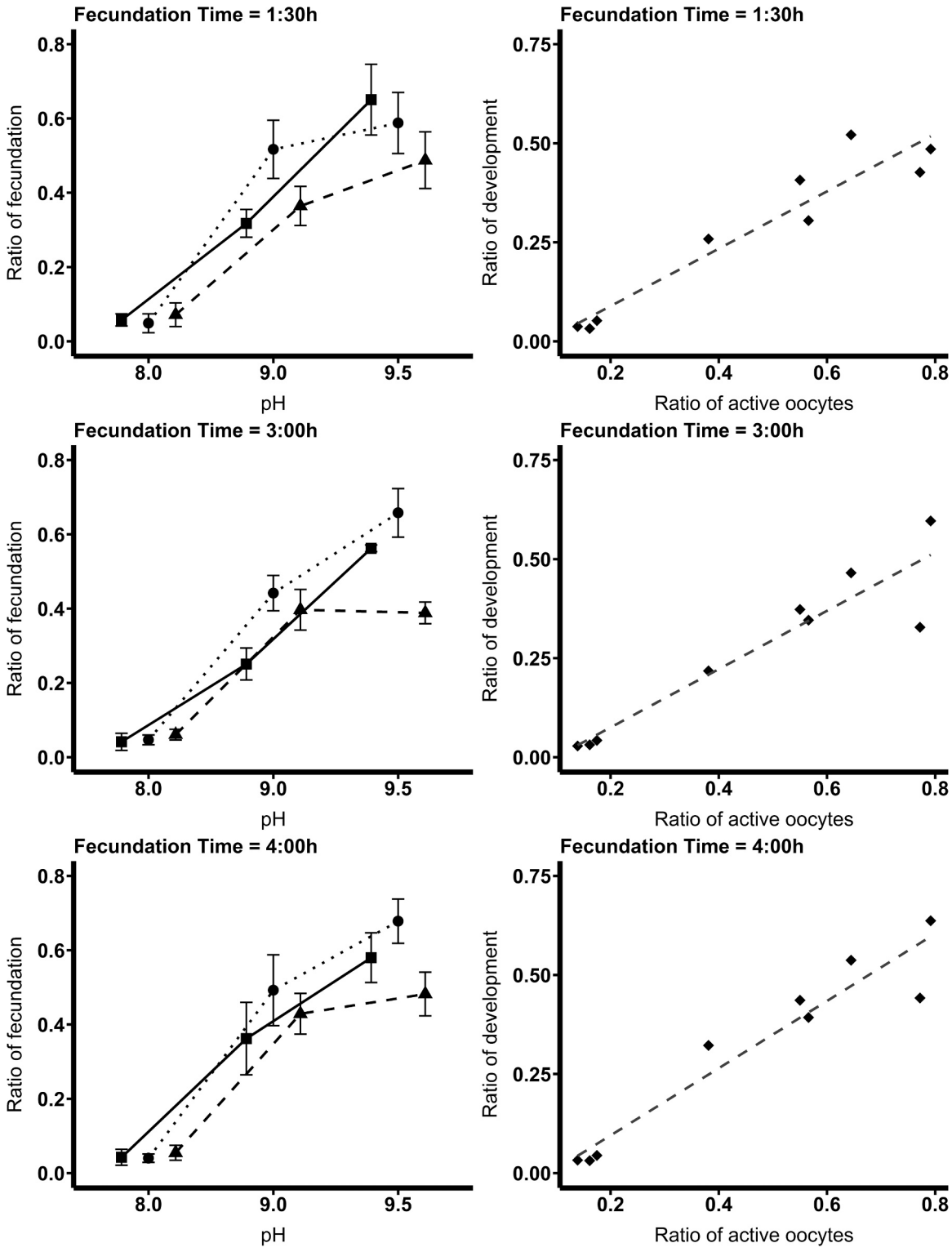

Fig. 3. *P. candei. Assay 2*: Influence of different combinations of factors on the larval production. A. Ratio of fertilization at different pH x activation x fecundation times combinations. B. Relationship between the ratio of development and the ratio of active oocytes. Different letters indicate significant differences ($p < 0.05$) among treatments.

4:00 h, when NaOH was used as alkalinizing agent (Guallart et al., 2020; Pérez et al., 2016). Nevertheless, in *P. candei* the ratio of non-spherical oocytes increased when AT = 5:00 h, suggesting that the overexposure to the alkalinising agent causes chemical damage to the oocytes (Moreau et al., 1990), including the degradation of signalling molecules such as the polyphosphoinositides (Borg et al., 1992). The assays 2 and 3 showed that the efficiency of the activation process varied among oocyte pools, probably depending of the initial level of maturation of the oocytes. This differential maturation also could explain the differential results observed in the relationship between the ratio of active oocytes (RAO) and the ratio of development (RD), when assays 2 and 3 were compared. The variation of the efficiency of the activation process, as

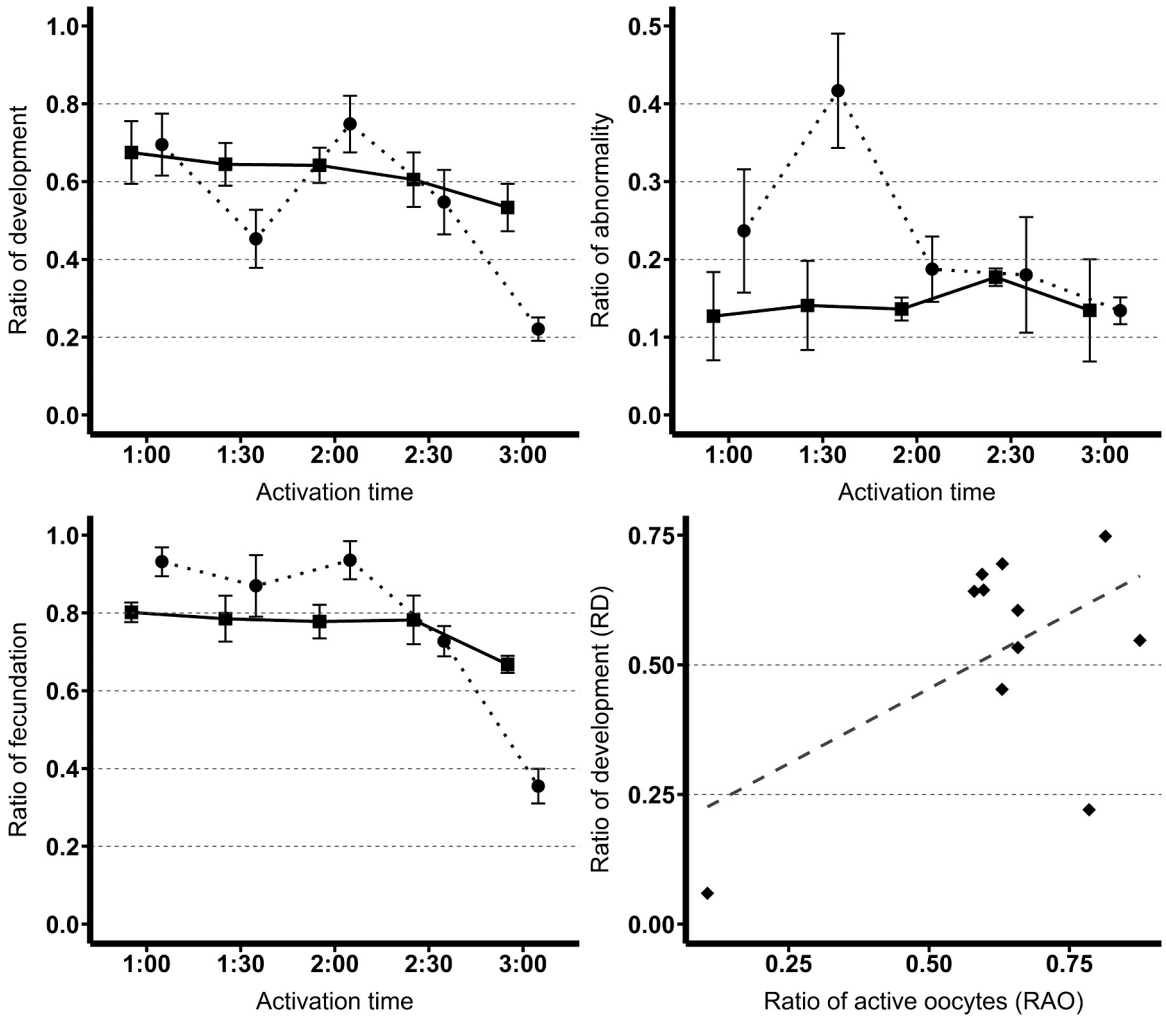

Fig. 4. *P. candei. Assay 3*: Study of the relationship between the ratio of development and the ratio of active oocytes. A. Ratio of (normal) development at different pH x activation times combinations. B. Ratio of abnormality at different pH x activation times combinations. C. Ratio of fertilization at different pH x activation times combinations. D. Relationship between the ratio of development and the ratio of active oocytes. Different letters indicate significant differences ($p < 0.05$) among treatments.

Table 4
P. candei. Assay 4. Longevity of the gametes after activation. Larvae production at different cross fertilizations and Times (hours, h) after the control fertilization. Abbreviations: O, oocytes; S, sperm; N, room temperature (20.2 ± 0.3 ºC); R: refrigeration temperature (5.2 ± 2.1 ºC). Control: Time = 0 h.

	0:00 h	4:00 h	8:00 h	12:00 h	24:00 h
Control	0.53 ± 0.01				
ON:SN		0.32 ± 0.10	0.09 ± 0.02[a]	0.00 ± 0.00[a]	N.A.
ON:SR		0.38 ± 0.01	0.16 ± 0.10[a]	0.01 ± 0.01[a]	N.A.
OR:SN		0.43 ± 0.05	0.28 ± 0.08[b]	0.08 ± 0.03[b]	N.A.
OR:SR		0.42 ± 0.04	0.27 ± 0.03[b]	0.07 ± 0.03[b]	N.A.

Different letters indicate significant differences ($P < 0.01$) among treatments within the same extraction time (column).

well in the relationship between RAO and RD, have been also reported in the Azorean limpet *P. aspera* by Castejón et al. (2020). Due to the vulnerability of the oocytes, and considering the results obtained in this study, it is recommended that a methodology to determine the quality of the oocytes before carrying out the activation process be implemented, being also necessary to confirm the reliability of the ratio of active oocytes as an indicator of the ratio of fecundation.

The fertilisation time (FT) did not show a clear influence on either the RD nor the ratio of abnormal larvae (RA), suggesting that FT did not influence polyspermy, as similarly observed in *P. aspera* (Castejón et al., 2020). The literature reported variability in the fertilisation periods: 3 h in *P. vulgata* (Fernández, 2015; Pérez et al., 2016), 1.5 h in *P. depressa* (Novoa, 2014), 1 h in *P. ferruginea* (Guallart et al., 2020) or 30 min in *P. ulyssiponensis* (Hodgson et al., 2007). This information supports that FT is not a determinant of the RD, but in our opinion it is recomendable to use FT around 24:00 h to ensure success in the production of larvae. This study also showed that incubation can be realized using a static method, which has been commonly used in the larval production of other marine gastropods such as abalone (Li, 2004; Pang et al., 2006; Leighton, 2008), the sea snail *Tegula funebralis* (Guzma et al., 2006), and the limpet *P. aspera* (Castejón et al., 2020).

The assay 4 showed that refrigeration can conserve a certain viability of the oocytes over time. This result reinforces the importance of the conservation of the quality of the oocytes to enhance the success of

fertilisation (Orton et al., 1956; Aquino De Souza et al., 2009). Refrigeration can help to maintain the cell freshness for a longer time (Guallart et al., 2020), giving the handler a broader worktime limit in scenarios of in vitro fertilisation. Different time and temperature ranges must be tested in future experiments to enhance the preservation of the gametes after extraction.

This study opens new perspectives for the culture of *P. candei* at early life stages and presents a solid foundation for future studies focused on the study of the larval biology of *P. candei* in laboratory. The study of the limpets' species early life is also necessary for the understanding the dispersal and distribution of the species, and consequently for fisheries management and purposes of conservation such as potential restocking plans (Crowe et al., 2002; Arnold, 2008; Purcell and Cheng, 2010; Loneragan et al., 2013). Further studies should focus on the aspects of larval production throughout the breeding season, settlement enhancement, and juvenile management etc.

In conclusion, this study presents a first approach towards the aquaculture of *P. candei*, having reached a ratio of larvae development between 0.65 and 0.75. However, further assays are necessary to enhance the production of larvae, as well as to study the larval development and metamorphosis requirements, before considering the scalation of this method to a full-fledged production plan.

CRediT authorship contribution statement

José M. Cañizares: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Diego Castejón:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing – review & editing, Visualization. **Ricardo Haroun:** Conceptualization, Validation, Writing – review & editing. **Natacha Nogueira:** Conceptualization, Validation, Resources, Writing – review & editing. **Carlos A.P. Andrade:** Conceptualization, Validation, Resources, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2021.100856.

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