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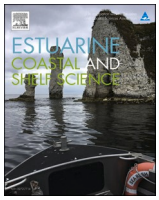

# Phenotypical variability affecting the commercial value of the stalked barnacle *Pollicipes pollicipes*: No evidence for epigenetic variation

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ABSTRACT

The stalked barnacle *Pollicipes pollicipes* is an important economic resource in Portugal and Spain. Two extreme phenotypes can be identified, based on their morphology. More elongated barnacles are associated with bad quality and have a lower commercial value.

The fishers perception about the existence, definition and causes for this phenotypical/quality variation was evaluated through a survey performed in Portugal and Galicia, Spain. The existence of two extreme commercial qualities was validated. Good quality barnacles were mainly defined as thick and short in both countries. In Spain (Galicia), the definition of bad quality animals corresponded mainly to the terms long and thin, while in Portugal, fishers used a wider variety of terms including watery, thin and soft. The characteristics of the rock and the hydrodynamics were the causes most referred by the fishers for this variation.

The morphological variation of *P. pollicipes* was described by the ratio between maximal rostro-carinal length (RC) and total height (TH): RC/TH values $> 0.4$ indicate good quality barnacles; and values $< 0.4$ indicate bad quality barnacles.

Although morphological variation between the two extreme qualities/phenotypes was found, no genetic (amplified fragment length polymorphism - AFLP) or epigenetic (methylation sensitive amplification polymorphism - MSAP) differences were detected.

## 1. Introduction

The economic value of marine exploited species is influenced by several factors such as the size (Santojanni et al., 2005), geographical location (Lahbib et al., 2010; Ramírez-Valdez et al., 2021), market factors (Natividad, 2016) and/or quality attributes (Reynolds and Wilen, 2000; Rocha et al., 2019). Quality attributes includes, for example, sex and variation in gonad colour in sea urchins (Rocha et al., 2019) and in mud crabs (Waiho et al., 2020), freshness and storage methods of several species of fishes such as sardines, cod and swordfish (e.g. Ababouch et al., 1996; Erkan and Özden, 2008; Ishimura and Bailey, 2013; Lee, 2014), and morphology in fishes (e.g. Lee, 2014; Sjöberg, 2015), mud crabs (Fazhan et al., 2020) and stalked barnacles (e.g. Lessard et al., 2003; Parada et al., 2012).

The most evident morphological variability in stalked barnacles of the genus *Pollicipes* is the variation in the peduncle. Two extreme phenotypes were described in the literature for *Pollicipes polymerus* Sowerby, 1833 and for *Pollicipes pollicipes* Gmelin, 1791 [in Gmelin, 1788–1792]. One phenotype was characterized as: stouter individuals (Chaffee and Lewis, 1988) with strong peduncles, relatively short and that attach to the substrate with a considerable basal area (Barnes and Reese, 1960) (*P. polymerus*); and barnacles with a smooth peduncle (Parada et al., 2012)

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containing a large amount of muscle (Rivera et al., 2014; Seoane-Miraz, 2015) (*P. pollicipes*). The other extreme phenotype was described as: slender individuals (Chaffee and Lewis, 1988) with a greatly elongated peduncle and a smaller attachment area of the peduncle to the substrate (Barnes and Reese, 1960) (*P. polymerus*); and barnacles with a wrinkled peduncle (Parada et al., 2012) that is thin, long and has a high water content (Seoane-Miraz, 2015) (*P. pollicipes*).

In *P. polymerus* this phenotypical variation was mainly related to wave exposure (Barnes and Reese, 1960) and to the relative position of the barnacles on the clump (Chaffee and Lewis, 1988). The barnacles with strong and short peduncles were associated with very wave-exposed locations, while those with elongated peduncle were associated with more sheltered places (Barnes and Reese, 1960). Also, slender individuals were generally located in the centre of the barnacles clumps of this species (Chaffee and Lewis, 1988). In *P. pollicipes,* this phenotypical variation was associated with differences in individual density, with locations with higher densities having thinner and longer animals (Cruz, 2000). However, this factor might be confounded with other factors such as hydrodynamics or predation (Cruz, 2000). Seoane-Miraz (2015) related the barnacles with the long peduncles as inhabiting shaded locations, as opposed to individuals in the sun which had more robust peduncles. However, these causes have not been experimentally tested.

Stalked barnacles of the genus *Pollicipes* are exploited all over the world. The most intensively exploited species is *P. pollicipes*, mainly in Portugal and Spain, but also in France (Molares and Freire, 2003; Cruz et al., 2022). The annual economic value of the *P. pollicipes* fishery in Europe is of EUR 10 million, representing 500t of landings and involving 2100 professional fishers (Aguión et al., 2022). In Spain (Galicia and Asturias) and also in the SW coast of Portugal, the economic value of this species was considered to be influenced by its quality/morphology, with elongated barnacles being associated with bad quality and having a lower commercial value (Parada et al., 2012; Rivera et al., 2016). In fact, following the fishers Local Ecological Knowledge (LEK), sections of the rocky coast have been classified according to their perceived quality/morphology of *P. pollicipes*, both in Galicia (Parada et al., 2012) and west Asturias (Rivera et al., 2014, 2016). Also, in the SW coast of Portugal, fishers have classified sections of the coast in relation to their perception on the quantity and quality of *P. pollicipes* (Carvalho et al., 2017). However, despite of the importance of the variation in quality/morphology for the fishery, we are not aware of any study that investigated whether fishers, in general, consider that there are individuals of *P. pollicipes* of different qualities, what is the definition of good and bad quality barnacles, and what is their perception on the causes of this quality variation. Furthermore, the fishers from Galicia have been concerned about the barnacles with the elongated morphology, since they have the perception that this morphology had become more abundant (Quinteiro et al., 2006). As a consequence, the fishers' guilds ("*cofradías*") had contacted the scientific community in order to obtain more information about this phenotype, namely if it was a different species.

Phenotypical variability can be related to genetic differences but can also be a result of environmental factors (Mokady et al., 2000). In a preliminary study carried out in Galicia, no evidence of genetic differences between the two *P. pollicipes* phenotypes was found (Quinteiro et al., 2006). However, by studying the expression of 5 genes in the peduncular muscle, a differential genetic expression for the two extreme *P. pollicipes* phenotypes was observed, namely an overexpression of 4 of the 5 studied genes in the peduncular muscle of the phenotype of barnacles with short and robust peduncles (Seoane-Miraz, 2015). This author suggested that the overexpression of these genes, mainly related to the muscular and cuticular integrity of the peduncle (guanine nucleotide-binding protein, chitin based cuticle attachment to epithelium, cuticular protein 11B and cuticular protein 47Ee genes), provides these barnacles the enough strength to maintain themselves attached to the rocky substrate. On the other hand, the phenotype of barnacles with long and thin peduncles showed a reduced expression of these genes (Seoane-Miraz, 2015).

Epigenetics is the study of hereditary alterations in the expression and genetic functions that cannot be explained by alterations in the DNA sequence (Richards, 2006; Bird, 2007; Bossdorf et al., 2008). DNA methylation is one of the main epigenetic mechanisms for the regulation of gene expression in eukaryotes. DNA methylation was described in mammals and other species as a mark repressing transcription, where the presence of DNA methylation at CpG-rich gene promoters, called CpG islands, would block transcription factor binding leading to gene silencing (Bird, 2002; Kaluscha et al., 2022). In vertebrates, gene bodies with substantially enriched DNA methylation are positively correlated with the level of gene transcription (gene expression), suggesting that methylation at these regions has a positive role in gene regulation (Keller et al., 2016). while there is evidence in some invertebrates that this correlation does not exist (Dixon and Matz, 2022). Among invertebrate species, methylation has been extensively studied in *Daphnia* spp. and in the Pacific oyster (*Crassostrea gigas*) where the role of methylation in gene regulation has been widely demonstrated (see Song et al., 2017; Kvist et al., 2018 references there). The role of methylation in gene expression has also been studied in other crustacean species such as the mud crab *Scylla paramamosain* (Jiang et al., 2020) and the Kuruma shrimp, *Marsupenaeus japonicus* (Wang et al., 2020).

The aims of the present study were: (1) to evaluate the perception of the fishers from Spain and Portugal about the existence of different commercial qualities of *P. pollicipes*, in order to describe the definition of two extreme qualities of barnacles (good and bad), and about the potential causes that may be determining this variation; (2) to characterize the morphometry of both extreme qualities/phenotypes of *P. pollicipes* from Spain (Galicia and Asturias) and Portugal (Alentejo) and (3) to determine the genetic and DNA methylation patterns of both extreme qualities/phenotypes of *P. pollicipes* from Spain (Galicia and Asturias) and Portugal (Alentejo).

## 2. Material and methods

The present study includes three parts: (1) a survey to professional fishers of *P. pollicipes*; (2) a morphological analysis; and (3) genetic/epigenetic analyses of this species.

### 2.1. Survey to fishers

The survey to the fishers (in Supplementary Material S.1.) was performed in Portugal and in Galicia (Spain). In Portugal, fishers from two marine protected areas, "Reserva Natural das Berlengas - RNB" and "Parque Natural do Sudoeste Alentejano e Costa Vicentina - PNSACV" (see Fig. 1), were interviewed. In Galicia, surveyed fishers belonged to 11 "*cofradías*"(Fig. 1).

Fishers were interviewed by telephone during the spring/summer of 2020. All interviews were conducted in their native language (Portuguese or Spanish). In Portugal, 52 professional fishers participated in the survey, 25 from RNB (which represents 63% of the professional fishing licences for *P. pollicipes* in RNB) and 27 from PNSACV (which represents 34% of the professional fishing licences for *P. pollicipes* in PNSACV). In Galicia, a total of 45 professional fishers from several "*cofradías*" participated in the survey: Vicedo (n = 4), A Coruña (5), Malpica (5), Laxe (2), Camelle (3), Camariñas (5), Muxia (1), O Pindo (5), Lira (5), Cangas (5), and Baiona (5). Based on official data from 2022, these 45 fishers represent 8% of the total number of *P. pollicipes* licences in Galicia, and approximately 17% of the active fishers (not all fishers with licence are active fishers, it has been estimated that only around half of the licences are actually in use, based on data from the *cofradía* of Bueu – unpublish data, Gonzalo Macho).

The fishers were questioned about (1) their opinion on the existence of stalked barnacles with different qualities, (2) their definition of both extreme qualities of stalked barnacles (good and bad) and (3) their


**Fig. 1.** Map with the sampled locations used for the morphological, genetic and epigenetic study (indicated in bold). Survey to professional fishers was run in several "*cofradías*" in Galicia, Spain – black dots (from North to South: Vicedo, A Coruña, Malpica, Laxe, Camelle, Camariñas, Muxia, O Pindo, Lira, Cangas and Baiona); and at RNB ("Reserva Natural das Berlengas") and PNSACV ("Parque Natural do Sudoeste Alentejano e Costa Vicentina") in Portugal.

perception on what is causing this variation. Questions 2 and 3 were open-ended questions and to analyse the respective answers, similar responses (in the original language) to each question were grouped into a single English term/expression representing the responses.

All interviews performed were confidential and anonymous and all the fishers gave their consent to answer to the survey and knew about the objective of the study.

### 2.2. Morphological analysis

*Pollicipes pollicipes* of the two extreme phenotypes (good quality – short and thick barnacles and bad quality – thin and long barnacles) were sampled by professional fishers or scientists in three locations: Cudillero (43°33′43.7"N 6°06′21.0"W), Asturias (hereafter called Cudillero) in July 2017; Baiona (42°07′06.5"N 8°52′00.2"W), Galicia (hereafter called Baiona) in October 2016; and Cape of Sines (37°57′46.49''N 8°53′10.04''W), Alentejo, Portugal (hereafter called Sines) in September 2017 (see Fig. 1). All samples were preserved in 99% alcohol and kept at −4°C until further analysis.

In the laboratory, 11/12 (from Baiona) and 19/20 individuals (from Sines and Cudillero) with a maximal rostro-carinal length (RC) of more than 16 mm were randomly selected from different clumps of stalked barnacles of each quality. The minimum size of 16 mm was defined to ensure that the analysed individuals were adults (minimum sexual maturity size (RC) is 12.5 mm, Cruz and Araújo 1999) and have some commercial value.

All individuals were measured with a calliper (precision 0.1 mm) to register RC and the total height (TH) in order to calculate the individual RC/TH ratio. The RC/TH ratio variability was analysed by permutational multivariate analysis of variance, PERMANOVA (Anderson, 2001) including two factors: 1) Quality (fixed factor with two levels: good quality and bad quality barnacles), 2) Location (random factor with three levels: Cudillero, Baiona and Sines). Analyses were based on Euclidean distances of untransformed data. Unrestricted permutation of raw data and Type III sums of squares were applied (Anderson et al., 2008). PERMANOVA was used to analyse univariate data due to the unbalanced design (different sample size among locations) and PERMDISP (Anderson, 2006) to test homogeneity of univariate dispersion. When appropriate, pair-wise a posteriori comparisons were conducted. The software PRIMER 6 & PERMANOVA+ (Anderson et al., 2008) was used to perform the morphological statistical analysis.

### 2.3. Genetics and epigenetics analyses

The genetic and epigenetic analyses of stalked barnacles were run considering the same samples that were used in the morphological analysis.

Genomic DNA was isolated from muscle tissue of the two qualities of barnacles of the three sampled populations (Cudillero, Baiona and Sines) using the E.Z.N.A®Mollusc DNA Kit (Omega Bio-Tek), following the manufacturers instructions. Subsequently DNA quality and concentration were checked with a Nanodrop-1000 spectrophotometer. DNA extractions were adjusted to a final concentration of 50 ng/μL and frozen until use.

Amplified fragment length polymorphism (AFLP) methodology was based on a modified version of Vos et al. (1995). For each individual, 50 ng of DNA were digested and ligated using 5 U of *Eco*RI and 3 U of *Mse*I (New England Biolabs). The obtained DNA fragments were ligated with specific adapters and subjected to two consecutive amplification rounds. A first pre-selective PCR, using *Eco*RI-A and *Mse*I-C preselective primers was followed by a second selective PCR with 6-FAM labelled *Eco*RI-ACT and *Mse*I-CAC selective primers.

A methylation sensitive amplification polymorphism (MSAP) protocol was adapted from Reyna-López et al. (1997). Briefly, each DNA sample was digested in parallel reactions with either *Eco*RI/*Hpa*II or *Eco*RI/*Msp*I endonucleases. The obtained DNA fragments were ligated with specific adapters and subjected to two consecutive amplification rounds. A first pre-selective PCR, using an *Hpa*II/*Msp*I + T and *Eco*RI + A

primer pair was followed by a second selective PCR with 6-FAM labelled *Hpa*II/*Msp*I + TAG and *Hpa*II/*Msp*I + TCC primers. A detailed protocol of the entire procedure is given in Morán and Pérez-Figueroa (2011).

PCR products, (AFLPs and MSAP) were loaded simultaneously with a GeneScan 500 ROX size standard (Thermofisher) into an ABI Prism 310 Genetic Analyzer Fragment analysis and AFLP scoring was performed using GeneMapper v.3.7 software (Thermofisher).

AFLP markers were scored as dominant binary markers 1 and 0, for fragment presence and absence, respectively and analysed with the R package MSAP (Pérez-Figueroa, 2013) using the option meth = FALSE. MSAP profiles were assessed from the resulting absence/presence matrix with the R package MSAP. Loci were categorized as non-methylated (NMT) on specimens amplifying bands for both *Hpa*II and *Msp*I digestions, internal cytosine methylated (ICM) or hemimethylated (HMM), if bands were respectively present only on either *Msp*I or *Hpa*II, or hypermethylated (HPM) whenever both bands were not present for a given specimen. The absence of genetic differentiation between phenotypes was checked. The option no.bands = "h" was used for the analysis assuming that HPA-/MSP- (no band for both isoschizomers) pattern represents full methylation of cytosines in the target (hypermethylation), ignoring the chance of genetic change in the target. Loci below a 5% error rate threshold and showing less than two occurrences of each state were systematically excluded from the analysis. Differences among phenotypes for each population were assessed with a multivariate Principal Coordinate Analysis (PCoA) and Analysis of Molecular Variance (AMOVA), using the R package MSAP (Pérez-Figueroa, 2013). To further assess whether locus-specific methylation is dependent on phenotype, Fisher exact tests were used to detect candidate loci among the Methylation-Susceptible Loci (MSL). After statistical adjustment of the resulting *p-values* according to Benjamini and Hochberg false discovery rate (FDR), only loci showing $p<0.05$ were selected (Benjamini and Hochberg, 1995).

## 3. Results

### 3.1. Survey to fishers

Most fishers from Galicia and Portugal, considered the existence of stalked barnacles with different qualities (96% and 100%, respectively).

When asked about a definition for good quality barnacles, 24% of the Galician fishers (11 of the 45 surveys performed) and 23% of the Portuguese fishers (12 of the 52 surveys performed), were not able to define them, consequently only answers of fishers that mentioned a definition were considered on the following analysis. The two terms that were more mentioned to define good quality barnacles were related to the morphometric characteristics “thick” (91% in Galicia and 63% in Portugal) and “short” (56% in Galicia and 50% in Portugal) (Fig. 2). Most of the Galician fishers also stated the term “red coloured” (53%). Both Galician and Portuguese fishers also mentioned the term “hard” as relevant (44% in Galicia and 38% in Portugal). Other expressions less used (≤15%) to define good quality barnacles were “dark coloured”, “yellow capitulum”, “heavier”, “tastier”, “less water”, “preserve longer time” and “sweeter” (Fig. 2).

Regarding the definition of bad quality barnacles, 22% of the Galician fishers (10 of the 45 surveys performed) and 31% of the Portuguese fishers (16 of the 52 surveys performed), were not able to define them, consequently only the answers of fishers that mentioned a definition were considered on the following analysis. The terms that were most mentioned by the fishers of Galicia to define bad quality barnacles were related to the morphometric characteristics “long” (86%) and “thin” (51%) (Fig. 3). The Portuguese fishers used a wider variety of terms to define bad quality barnacles, including the morphological characteristics “thin” (36%), “soft” (31%) and “long” (25%), but also the organoleptic term “watery” (39%), which was the one most frequently mentioned by these fishers (Fig. 3). Galician fishers also used the morphological term “soft” (40%) and the organoleptic term “watery”

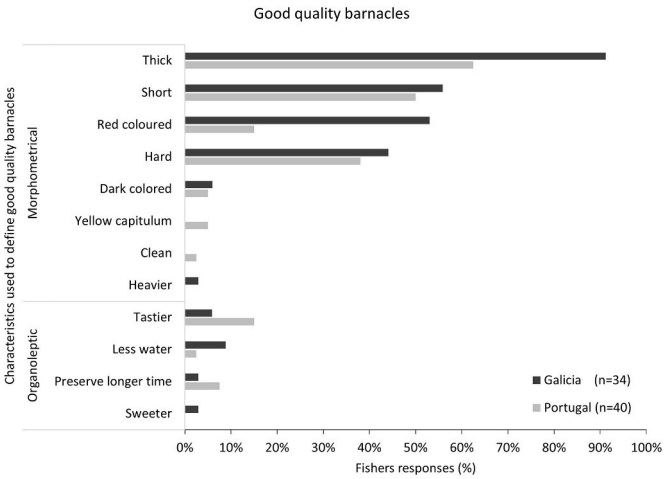


**Fig. 2.** Characteristics mentioned by Galician and Portuguese fisher to define good quality barnacles. The terms and expressions were grouped in one English term that better described the characteristic. The terms and expressions were mentioned in the native languages presented in supplementary material Table S1. Only the answers of fishers that mentioned a definition were considered (34 fishers in Galicia and 40 in Portugal).

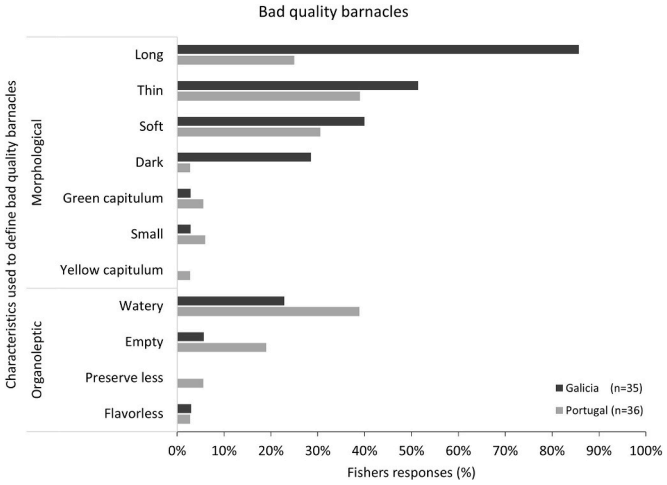


**Fig. 3.** Characteristics mentioned by Galician and Portuguese fishers to define bad quality barnacles. The terms and expressions were grouped in one English term that better described the characteristic. Terms and expressions mentioned in the native languages presented in supplementary material Table S2. Only the answers of fishers that mentioned a definition were considered (35 fishers in Galicia and 36 in Portugal).

(23%). Both Galician and Portuguese fishers also mentioned the terms (<30%): “dark”, “green capitulum”, “small”, “yellow capitulum” “empty”, “preserve less” and “flavourless” to define bad quality barnacles (Fig. 3).

When fishers were asked about their opinion on the causes of the variation in quality of the stalked barnacles, 31% of the Galician fishers (14 of the 45 surveys performed) and 8% of the Portuguese fishers (4 of the 52 surveys performed) were not able to explain them. Those who answered pointed out several causes, that differed slightly between the fishers of Portugal and Galicia. Almost the totality of the fishers from Portugal considered that the variation in the quality of stalked barnacles was related to some characteristics of the rock (96%, Fig. 4), being the hydrodynamics the second more referred cause (31%, Fig. 4). However, in Galicia there were no causes invoked by the large majority of the fishers, although the two most referred causes were the same: hydrodynamics (45%) and characteristics of the rock (46%) (Fig. 4). In

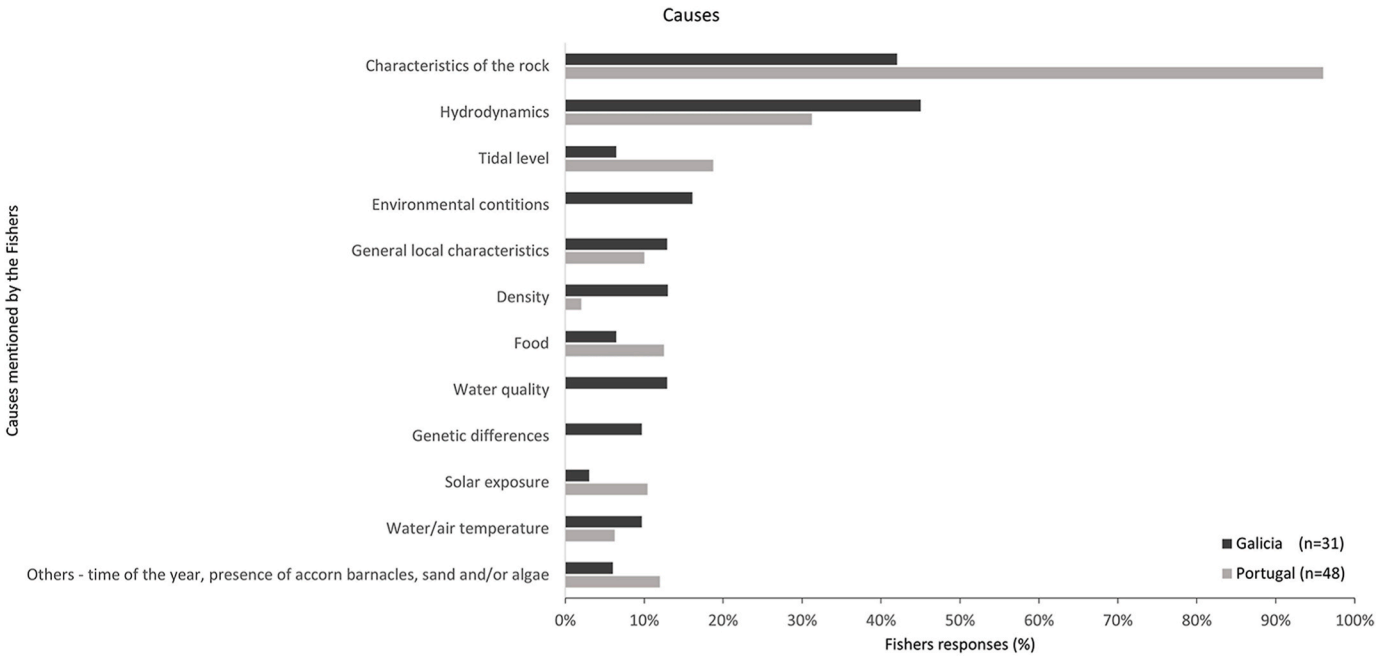


**Fig. 4.** Causes mentioned by Galician and Portuguese fishers to explain the morphological variation between good and bad quality barnacles. The identified causes were grouped in one English term/expression that better described the cause. causes mentioned in the native languages presented in supplementary material Table S3. Only the answers of fishers that mentioned a cause were considered (31 fishers in Galicia and 48 in Portugal).

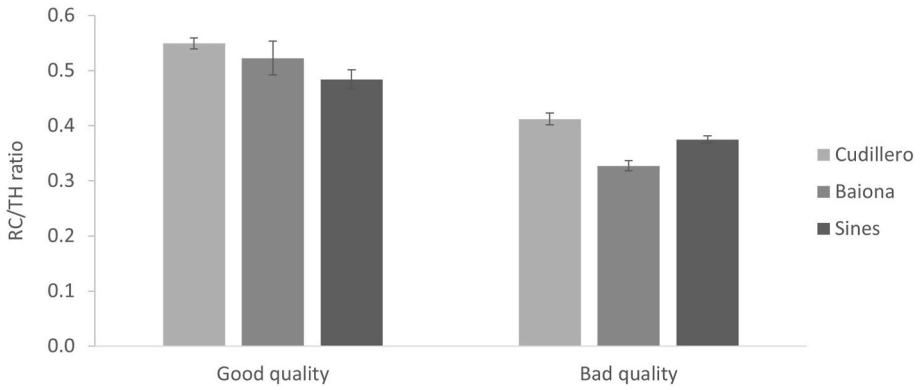


**Fig. 5.** *Pollicipes pollicipes* RC/TH ratio (mean ± SE; n = 19/20 for Cudillero and Sines and n = 12/11 for Baiona) for the good and bad quality barnacles from the three sampled locations (Cudillero, Baiona and Sines).

addition to the above referred causes, the fishers from Portugal and Galicia also referred as causes for this quality variation (<20%): tidal level, general local characteristics, density of barnacles, food, solar exposure, and water/air temperature (Fig. 4). However, another regional difference was the greater number of causes cited by fishers in Galicia compared to Portugal (Fig. 4). The fishers from Galicia added the environmental conditions, the water quality, and genetic differences to the list of possible causes of this quality variation (Fig. 4).

### 3.2. Morphological analysis

The average RC/TH ratio of good quality barnacles and bad quality

**Table 1**
PERMANOVA main test and pair-wise test on the RC/TH ratio in relation to "Quality" (Qu) and "Location" (Lo). Analyses were based on Euclidian distance of untransformed data. p-Values were obtained using 9999 random permutations. n = 12/11 for Baiona and n = 20/19 for Cudillero and Sines. Significant effects are indicated in bold ($p < 0.05$). PERMDISP test: F = 6.76 (Quality; $p < 0.05$).

| Source of variation | d.f. | MS | Pseudo-F | p |
|---|---|---|---|---|
| Qu | 1 | 0.515 | 35.40 | **0.039** |
| Lo | 2 | 0.034 | 9.52 | **0.000** |
| Qu x Lo | 2 | 0.013 | 3.81 | **0.029** |
| Res | 95 | 0.035 | | |
| *Pair-wise tests* | | | | |
| Qu x Lo | *Qu* | | | |
| | Good quality > Bad quality ($p<0.01$ in Cudillero, Baiona and Sines) | | | |

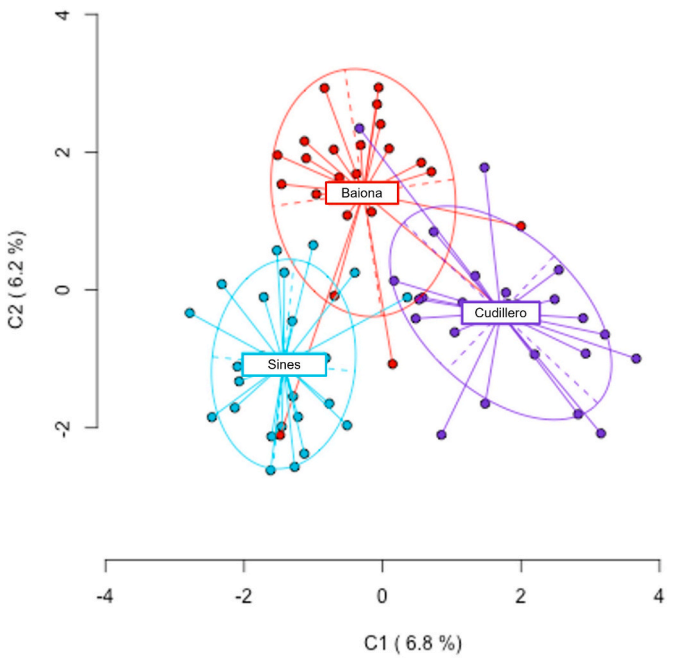


**Fig. 6.** Results of the Principal Coordinates Analysis (PCoA) with respect to the genetic differences detected among populations. The first two coordinates (C1 and C2) are displayed indicating the explained variance percentages in brackets. Scores represent individual samples. Labels indicate the centroids of each population. Ellipses represent the dispersion associated with each value with the long axis showing the direction of the maximum dispersion, while the short axis depicts the direction of minimum dispersion. In purple, barnacles from Cudillero (Asturias, Spain); in red, barnacles from Baiona (Galicia, Spain); in blue, barnacles from Sines (Alentejo, Portugal). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

barnacles ranged between 0.48 (Sines, n=20) and 0.55 (Cudillero, n=19) (Fig. 5), and between 0.33 (Baiona, n=11) and 0.41 (Cudillero, n=20) (Fig. 5), respectively.

The morphological analysis on the RC/TH ratio variability revealed a significant interaction of the factor quality with the factor location, but the RC/TH ratio was significantly higher for good quality barnacles when compared with low quality barnacles in all locations (Table 1). Homogenized dispersions were not observed among qualities (PERMDISP<0.05). A lower RC/TH ratio indicates longer barnacles.

### 3.3. Genetic and epigenetic analysis

A total of 72 individuals were analysed for AFLP, 31 from Cudillero (13 good and 18 bad quality), 26 from Baiona (13 good and 13 bad quality) and 15 from Sines (7 good and 8 bad quality), resulting in 229 loci. 99% of the loci were polymorphic. First, bad and good quality barnacles for each population were tested for differences. As no differences were found between phenotypes for each population, the samples were grouped for a comparison among populations. AMOVA showed significant differentiation among populations ($F_{ST}$ = 0.046, $p$<0.001). The principal coordinates analysis (PCoA, Fig. 6) clearly shows the among population differentiation. The first two principal coordinates (C1 and C2), account for 6.8% and 6.2%, respectively, of the total variance.

MSAP analysis detected a total of 222 loci. Since there are remarkable genetic differences among the three analysed populations, the differences in methylation between phenotypes were evaluated for each population although the results of presence and absence of bands were obtained globally, and were run simultaneously in GeneMapper. The number of Methylation-Susceptible Loci (MSL) in each population ranges from 43% to 61%. The relative frequency of the different states of methylation for the two phenotypes in each population is given in Table 2.

It can be observed that the frequency of unmethylated, hemimethylated, methylated states in the internal cytosine and full methylation are quite similar between phenotypes and populations. The absence of epigenetic variation between phenotypes was confirmed by both PCoA and the AMOVA. Genomewide methylation patterns were not statistically significant between phenotypes for each population (AMOVA, Table 2). The PCoA of the three populations with regard to the phenotypes is depicted in Fig. 7. The first two principal coordinates (C1 and C2), account for 15.2 to 12.2% and 12.2 to 8.4% respectively of the total variance. However, the clusters of each phenotype are overlapping and show no difference between phenotypes for any of the populations.

The single-locus analysis by means of Fisher exact test revealed up to 1 loci, 0 loci and 3 loci displaying significant ($p$<0.05) methylation differences between phenotypes in Asturias, Galicia and Portugal populations respectively. However, none of these loci remained significant ($p$<0.05) after FDR adjustment. Therefore, by means of the MSAP technique, no direct evidence of genome-wide methylation changes was detected between phenotypes.

## 4. Discussion

The survey on the professional fishers perception about the existence of different qualities of *Pollicipes pollicipes* revealed that the large majority of the fishers considered that there are differences in the quality of the harvested stalked barnacles. Most of the fishers from Galicia and Portugal had the same perception about the quality of the barnacles and generally use similar terms to define them, although there are slight regional differences. In general, both Galician and Portuguese fishers

**Table 2**
Frequency of polymorphic Methylation-Susceptible Loci (MSL). AMOVA results between phenotypes for each population. Frequency (%) of the different states of methylation for the sampled populations. Good = good quality morphotype, Bad = bad quality morphotype.

| State of Methylation | Cudillero | | Baiona | | Sines | |
|---|---|---|---|---|---|---|
| | Good | Bad | Good | Bad | Good | Bad |
| % polymorphic MSL[a] | 48% | | 61% | | 43% | |
| AMOVA | $F_{ST}$ = 0.001 ($p$ = 0.4158) | | $F_{ST}$ = 0.020 ($p$ = 0.1202) | | $F_{ST}$ = -7.744e$^{-05}$($p$ = 0.4577) | |
| HPA/MSP+ (unmethylated) | 14.0% | 13.3% | 14.8% | 17.3% | 14.9% | 13.5% |
| HPA/MSP- (hemimethylated) | 11.5% | 10.6% | 11.8% | 12.3% | 11.3% | 9.8% |
| HPA/MSP+ (internal cytosine methylated) | 12.1% | 11.0% | 18.0% | 16.0% | 10.7% | 11.4% |
| HPA-/MSP- (full methylation) | 62.3% | 64.9% | 55.1% | 54.2% | 62.9% | 65.2% |

[a] Methylation-susceptible loci.

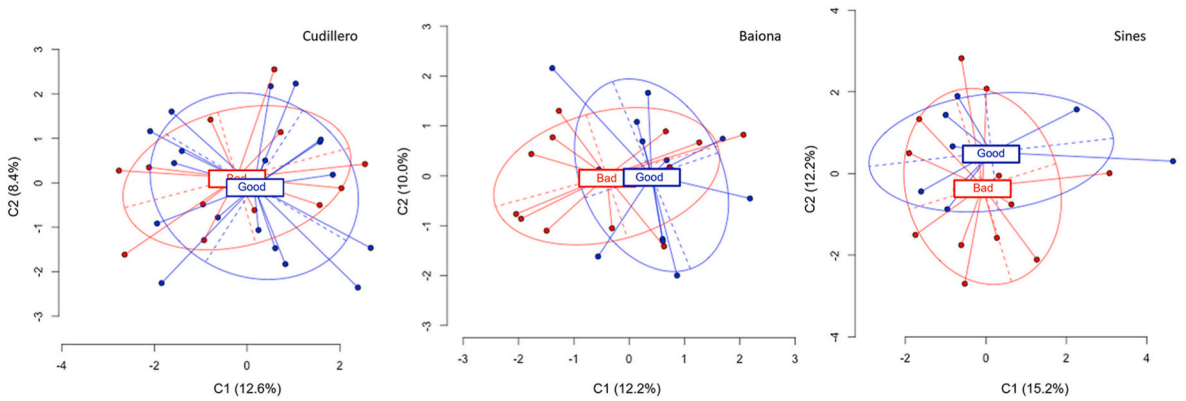


**Fig. 7.** Results of the Principal Coordinates Analysis (PCoA) with respect to the epigenetic differences detected between phenotypes in populations from Cudillero (Asturias, Spain), Baiona (Galicia, Spain) and Sines (Alentejo, Portugal). The first two coordinates (C1 and C2) are displayed indicating the explained variance percentages in brackets. Scores represent individual samples. Labels indicate the centroids of each morphotype. Ellipses represent the dispersion associated with each value with the long axis showing the direction of the maximum dispersion, while the short axis depicts the direction of minimum dispersion. In blue good quality barnacles and in red bad quality barnacles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

defined quality of barnacles privileging morphological terms instead of organoleptic characteristics such as taste.

The terms most referred in the present study by the fishers to define good quality barnacles in Galicia and Portugal were thick and short, but also hard in both regions and red coloured in Galicia. The same or similar terms were also associated with good quality of barnacles in other studies of *Pollicipes* (Barnes and Reese, 1960; Chaffee and Lewis, 1988; Lessard et al., 2003; Parada et al., 2012 Rivera et al., 2014), which indicates a consensus in this description.

Regarding the definition of bad quality barnacles, the terms that were more mentioned by the professional fishers of the present study were slightly different between regions. Most of the fishers in Galicia mentioned the terms long and thin, while there was no term referred in Portugal by the majority of fishers. In Portugal, the terms more used to define bad quality barnacles were "watery" and "thin" by 39% of the fishers, while "long" was mentioned by 25% of the fishers. The fact that "watery" was a relevant term to define bad quality barnacles in Portugal might be related to the fact that in Portugal the fishers call bad quality barnacles as "*percebe mijão*" ("pissing" barnacles), due to the fact that these barnacles have a high content in water and can squirt water when caught or eaten. In Galicia, bad quality barnacles are called "*picholón*" ("big dick") related to the most common term used, "long". Overall, the terms most referred by the fishers of Galicia and Portugal for bad quality barnacles or similar terms were also referenced in the literature regarding *Pollicipes* spp. quality (Barnes and Reese, 1960; Chaffee and Lewis, 1988; Parada et al., 2012).

In relation to what is causing the variation in quality in stalked barnacles, the fishers from Galicia and Portugal referred slightly different causes. Almost the totality of the fishers from Portugal agreed that the cause responsible for the quality variation was related to some characteristic of the rock, while the second most referred cause was the hydrodynamics. The answers given by the fishers of Galicia were more diverse than those from Portuguese fishers. The two most commonly identified causes were also hydrodynamics and characteristics of the rock, but there was a greater dispersion of answers across a higher number of potential causes. This pattern might indicate that the fishers of Galicia have been thinking more about what is causing this variation of quality. In fact, fishers from Galicia have considered that the morphology of *P. pollicipes* with an elongated stalk was becoming more abundant on the coast (Quinteiro et al., 2006), which might be a major concern for these fishers due to its lower price in the market (Parada et al., 2012). On the other hand, in Portugal, based on surveys conducted with fishers in RNB, the majority of fishers reported that there had been no change in the quality of *P. pollicipes* in the last 5 years (in relation to 2013 and 2018) (Sousa et al., 2020). Assuming that the size of the barnacles can also be an indication of quality, also in PNSACV, based on surveys carried out in 2013 (Cruz et al., 2015) and in 2016 (Carvalho et al., 2017), the situation seems similar, as the majority of fishers in both studies indicate that there have been no changes in the size of *P. pollicipes* in the last 5 years.

Several actions were taken in Galicia in the 2000s to deal with the issue of the elongated barnacles. Initially the biologists of the "*cofradías*" (see Macho et al., 2013) did several trials removing patches of elongated barnacles in the rocks to check if they were again recolonized by the same elongated barnacles or not. Results from these experiences were not conclusive (Alberto Garazo, Biologist from the cofradia of Bueu, personal communication). Due to the pressure of the "*cofradías*", the Fisheries administration in Galicia granted two projects, one to do a genetic study comparing the two morphologies and another one to look for processed products using elongated barnacles. As stated above, the first project found that both morphotypes were genetically the same species (Quinteiro et al., 2006). The second project aimed to produce processed products based on the bad quality barnacles and led to the creation of a company *Mar de Silleiro* formed by fishers from the "*cofradías*" of Baiona, A Guarda and Bueu, which was operational for 10 years. Nowadays, the elongated morphology is being exploited and commercialized, despite its lower commercial value (Miguel Verea, stalked barnacle harvester from Baiona, personal communication).

The identification that variation in hydrodynamics and in characteristics of the rock may be related to morphological/quality changes had also been mentioned in previous studies with *P. polymerus*: relatively short individuals, with strong peduncles, that attach with a considerable basal area, associated to locations with a strong wave exposure, while more elongated barnacles with a relatively small area of attach were related to more sheltered locations, and also with the presence of sand (Barnes and Reese, 1960); local conditions such as wave exposure to explain *P. polymerus* variation in body shape and size (Lessard et al., 2003). In contrary, in the Gaztelugatxe marine reserve, north of Spain, no significant relation was found between the total length of *P. pollicipes* and the degree of wave exposure (Borja et al., 2006), which can indicate that the occurrence of both phenotypes of *P. pollicipes* are not related exclusively with the degree of wave exposure. In Asturias, the quality of *P. pollicipes* was associated not only with wave exposure, but also with the configuration of the shore and the distance from the coast, with good quality barnacles associated with more exposed and convex areas that distance from the coast approximately 700m or more (Rivera et al., 2016). Other potential causes invoked to explain the morphological variation in these two species of the genus *Pollicipes* were: the position of

*P. polymerus* in the clump (Chaffee and Lewis, 1988); the density of *P. pollicipes* of the clump (Cruz, 2000); and the sun exposure (Seoane--Miraz, 2015). The overall conclusion is that the causes of this phenotypical variation in *Pollicipes* are still hard to understand, and more investigation is needed.

The RC/TH ratio expresses the relation between the height and width of a stalked barnacle. If the barnacle is thicker and shorter the RC/TH ratio is higher, and if the barnacle is longer and thinner the RC/TH ratio obtained is lower. As expected, the morphometric analysis on the RC/TH ratio variability on *P. pollicipes* of the two extreme qualities of the three sampled locations (Cudillero, Baiona and Sines) revealed that RC/TH ratio was significantly higher for good quality barnacle when compared with low quality barnacles, although both morphotypes differ in dispersion of RC/TH values. In the past, the commercial quality of *P. pollicipes* was defined by the relationship between capitulum height, capitulum width and the weight of the barnacle (Molares et al., 1987). Based on Ecological Fishers Knowledge, Lessard et al. (2003) considered that the configuration of the stalk is the most important attribute to classify *P. polymerus* quality, even more important than its weight. More recent studies used the ratio between the diameter of the capitulum base and the total height of the individuals to determine *P. pollicipes* quality (Parada et al., 2012). Based in the present study, we propose that the RC/TH ratio can be used to describe the morphological variation between the two extreme phenotypes of *P. pollicipes*: RC/TH ratio $>0.4$ – good quality barnacles, RC/TH ratio $<0.4$ – bad quality barnacles.

Regarding the genetic analysis, AFLPs were analysed, and although no differences were detected between both phenotypes, differences among the three analysed populations (Sines, Baiona and Cudillero) were detected. This is the first time that AFLPs were used to estimate the differences among populations of the genus *Pollicipes*. These results are partially consistent with data obtained with other genetic markers such as microsatellites where genetic differentiation among populations of juveniles of the same three geographical areas was detected (Parrondo et al., 2022). Contrarily, in the same study no spatial genetic differentiation of adults was found. Concordant or contradictory results based on the use of these two types of molecular markers have already been found in previous studies (e.g. Mariette et al., 2001; Gaudeul et al., 2004; Alacs et al., 2011), although comparisons of AFLPs and microsatellites are more common in studies to detect diversity in plants (Sønstebø et al., 2007).

The results on the MSAP analysis revealed that, although some methylation differences were found, no methylation differences were associated with the two extreme phenotypes studied in the three populations. In fact, although some significant single locus methylation differences were detected in two of the populations (Asturias and Portugal), these differences were not detected after multiple test correction. These results contradict the preliminary results obtained by Seoane-Miraz (2015). In recent studies, that used a similar methodology to the present study, with the Brown mussel *Perna perna* (Watson el al. 2018) and two species of reef corals (*Acropora cervicornis* and *A. palmata*) (Hackerott et al., 2023), it was found a relationship between phenotypic plasticity and variation in epigenetic DNA methylation. As the MSAP method is general, we cannot reject that other more specific DNA methylation approaches (see extensive review for marine organisms by Eirin-Lopez and Putnam, 2019) could detect differential gene expression mediated by methylation.

Considering the lower commercial value that seems to be associated with the bad quality barnacles (Parada et al., 2012 in Galicia; Cruz, 2000 and unpublished observations in Portugal), and the perception of Galician fishers that this quality of barnacles seems to be increasing in relation to the better quality ones, we recommend that the assessment of the quality/morphology of barnacles should be included as part of the monitoring of the state of this resource. On the other hand, in a previous study, although it was only carried out at one site (a big crevice in Cape Sardão, Alentejo, Portugal), it was observed that there was less sexual activity and less intense recruitment associated with more elongated barnacles (Cruz and Araújo, 1999; Cruz, 2000). Thus, in addition to the potential socio-economic impacts associated with a variation in quality, there may also be a potential variation in fundamental biological processes that should be better investigated in relation to the quality/morphology of the barnacles.

## 5. Conclusion

Professional fishers both from Portugal and Spain consider that there are *P. pollicipes* with different qualities, defining good quality barnacles as thick and short, and bad quality barnacles as long, thin, watery and soft. The fishers answers validated the definitions and terms that were used in previous studies to define extreme phenotypes in *Pollicipes* species.

Regarding the causes of this morphological/quality variation of *P. pollicipes*, the large majority of the professional fishers considered that this variation can be caused by characteristics of the rock and also by hydrodynamics.

Based on the results of the present study, we propose the use of RC/TH ratio to distinguish the two extreme phenotypes of *P. pollicipes*, in the Iberia Peninsula, where values $> 0.4$ indicates good quality barnacles and values $< 0.4$ indicates bad quality barnacles.

Differences between both phenotypes were detected when using the RC/TH ratio, but no epigenetic differences were found, using the MSAP methodology. Further studies are needed using other DNA methylation approaches and to investigate the causes of this variation.

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## CRediT authorship contribution statement

**A. Sousa:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **P. Morán:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **J.L. Acuña:** Writing – review & editing, Resources, Funding acquisition. **E. Vázquez:** Writing – review & editing, Funding acquisition. **T. Cruz:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Data curation, Conceptualization. **G. Macho:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Data curation, Conceptualization.

## 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.

## Data availability

Data will be made available on request.

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