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Deliverable 7.4

Guidelines to perform a marker-assisted selective breeding programme to produce marteiliosis-resistant cockle strains

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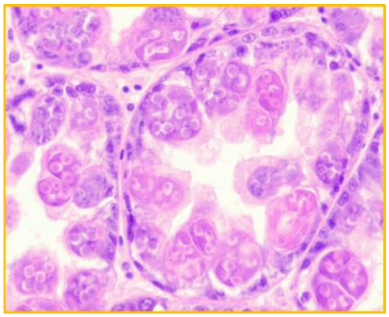

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Contents

1. Executive Summary

Marteiliosis, a disease due to infection with the protistan *Marteilia cochillia*, caused cockle fishery collapse in the southern rias of Galicia (NW Spain) and is considered the most serious pathological threat of Galician cockles. Considering the devastating effects of marteiliosis, the project COCKLES included the objective of stating the bases to perform a selective breeding programme to produce marteiliosis-resistant cockles. Procedures to culture cockles, both indoor (hatchery) and outdoor, have been settled in the action 7.1 of this project, while molecular markers of marteiliosis-resistance have been identified in the actions 7.2 and 7.3. This new knowledge has been integrated to devise the guidelines provided in this report to perform a marker-assisted selective breeding programme to produce marteiliosis-resistant cockle strains. These guidelines could serve as reference to plan selective breeding programmes to increase resistance to other diseases or to improve other traits.

A key issue determining the success of any genetic improvement programme is the choice of appropriate breeders. Results from actions 7.2 and 3.4 have shown that the cockle population of the inner area of Ría de Arousa has increased resistance against marteiliosis through natural selection. Therefore, a highly favourable position to start the selective breeding programme will be collecting cockles from the inner area of the Ría de Arousa and then farther choosing individuals among them to be used as breeders, using the genetic markers of marteiliosis-resistance as one of the main criteria. The proposed minimum number of breeders with which start the selective breeding programme is 400. The process to choose those breeders involves genotyping as many cockles from the inner side of the Ría de Arousa as possible, regarding 45 single nucleotide polymorphisms (SNPs) markers of marteiliosis-resistance, determining their sex, and selecting 200 females and 200 males with the most favourable genotypes. The procedures to take a biopsy from each cockle and how to use it to determine both sex and genotype are provided in the report. The procedures of broodstock conditioning, collecting larvae and culturing them in hatchery facilities as well as those of outdoor rearing are thoroughly described in the COCKLES' project reports by Joaquim *et al*. (2021) and Fernández *et al*. (2021). The cockle seed obtained through those processes may be

used in areas affected by marteiliosis either for ongrowing with commercial purposes or for restoring exhausted beds, while a batch of those seed (from 1,000 to 2,000 cockles) will be kept as the source of the breeders of the next generation of selection.

Surveys comparing the performance of the cockles of each selected generation with that of non-selected cockles deriving from the inner area of the Ría de Arousa and that of cockles deriving from a naïve population should be carried out to evaluate the effectiveness of the selective breeding programme and, thus, to decide whether keeping on, modifying or finishing the selective breeding programme. The genotype analysis that has to be performed to choose the breeders of each generation, focused on the 45 SNPs markers of marteiliosis-resistance, should be extended to cover other anonymous markers informing on the genetic variability in each generation, for which a procedure is provided. In case loss of genetic variability is detected, corrective measures should be adopted.

2. Introduction

Common cockle *Cerastoderma edule* populations are exposed to multiple stressors, some of them causing cockle mass mortalities. In the context of the project COCKLES, parasites and diseases affecting cockles through the Atlantic Area have been catalogued, identifying those most threatening among them (de Montaudouin *et al*., 2021). Particular attention deserves marteiliosis, a disease due to infection with the protistan *Marteilia cochillia* (Fig. 1), which caused unprecedented huge mortality of cockles leading to cockle fishery collapse in Ría de Arousa (Galicia, NW Spain) in 2012 (Villalba *et al*., 2014) and so did it in the rias of Pontevedra and Vigo in 2013 and 2014, respectively (Iglesias et al., 2015). Nowadays, marteiliosis is considered the most serious pathological threat of Galician cockles. Considering the devastating effects of marteiliosis, the project COCKLES devoted particular attention to this cockle disease, including to devise procedures to minimise the effects of marteiliosis. An important preliminary decision was to determine the strategic framework in which formulating those procedures because, compared to vertebrates, the types of measures to fight bivalve molluscan epizootics is much more limited, particularly those epizootics affecting wild populations or outdoor cultured individuals, as explained next. (1) Chemotherapy is not a current option because adding therapeutants in the open aquatic environment would be nonsense action due to the uncertainty of ingestion of the therapeutant by the affected or susceptible molluscs and its ecological consequences; inoculation or bath treatment of molluscs with therapeutants would be theoretically feasible, in case effective therapeutants were available, but of highly dubious cost-effectiveness. (2) Nowadays, immunisation of molluscs against specific pathogens is not feasible; vaccination, in the sense used for vertebrates, is useless for molluscs because they lack adaptive immune system (Fernández Robledo *et al*., 2019). Immune priming in molluscs, that is to say activation of the mollusc innate immune system to strengthen the immune response against pathogens, is feasible (Green & Motagnani, 2013; Lafont *et al*., 2017; Yang *et al*., 2020) but it remains at experimental level, lacking farther development, without having achieved industrial implementation thus far. (3) Eradication of marteiliosis from endemic areas does not seem feasible, as it has been proved for other protozoan-caused diseases of bivalve molluscs, such as bonamiosis of the flat oyster (Grizel *et al*., 1987; van Banning, 1991; Lynch *et al*., 2007).

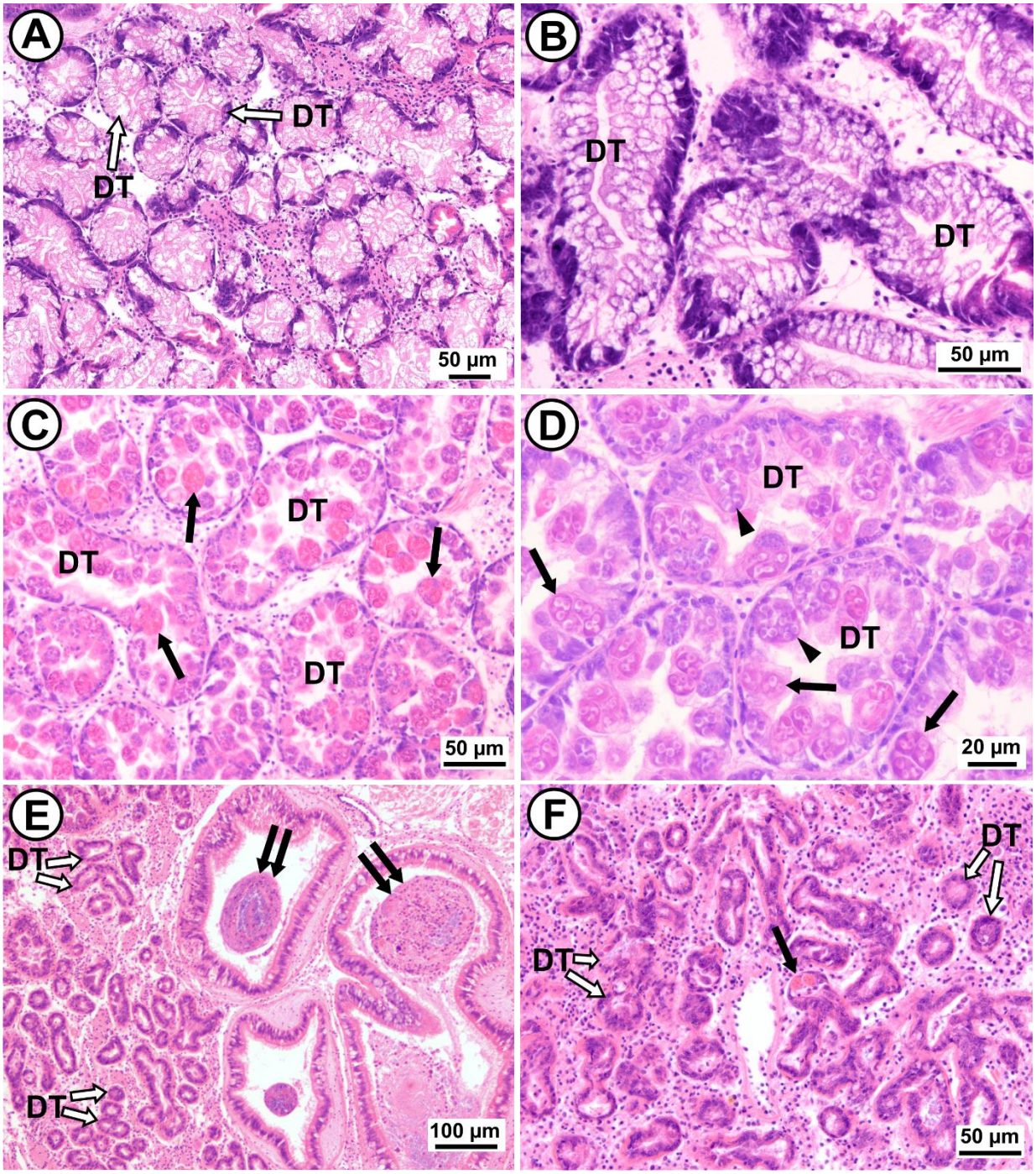

Fig. 1. Micrographs of histological sections (stained with Harris' haematoxylin and eosin) through the digestive gland of cockles *Cerastoderma edule*. Micrographs A and B correspond to a healthy cockle, while micrographs C-F to cockles infected with *Marteilia cochillia*. A and B: Digestive gland tubules (DT) showing normal, healthy appearance, with tall epithelium, where food digestion and absorption takes place. C and D: Digestive gland tubules (DT) of a cockle at advanced infection stage, with their epithelium heavily invaded by intermediate (arrowheads) and advanced (arrows) sporulation stages of *M. cochillia*, which heavily hamper food digestion and absorption. E and F: Digestive gland of a cockle at terminal infection stage, when most parasites have been released to digestive lumina and appear accumulated in large masses in the intestinal lumen (double arrows); the digestive gland tubules (DT) appear largely shrivelled, with metaplastic flat epithelium, thus nonfunctional, harbouring some remaining parasites (arrow).

(4) Increasing resilience to pathogens through selective breeding programmes has been shown as the most efficient way to minimise the effects of bivalve mollusc epizootics thus far (Ford & Haskin, 1987; Beattie *et al*., 1988, Ragone Calvo *et al*., 2003; Dove *et al*., 2013; Dégremont *et al*., 2015a, 2015b, 2015c, 2019, 2020; Smits *et al*., 2020). Consistently, mollusc industry and Administration frequently opted for this strategy, selective breeding for resistance, to fight a number of bivalve mollusc epizootics *(*Frank-Lawale *et al*., 2014; Lynch *et al*., 2014; Proestou *et al*., 2016; Sunila *et al*., 2016; Casas *et al*., 2017; Lapègue & Renault, 2018; Cruz *et al*., 2020). According to those four premises, the strategy to fight marteiliosis assumed in the project COCKLES was to increase cockle resistance to marteiliosis through selective breeding.

A key issue in the programmes aiming to increase disease-resistance in molluscs, as in any genetic improvement programme, is the criterion to choose the breeders. The pioneer programmes developed to increase disease-resistance in molluscs, actually to fight oyster diseases, used the intuitive criterion of survival after long exposure to disease for breeder choosing (Ford & Haskin, 1987; Ragone Calvo *et al*., 2003; Dove *et al*., 2013; Lynch *et al*., 2014). As more sophisticated molecular tools have been available, much research effort has been focused on the identification of molecular markers of disease resistance, through genomic (He *et al*. 2012; Meistertzheim *et al*., 2014; Nikapitiya *et al*., 2014; Nie *et al*., 2015; Wang *et al*., 2016; Gutiérrez *et al*., 2018; La Peyre *et al*. 2019; Vera *et al*., 2019; de Lorgeril *et al*., 2020; Farhat *et al*., 2020; Gutiérrez *et al*., 2020; Hasanuzzaman *et al*., 2020; Proestou & Sullivan, 2020) and proteomic approaches (Simonian *et al*., 2009; Fernández Boo *et al*., 2016; de la Ballina *et al*., 2018; Vaibhav *et al*., 2018; Smits *et al*., 2020b; Leprêtre *et al*., 2021). The recent development and increasing affordability of high-throughput sequencing technologies have facilitated the application of genomic tools in breeding programmes of aquatic species (Zenger *et al*., 2019; Nascimento-Schulze *et al*., 2021) and molluscs should not be an exception. Accordingly, research to identify molecular markers of resistance to marteiliosis has been accomplished through proteomic (Cao *et al*. 2021) and transcriptomic-population genomic approaches (Pardo *et al*., 2021) within the project COCKLES. The new knowledge on procedures to culture cockles, both indoor (hatchery) and outdoor (Joaquim *et al*., 2021; Fernández *et al*., 2021), and on genetic markers of marteiliosis-resistance (Pardo *et al*., 2021) have been

integrated to devise guidelines to perform a marker-assisted selective breeding programme to produce marteiliosis-resistant cockle strains, which are provided in the following sections of this report. These guidelines could serve as reference to plan selective breeding programmes to increase resistance to other diseases or to improve other traits.

These guidelines have to be considered as a first proposal that has been produced close to the end of the project COCKLES, taking advantage of the knowledge acquired along this project, but without testing every stage due to lack of time. Some stages of this proposal can be improved or optimise by farther testing. The proteins candidate markers of marteiliosis resistance identified through the proteomic approach (Cao *et al*., 2021) are not included in these guidelines because the procedure to quantify those proteins is too arduous; nevertheless, those (some or all) proteins could be integrated in the procedure as soon as alternative, quicker procedures to quantify them are available. One limiting factor is the lack of an effective procedure to induce cockle spawning (release of gametes) (Joaquim *et al*., 2021; Fernández *et al*., 2021), which discourages from attempting family designs and imposes the uncertainty of the number cockles that will effectively spawn.

3. Choice of breeders

A key issue determining the success of any genetic improvement programme is the choice of appropriate breeders to produce descendance as the base population for ensuring response to selection across generations in the medium-long term. Results from actions 7.2 and 3.4 have shown that the cockle population of the inner area of Ría de Arousa has increased resistance against marteiliosis through natural selection due to exposure to the disease since 2012 (Villalba *et al*., 2021a; Villalba *et al*., 2021b). Therefore, a highly favourable position to start the selective breeding programme will be collecting cockles from the inner area of the Ría de Arousa and then farther choosing individuals among them to be used as breeders, using the genetic markers of marteiliosis-resistance as one of the main criteria (e.g., genotyping the collected cockles and taking those cockles with the highest endowment of resistance-markers), but always considering other essential factors such as controlling inbreeding and maintaining genetic diversity in the broodstock.

3.1. Number of cockles, handling and tissue sampling for genotyping

Ideally, the higher the number of cockles collected from the inner area of the Ría de Arousa the better. However, two factors have to be considered to state the number of cockles to be collected: (1) the number of cockles to be genotyped will be limited by the genotyping costs; (2) a minimum of breeders is required to avoid inbreeding depression and maintain genetic diversity, as outlined before. Considering the uncertainty of the number of cockles that will spawn, a reasonable minimum number of breeders would be 400, distributed in 20 batches of ten females and ten males per batch, in order to set each batch in a different tank for final conditioning and collecting larvae. Those (minimum) 400 breeders (200 females and 200 males) have to be chosen as those with the most favourable genotypes among the collected cockles. The size of the collected cockles should be longer than 25 mm to allow easy handling. They have to be carried to the laboratory (or appropriate working facilities) minimising thermal and mechanical stress. In the laboratory, cockles should be kept in tanks with open seawater circuit. In case the working facilities were located in a marteiliosis-free area (which would make difficult the process), special measures should be adopted, such as using quarantine facilities, to avoid that viable cells of *Marteilia cochillia* (the aetiological agent of marteiliosis) reach the natural environment. Every collected cockle must be marked for individual identification, e.g. writing a number with indelible marker. A tissue biopsy has to be taken from each cockle for genotyping, for which tiny (ca. 2 mm side square) pieces of easily accessible organs (e.g., gills, syphon) or even haemolymph withdrawn from the adductor muscle with a needle coupled to a syringe (through a whole made, with a carpenter lime, in the shell margin close to the muscle) could serve. Nevertheless, a more convenient procedure involves taking a biopsy of gonad with a syringe through a hole made in the middle of the shell with a small drill. A first drop of the biopsy can be used to determine the sex by examining it with light microscopy (ovocytes and spermatozoa are easily distinguished); the remaining biopsy has to be preserved in ethanol (any concentration > 90%) for genotyping. Once a biopsy has been taken from every cockle, they have to be kept in favourable conditions until their genotypes are available; cockles could be transferred to the natural environment in the meantime, set in containers (e.g., mesh bags, perforated trays, baskets, lantern nets,…) hung from rafts, long lines, piers or any other

structure easily accessible. Another option would be keeping them in hatchery facilities. Considering that cockles should be initially collected before the beginning of the spawning season (late March or April) and that sex determination from a gonad biopsy requires that cockles have recognisable ovocytes or spermatozoa, the best period for collecting cockles from the inner area of the Ría de Arousa is February-early March, when most cockles have gonads in advanced gametogenesis or ripeness (Iglesias, 2006; Mahoney *et al*., 2020, Lorenzo Abalde *et al*., 2021).

3.2. Genotyping

A set of 60 single nucleotide polymorphism (SNPs) that showed signals of selection to marteiliosis or that were located in differentially expressed genes (DEGs), depending on the degree of marteiliosis infection, were identified as candidate markers of marteiliosis-resistance in the action 7.3 of the project COCKLES (Pardo *et al*., 2021). Three technical criteria were used to select the SNPs to be genotyped: (1) no genetic variation in the flanking regions to avoid interference with primer annealing; (2) flanking regions displaying a unique BLAST-alignment against the reference genome; (3) unlinked SNPs to have a panel as informative as possible. Subsequently, 45 of them (Table 1) have been validated in a common garden experiment using families from naïve and long-term affected populations (unpublished results) demonstrating their usefulness to select stocks resistant to marteiliosis for culture or restoring purposes in areas affected by marteiliosis, within the action 3.4 of the project COCKLES (Villalba *et al*., 2021b). The procedure to genotype the selected SNPs has been described in the final report of the action 7.3 of the project COCKLES (Pardo *et al*., 2021). Briefly, the protocol consists in a two-step reaction: the first involves the amplification by polymerase chain reaction (PCR) of an amplicon including the selected SNP and the second a mini-sequencing reaction using an internal primer adjacent to the SNP which extends the primer with a dideoxinucleotide complementary to the SNP variant. Flanking regions of 100 nucleotides in length of the selected SNPs were obtained from the edible cockle reference genome using a home perl script (201 nucleotides in total length). The primers corresponding to the 45 SNPs have been combined in two multiplex reactions (M1 for 25 SNPs and M2 for 20 SNPs) to facilitate and accelerate the process (Table 1).

Table 1. List of the 45 single nucleotide polymorphisms (SNPs) selected as markers of marteiliosis-resistance. The table provides information on the multiplex (either M1 or M2) to which corresponds each SNP, the identification of each SNP (SNP_ID), the chromosome (Chr) in which each SNP is located, their positions in the sequence of the *Cerastoderma edule* reference genome, the annotation of the gene in which each SNP is included, the forward (**F**) and reverse (**R**) primers of the PCR (1st genotyping step) for each SNP and the internal primer for 2nd genotyping step.

Multiplex	SNP_ID	Chr	Position	Annotation	Primers for PCR (1st step)	Internal Primer for 2nd step
M1	CE10	8	22273380	<250kb from gene: F-box only protein 21-like	**F**: ACGTTGGATGCACAAACGCATACCTGTCTG **R**: ACGTTGGATGAAGAGAAGTTTGGAAGCAGG	ACGTTGGATGAAGAGAAGTTTGGAAGCAGG
M1	CE5	13	8706747	Synaptojanin-1-like	**F**: CGTTGGATGTAGCATCTTGCATAGCAAAC **R**: ACGTTGGATGTCAACGAAATCAACTGGTTC	ACGTTGGATGTCAACGAAATCAACTGGTTC
M1	CE18	6	11581379	26S Proteasome non-ATPase regulatory subunit 9-like	**F**: ACGTTGGATGGTAGTCCTTTTCCTTGTCGC **R**: ACGTTGGATGTCCTGTGCATGTATTGAGGG	ACGTTGGATGTCCTGTGCATGTATTGAGGG
M1	CE6	15	19221254	Contactin-5	**F**: ACGTTGGATGGTAGCTTGGCAGGTAGGTAG **R**: ACGTTGGATGTTAGCGCTATCAGCGCTTTG	ACGTTGGATGTTAGCGCTATCAGCGCTTTG
M1	CE45	5	16875429	HMG box-containing protein C19G7.04-like	**F**: ACGTTGGATGTGGGCACGATGTTGCAGAAC **R**: ACGTTGGATGGGGTTTAATGCAGTGTCAAC	ACGTTGGATGGGGTTTAATGCAGTGTCAAC
M1	CE2	14	24563619	Receptor protein-tyrosine kinase	F: ACGTTGGATGGTATCACGATATCTGAACAC R: ACGTTGGATGCTCTCTGTACCCAGGTCTAT	ACGTTGGATGCTCTCTGTACCCAGGTCTAT
M1	CE8	3	10407569	<250kb from gene: 3'-phosphoadenosine-5'-phosphosulfate synthase	**F**: ACGTTGGATGAAACATGCATCTAGATGCGG **R**: ACGTTGGATGACCAATGTATGAAGACCAAG	ACGTTGGATGACCAATGTATGAAGACCAAG
M1	CE23	18	20951750	Cathepsin L	**F**: ACGTTGGATGGTATTTTTCAGATGTTGCG **R**: ACGTTGGATGAAGGCCTCCCATTCGGAGTC	ACGTTGGATGAAGGCCTCCCATTCGGAGTC
M1	CE14	3	4328109	Eukaryotic peptide chain release factor GTP-binding subunit ERF3B	**F**: ACGTTGGATGTGGTTTTCAGTGTGGTCTTC **R**: ACGTTGGATGTGGAAACAGCCCACTTAACG	ACGTTGGATGTGGAAACAGCCCACTTAACG

M1	CE33	8	19538893	Sulfotransferase family cytosolic 1B member 1	**F**: ACGTTGGATGCACACTTGTCCGCTATTTCC **R**: ACGTTGGATGTCCGAGCTAAAGAAGATCCG	ACGTTGGATGTCCGAGCTAAAGAAGATCCG
M1	CE3	13	11945836	Oxysterol-binding protein	**F**: ACGTTGGATGCTCCTTTCACTGAACTGAGC **R**: ACGTTGGATGCATATCTTAACATCAGCTACG	ACGTTGGATGCATATCTTAACATCAGCTACG
M1	CE22	2	31820629	Low quality protein: uncharacterized protein LOC111119482	**F**: ACGTTGGATGGTGTTTCAGACATTTGAGGAG **R**: ACGTTGGATGTGTGAAAAACTGAAGGCCCG	ACGTTGGATGTGTGAAAAACTGAAGGCCCG
M1	CE4	11	5177924	Deleted in lung and oesophageal cancer protein 1	**F**: ACGTTGGATGATAATGCCCTTCTGTCTGCC **R**: ACGTTGGATGCAGGTAGCAAAAGGGGAATC	ACGTTGGATGCAGGTAGCAAAAGGGGAATC
M1	CE51	10	37612300	Proton myo-inositol cotransporter-like isoform X1	**F**: ACGTTGGATGCCCAAAACATAGCATATGGC **R**: ACGTTGGATGCCCTGTTCACACAACTTGAG	ACGTTGGATGCCCTGTTCACACAACTTGAG
M1	CE54	13	2926422	CDGSH iron-sulphur domain-containing protein 3, mitochondrial	**F**: ACGTTGGATGCCCAAATTGAAAGAGTTTCC **R**: ACGTTGGATGGTTTTCTGCTCTTTCCGAAG	ACGTTGGATGGTTTTCTGCTCTTTCCGAAG
M1	CE39	3	28415984	Probable E3 ubiquitin-protein ligase HERC4	**F**: ACGTTGGATGGTTTACCTTGAAGGAAGGGC **R**: ACGTTGGATGGACTTCTGTTGCACGTCATC	ACGTTGGATGGACTTCTGTTGCACGTCATC
M1	CE9	12	28518329	<250kb from gene: Poly [ADP-ribose] polymerase	**F**: ACGTTGGATGAGCTGATGAATTCCTTCACG **R**: ACGTTGGATGTTATCGCGCATGCGTGAAAC	ACGTTGGATGTTATCGCGCATGCGTGAAAC
M1	CE1	10	15677896	Glutathione S-transferase sigma class protein	**F**: ACGTTGGATGAAACATGAAGTCGGTTCTCG **R**: ACGTTGGATGTTTGTTTCTTCGGCAGCAAC	ACGTTGGATGTTTGTTTCTTCGGCAGCAAC
M1	CE28	1	25594873	Kazal-like domain-containing protein	**F**: ACGTTGGATGTTCCAGTGGATGCATGTGAG **R**: ACGTTGGATGAGGCCCTGCTGATTCAACAC	ACGTTGGATGAGGCCCTGCTGATTCAACAC
M1	CE15	5	29151780	Hexosyltransferase	**F**: ACGTTGGATGGTGGCTCGATAAAGCACTGG **R**: ACGTTGGATGAAGTTAAATCCTCGGCGCTC	ACGTTGGATGAAGTTAAATCCTCGGCGCTC

M1	CE13	15	30613820	Golgin subfamily A member 2	**F**: ACGTTGGATGCTAGGTAACAGGTCGAAATT **R**: ACGTTGGATGTTTGTAATTCGCACTCAGGG	ACGTTGGATGTTTGTAATTCGCACTCAGGG
M1	CE27	13	24547378	3-Ketoacyl-CoA thiolase, mitochondrial	**F**: ACGTTGGATGTCTGAACGAGTTATTTCCCC **R**: ACGTTGGATGAACTCACTCCTTGAGACGAC	ACGTTGGATGAACTCACTCCTTGAGACGAC
M1	CE50	7	18666371	Core-binding factor subunit beta-like isoform X2	**F**: ACGTTGGATGCCTGAAGTACATGGAGAATG **R**: ACGTTGGATGGGTCTTTAAGCACTTATGACG	ACGTTGGATGGGTCTTTAAGCACTTATGACG
M1	CE12	18	16983304	Cysteine dioxygenase	**F**: ACGTTGGATGTGCAGACGACTCTGGAAAAC **R**: ACGTTGGATGAGGGCAGAACACATTATGGG	ACGTTGGATGAGGGCAGAACACATTATGGG
M1	CE42	8	31342192	Sodium/glucose cotransporter 4	**F**: ACGTTGGATGATCAACCAAATTCACACAG **R**: ACGTTGGATGTTGGAGAAGATTTCAGTGAC	ACGTTGGATGTTGGAGAAGATTTCAGTGAC
M2	CE40	6	15595766	Collagen pro alpha-chain	**F**: ACGTTGGATGTACTTCTCCTGGTTGACTCC **R**: ACGTTGGATGGGTGAATTTTTACAAGGACAG	ACGTTGGATGGGTGAATTTTTACAAGGACAG
M2	CE34	16	14033842	NFX1-type zinc finger-containing protein 1-like	**F**: ACGTTGGATGACCCATAATACAGTGAAGCC **R**: ACGTTGGATGTCCCTTACCATTTCGTTCGG	ACGTTGGATGTCCCTTACCATTTCGTTCGG
M2	CE11	11	30895196	<250kb from gene: Beta-N-acetylhexosaminidase	**F**: ACGTTGGATGCTCCTCTTTAGATACCACCC **R**: ACGTTGGATGAAATCGCTAACCGCTTTCGC	ACGTTGGATGAAATCGCTAACCGCTTTCGC
M2	CE31	3	33568023	RNA helicase	**F**: ACGTTGGATGTCATCTCCAAAGGAAGAGGG **R**: ACGTTGGATGGTATCCCCTAAGCATTTTCTC	ACGTTGGATGGTATCCCCTAAGCATTTTCTC
M2	CE37	15	8876009	Microsomal glutathione S-transferase	**F**: ACGTTGGATGAACACAAACATATCACAAG **R**: ACGTTGGATGAAAGGATGATGCAAGAATGG	ACGTTGGATGAAAGGATGATGCAAGAATGG
M2	CE48	6	28704888	Lactadherin-like	**F**: ACGTTGGATGGATGAGAATACGCCGGTAAC **R**: ACGTTGGATGTTCCAACCAAGCGGATTCAC	ACGTTGGATGTTCCAACCAAGCGGATTCAC
M2	CE7	18	17697950	IgGFc-binding protein-like	**F**: ACGTTGGATGACGACAGTTCTGTTCTAGTC **R**: ACGTTGGATGTGATTTCCTCGTGTATCAGC	ACGTTGGATGTGATTTCCTCGTGTATCAGC

M2	CE44	19	13393947	Nuclear receptor subfamily 4 group A member 2 (Fragment)	**F**: ACGTTGGATGAAAGGCGCTAAGTACGTCTG **R**: ACGTTGGATGAAATCGACAGAACTGGCACC	ACGTTGGATGAAATCGACAGAACTGGCACC
M2	CE25	2	24806853	Serine palmitoyltransferase-1	**F**: ACGTTGGATGGAACAGACACTGAGAAAAAG **R**: ACGTTGGATGGCTTGTTACCATTTAAGGTG	ACGTTGGATGGCTTGTTACCATTTAAGGTG
M2	CE38	10	30619312	monoacylglycerol lipase ABHD6-like	**F**: ACGTTGGATGAACCTGTTTGCTACTCTGGG **R**: ACGTTGGATGTGTTTGTGTCGTCTGCTTTC	ACGTTGGATGTGTTTGTGTCGTCTGCTTTC
M2	CE52	2	5582531	Cholesterol 25-hydroxylase-like	**F**: ACGTTGGATGTGACGTTTGGTTGGATCTTC **R**: ACGTTGGATGAACAGAGTGGAACAACGGAG	ACGTTGGATGAACAGAGTGGAACAACGGAG
M2	CE46	1	18985767	Cyclic nucleotide-binding domain-containing protein 2-like	**F**: ACGTTGGATGTTTTGGCTCTGTGAATGGCG **R**: ACGTTGGATGTTGTCAGTCCGAAAATGGCG	ACGTTGGATGTTGTCAGTCCGAAAATGGCG
M2	CE53	11	2619576	Metalloendopeptidase	**F**: ACGTTGGATGATAAACCACCAAATGATGGC R: ACGTTGGATGTGCACATGCGTACTGGAATG	ACGTTGGATGTGCACATGCGTACTGGAATG
M2	CE20	1	39526950	2-Aminoethylphosphonate--pyruvate transaminase	**F**: ACGTTGGATGATACGGCGTCTGTTACAAGC R: ACGTTGGATGAACGATGATCCTTGCGAGCC	ACGTTGGATGAACGATGATCCTTGCGAGCC
M2	CE16	16	19735623	Thymidylate kinase-like	**F**: ACGTTGGATGGCTGTATAACTGGCAAACAC **R**: ACGTTGGATGGCTACAGTTGCCAGACAAAG	ACGTTGGATGGCTACAGTTGCCAGACAAAG
M2	CE35	2	38523750	Regulator of chromosome condensation	**F**: ACGTTGGATGGTACAATCATGTAAGTTGAC **R**: ACGTTGGATGACAAGTGCACATGGGTATTG	ACGTTGGATGACAAGTGCACATGGGTATTG
M2	CE26	9	34714048	Cyclic AMP-responsive element-binding protein 3-like protein 3 isoform X2	**F**: ACGTTGGATGCAGTTTGCTTCAGACGACTC **R**: ACGTTGGATGAGGGCAGAAAACATTTTGGG	ACGTTGGATGAGGGCAGAAAACATTTTGGG

M2	CE49	14	17636263	Peptidylglycine monooxygenase	**F**: ACGTTGGATGGCCCAAATGACACTCTATTC **R**: ACGTTGGATGCTTCGTCAGAGTCGGTAAAG	ACGTTGGATGCTTCGTCAGAGTCGGTAAAG
M2	CE36	14	33337974	Sodium- and chloride-dependent glycine transporter 1-like	F: ACGTTGGATGTCGAATTAAAATTTATGTTG **R**: ACGTTGGATGGTTTTGTTAATGGACGGTTG	ACGTTGGATGGTTTTGTTAATGGACGGTTG
M2	CE30	16	3928743	Tumour necrosis factor ligand superfamily member 10	**F**: ACGTTGGATGCAGTGTGCGAGTTAGAAAGG **R**: ACGTTGGATGGGAACCTGAATTAAGCAACC	ACGTTGGATGGGAACCTGAATTAAGCAACC

4. Broodstock conditioning, larval and post-larval culture

Once the genotype of each cockle is available, a minimum of 400 cockles (equal number of females and males) have to be selected, prioritising the endowment of resistance-markers, to establish the broodstock. The chosen cockles will be distributed in batches of equal number of females and males (ten cockles of each sex in the case of a total of 400 cockles), for a minimum of 20 batches, setting each batch in a different conditioning tray. Therefore, larvae deriving from at least 20 parental sets will be expected; the number of cockles contributing to each spawning event will remain unknown with this design. Increasing the number of parental batches will reduce the probability of inbreeding depression and contribute to maintain genetic diversity thinking on a medium-long term breeding programme.

The procedures of broodstock conditioning, collecting larvae and culturing them in hatchery facilities (Fig. 2) as well as those of outdoor rearing (Fig. 3) are thoroughly described in the COCKLES' project reports by Joaquim *et al*. (2021) and Fernández *et al*. (2021). The cockle seed obtained through those processes may be used in areas affected by marteiliosis either for ongrowing with commercial purposes or for restoring exhausted beds.

5. Subsequent generations

A batch of the seed produced in the previous step (from 1,000 to 2,000 cockles) will be kept as the source of the breeders of the next generation of selection. These cockles will grow-out in an area heavily affected by marteiliosis and will be recovered, once they have overpassed 25 mm in length, to repeat the process of genotyping and choosing those with the most favourable genotypes regarding the marteiliosis-resistance markers to establish the broodstock of the next generation. This sequence will be repeated to produce new generations as far as it is needed. Various procedures could be used to rear those cockles set aside as source of the breeders of the next generation in an area affected by marteiliosis. They could be hung from a raft in baskets, trays or lantern nets; they could be placed in trays with sediment in a shellfish bed; alternatively, any other procedure allowing to easily recover the selected cockles could be used (Fig. 3).

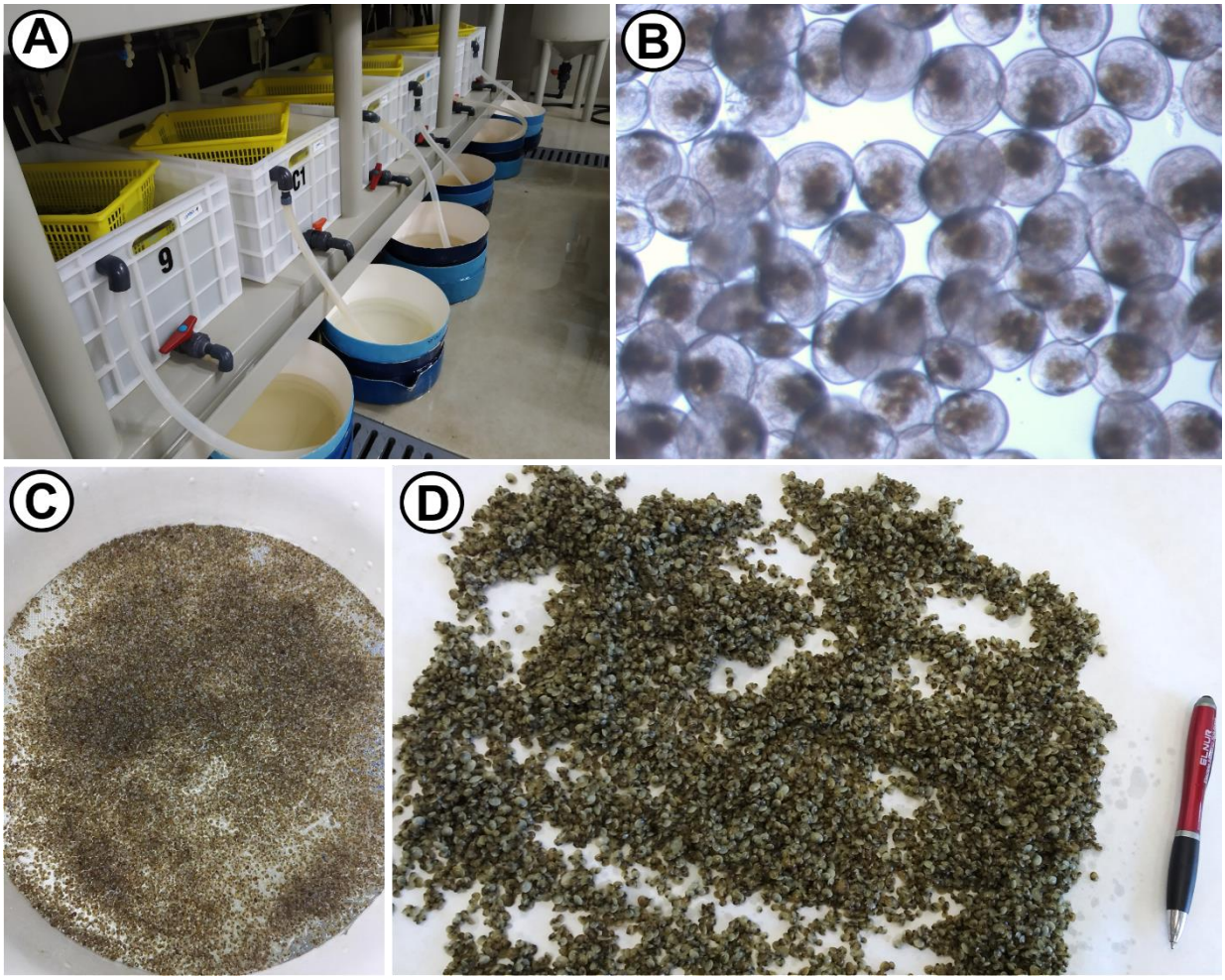

Fig. 2. Cockle culture at hatchery facilities. A: Broodstock conditioning. B: Fourteen days old cockle larvae. C: Cockle postlarvae at 1 mm mesh. D: Cockle seed ready for outdoor culture. Photographs courtesy of Damián Costas (ECIMAT-CIM-University of Vigo).

6. Monitoring effectiveness

Performing a selective breeding programme and keeping it on through generations consumes much funding and resources. Thus, information on its effectiveness is mandatory. Furthermore, as mentioned above, the cockle population of the inner area of Ría de Arousa has increased resistance against marteiliosis through natural selection, which involves that using cockles collected from that area, without any other choosing criterion, as breeders could yield highly marteiliosis-resistant cockles, as has been demonstrated in the pilot action 3.4 of the project COCKLES (Villalba *et al*., 2021b). Consequently, surveys comparing the performance of

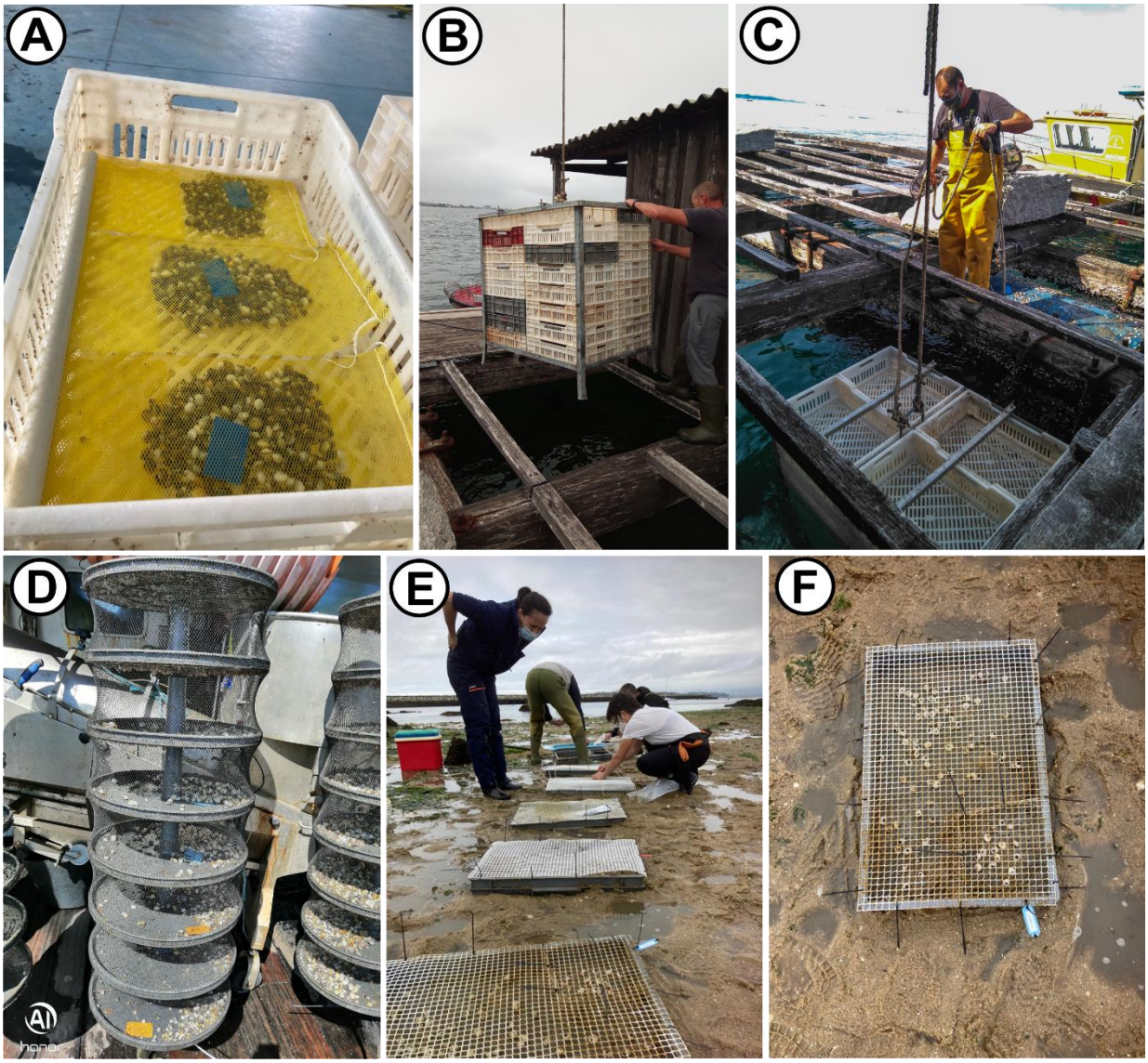

Fig. 3. Cockles growing out in easily accessible devices. A-C: Cockle seed set in trays to be hung from a raft. D: Cockle seed set in lantern nets. E-F: Cockle seed set in sediment within trays that are buried in a shellfish bed. Photographs courtesy of Eva Cacabelos (A-C, Hydrosphere, S.L.), María Ortega (D, CIMA) and Mónica Incera (E and F, CETMAR)

the cockles of each selected generation with that of non-selected cockles deriving from the inner area of the Ría de Arousa and that of cockles deriving from a naïve population should be carried out. The information from those surveys will serve to decide whether keeping on, modifying or finishing the selective breeding programme. The report of the pilot action 3.4 (Villalba *et al*., 2021b) could be used as a procedural guide to perform those surveys.

7. Monitoring genetic variability

Selective breeding programmes and some habits in mollusc hatcheries entail the risk of (do not necessarily cause) inbreeding depression (Nascimento-Schulze *et al*., 2021) along with loss of genetic diversity, which could reduce the response to selection and could lead to reduced biological fitness, thus causing counterproductive, undesirable effects. Keeping this risk in mind and taking advantage of the genotype analysis performed to choose the breeders of each generation, that genotype analysis should be extended to cover other anonymous markers informing on the genetic variability in each generation. In case loss of genetic variability is detected, corrective measures should be adopted, such as procedural changes to increase the number of parents contributing to each generation or outbreeding enhancement by including cockles out of the programme as breeders. The 12 microsatellites reported by Martínez et al. (2009) will be used for monitoring genetic diversity across generations of the selective breeding programme. Microsatellites are hypervariable markers holding high genetic diversity and they are very sensitive to losses of genetic diversity. Furthermore, microsatellite genotyping is cost-effective.

8. Literature cited

Beattie, J.H., Davis, J.P., Downing, S.L., Chew, K.K., 1988. Summer mortality of Pacific oysters. American Fishery Society, Special Publication 18: 265–268

Cao, A., Iglesias, D., Carballal, M.J., Villalba, A., 2021. Identification of protein markers of marteiliosis resistance. Report-Deliverable 7.2 of the project COCKLES. https://cockles-project.eu/public/uploads/downloads/08bb19cda0c40de6d14b96c180c43ad7.pdf

Casas, S., Walton, W., Chaplin, G., Rikard, S., Supan, J., La Peyre, J., 2017. Performance of oysters selected for dermo resistance compared to wild oysters in northern Gulf of Mexico estuaries. Aquaculture Environment Interactions. 9: 169–180. doi: 10.3354/aei00222

Cruz, A., Da Costa, F., Fernández-Pérez, J., Nantón, A., Fernández-Boo, S., Insua, A., Méndez, J. 2020. Genetic variability in *Ruditapes decussatus* clam combined with *Perkinsus* infection level to support founder population selection for a breeding program. PeerJ 8: e9728. doi: 10.7717/peerj.9728

de la Ballina, N.R., Villalba, A., Cao, A., 2018. Proteomic profile of *Ostrea edulis* haemolymph in response to bonamiosis and identification of candidate proteins as resistance markers. Diseases of Aquatic Organisms 128: 127-145. doi: 10.3354/dao03220

de Lorgeril, J., Petton, B., Lucasson, A., Perez, V., Stenger, P.L., Dégremont, L., Montagnani, C., Escoubas, J.M., Haffner, P., Allienne, J.F., Leroy, M., Lagarde, F., Vidal-Dupiol, J., Gueguen, Y., Mitta, G., 2020. Differential basal expression of immune genes confers *Crassostrea gigas* resistance to Pacific oyster mortality syndrome. BMC Genomics 21: 63. doi: 10.1186/s12864-020-6471-x

de Montaudouin, X., Arzul, I., Cao, A., Carballal, M.J., Chollet, B., Correia, S., Cuesta, J., Culloty, S., Daffe, G., Darriba, S., Díaz, S., Engelsma, M., Freitas, R., Garcia, C., Goedknegt, A., Gonzalez, P., Grade, A., Groves, E., Iglesias, D., Jensen, K.T., Joaquim, S., Lynch, S., Magalhães, L., Mahony, K., Maia, F., Malham, S., Matias, D., Nowaczyk, A., Ruano, F., Thieltges, D., Villalba, A., 2021. Catalogue of parasites and diseases of the common cockle *Cerastoderma edule.* UA Editora – Universidade de Aveiro, Aveiro, Portugal, 128 p. ISBN: 978-972-789-671-4. https://flipbooklets.com/2021/cockles/catalogue-of-parasites-and-diseases-2/

Dégremont, L., Garcia, C., Allen, S.K., 2015a. Genetic improvement for disease resistance in oysters: A review. Journal of Invertebrate Pathology 131: 226–241. doi: 10.1016/j.jip.2015.05.010

Dégremont, L., Nourry, M., Maurouard, E., 2015b. Mass selection for survival and resistance to OsHV-1 infection in *Crassostrea gigas* spat in field conditions: response to selection after four generations. Aquaculture 446: 111–121. doi: 10.1016/j.aquaculture.2015.04.029

Dégremont, L., Lamy, J.B., Pépin, J.F., Travers, M.A., Renault, T., 2015c. New Insight for the Genetic Evaluation of Resistance to Ostreid Herpesvirus Infection, a Worldwide Disease, in *Crassostrea gigas*. PLoS ONE 10: e0127917. doi: 10.1371/journal.pone.0127917

Dégremont, L., Maurouard, E., Rabiller, M., Glize, P., 2019. Response to selection for increasing resistance to the spring mortality outbreaks in *Mytilus edulis* occurring in France since 2014. Aquaculture 511: 734269. doi: 10.1016/j.aquaculture.2019.734269

Dégremont, L., Azéma, P., Maurouard, E., Travers, M.A., 2020. Enhancing resistance to *Vibrio aestuarianus* in *Crassostrea gigas* by selection. Aquaculture 526: 735429. doi: 10.1016/j.aquaculture.2020.735429

Dove, M.C., Nell, J.A., & O'Connor, W.A., 2013. Evaluation of the progeny of the fourth-generation Sydney rock oyster *Saccostrea glomerata* (Gould, 1850) breeding lines for resistance to QX disease (*Marteilia sydneyi*) and winter mortality (*Bonamia roughleyi*). Aquaculture Research 44: 1791–1800. doi:10.1111/are.12012

Farhat, S., Tanguy, A., Pales Espinosa, E., Guo, X., Boutet, I., Smolowitz, R., Murphy, D., Rivara, G.J., Allam, B., 2020. Identification of variants associated with hard clam, *Mercenaria*

mercenaria, resistance to Quahog Parasite Unknown disease. Genomics 112:4887–4896. doi: 10.1016/j.ygeno.2020.08.036

Fernández, R., Joaquím, S., Matias, D., Costas, D., Villalba, A., 2021. COCKLES' compilation of cockle culture experiences. Report-Deliverable 3.3 of the project COCKLES.

Fernández-Boo, S., Villalba, A., Cao, A., 2016. Protein expression profiling in haemocytes and plasma of the Manila clam *Ruditapes philippinarum* in response to infection with *Perkinsus olseni*. Journal of Fish Diseases 39: 1369-1385. doi:10.1111/jfd.12470

Fernández Robledo, J.A., Yadavalli, R., Allam, B., Pales Espinosa, E., Gerdol, M., Greco, S., Stevick, R.J., Gómez-Chiarri, M., Zhang, Y., Heil, C.A., Tracy, A.N., Bishop-Bailey, D., Metzger, M.J., 2019. From the raw bar to the bench: Bivalves as models for human health. Developmental and Comparative Immunology 92: 260–282. doi: 10.1016/j.dci.2018.11.020

Ford, S.E., Haskin, H.H., 1987. Infection and mortality patterns in strains of *Crassostrea virginica* selected for resistance to the parasite *Haplosporidium nelsoni* (MSX). Journal of Parasitology, 73: 368–376. doi: 10.2307/3282092

Frank-Lawale, A., Allen, S. K., Jr., Degremont, L., 2014. Breeding and domestication of Eastern oyster (*Crassostrea virginica*) lines for culture in the mid-Atlantic, USA: line development and mass selection for disease resistance. Journal of Shellfish Research, 33: 153–165. doi: 10.2983/035.033.0115

Green, T.J., Montagnani, C., 2013. Poly I:C induces a protective antiviral immune response in the Pacific oyster (*Crassostrea gigas*) against subsequent challenge with Ostreid herpesvirus (OsHV-1 µvar). Fish & Shellfish Immunology 35: 382–388. doi.org/10.1016/j.fsi.2013.04.051

Grizel, H., Bachere, E., Mialhe, E., Tige, G., 1987. Solving parasite-related problems in cultured molluscs. International Journal for Parasitology 17: 301–308. doi: 10.1016/0020-7519(87)90104-4

Gutierrez, A.P., Bean, T.P., Hooper, C., Stenton, C.A., Sanders, M.B., Paley, R.K., Rastas, P., Bryrom, M., Matika, O., Houston, R.D., 2018. A Genome-Wide Association Study for Host Resistance to Ostreid Herpesvirus in Pacific Oysters (*Crassostrea gigas*). Genes Genomes Genetics. 8: 1273-1280. doi: 10.1534/g3.118.200113

Gutierrez, A.P., Symonds, J., King, N., Steiner, K., Bean, T.P., Houston, R.D., 2020. Potential of genomic selection for improvement of resistance to ostreid herpesvirus in Pacific oyster (*Crassostrea gigas*). Animal Genetics, 51: 249–257. doi: 10.1111/age.12909

Hasanuzzaman, A.F., Cao, A., Ronza, P., Fernández-Boo, S., Rubiolo, J.A., Robledo, D., Gómez-Tato, A., Álvarez-Dios, J.A., Pardo, B.G., Villalba, A., Martínez, P., 2020. New insights into the Manila clam - *Perkinsus olseni* interaction based on gene expression analysis of clam hemocytes and parasite trophozoites through in vitro challenges. *International Journal for Parasitology* 50: 195-208. doi: 10.1016/j.ijpara.2019.11.008

He, Y., Yu, H., Bao, Z., Zhang, Q., & Guo, X., 2012. Mutation in promoter region of a serine protease inhibitor confers *Perkinsus marinus* resistance in the eastern oyster (*Crassostrea virginica*). Fish & Shellfish Immunology, 33: 411-417. doi: 10.1016/j.fsi.2012.05.028.

Iglesias, D., 2006. Estudio patológico de las poblaciones de berberecho *Cerastoderma edule* (L.) de Galicia. Doctoral thesis, University of Santiago de Compostela, Santiago de Compostela, Spain.

Iglesias, D., Villalba, A., No, E., Darriba, S., Mariño, C., Fernández, J., Carballal, M.J., 2015. Cockle *Cerastoderma edule* marteiliosis first detected in Ria de Arousa (Galicia, Nw Spain) has spread to other Galician rias causing mass mortality. In: 17th International Conference on Diseases of fish and Shellfish, Las Palmas de Gran Canaria, Spain. Book of Abstracts, European Association of Fish Pathologists, pp. 348.

Joaquim, S., Matias, A.M., Roque, C., Matias, D., 2021. Settling culture procedures at hatchery and outdoor stages. Report-Deliverable 7.1 of the project COCKLES. https://cockles-project.eu/public/uploads/downloads/6de8cc542309b8a36bd322ccd2d54675.pdf

La Peyre, J.F., Casas, S.M., Richards, M., Xu, W., Xue, Q., 2019. Testing plasma subtilisin inhibitory activity as a selective marker for dermo resistance in eastern oysters. Diseases of Aquatic Organisms 133: 127–139. doi: 10.3354/dao03344

Lafont, M., Petton, B., Vergnes. A., Pauletto, M., Segarra, A., Gourbal, B., Montagnani, C., 2017. Long-lasting antiviral innate immune priming in the Lophotrochozoan Pacific oyster, *Crassostrea gigas*. Scientific Reports 7: 13143: doi: 10.1038/s41598-017-13564-0

Lapègue, S., Renault, T., 2018. Les apports de la Génétique dans la filière ostréicole Française. Bulletin de la Académie Vétérinaire de France 171: 223–228. doi: 10.4267/2042/70141

Leprêtre, M., Faury, N., Segarra, A., Claverol, S., Degremont, L., Palos-Ladeiro, M., Armengaud, J., Renault, T., Morga, B., 2021. Comparative proteomics of ostreid herpesvirus 1 and Pacific oyster interactions with two families exhibiting contrasted susceptibility to viral infection. Frontiers in Immunology 11: 621994. doi: 10.3389/fimmu.2020.621994

Lynch, S.A., Armitage, D.V., Coughlan, J., Mulcahy, M.F., Culloty, S.C., 2007. Investigating the possible role of benthic macroinvertebrates and zooplankton in the life cycle of the haplosporidian *Bonamia ostreae*. Experimental Parasitology 115: 359–368. doi: 10.1016/j.exppara.2006.09.021

Lynch, S.A., Flannery, G., Hugh-Jones, T., Hugh-Jones, D., Culloty, S.C., 2014. Thirty-year history of Irish (Rossmore) *Ostrea edulis* selectively bred for disease resistance to *Bonamia ostreae.* Diseases of Aquatic Organisms 110: 113–121. doi: 10.3354/dao02734

Lorenzo Abalde, S., Cao, A., Iglesias, D., Carballal, M.J., Villalba, A., 2021. Cockle response to stress – Immune system. Report-Deliverable 5.2a of the project COCKLES. https://cockles-project.eu/public/uploads/downloads/9d848a3c71e0a2dea04166d7642f8233.pdf

Mahony, K., Egerton, S., Lynch, S., Culloty, S., Moura, P., Joaquim, S., Iglesias, D., Villalba, A., Correia, S., Freitas, R., Goedknegt, A., de Montaudouin, X., 2020. Cockle reproductive health. Report-Deliverable 4.3 of the project COCKLES. https://cockles-project.eu/public/uploads/downloads/bdf41d4e6dc6ab2520428dfe6c88f173.pdf

Martínez, L., Arias, A., Méndez, J., Insua, A., Freire, R., 2009. Development of twelve polymorphic microsatellite markers in the edible cockle *Cerastoderma edule* (Bivalvia: Cardiidae). Conservation Genetics Resources 1:107–109. doi: 10.1007/s12686-009-9026-7

Meistertzheim, A.L., Calvès, I., Roussel, V., Van Wormhoudt, A., Laroche, J., Huchette, S., Paillard, C. (2014). New genetic markers to identify European resistant abalone to vibriosis revealed by high−resolution melting analysis, a sensitive and fast approach. Marine Biology 161: 1883–1893. doi: 10.1007/s00227-014-2470-2

Nascimento-Schulze, J.C., Bean, T.P., Houston, R.D., Santos, E.M., Sanders, M.B., Lewis, C., Ellis, R.P., 2021. Optimizing hatchery practices for genetic improvement of marine bivalves. Reviews in Aquaculture 13: 2289–2304. doi: 10.1111/raq.12568

Nie, Q., Yue, X., Liu, B., 2015. Development of *Vibrio* spp. infection resistance related SNP markers using multiplex SNaPshot genotyping method in the clam *Meretrix meretrix.* Fish & Shellfish Immunology 43, 469–476. doi: 10.1016/j.fsi.2015.01.030

Nikapitiya, C., McDowell, I.C., Villamil, L., Muñoz, P., Sohn, S.B., Gomez-Chiarri, M., 2014. Identification of potential general markers of disease resistance in American oysters, *Crassostrea virginica* through gene expression studies. Fish & Shellfish Immunology 41: 27–36. doi: 10.1016/j.fsi.2014.06.015

Pardo, B.G., Fernández, C., Cao, A., Blanco, A., Pampín, M., Casanova, A., Iglesias, D., Vera, M., Carballal, M.J., Villalba, A., Martínez, P., 2021. Identification of candidate genes and genetic markers of resistance to marteiliosis. Report-Deliverable 7.3 of the project COCKLES. https://cockles-project.eu/public/uploads/downloads/e1b4171fe23dc7c6f5f56e07ab0d468e.pdf

Proestou, D.A., Sullivan, M.E., 2020. Variation in global transcriptomic response to *Perkinsus marinus* infection among eastern oyster families highlights potential mechanisms of disease resistance. Fish & Shellfish Immunology 96: 141–151. doi: 10.1016/j.fsi.2019.12.001

Proestou, D.A., Vinyard, B.T., Corbett, R.J., Piesz, J., Allen, S.K., Small, J.M., Li, C., Liu, M, DeBrosse, G., Guo, X., Rawson, P., Gómez-Chiarri, M., 2016. Performance of selectively-bred lines of eastern oyster, *Crassostrea virginica*, across eastern US estuaries. Aquaculture 464: 17–27. doi: 10.1016/j.aquaculture.2016.06.012

Ragone Calvo, L.M., Calvo, G.W., Burreson, E.M., 2003. Dual disease resistance in a selectively bred eastern oyster, *Crassostrea virginica*, strain tested in Chesapeake Bay. Aquaculture 220: 69–87. doi: 10.1016/S0044-8486(02)00399-X

Simonian, M., Nair, S.V., O'Connor, W.A., Raftos, D.A., 2009. Protein markers of *Marteilia sydneyi* infection in Sydney rock oysters, *Saccostrea glomerata.* Journal of Fish Diseases 32: 367−375. doi: 10.1111/j.1365-2761.2009.01022.x

Smits, M., Enez, F., Ferraresso, S., Dalla Rovere, G., Vetois, E., Auvray, J.-F., Genestout, L., Mahla, R., Arcangeli, G., Paillard, C., Haffray, P., Bargelloni, L., 2020a. Potential for genetic improvement of resistance to *Perkinsus olseni* in the Manila clam, *Ruditapes philippinarum*, using DNA parentage assignment and mass spawning. Frontiers in Veterinary Science 7: 579840. doi: 10.3389/fvets.2020.579840

Smits, M., Artigaud, S., Bernay, B., Pichereau, V., Bargelloni, L., Paillard, C., 2020b. A proteomic study of resistance to Brown Ring disease in the Manila clam *Ruditapes philippinarum*. Fish & Shellfish Immunology 99: 641–653. doi: 10.1016/j.fsi.2020.02.002

Sunila, I., Kenyon, H, Rivara, K., Blacker, K., Getchis, T., 2016. Restocking natural beds with remote set disease resistant oysters in Connecticut: a field trial. Journal of Shellfish Research 35: 115–125. doi: 10.2983/035.035.0113

Vaibhav, V., Thompson, E.L., Raftos, D.A., Haynes, P.A., 2018. Potential protein biomarkers of QX disease resistance in selectively bred Sydney Rock Oysters. Aquaculture 495: 144–152. doi: 10.1016/j.aquaculture.2018.05.035

van Banning, P., 1991. Observations on bonamiosis in the stock of the European flat oyster, *Ostrea edulis*, in the Netherlands, with special reference to the recent developments in Lake Grevelingen. Aquaculture 93: 205–211. doi: 10.1016/0044-8486(91)90232-V

Vera, M., Pardo, B.G., Cao, A., Fernández, C., Blanco, A., Gutiérrez, A., Bean, T., Houston, R., Villalba, A., Martínez, P., 2019. Signatures of selection for bonamiosis resistance in European flat oyster (*Ostrea edulis*): new genomic tools for breeding programs and management of natural resources. Evolutionary Applications 2019: 1781-1796. doi: 10.1111/eva.12832

Villalba, A., Iglesias, D., Ramilo, A., Darriba, S., Parada, J.M., No, E., Abollo, E., Molares, J., Carballal, M.J., 2014. Cockle *Cerastoderma edule* fishery collapse in the Ria de Arousa (Galicia, NW Spain) associated with the protistan parasite *Marteilia cochillia*. Diseases of Aquatic Organisms 109: 55–80. doi: 10.3354/dao02723

Villalba, A., Iglesias, D., Costas, D., Mariño, J.C., Domínguez, M., Cacabelos, E., Abella, E., Pampín, M., Martínez, P., Incera, M., Fernández, R., 2021a. Increased resistance against marteiliosis in the cockle *Cerastoderma edule* population of the inner area of the Ría de Arousa (Galicia NW Spain) through natural selection. In: 20th International Conference on Diseases of Fish and Shellfish – Abstract Book, European Association of Fish Pathologists, pp. 146.

Villalba, A., Iglesias, D., Costas, D., Mariño, J.C., Domínguez, M., Cacabelos, E., Abella, E., Pampín, M., Martínez, P., Incera, M., Fernández, R., 2021b. Pilot action demonstrating the

benefits of using selected cockle stocks resistant to marteiliosis for culture and restoration purposes in marteiliosis-affected areas. Report-Deliverable 3.4 of the project COCKLES.

Wang, K., Pales Espinosa, E., Tanguy, A., Allam, B., 2016. Alterations of the immune transcriptome in resistant and susceptible hard clams (*Mercenaria mercenaria*) in response to Quahog Parasite Unknown (QPX) and temperature. Fish & Shellfish Immunology 49: 163-76. doi: 10.1016/j.fsi.2015.12.006.

Yang, W., Tran, N.T., Zhu, C.H., Yao, D.F., Aweya, J.J., Gong, Y., Ma, H.Y., Zhang, Y.L., Li, G.L., Li, S.K., 2020. Immune priming in shellfish: A review and an updating mechanistic insight focused on cellular and humoral responses. Aquaculture 530: 735831. doi: 10.1016/j.aquaculture.2020.735831

Zenger, K.R., Khatkar, M.S., Jones, D.B., Khalilisamani, N., Jerry, D.R., Raadsma, H.W., 2019- Genomic selection in aquaculture: Application, limitations and opportunities with special reference to marine shrimp and pearl oysters. Frontiers in Genetics 9: 693. doi: 10.3389/fgene.2018.00693