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Bonamia-free flat oyster (*Ostrea edulis* L.) seed for restoration projects: non-destructive screening of broodstock, hatchery production and test for *Bonamia*-tolerance

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Abstract – Native (flat) oyster (*Ostrea edulis*) beds, once a major component of the North Sea, largely disappeared from the region in the late 19th century. Flat oyster restoration is taking place at a number of locations in the North Sea. When flat oyster beds are restored in areas where *O. edulis* is functionally extinct it is advised to treat these as disease-free areas. Adult oysters were collected in the Dutch Delta area which is infected with the pathogenic parasite *Bonamia ostreae*. The aim of this research was to obtain *Bonamia*-free seed from parents collected in a *Bonamia*-infected area. In addition, the oysters were analysed to identify candidate genomic regions related to bonamiosis tolerance and exposed to *Bonamia* in the field to assess survival compared to a naïve control group. With the aid of a non-destructive screening method, *Bonamia*-free broodstock were selected. These oysters produced *Bonamia*-free larvae and seed. For comparison, broodstock oysters were collected in the Dutch Wadden Sea, an area free of *Bonamia*. These oysters also produced *Bonamia*-free larvae and seed. To study if the Delta area oysters had developed a degree of resistance to the disease, while the naïve Wadden Sea oysters had not, seed of both groups was challenged in Lake Grevelingen where *Bonamia* occurs. Survival of the pre-selected *Bonamia*-free oysters was significantly higher than the naïve group. Samples of seed were analysed for association of candidate genetic markers related to bonamiosis tolerance. A higher percentage of individuals with tolerance-associated marker genotypes was found in the screened group compared to the naïve one. However, mortality of the naïve group could not be related to *Bonamia* presence. Further challenge tests are needed before firm conclusions regarding the genetic markers can be made. The results show that hatchery production of *Bonamia*-free and potentially *Bonamia*-tolerant flat oysters is possible.

Keywords: Flat oyster / *Ostrea edulis* / *Bonamia* / restoration / disease tolerance

1 Introduction

The restoration of habitat-forming species is being applied throughout the world's oceans to halt the loss of biodiversity, combat climate change and help sustain the provision of ecosystem services such as nutrient cycling or eutrophication control (Saunders et al., 2020). The flat oyster *Ostrea edulis* L. was a common species in the North Sea until about a century ago (Gercken and Schmidt, 2014; Houziaux et al., 2008; Olsen, 1883). However, intensive flat oyster fishery at the end of the 19th century caused the oyster population to decline rapidly (Gercken and Schmidt, 2014; Houziaux, 2008). Currently, the species is listed as 'threatened' or 'declining' by the

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Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR) (Haelters and Kerckhof, 2009). At present, only a few individuals remain in the North Sea (Kerckhof et al., 2018).

Several flat oyster restoration projects are currently being carried out in Europe (Pogoda et al., 2019). Efforts to restore the species and its habitat in the Dutch North Sea started in 2014 with a number of feasibility studies (Smaal et al., 2015: 2017; Kamermans et al., 2018a: 2018b). In 2016, restoration pilots started in a near shore area (Sas et al., 2016: 2018; Didderen et al., 2019a: Van den Brink et al., 2020), which was followed in 2018 by pilots further offshore (Didderen et al., 2018: 2019b: 2019c: 2020). The above mentioned feasibility studies identified factors that determine flat oyster presence, such as temperature, chlorophyll concentration, suspended sediment and sediment composition. The factors were linked to the present environmental conditions in the Dutch North Sea. It can be concluded that several locations in the Dutch North Sea are suitable for flat oyster restoration. Results from the different pilots cited above show survival, growth, larval production and recruitment of flat oysters.

Restoration pilots need oysters in order to start a bed. In the Dutch Delta area a local flat oyster population exists and is farmed with a production of ~ 3 million oysters per year (www.agrimatie.nl). This can be a source of flat oysters. However, the population is infected with *Bonamia ostreae* since the 1980s (Engelsma et al., 2010). Bonamiosis is a disease caused by the intracellular protistan parasite *Bonamia* sp. and can result in mortality of flat oysters (Engelsma et al., 2010, 2014). *Bonamia ostreae* was first observed in France in 1979 (Pichot et al., 1980) and is now present in most countries surrounding the North Sea, except for areas in Ireland, Scotland, Norway and Sweden (Sas et al., 2020). In the Netherlands, the parasite was introduced in 1980 by import of oyster stocks from France into the Oosterschelde area (Van Banning, 1982) and subsequently spread of the parasite to the adjacent Lake Grevelingen (Van Banning, 1991). The parasite can be detected in oysters throughout the year and at different life stages (Arzul et al., 2011; Engelsma et al., 2010). Although the complete life cycle is yet unknown, *B. ostreae* is thought to be transmitted directly (Engelsma et al., 2014). European flat oysters incubate their larvae in their pallial cavity for 8–10 days before releasing them into the water column (Waller, 1981). Arzul et al. (2011) showed that larvae present in flat oysters from Bay of Quiberon in South Brittany (France) were infected with *B. ostreae*. Larvae might thus contribute to the spread of the parasite during their planktonic life.

Distribution of *B. ostreae* can be very local. In Ireland, for example one bay can be infected while the adjacent bay is *Bonamia*-free (Culloty and Mulcahy, 2007). At locations where the disease has been present for a long period, tolerance seems to have been developed (Culloty et al., 2004; Lynch et al., 2014). Other countries, such as Norway, are *Bonamia* free (Mortensen et al., 2016). When flat oyster beds are restored in areas where *O. edulis* is functionally extinct it is advised to treat these as *Bonamia*-free areas (Pogoda et al., 2019).

In Europe, there are 12 hatcheries that produce flat oyster seed for aquaculture purposes (Colsoul et al., 2021; Kamermans et al., 2020). These hatcheries are either located in *Bonamia*-infected areas or *Bonamia*-free areas (Sas et al., 2020). Seed from *Bonamia* infected areas cannot be used in restoration project because of the risk of spreading the disease (Pogoda et al., 2019). Seed from *Bonamia*-free hatcheries has the disadvantage that the seed is vulnerable to the disease (Culloty et al., 2004; Lynch et al., 2014). If, for some reason, *Bonamia* will reach the restoration site, the oysters may suffer from high mortality. Therefore, the use of disease-free seed produced from disease-tolerant parents would solve this problem if tolerance is inheritable. This offers opportunities to produce oysters that are not infected with *Bonamia*, despite the parents having been exposed to *Bonamia*, potentially creating offspring with some tolerance to the disease.

In the Netherlands, *B. ostreae* was first observed in the Oosterschelde in 1980 and in Lake Grevelingen in 1988 (Engelsma et al., 2010). This implies that the oysters present in these waters have been exposed to the disease for a long period. Culloty et al. (2004) compared the performance of oysters that had been selectively bred for resistance to *B. ostreae* (Rossmore, Cork harbour, Ireland), and oysters from two areas where *Bonamia* had been present for a long time (Lake Grevelingen, the Netherlands and Brittany, France), with oysters from four naïve populations where *Bonamia* had not been detected (Lough Foyle, Ireland; Tralee, Ireland; Lough Kishorn, Scotland; Mull, Scotland). Strains from these seven populations were translocated to Cork Harbour (Ireland), Lake Grevelingen (the Netherlands) and Brittany (France). The field trials indicated that Rossmore and Lake Grevelingen oysters showed lower mortality compared to other populations. Culloty et al. (2004) conclude that previous exposure in these populations had conferred some reduced susceptibility to the parasite compared to naïve populations. In a follow up study, seed was produced in the hatchery of Roem van Yerseke with broodstock from long-term exposed populations in Lake Grevelingen and the Oosterschelde and a naïve population in Limfjord in Denmark. Seed of all three groups was reared for one year in Lake Grevelingen. Survival was best in seed from Lake Grevelingen (OYSTERECOVER, 2013). It was concluded that the oyster stock from Lake Grevelingen should be considered as a candidate stock for starting a breeding plan in the Netherlands. Although this stock had the highest overall prevalence of infection, it also had the greatest growth and survival rate indicating that it may have formed some local tolerance to the disease. In addition, Vera et al. (2019) identified SNP markers associated with *Bonamia* tolerance in flat oyster using distant naïve and tolerant European populations and these markers were recently validated in a narrower area of the North Sea (Sambade et al., 2022). Since most of these markers mapped at a single genomic region, they could constitute a useful tool to facilitate obtaining *Bonamia*-tolerant stocks.

The aim of this research was to obtain *Bonamia*-free seed from parents collected in a *Bonamia*-infected area. In addition, the oysters were analysed to identify candidate genomic regions related to bonamiosis tolerance and exposed to *Bonamia* in the field to assess survival compared to a naïve control group.

2 Materials and methods

2.1 Non-destructive screening of broodstock oysters

Broodstock oysters of around 5 years old were collected from the Delta area by the hatcheries of Roem van Yerseke and

Stichting Zeeschelp. Prior to screening they were taken out of their storage tanks and left dry at 8–14 °C for 24 h. The next day, the oysters were anaesthetised by placing them in 5 L containers using 50 g L^{-1} magnesium chloride dissolved in a mixture of fresh water (3 L) and seawater PSU 31 (2 L) at 15–20 °C to maintain salinity (Suquet et al., 2010). A small amount of algae (*Isochrysis galbana*) was added regularly to the water to stimulate filtration by the oysters and the water was aerated. After approximately 3 h in the anaesthetic solution, the status of the oysters was assessed. Oysters were considered anaesthetised when their valves remained open after exerting pressure. A piece of gill of approximately 4 mm^2 was cut with scissors and placed in a vial containing seawater. The vial and the oyster were given the same code number. Following the sampling, oysters were placed in separate containers with clean seawater until their valves closed. Then the valves were kept closed with a rubber band and a piece of paper with the number was slipped under the rubber band. The oysters were placed at 7 °C without water for 3-4 days, until the result of the *Bonamia* analysis (described below) became available. Based on the *Bonamia* analysis the oysters were then separated into two groups: *Bonamia* free and *Bonamia* infected. *Bonamia* free oysters were transferred to the hatcheries where larvae and seed were produced following Helm *et al.* (2004). The screening was carried out with five broodstock batches in the period 2018–2021. The two hatcheries subsequently provided larvae and seed produced from these screened non-infected broodstock for *Bonamia* analysis and field challenges.

2.2 Test of presence of *Bonamia* in gills *vs* rest of the body

For routine monitoring of *B. ostreae* in the flat oyster population in the Netherlands a pool of oyster tissue was collected from gill, mantle and digestive diverticulum (up to a total of ~25 mg tissue per individual oyster), below referred to as "standard method". In order to investigate whether the non-destructive method of sampling (gill tissue of approximately 4 mm^2) is providing a representative status of the presence of *Bonamia* in the oyster, both methods were compared with the real-time PCR as described below using 190 oysters collected in 2018, 2019 and 2021 during the routine monitoring for shellfish diseases from Lake Grevelingen.

2.3 Survival test when exposed to *Bonamia*

Flat oyster seed produced in 2018 by the Royal Netherlands Institute of Sea Research (NIOZ) and by Roem van Yerseke (RvY) were placed in the field in February 2020. The NIOZ seed was produced with broodstock from the Wadden Sea (Jacobs et al., 2020). This area is not known to be infected with *Bonamia* and the seed is therefore considered to be naïve. The RvY seed was produced at the hatchery of Roem van Yerseke with selected *Bonamia*-free broodstock from the Oosterschelde. At the start of the experiment, each group consisted of around 2000 individuals.While waiting for the possibility to transfer to an oyster culture plot Lake Grevelingen (from February 2020 until August 2020) the two groups were kept in one oyster basket (BST Oyster Supplies) each. These two baskets were attached to a FLUPSY (floating upwelling system) of Roem van Yerseke in the harbour of Yerseke (Oosterschelde). From August 2020 onwards the baskets were placed in two frames that kept four oyster baskets each suspended in a vertical way above a bottom culture plot for flat oysters in Lake Grevelingen. Each basket consisted of two individually numbered compartments and per group 1 to 7 baskets were used (see Supplementary Table 2). Stocking density was 74–114 oysters per compartment. In February 2021 a second group of oysters from the same 2018 NIOZ batch was transferred to the baskets in Lake Grevelingen. Stocking density was 82–148 oysters per compartment. Every 6 weeks the live oysters were counted and a sample of 60 individuals per group was placed at −20 °C for *Bonamia* and tolerance-associated markers analysis. Before storage a picture of the oysters was taken with a ruler in view. Shell width was determined of 30 individuals per group with the programme ImageJ. Survival was based on the counts and expressed as percentage of the previous count. Differences between groups were tested with *t*-test per date. The challenge test lasted until August 2021. Both the Oosterschelde and Lake Grevelingen are areas that show *Bonamia* infection (Van Banning, 1987; Haenen and Engelsma, 2020).

2.4 *Bonamia* analysis

Samples of oyster gill tissue, larvae and seed were homogenised with a TeSeE PRECESS 24 homogenizer (Bio-Rad) in tissue disruption tubes equipped with ceramic beads. DNA was extracted from the homogenised material and water samples using the MagNA Pure LC JE379 (Roche Diagnostics, Almere, Netherlands) with the MPLC DNA isolation kit 2 according to the protocol of the manufacturer or the NucliSens EasyMAG (bioMerieux Benelux bv, Zaltbommel, Netherlands) according to Specific protocol B of the manufacturer. The DNA was eluted in 50 µl and stored at −20 °C until further processing. The detection of genetic material of *Bonamia* by real time PCR was carried out based on the real time PCR described by Marty et al. (2006). The real-time PCR amplification reactions were performed using an ABI 7500 Fast Applied Biosystems Thermocycler (Applied Biosystems). All reactions were carried out in a total volume of 20 µl in a 96-wells plates (Bioplastics) consisting of 10 µl of TaqMan PCR Master Mix (Applied Biosystems), 0.4 µM of the forward-primer (5'-CCCGGCTTCTTAGAGGGACTA-3'; Applied Biosystems), 0.4 µM of the reverse-primer (5'-ACCTGTTATTGCCCCAATCTTC-3'; Applied Biosystems), 0.08 µM of the Minor Groove Binding probe (5'-6-FAM-CTGTGTCTCCAGCAGAT-NFQ-3'; Applied Biosystems), 0.25 µl Uracil-DNA Glycolase (UDG 5 U/µl; New Engeland Biolabs), 5 µl template DNA and distilled water to make up a final volume of 20 µl. Each real-time PCR run included non-template controls (with 5 µl distilled water as template) as well as negative and positive controls (extracted DNA of bivalve individuals known to be infected or free of *B. ostreae*). The following thermal conditions were used: 37 °C for 10 min; 95 °C for 10 min and 40 cycles of 95° for 3 s, 60 °C for 30 s. At the end of each cycle the fluorescence signal was collected during the annealing step (60 °C). As control of the integrity of the DNA extraction a parallel reaction was carried out for each

Table 1. Comparison of the standard method with the non-destructive method for detection of Bonamia by PCR.

		Standard method		
		Positive	Negative	Total
Non-destructive method	Positive	40	1	41
	Negative	11	138	149
	Total	51	139	190

sample using a Eukaryotic 18S rRNA Endogenous Control mix (Applied Biosystems). The threshold cycle (Ct) value, an increase of fluorescence above the background, was calculated by the Sequence Detection Software version 1.5 of the 7500 System Software (Applied Biosystems).

2.5 Genotyping of SNP markers associated with *Bonamia* tolerance

The 22 SNPs previously reported to be consistently associated with divergent selection for *Bonamia* tolerance (Vera et al., 2019) were evaluated to be genotyped in a single multiplex using a MassARRAY platform (Sequenom, San Diego, CA). Briefly, the technique consists of a two-step reaction: The first involves the PCR amplification of an amplicon which includes the selected SNP (i.e. locus-specific PCR reaction) and the second a single-base extension using mass-modified dideoxynucleotide terminators of an oligonucleotide primer that anneals immediately upstream of the polymorphic site (i.e. SNP) of interest (Oeth et al., 2009; Ellis and Ong, 2017). To design the set of primers for MassARRAY genotyping, the oligo-nucleotides of the oyster chip, where the SNPs are located (Gutierrez et al., 2017), were mapped in the flat oyster genome recently assembled and annotated (Gundappa et al., 2022; NCBI Bioproject: PRJNA772111). Then, regions of ±100 bp flanking the selected SNPs were retrieved and used for primer design (Supplementary Table 1). After *in silico* primer design evaluation, 18 SNPs could be included in a single multiplex reaction harnessing the distinct mass of extended primers adjacent to the SNP position of different mass (Supplementary Table 1). MALDI-TOF mass spectrometry analysis in an Autoflex spectrometer was used for allele scoring. This genotyping was conducted at the UCIM-University of Valencia Genomics Platform to validate 2b-RAD genotyping.

2.6 Analysis of genetic structure in the sample collection

The number of different genetic clusters (K value) in the sampling collection, composed by 381 individuals from seed produced by RvY (192) and the seed produced by NIOZ (189) collected during the challenge test in April 2021, was evaluated with STRUCTURE v 2.3.4 (Pritchard et al., 2000), a program that analyses linkage disequilibrium and Hardy-Weinberg equilibrium following a Bayesian clustering approach. For each of the possible number of clusters tested (from 1 to 10), the R package ParallelStructure v 1.0 (Besnier and Glover, 2013) was used with a burn-in of 100,000 iterations and 200,000 Markov Chain Monte-Carlo steps, as well as ten independent replicate runs to increase accuracy. To identify the most likely number of clusters (K), two different K estimators were used: deltaK (Evanno et al., 2005) and Mean LnP(K) (Pritchard et al., 2000). StructureSelector web-based software (Li and Liu, 2018) was used to obtain K estimators and CLUMPAK graphical outputs (Kopelman et al., 2015). This analysis was performed with the 16 most consistently related to bonamiosis tolerance SNPs.

3 Results

3.1 Screening method

In order to test whether the non-destructive method of sampling for *Bonamia* is comparable in sensitivity to the standard analysis (including a piece of gill, mantle and digestive tissue), as described above, 190 Lake Grevelingen oysters were tested with both methods. *Bonamia* genetic material was detected in 40 individuals out of the 190 oyster for each method: the standard sampling method and the non-destructive sampling method (Tab. 1). However, both methods had positive samples which were not detected by the other method. It should be noted that in all positive oysters only low amounts of *Bonamia* genetic material was detected (real time PCR Ct values $\geq$ 32), which might have caused the false negatives. It can be concluded that the non-destructive sampling is comparable for detecting a *Bonamia* infection.

The non-destructive screening of broodstock oysters identified 8–32% of the tested oysters as *Bonamia* infected (Tab. 2). The oysters that tested *Bonamia* negative were used to produce larvae and seed. All larval and seed batches were *Bonamia* free (Tab. 2).

3.2 Survival and growth in a *Bonamia*-infected area

For the first 8–12 weeks in both 2020 and 2021 survival was close to 100% for both origins (Fig. 1 and Supplementary Table 2). In 2020, survival of the *Bonamia*-naïve offspring (NIOZ) rapidly decreased between 19 May and 7 August, with only 25% survival. After that sampling date survival increased again. The survival of the pre-screened *Bonamia*-free broodstock (RvY) oysters also decreased, but much more gradually. For these oysters survival at the end of the challenge experiment was 73% (Fig. 1). In 2021, survival at the end of the experiment was higher than 2020 with 92% for the RvY oysters and 54% for the NIOZ oysters. In 2020, the highest mortality occurred in July, while June showed the highest mortality in 2021. The offspring of the pre-screened *Bonamia*-free broodstock (RvY) showed significantly higher survival than the offspring of *Bonamia*-naïve broodstock (NIOZ) in the second part of the season in both years (Fig. 1). The initial size

Table 2. Overview of number of broodstock oysters screened at Wageningen Marine Research; number of oysters without Bonamia infection used in hatcheries (RvY = Roem van Yerseke and ZS = Zeeschelp); negative Bonamia results for larval batches released and seed produced by these pre-screened broodstock oysters. Number of pools for pooled samples is presented in parenthesis.

Screening date	Total # oysters	# Neg	Sampling date	# Larvae batches	# Neg	# Seed	# Neg	Hatchery
30 April 2018	149	137	June–August 2018	9	9	(2)	(2)	RvY
30 April 2018	149	137	28 July 2019	12	12	(3)	(3)	RvY
7 October 2019	200	136	–	–	–	–	–	RvY
11 May 2020	145	105	June and July 2020	9	9	240 (56)	(56)	ZS
12 April 2021	182	163	13 July 2021	–	–	145 (27)	(27)	RvY
17 May 2021	180	149	August 2021	–	–	150 (30)	(30)	ZS

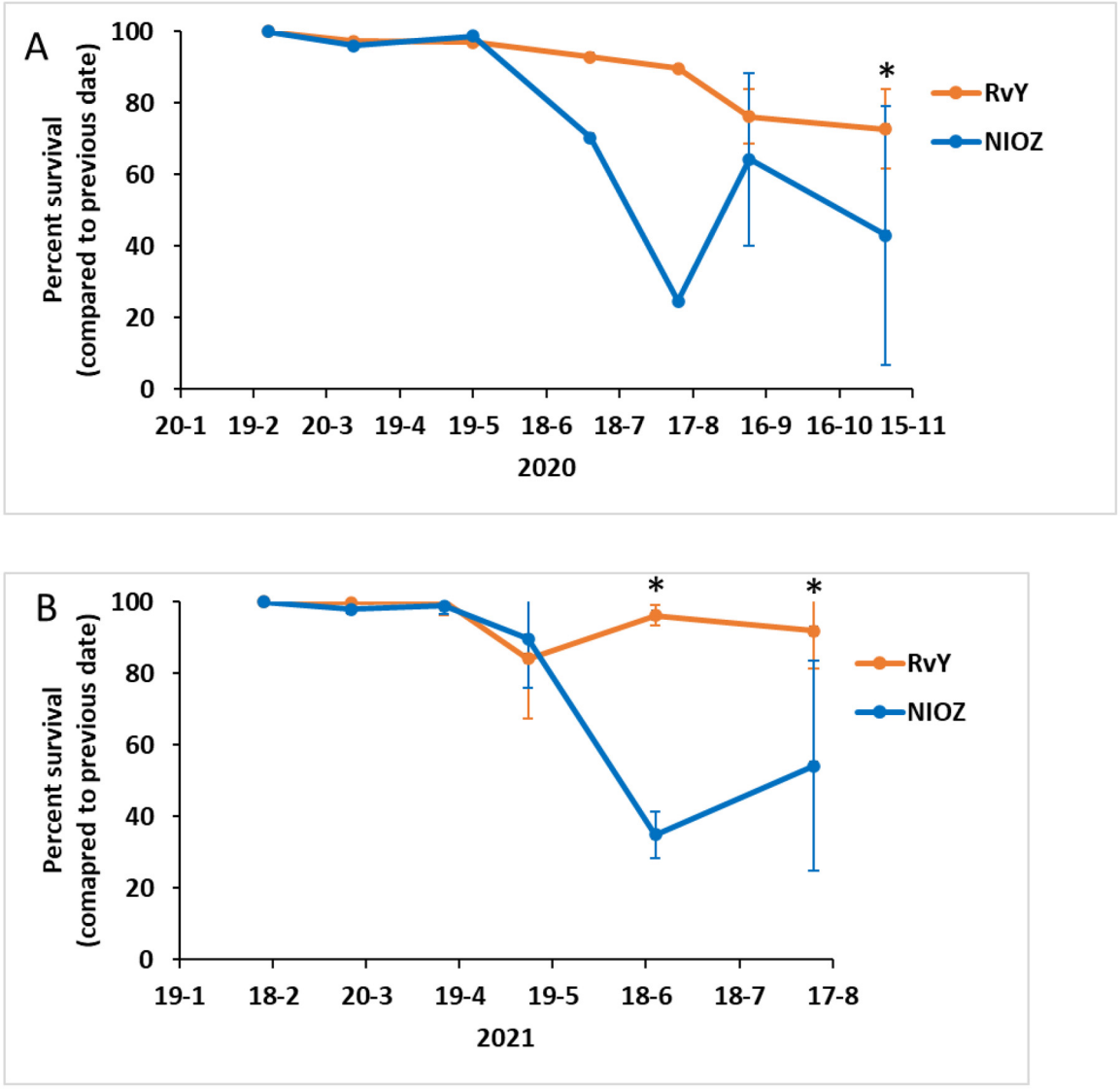

Fig. 1. Percentage survival with SD compared to previous date of hatchery produced *O. edulis* from two broodstock origins (pre-screened Bonamia free = RvY and Bonamia naïve = NIOZ) in 2020 (A) and 2021 (B). From February until August 2020 the two groups were kept in the harbour of Yerseke (Oosterschelde) in one basket each. From August onwards 1 to 7 baskets per group with two compartments each were placed in Lake Grevelingen. * = significant difference between the two groups, $P < 0.05$.

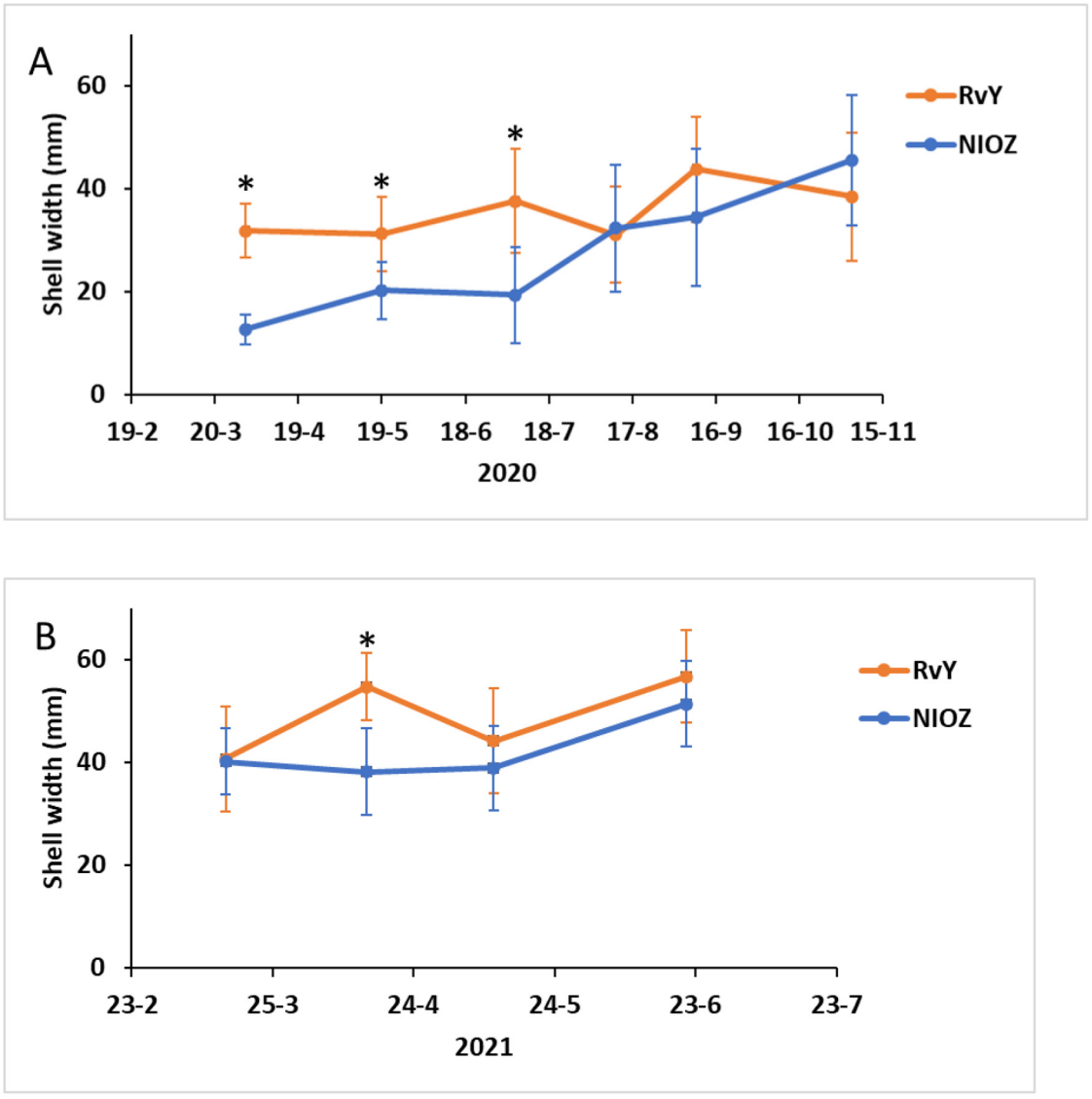

Fig. 2. Average shell width with SD ($n = 30$) of hatchery produced *O. edulis* from two broodstock origins (pre-screened Bonamia free = RvY and Bonamia naïve = NIOZ) in 2020 (A) and (2021 (B). From February until August 2020 the two groups were kept in the harbour of Yerseke (Oosterschelde). From August onwards they were placed in Lake Grevelingen. * = significant difference between the two groups, $P < 0.05$.

of the NIOZ oysters was significantly smaller than the RvY oysters (Fig. 2). However, from August 2020 onwards the average sizes were comparable, except for April 2021, when RvY oysters were larger.

3.3 SNP markers associated with *Bonamia* tolerance

The genotypes of the 381 individuals studied for the 18 SNP markers are shown in Supplementary Table 3. Nine individuals (five from RvY and four from NIOZ) showed missing genotyping call in ≥ 6 SNPs (corresponding with ≥ 1/3 of molecular markers analysed) and they were discarded. Therefore, the number of individuals used for population genetics analyses was finally 372.

Following the deltaK method proposed by Evanno et al. (2005) and the Mean LnP(*K*) described by Pritchard et al. (2000), the best *K* value in the samples analysed was 2. The *Bonamia*-free sample, coming from the seed produced with pre-screened broodstock from a long-term affected area by RvY (187 individuals: 48 "orange"/139 "blue"), showed much higher proportion of individuals belonging to the "orange" cluster than the sample coming from seed produced with broodstock from a naïve area by NIOZ (185 individuals, 7 "orange"/178 "blue") and the difference was highly significant (exact test *P*-value < 0.00001) (Fig. 3).

3.4 *Bonamia* presence in challenged oyster

Samples were analysed for presence of *Bonamia* at three sampling dates over a period of 8 months. All samples were negative for *Bonamia* (Tab. 3). This is surprising as it was expected that the NIOZ group, coming from a naïve area and

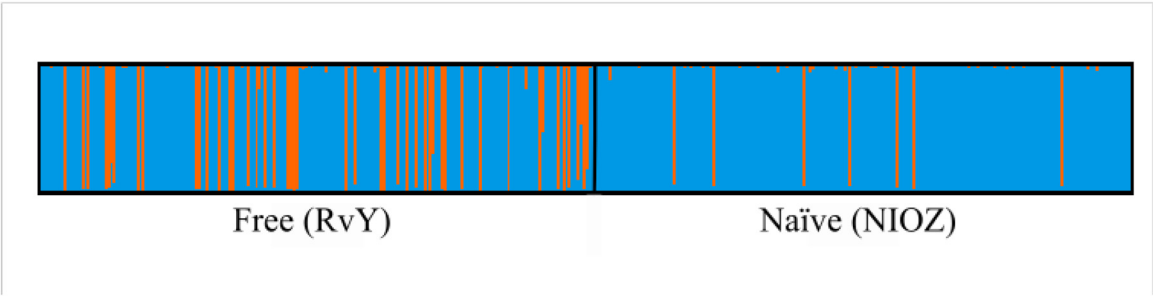

Fig. 3. STRUCTURE plot using the 18 SNP markers associated with resilience to bonamiosis in the sampled individuals from seed produced with pre-screened broodstock from a long-term affected area by RvY and the seed produced with broodstock from a naïve area by NIOZ collected during the challenge test in April. Each vertical bar represents one individual, and the colour proportion for each bar/individual represents the posterior probability of its assignment to the different clusters (K) inferred by the program. Individuals pertaining to these two clusters, "orange" and "blue", showed most of them a full "orange" or "blue" constitution, and further, they appeared intermingled in both samples at very different frequencies.

Table 3. Results of Bonamia analysis of hatchery produced O. edulis from two broodstock origins (pre-screened Bonamia free = RvY and Bonamia naïve = NIOZ) challenged in Lake Grevelingen.

Sampling date	RvY (n = 30)	NIOZ (n = 30)
4 November 2020	Negative	Negative
14 April 2021	Negative	Negative
21 June 2021	Negative	Negative

with a lower percentage of genetic variants associated with *Bonamia* tolerance, should be expected to become infected with *Bonamia*. As a result, a relationship between individual presence, or absence, of markers and level of *Bonamia* infection could not be established.

4 Discussion

The results showed that the non-destructive screening method provides information on the status of the oyster with regard to *Bonamia* infection. In addition, *Bonamia* negative broodstock always produced *Bonamia* negative larvae and seed. De Melo et al. (2021) produced disease free seed of Pacific oysters (*Crassostrea gigas*) through after spawn testing of broodstock. Unlike *C. gigas*, *O. edulis* cannot be strip spawned. Therefore, a non-destructive method was developed. This provides opportunities for *Bonamia*-free hatchery production with broodstock from a *Bonamia*-infected area. A prerequisite is that production takes place in a *Bonamia*-free zone of the hatchery, as Flannery et al. (2016) suggested that larvae may be able to acquire the pathogen from the water column during filter feeding. Water treatment in the hatcheries involved filtration (5 μm) and UV.

The challenge experiment with pre-screened *Bonamia* free and *Bonamia* naïve flat oysters showed that the survival of the naïve group was less than the *Bonamia*-free group. In addition, the naïve group showed a significantly lower proportion of the "orange" cluster associated with *Bonamia* tolerance in previous studies (Vera et al., 2019; Sambade et al., 2022). However, none of the oysters became infected with the parasite during exposure in Lake Grevelingen according to the PCR test performed. There are some hypotheses that could explain this inconsistency. Firstly, the parasite was not present in the area. This seems unlikely since the area is long-term affected (Fig. 4). In 2020, no sampling was performed due to COVID-19 restrictions, but in 2021 *Bonamia* was detected in the oysters during the regular sampling program indicating that the parasite is still present in the area. Secondly, the test sensitivity could be too low. However, the PCR methodology itself is very sensitive, and the controls used in the DNA extraction and PCR test did not show any failures or reduced sensitivity. Thus it would be expected that if *Bonamia* was present it should have been detected, also from weakly infected oysters. Thirdly, the oysters did not contract the disease. It is largely unknown when *B. ostreae* disperses to infect other oysters. The naïve oysters were introduced in Lake Grevelingen in two batches and contraction of the disease perhaps took place in the period that naïve oysters were not present (7 December 2020 to 5 February 2021). Furthermore, it is unknownhow long it takes for the oysters to become infected and how, once infected, the proliferation of the parasite progress. From cohabitation experiments in which naive oysters are exposed to (heavily) infected oysters can be deduced that it takes several months before the parasite can be detected (Culloty et al., 2004; Lynch et al., 2005; Lallias et al., 2008; Ronza et al., 2018). The naïve oysters were deployed in Lake Grevelingen for four months in 2020 (August–December) and in 2021 for 6 months (February–August). This time may not have been long enough to get infected and/or development of the infection to a detectable level. Normally spawning stress causes more mortality in infected oysters (Engelsma et al., 2010), probably leading to increase *Bonamia*-free oysters as a result of dying. The question is if this is coinciding with the (yet unknown) time that *Bonamia* spreads. As shown in Figure 4 the percentage of infected *B. ostreae* infected flat oysters in 2021 was 26%. This was however the overall results from Lake Grevelingen. But this spread is not homogeneous, between the sites the distribution is more patchy. This might have

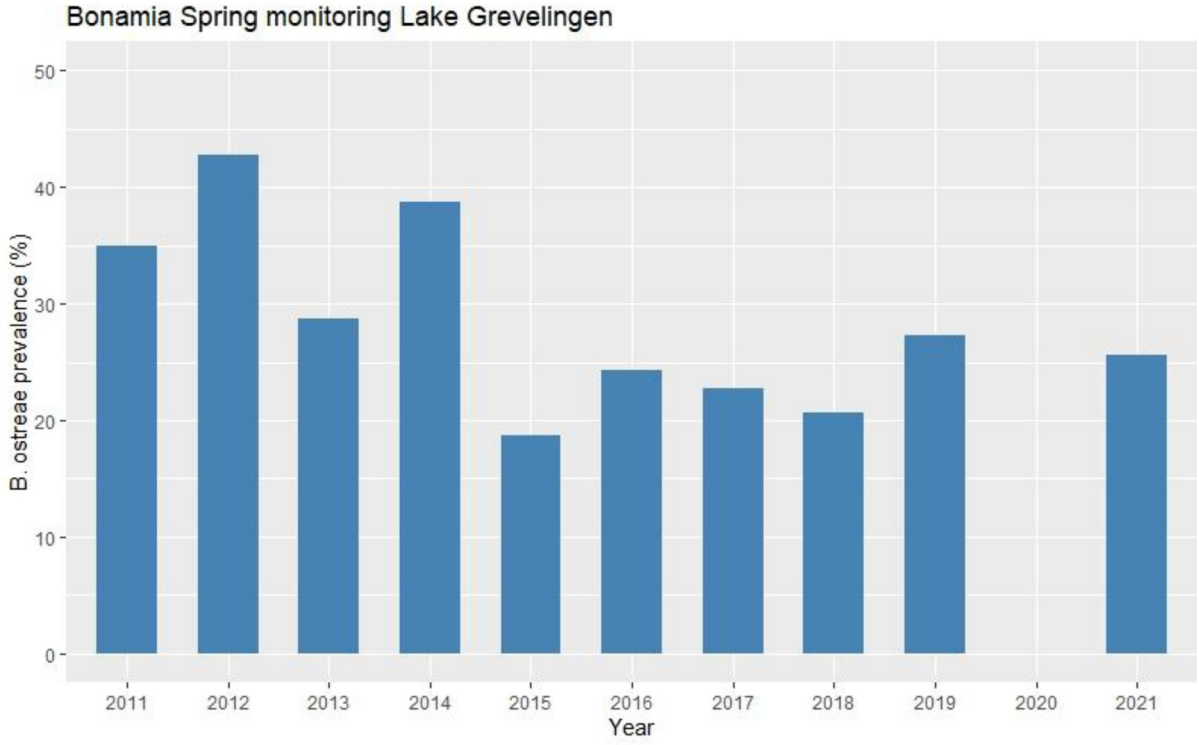

Fig. 4. Bonamia ostreae prevalence (%) in the Ostrea edulis population in Lake Grevelingen during the annual spring monitoring. Due to COVID-19 restrictions no sampling was carried out in 2020.

contributed to a lack of infection in the challenge population. Also keeping the oysters in suspended culture above the bottom may have reduced the likelihood of getting infected.

Our conclusion is that the much higher mortality of the naïve oysters compared to the prescreened oysters was most likely caused by other factors such as a potential difference in condition at the start of the experiment.

The analysis of the genetic markers is a follow-up of the research of Sambade et al. (2022). In that study, the same groups of hatchery-produced seed from screened *Bonamia*-free broodstock and seed from *Bonamia*-naïve broodstock (from the Dutch Wadden Sea) were analysed for the potential association of the consistent outlier SNP loci reported by Vera et al. (2019) with bonamiosis resistance. In addition, the screened *Bonamia*-free broodstock that produced the seed was also analysed. The pre-selected *Bonamia*-free oysters and seed showed a higher percentage of individuals with the tolerance genetic constitution ("orange"), while the naïve oyster seed showed a lower percentage. The present study also detected a significant lower percentage of "orange" individuals in the naïve group as compared to the offspring from screened *Bonamia*-free broodstock. The higher percentage of "orange" genotypes associated with *Bonamia* tolerance of pre-screened *Bonamia*-free seed is corroborated by a higher survival of that group. These results give additional support to previous findings on the association of a specific genomic region with *Bonamia* tolerance (Sambade et al., 2022), but at the same time they cannot discard that other genomic regions could be involved on tolerance since association was detected at the population level. Unfortunately, none of the oysters tested positive for *Bonamia*. Thus, the desired quantitative information on parasite load could not be correlated with genotyping information in oysters from the field challenge, information which is crucial to establish the association at individual level. Further challenge tests are needed before firm conclusions regarding the genetic markers can be made.

If it is assumed that higher survival of pre-screened *Bonamia*-free seed is related to the higher percentage of the "orange" constitution related to bonamiosis tolerance, the question remains whether this is resistance or tolerance. Holbrook et al. (2021) consider disease-resistance to be reduced susceptibility to infection by the parasite, or active suppression of the parasites ability to multiply and proliferate. They define disease-tolerance as the retention of fitness and an ability to neutralise the virulence of the parasite. Tolerance also includes the possibility of maintaining health despite being infected. Disease-resilience is identified as the ability to recover from illness. Råberg et al. (2008) states that resistance should reduce the prevalence of the parasite in the host population (causing selection in both the parasite and the host), while tolerance should have a neutral or positive effect on parasite prevalence. Since *Bonamia* prevalence in Lake Grevelingen is not declining over time, the higher survival and presence of candidate genetic variants found in the pre-selected *Bonamia*-free oysters compared to the naïve oyster indicate tolerance and not resistance.

Producing *Bonamia*-free oysters with broodstock originating from a *Bonamia*-infected area provides disease-free

oysters that may also have developed tolerance to the disease. This is very useful for restoration projects since transfer of diseases is not wanted, but protection against disease is desired, in case it does show up in a newly established bed.

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Author contribution statement

Pauline Kamermans: Conceptualization, Methodology, Funding acquisition, Investigation, Supervision, Formal analysis, Writing – Original draft preparation. Ainhoa Blanco: Methodology, Investigation, Writing – Review & Editing. Pim van Dalen: Methodology, Investigation. Nienke Bakker: Resources. Marc Engelsma: Validation, Investigation, Formal analysis, Writing – Original draft preparation. Pascalle Jacobs: Resources, Writing – Review & Editing. Marco Dubbeldam: Resources. Inés M. Sambade: Investigation, Formal analysis, Writing – Original draft preparation. Manuel Vera: Investigation, Formal analysis, Writing – Original draft preparation. Paulino Martinez: Investigation, Supervision, Writing – Original draft preparation. The authors declare no conflict of interest.

The Ministry of Agriculture, Nature and Food safety, PRW and DRN were not involved in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Supplementary Material

Supplementary Table1. Information about SNPs markers used in the present study. Name of the SNP marker within Sequenom multiplex (SNP marker), name of the marker used by Vera et al. (2019) (Vera et al. IDs), genomic region where marker is located (Sequences, SNP marker and its variants are shown between brackets), left primer, right primer and extension primer for the Massarray Sequenom multiplex are shown. The SNPs removed are highlighted in red.

Supplementary Table 2. Number of oysters counted in BST baskets attached to a FLUPSY or to a frame above a bottom plot (numbered compartments) and the number of oysters collected for analysis in 2020 (A) and 2021 (B).

Supplementary Table 3. Genotyping from the 381 initial individuals. "-" indicates no genotyping call. The criteria for eliminating individuals was 6 or more SNPs markers with no genotyping call. Individuals removed from genetic analyses are shown in red.

The Supplementary Material is available at https://www.alr-journal.org/10.1051/alr/2023005/olm.

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