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Successful rearing of *Ostrea edulis* from parents originating from the Wadden Sea, the Netherlands

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ABSTRACT

Since 2017, the European flat oyster (*Ostrea edulis*) reappeared in the Wadden Sea after decades of absence. In several countries, restoration and reinforcement projects have been initiated. Better insight in *Ostrea* behaviour is needed to ensure sustainable conservation in the future. In order to acquire substantial numbers of oysters for eco-physiological and transplant experiments without threatening the small local population, indoor rearing facilities were set-up at Royal NIOZ. Here, a broodstock of 38 adults originating from the western Wadden Sea, produced over 21 million larvae in 10 batches within 3 months (Sept-Nov 2018). Release of larvae and rearing took place at a water temperature of 19.7 ± 0.6 °C and a salinity of 30.5 ± 0.5 PSU. Outbreaks of *Vibrio* bacteria did not occur. The growth rate before settlement was 1.5–1.7 μm per day, which was at the low end of growth rates reported in previous studies. The first competent larvae were observed 17–22 days after release. Size at settlement varied between 255−325 μm. Survival until settlement was 0.96−0.98 per day. After settlement, growth rates increased to 30−47 μm per day, with batches that were released in September having a significantly higher growth rate and initial size compared to larvae that were released later. Mortality from release to the end of the experiment (Feb 2019) was 0.04−0.05 per day. A cryopreservation trial did not result in living larvae after thawing, most likely due to their advanced development when preserved. The larvae and juveniles were negatively tested for the parasite *Bonamia ostrea* and can thus be used in oyster conservation and restoration projects. Recommendations for future breeding attempts include the use of a higher water temperature and slightly higher food concentrations.

1. Introduction

With a world population that is expected to reach 9.7 billion people by 2050 (United Nations, 2019), an increase in sustainable aquaculture of shellfish is considered one of the ways to feed humankind (Food and Agriculture Organization (FAO), 2018). Shellfish aquaculture generally makes use of recently settled juveniles ('spat') collected from the wild as starting material. With natural populations of shellfish becoming increasingly depleted due to over-exploitation, aquaculture more and more relies on hatcheries to provide these juveniles (Dubert et al., 2017). Next to contributing to food production, shellfish hatcheries can also play a role in providing material to restock decimated or locally extinct populations (Grant et al., 2017).

The native oyster, *Ostrea edulis,* can be considered as a key stone species in marine ecosystems because it provides habitat, shelter and food for many other species and improves water clarity by suspension feeding. Historically, oysters have been a highly valued food source for humans as well (Lotze, 2007). In western Europe, large areas of the North Sea, the Wadden Sea, and the coastal zones were covered by *Ostrea edulis* at the end of the 19th century (Gercken and Schmidt, 2014; Olsen, 1883). Stocks declined since the 1920s, this downturn has been attributed to over-exploitation and cold winters (Laing et al., 2006). At present, this species is relatively rare in western Europe. Factors that have been identified as potentially hampering restoration and re-enforcement of self-sustainable oyster populations include the presence of the parasite *Bonamia ostrea* and lack of suitable settling substrate due to habitat degradation (Bromley et al., 2016).

Restoration projects have been initiated in many countries including

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the UK, France, Germany, Sweden (Native Oyster Restoration Alliance, 2019). In addition to diseases and habitat destruction, recovering or reintroduced populations have to deal with new challenges. Climate change results in increasing ocean temperatures, and in coastal seas, in changing salinity and turbidity, dramatically altering marine ecosystems (Harley et al., 2006). In addition, the presence of new invasive species, including the Pacific oyster, *Magallana gigas* (formerly known as *Crassostrea gigas*) (World Register of Marine Species, 2020; Zwerschke et al., 2017), pose a potential threat to the native *Ostrea edulis*.

In western Europe, the Pacific oyster was first used in aquaculture in the 1960s and rapidly spread throughout northwestern Europe (Troost, 2010). Initially, it was not expected that the presence of the Pacific oyster could hamper the recovery of *Ostrea edulis* since the first species mainly occurs in the intertidal while *Ostrea* was confined in the past to the subtidal (Reise, 1998). Both species are reported to co-occur (Bodvin et al., 2010; Zwerschke et al., 2017) and competition for food and space between the two species is very likely (Bodvin et al., 2010; Trimble et al., 2009). In addition, the Pacific oyster might act as a carrier or reservoir for the parasite *Bonamia ostrea*, without suffering from the disease itself (Lynch et al., 2010).

The life cycle of *Ostrea edulis* differs from most other bivalves. The native oyster is a protandric hermaphrodite; the animals start their reproductive life as male and then spawn alternatingly as female and male, with sex-changes that can occur within a season (*in* FAO, 2018). Spawning is stimulated at temperatures above 15 °C, although for some southern populations spawning is said to commence at temperatures as low as 12−13 °C while in Norwegian fjord temperatures has to be as high as 25 °C (*in* FAO, 2018). Eggs are released into the pallial cavity of the female where they are fertilised by externally released sperm and incubated on the gills. The number of eggs per female increases with size and is estimated to be $0.1–1.5 \times 10^6$ larvae per female (Cole, 1941; Walne, 1964).

Fertilised eggs are incubated for 8–10 days before being released into the water column. Larvae remain pelagic until are competent to settle. By then the larvae, now called pediveligers, have a so-called eyespot and a foot (Mesías-Gansbiller et al., 2013) enabling them to descend and find suitable settling substratum. The development from release to competence depends on the available food and water temperature (Robert et al., 1988). Once settled, the larvae undergo metamorphosis. During this time, the larvae do not feed, and their feeding modus changes from a velum into gills (Bayne, 1971). Arzul and colleagues (2011) demonstrated that already at the larval phase *Ostrea* can become infected with *Bonamia ostrea,* suggesting vertically transmittance from adult to offspring during the incubation period. During the pelagic phase of the larvae infected larvae can thus contribute to the spread of the parasite (Arzul et al., 2011).

Until recently, European oysters only occurred in the southwestern part of the Netherlands (in the Oosterschelde estuary, Lake Grevelingen and Lake Veere) where *Bonamia* is prevalent (Engelsma et al., 2010). In 2015, oysters were discovered to be present nearby in the coastal part of the Dutch North Sea (Christianen et al., 2018). In 2017, the presence of several *Ostrea edulis* individuals was reported for the Dutch Wadden Sea (Van der Have et al., 2018) for which the disease-status is unknown. In 2018, 6000 kg of adult disease-free European oysters, originating from Norway, were launched in the North Sea as part of pilot projects (World Wildlife Fund, 2018). Both in the southwestern part of the Netherlands as in the Wadden Sea, Pacific oysters are numerous (Fey et al., 2010; Smaal et al., 2009).

In the Netherlands, there are initiatives to couple the construction of wind turbines with the building of native oyster reefs in the Dutch part of the North Sea. There is a general agreement that only disease free (or resistant) oysters are to be used in such projects. There is thus an urgent need for oysters free of *Bonamia* to supply these initiatives. At the same time, a better understanding is needed of how *O. edulis* is coping in a modified and rapidly changing environment. This information can be gained from controlled laboratory and field experiments. To obtain animals for experiments without threatening the small local population in the Dutch Wadden Sea, indoor rearing facilities were set-up at Royal NIOZ, Texel, The Netherlands. Better insight in *Ostrea* behaviour increases the chance of success of restoration projects and is needed to ensure sustainable conservation of this species in the future.

2. Methods

2.1. Hatchery & nursery conditions

All activities described in the current study took place in a temperature and light controlled room ('climate-room') at the NIOZ Royal Netherlands Institute for Sea Research on Texel (Fig. 1). The seawater that was used was sourced locally and passed through a series of sand filters inside the building before entering the climate-room. The sand filters removed most of the algae and no further water treatments were applied.

Inside the climate room, 4 conical tanks (180 L each) were placed. These tanks were used to rear the newly released larvae (paragraph 2.3). Seawater flowed through these tanks, renewing the water volume 3 times a day. From the bottom of the tank, air bubbles gently aerated the water and kept larvae in suspension. Tanks were cleaned 3 times a week as described below.

In addition to the conical tanks there was one tank (1500 L) containing the broodstock, one 'downwelling' tank to contain competent larvae (1500 L) and an 'upwelling tank' (1500 L) for settled juvenile oysters. In the broodstock tank, the oysters were placed in a shallow container on top of the tank, by means of an airlift the water was recirculated over the oysters.

Seawater supply resulted in a turnover time of the tank of once a day. In both the down-as the upwelling tanks an aquarium pump (Eheim 1260210, capacity of 3400 L h^{-1}) was placed to direct the water flow down or up respectively. To maintain a fully mixed tank, two additional pumps (Sicce Voyager 3) per tank were placed.

Water temperature, oxygen, pH and salinity were measured regularly using a Hach multi-parameter meter. Breakdown of oyster faeces and algae can result in enhanced ammonia concentrations, which can potentially be toxic for oysters. Ammonia is converted into nitrite and nitrate by specialised bacteria. The concentration of NO_2 and total ammonium ($NH_4 + NH_3$) was checked on a regular basis, generally, before tanks were cleaned (COLOMBO test). Concentrations were estimated based on a colour change of the water after adding reagents. The range of concentrations of the test were between 0−2 mg L^{-1}, with steps of 0.25 for N-NO_2 and 0−5 mg L^{-1} with steps of 0.25 for N-NH_{total}. Apart from the conical tanks, all other tanks were cleaned weekly applying a strict cleaning regime that included the use of diluted, commercially available, chlorine. In addition to the tanks, food containers and tubes were also cleaned. After cleaning, all material was rinsed with fresh water and allowed to dry completely before use.

2.2. Broodstock

Native oysters were found in the 'Eijerlandse gat' near the east coast of the island Texel, the Netherlands (Fig. 1) and brought to the laboratory between July to October 2017. In total 38 oysters were collected, the shell length of the oysters ranged between 59 and 130 mm. During this time, the oysters were maintained in flow-through aquaria at 18 °C. They were fed commercially available frozen algae (Acuinuga) in a ratio of 6 % of dry flesh mass in dry mass of algae per day (% DW/DW) (Utting and Millican, 1997). Dry mass of oyster flesh was estimated using a length- dry mass relation reported for *Magallana gigas* (Kobayashi et al., 1997):

$$DW = aL^b \quad (1)$$

DW is dry flesh mass in g, L is shell length in mm and the constants

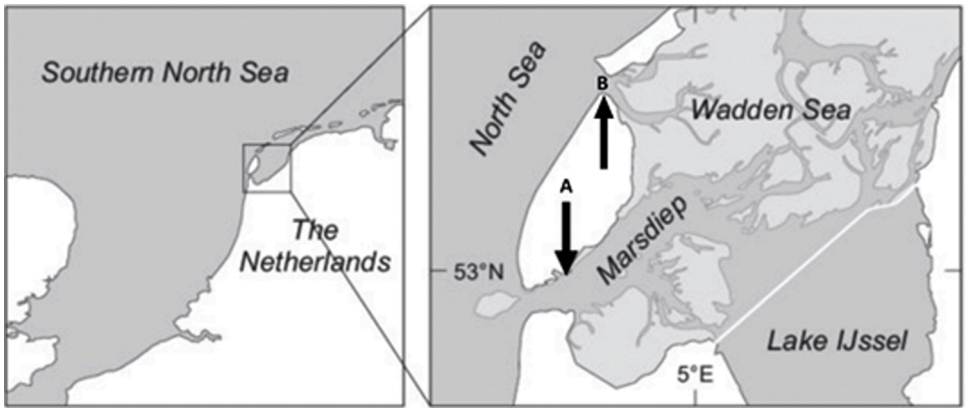

Fig. 1. Map of the area, including the location of the Royal NIOZ (A) and the Eijerlandse gat (B). Figure adapted from Brandsma et al. (2012).

$a = 3.0 \times 10^{-7}$ and $b = 3.45$.

Clearance rates at *in situ* temperatures were estimated following the relation between flesh dry mass and clearance reported by Haure et al. (1998):

$$CR = cDW^{d} \quad (2)$$

In this relation CR is the individual clearance rate (L h^{-1}), c = 0.423 and d = 0.921 are constants.

In October 2017, the water- and room temperature was lowered to 12 °C and the food ratio to 3% DW/DW. Previous research showed that under these conditions oyster gonad development and maturation takes place while shell growth ceases (Utting and Millican, 1997). Pre-conditioning of the broodstock started at the beginning of August 2018 by increasing room- and water temperature to 18 °C in 2 weeks and raising the food concentration to 6 % DW/DW in the same period.

A mixture of fresh live algae obtained from 'Foundation Seashell' replaced the commercial food. Algae were grown as continuous monocultures in plastic upright bags of 260 L, under natural light and room temperatures in a greenhouse. Seawater was filtered, pasteurised and enriched with CO_2 and L_1 medium. Algae were then mixed in the approximate ratio of *Chaetoceros calcitrans/muelleri* (20 %), *Skeletonema costatum* (20 %), *Thalassiosira weisfloggi* (10 %), *Isochrysis galbana* (20 %), *Rhodomonas balthica* (20 %) and *Tetraselmis suecica* (10 %). This mixture of species should provide all essential polyunsaturated fatty acids (PUFA) and sufficient protein important for both conditioning of broodstock as well as larvae growth (*e.g.* Castell, 1986). Algae were centrifuged and transported in a cooled container to Texel weekly, and stored immediately at 4 °C on arrival.

Cell counts were performed using a Neubauer hemocytometer. Dry weight of each batch of algae was established by filtering a known volume of algae mixture over a pre-weighted Whatman GFF filter. The filters were dried (12 h, 60 °C) and re-weighed. Algae were automatically dispensed into the oyster tanks in a semi-continuous mode of two hours on, two hours off. In the climate room, the light was on for 16 h, and off for 8 h, as soon as there were competent larvae present, during the 16 h of light the room was only dimly lit.

When the temperatures had reached 18 °C, a sieve (100 μm) was attached under the outflow of the oyster tank to capture larvae when released. The sieve, which was submerged in water, was checked and cleaned once a day, as soon as the first larvae were collected the sieve was checked twice a day. On 7 September 2018, four weeks after the conditioning of the adults had started, the first larvae were detected. In first week of December, the water temperature was lowered from 18 to 15 °C. The last larvae were released on 11 December 2018.

2.3. Larvae & juvenile oysters

Released larvae were retrieved on a sieve. Each time the sieve was emptied, a subsample was counted using a modified Bogorov counting chamber. The presence of larvae was erratic, in first instance larvae were placed in the same conical tank until the capacity of 1×10^6 per tank was reached. Larvae here were different in age. Later, only larvae that were released within two days of each other were placed in the same tank. These larvae were of comparable age. All larvae in a tank were called a 'batch'.

The larvae were given the same food as the brood stock, but food concentration was based on cell numbers. Cell densities aimed for were 20.000 algal cells per ml. The cell densities in the tank were not counted, the supply rate was set equal to the loss of algae through renewal of seawater, resulting in a continuous concentration.

Once a week, larvae were counted and for a subsample (± 25) shell length was measured using an invert microscope (Zeiss Axioplan 2) with a measuring ocular when the larvae were small, and a binocular (Zeiss 2000) with a measuring ocular when larvae were bigger (± 1 mm). The number of larvae with a visible foot and or eyespot was recorded. Mobility and the presence of full stomach and faeces was examined since this is considered as a sign of good health/metabolism (M. Dubbeldam, pers. comm.).

Competent larvae were transferred to the 'downwelling' tank. In the 'downwelling' tank, larvae were placed in a sieve (250 μm) with at the bottom a thin (1 mm) layer of settlement material consisting of fine shell fragments (300 μm) (microbrisure, 'Entre Mer et Terre', France). This shell material in the sieve was placed in the downwelling tank approximately one week prior to the expected placement larvae. The decision to place the larvae in a settlement tank was made based on a percentage of larvae with an eyespot >50 %. Since larvae were already crawling on the wall of the conical tank, for the next batches the decision to transfer was made earlier, when 25−30% of the larvae had an eyespot.

Once settled, larvae were regularly counted and measured again. As they grew, the mesh size of the sieves also increased, minimising clogging of the sieves (250−600 μm). For the same reason, the oldest batches were subsequently transferred to the upwelling tank in sieves with a larger mesh size (800−1000 μm). In both the downwelling and the upwelling tank, algal cell densities provided were 40.000 cells ml^{-1}. In the first week of February 2019, the oysters were counted and measured for the last time.

Ten batches of larvae were distinguished (A–J) (Table 2), the first batch was sacrificed for *Bonamia* testing as soon as there was a new batch of larvae. Batch B and C were sieved over 250 μm sieve on 5 October 2018, all larvae smaller than 250 μm were redefined as batch D. When the batches H–J were released, the conical tanks had already reached their maximum capacity of 1 million larvae per tank. These newly released larvae were placed in one of the water reservoirs, provided with food and two seawater pumps. Because the survival of batch H was extremely low, it was decided to sacrifice the larvae of batch I for *Bonamia* testing rather than to attempt to keep them alive. With

exception of the larvae that were released on 11 December 2018, the larvae of batch J were used for the cryopreservation trial (Section 3.4).

After release into the water, the larvae were counted and measured before placement into the settlement tank only (batches B–D) or on a regular basis (batches E–G). A growth rate before settlement could therefore be calculated only for the batches E–G. For batches B–C, larvae were considered 'competent' when >50 % had an eyespot. The growth rates after settlement were calculated for a period of 50 days, from the beginning of settlement.

It was assumed that growth (shell length) before settlement was linearly related to time (days) according to: shell length = a*day + b +ε, in which *a* is the slope (or growth rate (μm day^{-1}), *b* is intercept (or estimated length after release (μm) and ε an error term. Whether there were differences in growth rates between batches was investigated by comparing different linear models for which 1. all batches have the same slope and intercept, 2. batches differ in their intercept, 3. batches differ in their slope and intercept. Models were compared using ANOVA's (R Core Team, 2018), with $p < 0.05$. Size data after settlement was ln-transformed before analysis.

Mortality (M) and survival (S) rates per day were calculated according to Eqs. (3) and (4) respectively:

$$M = \ln\left(\frac{N_{end}}{N_{start}}\right) \Big/ - t \quad (3)$$

In Eq. (3), *N* is the number of larvae counted per batch at the *start* of a period (N_{start}) and the *end* of a period (N_{end}), *t* is the time in days between start and end.

$$S = 1 - M \quad (4)$$

2.4. Cryopreservation trial

A first trial was performed, using readily available chemicals and instruments, to test the possibility of cryopreservation of *Ostrea* larvae. The test protocol was based on published methods on cryopreservation of *Magallana gigas* oyster larvae (Labbé et al., 2018; Suquet et al., 2014).

Here, two freezing times (2 and 4 h in −80 °C), two preservatives (DMSO and Glycerol) and two concentrations per preservatives (15 and 30 %) were applied, with 4–5 vials per treatment. Each vial contained seawater and a preservative, with a total volume of 2 mL, and a final concentration of larvae of 5.000 mL^{-1}.

For this trial, newly released larvae were sieved over 100 μm, counted and mixed with 0.2 μm-filtered seawater. The larvae were gently mixed and pipetted, using a cut off pipette tip, into cryovials, which were prefilled with preservative. The vials were placed in freezing containers (Mr. Frosty™), which, according to the manufacturer can achieve a cooling rate of approximately 1 °C min^{-1}.

The two freezing containers (Mr. Frosty™) were placed in a −80 °C freezer for 2 and 4 h respectively. After the required time, the vials were removed from the freezing container and transferred to −150 °C.

The remaining larvae of this batch (that served as a control) were placed in three gently air bubbled aquaria of 3 L each, in the climate room (18 °C) in dim light. The aquaria contained GFF filtered seawater, the total number of larvae the aquarium was 5.000. The larvae were fed a mixture of algal species in a concentration of 20.000 cells ml^{-1}.

The following day, one vial per treatment was thawed to check for viable larvae. Thawing was done by placing the vials in a water bath of 39 °C in dim light. As soon as the content of the vials was thawed, the outside was wiped with 70 % ethanol and the content was added to 200 mL GFF filtered seawater, the defrosted larvae remained at room temperature (18−20 °C) to acclimatise for 1 h before transferring them to the climate room and treated in the same way as the control group.

For the treatments as well as for the controls, a subsample was counted for living larvae at day 2 and 7 after thawing.

2.5. Bonamia presence/absence

Subsamples of each batch were taken to examine the presence of the parasite *Bonamia ostrea*. Batches were also repeatedly sampled throughout the rearing period. This resulted in 39 samples of both larvae and juvenile oysters. Subsamples varied in numbers from 1000 to 100.000 for larvae and from 5 to 25 for juveniles. Subsamples were conserved in 70 % ethanol when shell length was <10 mm and frozen (-20 °C) when >10 mm. The presence of *Bonamia ostrea* DNA in larvae can reliably be identified using real time PCR (Arzul et al., 2011). Detailed methods are described in Engelsma et al. (2010). Analysis were performed by Wageningen Bioveterinary Research, Lelystad.

3. Results

3.1. Hatchery & nursery conditions

Water quality parameters were measured at regular intervals from the end of the pre-conditioning period of the broodstock until the end of the rearing period in December. Water temperature, salinity and pH were constant with less than 5 % variability throughout (Table 1). During the course of the rearing period, the oxygen sensor failed. Based on the measurements made at the start and on the fact that the tanks were well mixed, it is assumed that oxygen concentrations were always at least saturating. The presence of potentially toxic nutrient concentrations (NO_2 and NH_{total}) were always between 0−0.5 mg L^{-1} for N-NO_2 and between 0−0.25 mg L^{-1} for N-NH_{total}.

3.2. Broodstock

All adult oysters survived after arrival at the laboratory. Most of these adults showed new shell growth, with the exception of the largest individuals. When algae were dispersed into the tank, the shells of all oysters were regularly open during times, indicating that they were feeding (R. Witbaard, pers. comm.).

3.3. Larvae & juvenile oysters

First larvae were collected on 7 September, 24 days since the start of the conditioning of the broodstock, and the last larvae on 11 December 2018. Larvae that might have been released after this date were not collected and spawning was stopped by lowering the temperature to 15 °C. At least 21 million larvae were released.

Growth rate before settlement was linear, varying between 1.5–1.7 μm per day (Table 3). Growth rates of the three batches did not significantly differ ($F_{2, 47} = 0.48$, $p = 0.62$). The initial size for batch E was low compared to the other two batches (Fig. 2).

When transferring the larvae from batches B and C to the settlement tank, it was noticed that large numbers of competent larvae were crawling on the wall of the conical tank. These larvae were included in the calculation of the percentage survival until settlement (Table 3). These larvae were gently removed from the wall of the tank by means of a freshwater jet, but the survival of these larvae after placement in the settlement tank was negligible (data not given). In the batches E-F-G,

Table 1
The average water quality parameters (water temperature (°C), salinity (practical salinity unit) and pH (-)) during the rearing period from the end of the pre-conditioning period in August to the end of the experiment in December 2018. B = broodstock, C = conical, DW= 'downwelling' and UP='upwelling' tank.

Tank	Water temp (°C)	Salinity (PSU)	pH (-)
B	20.0 ± 0.7	30.7 ± 0.4	8.1 ± 0.1
C	19.4 ± 0.3	30.6 ± 0.4	8.1 ± 0.0
DW	19.6 ± 0.7	30.4 ± 1.0	8.1 ± 0.0
UW	20.2 ± 0.9	30.0 ± 1.0	8.1 ± 0.0

Table 2
Description of batches of larvae distinguished, hatching date per batch, number of larvae per batch and their fate. Larvae were either, transferred to a settling tank when competent, were sacrificed for Bonamia tests or used in the cryo-preservation trial.

Batch	Hatching date	# Larvae hatched	Fate
A	7/9/18	5830	Bonamia test
B	11/9/18	150.000	Settled
C	13/9/18	100.480	Settled
D	11/9 & 13/9/18		Settled
E	11/10/18	974.004	Settled
F	12/10/18	1.400.053	Settled
G	13/10/18	923.280	Settled
H	16/10 to 11/11/18	4.824.887	Bonamia test
I	20/11/18	90.000	Bonamia test
J	6/12 to 11/12/18	6.412.722	Cryo trial, Bonamia test

only a negligible number of larvae crawled or settled on the wall of the conical tanks.

The duration from release until placement in the settlement tank varied between 25–38 days for the first three batches (B, C & D) to 41–43 days for the last ones (E, F & G) (Table 3).

The number of larvae that survived until placement in the settlement tank differ between the batches (Table 3), but also number of days between release and settlement differed. Survival varied between 0.96 and 0.98 per day.

The average size at settlement was 255−325 μm (Table 3). The largest larvae were found in batch D (447 μm), where larvae that stuck to the wall were scraped off, measured, counted and added to the settlement tank. This resulted in an overestimation of the size at settlement and most likely to the mortality rate, as it is expected that survival of already settled larvae is low. For this batch, therefore no survival rate is included in the results (paragraph 2.3).

Growth rate after settlement varied between 30−47 μm per day (Table 3, Fig. 3) and was much higher compared to the growth rate before settlement. The average growth rate per day for batches that hatches in October (E, F & G) was lower compared to the growth rate of the earlier batches (B, C & D) (Table 2).

There was no significant difference between the growth rate and initial size for the batches B, C and D ($F_{4,\ 37} = 0.32$, $p = 0.38$ and $F_{2,\ 39} = 2.07$, $p = 0.14$), nor between batches E, F and G ($F_{4,\ 14} = 1.89$, $p = 0.17$ and $F_{2,\ 16} = 1.72$, $p = 0.21$). Both the size at settlement ($F_{1,\ 41} = 61.6$, $p < 0.0001$) and the growth rate ($F_{2,40} = 47.6$, $p < 0.0001$) were lower for the later batches E, F and G compared to the early batches B, C and D (Table 3, Fig. 4). For the batches B, C and D, growth rate levelled off around day 75 (Fig. 3). At the beginning of February, the largest average oyster shell length is 14.5 mm (batch B), while the smallest average size was found in batch F (Table 3).

The mortality rate from release to placement in the settlement tank was between 0.01 and 0.04 per day (batches E, F & G only). From settlement to the beginning of February, mortality was very low for batches B and C (0.004 and 0.008 per day respectively), compared to the other batches (between 0.03 and 0.07 per day). Mortality rates for the period between release and the end of the pilot was 0.04−0.05 per day (batches E, F & G only).

3.4. Cryopreservation

The count of larvae at day 2 and 7 after thawing revealed that for all treatments no larvae survived freezing, while in the controls the average number of larvae per aquarium was 5087 ± 1574 and 2360 ± 1376, on day 2 and 7 respectively.

3.5. Bonamia

From the 39 samples prepared for analysis, two samples gave a signal that was too low to perform a PCR, these samples were rejected. All other samples (37) tested negative for the presence of *Bonamia*.

4. Discussion

4.1. General release process

In three months, 21 million larvae were released from 38 adult individuals. Oysters reproducing as female can produce 0.1–1.5 × 10^6 eggs per year (Walne, 1964) and not all eggs will be fertilised. In the

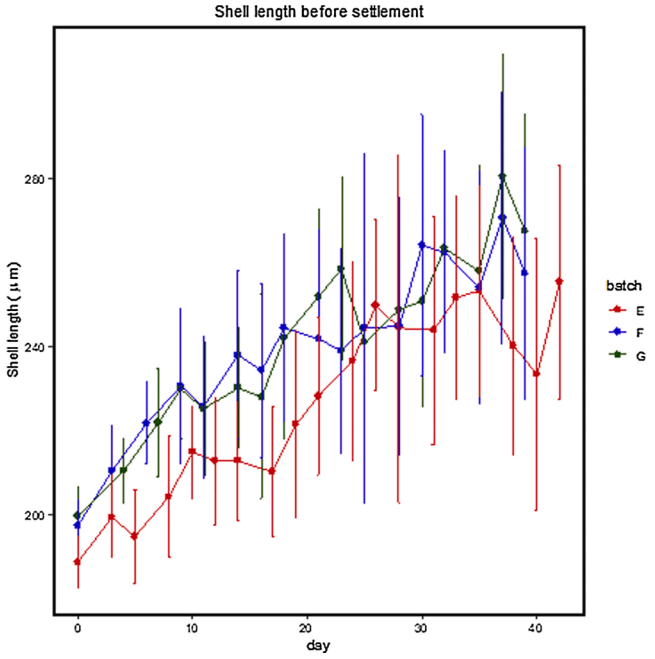

Fig. 2. Shell length (μm) ± standard deviation of oyster larvae before settlement for the batches E-F-G.

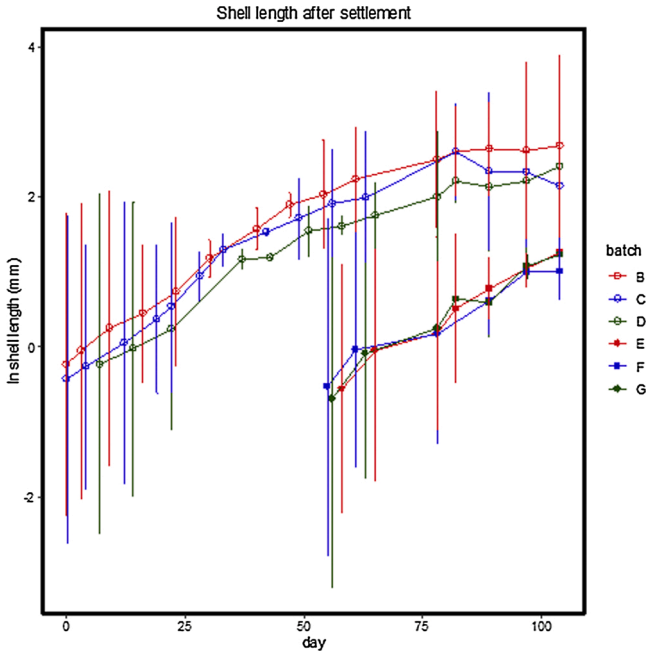

Fig. 3. Shell length (mm, ln-transformed) of juvenile oysters after settlement for the batches B-C-D-E-F-G.

Table 3
Characteristics of the different batches of oyster larvae and juveniles.

Batch	Before settlement				After settlement			
	Nrs in settlement tank	Days between hatching-settlement	Survival rate (d^{-1}) until settlement	Growth rate (µm day^{-1})	Size (µm) at settlement	Nrs that survived until end	Size (mm) end	Growth rate (µm day^{-1})*
B	2144	27	0.97		325 ± 15	1360	14.5 ± 3.4	45
C	5040	25	0.97		299 ± 29	2020	10.0 ± 3.3	47
D	93600	38				5360	11.6 ± 3.4	43
E	515700	43	0.98	1.5	255 ± 28	2430	3.5 ± 1.2	38
F	313200	42	0.96	1.5	257 ± 30	14460	2.7 ± 0.7	30
G	558900	41	0.98	1.7	267 ± 28	3460	3.4 ± 1.0	38

* The growth rate after settlement was based on the first 50 days after placement in settlement tank.

current study, the sex ratio of the broodstock remains unknown, but based on the maximum eggs per female per year at least 14 females took part in the reproduction process. From mid-October to the end of November 2018, the conical tanks were occupied by previous batches. During that time, newly released larvae were placed in water reservoirs of 1500 L using two seawater pumps to keep the water aerated and mixed. Larvae showed no signs of disease (see below), but survival in these tanks was very low. It is assumed that the larvae were crushed and died when they encountered the pumps.

Disease outbreaks of *Vibrio* spp. bacteria are considered the bottleneck for successful release and nursing of bivalve larvae (Dubert et al., 2017). A *Vibrio* infection can be detected under the microscope as 'swarms' of bacteria around the larvae. Infected larvae show reduced motility or erratic swimming behaviour, eventually resulting in high mortality a few hours after infection (Beaz-Hidalgo et al., 2010). In the current study, all batches of larvae were regularly inspected under the microscope, and signs of a *Vibrio* outbreak as described above were never detected. Since the presence of *Vibrio* in the water was not tested, it cannot be excluded that *Vibrio* were present in low densities and still had a detrimental effect on survival. However, with the exception of the larvae that were kept in water reservoirs with seawater pumps, the mortality rates were comparable to other studies (paragraph 4.2). It is therefore concluded that *Vibrio* bacteria were never present in high densities and that their potential impact on mortality in this study was low. The risk of an outbreak increases under conditions of low salinity (<25‰) and high temperatures (>26 °C) (Davis and Calabrese, 1969). *Vibrio* outbreaks might have been prevented due to the relatively low water temperature, high salinity (Table 1), a flow-through system and a strict cleaning protocol (González-Araya et al., 2012).

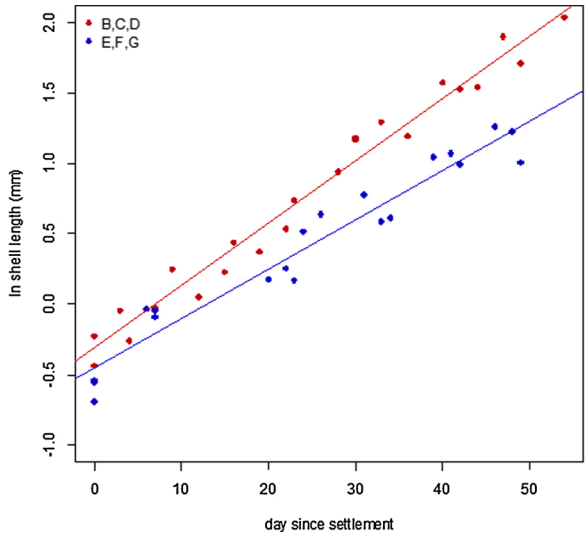

Fig. 4. Shell length (mm, ln transformed) during the first 50 days after settlement for the batches that hatched in September (B, C & D, red) and in October (E, F & G, blue). Lines (ln y = a + b ln x) indicate the linear regression of shell length and day. Red line: a=-0.31 ± 0.04, b = 0.042 ± 0.001, $R^2 = 0.98$ ($p < 0.001$). Blue line: a=-0.46 ± 0.06, b = 0.035 ± 0.002, $R^2 = 0.94$ ($p < 0.001$).

High concentrations of ammonia can be toxic to oysters (Postma et al., 2002). Bioassays with Pacific oysters indicated that the no observed effect concentration (NOEC) for total ammonia (NH_{total}) for embryos and larvae is < 2.8 mg N L^{-1} at a pH of 7.9–8.2 (Geffard et al., 2002). For adults a comparable NOEC of < 3 mg N L^{-1} was reported (Postma et al., 2002). In the current study, the measured concentrations of N-NH_{total} were always far below the NOEC concentration for Pacific oysters. The NOEC is unknown for native oysters and could be different. In addition, these NOEC are based on short-term exposure (<48 h) to high concentrations, the NOEC for long-term exposure for oysters are unknown but will be considerably lower (*e.g.* Boardman et al., 2004). Experiments using other bivalve species indicated that animals, in response to elevated concentrations, close their valves, which is expected to result in reduced growth rates when concentration remain elevated for prolonged periods (Boardman et al., 2004). It is recommended, that in future studies to measure the concentration of N-NH_{total} with a higher accuracy.

4.2. Larvae & juvenile oysters

The main factors that influence growth and development in larvae are temperature, food quantity and quality, both during broodstock conditioning and larval growth. Robert et al. (2017) investigated the growth and survival of *Ostrea edulis* larvae at 15, 20, 25 and 30 °C water temperature and concluded that at 15 °C growth was slowest and stagnated after the first week. Davis and Calabrese (1969) concluded that growth and survival of larvae was optimal at water temperatures ranging from 17.5 to 27.5 °C, while Roberts and co-auteurs (2017) concluded that the optimum temperature was higher (25−30 °C). However, care should be taken at high temperatures (>27.5 °C) since it increases the risk of *Vibrio* outbreaks, resulting in poor survival of the larvae and juveniles (Davis and Calabrese, 1969). In the current study, the water temperature during the pelagic phase of the larvae was 19.4 °C, which is below the optimum temperature for growth.

Most studies investigate the number of competent larvae after a fixed time. In these studies, the first competent larvae were recorded after 11–17 days in a temperature range of 18−22 °C (Acarli and Lok, 2009; González-Araya et al., 2012; Jonsson et al., 1999; Mesías-Gansbiller et al., 2013). In the current study, the first competent larvae, identified by the presence of an eyespot, were observed after 17–22 days after release. Larvae, however, were transferred to the settlement tanks only as soon as 30−50% of larvae in a batch had an eyespot. The time from release to 'settlement' varied between 25 and 43 days. Competent larvae were thus already present in the conical tanks, but these larvae were withheld from settlement substrate until more larvae were 'competent'. Generally, in bivalves, settlement and metamorphosis can be delayed when suitable substratum is not available (Pechenik et al., 1990). Under

natural conditions, delayed settlement might result in lower numbers of settled larvae due to high predation pressure on pelagic larvae (Rumrill, 1990). A recent study however indicated that mortality of planktonic larvae is lower than widely assumed (White et al., 2014). In the current study, the mortality rates from release to settlement ranged from 0.012 to 0.036 d^{-1}. Robert et al. (2017) studied the effect of temperature and food concentration on larval performance. The highest mortality rates were found for unfed larvae (calculated for a period of 6 days after hatching) and at a water temperature of 15 °C (calculated after 13 days), which was the lowest temperature investigated. Mortality rates were 0.019 and 0.018 per day respectively. Berntsson et al. (1997) reported mortality rates between 0.001 and 0.095 d^{-1}. The highest rates were found after the first 12 days at a water temperature of 21 °C. The mortality rates reported in the current study are thus within the range of rats reported by others. There is no indication that the prolonged delay of settlement in the current study resulted in higher mortality rates.

In previous studies, where water temperature ranged between 20−22 °C, size at settlement of *Ostrea edulis* varied between 204 and 303 μm (Berntsson et al., 1997; González-Araya et al., 2012; Jonsson et al., 1999). In the current study, larvae in batch E were on average the smallest with 255 μm and those in batch the largest with 325 μm (Table 3). As argued before, the larvae in batch A (and B) were ready to settle for some time before they were transferred to the settlement tanks. Growth rate in the current study was calculated for batches E–G only and with 1.5–1.7 μm per day, these rates were at the low end of growth rates recorded in previous studies (1.4–9.3 μm d^{-1}) (Acarli and Lok, 2009; Berntsson et al., 1997; González-Araya et al., 2012; Jonsson et al., 1999). Both size at settlement and the growth rate until settlement was variable between studies as well as within studies. Within the narrow temperature range of 20−22 °C diet of broodstock and larvae were the main explanatory variables (Acarli and Lok, 2009; Berntsson et al., 1997; González-Araya et al., 2012; Jonsson et al., 1999). Berntsson et al. (1997) and Jonsson et al. (1999) found a correlation between larvae that had a high content of polyunsaturated fatty acids (PUFA), growth rate and settlement success. In both studies, the authors could however not link the content of fatty acids to a specific diet used to condition the broodstock (Berntsson et al., 1997) nor to the algal composition given to the larvae (Jonsson et al., 1999). The authors concluded that there is considerable variation in the biochemical composition of algal mixtures during the course of the experiments due to differences in temperatures, light and nutrients. Robert et al. (2017) studied the influence of food concentration on larval performance and concluded that the optimal concentration was 25 cells μl^{-1} in a two-species diet. In the current study, algal cell densities provided to the pelagic larvae was 20.000 cells ml^{-1}, which is slightly below the optimum described by Robert et al. (2017). The food quality was not investigated and the content of PUFA was unknown. However, unlike most other studies, in the current study a mixture of six species was used for both adult broodstock, larvae and juveniles. A mixture of at least 2–3 algae is assumed to provide the optimum diet (Castell, 1986; Utting and Millican, 1997). It cannot be excluded that there have been periods of sub-optimal food quality and quantify conditions in the current study, resulting in slow growth and development. First, at the start of the experiment, flocculation of algal species was observed. This was due to prolonged exposure of the algae to 20 °C in the climate-controlled room, which most likely affected the quality of the food. This was solved by refilling the algal containers more often, reducing the exposure time to high temperatures, thereby minimising the deterioration of food quality. Second, cell densities were kept constant, but dry mass varied throughout the rearing period between 42–326 pg dry mass per cell. This variation was most likely due to different ratio of species, varying quality of algal cells and (observed) variation in cell size per species.

The growth rates after settlement were much higher compared to before settlement (Table 3). This is easily explained, as during metamorphosis there is a change of feeding apparatus from a velum to gills, which allow for more efficient feeding (Urban, 2002). Davis and Calabrese (1969) investigated the effect of temperature on growth of *Ostrea* larvae after settlement and recorded a shell length of 1.9 mm for their juveniles 30 days after settlement at 20 °C. In the current study, the juveniles had reached a length between 1.8 and 3.3 mm after 30 days (Fig. 3). There were differences in larval and juvenile performance between batches that were released in September and batches that were released in October. Differences in larval performance with time have been reported before (Rödström, 1989 *in* Berntsson et al., 1997; Walne, 1964). These differences were related to depletion of food reserves in the broodstock, resulting in smaller newly released larvae (Walne, 1964), which translated in slower growth and development. Mortality, calculated from release to the end (0.04-0.05 d-1), was comparable to the rate reported by Cole (1941) (0.038 d-1) calculated over a period of 365 days since release.

4.3. Cryopreservation

For all treatments, the survival of the cryopreserved *Ostrea edulis* larvae 2 days after thawing was zero. In the current study, the larvae were collected directly after being released and were approximately 1–2 weeks old. The protocol tested in the current study was based on studies using *Magallana* oyster larvae (Labbé et al., 2018; Suquet et al., 2014). The larvae in these studies were much younger; here larvae were frozen within 48 h post-fertilization, and best results were shown for larvae 20−24 h after fertilization (Labbé et al., 2018). Survival 12 days after thawing was 9.4 % compared to 52.5 % for untreated larvae. Others mentioned that later development stages of oysters are generally more resistant to cryoprotectant toxicity and freezing than earlier ones and that failure was due to an inaccurate freezing protocol (Horváth et al., 2012). Cryopreservation of 10-day old larvae is very challenging and would require the development of specific protocols.

4.4. Bonamia

The 39 samples were collected throughout the rearing process, from newly released larvae to settled juveniles, for the analyses of presence/absence of *Bonamia ostrea*. Two samples had to be rejected, these samples consisted of the smallest larvae, and yielded not enough material for a reliable test. The absence of a parasite or pathogen cannot be demonstrated but can only be estimated with a given statistical certainty. For a reliable estimate of the absence of *Bonamia ostrea* in a population of adults, generally 150 adults are sacrificed (pers. comm. M. Engelsma). In the current study, the total number of animals tested far exceeded this minimum. Real-time PCR, as was applied in the current study can result in a false positive result, for example when DNA of a degraded pathogen is present or in a false negative result, due to inhibitory factors present in tissue of oysters (Corbeil et al., 2006). However, the percentage of false negatives and positives is low (<2 %, Marty et al., 2006). With a high degree of certainty, it can thus be concluded that *Bonamia ostrea* is absent in the juvenile oysters cultured in the current experiment.

5. Conclusion

The present study reports on a successful attempt to breed *Ostrea edulis* from recently discovered adults in the Dutch Wadden Sea. In a period of three months, 21 million larvae were released. Survival during the larval stage was comparable with previous studies, but growth was at the low end. After settlement, mortality was low and growth considerable. Recommendation for future attempts are an increase in pre-settlement water temperature and food concentration. Although there is no indication that withholding settlement material to competent larvae resulted in a lower settlement success, it is worth the effort to find a way of sorting the 'competent' larvae from the pelagic ones and placing these competent larvae in settlement tanks immediately. In the current study, placing newly released larvae in conical tanks at a

maximum density of 5.5 larvae ml^{-1} and using air to keep larvae suspended was a success and can be recommended. In contrast to many other studies (Beaz-Hidalgo, et al., 2010; Dubert et al., 2017; Sicuro, 2015), in the current experiment no additional measures were taken to control *Vibrio* other than the use of a flow-through systems and a strict cleaning protocol (Section 2.1) and *Vibrio* outbreaks did not occur.

Although it should be possible to produce larvae in a laboratory setting year-round, it might be convenient and cost-effective to breed larvae for a short period and then keep these larvae for later use. Cryopreservation is a promising technique, as it has proven to result in viable larvae after thawing for several bivalve species including Pacific oyster, mussels and clams (Paredes et al., 2013; Simon and Yang, 2018; Suquet et al., 2014). The advanced development stage of *Ostrea* larvae might however prove to be a bottleneck in freezing larvae of this species.

The broodstock animals originated from one location in the Wadden Sea (Fig. 1), these animals were not tested for the presence of *Bonamia ostrea*. As it is assumed that *B. ostrea* is transmitted from adult to eggs or larvae during brooding (Arzul et al., 2011) the absence of *B. ostrea* in the offspring suggests that *Bonamia* is absent in the broodstock in the laboratory and therefore also absent in at least part of the Wadden Sea population originating from the Eijerlandse Gat location. The juvenile oysters bred in the current study can be used for restoration projects as well as for experiments in areas where *B. ostrea* is absent or where the presence of *Bonamia* is unknown. With the described successful hatching and rearing experiment, a first important step is made in the goal to achieve sustainable populations of native oysters in Dutch waters.

Author statement

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Declaration of Competing Interest

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

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