Initiation of a Bras d'Or lake oyster breeding program and broodstock management for resistance to MSX

Aquaculture Collaborative Research and Development Program (ACRDP) 2005-2008

Part 1. Initiation of a Bras d'Or lake oyster breeding program for resistance to MSX

Part 2. Broodstock management for the Bras d'Or lake oyster breeding program - resistance to MSX

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by

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With the decline of the Bras d'Or lake oyster (*Crassostrea virginica*) populations, driven by habitat degradation, excessive fishing and outbreaks of oyster parasites, stock enhancement is one approach among other management options, supported by many stakeholders including DFO and First Nations. Aquaculture and hatcheries technologies can stabilize and improve tolerance of oyster stocks to MSX disease but the potential for decrease in genetic diversity must be addressed. The first R&D project was to initiate a breeding program for MSX tolerance with a rotational breeding plan, performing crossing of oysters from specific sites and testing the progenies in field sites within the Bras d'Or lake. However, the direct and/or indirect effects of the MSX parasite on the gametogenesis and spawning of the oyster were not clear and MSX infection impeded on the abilities of adult oysters to properly reproduce. Temperature and salinity are two factors influencing the activity of the MSX parasite. A second R&D project proposed to identify critical time-temperature-salinity combinations to ensure proper gametogenesis and spawning of MSX infected oyster broodstock, and to make recommendations for ongoing MSX resistant oyster breeding program and for future restoration programs.

RÉSUMÉ

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Avec le déclin des populations d'huîtres (*Crassostrea virginica*) du lac Bras d'Or, causé par la dégradation de l'habitat, la pêche excessive et l'apparition de parasites d'huîtres, l'amélioration des stocks est une approche, parmi d'autres options de gestion, soutenue par de nombreuses parties prenantes y compris le MPO et les Premières Nations. Les technologies d'aquaculture et d'écloseries peuvent stabiliser et améliorer la tolérance des stocks d'huîtres à la maladie MSX, mais le risque de diminution de la diversité génétique doit être adressé. Le premier projet de R & D était de lancer un programme de sélection pour la tolérance au MSX avec un plan d'amélioration en rotation, en effectuant des croisements d'huîtres provenant de sites spécifiques et en testant les descendances dans les sites du lac Bras d'Or. Toutefois, les effets directs et /ou indirects du parasite MSX sur la gamétogenèse et les pontes de l'huître ne sont pas clairs et l'infection du MSX entrave les capacités des huîtres adultes à se reproduire correctement. La température et la salinité sont deux facteurs qui influencent l'activité du parasite MSX. Un deuxième projet R & D a proposé d'identifier les combinaisons critiques de durée-température-salinité pour assurer la gamétogenèse et la ponte adéquates des géniteurs infectés par le MSX, et de formuler des recommandations pour les programmes d'élevage d'huîtres résistantes au MSX et pour les programmes de restauration.

NIKANATUEK

Vercaemer B., A. McIsaac and P. Drinnan, 2010. Initiation of a Bras d'Or lake oyster breeding program and broodstock management for resistance to MSX. Can. Tech. Rep. Fish. Aquat. Sci. 2871: vi + 40 p.

Pemi-aji-tkle'jijik mn'tmu'k *(Crassotrea virginica)* Pitu'poq, tela'tekek winamukwa'tasik samqwan, awsami-ktanujik aqq ksnukwaqn, na kisutasik kisite'taqn ta'n tli-aji-wla'sitew telikwenuj, welte'tmi'tij alsusultijik we'kaw DFO aqq L'nu'k. Kina'masuti wjit etlikwenikemk samqwan-iktuk aqq ta'n telikwenuj mn'tmu'k apoqnt‡tew aqq wla'tew ta'n mn'tmu'k tel-kaqamutmi'tij ksnukwaqn teluisik MSX na's‡k amujpa tmk wesku'tasik ta'n tli-apsa'sitew wetapeksultijik. Amskwesewey lukwaqn R&D kisa'tu'tip na ta'n tli-kwenaten mn'tmu'k kaqamutmi'tij ksnukwaqn teluisik MSX , ewe'wa'tijik mn'tmu'k weja'la'tijik keknue'kl etlikuti'tij aqq wetnu'kwatmi'tij kisikwenanew mn'tmu'jk keknue'kl etekl Pitu'poq. Na's‡k mu welnmitu'tik‡p ta'n MSX tela'toq teli-sika'ta'tij mn'tmu'k aqq ksnukwaqn wejiaq MSX wina'toq ta'n teli-sika'ta'tij mn'tmu'k. Ta'n telp‡tek aqq tel-salawapua'q samqwan na tapu'kl koqoe'l we'tuo'tmi'tij juji'jk ta'n wejiaq ksnukwaqn teluisik MSX. Na ta'puewey lukwaqn R&D kisutmi'tip na kwilmnew keknue'kl ta'n samqwan telp‡tek aqq tel-salawapua'q kulaman kisi-sika'ta'titaq mn'tmu'k ta'n ki's ala'tu'tij ksnukwaqn, aqq kisutmnew ta'n ne'kaw tla'ten teli-sika'ta'tij mn'tmu'k kaqamutmi'ti.

INTRODUCTION

The American oyster (*Crassostrea virginica*) is an economically, ecologically and culturally important species in Cape Breton, Nova Scotia, but populations have been in decline due to over-fishing, degradation of habitats and by the appearance of the MSX parasite (*Haplosporidium nelsoni*) in the Bras d'Or lake (Pitu'paq) in 2002 (Stephenson et al., 2003). Until then, the MSX disease, which decimated oyster stocks in Delaware Bay and Chesapeake Bay in the late 1950's (Ewart and Ford, 1993), had never been present north of Maine. MSX or "Multinucleate Sphere X", a microscopic protistan parasite with an unknown intermediate host or reservoir of infection, causes tissue damage, which weakens the oysters, and leads to mass mortalities in *C. virginica*. In 2007, an outbreak of Malpeque disease (named after Malpeque Bay on Prince Edward Island, where it was first observed in *C. virginica* in 1915) was observed in one area of the Bras d'Or lake and represents the first incidence of Malpeque disease reported since the 1960s. This disease is caused by an unknown pathogen and it took several decades (~40 years) for the oyster beds in PEI to develop tolerance to the disease but this was followed by recovery of most stocks in the Gulf of St. Lawrence.

Rejuvenation of depleted private leases and public beds through seeding and cultivation programs has been proposed as part of the solution by DFO, Eskasoni Fish and Wildlife Commission (EFWC), the Unama'ki Institute of Natural Resources (UINR) and other stakeholders. Importation of oysters from outside of the Bras d'Or lake is presently not permitted; it has been prohibited since the 1950s in order to protect the native oysters from exposure to Malpeque disease. Furthermore, there is recent molecular evidence that the Bras d'Or lake oyster is a population genetically discrete from oysters found in the Gulf of St. Lawrence (Vercaemer et al., 2010). Therefore, aquaculture of American oyster must currently rely solely on resident populations for culture and future enhancement activities.

1. PART ONE

Since the confirmation of the MSX oyster disease in October 2002, the Shellfish Health Unit (SHU), DFO Moncton, and the Nova Scotia Department of Agriculture and Fisheries have worked intensely on the testing of shellfish for MSX to provide scientific advice for disease management within Nova Scotia and a contingency plan for Eastern Canada (McGladdery and Stephenson, 2005). Based on the results to date, the Bras d'Or lake is considered positive for MSX. However, there remain within the lake, areas where MSX had not yet been detected and it is unknown whether these populations harbour a natural resistance or have not yet been exposed to the disease or if the environmental conditions are not conducive to the development of the parasite. In the fall of 2004, the Bras d'Or Stewardship Society received funding from Enterprise Cape Breton Corporation (ECBC) to implement a "Bras d'Or lake oyster enhancement project". Juvenile oysters collected from the wild and believed to be exposed to MSX but not expressing the disease, were seeded in Public areas to be monitored for disease and performance over the next number of years. The Aquaculture Association of Nova Scotia (AANS) submitted a proposal, "Analysis of prevalence, incidence and transmission of MSX within populations of the oyster *Crassostrea virginica* of the Bras d'Or lake, Nova Scotia, to provide baseline information in support of a recovery strategy for commercial culture and harvest sectors" to ECBC and NRC-IRAP in which disease-host relationships and environmental parameters will be studied.

Along with these proposed disease management/studies and oyster enhancement initiatives, breeding for disease resistance/tolerance is seen by the different stakeholders (DFO, oyster growers) and titleholders (Mi'kmaw communities) as another long term strategy for the recovery of the Bras d'Or oyster. Stakeholders looked to the United States where MSX has devastated local oyster populations. Research and development in those areas has been directed at the creation of a pedigree of oysters resistant to MSX for use in enhancement initiatives. *C. virginica* has been selected for MSX resistance in Delaware Bay, USA, for 5 generations and selected lines have been shown to survive up to 9 times better than unselected stocks (Ford and Haskin, 1987). It has also been observed that some stocks in Delaware Bay have become resistant to MSX (Haskin and Ford, 1979) but waiting for natural resistance to develop in the Bras d'Or lake is not a realistic option for oyster growers. After 3 years of disease challenge in the Bras d'Or lake, it was suggested that the timing for the initiation of a selection program was opportune.

The oysters still surviving in the MSX affected areas appeared to be exhibiting a level of tolerance and should be considered as a prime broodstock for initiating a MSX resistance breeding program. In addition, they are physiologically adapted to the particular environment of the Bras d'Or lake and would at this point be the most suitable candidates for such a program. However, it should be noted that disease resistance does not necessarily prevent infection but allows oysters to restrict parasite development (i.e. increase tolerance) and thus reach market size.

Project objectives

The goal of this R&D project was to initiate a breeding program for MSX resistance in the Bras d'Or lake oyster population with the following objectives:

1) initiate a rotational breeding plan with oysters from specific sites within the Bras d'Or lake
2) perform crosses in parallel at the Eskasoni Fish & Wildlife Commission (EFWC) hatchery by the Unama'ki Institute of National Resources (UINR) in Eskasoni and at the Bedford Institute of Oceanography (BIO) quarantine
3) test the progenies in field sites
4) consider impacts of future breeding in Quarantine to obtain hybrids with Bras d'Or lake oysters and oysters from the Gulf region, resistant to Malpeque disease, and from the US, the latter showing MSX resistance
5) make recommendations for an expansion/ continuation of the breeding program and for future restoration programs.

1.1. Materials and methods

1.1.1. Description of work and experimental protocol

The work was scheduled to be done in parallel at the EFWC hatchery in Eskasoni and at BIO in Dartmouth to take advantage of the respective expertises (scientific R&D research capacity, quarantine, Traditional Ecological Knowledge, field capacities). Advice from international experts on breeding for resistance (Drs. Stan Allen, Pierre Boudry, Ximing Guo and Susan Ford) was also solicited.

An application for the transfer of oysters from Cape Breton to the BIO quarantine associated with this project had been filed with the Nova Scotia Introduction and Transfers Committee (NSITC). In the BIO quarantine, all quarantine protocols are strictly followed and controlled by the Quarantine supervisor. All

outgoing water is treated with bleach and incoming water can be UV treated for larval and spat rearing in static tanks. Groups/lots can be raised separately with a partition between tanks to prevent horizontal contamination. All necessary equipment (buckets, beakers, screens) is also allocated to a specific tank. However, in spite of these measures listed above, the NSITC could not approve the BIO Quarantine at the time of the project. The issues of primary concern were the unknown treatment efficiency of effluent water to prevent release of MSX and of influent water to prevent exposure of animals to Malpeque disease and/or unknown pathogens that may be introduced into the Bras d'Or lake once the progenies are transferred for testing. Because approval for the BIO Quarantine could not be obtained on time, hatchery work was carried out solely at the Eskasoni site.

Much effort was put into the development of the hatchery and a nursery unit to complete the shellfish section of the Marine Research Laboratory.

Initially, 50 pre-conditioned surviving oysters from Washabuck, Whycocomagh and Crane Cove (MSX infected sites) and 50 "naïve" oysters from Chapel Island where MSX had not yet been detected were targeted for collection in the spring of 2005. Indeed, to optimize the selective program, broodstock should be collected where the selective pressure (MSX) was the most intense. Oysters from the first two sites were showing high mortalities and, at the time, were the most affected by the MSX parasite. However, oysters from high MSX infestation areas could not be transferred to a lower infestation area (such as Crane Cove in Eskasoni). Hence, Gillis Cove, with mortalities and MSX infection similar to Crane Cove and history of continuous transfers between these two sites, was used as an alternate site (Figure 1). In the spring of 2005, oysters were transferred from Gillis Cove and Chapel Island to the UINR in Eskasoni for further conditioning and breeding. Other sites could be chosen in later years based again on the status of the MSX prevalence and the authorized movements of oysters in the Bras d'Or lake (according to results from the Shellfish Health Unit of the Department of Fisheries and Oceans in Moncton).

1.1.2. Selective breeding design

A rotational line crossing which has been recommended to avoid inbreeding depression by minimizing the increase in level of inbreeding per generation (Hershberger et al., 1984) was utilized. Other selection designs such as within family selection, between family selection or a combination of both (where the best individuals of the best families are selected) were unpractical as they require the

maintenance of a large number of known families (Mallet, 2004). However, different spawning groups could be considered as lines in a rotational design (Newkirk, 1996). This design has also been recommended in a recent review of the MSX literature (Mallet, 2004).

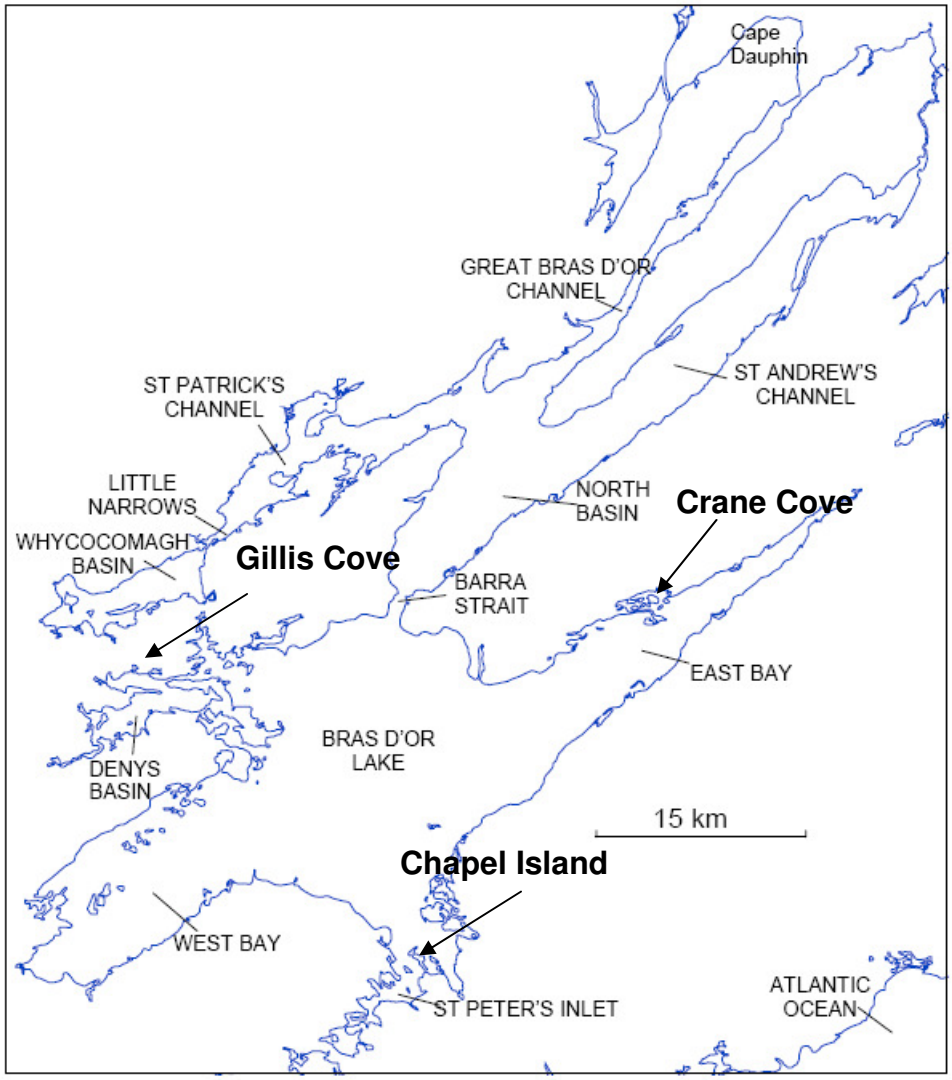


Figure 1. Collection sites, Bras d'Or lake, Nova Scotia: Gillis Cove 45° 54.687'N 61°3.266'W, Crane Cove 45°57.206'N 60°34.647'W and Chapel Island 45°41'33.52"N 60°47'00.27"W.

The rotational crossing of three MSX groups (A, B and C) and regular mass spawning of the naïve group (control D) is schematized in Figure 2. Females and males oysters from the different groups were crossed, only females and males of the

control group D were crossed within the same group. Then the males and females of the different lines were rotated at every generation to minimize inbreeding.

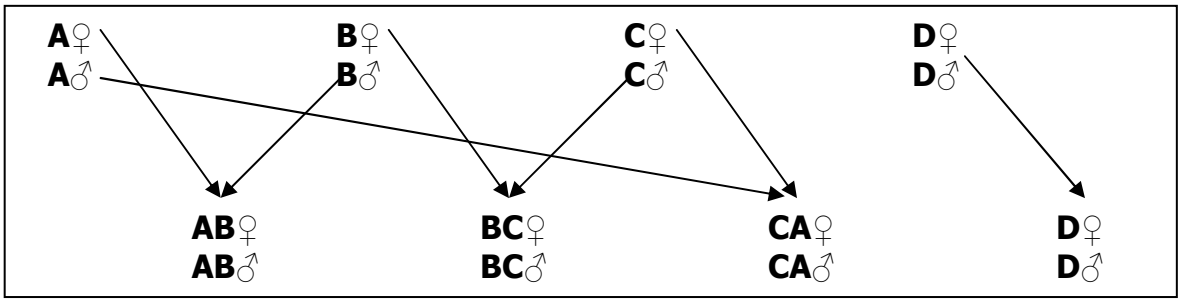


Figure 2. Schematics of the first round of crosses

1.1.3. Crosses and rearing

All materials involved in the spawning and rearing of larvae (screens, plungers, temperature probes, containers, buckets and tanks) were carefully cleaned with bleach and rinsed prior to use. Phytoplankton cultures were produced aseptically and followed standard hatchery and nursery protocols.

For each spawning group/lot, four principles were targeted:

1. Maximize number of parents (50 as a target number).
2. Get an equal contribution from each spawner: Eggs were divided into equal quantities for fertilization by individual males. After fertilization, zygotes were pooled into a common container for larval rearing.
3. Grade larvae gently.
4. Maintain accurate records.

Progenies were raised in the UINR/EFWC oyster nursery following standard protocols and then transferred to their respective original MSX-infected field sites along with a sub-sample of the control group: Crane Cove, Washabuck, Whycocomagh and also an area close to St. Patrick's Channel (highest MSX-infected field site) for field grow-out and survival tests.

1.1.4. Pedigree analysis and MSX testing

Tissue samples from parents and sub-samples of progenies were taken for subsequent DNA analysis. 10 to 12 polymorphic DNA markers (microsatellites) were available in the literature and have already been optimized at the molecular laboratory at BIO. They can be used to establish pedigree, insure genetic variability

and minimize inbreeding and for assessing overall survival and family differences in progenies survival.

In addition, tissue samples from broodstock and from sub-samples of progenies from the different crosses, groups and lots were taken prior to transfer to the field sites and at regular post-transfer intervals for assessing MSX prevalence. The histological and/or PCR analysis were done at the SHU laboratory in Moncton following OIE (Office International des Epizooties) protocols.

1.2. Results and discussion

1.2.1. Spawning and hatchery performance

The first round of crosses was performed on July 14, 2005 with 60 oysters, three groups of 20 oysters from Gillis Cove, Crane Cove and Chapel Island. Each oyster was carefully scrubbed, air-dried for 30 min and placed in an individual container with 27°C filtered, UV treated sea water. Every hour, water was changed to maintain proper conditions. Because spawning could not be achieved with this thermal induction (after 4 hours), oysters were opened, examined, separated according to sex and evaluated. Out of 60 oysters, 24 (10 from Gillis Cove, 2 from Crane Cove and 12 from Chapel Island) were used for this first round of spawning (Table 1). Stripped eggs were carefully rinsed through a 100 µm screen and sperm through a 20 µm screen to remove debris. Eggs from the same origin were pooled, gently mixed and divided into the same numbers of beakers as males participating in the cross. Eggs were regularly checked until they became completely rounded. Sperm was then added, beakers were gently swirled and then let sit for 5-10 min.

<u>July 14, 2005</u>
Gillis Cove ♀ x Gillis Cove ♂: 5 females x 5 males
Crane Cove ♀ x Gillis Cove ♂: 2 females x 5 males
Chapel Island ♀ x Chapel Island ♂: 6 females x 6 males (control group)

The first batch GC x GC was divided into two subgroups A and B to reduce the density of larvae in the rearing tanks. The last batch (control group) was divided into 2 subgroups A (“early” fertilization) and B (“normal” fertilization).

This first spawning was carried out to optimize the hatchery set-up (5 x 100L conical tanks + 1 x 100L spare for water change, see Figure 3). The remaining crosses would be performed once the larvae moved out of the conical tanks.

The larvae were fed (400 mL per tank of 1:1 T-Iso:Chaetoceros) and developed normally. The five groups' performance during the first week in terms of number and sizes are presented in Figures 4a, 4b, 5 and 6. After one week, fertilization performed "early" in the control group (CI x CI subgroup A) did yield more live larvae than the "normal" fertilization (Figure 6).

ID	No.	Sex	Shell Length	Shell Width	Shell Height	Comments
GC1	1	M	116	70	32	95-100% active
GC2	2	M	96	67	40	lots - 95-100% active
GC4	3	M	84	58	26	lots - 95-100% active
GC3	5	F	96	76	35	some misshapen
GC5	6	F	142	60	35	only a little mishapen, eggs full
GC6	9	M	79	62	22	lots - 95-100% active
GC7	14	F	132	82	40	only a little mishapen
GC8	17	F	117	74	31	few misshapen
GC9	16	M	73	50	20	lots - 95-100% active
GC10	20	F	108	78	29	some misshapen
CC1	1	F	82	56	33	some misshapen
CC2	2	F	62	48	24	most misshapen
CI1	1	M	78	60	29	not as active as previous
CI2	2	M	69	51	21	active-lots
CI3	3	M	73	53	27	active-lots
CI4	4	F	81	58	22	most are slightly elongated but full
CI5	5	F	74	60	25	some are slighly elongated but full
CI6	6	F	79	52	21	some mishapen
CI7	7	M	67	51	20	active - lots
CI8	8	M	84	62	30	less active than other males
CI9	9	M	84	64	28	less active than other males
CI10	10	F	73	64	20	some mishapen
CI11	11	F	91	66	29	eggs full - some mishapen
CI12	12	F	82	65	31	eggs full - some mishapen

Table 1. Parental oyster's characteristics - July 14, 2005 spawn

Figure 3. Larval rearing set-up, Marine Research Laboratory, EFWC, Eskasoni, NS.

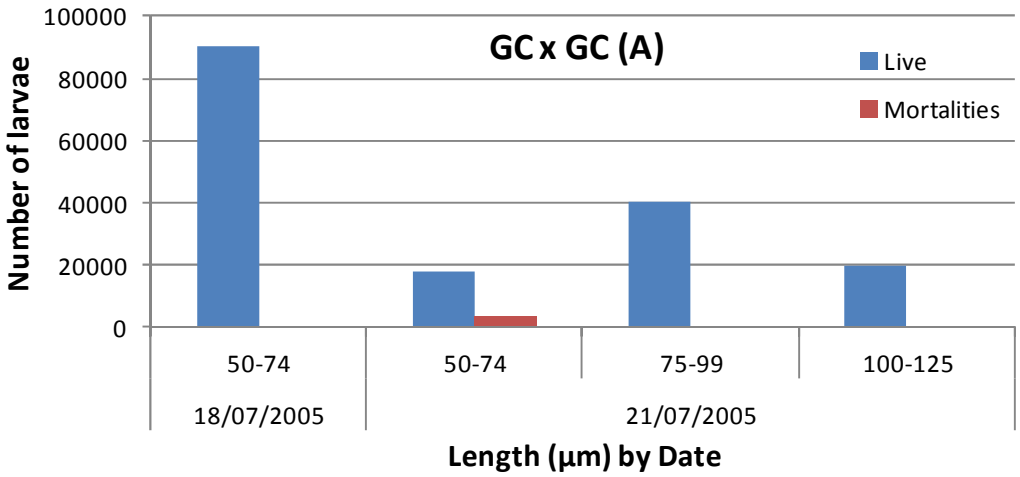


Figure 4a. Larval performance of Gillis Cove ♀ x Gillis Cove ♂ (5 females x 5 males - subgroup A).

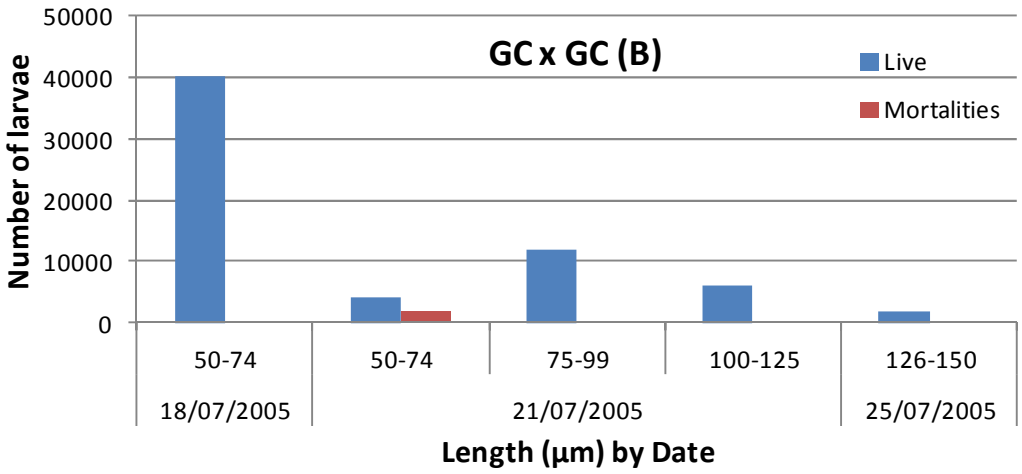


Figure 4b. Larval performance of Gillis Cove ♀ x Gillis Cove ♂ (5 females x 5 males - subgroup B).

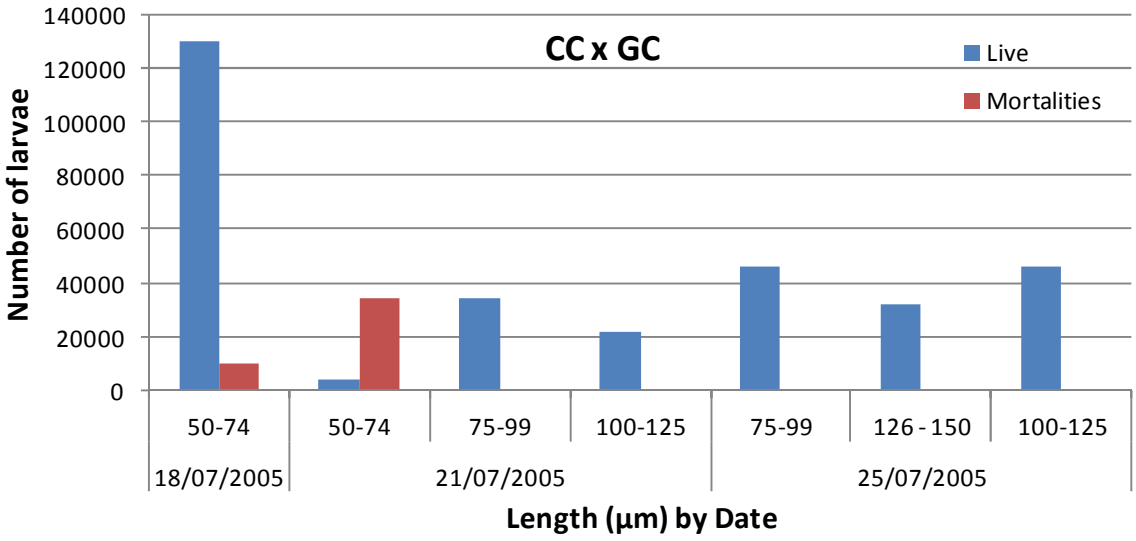


Figure 5. Larval performance of Crane Cove ♀ x Gillis Cove ♂ (2 females x 5 males).

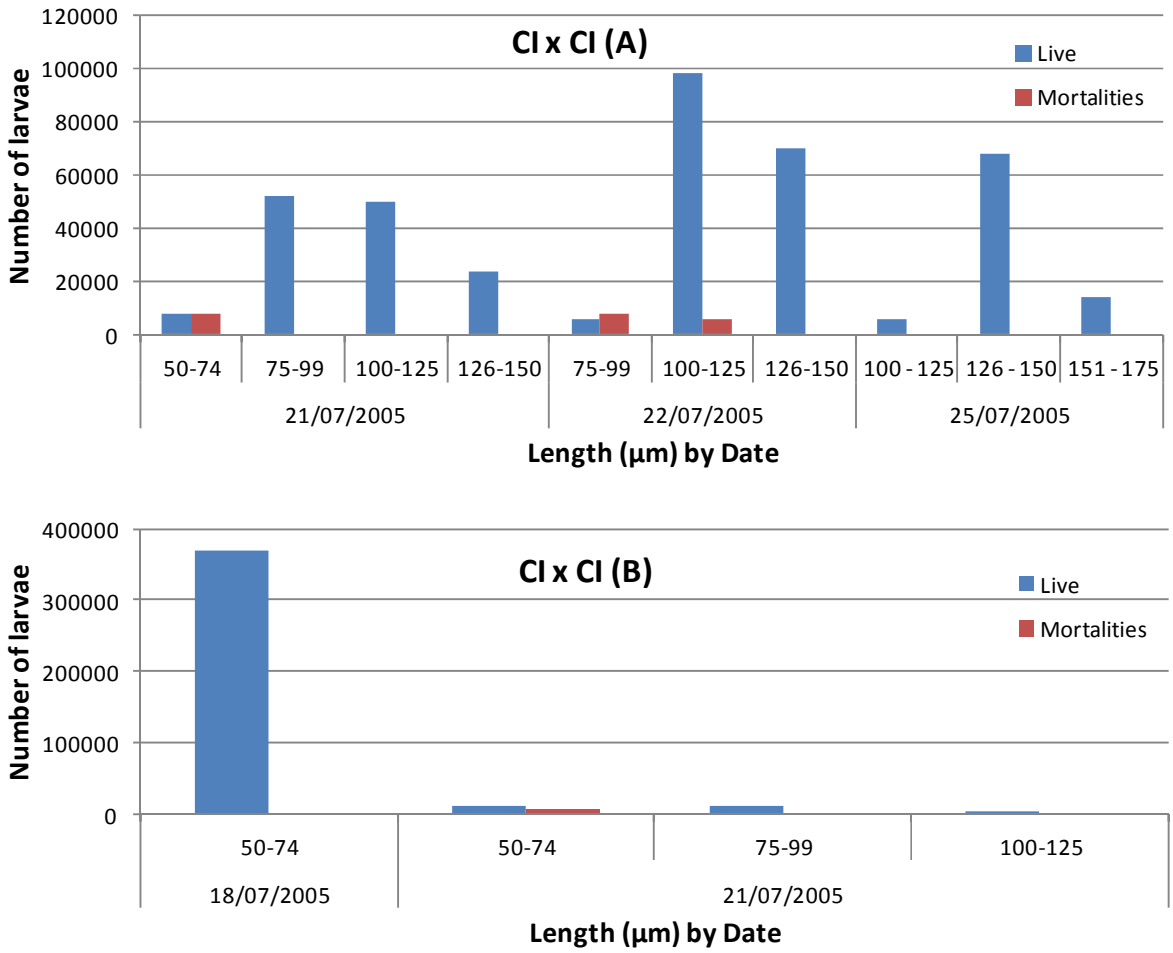
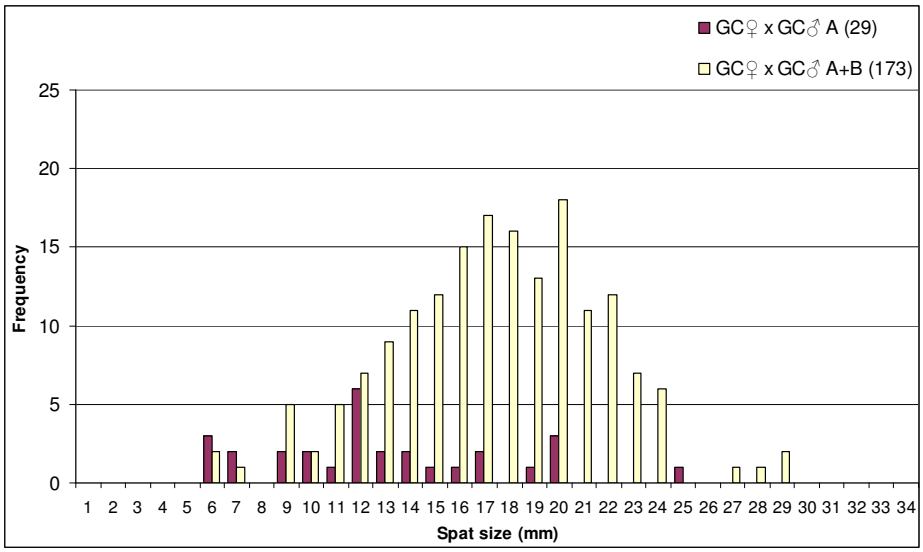


Figure 6. Larval performance of control group Chapel Island ♀ x Chapel Island ♂ (6 females x 6 males - subgroups A and B).

Very limited culling (elimination of the smallest growing larvae) was done to preserve genetic diversity, as it was already restricted by the limited number of parents that actually participated into the different crosses. Once larvae were ready to metamorphose and settle (>250µm), they were transferred to tanks with hanging Vexar sheets (1m x 1m) where they remained until sampled. One additional tank was used to accommodate a mix of the July 14 crosses. Figures 7 to 10 display the variability in terms of spat size distribution among the different crosses. The GC x GC subgroup B seems to have performed better in term of size than subgroup A (Figure 7), much better than the GC x CC group (Figure 8) and as well as the control group CI x CI (Figure 9). The spat from mixed origins, in the additional tank, performed above all other groups (Figure 10); it would be interesting to follow-up

their performance in the field as growth and survival vary greatly among families (GIonet, 201O). Subsequent genetic sampling could indicate their pedigree.

a.



b.

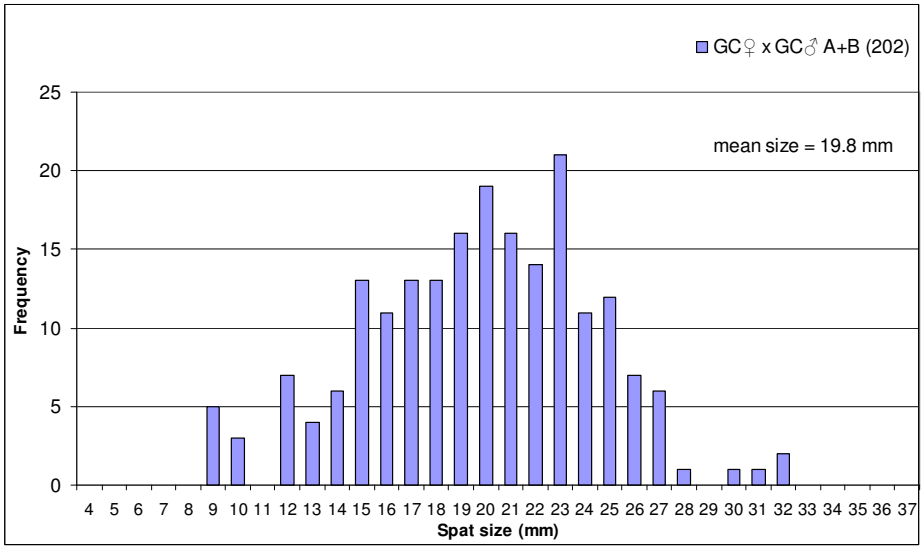


Figure 7. Spat size distribution and mean size of 6 month old oysters from GC x GC cross **a**. subgroups A and B. **b**. pooled subgroups (July 14, 2005 spawn, measured on January 19 (subgroup A) and January 20, 2006 (subgroup B). Number in brackets represents number of spat measured.

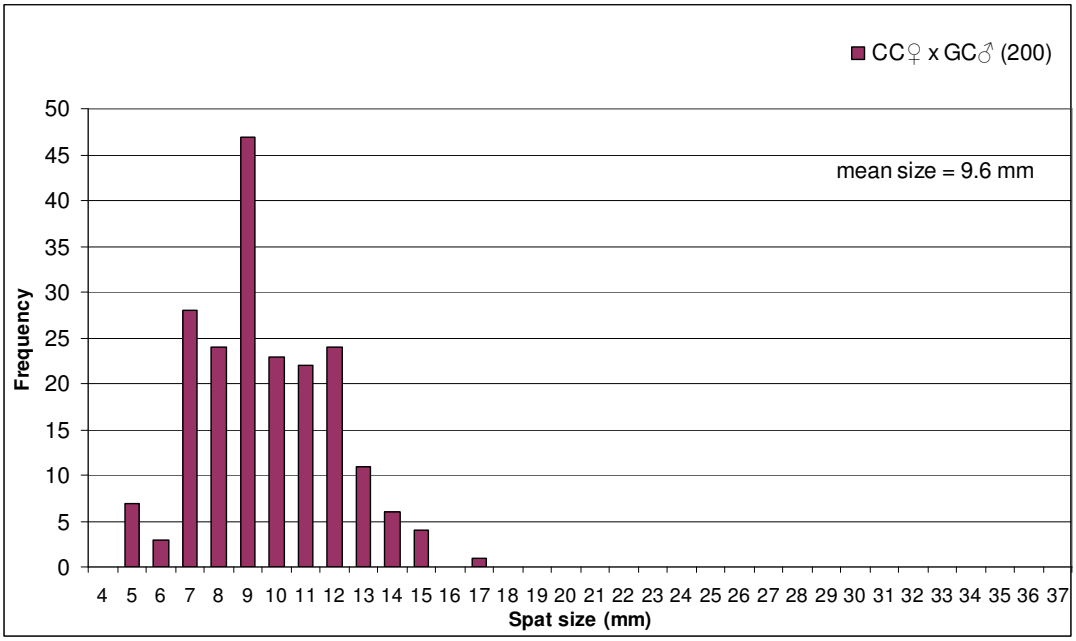


Figure 8. Spat size distribution and mean size of 6 month old oysters from CC x GC cross (July 14, 2005 spawn, measured on January 17, 2006). Number in brackets represents number of spat measured.

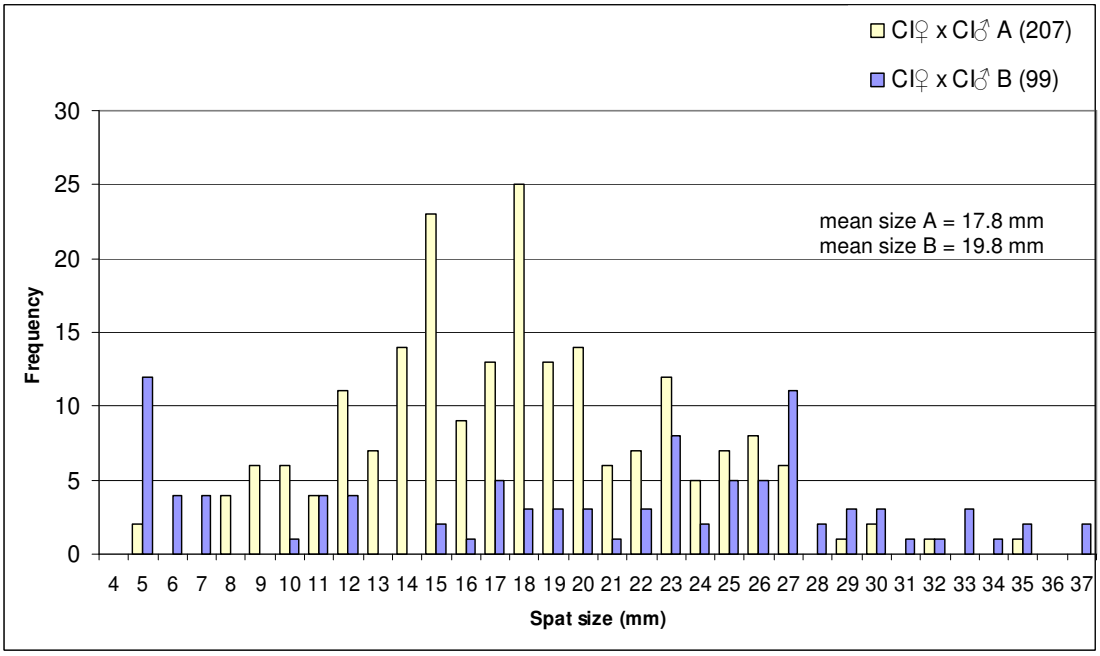


Figure 9. Spat size distribution and mean size of 6 month old oysters from CI x CI cross (July 14, 2005 spawn, measured on January 20, 2006). Numbers in brackets represent numbers of spat measured.

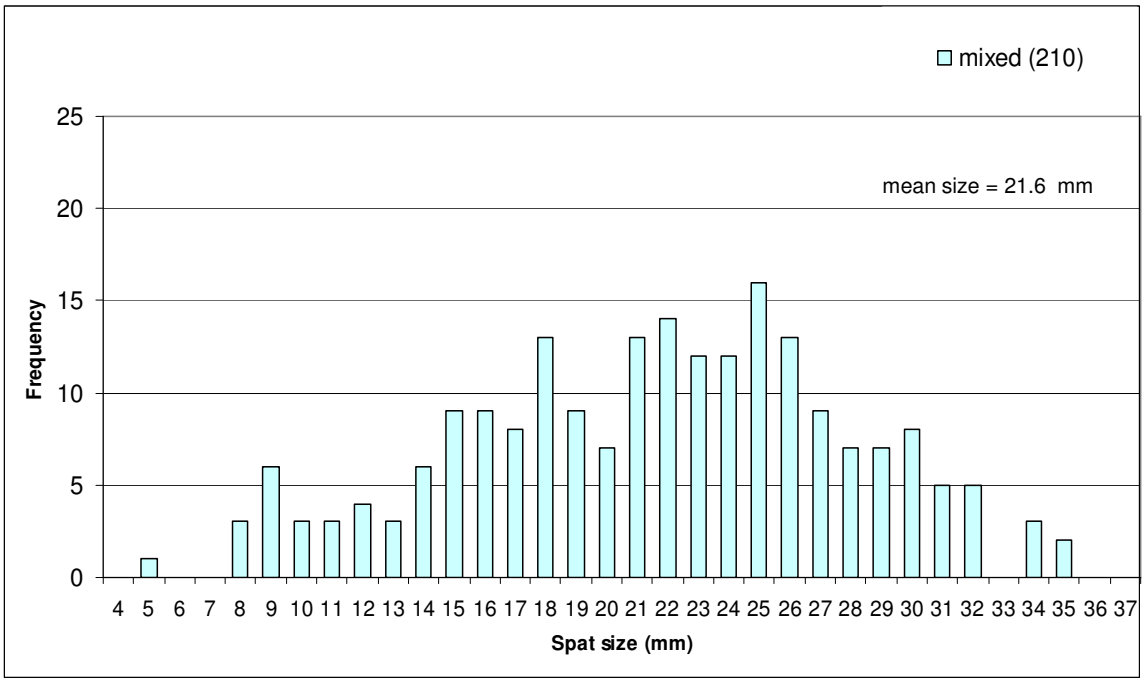


Figure 10. Spat size distribution and mean size of 6 month old oysters of mixed origins (July 14, 2005 spawn, measured on January 24, 2006). Number in brackets represents number of spat measured.

With a first successful round of oyster progenies completed, a second round of spawning was initiated in January and February 2006 in the same facility, following the same procedures for thermal induction, stripping (if necessary) achieve, and larval rearing:

<u>January 23, 2006</u>
Crane Cove ♀ x Crane Cove ♂ (3 females x 1 male). One female spawned naturally ~$2.3x10^6$ eggs.

From January 25 (Day 2) on, larvae were fed a mixed *Pavlova/T-Iso/Chaetoceros* diet to satiety. Figure 11 summarises the performance of that particularly successful cross (>500,000 spat produced, more than the total of all crosses from July 14, 2005). Partial transfers to spat tank took place on Feb. 13, 15 and 20 when larvae were >250µm. Metamorphosis occurred as early as Day 21 after fertilization. Final transfer to spat tank occured on Feb. 24, 2006. Figure 12 shows the distribution of spat size and the mean size of 6 month old oysters of the Crane Cove ♀ x Crane Cove ♂ cross from January 23, 2006, measured on August 3, 2006, prior to their transfer to the field. While there were a lot of spat produced in January

2006, their mean size was smaller (15.4 mm) than the crosses performed earlier in July 2005, with the exception of the CC x GC, probably due to the rearing conditions that could have been less optimum during the winter. Additionally, the CC (Crane Cove) stock may not perform as well as the GC (Gillis Cove) stock in general.

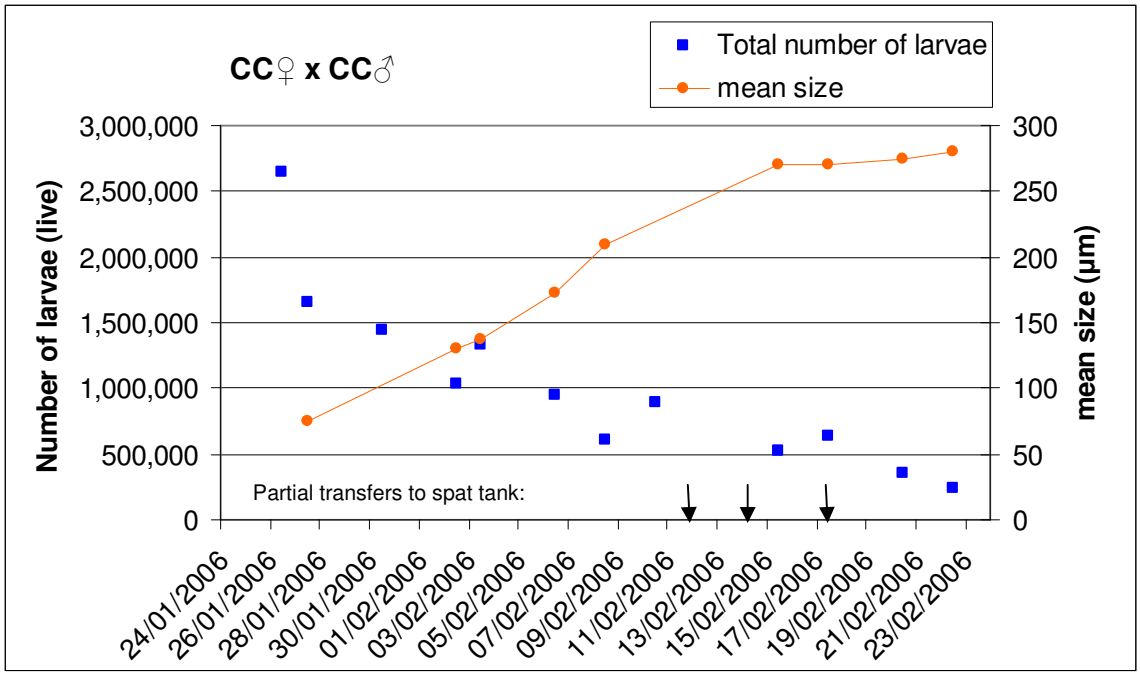


Figure 11. Larval performance of Crane Cove ♀ x Crane Cove ♂ cross from January 23, 2006.

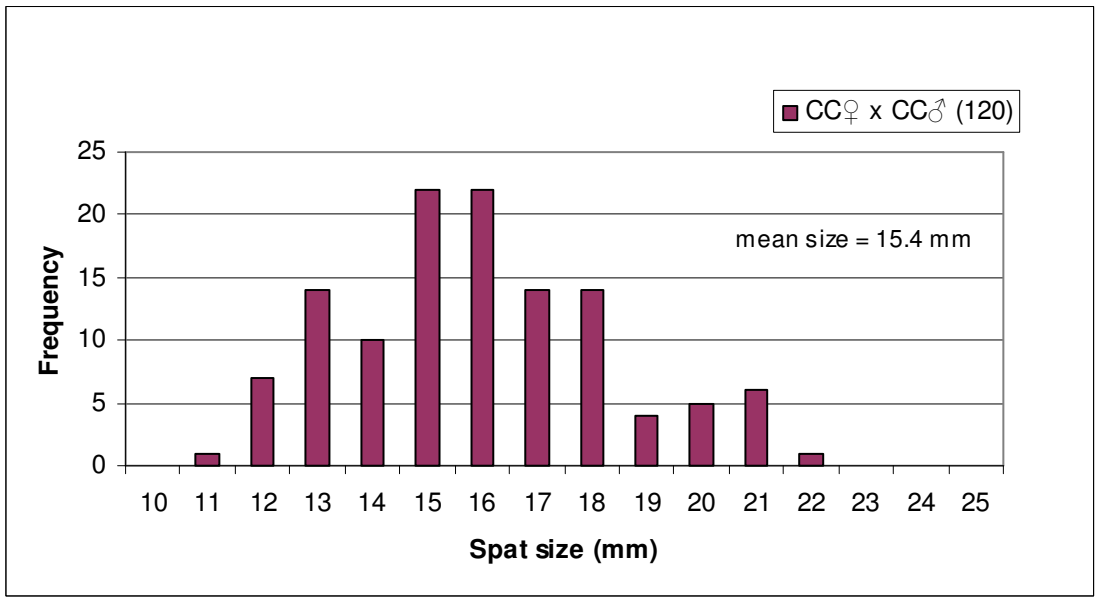


Figure 12. Spat size distribution and mean size of 6 month old oysters Crane Cove ♀ x Crane Cove ♂ cross from January 23, 2006, measured on August 3, 2006. Number in brackets represents number of spat measured.

At 6 months of age, spat produced from the CC x CC (January 23, 2006) were transferred for MSX resistance testing in Crane Cove, in Eskasoni and Nyanza Bay, on August 18 and 22, 2006, respectively.

<u>February 8, 2006</u>
Chapel Island ♀ x Chapel Island ♂
Gillis Cove ♀ x Crane Cove ♂
Crane Cove ♀ x Gillis Cove ♂
Crane Cove ♀ x Crane Cove ♂ (repeat of January cross)
Gillis Cove ♀ x Gillis Cove ♂

From February 10 (Day 2) to February 16 (Day 8), larvae were fed a mixed Pavlova/T-Iso diet and from February 17 on, a mixed *Chaetoceros/Tiso/Pavlova* diet. The following Figure 13 summarizes the performance of those five crosses.

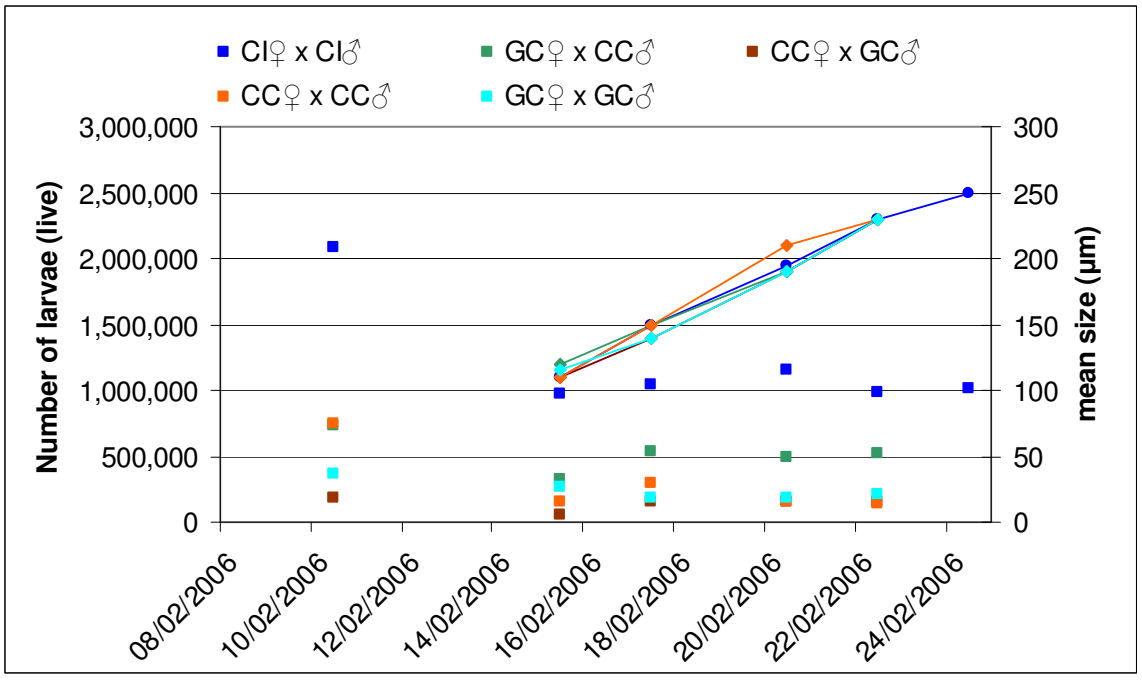


Figure 13. Larval performance of the different crosses performed on February 8, 2006.

Again, the CI x CI, control group, produced lots of larvae and subsequently spat (~1,000,000) as seen earlier. As oyster larval growth in a hatchery is multifactor

dependant, the differences in survival between the January and February spawns could be attributed to diet, temperature and other environmental conditions. Other factors being equivalent, the water temperature between Day 4-7 was ~4°C higher in February compared to January (Figure 14) and although the mean larval growth rates were higher (very similar between February crosses, average of 16.39 µm/day, see Figure 13) than for the January cross (10.83 µm/day, see Figure 11), higher temperature may have caused some stress and potential bacterial infection. Nonetheless, the larval growth rates observed in the UINR/EFWC hatchery were very good. Growth rates of 6 families/pools of *C. virginica* larvae raised in Shippagan, NB in 2005 varied from 7.32 to 13.39 µm/day (Gionet, 2010).

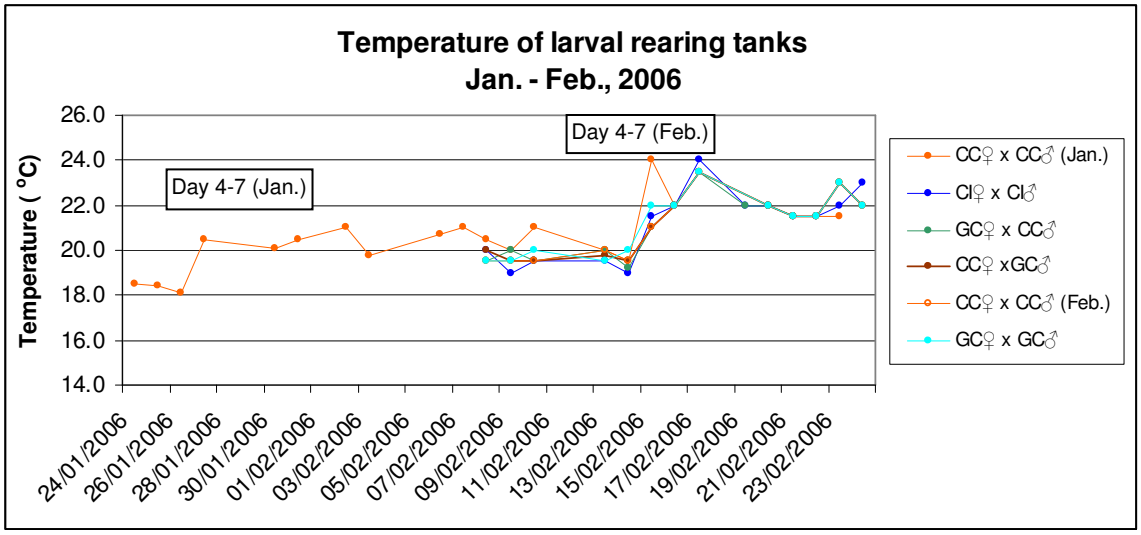


Figure 14. Temperature (°C) of the different larval rearing tanks.

Although some spawning and larval rearing conditions need to be standardized (e.g. temperature), the nine crosses performed at the UINR/EFWC hatchery were very successful, as well as the larval rearing. The principal limiting factor was the availability of properly conditioned MSX tolerant broodstock.

1.2.2. Testing of spat for MSX tolerance

As the different progenies were transferred from the nursery to the field sites, tissue were sampled for MSX testing and future DNA profiling. The results were 100% negative for MSX.

Survival, growth and subsequent MSX testing of the different spat groups are currently being performed and evaluated.

This initiation of a rotational breeding plan, with oysters from specific sites within the Bras d'Or lake, was successfully performed as an R&D project by EFWC at the new UINR's research facility in Eskasoni. Many more crosses and field testing clearly need to be completed to achieve a fully fledged breeding program for tolerance to the MSX parasite. Future crosses to obtain hybrids with Bras d'Or lake oysters and oysters from the Gulf region, resistant to Malpeque disease, and from the US, the latter showing MSX resistance, could only be considered once a new or renovated quarantine facility is approved in Nova Scotia. There are various MSX-resistant lines developed by Rutgers University, NJ, including CrosBreed®: Delaware Bay (DB) origin, long selected for MSX and (since 1992) Dermo resistance, DEBY: Delaware Bay origin, selected for dual disease resistance in lower York River, VA, NEH (submitted to the US Patent and Trademark Office): Long Island origin, selected for both MSX and (since 1980) and/or ROD (*Roseovarius* Oyster Disease, previously known as Juvenile Oyster Disease - JOD) resistance, WHS: hybrid between NEH and DB selected lines (Rawson et al., 2010).

Nevertheless, there is still considerable debate around the idea of importing resistant strains for hybridization with oysters from the Bras d'Or lake. These resistant oysters could transfer diseases, such as Dermo, or unknown diseases, and they are also not physiologically adapted to the northern environmental conditions of the Bras d'Or lake. This option is therefore considered premature at this time.

The main challenge of the oyster breeding project was to obtain proper conditioning of the surviving broodstock. Very few individuals (e.g. 24 out of 60 in July 2005) could produce sufficient and/or viable gametes to warrant fertilisation. In fact, infection by MSX interferes with the gamatogenic cycle of oysters and may reduce fecundity (Ford and Figueras, 1988). Many surviving oysters, conditioned in the laboratory but not used in spawning events, did show signs of disease and often died after a few months. It seemed that the MSX parasite had direct and/or indirect effects on the gametogenesis and impeded the proper spawning of the selected oysters, prior to general weakening and eventual death of the oyster. This difficulty had to be addressed and was the objective of a second R&D project.

2. PART TWO

Since the onset of the MSX oyster disease in October 2002, several disease management/studies and oyster enhancement initiatives have been undertaken, including the ACRDP project: “Initiation of a Bras d’Or lake oyster breeding program for resistance to MSX“ (see Part One above). A selective breeding program for disease resistance is seen by the different stakeholders (DFO, oyster growers, Mi’kmaw elders) as another long-term strategy for the recovery of the Bras d’Or oyster. This R&D project was performed in 2005/06 by EFWC at the UINR’s research facility in Eskasoni and initiated a rotational breeding plan with oysters from specific sites within the Bras d’Or lake. Crosses were performed in the new oyster hatchery and progenies tested for pedigree and disease status.

During the course of the project, it became evident that the surviving oysters collected from MSX infected sites did not optimally condition, even though water and food quantity and quality standards were met: their gonads were only partially full and they did not naturally spawn during a thermal shock in July 2005 or in January and February 2006, with the exception of one individual. Gametes had to be stripped and then fertilization was performed according to the experimental protocol. Finally, larvae were raised and their performance was comparable to that of larvae raised in standard North-American oyster hatcheries. The direct and/or indirect effects of the MSX parasite on the gametogenesis and spawning of the oyster are not clear but, overall, MSX infection impedes on the abilities of an adult oyster to properly reproduce. Conditioning oysters selected for the R&D breeding program in Eskasoni showed very low condition factor and poor gonad development (P. Drinnan, pers. obs.). Ford et al. (1990) and others indicated that *H. nelsoni* parasitism reduces the energy available to oysters and that the resulting overall metabolic depression causes impaired gametogenesis. Similar interference with proper gametogenesis is likely the cause of the exceptionally low oyster spat fall in the past years in Gillis Cove, an area which tested positive for MSX and which is well known in the Bras d’Or lake for its otherwise remarkably good spat fall. Previous studies have shown that gametogenesis in *C. virginica* is characterized by a general lack of development, maturation, and spawning in MSX infected areas, such as the lower Chesapeake Bay (Barber, 1996).

Temperature and salinity are two factors influencing the activity of the MSX parasite *H. nelsoni* (Haskin and Ford, 1982; Ford, 1985). Temperatures below 5^{o}C or

above 20°C have been reported to control infection. Previous research has shown that *H. nelsoni* is inactive or absent at low salinity (10 ppt or lower) and low salinity immersions of oysters have been used as a control measure in Delaware Bay and Chesapeake Bay. However, gametogenesis is also retarded at salinities below 5 ppt.

Advice from international experts on MSX (Dr. Susan Ford from Rutgers University and Dr. Eugene Burreson from the Virginia Institute of Marine Science) has been solicited and resulted in a productive correspondence regarding the effects of temperature and salinity on the parasite and oyster gametogenesis.

The second research project proposed to precisely determine the time-temperature-salinity combinations needed for appropriate gametogenesis and spawning in MSX infected broodstock. This is critical to (1) ensure the success of the on-going breeding program for resistance to MSX initiated in Eskasoni for the Bras d'Or lake oysters, and (2) refine timing and zoning of oyster management activities within the lake.

Project objectives

1. Identify critical time-temperature-salinity combinations to ensure proper gametogenesis and spawning of MSX infected oyster broodstock,
2. Make recommendations for ongoing MSX resistant oyster breeding program and for future restoration programs.

2.1. Materials and methods

2.1.1. Description of work and experimental protocol

The work was done through EFWC in the UINR hatchery facility in Eskasoni in consultation with DFO biologists and Hatchery manager at BIO to take advantage of the respective expertises (scientific R&D research capacity, hatchery, Traditional Ecological Knowledge, field capacities).

Experimental design:

Adult oysters were collected from two MSX infected stocks (Crane Cove, Eskasoni and South Denys Basin near Lewis Island (Figure 15)) in September 2006, transferred to the oyster hatchery, acclimated and subsequently divided into the following treatment tanks:

Tank 1: Low salinity (10 ppt; 20°C)
Tank 2: Medium salinity (15 ppt, 20°C)
Tank 3: Low salinity-High temperature (10 ppt; 25°C)
Tank 4: High temperature (20 ppt, 25°C)
Tank 5: Control (20 ppt; 20°C)

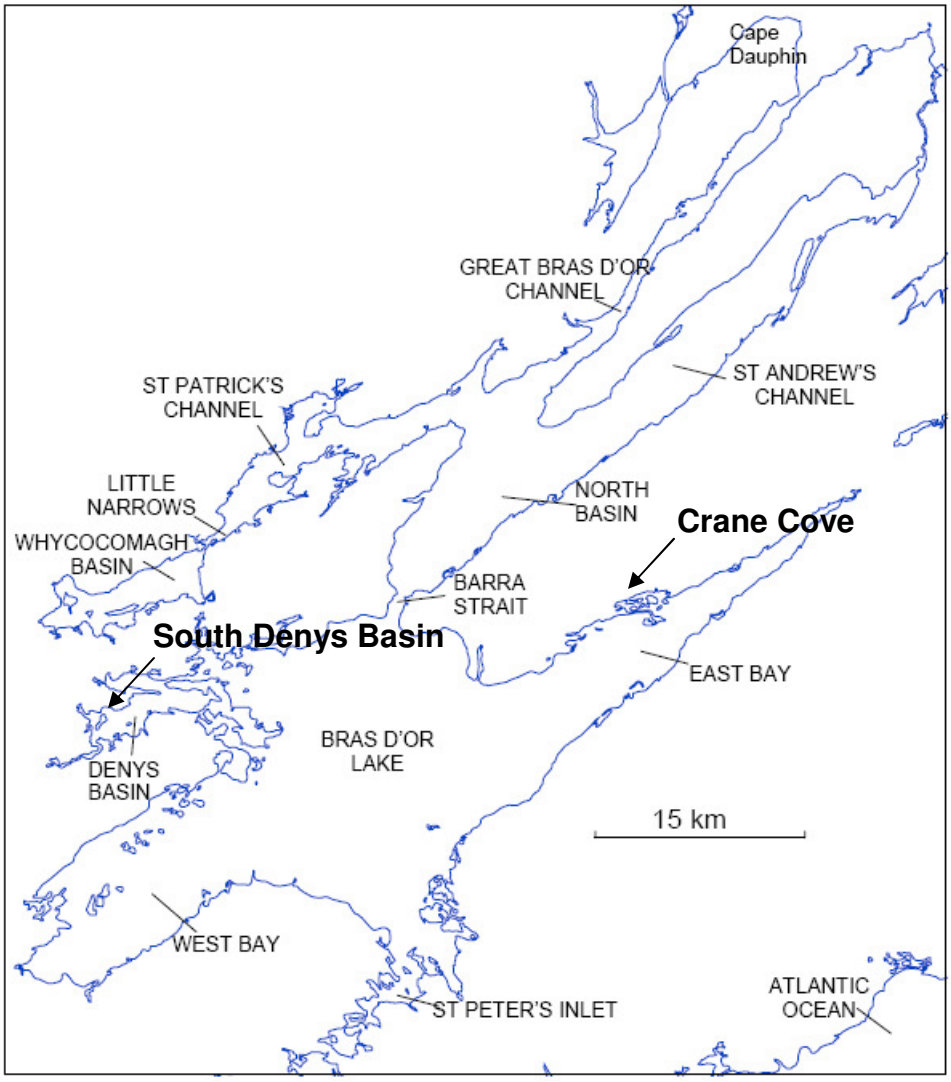


Figure 15. Sampling locations: Crane Cove, in Eskasoni (45° 57' 19"N, -60° 35' 10"W) and South Denys Basin, near Lewis Island (45°52'36" N, -61°05'22"W).

After a 4 week artificial winter using refrigerated water to mimic the start of a reproductive cycle, oysters were gradually exposed, over a 3 week period, to the treatment conditions and conditioning began in October (diet supplemented with cultured phytoplankton). Temperature and salinity were recorded continuously for each tank.

2.1.2. Gonad development and MSX prevalence

For each stock, a subsample of 20 oysters was examined upon arrival, at the beginning of the acclimation period and every 2 weeks for gonad development and MSX infection for each tank/treatment (unless specified, see Tables 1 and 2). A total of 1066 (533 x 2 stocks) oysters were measured (length, width and height) with a calliper and sexed, induced for spawning on 4 two-week interval occasions (see following paragraph), then shucked, sectioned across the visceral mass and gill, fixed in Davidson's solution and processed for histological examination according to Ford and Haskin (1982). Gonad development (i.e. maturity and fullness) was assigned to the following stages:

Maturity 1 = immature gametes
Maturity 2 = maturing gametes
Maturity 3 = mature gametes / spawning
Fullness 1 = gametes small with large interstitial spaces within gonad
Fullness 2= gametes developing with less interstitial space within gonad
Fullness 3= gametes developed with little to no interstitial space

Each oyster was also rated according to *H. nelsoni* infection and assigned to one of the following 4 categories: none, gill infected, light systemic and advanced systemic.

2.1.3. Spawning

The sub-sample of 20 oysters from each treatment was induced separately (in individual containers) for spawning at 2 weeks (December 4, 2006), 4 weeks (December 18, 2006), 6 weeks (January 1, 2007) and 8 weeks (January 16, 2007) during the conditioning/treatment period by thermal shock. Egg production was quantified and sperm quality assessed. Oysters were stripped of their gametes if necessary. Each shucked oyster was then processed for MSX prevalence and gonad development as described above and a piece of tissue preserved in 95% ethanol for future DNA fingerprinting.

2.2. Results and discussion

Note: The MSX prevalence results determined by histology will be published in a separate document.

Sampling date	Crane Cove							Total
21/09/2006	**Pre-treatment (winterization)**							
	Male	1						1
	Female	17						17
	Undetermined	2						2
	total	**20**						**20**
24/10/2006	**Pre-treatment (acclimation)**							
	Male	1						1
	Female	2						2
	Undetermined	17						17
	total	**20**						**20**
	Treatment tanks		**1** 20°C 10 ppt	**2** 20°C 15 ppt	**3** 25°C 10 ppt	**4** 25°C 20 ppt	**5** 20°C 20 ppt	
14/11/2006	Male							
	Female							
	Undetermined		20	20	20	20	20	100
	total		**20**	**20**	**20**	**20**	**20**	**100**
04/12/2006	Male				5	5	6	16
	Female				11	4	10	25
	Undetermined		20	18	1	8	3	50
	dead					1	1	2
	total		**20**	**18**	**17**	**18**	**20**	**93**
	Sex-ratio M:F (%)				*31:69*	*56:44*	*38:62*	
18/12/2006	Male		10	6	8	1	7	32
	Female		10	10	9	9	12	50
	Undetermined			2		4		6
	dead			2	3	6	1	12
	total		**20**	**20**	**20**	**20**	**20**	**100**
	Sex-ratio M:F (%)		*50:50*	*38:62*	*45:53*	*10:90*	*37:63*	
01/01/2007	Male		3					3
	Female		6					6
	Undetermined		7	17	19	16	12	71
	dead		4	3	1	4	8	20
	total		**20**	**20**	**20**	**20**	**20**	**100**
	Sex-ratio M:F (%)		*33:67*					
16/01/2007	Male		6	1	7	5	3	22
	Female		10	14	8	14	13	59
	Undetermined		1		1			2
	dead		3	5	4	1	4	17
	total		**20**	**20**	**20**	**20**	**20**	**100**
	Sex-ratio M:F (%)		*38:62*	*7:93*	*56:44*	*47:53*	*19:81*	
Total		***40***	***99***	***100***	***96***	***98***	***100***	***533***

Table 2. Crane Cove stock: Sampling regime, numbers of oysters sampled and determination of sex ratio by treatment tanks 1 to 5.

Sampling date	South Denys Basin							Total
21/09/2006	**Pre-treatment (winterization)**							
	Male							0
	Female	2						2
	Undetermined	18						18
	total	**20**						**20**
24/10/2006	**Pre-treatment (acclimation)**							
	Male							0
	Female	1						1
	Undetermined	19						19
	total	**20**						**20**
	Treatment tanks		**1** 20°C 10 ppt	**2** 20°C 15 ppt	**3** 25°C 10 ppt	**4** 25°C 20 ppt	**5** 20°C 20 ppt	
14/11/2006	Male		1					1
	Female		17					17
	Undetermined		2	20	20	20	20	82
	total		**20**	**20**	**20**	**20**	**20**	**100**
04/12/2006	Male		6		5	6		17
	Female		7		10	10		27
	Undetermined		5	20	1	2	20	48
	dead		1					1
	total		**19**	**20**	**16**	**18**	**20**	**93**
	Sex-ratio M:F (%)		*46:54*		*33:67*	*38:62*		
18/12/2006	Male		3	2	6	1	10	22
	Female		6	13	7	8	6	40
	Undetermined		4	2	1	6		13
	dead		7	3	6	5	4	25
	total		**20**	**20**	**20**	**20**	**20**	**100**
	Sex-ratio M:F (%)		*33:67*	*15:85*	*46:54*	*11:89*	*63:37*	
01/01/2007	Male		9	6	4			19
	Female		7	9	9			25
	Undetermined		1		3	20	20	64
	dead		3	5	4			12
	total		**20**	**20**	**20**	**20**	**20**	**100**
	Sex-ratio M:F (%)		*56:44*	*40:60*	*31:69*			
16/01/2007	Male		9	5	9	8	5	36
	Female		9	12	7	8	9	45
	Undetermined		1			2	1	4
	dead		1	3	4	2	5	15
	total		**20**	**20**	**20**	**20**	**20**	**100**
	Sex-ratio M:F (%)		*50:50*	*29:71*	*56:44*	*50:50*	*36:64*	
Total		***40***	***99***	***100***	***96***	***98***	***100***	***533***

Table 3. South Denys Basin stock: Sampling regime, numbers of oysters sampled and determination of sex ratio by treatment tanks 1 to 5.

Oysters were acclimated to the laboratory conditions from September 21 to October 17, 2006. After this initial acclimation, the oysters were divided in 5 groups and gradually acclimated to their respective tank treatment over a 3 week period (Figure 16). The treatment conditions were relatively well maintained over time and ensured that the oysters could properly condition. However the 25°C temperature was particularly difficult to maintain in the UINR/EFWC wet lab facility in the winter time and there was some (~5°C) variation in Tanks 3 and 4 (Figure 16).

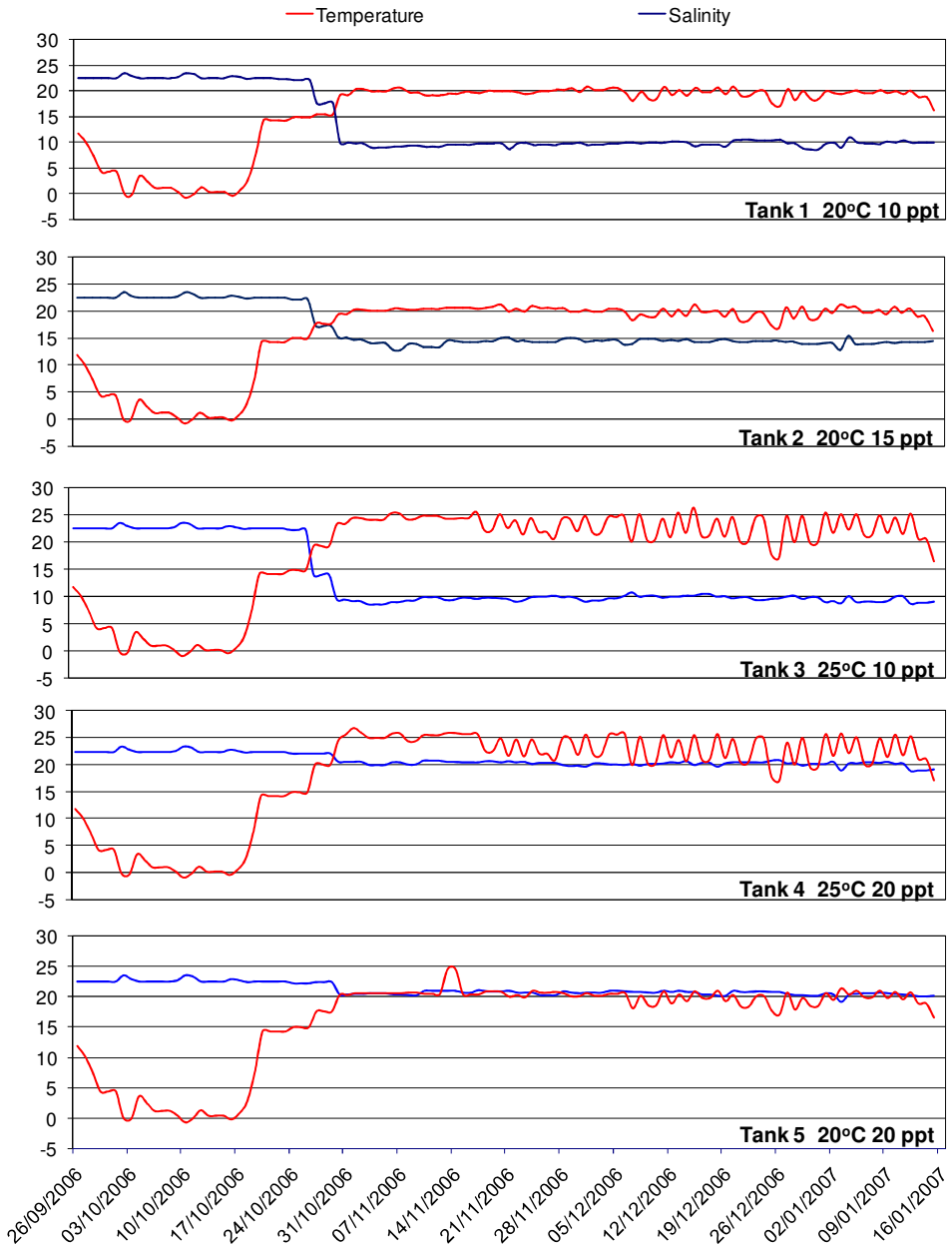


Figure 16. Temperature and salinity regimes in the 5 treatment tanks (Tank 5 is a control tank)

At each sampling and/or spawning attempt (September 21, October 24, November 14, December 4, December 18, January 1 and January 16), the sub-sample of 20 oysters was measured (Appendix 1) and the gonads visually assessed for maturation. This assessment was then compared with the gonad development results determined by histology. Figures 17 and 18 summarize the successive gonad development for the Crane Cove and South Denys Basin stocks, respectively, including mortalities, for the entire duration of the experiment.

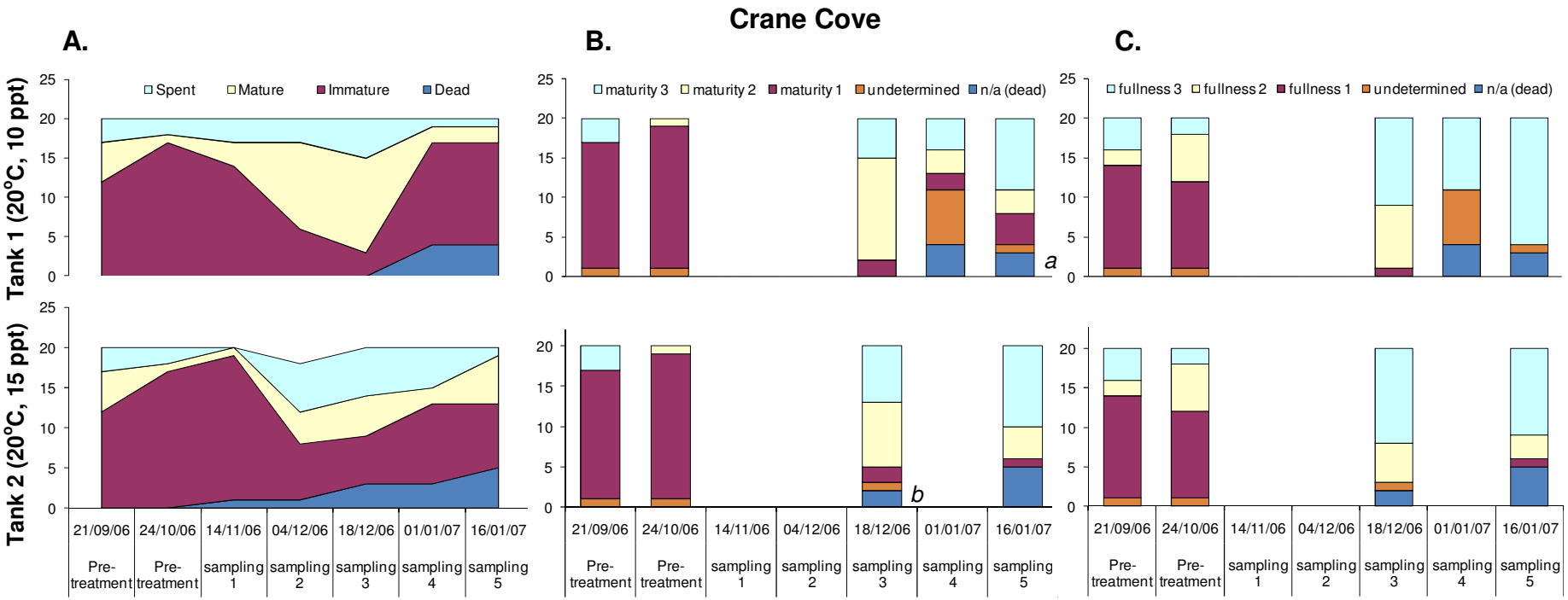
Mortality was nil or minimum during the artificial winter and during the acclimation period for both stocks but increased over time to reach a maximum of 40% in some treatments. On average there was a similar mortality between the Crane Cove (10.3%) and South Denys Basin (10.75%) stocks.

The 4 week artificial winter and following acclimation successfully allowed oysters to acquire the resting stage necessary to start a new reproductive cycle. The visual condition index as well as the maturity and fullness levels changed to "immature" or decreased during that period.

Overall, the assessment of maturation by visual examination of the gonad during the experimental period was relatively conservative as the histological examination results revealed more oysters maturing or having reached the mature stage over time. This was systematic for each treatment throughout the experiment and thus was used as a conservative indication only when histological results were missing.

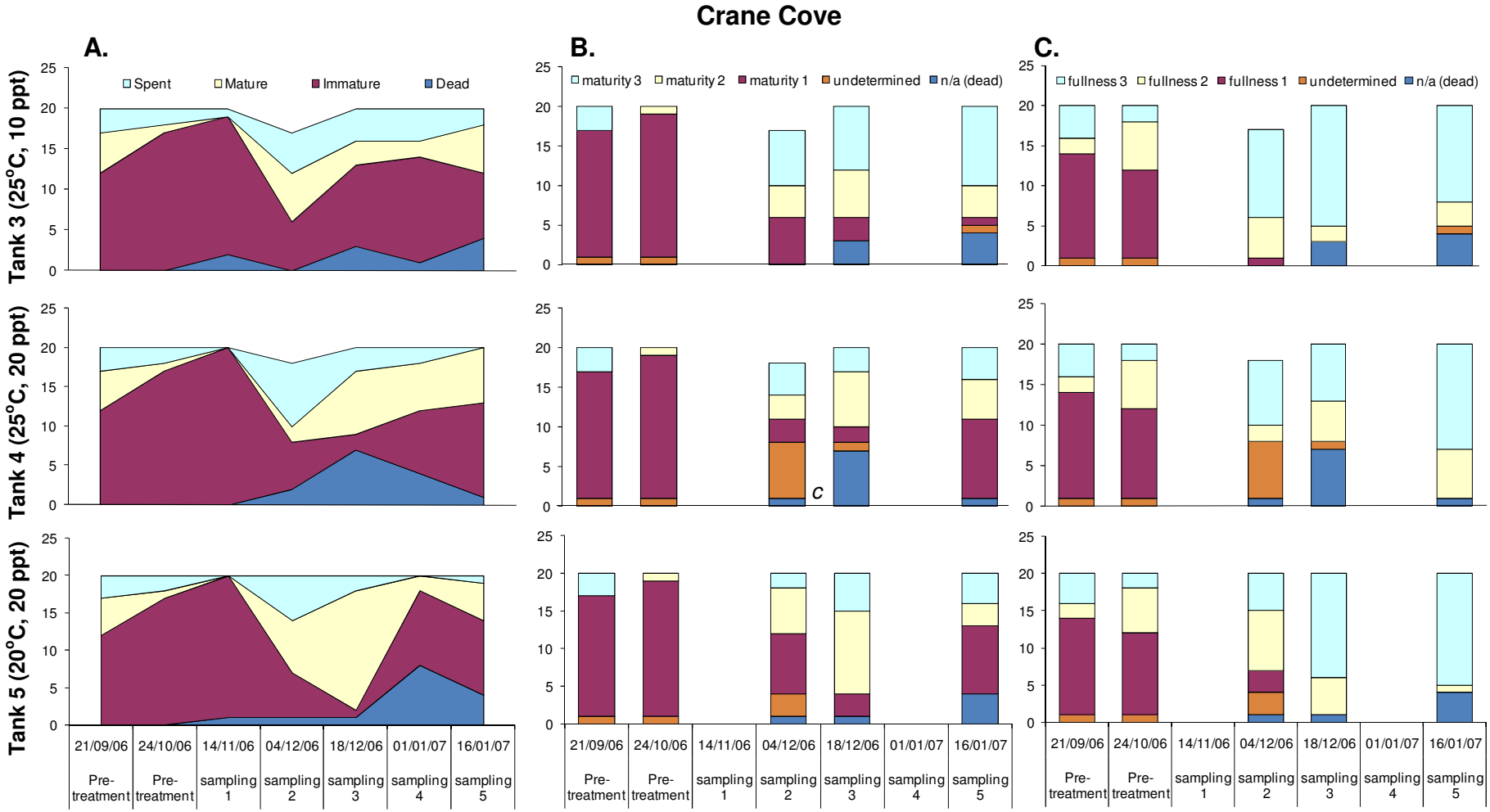
Maturity was most successfully achieved the third period (sampling on December 18, 2006, 4 weeks within conditioning) for the Crane Cove group, where maturity implicated 84.6% of the live oysters in the sample of oysters treated in Tank 4 (25^{o}C, 20 ppt) and 85% in Tank 1 (20^{o}C, 10 ppt), percentages closest to the control group (90%). However, 35% mortality was recorded for that period in Tank 4 but none in Tank 1. Tank 4 continued to show a relatively high percentage of maturity for the two subsequent periods (50% and 36.8%) with less mortality than previously seen and with a higher maturity than the control group. When comparing treatments with the same temperature, 20^{o}C, but different salinities, 10, 15 and 20 ppt (Tank 1, 2 and 5 respectfully), it seems that broodstock mortalities were similar but that spawning was still occurring at 8 weeks into the treatment (Figure 17, panel B) for lower salinities and the control group consisted of spent oyster or oysters maturing possibly for a second time.

The treatment in Tank 3 (25^{o}C, 10 ppt), which represents the most extreme conditions compared to the control tank, showed the lowest maturity (41.1%) on



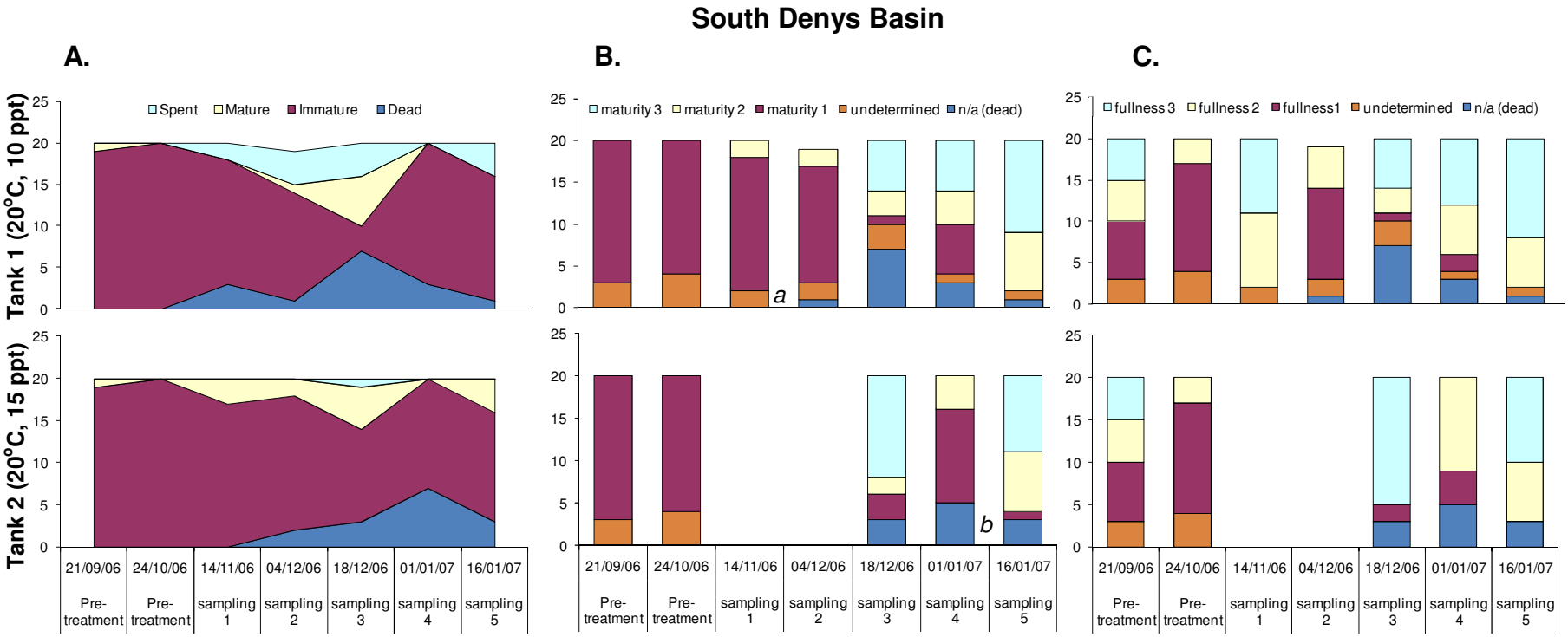
a. 1 out of 4 and *b.* 1 out of 3 dead oysters could be indexed for gonad development

Figure 17. Maturation determined by external observation (A) and gonad development (B and C) determined by histology for Crane Cove stock conditioning under different treatments. Missing bars represent histological slides that could not be read.



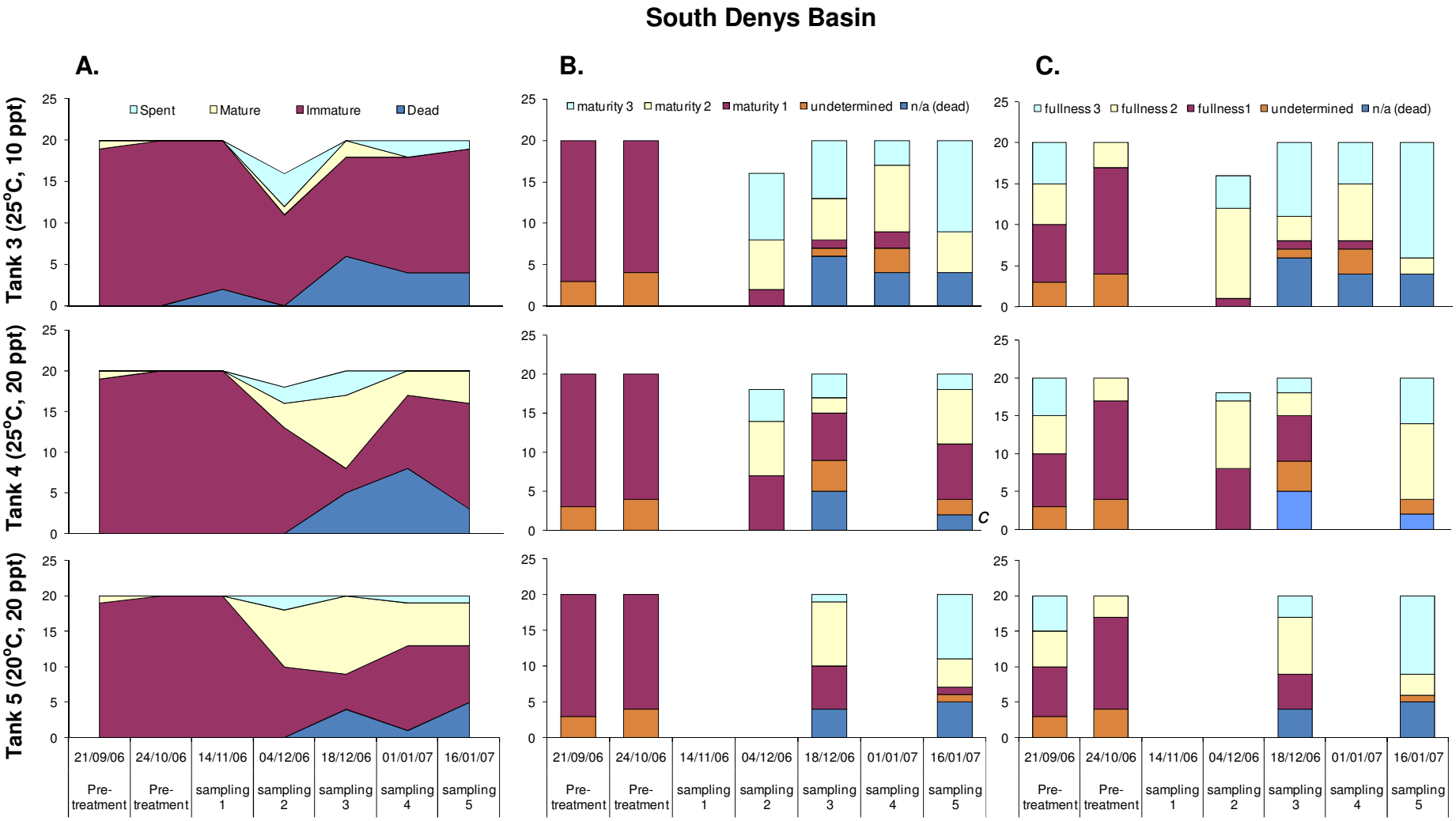
c. 1 out of 2 dead oysters could be indexed for gonad development

Figure 17. (continued)



a. 3 out of 3 and *b.* 2 out of 7 dead oysters could be indexed for gonad development

Figure 18. Maturation determined by external observation (A) and gonad development determined by histology (B and C) for South Denys Basin stock conditioning under different treatments.



c. 1 out of 3 dead oysters could be indexed for gonad development

Figure 18. (continued)

the third sampling but overall, oysters in terms of maturity and survival performed almost as well as the other treatments.

For oysters from South Denys Basin, 80% of live oysters were mature in Tank 4 (25°C, 20 ppt) and 78.9% in Tank 1 (20°C, 10 ppt) compared to 68.75% in the control tank. However there were some mortalities in both tanks, 25% and 35%, respectively. Again, oysters from the treatment Tank 3 (25°C, 10 ppt) showed the lowest maturity of all treatments (10%). The oysters from the control group showed an overall maturity of 50.0% over the treatment period, almost 10% lower than oysters from the Crane Cove control group (59.09%).

It should be noted that the maturity of a few oysters could not be determined through histology as cuts were improperly performed, but these were minimal. However, we should stress here that the quality of the histological slides is of key importance to properly assess the maturity of different oyster groups.

Overall, oysters from South Basin were smaller (volume index varied from 52 to 95) than oysters from Crane Cove (volume index varied from 78 to 113) (see Appendix 1) and this could explain the overall maturity and fecundity difference (Hoffman et al., 1994).

Sex ratio was calculated when sex could be determined and there appears to be a bias against males (i.e. the sampled oysters were mostly female); however it seems that an equilibrium was reached toward the end of the conditioning period with the exception of the Crane Cove Tank 2 sampling (Tables 2 and 3). Ford et al. (1990) found an increased proportion of females in MSX infected oysters compared to uninfected oysters (3:1) but no evidence of differential infection or mortality. They also suggest that gametogenesis is inhibited more in male than in female oysters.

Spawning could only be achieved by stripping for all treatment groups but there was a high degree of fertilization. In fact, because of gametic incompatibility in eastern oyster, up to 50% of crosses can show low or delayed fertilisation (Gaffney et al., 1993).The different egg yields did fit well to the resulting larval counts (results not presented here).

The best maturity levels and best spawning attempts were observed 4 weeks into conditioning. Higher temperature seemed to have increase gametogenesis but

also increased mortality for both groups of treated oysters. Lower salinities tended however to delay spawning. Overall, when selecting treatment to improve oyster maturity and gametogenesis while repressing MSX, it is recommended to use lower salinities while maintaining appropriate temperature and food supply.

Conclusion and future directions

These two R&D projects complement the initiatives proposed for enhancement of the Bras d'Or lake oysters after three years of exposure to the MSX disease. The newly constructed UINR hatchery, operated by EFWC, showed much promise, having successfully spawned in 2005 and 2006, three groups of oysters that were exposed to the MSX disease and performed nine crosses within this project as well as many others. When selecting MSX tolerant broodstock for conditioning, it cannot be understated that we are dealing with infected (therefore live but seriously ill) oysters and this is a major factor in proper conditioning. In fact, broodstock management for disease tolerant selection programs is generally clearly identified as a knowledge gap. Treatment of broodstock is recommended for improving spawning success. Higher temperature during the conditioning period led to higher maturity in the two stocks tested but also to increased mortalities.

With the use of optimally conditioned males and females from separate lines rotated at each generation, the production of multiple selected lines would work well with the expectation of high heritability for MSX tolerance. It is critical to pursue these efforts to reach a fully fledged oyster breeding program for tolerance to MSX. However, financial support has been identified early on as a critical limitation, in particular if the breeding program is expanded to include restoration projects.

These R&D projects also provided a significant opportunity to foster the collaboration between DFO, Cape Breton First Nations and the oyster industry. In the spirit of Scientific Research and Information Exchange proclaimed in the Memorandum Of Understanding signed in 2004 between UINR and DFO, this collaborative project facilitated and accelerated the process of technology transfer to the oyster industry and increased the scientific capacity for a critical oyster aquaculture R&D issue. For instance, preparation of tissues and slides for histology was done at the laboratory in Eskasoni. And with training in histological analysis, and by following protocols used by the DFO-SHU laboratory in Moncton, technicians and biologists were able to perform full histological analyses.

Future breeding to obtain hybrids with Bras d'Or lake oysters and oysters from the Gulf region (resistant to Malpeque disease) and from the US (showing MSX and/or Dermo resistance) can only be considered once an approved quarantine unit is built in Nova Scotia and genetic and disease impacts evaluated. Also, regulations for quarantine units are changing and the new time frames of the OEI under which MSX is reported, may not be realistic. In addition, there could be a levy imposed on oysters bred from US lines, such as Rutgers MSX-resistant, and also some inbreeding or performance issues (Mallet, 2004).

Selective breeding for enhanced MSX resistance in oysters from the Bras d'Or lake is possible and will improve, relatively quickly, the survival of cultured and natural populations. Selected lines restrict the development of lethal infections (Haskin and Ford, 1979), the oysters typically display no mortality caused by the disease during the first 2-3 years (until they reach market size), however, the oysters will become infected and eventually die with MSX disease, but only after 5-6 years (S. Ford, pers. comm.). In addition, it should be possible to select simultaneously for increased disease resistance and increased growth (Mallet, 2004). However, special attention to breeding structure, through detailed pedigree records and genotyping, should be a priority to avoid effects of inbreeding. This traditional approach to selection could benefit from the research on DNA markers to refine disease resistance lines. In the US, Wang and Guo (2008) identified disease-resistance DNA markers in the eastern oyster based on their family-based association with resistance to both Dermo and MSX diseases.

An even more effective selection could be achieved once the MSX disease transmission is known. Attempts to pass it from infected to uninfected oysters or to inject the parasite into the oyster in laboratory conditions have failed in the US and this represent a major constraint for any selective breeding to MSX resistance. For example, resistance to *Bonamia*, a parasite of the flat oyster *Ostrea edulis* has been achieved through injection (Naciri-Graven et al., 1998) while natural resistance has still not occurred. In addition, with a group of Eastern oysters experimentally injected with the Dermo parasite *Perkinsus marinus*, Zhang et al. (2008) were able to map quantitative trait loci (QTLs) conferring resistance to Dermo.

In the US, the Delaware Bay Ecology of Infectious Diseases (EID) group has been working extensively on interactions between oyster population genetic/dynamics – environment – parasite and on how climate change might modify these interactions (Hoffman et al., 2009). Their laboratory, field and modelling studies on Delaware Bay may have applications that could extend to other systems, such as the Bras d'Or lake. For example, the issue of true or putative disease refugia has significant management implications. Oyster populations affected by the MSX disease usually contract to low salinity areas where susceptible genotypes persist. In this scenario described by Hoffman et al. (2009) the majority of the population survive in these refugia; only a few highly selected individuals develop resistance and the population undergo rapid genetic shift. In the Chapel Island control group, because the MSX parasite can be detected with the sensitive PCR diagnostic method, the parasite is considered present but it does not lead to infection detected by histological method. There may be true low-salinity disease

refugia remaining in the lake where the parasite is not present (or not detected by PCR). The existence of disease refugia means that the overall system may not be able to develop true MSX-resistant populations, unless through large-scale strong selective events such as the epizootic that occurred in Delaware Bay in 1984-1986 (Hoffman et al., 2009). The EID group also developed models and simulations indicating that, alternatively, long term sustained input of selected larvae and/or adults (e.g. enhancement/ restoration projects) will result in the establishment of new traits in the oyster population (Hoffman et al., 2009).

Another critical management issue pertaining to these efforts in restoring oyster populations is the removal, through fishing, of oysters (usually the largest/oldest) with traits that are beneficial to the long-term survival of the resource. This has to be restricted or prohibited as the establishment of resistance characteristics would be greatly reduced.

Lastly, if climate-induced changes result in increased temperature and lower salinity, as predictions of increased temperature and rain fall seem to lead to, we might observe lower MSX infection levels in the Bras d'Or lake oysters in the future but we should keep in mind that other factors such as circulation (i.e. for larval dispersion) and food supply might be altered as well.

The complex MSX resistance question needs to be addressed further. In working collaboratively, and in a timely manner, within the region and internationally with the US mid-Atlantic region, valuable experience and information could be gained to restore and improve the health and competitiveness of both the EFWC aquaculture operation and the sustainability of the Bras d'Or lake oyster industry as a whole, which is currently facing multiple disease challenges.

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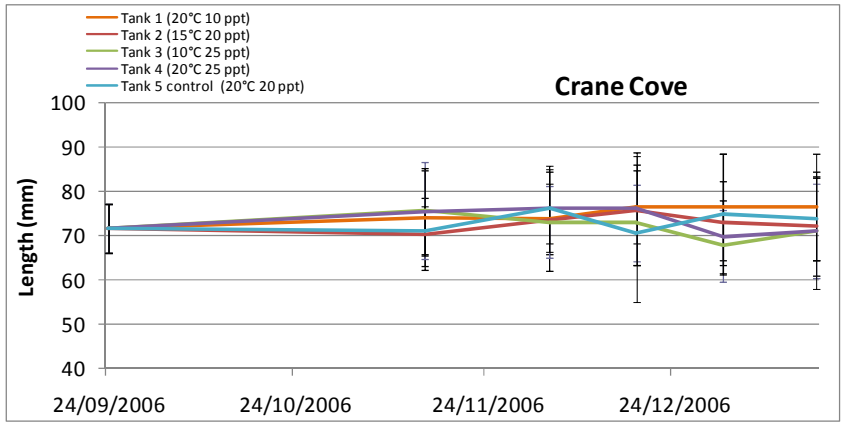
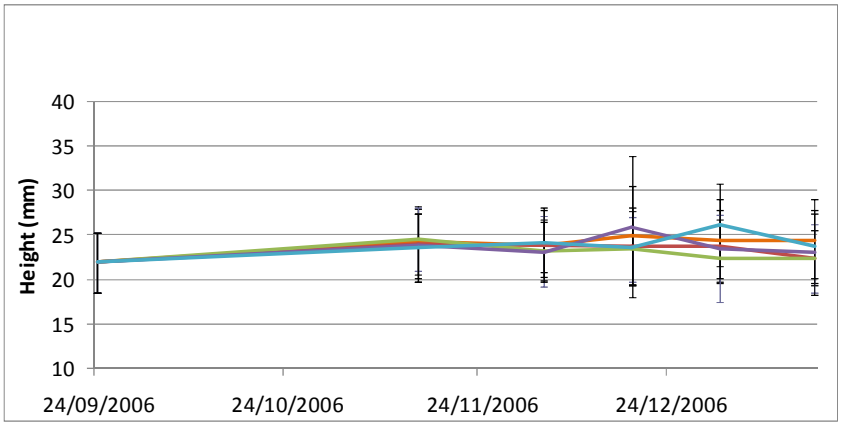
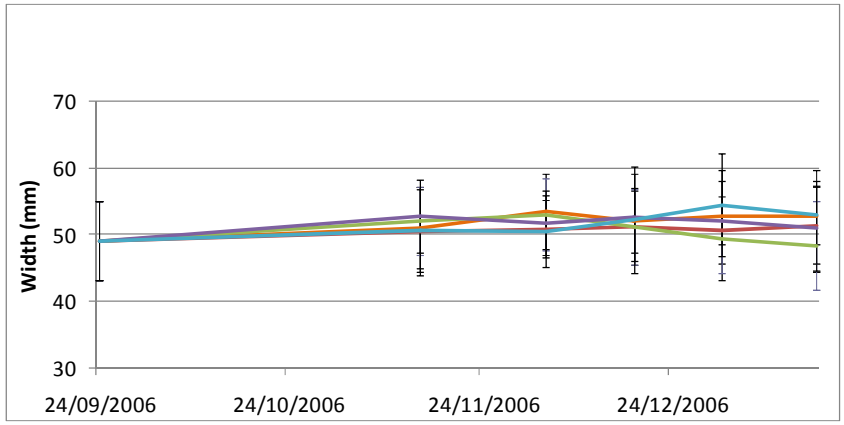
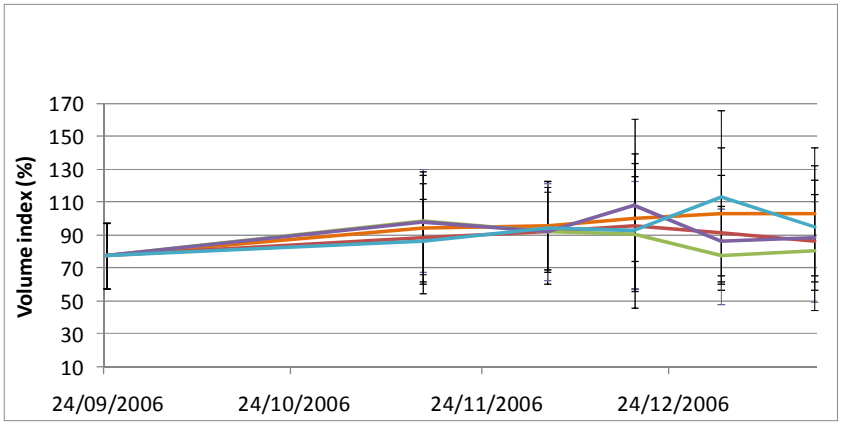
Appendix 1. Measurements of sampled oysters from Crane Cove and South Denys Basin stocks.









Appendix 1. (continued)