MATERIAL RECOPILADO

Induction of triploidy in Atlantic cod (*Gadus morhua*) by cold shocks

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Background and objective

The production of triploid sterile fish enables avoidance of the adverse effects of early maturation in some aquacultural species, and can be seen as a technique for conservation of natural populations.

The objective of this preliminary study was to induce triploidy in Atlantic cod (*Gadus morhua*) using cold shocks.

Materials and methods

Brood - stock 3-4°C

♂ 4x0.5 ml
♀ 200 ml

Two identical experiments were carried out at Tromsø Aquaculture Research Station during March-April 2004. Gametes from Northeast Arctic cod were collected concurrently as routine stripping of brood-stock, and the following cold-shock protocol was adopted:

For ploidy analysis, larvae were thawed in 0.4 ml of propidium iodide solution, carefully desegregated using a pipette, and filtered through a 20 µm mesh (Peruzzi and Chatain, 2003). The DNA content of up to 50 larvae per treatment was measured by the use of a Becton Dickinson FACSCalibur flow cytometer (University Hospital of North Norway).

Fertilisation 6°C

+ 100 ml seawater
↓ 1 min
+100 ml seawater
↓ 1 min
+200 ml seawater
↓ 3 min

CONTROL

10min
20min
30min
40min
50min
60min
p.f.

Cold shock

-1.7±0.1°C

Incubation 6°C

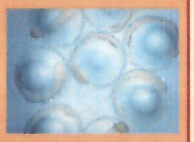
Control

1 h: 10min/1h, 20min/1h, 30min/1h, 40min/1h, 50min/1h, 60min/1h

2 h: 10min/2h, 20min/2h, 30min/2h, 40min/2h, 50min/2h, 60min/2h

1 d p.f.
activation rate

~50 dd
embryonation



survival at hatching

Sampling

larvae rinsed repeatedly in distilled H_2O

Storage -80°C

Results

Mean survival of the three replicates was estimated as egg survival at embryonation (Figure 1) and larval survival at hatching (Figure 2). Treated groups performed poorer than sham control at hatching in Experiment I, whereas in Experiment II, some of the treated groups showed enhanced survival (Figure 3). No triploid larvae were observed in the first experiment, and only low rates of triploidy (0-14%), irrespective of the timing or duration of the treatment, were found in Experiment II (Table I). In Experiment I, a low number of tetraploid larvae was also detected : 4% in 30min/1h, and 2% in 60min/2h. Typical nuclear DNA content of mixed samples of diploid/triploid and diploid/tetraploid larvae are shown in Figure 4a and 4b, respectively.

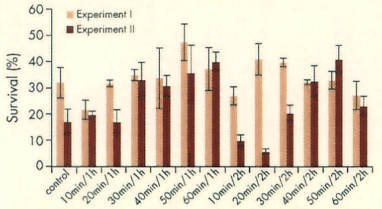


Figure 1. Egg survival at embryonation (approximately 50 dd) relative to the initial number of eggs.

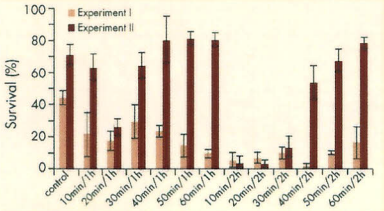


Figure 2. Larval survival at hatching relative to the number of embryonated eggs.

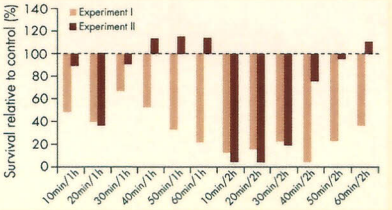


Figure 3. Survival of the treated groups at hatching relative to control.

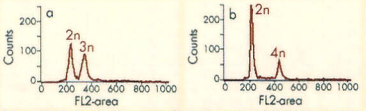


Figure 4. Nuclear DNA content of mixed samples of diploid/triploid (a) and diploid/tetraploid (b) larvae measured by flow cytometry.

Treatment	N		Triploid rate (%)		Triploid yield (%)	
	Expl	Expll	Expl	Expll	Expl	Expll
Control	62	58	0.00	0.00	0.00	0.00
10min/1h	50	50	0.00	4.00	0.00	2.52
20min/1h	50	50	0.00	0.00	0.00	0.00
30min/1h	50	50	0.00	2.00	0.00	1.28
40min/1h	50	50	0.00	0.00	0.00	0.00
50min/1h	50	50	0.00	4.00	0.00	3.25
60min/1h	39	50	0.00	0.00	0.00	0.00
10min/2h	41	7	0.00	14.29	0.00	0.44
20min/2h	34	3	0.00	0.00	0.00	0.00
30min/2h	49	50	0.00	2.00	0.00	0.27
40min/2h	29	50	0.00	0.00	0.00	0.00
50min/2h	41	50	0.00	0.00	0.00	0.00
60min/2h	50	50	0.00	8.00	0.00	6.24

Table I. Number of analysed larvae (N), triploid rate, and triploid yield for the two experiments (Expl and Expll).

Concluding remarks

Cold shocks of -1.7±0.1°C for duration of 1 to 2 hours induced only low rates of triploidy in Atlantic cod.

Future experiments will be conducted to investigate the effect of longer cold shock treatments on triploidy rate, as well as to compare such results with those from heat-shock and hydrostatic pressure experiments.

References

Peruzzi, S. and Chatain, B., 2003. Induction of tetraploid gynogenesis in the European sea bass (*Dicentrarchus labrax* L.). Genetica **119**: 225-228.

Layout: François Simond

Development of microsatellite markers in *Pagellus bogaraveo* and their usefulness in other five species with interest in aquaculture.

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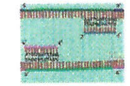
INTRODUCTION

Microsatellites are very useful genetic markers in aquaculture. However these markers are usually species specifics and therefore as many different loci sets as species under study are needed. In this work a set of 15 microsatellites previously developed from a *Pagellus bogaraveo* genomic library was tested by a cross-species amplification in other related species currently exploited such as *Sparus aurata* and *Dicentrarchus labrax*, and in others of aquacultural potential interest like *Diplodus puntazzo*, *Dentex dentex* or *Pagellus erythrinus*. The final idea is to avoid the development of new microsatellite sets for the new aquacultural species, and also to obtain a single multilocus set that allows us to analyse wild populations and the management and improvement of farmed stocks.

Pagellus bogaraveo

15 microsatellite loci set

The microsatellite loci battery was tested in other 5 species



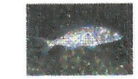
Sparus aurata

Dicentrarchus labrax

Diplodus puntazzo

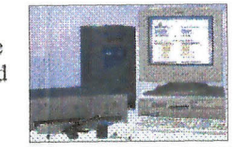
Pagellus erythrinus

Dentex dentex



PTC-200 (Mj Research, Inc.) thermal cycler

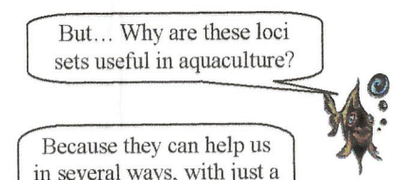
The cross-species amplifications were carried out on a thermal cycler, varying the annealing temperature and the [$MgCl_2$], and visualized on an automated DNA sequencing system.



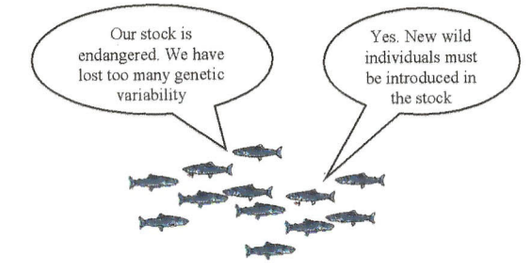
OpenGene Automated DNA sequencing system (Visible Genetics Inc.)

Table I: PIC (*Marshall et al., 1998*) and mean number of alleles (between brackets).

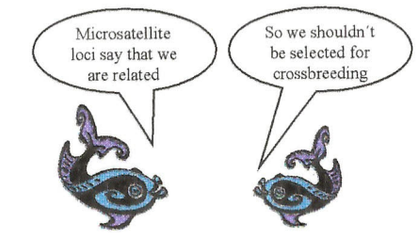
Loci	*P. bogaraveo*	*D. puntazzo*	*S. aurata*	*D. dentex*	*D. labrax*	*P. erythrinus*
Sample size	31	24	26	31	18	24
PbOviD102	0.85 (2)	0.91 (16)	0.89 (16)	0.76 (7)	0 (1)	0.93 (21)
PbOviB2	0.88 (14)	0 (1)	0.59 (6)	0.58 (9)	0.10 (2)	
PbOviA5	0.9 (19)		0.20 (4)		0.05 (2)	
PbOviA3	0.88 (15)	0.85 (13)	0.51 (4)			
PbOviD106	0.75 (7)	0.88 (14)	0.79 (10)			
PbOviD108	0.45 (4)		0.04 (2)			
PbOviD22	0.83 (12)		0.63 (7)			
PvOciC103	0.76 (8)	0.26 (6)	0.14 (3)			
PbOviD101	0.84 (11)				0.22 (4)	
PbOviD114	0.77 (9)	0.86 (12)				
PbOviD20	0.81 (9)					
PbOviD21	0.82 (10)					
PbOviC109					0.68 (5)	
PbOviD23					0 (1)	
PbOviD110		0 (1)				



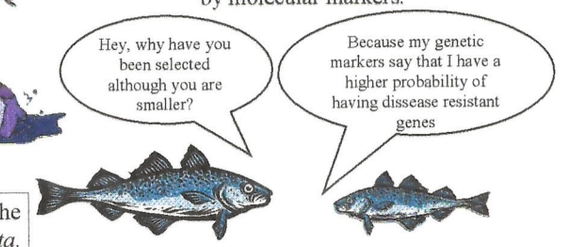
Assesing the evolution of the genetic variability in farmed stocks.



Classifying individuals of unknown pedrigree to avoid an increase of the inbreeding.



Selecting interesting phenotypes assisted by molecular markers.



CONCLUSIONS

This set of microsatellite markers is helpful both in population genetic studies and in the management and improvement of farmed stocks in *P. bogaraveo*, *D. puntazzo* and *S. aurata*. However, new microsatellite markers should be added to the battery to increase the resolutive power in *D. dentex*, *D. labrax* and *P. erythrinus*.

This work is supported by a FICYT grant for Jorge Álvarez and a FPU grant for David bernardo.

How to make females out of males

S. van Nes Ø. Andersen

the effect of stimulation of P450-aromatase on the sex differentiation in Atlantic halibut

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Introduction

Gonadal development in lower vertebrates seems to be mainly controlled by the enzyme P450-aromatase, which transforms testosterone into estrogen. This process is temperature-sensitive, which opens for the possibility to sex reverse Atlantic halibut for aquaculture purposes without the use of hormones or genetic manipulation.

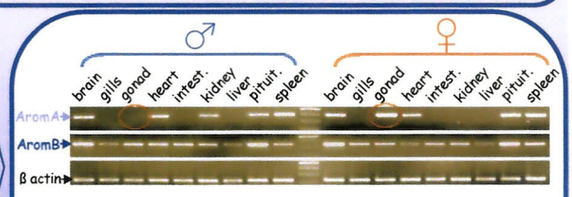
Here we report on the identification of two Atlantic halibut isoforms of P450-aromatase and their temporal and spatial distribution.

These results allow further investigation and understanding of the molecular basis of sex differentiation in Atlantic halibut

Results

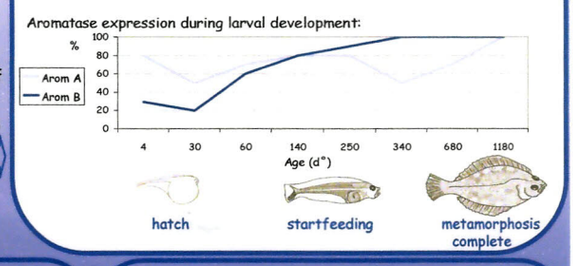
Two full-length cDNAs encoding distinct P450aromA and aromB isoforms were isolated.

The tissue specific expression patterns were overlapping, but aromA clearly predominated in the ovary, whereas aromB showed higher expression in brain and pituitary.



The temporal expression analysis also revealed individual differences in expression pattern in early developmental stages (demontrated as % of animals showing expression on each stage).

A very early activity of the two aromatase genes studied was found as compared to initiation of the histological sex differentiation



Discussion & Conclusion

The differences in the spatial and temporal expression patterns of aromA and aromB suggest that they have distinct functional roles.

The detection of both variants of aromatase at early developmental stages reveals that the onset of molecular sex differentiation clearly precedes any morphological development of the gonads.

The early onset of molecular sex differentiation suggests that in order to accomplish sex reversal by temperature treatment, the treatment should be initiated at a early developmental stage.

In progress

A temperature experiment is nearly completed, and in the near future we will reveal the exact effect of temperature on aromatase by quantitative expression analysis of the two aromatase genes using Real-Time PCR. In parallell, the temperature experiment will show the effect on phenotype and to what degree sex reversal of Atlantic halibut is possible by means of thermal treatment.

The understanding and possibly accomplishment of sex reversal by thermal treatment has potential to be of great importance for the Atlantic halibut aquaculture industry.

Methods

Cloning of the two different aromatase cDNAs was conducted by RACE-PCR strategies.

The PCR products were inserted into pGEM-T vector and sequenced in both directions.

The spatial gene expression analysis was performed by qualitative RT-PCR on a variety of tissues in sexually mature halibut as well as a temporal expression analysis from hatch to metamorphosis.

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Gonadal cycle of the cockle *Cerastoderma edule* of Galicia (NW Spain)

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CONSELLERÍA DE PESCA E ASUNTOS MARÍTIMOS

Introduction

The edible cockle *Cerastoderma edule* is one of the most important infaunal bivalve mollusc resources exploited in Galicia. The knowledge of the reproductive cycles of marine bivalves is essential to develop management strategies to guarantee the sustainability of fisheries. In this study the gonadal cycle of *Cerastoderma edule* from four different natural beds of the coast of Galicia was studied using histological methods. The influence of disseminated neoplasia in the gonadal development was also evaluated in one of the studied populations in which this pathological condition was more prevalent.

Material and methods

Four natural beds (Fig. 1) were considered in this study, three in the *Rías Baixas* (southern estuaries of the Galician coast): *Carril, Camaxe, Misela;* and another in the *Rías Altas* (northern estuaries): *Villabril*. Random samples of 25 cockles were taken monthly -from 1999 to 2000 in the southern populations, and from 2000 to 2001 in *Villabril*-, carried to the lab and processed using histological techniques. Histological cross-sections of cockles were examined by light microscopy for analysis of the gonad condition and diagnosed for disseminated neoplasia. Gonad development was determined using a scale with 7 different gametogenic stages: 0, *Resting stage*; 1, *Initiation of gametogenesis*; 2, *Developmental stage*; 3, *Ripe stage*; 4, *Spawning*; 4B, *Redevelopment*; and 5, *Reabsorption*.

Disseminated neoplasia effects were studied in Misela, where the prevalence was high. The disease was staged from 0 to 4 -higher numbers indicating more advanced disease stages (Fig. 2)- (Mix, 1983).

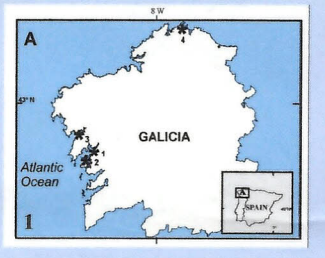
Results and discussion

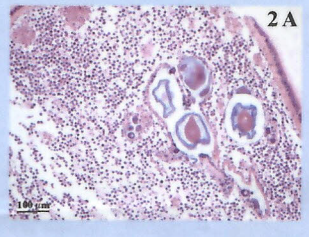
The general pattern of gonadal cycle found in cockle populations of the 3 sampled beds of Rías Baixas (Fig. 3A) involved a resting stage and initiation of gametogenesis in late autumn-early winter, a rapid development of gonads from January to March and the achievement of ripeness at late March-early April. Partial spawning and redevelopment of gonads occurred during the spring and summer. A reabsorption phase took place at late summer-early autumn .

Gonadal cycle of the Galician northern seabed Villabril in 2001 (Fig. 3B) was slightly different and delayed in the time, compared to the data from the southern estuaries in 1999-2000. Beginning of gametogenesis took place in late autumn-early winter.

Gametogenesis progressed slowly so most of the population was not ripe until May. Spawning began at June and continued -simultaneously with gonad redevelopment- until mid October .

It seemed that there was a delay in the gametogenesis in the northern estuaries (ripeness in May) *versus* the southern populations (ripeness in April) but the avaliable data correspond to different years. Therefore this should be further assessed.





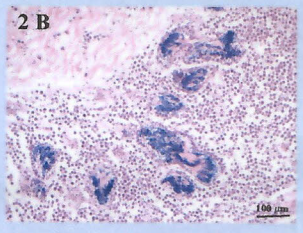


Fig. 1: Map of Galicia showing the location of the sampled beds: *Carril* (1), *Camaxe* (2) and *Misela* (3) in the *Rías Baixas*; and *Villabril* (4) in the *Rías Altas*. **Fig. 2:** Cockle histological sections (H&E) showing high intensity levels (stage 4 of the Mix scale) of disseminated neoplasia affecting female (2A) and male (2B) gonadal tissue.

Cockles from Misela (Fig. 4) with high intensity levels of disseminated neoplasia (4) had higher proportion of resting (29 %) and reabsorption gonad stages compared to unaffected (0) cockles (only 5% of individuals showed resting gonads). A significant association between disease intensity and gonad condition was detected (TABLE 1: $\chi 2$=39.556; DF=24; P=0.024).

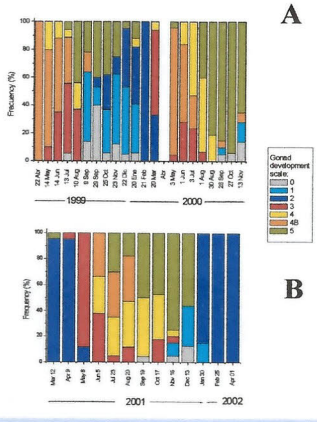


Fig. 3: Annual gonadal cycle pattern of cockles from Misela (A), in the Rías Baixas; and Villabril (B) -Rías Altas-.

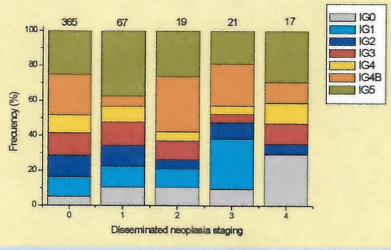


TABLE 1: Chi-square test results.

INTENSITY LEVELS OF AFECTION	N	DF	Chi-square	P	
0 vs 1 vs 2 vs 3 vs 4	436	24	39,556	0,024	*
0 vs 1	432	6	13,902	0,031	*
0 vs 2	394	6	2,803	0,833	n.s.
0 vs 3	349	6	7,660	0,265	n.s.
0 vs 4	302	6	18,883	0,005	**
1 vs 2	96	6	9,954	0,191	n.s.
1 vs 3	88	6	10,881	0,092	n.s.
1 vs 4	84	6	6,923	0,328	n.s.
2 vs 3	40	6	2,776	n.s.	
2 vs 4	36	6	6,625	n.s.	
3 vs 4	39	6	9,365	0,154	n.s.

Fig. 4: Cockle distribution according to gonadal condition and disseminated neoplasia intensity. Numbers above the bars indicate the number of cockles in every stage of neoplasia intensity.

References

Mix, M.C. 1983. Haemic neoplasm of bay mussels, Mytilus edulis L., from Oregon: occurrence, prevalence, seasonality and histopathological progression. Journal of Fish Diseases 6: 239-248.

Acknowledgements

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AQUACULTURE EUROPE 2004 Barcelona, Spain, October 20-23

FINDING MY PARENT: HOW MANY GENETIC MARKERS ARE NEEDED?

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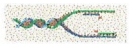
ABSTRACT: In this work, we evaluated the minimum number of microsatellite *loci* needed to obtain high percentages of correct assignment. By software simulations, we determined that 7-9 highly polymorphic markers would achieve 100% of accuracy.

RESUMEN: Se evaluó el número mínimo de *loci* microsatélites necesarios para obtener elevados porcentajes de asignación correcta. A través de simulaciones informáticas consideramos que 7-9 *loci* microsatélites con elevados niveles de polimorfismo permiten obtener porcentajes del 100% de acierto.

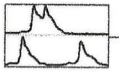
INTRODUCTION:

Several breeding programmes implement artificial selection on phenotypic variation to obtain varieties with high commercial value, however inbreeding effects resulting from selection based on phenotypic variation could produce a drastic reduction of productivity in few generations. Unequivocal pedigree information allows to avoid those effects. Nowadays, several hatcheries rear the progenies from different families at separate tanks, but this method is laborious and expensive, also introduces environmental variance. Another method is the use of physical markers (fins clipped, heat- and cold-branded) but a minimum corporal size of individual is required. Microsatellite markers can be fast and easily analyzed from a little piece of tissue, and do not show the aforementioned problems. But, how many microsatellite *loci* are needed to obtain high percentages of correct assignment? Efficiency of microsatellite *loci* depends on genetic variability of stocks. In this work, we evaluated which is the lowest number of microsatellite markers needed according to their variability levels (PIC: Polymorphic Information Content) to obtain unambiguous pedigree information.

DNA extraction

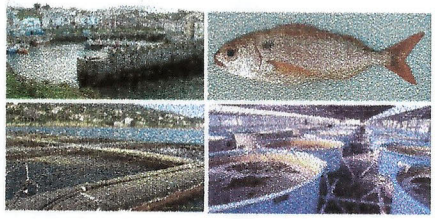


PCR amplification



Fragment analysis of 15 microsatellite *loci*.

- 5 *loci* PIC<0.5
- 5 *loci* 0.5<PIC<0.75
- 5 *loci* PIC>0.75

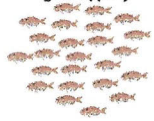


Ten randomly selected parents: 5 males and 5 females to create by simulation 25 families(10 descendants for family).

Genotypes of 34 Blackspot seabream individuals for the 15 microsatellites set

Assignment process

Cervus searches the most likely parental couple for each offspring by genotype comparisons.



(250 descendants with their genotypes)

Correct assignment

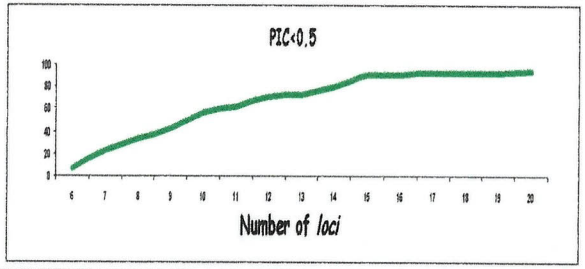
5 *loci* PIC<0.5	5 *loci* 0.5<PIC<0.75	5 *loci* PIC>0.75
2%	60.4%	95.2%

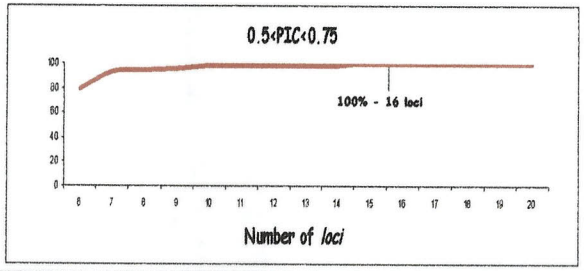
How many *loci* are needed to reach a 100% of correct assignment?

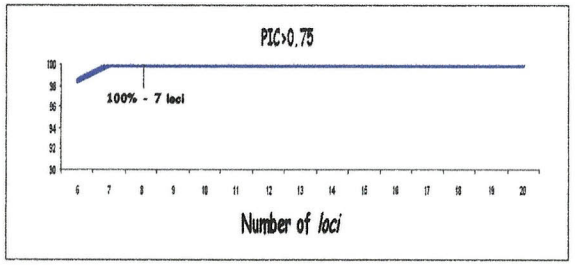
New *loci* generated by simulation using random reorganization

20 *loci* PIC<0.5 | 20 *loci* 0.5<PIC<0.75 | 20 *loci* PIC>0.75

Assessment of the minimum number of *loci* to reach a complete correct assignment







CONCLUSIONS:

7-9 highly polymorphic *loci* are enough to obtain high percentages of correct assignment.

However, level of polymorphism of microsatellites changes among samples, therefore one highly polymorphic *loci* at sample 1 could show low variability at sample 2. Thus, first of all, we must select a wide number of *loci* (12-15), and new markers should be added up to obtain a large enough battery of microsatellites, in order to have always at least 7-9 highly polymorphic loci available for any sample analysed.

The high values of correct assignment obtained will allow to rear the families in a common tank which removes the need of extensive hatchery space and could avoid statistical problems because of environmental variance. Also, since individual identification is possible, the mating can be controlled to minimize the inbreeding effects, preventing the crossbreeding between related individuals.

Pressure induced tetraploid gynogenesis in the European sea bass, *Dicentrarchus labrax* L.

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Introduction

Tetraploidy in fish is commonly produced by disrupting the first cleavage with thermal or hydrostatic pressure shocks in eggs fertilized with normal sperm. Viable tetraploids have been produced by these methods in a number of fish species including sea bass, *Dicentrarchus labrax* (Barbaro *et al.*, 1998; Francescon *et al.*, 2004).

In this work, we investigated a novel technique to induce tetraploidy in fish by pressure-blocking second meiosis and first mitosis in sea bass eggs fertilized with homologous-irradiated sperm.

Materials & methods

The experiments were conducted twice using a pool of gametes from two male and two female *D. labrax* breeders at each time. Sperm inactivation, artificial insemination and pressure treatments followed Peruzzi and Chatain (2000). Untreated groups included haploid (n) and diploid (2n) controls.Treated eggs were first pressure shocked (8.500 psi for 2 min) at 6 min a.f., and then at 65, 70 or 75 min a.f. respectively. Survival of de-veloping eggs and viable larvae was recorded between gastrulation and hatching and expressed as relative to control (RC) after adjustment of the latter to 100%. Ploidy level was determined on 1-2 days old hatched larvae by flow cytometry (Peruzzi and Chatain, 2003).

Results

The hatching rate of diploid control groups within the two replicates at 24 h a.f. was 83% and 58%, respectively. Pressure treatments generally resulted in a sharp decrease of egg survival at day 1. In Replicate 1, only eggs double-shocked at 6 and 65 min a.f. yielded viable larvae. In unshocked haploid control groups, larvae exhibited a typical haploid morphology and did not developed beyond hatching stage. Their haploid state was further confirmed by flow cytometric analysis. Percent survival relative to diploid controls (RC) and ploidy level of larvae from control and pressure-shocked groups are reported in Table 1 and Figure 1 respectively.

Replicate/ Treatment	Survival (%)	Ploidies (%)				Sample size
		Haploid	Diploid	Tetraploid	Mosaic	
Replicate 1						
n control	0	100	0	0	0	10
2n control	100	0	100	0	0	10
6'+65' a.f.	6	0	0	95	5	44
6'+70' a.f.	0	-	-	-	-	-
6'+75' a.f.	0	-	-	-	-	-
Replicate 2						
n control	0	100	0	0	0	10
2n control	100	0	100	0	0	10
6'+65' a.f.	4	0	93	7	0	29
6'+70' a.f.	8	0	100	0	0	20
6'+75' a.f.	12	0	100	0	0	13

Table 1. Percent survival (RC) at hatching and ploidy level of larvae from control and pressure-shocked groups. Sample size indicates the number of larvae analysed by flow cytometry.

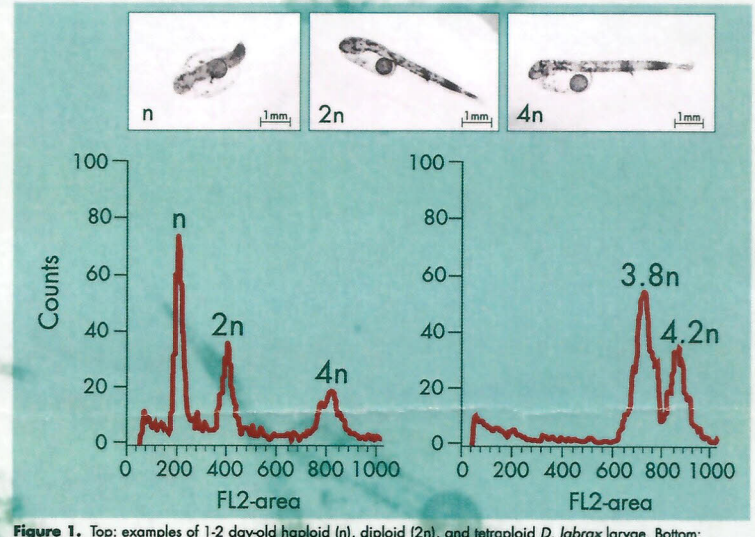

Figure 1. Top: examples of 1-2 day-old haploid (n), diploid (2n), and tetraploid *D. labrax* larvae. Bottom: nuclear DNA content of 1-2 day-old *D.labrax* larvae as measured by flow cytometry. (A) cell suspension from one tetraploid (4n) larva with added haploid (n) and diploid (2n) controls ; (B) cell suspension from one mosaic (3.8n /4.2n) larva. DNA values are reported in arbitrary units expressed as fluorescence channel numbers (FL2-Area).

Conclusions

Variable levels of tetraploid gynogenetic sea bass larvae were produced by meiosis II and mitosis I blocking in gynogenetically activated eggs using pressure shocks. This variability was possibly due to different egg quality between the two experiments, as indicated by the hatching rate of controls, and to individual responses of females to pressure treatments. Moreover, the occurrence of low percentages of mosaic larvae could be indicative of sub-optimal conditions for the suppression of first mitosis.

Further studies are needed to validate the method, and to improve the rate of tetraploidization possibly by application of newly developed protocols for mitotic manipulation in this species (Francescon *et al.*, 2004).

Potential applications

- **This technique could provide an alternative approach for the induction of tetraploidy in sea bass.**
- **Tetraploids can be useful for mass production of triploid fish by mating them with normal diploids.**

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Layout: Frøydis Strand

SURVIVAL AND LARVAL GROWTH OF SEA URCHIN *ARBACIA PUNCTULATA* (LAMARCK, 1816) (ECHINODERMATA: ECHINOIDEA) AT TWO SALINITIES AND FIVE MICROALGAE DIETS

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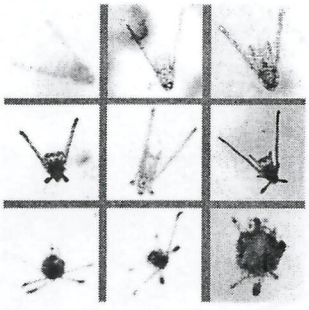
ABSTRACT

Ten sea urchins (*Arbacia punctulata)* catched on Cubagua island (10°49"40'N 64°10"40'O) spawned spontaneously in the lab. The females and male gametes were put in (100:1 oocytes/sperms) glass- 3 l recipients filled with filtered seawater and moderated aeration at 30 ± 1 °C. The fertilized eggs were measurement and continuously observed till embryonic development was completed. The echinoplutei larvae (3 ind/ml) were distributed randomly into 50 plastic- 18 l recipients (25 recipients to 30 psu and 25 to 40 psu) with filtered seawater, continuous aeration and fed on *Tetraselmis chuii, Nannochloropsis oculata, Isochrysis galbana, Chaetoceros gracilis* and *C. calcitrans* under natural photoperiod. The water of the recipients was partially renewed (75%) everyday. Larval anatomic development aspects, daily survival and growth was determinate. The growth was obtained through postoral arms and body length measurement, and body diameter during metamorphosis. During planktonic larval phase, each diet was statistically different between salinities, only *I. galbana* diet was similar for both salinities. The relative growth was allometric negative for *N. oculata* at 40 psu, isometric (I) for larvae fed on *N. oculata* at 30 psu and *I. galbana* at two salinities and allometric positive fed on *C. gracilis* y *C. calcitrans* at both salinities. In this study *A. punctulata* larvae develop fast in 25.8 – 30.8 °C range, starting metamorphosis at fourteen days and be completed at thirty day after fecundation. Statistical differences were detected (F=23.58, p<0.05) in the body growth after the settlement between two salinities, showing that growth was better for larvae at 30 psu, and the final diameter body in the experience was 3.14 ± 0.44 mm. The final percentage rate of planktonic larvae was the highest with *I. galbana* (58.33%) and for juvenile was 6.48 % fed on *C. gracilis* at 40 psu in the two cases.



psu	Diet	Growth rate (µm/d)
30	*Tetraselmis chuii*	- *
40	*T. chuii*	-10.62 *
40	*Nannochloropsis oculata*	6.20 *
40	*Chaetoceros calcitrans*	15.52
30	*C. gracilis*	20.34
30	*Isochrysis galbana*	21.08
30	*N. oculata*	25.30*
30	*C. calcitrans*	22.84
40	*I. galbana*	21.96
40	*C. gracilis*	22.34

*not development completed

INTRODUCTION

There are four species of sea urchin in Venezuela with aquaculture potential: *Lytechinus variegatus, Echinometra lucunte, Arbacia punctulata and Tripneustes ventricosus*, due to their relatively fast growth and their capacity to produce big amounts of gonads in a short time period and their easy adaptability to enclosed systems (Lawrence y Balzhin 1998).

The objective of this research was to determine the growth and survival of the larvae of the sea urchin *Arbacia punctulata* fed on five microalgaes, *Tetraselmis chuii, Nannochloropsis oculata, Isochrysis galbana, Chaetoceros gracilis y C. calcitran* and a mixture of them, and at two salinities, 30 and 40 psu, as a contribution of the knowledge of this resource in Venezuela.

RESULTS AND DISCUSSION

The survival rates were higher at 40 psu that at 30 psu due that the similarity with their environmental natural salinity.

N. oculata y *T. chuii* have lower lipid content that the other microalgae and it caused lower survival rate.

It has been recommended I galbana as better diet microalgae

The juvenile survival rates after 49 days were 4.98 % at 30 psu and 6.48 % at 40 psu, fed on *C. gracilis*, similar to the 5% reported by Grosjean *et al.* (1998)

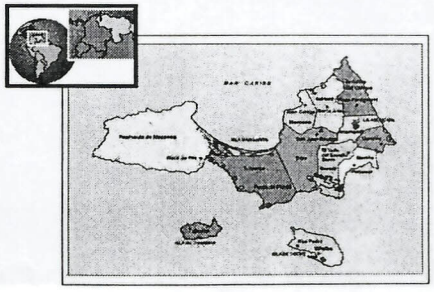
A. punctulata larvae develop fasted in 25.8 – 30.8 °C range, starting metamorphosis at fourteen days and it be completed at thirty days after fecundation.

Statistical differences were detected (F=23.58, p<0.05) in the body growth after the settlement between two salinities, showing that growth was better for larvae at 30 psu, and the final diameter body in the experience was 3.14 ± 0.44 mm.

RESUMEN

Diez erizos (*Arbacia punctulata)* capturados en la isla de Cubagua (10°49"40'N 64°10"40'O) desovaron espontáneamente. Los gametos femeninos y masculinos fueron colocándolos (100:1 óvulos/espermas) en un recipiente de vidrio con 3 l de agua de mar filtrada y aireación moderada a 30 ± 1 °C. Los huevos fertilizados fueron medidos y observados continuamente hasta completar su desarrollo embrionario. Las larvas equinopluteus (3 ind/ml) fueron distribuidas aleatoriamente en 50 envases de plástico con 18 l de agua de mar filtrada, aireación continua (25 envases a 30 psu y 25 a 40 psu), se alimentó con *Tetraselmis chuii, Nannochloris oculata, Isochrysis galbana , Chaetoceros gracilis* y *C. calcitrans* bajo fotoperiodo natural. Se realizó un recambio de agua diario del 75% del total del volumen. Se determinó aspectos anatómicos del desarrollo larval, la sobrevivencia diaria y el crecimiento midiendo la longitud de los brazos postorales, longitud del cuerpo y durante el proceso de metamorfosis el diámetro del cuerpo. Durante la fase planctónica de las larvas, cada dieta fue estadísticamente diferente entre las salinidades, solo la dieta a base de *I. galbana* fue similar a ambas salinidades. El crecimiento relativo fue alométrico negativo para *N. oculata* a 40 psu, isométrico (I) para las larvas alimentadas con *N. oculata* a 30 psu e *I. galbana* en ambas salinidades, y alométrico positivo en las alimentadas con *C. gracilis* y *C. calcitrans* a ambas salinidades. En este estudio las larvas de *A. punctulata* se desarrollaron rápidamente en un rango de temperatura entre 25,8 - 30,8 °C iniciando la metamorfosis a los 14 días y completándola 30 días después de la fecundación. Se detectó diferencias estadísticas (F=23.58, p<0.05) en el crecimiento del cuerpo después de la fijación de las larvas entre las dos salinidades, indicándose que el crecimiento fue mayor para las larvas a 30 psu, con un diámetro del cuerpo al final de la experiencia de 3,14 ± 0,44 mm. El porcentaje de la sobrevivencia final de las larvas planctónicas fue mayor con *I. galbana* (58.33%) y para los juveniles fue de 6.48 % con *C. gracilis* en ambos casos a 40 psu.

Diet	psu	Survival (%)
Nannochloropsis oculata	30	0
N. oculata	40	0
Tetraselmis chuii	30	0
T. chii	40	0
Isochysis galbana	30	23.33
I. galbana	40	58.33
Chaetoceros gracilis	30	21.66
C. gracilis	40	55.00
Chaetoceros calcitrans	30	25.00
C. calcitrans	40	51.66



Isla de Margarita, Venezuela.

NEW MICROSATELLITE MARKERS FOR THE SENEGAL SOLE (*Solea senegalensis* Kaup)

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Abstract

Senegal sole is a flatfish of high interest in aquaculture. However, little information is available about the genetics characteristics of this species. Microsatellite markers have been identified in a large variety of fish species and are widely used in population genetic studies. Here we report the isolation and characterization of ten new microsatellite loci in Senegal sole and their application to the study of a both wild and reared broodstock

Resumen

El lenguado senegalés es una especie de gran interés en acuicultura. Sin embargo se conoce muy poco sobre sus características genéticas. Los microsatélites se han utilizado como marcadores genéticos en gran variedad de especies marinas y se usan para estudios de genética de poblaciones. En el presente trabajo se muestra el aislamiento y caracterización de diez nuevos microsatélites en lenguado senegalés y su aplicación al estudio tanto de poblaciones salvajes como individuos en cautividad.

A microsatellite-enriched library was built using biotin-labelled microsatellite oligoprobes and streptavidine-coated magnetic beads (Reddy et *al*, 2001).

Primers were designed for 10 microsatellite-bearing clones. Mostly microsatellite DNA amplification reactions were carried out in a multiplex PCR and analysed by GeneScan v3.1.2 software (Table I).

Allele number and heterozygosities were estimated in 21 individuals from a reproductive broodstock and 15 wild specimens. In each population, exact test of Hardy-Weinberg equilibrium (HWE) as well as the observed and expected heterozygosities were calculated using Arlequin v2.5.

TABLE II

	CULTURED					WILD				
Locus	*k*	*N*	*Ho*	*He*	HW	*k*	*N*	*Ho*	*He*	HW
SseCA39	10	21	0.76	0.87	0.461$_{ns}$	8	15	0.80	0.85	0.125$_{ns}$
SseGATA23	5	21	0.52	0.70	0.007**	4	15	0.53	0.71	0.321$_{ns}$
SseCA28	13	21	0.91	0.90	0.500$_{ns}$	11	15	0.80	0.91	0.264$_{ns}$
SseGATA21	8	21	0.67	0.72	0.011*	7	15	0.47	0.67	0.199$_{ns}$
SseCA47	10	20	0.30	0.85	0.001***	6	15	0.27	0.78	0.001***
SseGATA26	7	19	0.58	0.76	0.019*	5	15	0.40	0.66	0.056$_{ns}$
SseGATA67	11	21	0.81	0.90	0.058$_{ns}$	16	15	0.87	0.93	0.234$_{ns}$
SseGATA9	6	21	0.19	0.73	0.001***	8	15	0.53	0.87	0.005**
SseGATA38	14	21	0.67	0.76	0.187$_{ns}$	9	13	0.66	0.78	0.510$_{ns}$
SseGATA3	20	20	0.65	0.97	0.001***	16	15	0.67	0.94	0.007**

k, number of alleles by locus in each population; *N*, number of specimens analysed; *Ho*, observed heterozygosity; *He*, expected heterozigosity; HW, *P*-value to Hardy-Weinberg equilibrium

Four loci (SseCA39, SseCA28, SseGATA67 and SseGATA38) were found under HWE whereas another three loci (SseCA47, SseGATA9 and SseGATA3) displayed a significant ($P < 0.05$) departure from HWE in both population. These significant deficits of heterozygous genotypes are consistent with the presence of null alleles. These three markers showed an estimated null allele frequency (Cervus v2.0) of 0.46, 0.22, 0.16 and 0.47, 0.58, 0.18 in the wild and cultured population, respectively (Table II).

The other markers did not exhibit null allele frequency higher than 0.12 under any circumstance. Furthermore, three loci (SseGATA23, SseGATA21 and SseGATA26) did not fit to HWE in the cultured population. The deficit of heterozygotes in this population could be explained by a higher level of inbreeding due to a founder effect and subsequent low average population size in the broodstock maintained in captivity.

In the cultured population, the observed and expected heterozygosities varied from 0.19 to 0.91 and 0.72 to 0.97, respectively. In the wild population the observed and expected heterozygosities ranged from 0.27 to 0.87 and 0.66 to 0.94, respectively (Table II).

TABLE I

LOCUS	REPEAT MOTIF	PRIMER SEQUENCES (5'-3')	*k*	ALLELE SIZE RANGE (bp)	*Ta*	MPCR (μM)
SseCA39	(CA)13	F: TCTCTTTATCTTCCAGCGCGGTGTTCACCTCA R: FAM-CGCTGAGACGGATGTTAGAGGGAAAT	10	133-189	60	0.25/0.51
SseGATA23	(GATA)3(GACA)3(GATA)9(GGTA)3(GATA)3(GACA)3(GATA)3	F: CACCTATTGACTTGGCATGACAGGGTTCCTTCT R: 5-TATGGGTGCATTCACCCAGCTCCTTCT	6	176-200		
SseCA28	(CA)17	F: AGAGACAGAGTACACAGGAGGGAGTGCAG R: FAM-TTTCACATCCTGGTGTCACTCA	14	113-157	68	0.40/0.56
SseGATA21	(GATA)16(GATC)3(GATA)4(GAAA)3(GATA)3(GATC)3(GATA)3	F: GAAAAAGCCAAAACCATCAGTTGATCCCAAAG R: 5-GTAAATATAATTGGGCTCCCAACGGAATTTCA	12	177-261		
SseCA47	(CA)12(TA)3(CA)4	F: CTTGTTATGTAAGTCAAGGTCCATGTGTCGAGT R: FAM-AAAATTC AAAATGGACGGCAAGATAAAGC	10	149-186	60	0.52/0.52
SseGATA26	(GATA)12	F: GTGTTAGACAGACAGATAGATAGCTGTTAGCA R: 5-TGTAATGTAAAATCTCTGACTGTAGTTTCAC	8	188-240		
SseGATA67	(GATA)17	F: GGGGAAGGTGGGATAAATGGATGATTCA R: 5-atTGATTAC AAACACCACCTGGAAAG	17	124-208	60	0.40/0.40
SseGATA9	(GATA)3nnn(GATA)3nnn(GATA)4	F: GGTTAAACATTTACCCTTAACCCTTAGAACA R: FAM-CCATTCTGGAGGAGACACTACATTTCAAAG	12	243-445		
SseGATA38	(GATA)20	F: ATATCATCAGGCAGTAAATGGATGATTTGA R: 5-GCAATCTGCAAACTGACCACTAGATGACCAGT	17	126-232	68	0.40
SseGATA3	(GGTT)4(GGTA)8(GATA)6(GATCGATA)3(GATA)2	F: GGACCCTAATCTCCTGTGGCTGGACGTAACAAGC R: FAM-CTGGCTGTGGCTGGACGTAACAAGG	30	227-305	65	0.40

Characteristics of 10 microsatellite loci for the marine flatfish Senegal sole (Solea senegalensis Kaup). Locus designation, repeat motif (referring to sequenced allele), primer sequences (5'-fluorescent primer label indicated, FAM or HEX), total number of alleles (*k*) and observed size range overall, final primer pair concentration ratio in each multiplex PCR or single PCR

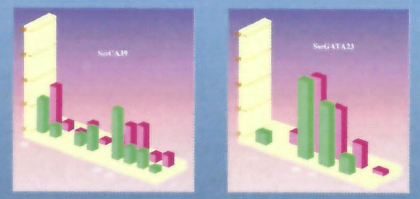
The primers were further tested in a pair of individuals from a range of flatfish species (*Solea solea, Solea lascaris, Solea kleinii, Sypnatura lusitanica* and *Microchirus azevia*). Three loci product fragments within the expected size range (+). Of these, the locus SseCA28 showed polymorphism (P) in all species investigated, whereas the loci SseGATA26 and SseGATA67 did it only in the most related species *Solea solea*. These results highlight the possibility of some of them being used in population genetic studies in other flatfish species (Table III).

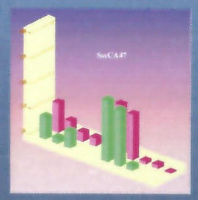
TABLE III

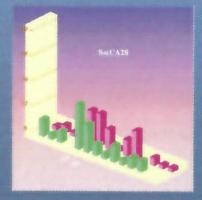
LOCUS	*Solea solea*	*Solea lascaris*	*Solea kleinii*	*Sypnatura lusitanica*	*Microchirus azevia*
SseCA39	–	–	–	–	–
SseGATA23	–	–	–	–	–
SseCA28	+ P	+P	+P	+P	+P
SseGATA21	–	–	–	–	–
SseCA47	–	–	–	–	–
SseGATA26	+P	+	+	+	+
SseGATA67	+P	+	+	+	+
SseGATA9	–	–	–	–	–
SseGATA38	–	–	–	–	–
SseGATA3	–	–	–	–	–

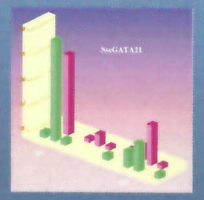
The number of alleles for the 10 loci ranged from five to 20 in the cultured population, whereas in the wild population ranged from four to 16.
Each loci showed from one to 3 more frequent alleles in both populations. The fact that these alleles occupied an intermediate position suggests that the loci investigated fitted to a stepwise mutation model (Figure 1).

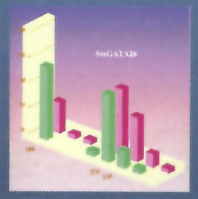
FIGURE 1

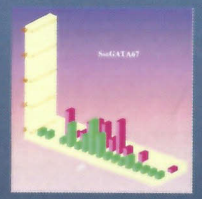


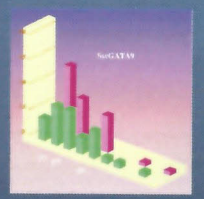


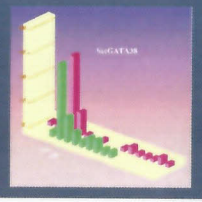












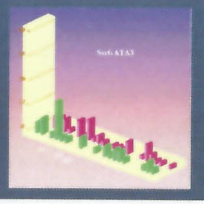


Figure 1 shows the allele size frequency (%) in wild (green) and cultured (purple) populations. The more frequent size are pointed for each locus analysed.

LARVAL GROWTH AND SURVIVAL OF BLACK URCHIN *ECHINOMETRA LUCUNTER* (ECHINOIDEA: ECHINOMETRIDAE) FEEDING ON *CHAETOCEROS GRACILIS* AND *ISOCHRYSIS GALBANA* MICROALGAE

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ABSTRACT

Thirty sea urchins (*Echinometra lucunter*) with 45.83 ± 17.52 mm average diameter, were injected with potassium chloride (50 M) directly into celomic cavity. After two minutes, 90 % of urchins spawned (55% females and 35 % males) the let 10 % never spawned. The fertilization rate was 87.01 ± 12.63 % (1:100 oocytes/sperma) at 29 ± 2 ºC. The fertile eggs were put in 27 - 18 l recipients (2 eggs/ml), with aeration from the bottom. It was added *Chaetoceros gracilis* into nine recipients, *Isochrysis galbana* into others nine recipients and a mixture of both microalgae into the last nine recipients (20000 and 60000 cell/ml for each microalgae, with a rate 1:1 for the mixture). The pH, salinity, temperature and larval survival were determinated daily. The experience concluded when post-metamorphic phase was completed. The embryonic development time was 16.25 ± 0.15 h till the prism stage at pH 8.44 ± 0.06; 36 ± 1 psu and 28 ± 1.4 °C. The two arms larval stage was reached at 24 h: 33 min, with a total length of 190 ± 16.25 µm fed on *C. gracilis;* 152 ± 19.02 µm with *I. galbana* and 182.4 ± 14.06 µm with the mixture. The larvae next to metamorphosis reabsorbed the arms and took the characteristic shape of juvenile urchin at 12 days with 670.2 ± 22.2 µm fed on *C. gracilis;* 665 ± 12.09 µm fed on *I. galbana* and 670 ± 14.2 µm fed on the mixture. The accumulated survival till juvenile stage was 14.68 ± 3.80 % fed on *C. gracilis*, producing statistical differences (p>0.05) in relation to others treatments. It concluded that *E. lucunter* urchin is an excellent prospect to be commercially cultured due to short embryonic (16 hours) and larval development time (12 days) and a good survival rate feeding on monoculture (*C. gracilis* and *I. galbana*) as well as on mixture diets, being recommended the use of *C. gracilis* microalgae.

INTRODUCTION

There are four species of sea urchin in Venezuela with aquaculture potential: *Lytechinus variegatus, Echinometra lucunte, Arbacia punctulata and Tripneustes ventricosus*, due to their relatively fast growth and their capacity to produce big amounts of gonads in a short time period and their easy adaptability to enclosed systems (Lawrence y Balzhin 1998).

The objective of this research was to determine the embryonic development time, and growth and survival of the larvae of the sea urchin *E. lucunter* fed on two microalgaes, *Chaetoceros gracilis, Isochrysis galbana* and a mixture of them, as a contribution of the knowledge of this resource in Venezuela.

Conclusions

It was concluded that *E. lucunter* urchin is an excellent prospect to be commercially cultured, due to short embryonic (16 hours), and larval development time (12 days). Moreover it has a good survival rate feeding on monoculture (*C. gracilis* and *I. galbana*) as well as on mixture diets, being recommended the use of *C. gracilis* microalgae.

RESUMEN

Treinta erizos de mar (*Echinometra lucunter*) con diámetro promedio de 45.83 ± 17.52 mm. Fueron inyectados individualmente con 50 µL/g de cloruro de potasio (50 M) directamente a la cavidad celómica. A los dos minutos se observó el desove del 90 % de los erizos (55% hembras y 35 % macho) el 10 % restante nunca desovó. La fertilización fue de 87.01 ± 12.63 % (1:100 óvulos/espermios) a 29 ± 2 ºC. Los huevos fértiles se colocaron en 27 envases plásticos de 18 l de capacidad (2 huevos/mL), con aireación de fondo. A nueve envases se les suministró *Chaetoceros gracilis*, a otros nueve *Isochysis galbana* y a los nueve restantes la mezcla de ambas microalgas. En todos los casos la densidad de microalgas fue entre 20000 y 60000 cel/ml (relación 1:1 para la mezcla). Diariamente, se determinó pH, salinidad, temperatura y la sobrevivencia larvaria. La experiencia concluyó cuando se completó la fase post-metamórfica. El tiempo del desarrollo embrionario fue de 16.25 ± 0.15 horas, hasta el estadío de prisma a pH 8.44 ± 0.06; 36 ± 1 psu, y 28 ± 1.4 °C. El estadio larval de dos brazos, se alcanzó en 24 h: 33 min, con una longitud total de 190 ± 16.25 µm para las alimentadas con *C. gracilis;* 152 ± 19.02 µm con *I. galbana* y 182.4 ± 14.06 µm con la mezcla. Las larvas próximas a la metamorfosis reabsorbieron los brazos tomando la forma característica de un erizo juvenil en 12 días, con un tamaño de 670.20 ± 22.20 µm con *C. gracilis;* 665 ± 12.09 µm con *I. galbana* y de 670 ± 14.20 µm para la mezcla. La sobrevivencia acumulada hasta la obtención de juveniles fue de 14.68 ± 3.80 % con *C. gracilis*, significativamente (p>0.05) más alta que las observadas con las dos dietas restantes (5.39 ± 1.23; 13.70 ± 2.60). Se concluye que el erizo *E. lucunter* es un excelente candidato para ser cultivado comercialmente debido a su corto tiempo de desarrollo embrionario (16 horas), larval (12 días) y buen porcentaje de sobrevivencia con dietas tanto monoespecíficas (*C. gracilis* e *I. galbana*) como mixta, siendo en este caso en particular recomendable el uso de *C. gracilis*.

Embryonic time development of black urchin *E. lucunter* at 28.1 ± 1.4 °C.

Stage	Time(h:min)
2 cells	0: 99
4 cells	1: 16
8 cells	1: 50
16 cells	2: 20
32 cells	2: 45
64 cells	3: 39
Blastule	4: 58
Gastrule	11: 34
Prisma	16: 25
Pluteus	24: 33

Larval survival rate and juvenile setting of black urchin *E. lucunter* feeding on *C. gracilis, I. galbana* and the mixture of both microalgae.

	C. gracilis	SR %	*I. galbana*	SR%	Mixture	SR%
Initial Larvae	32000		32000		32000	
Day 1	17065	53 ± 2	11200	35 ± 2	20256	63 ± 1
Day 11	4698	15 ± 4	1924	5 ± 1	4384	14 ± 3
Juveniles	3288	10 ± 2	962	3 ± 1	2117	7 ± 1

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KOUSHA Armin	Azad University	Iran
KRISTJANSSON Jakob	PROKARIA	Iceland
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MIRES Dan	The Israeli Journal of Aquaculture	Israel
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MOHD-ADNAN Adura	National University of Malaysia	Malaysia
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MORTENSEN Stein	Institute of Marine Research	Norway
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OLSEN Cecilia	NTNU	Norway
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announcement &
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aqua
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2005

Optimising the Future

AQUA NOR

AQUACULTURE EUROPE 2005

August 5-9, 2005

In conjunction with

AQUA NOR 2005

August 9-12, 2005

Trondheim, Norway

Organised by:
European Aquaculture Society (EAS)
Nor-Fishing Foundation

Scope and objectives

The last half century has seen major developments throughout the world in the farming of aquatic organisms for food. In Europe, trout, and other freshwater fish farming has continued to develop, although developments in sea fish farming have predominated with cage culture of salmon, sea bass and sea bream hugely increasing the availability of these species in the market place. Although a range of alternative approaches (e.g. extensive culture in enclosed bays, and ranching/stock enhancement in fjords and the open sea) were considered, for various biological, political and commercial reasons significant developments have been limited to intensive culture in cages and tanks.

These successes are attributable to the ingenuity and perseverance of scientists, engineers and 'entrepreneurs', who have developed and applied methods for rearing these species through the whole of their life cycle on a commercial scale. As the problems of culture have become increasingly understood, the application of science has developed from an initial empirical approach through to a more sophisticated investigative approach employing advanced techniques and methodologies in a range of biological and other disciplines [as well illustrated at AE2004]. The result is not only a commercially successful industry, but a sound scientifically-generated information base that will underpin future developments. These developments are directed not only at improving the efficiency of culture methods for established species to combat downward pressure on prices but also at establishing methods for new species that will enable new markets and production opportunities to be exploited.

Despite these achievements, uncertainties remain over the future of the aquaculture industry. Public acceptance has been undermined by adverse publicity regarding the environmental impact and ethics of intensive aquaculture as well as product quality and safety. In the past, commercial imperatives necessitated that scientific investigations were focussed primarily on the removal of bottlenecks in production and it is only relatively recently that this restricted approach has given way to a recognition of the wider range of issues on which the long-term future of aquaculture will depend.

Economic sustainability will depend on continuing technical developments but also, on a wide range of factors including improved understanding of market dynamics and issues of governance. The future of the aquaculture industry also depends, however, on a rigorous application of scientific principles to issues of **environmental sustainability** and **ethics**. Neglecting this full range of issues will not only impair the ability of the industry to efficiently deliver high quality products but, perhaps more importantly, will undermine the public acceptability on which the future of the industry depends. The need for this change in emphasis has already been recognised and research projects to address these issues are underway. This conference aims to provide a forum for the dissemination of the latest advances in these areas.

This European and international meeting will bring together participants from as many as 40 countries to address the key issues and discuss some of the most advanced scientific results and technological tools. Aquaculture Europe 2005 will be organised through plenary sessions reviewing key themes, and parallel sessions consisting of

selected oral presentations and poster sessions reflecting those themes and key issues for the future development of aquaculture. Although emphasis will be given through the plenary sessions to **"Lessons from the past to optimise the future",** other aspects of aquaculture development will also be approached during parallel sessions.

Immediately following the conference the Aqua Nor trade show, the largest aquaculture trade exhibition, will open its doors from August 9-12. Conference delegates have free access to this event.

Over the years, "Aquaculture Europe" has established its truly international scope as one of the major aquaculture events of the year. The conference programme being put together is formulated to stimulate and encourage broad debates. The event is based at the first-class facilities of the Norwegian University of Science and Technology (NTNU) in the interesting city of Trondheim. We hope to see you all - academics, farmers, suppliers, buyers, distributors, managers, regulators, policy/decision makers - in August 2005 .

Conference format

The conference programme, running over three days, will consist of plenary sessions introducing topics. Late mornings and afternoons will be split into three parallel sessions consisting of selected oral presentations and poster sessions.

Preliminary conference programme (could be subject to change)

Plenary sessions

These will be held during the first part of the morning and will address the following key themes:

1. **Applying lessons learned from established species.**
2. **The capacity of the environment to support predicted growth.**
3. **The contribution of aquaculture to sustainable fisheries.**
4. **Developments in environmentally-friendly systems.**
5. **Ethics: issues and their communication.**

Parallel sessions

These will be held late morning and afternoon. They will include themes #2-5 above. The bullet points indicate some of the research fields relevant to the session titles. Contributions on all cultured species are welcomed for each topic.

A. The capacity of the environment to support predicted growth

- Measuring carrying (productive, assimilative) capacity
- Manipulating carrying capacity (upwelling, reefs)
- Multiple use and ICZM issues
- Aquaculture as a tool in environmental protection
- Biodiversity issues
- Scientific basis for legislation

B. The contribution of aquaculture to sustainable fisheries

- Stocking strategies in freshwater and marine environments
- Implications for rearing methodology (behavioural, genetic, disease issues)
- Cost benefit and ownership issues

C. Developments in environmentally-friendly systems

- Offshore, recirculation and integrated systems
- Effluent systems
- Farm design
- Bio-fouling prevention
- Escape prevention

D. Ethics: issues and their communication

- Scientific basis for good welfare practice / legislation
- Slaughter methods
- Manipulated strains
- Communication initiatives/strategies

E. Health management

- Disease prevention (vaccination strategies, immuno-stimulants, selection)
- Stress control
- Bio-security and containment (etiology, vectors & latent carriers, transport/movement)

F. Sustainable feed resources

- Alternatives to fish meal/oil: effects on performance, quality and consumer perceptions
- Projected availability of high quality fish meal and oil
- Cost benefit analysis of alternatives

G. Reliability of hatchery production

- Quality criteria / viability indicators for all facets / life stages
- Broodstocks management and gamete quality
- Water management (including microbes)
- Feed quality (live and formulated)

H. Towards economic sustainability

- Market dynamics
- Emerging new species
- Product quality and safety
- Governance & self-regulation
- Socio-economic aspects

We welcome **other contributions** not directly covered by the above topics.

EU Forum on the 6th & 7th Framework Programmes of RTD

Representatives of the European Commission Directorate General Fisheries will present the latest news on the 6th Framework programme (2002 – 2006) and an update on the development of the 7th Framework Programme of research and technological development (2007-2011), especially concerning aquaculture and fisheries. Following the presentation, the floor will be open for discussion.

A special Aquaculture Innovation Network event

On the first day of the Aqua Nor exhibition (August 9), EAS through its participation in the CNS-INTRANS EU funded network (www.aquainnovation.net) is organising a special event in the frame of the goals of this network, including assistance of companies in developing worthwhile collaborations, accessing information about innovation and supporting and guiding technology transfer. Interest in this event can be indicated on the registration form enclosed.

Posters

The posters will, as usual, be presented in the vicinity of the conference area. Authors are encouraged to emphasise potential applications of their work for the end user. Contributions for the poster sessions are encouraged for all aspects of aquaculture. Several sessions with authors present are planned.

Language

The official language of the conference is English. An abstract in Norwegian of the poster presentations is encouraged.

Submission of titles

Interested contributors to the conference are hereby invited to submit a short 10-15 lines preliminary outline (preferably by e-mail to: ae2005@aquaculture.cc or 3 copies by post) of the oral or poster contribution they would like to present, to the EAS conference secretariat prior to **January 15, 2005**.

Once confirmed, and to minimalise the problem of 'non-attendance' of scheduled presentations, the extended abstract will need to be accompanied by the registration fee or by a non-refundable submission fee of € 55 per presentation, which can be subtracted from the registration fee upon settlement at a later date.

The trade exhibition (Aqua Nor)

The Aqua Nor 2005 trade show will take place from August 9-12, immediately following the conference. Aqua Nor has been organised since 1979 and is the leading exhibition of its kind, comprising indoor and outdoor displays of the latest products, services, research and developments in aquaculture. Organised bi-annually, it is the primary meeting place for fish farmers, suppliers and others involved in the fresh and processed seafood selling chain linked with aquaculture. Aqua Nor 2003 attracted 20.000 visitors from 50 countries and 492 firms were represented.

Accompanying persons programme

Accompanying persons will have full opportunity to explore the city and the area. Trondheim is also the ideal starting point to the old mining town of Røros which is on the UNESCO's World Heritage list, splendid mountain scenery, fjords, coastal regions, etc. A detailed programme will be provided in the second announcement.

The meeting place

Trondheim is Norway's third largest city and was the first capital (in 997) of Norway. It is situated on the Nidelv River in very lush surroundings. Trondheim is a lively university town (with more than 10 000 students) and has a rich medieval history. The Nidarøs cathedral is one of the largest medieval buildings in Scandinavia.

The city is easily accessible by air (through Trondheim airport at 32 km) with a number of regular direct and indirect (stopover in Oslo or Bergen) international flights, rail, bus, ferry and the coastal steamer.

Trondheim enjoys a pleasant climate with average temperatures around 18°C in August. General information on Trondheim may be found at:
http://www.trondheim.com/engelsk/

Further information

The second announcement with detailed information and the provisional programme will be mailed in April 2005 to those persons who have returned the (pre) registration form. More details will also be available at: http://www.easonline.org

Practical information

Conference secretariat and registration for delegates

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E-mail: mailbox@nor-fishing.no
http://www.nor-fishing.no

Conference venue

Norwegian University of Science and Technology (NTNU), Trondheim, Norway

Special Aquaculture Innovation Event venue

Nidarøhallene, Trondheim, Norway

Committees

ORGANISING COMMITTEE
Chair: Elin Kjørsvik (Norwegian University of Science and Technology (NTNU) and EAS, Norway)
Members: Peter Gullestad (Fisheries Directorate, Norway), and Alistair Lane (EAS, Belgium)

PROGRAMME COMMITTEE
Chairs: Rosa Flos (Universitat Politècnica de Catalunya, Spain and EAS) and Bari Howell (UK).
Members: Mark Burdass (Sparsholt College, UK), Philippe Goulletquer (IFREMER, France), Kjell Maroni (FHL, Norway), Paolo Melotti (University of Camerino, Italy) and Josianne Støttrup (Danish Institute for Fisheries Research, Denmark)

LOCAL ARRANGEMENTS COMMITTEE
Chair: Jan O. Evjemo (NTNU, Norway)
Members: Alexandra Neyts (NTNU, Norway)

Registration

Please send your completed registration form and accompanying payment in Euro to:
Aquaculture Europe 2005 - EAS Conference secretariat
Slijkensesteenweg 4, B-8400 Oostende, Belgium
Tel. +32 59 32 38 59; Fax +32 59 32 10 05; E-mail: ae2005@aquaculture.cc

Registration fees

Conference registration fees

	received before 15/03/05	received between 16/03-15/05/05	received between 16/05-30/06/05	received after 01/07/05
EAS Members STANDARD[1]	€ 375	€ 420	€ 470	€ 500
EAS Members REDUCED[2]	€ 250	€ 300	€ 350	€ 380
Non-member	€ 460	€ 500	€ 550	€ 585

[1] Also applicable to WAS Members. Annual membership of EAS for 2 years is ***standard*** €210 with the journal Aquaculture International, €115 without and €475 for Institutional members. ***Reduced*** Membership fee for one year is respectively €90, €40 and €150. EAS Life Membership is €720.
[2] Applies to current EAS Student members and to EAS members eligible for the reduced membership.
[1,2] For details and conditions on EAS membership please see rear of registration form or visit www.easonline.org

Conference registration fees include:

- conference documents
- admission to all conference sessions; poster sessions; Aqua Nor exhibition* (Aug 9-12)
- coffee/tea served during breaks
- scheduled get-together
- lunch on Saturday, Sunday and Monday**

*Included in spouse registration **Not included in spouse registration

Spouse registration fees

Price: Settled prior to June 1: €100. After June 1, it becomes €110. It includes scheduled get-together (on August 5) and farewell dinner (on August 8)

Cancellations

Full refunds of registration fees less 20% will be made on written requests received prior to April 1, 2005. Refunds less 30% on cancellations received between April 1, 2005 and June 30, 2005. After this date refunds will not be possible. All refunds will be processed after the conference. Only cancellations in writing received at the conference secretariat will be considered.
In the event of any unforeseen circumstances, EAS reserves the right to change the date and venue of the conference or to cancel it.

Accommodation

Accommodation in Trondheim will be available in all price categories, although early booking is highly recommended. More details on prices and contact information for the hotel agency will be available at www.easonline.org and in the 2nd announcement.

Sponsors - Supporting Societies

Support has already been confirmed by NTNU. Sponsors have been solicited and will be confirmed in the second announcement.
The following societies have already expressed their support:
Association of Scottish Shellfish Growers (AASG) - Dutch Aquaculture Society (NGvA) - Egyptian Aquaculture Society (EgAS) - European Bureau for Conservation & Development (EBCD) - Federation of European Aquaculture Producers (FEAP) - Federation of Greek Maricultures (F.G.M.) - Panhellenic Soc. of Technologists Ichthyologists (PASTI).

Organisers

Aquaculture Europe 2005 - Aqua Nor is jointly organised by the European Aquaculture Society (EAS) and the Nor-Fishing Foundation (NFF).

EUROPEAN AQUACULTURE SOCIETY (EAS)
The EAS is an international non-profit association dedicated to the promotion of contacts and the exchange of information amongst all involved or interested in aquaculture, at the European level as well as beyond. Now in its 29th year, the society's primary activities include publication of a quarterly magazine; publication of a series of special publications; publication of an international scientific journal; development and co-ordination (with the Federation of the European Aquaculture Producers (FEAP) of the AquaFlow network; initiatives for sustainable aquaculture development; organisation and co-sponsorship of aquaculture symposia and workshops (including the Aquaculture Europe events); services to the members.

THE NOR-FISHING FOUNDATION
Nor-Fishing has been organised as an international fishing fair since 1960. In 1992 Nor-Fishing was established as a foundation with its Board of Directors appointed by the Royal Department of Fisheries, the Norwegian Trade Council, the Municipality of Trondheim, the Norwegian Fisherman's Association and the Norwegian Fish Farmers' Association. The Nor-Fishing Foundation owns both the Nor-Fishing exhibition and the major international aquaculture show, Aqua Nor. The Nor-Fishing Foundation has established a separate exhibition management in Trondheim geared to take care of both the Nor-Fishing and the Aqua Nor events.

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Optimising the Future

INTERNATIONAL CONFERENCE AND EXHIBITION

Trondheim, Norway

organised by

EUROPEAN AQUACULTURE SOCIETY (EAS)
NOR-FISHING FOUNDATION

Aquaculture Europe 2005 Conference
August 5-9, 2005

AquaNor 2005 Exhibition
August 9-12, 2005

Our ref: 2004/176

Date as postmark

Dear Madam, Dear Sir,

We are pleased to invite you to participate in the Aquaculture Europe 2005 Conference, which will take place in Trondheim, Norway, from August 5-9, 2005.

The conference theme "**Lessons from the Past to Optimise the Future**" is focussing on how we can build on some major achievements over the last half-century, to address certain uncertainties for the future of the aquaculture industry. In the past, commercial imperatives necessitated that scientific investigations were focussed primarily on the removal of bottlenecks in production and it is only relatively recently that this restricted approach has given way to recognition of the wider range of issues on which the long-term future of aquaculture will depend.

The Plenary sessions (during each of the 3 conference days) will address issues related to the overall conference theme – with particular emphasis on economic and environmental sustainability and ethics. The parallel and poster sessions (some with authors present) will further develop these three central ideas.

In addition, a special event on **innovation and technology transfer in aquaculture** is being scheduled on the opening day (August 9) of Aqua Nor 2005, i.e. the world's largest aquaculture exhibition.

On behalf of EAS we have the pleasure to enclose the first announcement and guidelines for submitting presentations related to this international event. May we kindly ask you to distribute this information among interested people?

Please do not hesitate to contact us for further information. We very much look forward to welcoming you in Trondheim.

Yours sincerely,

A. Lane
EAS Executive Director

Conference secretariat: European Aquaculture Society
Slijkensesteenweg 4, B-8400 Oostende, Belgium
Tel.: +32 59 32 38 59
Fax: +32 59 32 10 05
E-mail: ae2005@aquaculture.cc
http://www.easonline.org

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AQUACULTURE EUROPE 2005

Optimising the Future

Trondheim, Norway, August 5-9, 2005

Call for contributions and reply form

(to be returned before January 15, 2005)

Please make a copy if you want to submit more than one presentation.

Presenting author information:

Mr./Ms./Dr. Last Name ..... First Name .....
Title and/or Position .....
Institution/Company .....
Street .....
Postal code ..... City .....
Region/State ..... Country .....
Tel ..... Fax ..... E-mail .....

☐ Plans to register for the conference at a later date. Please send final announcement when available by ☐ Mail ☐ E-mail
☐ Would like to advertise in programme
☐ Would like to contribute as sponsor

☐ Would like to give a presentation

- Preferred presentation form **(please tick only ONE):**
  The final selection of contributions for Oral or Poster will be made by the programme compilers.

☐ oral ☐ poster ☐ oral or poster (no preference)

- title of proposed presentation: .....
  .....
  .....
  .....

- authors of proposed presentation. Underline the name of the presenting author:
  .....
  .....

- to be treated in the following session (please tick only **ONE**):

☐ **A – Capacity of the environment to support predicted growth**
☐ **B – Contribution of aquaculture to sustainable fisheries**
☐ **C – Developments in environmentally-friendly systems**
☐ **D – Ethics: issues and their communications**
☐ **E – Health management**
☐ **F – Sustainable feed resources**
☐ **G – Reliability of hatchery production**
☐ **H – Towards economic sustainability**

☐ **Miscellaneous / Other (please specify)** .....

I'm sending a brief outline of the presentation in max 250 words (10-15 lines) - **please tick only one:**

☐ by post (3 paper copies) **OR**
☐ by e-mail to ae2005@aquaculture.cc

Once accepted, the submission of the extended abstract will need to be accompanied
- by a non-refundable submission fee of € 55 per presentation, which can later on be subtracted from the registration fee
  or
- by the completed registration form and settlement of the full registration fee

Signature: Date: ...../...../.....

To be returned prior to January 15, 2005 to:
EAS Conference Secretariat - Aquaculture Europe 2005, Slijkensesteenweg 4, B-8400 Oostende, Belgium.
Tel. +32 59 32 38 59; Fax +32 59 32 10 05; E-mail: ae2005@aquaculture.cc; http://www.easonline.org

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AQUACULTURE EUROPE 2005 – August 5-9, 2005

CONFERENCE REGISTRATION FORM

Please use one form per participant (+ possibly spouse)

Please complete (type or use capital letters) and return to:
EAS Conference Secretariat, Slijkensesteenweg 4, B-8400 Oostende, Belgium.
Fax. +32 59 32 10 05; Tel. +32 59 32 38 59; E-mail: ae2005@aquaculture.cc

The undersigned wants to be registered as a participant to the above meeting:

Badge Information (As you want your badge to read - no titles please)

Last Name ..... First Name .....

Company/Institution .....(max 40 characters, white spaces included)

Country .....

Mailing information: ☐ Dr. ☐ Mr. ☐ Ms. Position:.....

Company/Institute .....

Street .....

Postal Code ..... City.....

Region/State ..... Country.....

Tel ..... Fax ..... E-mail .....

(include country and area code)

Registration Conference:	**Not a member of EAS? Save by joining! Please see below and rear.**				
Conference fees	received before 15/03/05	received between 16/03-15/05/05	received between 16/05-30/06/05	received after 01/07/2005 or on-site	**EAS/WAS membership N°:**
☐ **EAS Members STANDARD**[1]	€ 375	€ 420	€ 470	€ 500	.....
☐ **EAS Members REDUCED**[2]	€ 250	€ 300	€ 350	€ 380	€ _____
☐ **Non-member**	€ 460	€ 500	€ 550	€ 585	
☐ **Spouse registration**[3]: ☐ Mr. ☐ Ms. ☐ Mrs. Full Name: _____			prior to June 1	€ 100	€ _____
			after June 1	€ 110	
☐ **I / We will attend the get-together on Friday evening, August 5** (free for conference participants and spouses, but pre-registration required)					**YES / NO** please circle
☐ **Conference dinner on Monday, August 8 (early booking recommended)**				€ 75	€ _____
☐ I am interested in the ***Aquaculture Innovation Network*** event, August 9, 2005. Details will in due time be posted at www.easonline.org					
☐ **Submission payment** for my presentation(s) has been paid previously and is now **subtracted**				– € 55 x __	– € _____
EAS membership:					
☐ I apply for the EAS membership for the next year/ 2 years, which enables me to benefit from the member registration rate on all EAS and WAS conferences during membership period. For rates and the other benefits of the EAS membership please see overleaf (II)					€ _____

[1] *Also applicable to full WAS members.*
[2] *Only applicable to EAS members with age limit 30 (in this case please provide copy of ID proving age), and/or to current EAS Student members and/or to EAS Members coming from certain countries. Please see rear for current list of countries and more information on joining rates.*
[3] *Spouse fee includes admission to the get-together and farewell dinner.*

TOTAL EURO REMITTED € _____

Payment Method – all fees must be paid to the order of Aquaculture Europe 2004 without charges for the beneficiary. Payments are accepted in EURO only. Registration is only valid upon receipt of the registration fee. Note that cheques are not accepted!

O **bank transfer:** for details please see overleaf (I)

O **credit card:** O Diners O Mastercard O Visa

Card No. | | | | | | | | | | | | | | | | Expiry date ...../.....(mm/yy)

Name cardholder: ..... Date:..... Signature .....

Cancellation policy: see announcement and call for papers

Upon payment, confirmation of registration will be sent.
Registered office: c/o VLIZ, Vismijn, Oostende

I For payments by bank transfer:

Bank transfer in EURO to account EAS-Aquaculture Europe 2005, no. 777-5918867-03 (Dexia Bank, Pachecolaan 44, B-1000 Brussels, Belgium. B.I.C. code: **GKCCBEBB**, IBAN BE21 7775 9188 6703). For smooth registration it is **essential** that **the name of the participant appears first in the section "Message for the recipient"** of the bank transfer instructions !
The beneficiary should be referred to as 'EAS-Aquaculture Europe 2005, c/o EAS Conference Secretariat, Slijkensesteenweg 4, BE-8400 Oostende, Belgium.'

II EAS membership

The EAS membership categories and fees are:

	Standard **2 years**	Reduced rate** **1 year**
EAS Individual* Membership	Euro 115	Euro 40
EAS Individual Membership, incl. AI	Euro 210	Euro 90
EAS Institutional* Membership	Euro 475	Euro 150
EAS Life Membership* **	Euro 720	/

*Peer-reviewed scientific Journal "Aquaculture International" not included.
**Reduced membership fees are available for:
- Individuals if aged 30 or under (proof of age required). Also for individuals residing in European and non-European Mediterranean countries with Gross National Income (GNI) lower than US$ 8000 per capita. For 2004 these countries are: Albania, Algeria, Belarus, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, Egypt, Estonia, Hungary, Latvia, Lebanon, Lithuania, Libya, Macedonia, Moldova, Morocco, Poland, Romania, Russia, Serbia and Montenegro, Slovak Republic, Syria, Tunisia, Turkey, Ukraine.
- Institutes if based in one of the above mentioned countries

***Individual Life Membership offers the general EAS benefits (AI not included) for full lifetime duration

The **EAS membership** includes the following **BENEFITS**:

Automatic receipt of:

-**"Aquaculture Europe** (quarterly publication). This magazine covers all aspects of aquaculture. Each issue includes a special up-to-date article, an illustrated report on aquaculture in a certain country and news on progress in aquaculture development worldwide. You will also find details on latest and future international symposia, conferences, courses and publications as well as information from the members. Institutional members receive 2 copies.

-**"World Aquaculture"** (quarterly publication of the World Aquaculture Society)

-**"Aquaculture International" (AI):** bimonthly journal comprising research papers, short communications, technical notes and review papers. It is only included in the individual membership category and if opted for it.

-**An annual members list and free listing in it**

Other services for EAS members include:

- **access to the members' part of the EAS web site, including an on-line members' directory, networking part, etc...**
- **reduction on:**
  - EAS publications (full list available at www.easonline.org)
  - WAS publications
  - Kluwer Aquaculture Books
- **reduced subscription rates of up to 20% on various magazines/newspapers/web sites :**
  - Fish Farming International
  - Fish Farmer
  - Fish Farming Today
  - Havbruk
  - Eurofish
  - ContactDirect.com
- **reduced registration fees** (membership rate) at EAS or WAS co-organised meetings. Institutional members are entitled to a reduced registration rate (member rate) at EAS or WAS co-organised meetings for **up to 3 persons from the institute or company.**

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Positive Correlation Between Genetic Variation and Growth Rate in Farmed European Eel, *Anguilla anguilla*

J M Pujolar, G E Maes & F A M Volckaert

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Ch. de Bériotstraat 32, B-3000 Leuven, Belgium

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SUMMARY

We provide evidence for a positive correlation between genetic variability and growth rate in the European eel (*Anguilla anguilla*). More heterozygous individuals show a significantly higher length and weight increase and a higher condition index in comparison with more homozygous individuals.

RESUMEN

Nuestro estudio muestra una correlación positiva entre el nivel de variabilidad genética y la tasa de crecimiento en la anguila europea (*Anguilla anguilla*). Los individuos con una heterozigosidad más alta presentaban un crecimiento más rápido, tanto en longitud como en peso, en comparación con individuos homozigotos .

EXPERIMENT DESIGN

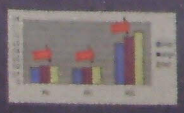
Glass eel individuals were raised at the facilities of Royaal BV (Helmond, Holland) in 2 separate batches. Individuals grown for one year at the farm presented a differential growth rate that produced a substantial range of sizes, including small (slow growth), large (regular growth) and fast-growth individuals.

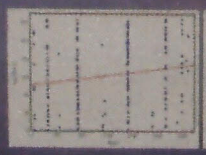
We tested for a possible correlation between GROWTH – measured as length and weight increase – and GENETIC VARIABILITY – measured as multi locus heterozygosity (MLH) using 13 ALLOZYMES and 8 MICROSATELLITES

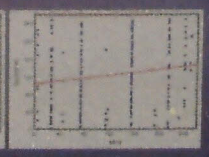
RESULTS

> 1. Fast-growth individuals show the highest genetic variation in comparison with slow or regular growth individuals

> 2. Significant positive correlations are observed between MLH / length increase and MLH / weight increase







CONCLUSION

In conclusion, our results provide good evidence for a heterozygozity fitness correlation in farmed European eel individuals. Multi-locus heterozygosity is positively correlated with growth, so that individuals attaining a larger size presented a higher genetic variation in comparison with smaller individuals

ACKNOWLEDGEMENTS

The work was funded by the EU contract EELREP (QLRS-2001-01336)

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DETERMINATION OF RFLPS IN GENES RELATED TO GROWTH IN GILTHEAD SEA BREAM (SPARUS AURATA)

I. Sánchez, I. Cross and L. Robaina*

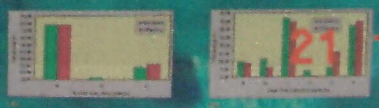
Laboratorio de Genética, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Polígono Río San Pedro, E-11510, Puerto Real, Cádiz, Spain;

e-mail: laurena.robaina@uca.es

Introduction

Materials and methods

Results and discussion



21 10 2004

PARENTAGE DETERMINATION IN SEA BREAM (*Sparus aurata*) USING MICROSATELLITE GENETIC MARKERS

K. Parati, S. Ben Jemaa, R. Alvarado, G. Bongiorni, V. Aquarone, A. Gallo
[illegible]
Poster created by [illegible]

INTRODUCTION

In fish progeny test it is necessary to keep together the eggs of each family, until the juvenile size (10 g) permits the use of electronic tags (microchip). The families may be created using artificial reproduction but for sea bream (*Sparus aurata*) is very difficult to obtain individual eggs and therefore it is seldom possible to use frozen or fresh milt. An alternative approach may be the use of genetic markers.
The aim of this paper is to test the opportunity to obtain families using natural reproduction and a small broodstock group (in a tank of 5 m³).
A genetic parental assignment protocol will be tested.

MATERIALS AND METHODS

Two small broodstock groups (A, B) are kept in two tank (5 m³).

- **DNA EXTRACTION:** *The larval DNA was extracted with PrepMan Ultra (Applied Biosystem)*
- **DNA AMPLIFICATION:** *Four PCR reaction multiplex: The dinucleotic microsatellites used for the analysis were the following: Sau0705, Sau0706, Sau1284, Sau1285, Sau1286, Sau0508NRA, Sau8521NRA, Sau2410NRA, Sau6408NRA, Sau4140NRA. Specific fluoroforesc labeled primers forward of 11 microsatellites loci (Ned, Fam or Vic).*

TRIPLEX A	TRIPLEX B	TRIPLEX C	TRIPLEX D
[illegible]	[illegible]	[illegible]	[illegible]

- **ELECTROPHORESIS** *by capillary sequencer ABI PRISM 3100*

- **STATISTICAL ANALYSIS:** *For each locus the heterozigosity (Ha) was computed according to: Ha = 1-Σki=1 pi2 where: k = number of alleles, pi = frequency of i-mo allele.*

RESULTS

The mean of observed heterozigosity, for all the microsatellites, was 0.80.
Only two multiple assignments were obtained in group A, with the progeny derived from only four parentages: 2 males and 2 females.
Only one multiple assignment were obtained in group B, with the progeny derived from only four parentages: 2 males and 2 females.

21 10 2004

CONCLUSION

- A small broodstock group can not be used (to create families) because of the reduced number of parents in the progeny.
- The set of genetic markers tested is sufficient to guarantee a parentage assignment.
- The reduced number of parentages in the two progeny may be explained by: (1) reduced sexual functionality of females used, (2) low sexual females stimulation. Therefore new experiment is necessary.

Pedigree evaluation of spotted halibut hatchery progenies by means of microsatellite DNA markers

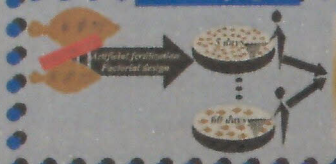
Maria del Mar Ortega-Villaizán Romo [1], M. Aritaki[2], M. Nakajima[2] and N. Taniguchi[1]
[1] Laboratory of Applied Population Genetic Informatics, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan
[2] National Centre for Stock Enhancement-Miyako Station, Miyako, Iwate, Japan
e-mail: mariaa@bios.tohoku.ac.jp

I. Abstract

Spotted halibut, *Verasper variegatus*, belongs to the righteye flounders, family Pleuronectidae. It is a distributed along Japan, from north Honshu to Okinawa. However, at the present time its abundance situation is rather unstable, being its catches considerably decreased since 1984. Consequently, this species became one of the target species of the Japanese National Center for Stock Enhancement. The stock enhancement program of spotted halibut has been carried out since 1993. However, the genetic variability of the released juveniles have not been tested so far. The main objective of this study was to evaluate the family composition of the hatchery-offspring produced to be released, as well as the effectiveness of the newly developed microsatellite DNA markers to the pedigree determination.

"Spotted halibut", *Verasper variegatus*, pertenece a la familia Pleuronectidae ("righteye flounders"). Su distribución se extiende a lo largo de Japón, desde Honshu hasta Okinawa. Sin embargo, actualmente su situación es más inestable, ya que su captura ha venido disminuyendo desde 1984. En consecuencia, esta especie se convirtió en una de las especies objetivo del Centro Nacional de Repoblación Japonés. El programa de repoblación de "spotted halibut" se ha estado llevando a cabo desde 1993, sin embargo, la variabilidad genética de los juveniles liberados al mar no se ha comprobado todavía. El principal objetivo de este estudio fue evaluar la composición familiar de la descendencia de criadero producida para ser liberada al mar, así como la efectividad de los marcadores microsatélites de ADN desarrollados recientemente, en la determinación del pedigrí.

II. Sampling



III. Microsatellite markers

10 microsatellite loci were used to genotype all the broodstock (30 ind.)

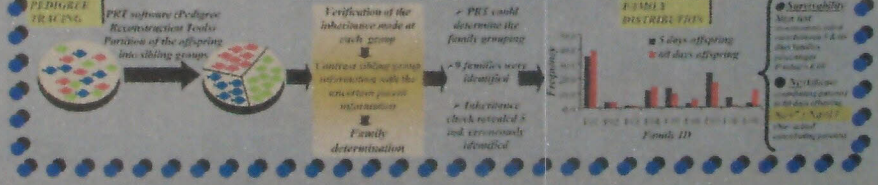
	PIC	A	Ho
Vsa2	0.832	16	0.87
Vsa4	0.817	5	0.73
Vsa5	0.909	25	0.93
Vsa6	0.723	5	0.77
Vsa8	0.761	9	0.80

	PIC	A	Ho
Vsa9	0.816	6	0.68
Vsa11	0.722	11	0.79
Vsa13	0.917	24	0.94
Vsa14	0.829	11	0.85
Vsa15	0.918		0.95

PIC = Polymorphism Information Content
A = number of alleles
Ho = observed heterozygosity

Selected loci for pedigree tracing

IV. Pedigree tracing analysis and results



V. Conclusions

1) The three loci (Vsa2, Vsa13, Vsa14) showed high efficiency to trace the pedigree of the hatchery offspring using the software Pedigree Reconstruction Tools.
2) This methodology could be applied in the future identification of the recaptured stock.
3) High family survivability was observed in seven both growth stages. In the future, family survivability will be examined in the recaptured samples, in order to assess the effectiveness of the enhancement program.
4) Separately, the fertilization procedures should be reconsidered in order to avoid the high variance in family size observed in the offspring.

21 10 2004

FINDING MY PARENT: HOW MANY GENETIC MARKERS ARE NEEDED?

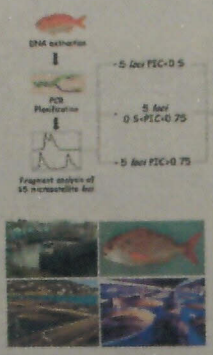
Piñera, J.A.[1]; Bernardo, D.; Blanco, G; Vázquez, E; Sánchez, J.A.
Universidad de Oviedo. Departamento de Biología Funcional. Laboratorio de Genética Acuícola. 33071 Oviedo
[1]email: jorgeap@correo.uniovi.es

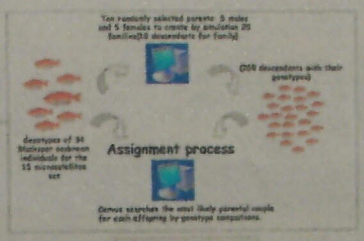
ABSTRACT: In this work, we evaluated the minimum number of microsatellite *loci* needed to obtain high percentages of correct assignment by software simulations, we determined that 7-9 highly polymorphic markers would achieve 100% of accuracy.

RESUMEN: Se evaluó el número mínimo de *loci* microsatélites necesarios para obtener elevados porcentajes de asignación correcta. A través de simulaciones informáticas consideramos que 7-9 *loci* microsatélites con elevados niveles de polimorfismo permiten obtener porcentajes del 100% de acierto.

INTRODUCTION:

Several breeding programmes implement artificial selection on phenotypic variation to obtain varieties with high commercial value, however inbreeding effects resulting from selection based on phenotypic variation could produce a drastic reduction of productivity in few generations. Unequivocal pedigree information allows to avoid these effects. Nowadays, several hatcheries rear the progenies from different families or separate tanks, but this method is laborious and expensive, also introduces environmental variance. Another method is the use of physical markers (fins clipped, heat- and cold-branded) but a minimum corporal size of individual is required. Microsatellite markers can be fast and easily analysed from a little piece of tissue, and do not show the aforementioned problems. But, how many microsatellite *loci* are needed to obtain high percentages of correct assignment? Efficiency of microsatellite *loci* depends on genetic variability of stocks. In this work, we evaluated which is the lowest number of microsatellite markers needed according to their variability levels (PIC: Polymorphic Information Content) to obtain unambiguous pedigree information.





Correct assignment

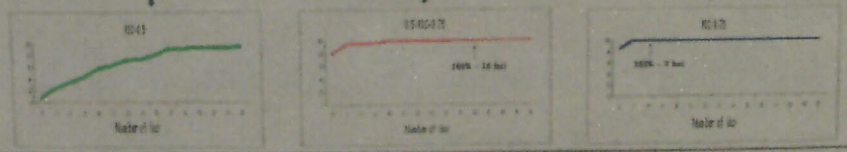
5 *loci* PIC<0.5	5 *loci* 0.5<PIC<0.75	5 *loci* PIC>0.75
2%	60.4%	98.2%

How many *loci* are needed to reach a 100% of correct assignment?

New *loci* generated by simulation using random reorganization

20 *loci* PIC<0.5	20 *loci* 0.5<PIC<0.75	20 *loci* PIC>0.75

Assessment of the minimum number of *loci* to reach a complete correct assignment



CONCLUSIONS:

7-9 highly polymorphic *loci* are enough to obtain high percentages of correct assignment

However, level of polymorphism of microsatellites changes among samples, therefore one highly polymorphic *loci* of sample 1 could show low variability at sample 2. Thus, first of all, we must select a wide number of *loci* (15-20), and new markers should be added up to obtain a large enough battery of microsatellites, in order to have always at least 7-9 highly polymorphic loci available for any sample analysed.

The high values of correct assignment obtained will allow to rear the families in a common tank which removes the need of extensive hatchery space and could avoid stress of specimens having to be reared in individuals environmental variance. Also, since individual identification is possible, the mating can be controlled to minimize the inbreeding effects, preventing the crossbreeding between related individuals

This work is supported by FFU grant for David Bernardo and a FICYT grant for Jorge A. Piñera.

21 10 2004

Rapid identification of several microalgae of commercial Importance based on nuclear rDNA sequences.

Annie Hervé, Roland Le Guedes, Nathalie Schreiber, Jean-Paul Cadoret

Ifremer

Abstract

Nuclear rDNA sequences of 10 microalgae used in aquaculture were determined in order to offer a tool in rapid species identification. We undertake the collection of public available sequences we performed the sequencing of selected nucleotide sequences. Phylogenetic trees were constructed with the completion of these sequences and best tree representations were tested against alternative trees. Beyond the phylogenetic assignment, a complementary RFLP pattern was mapped to give a basis for unequivocal thinking of cultured microalgal clones. These data are transparent to the publicly available sequences in order to show their place in the genetic tree of microalgae.

Introduction

Methods

Results and discussion

21 10 2004

Induction of triploidy in Atlantic cod (*Gadus morhua*) by cold shocks

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[2] University Hospital of North Norway (UNN), Department of Immunology and Transfusion Medicine, PB 35, N-9038 Tromsø, Norway

Background and objective

The production of triploid sterile fish provides evidence of the adverse effects of early maturation in some aquacultural species, and can be used as a technique for conservation of natural populations.

The objective of this preliminary study was to induce triploidy in Atlantic cod (*Gadus morhua*) using cold shocks.

Materials and methods

Brood - stock 3-4°C

Fertilization 6°C

Incubation 6°C

Cold shock

1.7±0.1°C

1 h

2 h

Sampling

Storage -80°C

Results

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Table 1.

Concluding remarks

Cold shocks of -1.7±0.1°C for duration of 1 to 2 hours induced only low rates of triploidy in Atlantic cod.

Future experiments will be conducted to investigate the effect of longer cold shock treatments on triploidy rate, as well as to compare such results with those from heat-shock and hydrostatic pressure experiments.

21 10 2004

Randomly Amplified Polymorphic DNA Analysis of Four Different Populations of the Indian Major Carp *Labeo rohita* (Hamilton)

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Introduction

- Rohu (*Labeo rohita*) is indigenous in South and South-East Asia
- Most popular aquacultured species in Bangladesh.
- Information on genetic structure of this important fish species is very scarce
- It is essential to understand the genetic composition of the rohu populations for future supporting programs on management of the fishery stocks, breeding program, enhanced genetic quality and conservation of their diverse gene pools

Objectives

- To assess the intra- and inter-population genetic variation in river and hatchery populations.
- To study the relatedness among the populations.

Materials and Methods

- Rohu fry were collected from three rivers viz. the Padma, the Jamuna, the Halda and one hatchery
- Genomic DNA was extracted from a small piece of fin tissue.
- Six decamer primers (screened from 45) was used for analysis of all the samples
- RAPD amplifications were performed following Williams *et al.* (1993) with some modifications.
- PCR products were separated on 1% agarose gel
- RAPD data were analyzed using POPGENE (version 1.31) (Yeh *et al.*, 1999), TFPGA, Lynch (1991).

Results

Random primers with the number and size ranges of bands

RAPD profiles of *L. rohita*

Intra- and inter-population genetic scores

	Halda	Jamuna	Padma	Hatchery
Halda	[illegible]			
Jamuna	[illegible]	[illegible]		
Padma	[illegible]	[illegible]	[illegible]	
Hatchery	[illegible]	[illegible]	[illegible]	[illegible]

Estimates of genetic variation

Parameters	Halda	Jamuna	Padma	Hatchery
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Population differentiation, gene flow and genetic distance

[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]

Dendrogram showing population relationship

Halda

Jamuna

Padma

Hatchery

Conclusions

- The genetic variability in the hatchery population was found to be lower than the river populations probably due to inbreeding and genetic drift.
- The overall population differentiation indicates little genetic differentiation between all river populations and the hatchery
- The higher genetic distance between the Halda and the Jamuna or between the Halda and the Padma populations may be explained by the presence of geographical barrier between each pair of them.
- The genetic variation exists in the Halda population was relatively more than the populations of the other rivers

Acknowledgement

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References

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21 10 2004

COMPARATIVE STUDY OF DIFFERENT NATURAL BENTHIC DIETS FOR ABALONE POSTLARVAE

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[2] Cawthron Institute, New Zealand., [3]Fachbereich Biologie der Universität, Germany.

Introduction

Material and Methods

RESULTS

CONCLUSIONS

Acknowledgements

21 10 2004

Rapid Diagnostic PCR for Epizootic Ulcerative Syndrome

Heather McLetchie[3]

N Buller[1], B Jones[2], S Fenwick[1], P Nicholls[2], D Alderman[4]

Introduction:

A. invadans is a fungus belonging to the phylum Oomycetes (water molds) and is a pathogen of fresh and brackish water fish. It is the causative agent of Epizootic Ulcerative Syndrome (syn. Red Spot Disease, Mycotic Granulomatosis, Ulcerative Mycosis).

It is one of the most destructive diseases in Asian aquaculture and is endemic throughout Australia.

The disease causes high mortality (>80%) or large ulcerative lesions (Figure 1) which render the fish unsaleable.

Presently the Office International des Epizootes (OIE) recommends a diagnosis based on clinical signs and confirmed by histopathology. This requires a certain amount of specialist knowledge and rely on morphological features which can be open to misinterpretation. The OIE is interested in a practical molecular diagnostic test which can be used on clinical samples.

Aim:

To develop a specific and sensitive diagnostic PCR for *A. invadans*

Methods:

Total genomic DNA was extracted (DNAzol reagent, Invitrogen, Paisley, Scotland) from an *A. invadans* culture, grown in Glucose Peptone broth and quantified by spectrophotometry.

Sequences from GenBank spanning the 18S ribosomal RNA gene, internal transcribed spacer (ITS) 1, 5.8S rRNA gene, ITS2, large subunit (LSU) rRNA gene (28S rRNA) were used to design a number of primers for further evaluation.

Results:

The chosen primer pair produced a product size of 554bp. The band was sequenced and matched 100% with known *A. invadans* (ITS region) in GenBank. The PCR was tested for specificity against different oomycetes with >90% homology to *A. invadans*, *A. dataci* (syn. *S. parasitica*), *Saprolegnia parasitica* (97% and is often found in conjunction with EUS), *Achyla* sp. (96%), *Pythium* (94%) and common laboratory contaminants *Aspergillus* and *Penicillium* sp.

The PCR appears highly specific for *A. invadans* (Figure 2).

The PCR is sensitive enough to detect DNA at 18 pg and can detect EUS in clinical tissue samples.

Electrophoretically separated product showing expected size of 554bp. Lane 1: 100bp DNA marker (band not included). 2: EUS positive, clinical sample. 3: *A. dataci*. 4 & 5: *Saprolegnia parasitica*. 6: *Achyla* sp. 7: *Pythium sulcatum*. 8: *Aspergillus* sp. 9: *Penicillium* sp. 10: negative control.

Conclusion:

The PCR is specific and sensitive enough to detect EUS from clinical samples. This is important for the aquaculture industry, offering a rapid diagnostic test that can be used for export certification.

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21 10 2004

Genetic diversity in European populations of mussels *Mytilus*

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[2] - Department of Biological Sciences, University of Hull, Hull, HU6 7RX, UK

Introduction

Marine mussels *Mytilus* belong to a group of benthic species crucial to the coastal ecosystems in Europe. *Mytilus edulis* and *M galloprovincialis* are important for the cultivation industry and their annual production exceeds 500000 tonnes in Europe (Smaal 2002). Collection of spat and subsequent translocations for culture leads to introductions of populations to new sites. This can be cause of introgression (Smietanka *et al.* 2004). Identification of mussel populations and stocks, and their genetic characteristics is important for management and biodiversity protection in Europe.

Materials and methods

Nuclear DNA markers and mitochondrial DNA was used for identification of the Mytilus taxa and their hybrids in populations from various coastal areas in Europe. A nuclear DNA marker *Me15/16* adhesive protein gene, internal transcribed spacer (ITS) regions between the *18S* and *28S* rRNA genes and *EF-1ab* diagnostic for the three taxa *M. edulis*, *M. trossulus* and *M galloprovincialis* were used. The nuclear DNA polymorphism was analysed by PCR amplification with specific primers. Mitochondrial DNA mtDNA coding region *ND2-COIII* and Dloop were studied by PCR, RFLP and sequencing.

Results

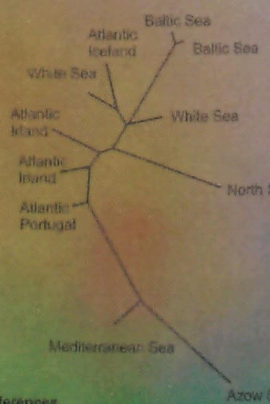
With the molecular methods, presence of pure *M edulis* was observed in the White Sea (Kartesh and Chupa Inlet, Kandalaksha Bay, Russia) and Iceland (Reykjavik). *M edulis* populations with low frequency of *M galloprovincialis* and *M trossulus* alleles was present in Netherlands (Grevelingen, Oosterschelde, North Sea). *M galloprovincialis*, *M. edulis* and their hybrids occurred in Ireland (Friend's Cove, Galway). *M galloprovincialis* populations were observed in the Sea of Azov (Black Sea), Mediterranean (Villefranche-sur-Mer, France) and Portugal (Lisbon). *M trossulus*, *M edulis* and their hybrids were found mainly in the Baltic Sea. Populations from Danish Straits contained much higher frequency of *M. edulis* than populations from the inner Baltic. The Baltic populations of mussels did not reveal presence of *M trossulus* specific mtDNA. Despite strong differentiation of mtDNA and isolation between the *Mytilus* taxa on macro-geographic scale, strong mitochondrial gene flow between populations and introgression in the hybrid zones occur on a more local geographic scale in Europe.

Discussion

Possible influence of mussel culture on geographic distribution of the *Mytilus* taxa and their hybrids is considered. The Baltic populations are a valuable reservoir of genetic biodiversity in European *Mytilus* and have not been cultured. Baltic populations of mussels do not contain mtDNA characteristic for *M. trossulus* due to strong introgression from the North Sea *M. edulis*, which is a natural process. Genetic characterization of mussel populations can also be useful for monitoring and predictions of diversity changes at the intra-specific and inter-specific levels with implications for understanding biodiversity changes in the coastal ecosystems in Europe.

The frequencies of *Me15/16* alleles diagnostic for *Mytilus edulis*, *M. trossulus* and *M. galloprovincialis*

ML tree based on *ND2-COIII* haplotype frequencies



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21 10 2004

Genetic Selection for Shell Height and Live Weight in the Chilean Blue Mussel (*Mytilus chilensis* Hupe 1854) Grown under Different Environmental Conditions

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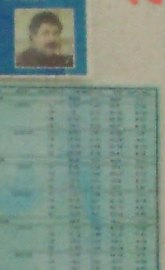
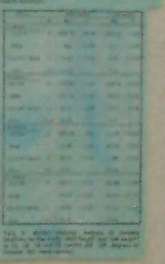
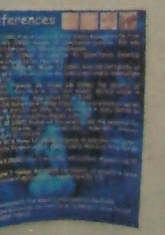
E mail: [illegible]
[2]Instituto de Fomento Pesquero, Centro Mariculturá Huelhue, Marisao Rural s/n, Chiloé, Chile.







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How to make females out of males

the effect of stimulation of P450-aromatase on the sex differentiation in Atlantic halibut

Selveig van Nes and Øivind Andersen, AKVAFORSK, Norway

21 10 2004

Introduction

Gonadal development in lower vertebrates seems to be mainly controlled by the enzyme P450-aromatase, which transforms testosterone into estrogen. This process is temperature-sensitive, which opens for the possibility to sex reverse Atlantic halibut for aquaculture purposes without the use of hormones or genetic manipulation.

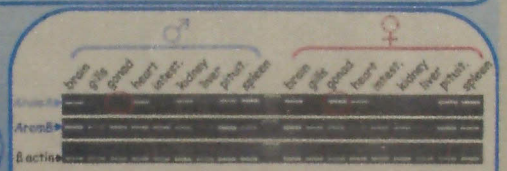
Here we report on the identification of two Atlantic halibut isoforms of P450-aromatase and their temporal and spatial distribution.

These results allow further investigation and understanding of the molecular basis of sex differentiation in Atlantic halibut

Results

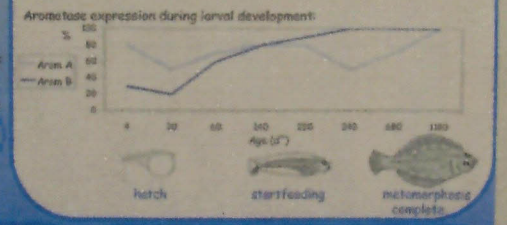
Two full-length cDNAs encoding distinct P450aromA and aromB isoforms were isolated.

The tissue specific expression patterns were overlapping, but aromA clearly predominated in the ovary, whereas aromB showed higher expression in brain and pituitary



The temporal expression analysis also revealed individual differences in expression pattern in early developmental stages (demonstrated as % of animals showing expression on each stage).

A very early activity of the two aromatase genes studied was found as compared to initiation of the histological sex differentiation



Discussion & Conclusion

The differences in the spatial and temporal expression patterns of aromA and aromB suggest that they have distinct functional roles.

The detection of both variants of aromatase at early developmental stages reveals that the onset of molecular sex differentiation clearly precedes any morphological development of the gonads.

The early onset of molecular sex differentiation suggests that in order to accomplish sex reversal by temperature treatment, the treatment should be initiated at a early developmental stage.

In progress

A temperature experiment is nearly completed, and in the near future we will reveal the exact effect of temperature on aromatase by quantitative expression analysis of the two aromatase genes using Real-Time PCR. In parallel, the temperature experiment will show the effect on phenotype and to what degree sex reversal of Atlantic halibut is possible by means of thermal treatment.

The understanding and possibly accomplishment of sex reversal by thermal treatment has potential to be of great importance for the Atlantic halibut aquaculture industry.

Methods

Cloning of the two different aromatase cDNAs was conducted by RACE-PCR strategies.

The PCR products were inserted into pGEM-T vector and sequenced in both directions.

The spatial gene expression analysis was performed by qualitative RT-PCR on a variety of tissues in sexually mature halibut as well as a temporal expression analysis from hatch to metamorphosis.

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Using DNA markers for trace ability in Aquaculture species

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The need for Trace-ability

- Trace-ability schemes can to trace a fish from any point in the production chain to any other point in the production chain
- Trace-ability is one not because
  - Consumers demand information on origin and production chain of food products they eat
  - Is fish caught in the wild or farmed?
  - If disease or toxins detected in market fish, trace fish to origin
- DNA markers for trace-ability?
  - can be genotyped by taking a sample from the fish or fish product at any stage along the production chain
  - Non destructive
  - Assignment of fish to parents or populations with a high degree of accuracy with DNA markers has been demonstrated

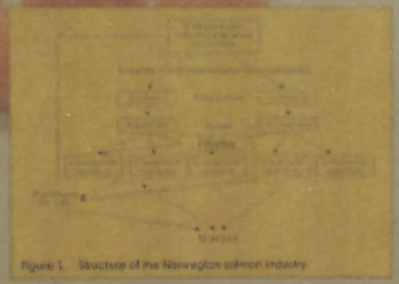

Figure 1. Structure of the Norwegian salmon industry

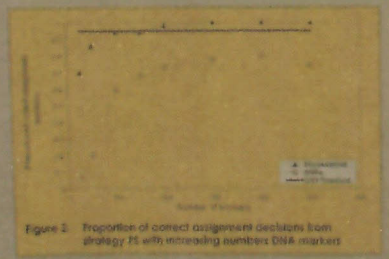

Figure 2. Proportion of correct assignment decisions from strategy F5 with increasing numbers DNA markers

Strategies for trace-ability using DNA markers

- We evaluated three strategies for trace-ability using DNA markers, in a simulation of the Norwegian salmon industry (Fig. 1)
  - Assign market fish to full sib families (grow out Ref. F5)
  - Assign market fish to parents (multiplier tier) of origin (PAR)
  - Assign market fish to grand parents (nuclei tier) (GRAND)
- Both Single Nucleotide Polymorphism markers (SNPs, 2 alleles per locus) and microsatellites (10 alleles per locus) tested
- Aim: Determine number of DNA markers required to achieve 95% of correct assignment decisions for GRAND, PAR and F5)

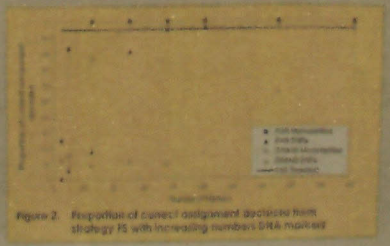

Figure 2. Proportion of correct assignment decisions from strategy F5 with increasing numbers DNA markers

Conclusions

- We were able to achieve 95% of correct assignment decisions in all strategies, figures 2 and 3.
- GRAND strategy (tracing market fish back to grandparents) with SNPs is cheapest option (assuming cost of genotyping a SNP is 5 times less than a microsatellite)
- but to achieve full trace ability, most complicated logistics
  - For example nucleus must record offspring from which matings sold to which multipliers.

21 10 2004

EFFICACY OF AN ELECTROMAGNETIC ANTIFOULING DEVICE FOR NET CAGE

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INTRODUCTION

In mariculture fouling causes the daubing of cages, the obstruction of nets and the increase in weight of the entire structures. As a consequence, frequent cleaning and substitution of nets determine the enhance of the management costs.

In order to reduce cost and/or the use of biocide a trial was carried out to investigate the efficiency of an electromagnetic device to limit the development of fouling on cage nets placed in North Adriatic Sea.

Table I Electrical parameters as function of net and cathode weight

CATHODE		CURRENT		VOLTAGE	WEIGHT INCREASE	
Metal	Form	Aver	Cathode	Average	Net	Cathode
		A	A/m²	mV	%	%
Steel	Network	2.5	12.8	418	29	54
Steel	Network	4.1	9.2	567	28	10
Copper	Linear	41.3	51.2	464	34	25
Steel	Grind	5.8	219.9	601	41	34
Copper	Linear	2.3	113.2	579	44	26
Steel	Grind	7.4	91.7	533	53	64

MATERIALS AND METHODS

The electromagnetic devices were immersed in a raceway supply with brackish water (Venice Lagoon) (Picture 1.)

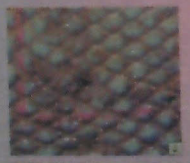
Antifouling system was composed by a plastic frame supporting a cage net, a cathode (C) and an anode (A). C and A formed a pile generating electrical field and electrolysis.

Several variations of device were tested, particularly with different distance between electrodes, shape of C (linear, network or grind) and metal (steel, copper or zinc) used to make C or A.

The developing of fouling was assessed as wet net (20" dripped) weight increase (%). Data were used also to evaluate the correlation among net and C weight raising and some experimental parameters.

Table II Correlation level of wet net and cathode weight increase with some parameters

PARAMETER	NET WEIGHT	CATHODE WEIGHT
Cathode surface	-0.50 (P<0.05)	-0.47 (ns)
Cathode weight	0.02 (ns)	-
Frame weight	0.71 (P<0.05)	0.72 (ns)
Medium current	0.37 (ns)	-0.09 (ns)
Cathode current	0.32 (ns)	0.23 (ns)
Voltage	0.39 (ns)	-0.02 (ns)



RESULTS AND DISCUSSION

- The control system without pile showed a weight increase (biofouling Pictures 2-5) equal to 94%, whereas the presence of the metal C even if not power supplied limited this increase below 50% (grind-steel: 48% and copper-linear: 44%).
- With regard to form and material of C, close piles in grind-steel gave lower net wet increase than copper ones (Table I).
- The limestone formation caused weight increase specially for steel devices and limited the efficiency of the electromagnetic devices (Picture 4).
- C surface (negative) and frame weight (positive) showed the highest correlation values with net weight increase (Tab. II). Wide C surface appeared able to avoid fouling developing, probably due to a higher production of inhibitor electrolytes.
- Electrical parameters were not significantly correlated with net weight.
- The antifouling efficiency of pile depends on its shape (correlation between cathode surface and weight) which should be the widest as possible at the same weight, resulting in lowest both fouling and limestone weight increase.

SUMMARY

Data obtained from this research showed that the use of an electromagnetic device can reduce fouling development both pile (current) and only metal made (not powered supplied). In the case of the pile, the best characteristics should be direct current powered, A and C in close form, shape and metal for C grind and steel respectively, C with a wide surface.

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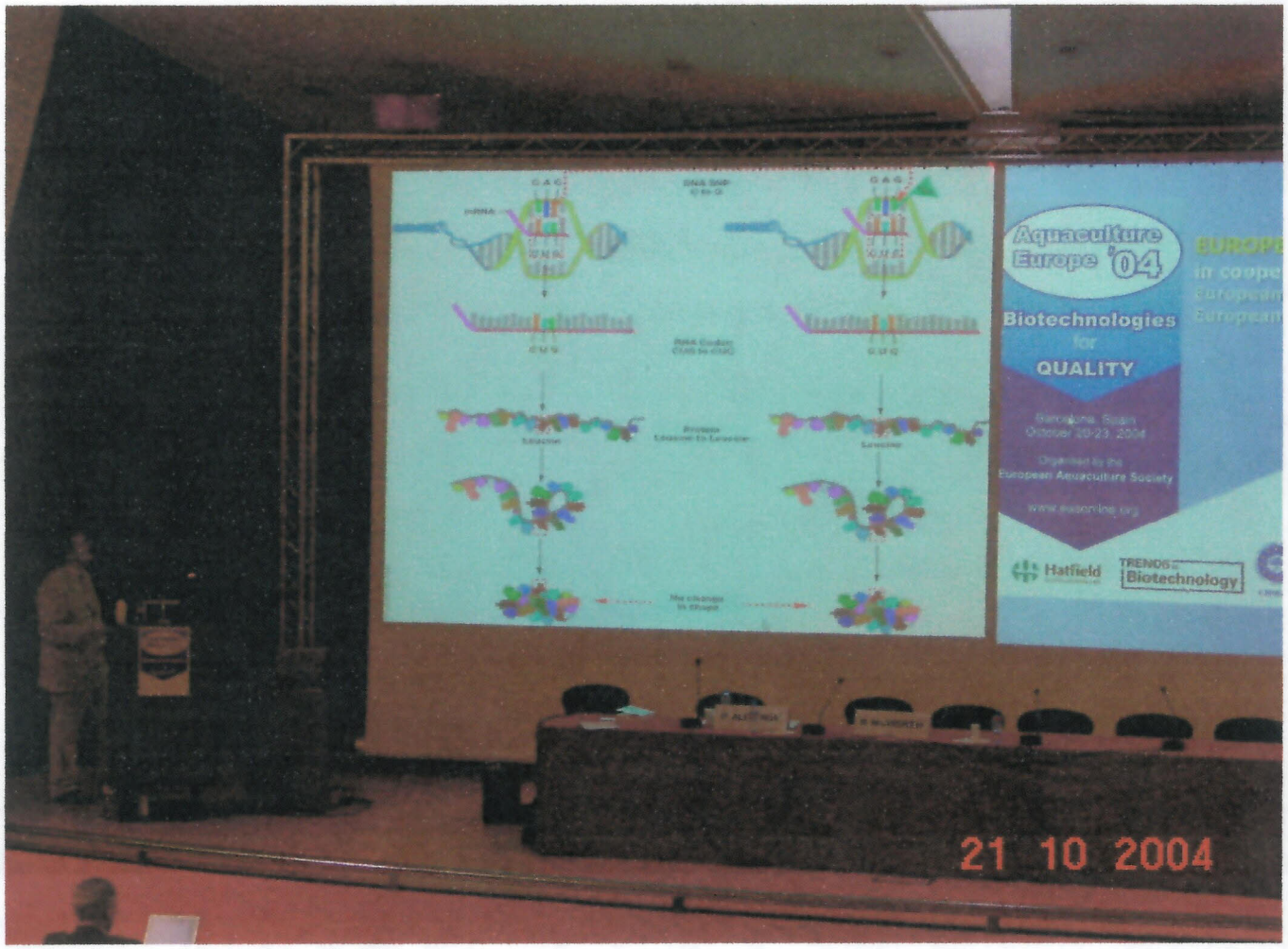
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Single Nucleotide Polymorphism
• SNP's now widely available and used in sequenced species.
• Many competing methodologies.
• Readily automated system possible to look at 1000's using chip technology.
• Not widely available in most commercial fish species- lack of sequence information.
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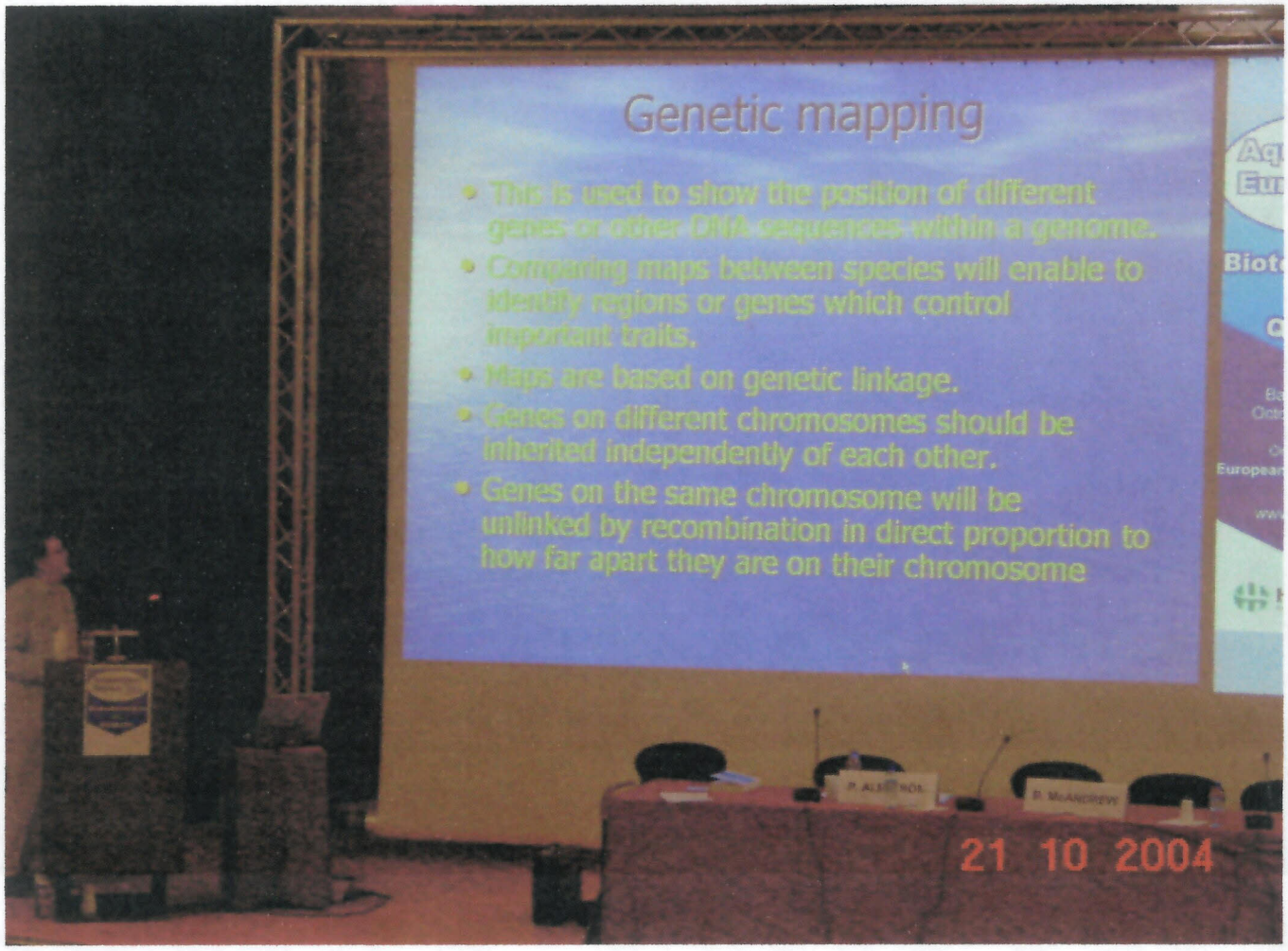
Genetic mapping
• This is used to show the position of different genes or other DNA sequences within a genome.
• Comparing maps between species will enable to identify regions or genes which control important traits.
• Maps are based on genetic linkage.
• Genes on different chromosomes should be inherited independently of each other.
• Genes on the same chromosome will be unlinked by recombination in direct proportion to how far apart they are on their chromosome
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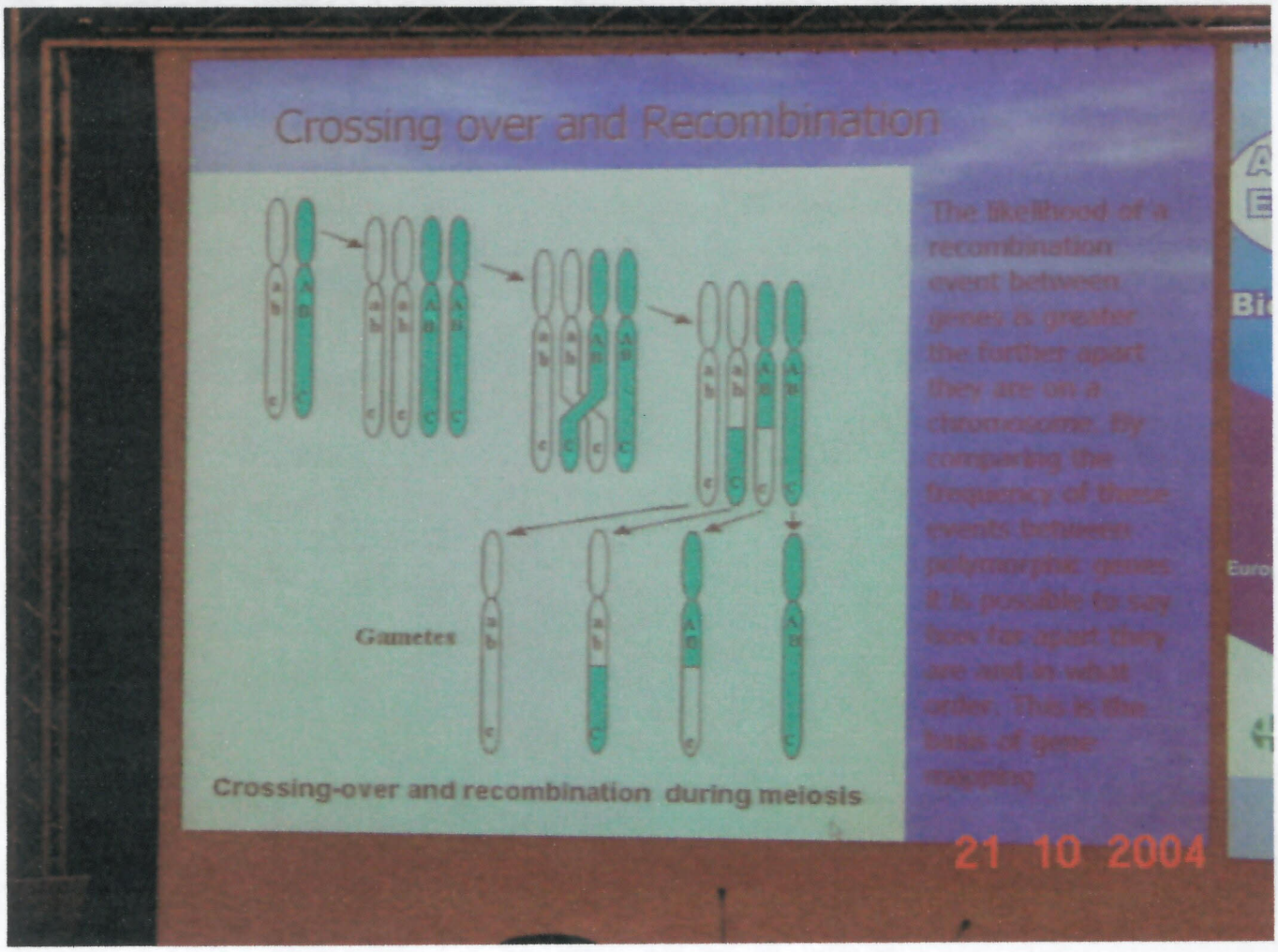
Crossing over and Recombination
Gametes
Crossing-over and recombination during meiosis
The likelihood of a recombination event between genes is greater the further apart they are on a chromosome. By comparing the frequency of these events between polymorphic genes it is possible to say how far apart they are and in what order. This is the basis of gene mapping
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Breeding Programmes in Aquaculture
• Significant gains have been achieved e.g. growth performance, age at maturity >5%/yr
• Most eggs supplied to Atlantic salmon farms now come from selected lines.
• Evidence that return on investment is substantial ratio 1: 15-40.
• However, this approach is not suited to all species or commercially viable for many companies
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Breeding Programmes in Aquaculture

- Broodstock replacement usually based on some form of mass selection not sustainable prone to genetic degradation (inbreeding & genetic drift).
- Selection based on pedigree information is potentially more sustainable than common mass selection approaches.

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Breeding Programmes in Aquaculture

- Biology of marine species (mass spawning) or hatchery requirements (specialised incubators & live food) and high mortality levels make family units impractical.
- Need for new approaches to match the biology of the fish and commercial reality.
- Molecular markers-genetic fingerprinting open up new possibilities.

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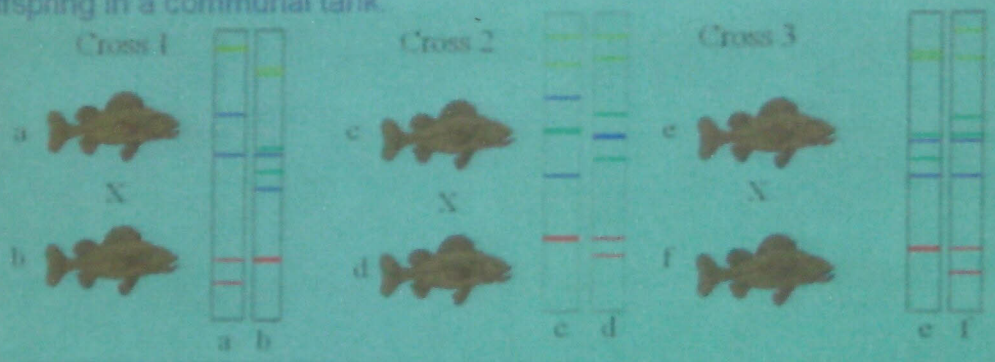
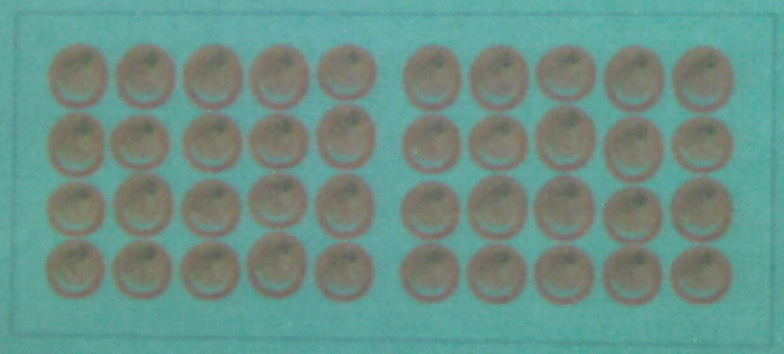
If genotype of parents is know we can be confident that we can identify their offspring. Therefore possible to combine eggs or embryos and rear offspring in a communal tank.





All families mixed at egg stage. Family sizes and environmental factors are standardised

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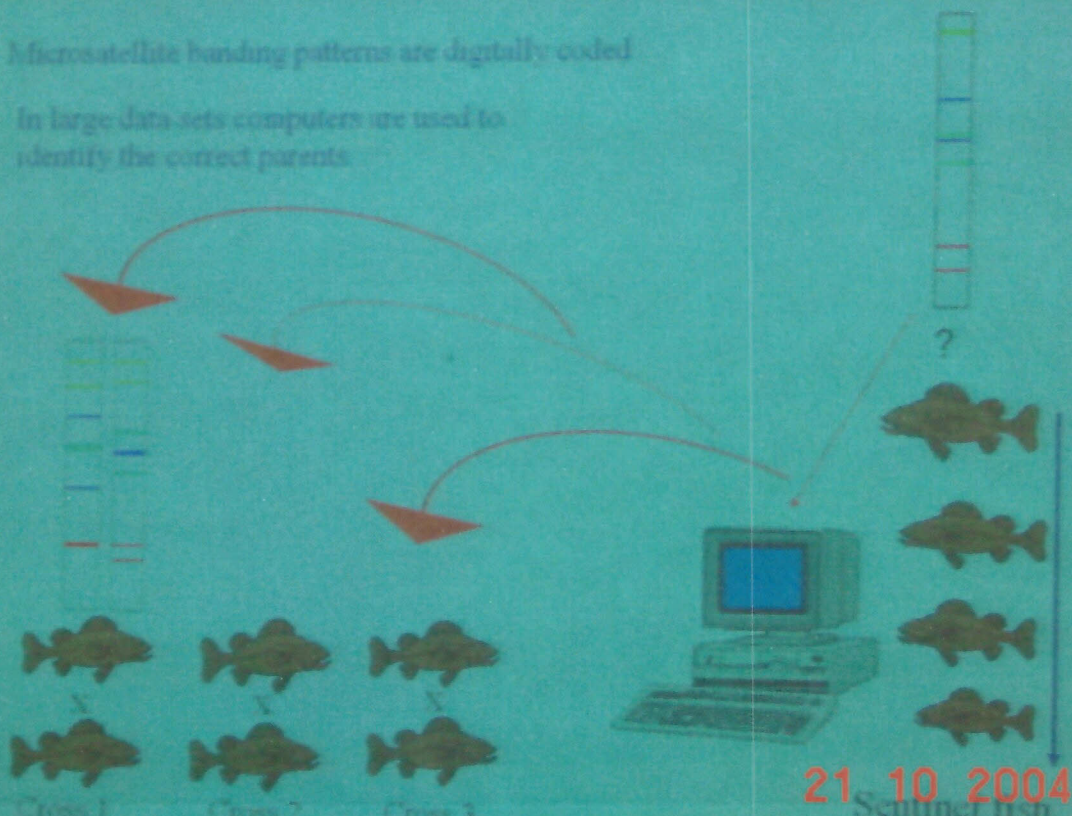
Microsatellite banding patterns are digitally coded

In large data sets computers are used to identify the correct parents



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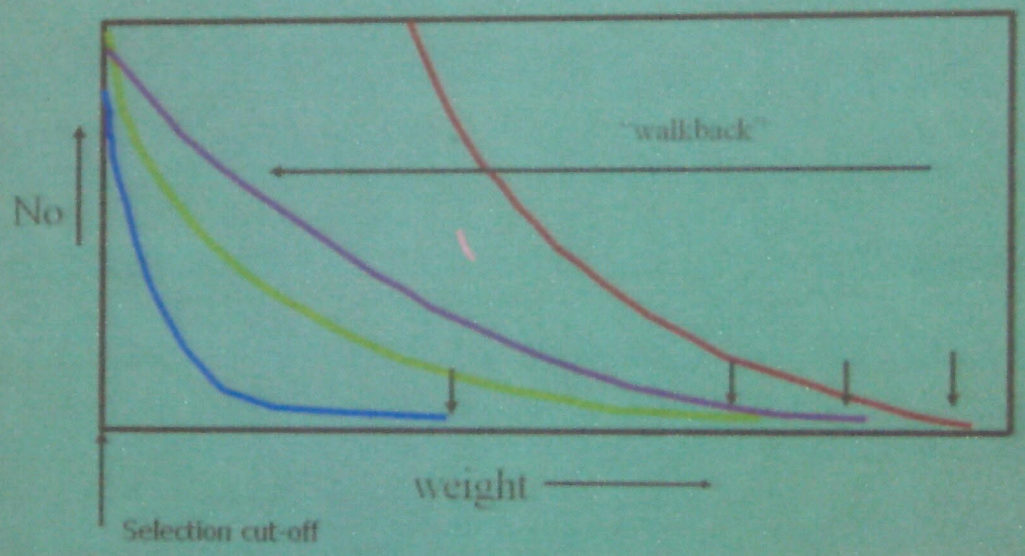
Sentinel fish

The fish are genotyped starting with the largest and we "walkback" into the population until we find the largest from the next family and continue to do this until we have enough different families represented to retain the genetic diversity of our broodfish.

This particular population is dominated by the red family.



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Applications

- Markers for parentage assignment
- Enables small companies to engage in controlled broodstock replacement.
- Application of communal rearing strategies will enable the selection of better fish.

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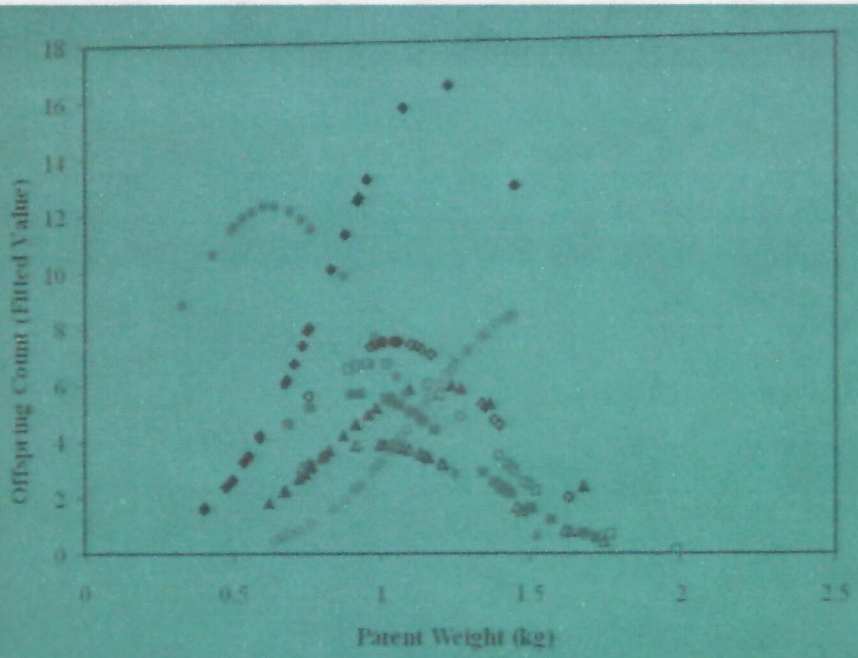

Parentage data on cohorts within *Sparus aurata* hatchery showed that 1kg broodstock are the optimum size as they contribute more offspring.

(Brown et al 2004)

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Limitations

- Mass selection restricts the traits that can be selected.
- Post harvest quality traits or disease resistance require sib analysis- selection on best families not individuals.
- Trait identification still difficult in most farmed fish as "value" needs to be established.
- This is where QTL approach will help to improve potential selection gains.

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opportunities

- Companies with established breeding programmes and measuring traits will be at an advantage-identify families that contain individuals that are segregating for a given trait e.g. mortality-survivor.
- Analysis of such families by geneticists will enable markers or genes associated with the trait to be identified.

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opportunities

- Once a QTL(s) has been identified it will need to be assessed.
- What is the contribution of the QTL(s) to the overall trait.
- Is the QTL stable and at what level, family, generations, strain, species.
- Need to incorporate this trait alongside others to generate a genetic merit or EBV.
- Microarrays identify gene expression profiles under different conditions. May identify genes as possible QTL or candidate.

21 10 2004

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Short communication

Induction of triploid embryos by heat shock in the Chilean northern scallop *Argopecten purpuratus* Lamarck, 1819

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Abstract Triploidy in embryos of the Chilean northern scallop, *Argopecten purpuratus* Lamarck, 1819, was induced with heat shock. Ploidy levels were assessed by chromosome counting after 3 h from embryos at the 8–16 cell stage of development. The efficiency of induction varied with the time of treatment post-insemination and the temperature of the shock. The duration of the shock showed a positive correlation with the percentage of triploids, the highest of which was obtained using 31°C for 15 min applied 10 min after insemination (66.7 %) but with low percentage of survival embryos after 3 h (26.4 %) and 18 days (11.2 %). The optimal procedure for triploidy induction (62.7 %) and survival after 3 h (62 %) and 18 days of culture (30.5 %) was the heat shock of 31°C for 10 min applied 10 min after fertilisation. We observed no significant difference in growth of shell length between controls and treated larvae after 18 days of culture.

Keywords *Argopecten purpuratus*; triploidy; genetics; Chile

M94013
Received 2 June 1994; accepted 2 November 1994

INTRODUCTION

The Chilean northern scallop *Argopecten purpuratus* Lamarck, 1819 is a functional hermaphrodite with external fertilisation and is a highly valued seafood (Padilla 1979; DiSalvo et al. 1984; Wolff 1988; Illanes 1990). The diploid chromosome number in this species is 32 (Von Brand et al. 1990).

Triploid organisms contain three genomes instead of the usual two. Chemical, pressure, and thermal treatments have been reported to induce triploidy in bivalves (Beaumont & Fairbrother 1991). To induce triploidy in bivalves, heat shock treatments at meiosis I or II are advantageous because of the low cost, the safe handling, and the large amount of eggs that can be treated (Thorgaard 1986; Gosling & Nolan 1989). Heat has been used successfully to induce triploidy in *Crassostrea gigas* (Quillet & Panelay 1986; Yamamoto et al. 1988), *Tapes semidecussatus* (Gosling & Nolan 1989), *Mytilus edulis* (Yamamoto & Sugawara 1988; Beaumont & Kelly 1989) and *Mytilus galloprovincialis* (Scarpa et al. 1994). Among the species of scallops that have been induced by cytochalasin B (CB) treatment to produce triploids are *Argopecten irradians* (Tabarini 1984), *Chamys nobilis* (Komaru et al. 1988), and *Chlamys varia* (Baron et al. 1989). In this paper we present the results from two preliminary experiments on the induction of triploid embryos in *A. purpuratus* through the application of heat shocks, and investigate the appropriate combination of temperature, time after-insemination, and duration of the shock.

MATERIALS AND METHODS

Scallops and gametes

Mature adult scallops from Tongoy Bay (northern Chile) were induced to spawn by exposing them to high concentrations of cultured microalgae

(DiSalvo et al. 1984) in the Quempillén laboratory, Chiloé Island, southern Chile. The scallops were rinsed in clean sea water and placed in 2-litre buckets with filtered (1 micron) and UV-sterilised sea water (FSW) and were continuously monitored to avoid self-fertilisation. Replication of the experiment was carried out using a different sample of progenitors. The insemination was done at 20°C, and 10 min after sperm addition, the eggs were rinsed and resuspended in 2 l FSW.

Triploid induction

About 4.8 million activated eggs were distributed in 300 ml baskets with 20 µm mesh screen in the bottom. These plastic baskets were maintained at 20°C for 30 min before the treatments. Three temperatures (23, 27, and 31°C), three times of post-activation (10, 20, and 30 min) and two periods of duration (10 and 15 min) were applied, resulting in 18 treatments with two controls held at 20°C in similar plastic baskets. After the treatments the activated eggs were rinsed and placed in 10 l buckets at 20°C. After 3 h the embryos, at the 8 to 16 cell divisions stage, were fixed in colchicine 0.05 % for 90 min, followed by a 30 min hypotonic shock 3:1 (sea water : distilled water) and Carnoy 3:1 (methanol : glacial acetic acid) fixation, changed three times at 4°C. The vitellus was removed following Von Brand et al. (1990). After 3 h, a direct count was made by microscope from a random sample of 1500 eggs of each treatment. From these counts the percentages of abnormal and non-developed eggs were estimated.

Ploidy examination

Triploidy level was determined by counting chromosomes stained with acetic-orcein (2 % at 50°C) on randomly examined metaphases from 30 embryos in each treatment ($2N = 32 \pm 2$ and $3N = 48 \pm 2$) using the "drop" technique, for induction rate estimation.

Growth and survival

In both experiments, after the induction of triploidy the embryos from each treatment were placed in 10 l plastic buckets containing FSW at 20°C, at a density of 150 individuals per ml. After 24 h (D larvae stage) the density was adjusted to 8 larvae per ml. Every other day the cultures were filtered, counted, and 3 random samples of 30 larvae from each bucket measured along the longest shell axis until the larvae reached the pediveliger stage. All larvae cultures were fed *Isochrysis galbana* T. at a ration of 60 cells per µl.

Data analysis

A three-way analysis of variance (temperature, time of post-activation, and duration of treatment) using SYSTAT 5.1 (Wilkinson 1991) was performed. Arcsine transformed data (Sokal & Rohlf 1981) was analysed with data expressed as percentages.

RESULTS

Triploidy induction

The percentage of triploid embryos in each treatment for both experiments are shown in Table 1. No triploid embryos were detected in the controls. Triploid individuals were obtained in all the treatments. The percentages of triploidy induction in both experiments ranged from 9.8 to 66.7% over all treatments. A significant effect was found for the variables "temperature" ($F = 143.745$, $P = 0.001$), "time of post-activation" ($F = 62.473$, $P = 0.001$) and "duration of treatment" ($F = 10.797$, $P = 0.004$), on the percentage of triploids. A positive effect of duration of the heat shock on the porcentage of triploids was observed, where the 15 min treatment produced more triploids than the 10 min shock. Also, targeting meiosis I (10 min post-activation) was more efficient in triploid production in both experiments. A high proportion of aneuploids were observed in treated larvae in both experiments.

Larval development

Percentages larval survival after 3 h and after 18 days (3 days before settlement) of culture for all treatments and both experiments are reported in Table 1. A significant effect ($P < 0.05$) of "temperature", "time after insemination", and "duration of treatment" was detected on the percentage of survival at 3 h and 18 days of larvae culture. The percentages of survival obtained for the larvae in the treatments were lower than in the controls (Table 1) but still in line with those reported in the literature for the same species (DiSalvo et al. 1984). No significant differences for growth between the two experiments were detected using analysis of covariance ($F = 1.276$, $P > 0.05$). Daily growth rates averaged 8.2 µm per day. The growth of the different groups induced with 31°C and controls are presented in Fig. 1 fitted by linear regressions (Downing & Allen 1987).

DISCUSSION

The results obtained from this preliminary study show that all the treatments were effective in producing triploid embryos in *A. purpuratus*, reconfirming the effectiveness of heat shock to induce triploidy in bivalve molluscs (Scarpa et al. 1994). Longer treatment times (15 min) produced higher percentages of triploids in the present study; several authors using heat treatment have also found an increased production of triploids with longer times of induction (Quillet & Panelay 1986; Yamamoto & Sugawara 1988; Yamamoto et al. 1990). The optimal procedure for triploidy induction and percentage survival at 3 h and at 18 days of culture was 31°C for 10 min and applied 10 min after insemination (meiosis I). The highest levels of triploidy (65.3–66.7 %) obtained with the highest temperature tested in the present study are comparable with those reported for other bivalve species, *C. gigas* (51–64 %, Quillet & Panelay 1986), *T. semidecussatus* (56 %, Gosling & Nolan 1989) and *Mytilus galloprovincialis* (81.2 %, Scarpa et al. 1994), but less effective compared with chemical (CB) treatment in other species of scallops with percentage of triploids ranging from 66 to 94% in *Argopecten irradians* (Tabarini 1984), 88.2% in *Chlamys nobilis* (Komaru et al. 1988); 78.5% in *Chlamys varia* (Baron et al. 1989). According to Allen (1987) one reason for these differences in ploidy production is that heat shocks inhibit all development and only those eggs that were in a specifc stage of cell division will be affected by the high temperature—whereas CB does not appear to arrest development of eggs; so as they reach the vulnerable stage of cell divison they are affected by the chemical.

The high proportion of aneuploidy in the treated larvae might be a cause for the drastic reduction in survival in the cultures, especially at 31°C.

Larval growth rates in triploidy experiments have been carried out by Downing & Allen (1987) in *C. gigas* and by Baron et al. (1989) in *Chlamys varia*; these authors, as in the present study with *A. purpuratus*, observed no significant difference for growth in length between control and treated larvae.

In the present study we estimated the percentage of triploidy production from chromosome counting of randomly examined metaphase of 3-hour-old embryos. Further studies need to examine the ploidy of the spat to verify these results because some authors have reported that the percentage of

Table 1 Percentages of triploids in 3-h old embryos for the two experiments (% EXP1) (%EXP2), and percentage of survival for each experiment (% SUR1) (%SUR2) estimated 3 h after insemination and after 18 days of culture (% SUR18-1) (% SUR18-2) in the controls and in the different temperatures (T°C), times (in minutes) after insemination (TAI), and shock duration (SHD) treatments (in minutes).

T°C	TAI	SHD	% EXP1	% EXP2	% SUR1	% SUR2	% SUR18–1	% SUR18–2
23	10	10	16.3	13.7	68.6	70.3	43.1	42.6
23	10	15	20.0	18.4	62.0	60.1	41.3	40.7
23	20	10	14.0	11.3	78.2	64.9	36.8	38.4
23	20	15	16.7	13.6	63.6	59.6	30.3	31.5
23	30	10	10.0	9.5	80.8	86.8	43.6	47.2
23	30	15	13.3	9.8	93.0	79.7	37.2	24.1
27	10	10	26.7	29.9	79.2	88.3	33.7	34.0
27	10	15	30.0	32.7	76.4	72.5	27.3	36.6
27	20	10	43.3	32.0	87.2	73.4	32.1	20.1
27	20	15	43.3	37.2	75.6	69.6	27.3	22.7
27	30	10	26.7	25.1	81.4	84.9	42.6	36.4
27	30	15	33.3	32.4	38.6	66.2	33.2	24.2
31	10	10	60.0	65.3	62.4	61.5	31.6	29.3
31	10	15	66.7	63.8	26.4	30.4	11.2	16.5
31	20	10	26.7	33.5	20.0	18.7	12.1	9.3
31	20	15	30.0	41.2	15.0	15.8	6.1	4.8
31	30	10	13.0	18.2	21.0	16.7	14.1	11.3
31	30	15	20.0	26.8	7.4	11.6	5.3	4.1
Control 1		0.0	0.0	87.2	91.2	55.9	51.8	
Control 2		0.0	0.0	81.4	88.5	50.6	53.4	

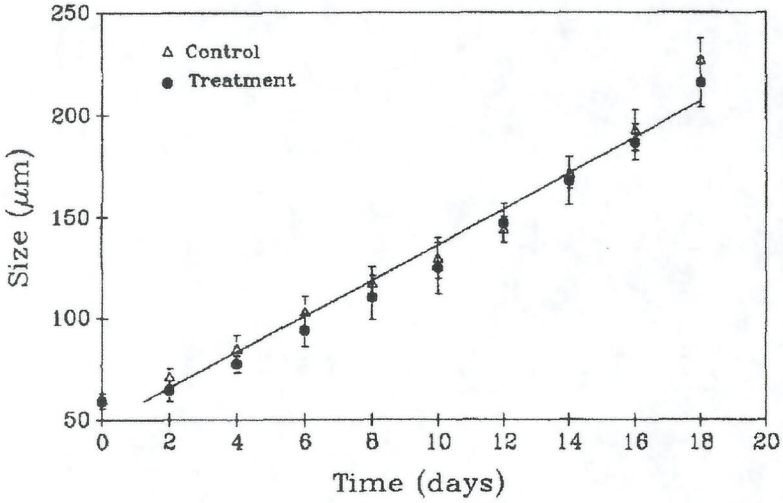

Fig. 1 Growth curves of controls and treated (31°C, 10 min duration and 10 min post insemination) larval population (*A. purpuratus*) cultured under 20°C over 18 days. Data from the two experiments grouped together. Vertical bars correspond to 95% confidence intervals.

polyploid larvae is sometimes reduced during the larval stage in other bivalve species (Uchimura et al. 1989). Further studies, including a combination of heat and caffeine (Yamamoto et al. 1990) to induce triploidy in *A. purpuratus* are in progress.

ACKNOWLEDGMENTS

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Journal of Shellfish Research, Vol. 23, No. 3, 753–757, 2004.

SELECTION RESPONSE FOR GROWTH RATE (SHELL HEIGHT AND LIVE WEIGHT) IN THE CHILEAN BLUE MUSSEL (*MYTILUS CHILENSIS* HUPE 1854)

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ABSTRACT The parental stock was taken from the 1999 natural spatfall of *Mytilus chilensis* collected in the Yaldad Bay of southern Chile. From the 22-mo-old cohort 5,688 mussels were monitored for live weight and shell length. Selection was carried out by applying a selection intensity of 1.755 for the trait "live weight". Five selected lines and five lines of an unselected control group were conditioned in seven 150-L tanks. Juveniles from the 3 selected and 2 control lines were individually tagged and transferred to three geographically distant mussel farms in southern Chile. Live weight and shell length were monitored after 10, 14, 18, and 22 months of age in all experimental mussels. The ANOVA results showed a significant difference, in both traits, between the selected and control groups at every age and location. Realized heritabilities for the trait "live weight" ranged between $h^2 = 0.35$ and $h^2 = 0.54$, whereas those for the trait "shell height" ranged between $h^2 = 0.32$ and $h^2 = 0.49$. Genotype-environment interactions were not apparent for either trait, indicating that similar selection pressures result in similar phenotypic changes for these traits across environments. These results suggest that mass selection for the improvement of the traits live weight and shell height would be effective in the Chilean mussel broodstocks.

KEY WORDS: mussel, *Mytilus chilensis*, selection response, growth, Chile

INTRODUCTION

Mussel aquaculture has enormous potential in southern Chile mainly because the numerous sheltered bays and estuaries along its coastline are free from industrial pollution. Fertilizers and pesticides are rarely applied in local agriculture, and domestic pollution, because of the low human population densities in southern coastal areas. (Winter et al. 1984, Toro & Chaparro 1990).

The Chilean blue mussel *Mytilus chilensis* (Hupe 1854) is an economically important bivalve in southern Chile. Its culture began in 1943 in the area of Quellón located southeast of Chiloé Island (Navarro & Gutierrez 1990, Aiken 1993) and the aquaculture production of this species increased from 3,864 t in 1993 to 41,406 t in 2001 (SERNAPESCA 2002). These values indicate an increase of 971.5 % (37,542 t) for mussel aquaculture between 1993 and 2001. Greater production space (hectares) under cultivation rather than higher outputs per unit area accounts for most of this increased production (Sanchez 2002). However, social, ecological, and economical pressures will eventually promote a greater efficiency of these aquaculture production systems. There are various means to achieve this goal: improved husbandry, nutrition and disease control, and improvement by genetic means. The potential for improving performance by genetic means is unquestionably substantial. There are several reports in the literature addressing the importance and potential of genetics in aquaculture (Moav 1976, Newkirk 1983, Gjerde 1986, Guiñez 1988, Toro & Newkirk 1990, Gjedrem 1999, Koment 2002, Beaumont & Hoare 2003). Increasing production efficiency, reducing generation time, and/or improving survival rate will reduce the negative effects of short growing seasons in high latitudes (Newkirk 1980).

Growth rate has implications for the fitness of individuals and also is considered the most economically important trait to be improved in a selection program (Beaumont & Hoare 2003). Traits of economic importance are usually determined by a large number of genes each having a small contribution and under considerable environmental influence (Newkirk 1980, Lande 1982). Thus, these traits are best studied through the quantitative genetic theory that reduces the most important features of complex genetic systems to a few variables that can be estimated from phenotypic measurements (Falconer 1989, Lande 1982).

Due to the high market demand for this species, the culture of *M. chilensis* in Chile has been increasing rapidly in the past years (Winter et al. 1984, Navarro & Gutierrez 1990, SERNAPESCA 2002, Sanchez 2002), and because of this mussel aquaculture development, estimates of genetic variation (i.e., heritability) and response to selection pressure are not only important from the ecological point of view but also for practical management purposes (Mallet et al. 1987, Koment 2002). The type of selection scheme that is appropriate depends upon the relative magnitude of the variation of that trait, due to differences in genotype and in environmental factors (Falconer 1989). These mussels are marketable at a shell length of about 55 mm, which is attained after 14 to 16 mo of growth in suspended culture (Winter et al. 1984, Kino & Valencia 1990). Therefore, a reduction in the time required to attain commercial size (12 mo) could be an important benefit in the culture of this mussel.

Very little has been published on the population genetics of *M. chilensis* and there are no reports on genetic improvement of the Chilean blue mussel. Control of the complete life cycle, which is required for any type of direct genetic improvement (Hershberger et al. 1984), has been accomplished only recently (Toro et al. 2004).

A high intrapopulation variation in the growth rate of *M. chilensis* has been reported in the literature (Winter et al. 1984, Navarro & Gutierrez 1990); however, only a few reports exist on genetic parameters (Toro & Paredes 1996) or genetic manipulation (Toro & Sastre 1995) in *M. chilensis*. A review of the literature shows that heritability estimates in blue mussels have been mainly based on half and/or full-sib correlation (Stromgren & Nielsen 1989, Toro et al. 2004), however, the most appropriate way in a commercial context to determine the heritability of a trait is to carry out selection trials and measure the response to selection for the trait (Beaumont & Hoare 2003). To ensure similar rearing conditions for the selected populations, a control group should also be spawned and their offspring reared in identical environments.

A high heritability value for a particular trait indicates that a

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large proportion of the trait is associated with additive gene action, and that genetic progress can therefore be achieved through mass selection (Newkirk et al. 1977). In this study, we consider that h^2 estimates using realized heritability analysis to obtain a more accurate estimation of its values among different rearing locations. To date, there have been no reports on growth and survival of hatchery produced seed of *M. chilensis*. This study represents the first attempt to produce large numbers of juveniles and adults under laboratory conditions and corresponds to a portion of a large-scale breeding program to improve the growth rate of the Chilean mussel *Mytilus chilensis*.

MATERIAL AND METHODS

Adult mussels from a 1999 naturally settled cohort (*Mytilus chilensis* Hupe 1854) were collected from a mussel farm located in Yaldad Bay, Chiloé Island (43°08′S; 73°44′W), southern Chile during October 2001 (prior to the spawning season). At 22 mo of age, a frequency distribution for the trait "live weight" from a random sample (N = 5,688) of the cohort of mussels was carried out. The trait presented a normal distribution ($P > 0.05$, Kolmorov Smirnov test, $P > 0.05$). A selection intensity of 1.755 was applied, corresponding to the largest 10% of the trait distribution. A similar number of average-size animals were segregated as control-line parents (Table 1).

Spawning was conducted over a 2-d period. The mussels were kept out of water for about 4–5 h before attempting spawning by thermal shock. They were rinsed in clean seawater and the three selected lines (N = 200 each) and 2 control lines (N = 200 each) were placed in ten 150-L tanks with filtered (1-μm) and UV-treated sea water (FSW) at 18°C. They were then continuously monitored until they spawned at which time they were removed. The day after the spawning the water of each tank was filtered through a 45-μm mesh. The embryos collected from each family were placed into a 200-L fiberglass tank containing 1-μm filtered and UV-treated fresh seawater (FSW) at 16 ± 1°C, and a density of 100 individuals per mL.

After 48 h the density was adjusted to 5 larvae per mL. A high cell concentration (100,000 cells/mL) of the microalgae *Isochrysis galbana* (Parke 1949) and *Chaetoceros gracilis* (Schuett) were used as food (Toro & Paredes 1996). Every day the water in each tank was passed through a 45-μm "nitex" screen to retain the larvae. Each beaker was rinsed with fresh water followed by seawater. The larvae were then resuspended in FSW and algal food was added daily at the desired cell concentration.

TABLE 1.

***Mytilus chilensis*. Basic statistics for the traits "live weight" (g) and "shell height" (mm) for the 5.688 individuals from the 22 months old cohort used as a base population in the selection program.**

N	Population 5688	Control 400	Selected 600
Live weight			
Mean (g)	18.47	18.42	26.50
Standard Deviation	4.27	0.89	2.54
Coefficient of Variation (%)	23.11	4.83	9.50
Shell height			
Mean (mm)	63.05	63.41	69.73
Standard Deviation	5.24	3.11	3.95
Coefficient of Variation (%)	8.31	4.90	5.66

The juveniles from each selected and control lines were settled on netlon mesh and after 2 weeks of growth in the laboratory, transferred to the field (Quetalmahue) until they were ready for tagging (5 mm). After 15 weeks of growth in the field, the juveniles from each line were individually labeled and transferred randomly in pearl nets to 3 different aquaculture farming sites. Hueihue Bay, located 500 m from a commercial oyster farm; and Quetalmahue Gulf and Putemún Channel, located around a mussel farm, all of them within the Chiloé Island (Fig. 1).

Live weight and shell height were monitored at 10, 14, 18, and 22 mo of age using an A&D portable balance (±0.01 g) and vernier calipers (±0.1 mm).

Analysis of Variance (ANOVA) including the effects of location, group (selected and control), and their interactions were carried out for live weight and shell height at the four life stages using the SYSTAT 5.1 statistical package (Wilkinson 1991). Log-transformations were used to normalize the data where appropriate. The response to selection and realized heritability estimates and their standard errors were calculated according to Falconer (1989), Becker (1992), and Hadley et al. (1991). A statistical comparison ($P \leq 0.05$) of differences in heritability values (Student's t test) was done.

RESULTS

Growth data at 10, 14, 18, and 22 mo of age for the traits live weight and shell height at 3 locations are presented in Table 2. From the mean of each group (control and selected) it can be seen that there are differences in both traits among the three different locations. The ANOVA results (Table 3) show that for live weight

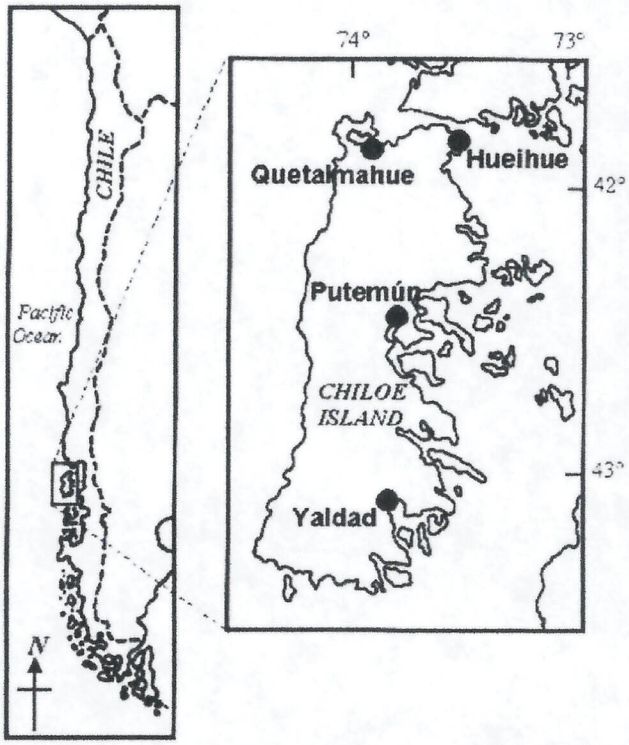

Figure 1. *Mytilus chilensis*. Aquaculture farms (Hueihue, Quetalmahue, Putemún) where the experimental mussel grew up and location of the natural population used as a source of the parental stock (Yaldad) (●).

TABLE 2.

Mytilus chilensis. **Basic statistics for the traits "shell height" and "live weight" in the F1 of the selected and control groups at 10, 14, 18, and 22 months old maintained in the 3 mussel farms: Hueihue, Putemún, and Quetalmahue, (mean: average; SD: standard deviation).**

Farm	Group	Age (months)	Live Weight (g)		Shell Height (mm)	
Hueihue			Mean	SD	Mean	SD
	Control	10	5.35	2.22	36.73	5.39
		14	9.00	3.25	44.68	2.28
		18	11.81	3.10	49.28	4.55
		22	19.20	5.29	56.37	4.35
	Selected	10	6.55	1.51	40.89	4.50
		14	10.36	2.36	48.23	4.30
		18	12.30	2.69	52.78	4.15
		22	22.34	4.18	58.42	4.28
Putemún			Mean	SD	Mean	SD
	Control	10	5.53	2.35	37.60	5.87
		14	9.02	2.89	45.12	5.13
		18	12.10	3.01	49.90	4.37
		22	18.77	6.16	56.50	5.47
	Selected	10	6.07	1.88	39.76	5.10
		14	9.15	2.40	46.03	5.18
		18	12.41	3.17	50.72	4.81
		22	22.80	4.60	59.93	5.75
Quetalmahue			Mean	SD	Mean	SD
	Control	10	7.58	2.52	42.68	5.39
		14	12.38	3.39	50.27	5.31
		18	15.20	3.62	54.20	4.76
		22	21.52	4.48	58.24	5.04
	Selected	10	8.14	2.11	44.99	4.50
		14	13.50	2.81	52.50	4.54
		18	16.20	3.37	55.90	4.55
		22	24.38	4.09	61.28	4.37

TABLE 3.

Mytilus chilensis. **Analysis of variance (ANOVA) for the traits "shell height" and "live weight" at 10, 14, 18, and 22 months old.**

Effect	df	Shell Height		Live Weight	
		MS	P	MS	P
10 Months					
Location	2	4586.79	<0.05	673.52	<0.05
Group	1	368.00	<0.05	22.10	<0.05
Location * Group	2	75.93	>0.05	8.47	>0.05
Error	1507	29.33		5.32	
14 Months					
Location	2	2727.43	<0.05	1047.90	<0.05
Group	1	212.36	<0.05	54.22	<0.05
Location * Group	2	66.05	>0.05	14.71	>0.05
Error	1016	26.66		9.70	
18 Months					
Location	2	1552.06	<0.05	11.34	<0.05
Group	1	96.84	<0.05	40.85	<0.05
Location * Group	2	46.96	>0.05	7.44	>0.05
Error	719	20.81		10.52	
22 Months					
Location	2	348.51	<0.05	382.84	<0.05
Group	1	1465.70	<0.05	2030.38	<0.05
Location * Group	2	30.38	>0.05	22.92	>0.05
Error	747	23.75		23.26	

df, degrees of freedom; MS, mean squares.

TABLE 4.

***Mytilus chilensis.* Realized heritability estimates (h^2) and their standard errors (±SE) for the traits "shell height" and "live weight" at 22 months old grown at 3 geographically separated mussel farms.**

	Shell Height h^2 SE	Live Weight h^2 SE
Hueihue	0.32 ± 0.017	0.38 ± 0.020
Putemun	0.49 ± 0.024	0.54 ± 0.018
Quetalmahue	0.48 ± 0.016	0.35 ± 0.015

and shell height, there is a significant effect of "location" (three locations) and "group" (selected and control) sources of variance. There were no significant effects of the location by group interaction term. A significant response to selection for the direct selected trait "live weight" was found at all ages monitored (see Table 3). The standardized responses fluctuated between 0.77 and 0.05 units of SD from the control. A correlated response for the trait 'shell height' was detected (see Table 3). The response measured as standardized Index did not differ significantly among the different localities ($P > 0.05$).

The realized heritability estimates for the traits "live weight" and "shell height" at 22 months of age exhibited values significantly greater than zero in all three locations (Table 4). Heritabilities for live weight did not differ significantly between Hueihue and Quetalmahue locations ($P > 0.05$) while Putemún and Quetalmahue showed similar heritabilities for shell height ($P < 0.05$); therefore even when growth rates differed significantly among environments, the additive genetic variance did not vary from location to location, with the exception of Putemún and Hueihue for live weight and shell height respectively.

DISCUSSION

Live weight and shell height varied significantly among environments in which the offspring were raised (see Table 2 and 3). This implies that environmental variation plays a large role in determining these growth traits. The location × group interaction (G X E) was not significant for either of the two traits (see Table 3), meaning that although there was significant variation among environments for each trait, the overall rank of each group in each environment was unchanged across sites.

There was a significant and steady response to selection in both traits across the environments. These results are in accordance with those reported by Crenshaw et al. (1991), for *Argopecten irradians concentricus*; Bustos et al. (1991) and Toro et al (1994), for *Ostrea chilensis;* and Nell et al (2000), for *Saccostrea glomerata* were a positive response to selection was found. Toro and Newkirk (1991) working with *O. chilensis* reported also a significant response to reduce the same trait, comparing a low selected line against an unselected control line.

The realized heritability estimates in both traits 22 months of age (see Table 4), were significant across the three environments measured, although, no significant genotype x environment interaction for these traits where detected (see Table 3). Therefore, a selection intensity applied to these traits results in predictable changes in the phenotype values of the traits among environments (Falconer 1989). There are no reports on realized heritabilities on adult mussels, however, the heritability values obtained here are lower than those reported by Newkirk & Haley (1982), in *Ostrea edulis*; Lannan (1972), in *Crassostrea gigas*; Stromgren & Nielsen (1989) and Losee (1978), in *Mytilus edulis.* However, they are in accordance with those reported by Toro & Newkirk (1990), in *O. edulis*; Toro & Newkirk (1991), in *O. chilensis*; Wada (1986), in *Pinctada fucata martensii* (h^2 = 0.47 & 0.35 for shell width and shell convexity respectively); Hadley et al. (1991), in *Mercenaria mercenaria* (h^2 = 0.43 for growth rate); and also in line with values for production traits in livestock (Van Vleck 1987).

Using the realized heritability values obtained and applying a selection intensity of 1.755 that is equivalent to select the higher 10% of the population for the trait, the estimated mean change in live weight ranges between 2.6 % to 4.0 % and for shell height between 2.9 % to 4.5 % per generation. These values of response to selection for increasing the traits are lower than those given by Mallet et al (1986) and Stromgren & Nielsen (1989) for *M. edulis* and Newkirk & Haley (1982) for *O. edulis*; these authors reported a large response to selection for growth to market size with an average of 23 % of gain over the controls.

The correlated response found for the trait "shell height" suggests a positive genetic correlation between the two traits. Very high genetic correlations between live weight and shell height have been reported (r_a = 0.995) for *O. edulis* (Toro & Newkirk 1990) and for *M. edulis* juveniles (Nielsen 1985).

The estimates of heritability for traits of economic importance such as live weight and shell height is essential in a long term breeding program to enhance the growth rate of the Chilean mussels. The quantitative genetic parameter estimates should be obtained under natural conditions and their constancy measured among different environments to be able to predict potential responses to selection (Mitchell-Olds & Rutledge 1986). Heritability values are essential for the purpose of predicting the genetic gain by selection. In common with previous studies of bivalves, our experiments demonstrate significant genetic variation in live weight and shell height, which may have implications for the biology of natural populations of *M. chilensis* as well as for commercial culture.

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RESEARCH ARTICLE

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Genetic variation among life-history stages of mussels in a *Mytilus edulis–M. trossulus* hybrid zone

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Abstract The mussel species *Mytilus edulis* L. and *M. trossulus* Gould coexist and hybridize throughout a large area that includes the north coast of Maine and Atlantic Canada. Previous studies provided genetic evidence for limited hybridization between the two species for mussels > 15 mm. The present study used two genetic markers (*ITS*, *Glu-5′*) to examine the genetic composition of early life-history stages by sampling veliger and pediveliger larvae, juveniles (< 2.0 to 15.0 mm shell length) and adults (> 15 mm shell length) in Trinity Bay, Newfoundland, during three consecutive years (1995–1997) to determine if differential mortality limits the relative abundance of hybrids. The relative frequency of the two species and the different hybrid genotypes was similar among the larvae, juveniles and small adult mussels. The double hybrid genotype (F_1-like) was the rarest genotype observed. There was no evidence for differential mortality during the early life-history stages, and factors limiting production of hybrids appear to operate before the late larval stage. The observed frequency of hybrids is probably due to a combination of pre- and postzygotic reproductive isolating mechanisms operating early in the mussel's life history. *M. trossulus* dominated the early life-history stages, possibly due to a higher population density and a greater reproductive output than *M. edulis*. Differential mortality may explain the observed decrease in frequency of *M. trossulus* and increase in frequency of *M. edulis* with increasing shell length. A similar frequency of hybrid mussels from larvae to the size class of 55 mm shell length may indicate a rate of mortality intermediate between the two parental species. The *M. edulis–M. trossulus* hybrid zone appears to be maintained by reproductive isolating mechanisms limiting the production of hybrids and life-history differences that allow the two species to coexist.

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Introduction

Hybrid zones have attracted a great deal of interest because they represent the interaction between adaptation, reproductive isolation and speciation (Barton and Hewitt 1989; Harrison 1993; Arnold 1997). Much insight into the speciation process can be gained by studying the origin, maintenance and fate of hybrid zones. Natural hybridization involves incomplete reproductive isolation, and hybrid zones have therefore been particularly useful for understanding the evolution of pre- and postzygotic isolating mechanisms that form the basis for reproductive isolation and speciation (Orr and Presgraves 2000; Marshall et al. 2002). Hybridization may represent a transitory phase leading to an increase in reproductive isolation between species and a reduction in the rate of hybrid formation. Low survival and/or fertility of hybrids may select for increased prezygotic isolating mechanisms, a process known as reinforcement (Marshall et al. 2002). However, if hybrids are viable and fertile, then hybrid zones represent a means through which genes can introgress between species. Although its importance is unknown, introgression may be a source of novel genetic variation for adaptation (Arnold and Hodges 1995; Arnold 1997).

Hybrid zones often occur as narrow regions where two allopatric species come into contact and interbreed. Much research has investigated the width, location and stability of hybrid zones, as well as clinal variation of phenotypic and genetic characteristics as a means of

understanding factors that maintain hybrid zones (Barton and Hewitt 1989). Some hybridizing species occur as a mosaic of parental species and hybrids coexisting over large areas consisting of patchily distributed habitats (Rand and Harrison 1989; Ross and Harrison 2002). A mosaic hybrid zone may result in increased opportunities for hybridization and introgression, because geographic proximity increases contact between species and hybrids. Regardless of the geographic structure of a hybrid zone, recent studies have focused on assessing the relative importance of endogenous genetic and exogenous ecological factors in maintaining hybrid zones (Arnold and Hodges 1995; Arnold 1997).

The "Tension Zone" model proposes that hybrids have consistently low fitness due to endogenous genetic incompatibilities and that hybrid zones are maintained by a balance between the formation of hybrids and selection against them (Barton and Hewitt 1989). However, this simple model has been criticized because there are many examples of hybrid genotypes with intermediate fitness, equal fitness or even greater fitness than the parental genotypes (Arnold and Hodges 1995). There are also examples in which the relative fitness of parental and hybrid genotypes depends on the environment (Arnold and Hodges 1995; Arnold 1997). Thus the maintenance of hybrid zones probably results from an interaction between endogenous and exogenous factors. An alternative model to the tension zone model suggests that genetic incompatibility can select against certain hybrid genotypes, while other hybrid genotypes are favored under specific environmental conditions (Arnold 1997).

An understanding of the structure of hybrid zones requires the determination of the relative fitness of parents and hybrids under a variety of natural conditions. Additional information can be obtained from experiments in which fitness differences are estimated from laboratory-produced hybrids. More commonly, basic information on the genetic structure of a hybrid zone is obtained from the distribution of genetic variation between parental species and their natural hybrids as defined by genetic markers. Such information often provides the first indication that hybridization is occurring between two species (Boecklen and Howard 1997). Furthermore, determining genetic variation with several genetic markers allows hybrid zones to be characterized on a continuum from unimodal to bimodal based on the frequency distribution of parental and various types of mixed hybrid genotypes (Jiggins and Mallet 2000). Unimodal hybrid zones have a greater frequency of individuals with mixed hybrid genotypes compared with parental genotypes and are associated with a lack of prezygotic isolation mechanisms. In contrast, bimodal hybrid zones contain more parental than mixed hybrid genotypes and often result from strong prezygotic isolation mechanisms, primarily through assortative mating, although both unimodal and bimodal hybrid zones exhibit similar levels of postzygotic genetic incompatibility, suggesting that postzygotic mechanisms alone cannot generate bimodal hybrid zones (Jiggins and Mallet 2000). These observations further suggest that assortative mating combined with selection against certain hybrid genotypes may be the primary mechanism for maintaining bimodal hybrid zones (Jiggins and Mallet 2000).

Species of mussels in the *Mytilus edulis* complex are often found sympatrically, and there is always hybridization between them (Gosling 1992). Some of these contact zones are located on the Pacific coast of North America, between *M. galloprovincialis* and *M. trossulus* (McDonald and Koehn 1988; Heath et al. 1995; Rawson et al. 1999), in southwest England and France, between *M. edulis* and *M. galloprovincialis* (Skibinski et al. 1978; Edwards and Skibinski 1987; Gardner et al. 1993; Gardner 1994; Daguin et al. 2001; Bierne et al. 2002b, 2003a; Hilbish et al. 2002), and in the Baltic Sea, between *M. edulis* and *M. trossulus* (Väinölä and Hvilsom 1991; Riginos et al. 2002). In the western Atlantic, there is evidence that interbreeding between *M. edulis* and *M. trossulus* is limited in natural populations (Koehn et al. 1984; Varvio et al. 1988; Bates and Innes 1995; Saavedra et al. 1996; Comesaña et al. 1999; Rawson et al. 2001; Toro et al. 2002), despite their sympatric occurrence and the successful production of viable hybrids in laboratory crosses (Zouros et al.1992, 1994).

The *M. edulis–M. trossulus* hybrid zone of eastern North America extends from northern Newfoundland to northern Maine (Koehn et al. 1984; Saavedra et al. 1996; Comesaña et al. 1999; Penney and Hart 1999; Rawson et al. 2001; M. Miranda, unpublished data). Most sites consist of various mixtures of the two species and their hybrids, producing a bimodal distribution of parental and mixed hybrid genotypes (Bates and Innes 1995; Saavedra et al. 1996; Comesaña et al. 1999; Rawson et al. 2001). Although some areas appear to be dominated by one species or the other on both a macro- and micro-geographic scale, no environmental factors have been identified that could definitively explain the observed distribution. However, variations in salinity, temperature and wave exposure may play a role in some stages of the life history (Bates and Innes 1995; Gardner and Thompson 2001; Rawson et al. 2001; Qiu et al. 2002).

Information from four diagnostic nuclear genetic markers and mtDNA variation in samples from Trinity Bay, Newfoundland, collected during July 1997 showed that *M. trossulus* dominated the smaller size classes (15–20 mm SL), while *M. edulis* dominated the larger size classes (>40 mm SL) (Comesaña et al. 1999). The relative frequency of hybrids was independent of shell length (SL), and most of the hybrids were backcross genotypes. Only five of the 400 individuals sampled had a genotype that could be classified as F_1. The low frequency of F_1 individuals is consistent with strong prezygotic isolation (Jiggins and Mallet 2000), and differences in the gametogenic cycles of the two species may contribute to reproductive isolation (Toro et al. 2002). The Toro et al. (2002) study, which was also done

at the same sites in Trinity Bay, Newfoundland, in 1996 showed that *M. edulis* spawned over 2–3 weeks in July, while *M. trossulus* and hybrids spawned in late summer to early autumn. However, postzygotic genetic incompatibility may also play a role in limiting the occurrence of F_1 genotypes, particularly during the early life-history stages. It is possible that genetic incompatibility may be expressed at metamorphosis, a critical stage at which larvae settle from the plankton.

Recently developed genetic markers allow the genotyping of individual mussel larvae, extending genetic information on species composition and hybridization to the earlier life-history stages (Toro 1998; Martel et al. 2000; Bierne et al. 2003b; Gilg and Hilbish 2003a, 2003b; Wood et al. 2003). The specific question addressed in the present study was: does differential mortality during the early life-history stages (larvae and juveniles) explain the high frequency of *M. trossulus* and low frequency of *M. edulis* and hybrids observed in the small adult size classes (15–20 mm SL) by Comesaña et al. (1999)?

Materials and methods

Study sites and sampling

Adult mussels (*Mytilus edulis* Linnaeus, 1758; *M. trossulus* Gould, 1850) were collected subtidally by SCUBA at a depth of approximately 1.5 m below mean low tide at two locations, Chance Cove and Bellevue, in Trinity Bay (47.62°N; 53.75°W) on the east coast of Newfoundland, in October 1995, 1996 and 1997. Dalley et al. (2002) provide a summary of oceanographic conditions in Trinity Bay. At each location, two sites were sampled: one exposed to wave action (Chance Cove exposed, CE; Bellevue exposed, BE) and another protected and sheltered from wave action (Chance Cove protected, CP; Bellevue protected, BP) (see Fig. 1 in Comesaña et al. 1999). Exposed and protected sites at each location are separated by about 2 km and the Bellevue and Chance Cove locations are separated by about 16 km. Haphazard samples of approximately 200 adult mussels (15.0–98.6 mm SL) were taken from each mussel bed at each site. The mussels were brought alive to the laboratory, shell length was measured with an electronic caliper (±0.01 mm), and the mussels were dissected. A small piece of mantle edge tissue (approximately 200 mg) was removed, placed in a 1.5-ml Eppendorf tube, fixed with 95% ethanol and stored at −20°C until DNA extraction.

Mussel juveniles (<2.0 to 15.0 mm SL) were collected from brown filamentous algae and from within mussel clumps at each of the sites described above during October and December 1995, September, October, and December 1996, and September and December 1997. The samples were placed in 95% ethanol and refrigerated (4°C) until DNA extraction. Larvae were qualitatively sampled in plankton tows (20 μm mesh) at the same sites sampled for adult mussels at Bellevue and Chance Cove, Newfoundland, during 1996 (19 July, 9 August, 3 and 19 September, 11 October), 1997 (2 September) and 1998 (1 August). Plankton samples were fixed with 95% ethanol and stored at 4°C until DNA extraction.

DNA extraction

Approximately 50–100 mg of mantle-edge tissue from each mussel (juvenile or adult) was coarsely chopped and digested overnight in 500 μl lysis buffer (50 mM Tris-HCl, pH 8.0, 1.0% SDS; 25 mM EDTA) with 200 μg proteinase K (Sigma) at 37°C. The digest was then extracted twice with 500 μl phenol–chloroform–isoamyl alcohol (24:24:1) followed by precipitation in 95% ethanol at −20°C. The extracted DNA was resuspended in 200 μl ultra-pure sterile distilled water.

For mussels of 2–5 mm SL, the whole flesh was removed from the shell and used for DNA extraction. For very small individuals (<2 mm SL), the whole mussel was used. DNA was extracted from individual larvae collected from plankton tows. For each sample, the largest larvae were selected to ensure that sufficient DNA could be extracted. *Mytilus* spp. larvae were the dominant bivalve species in the samples, and the species-specific primers ensured that only *Mytilus* spp. larval DNA was amplified. The DNA extraction procedure for larvae and newly settled juveniles was similar to that described for the mantle tissue, except that the shell length of each individual was first measured with a graduated eyepiece fitted to a stereomicroscope at ×40 magnification and the individual was isolated using a Pasteur pipette. Each individual was then placed in a separate 1.5-ml Eppendorf tube and washed twice in 0.5 ml distilled water before DNA extraction. In addition, 60 D-stage larvae from each of the two parent species and hybrids from laboratory cultures were analyzed in order to confirm species-specific genetic markers. For both genetic markers, the cultured larvae produced genotype patterns identical to samples from adult tissue for each species and hybrids.

Genetic markers

Genetic information was collected for two nuclear-DNA species-specific markers using the polymerase chain reaction (PCR). *ITS* and *Glu-5'* are co-dominant genetic markers producing two specific *M. edulis* and *M. trossulus* patterns and distinct patterns for hybrids. The *ITS* marker, developed by Heath et al. (1995), is based on the internal transcribed spacer (*ITS*) regions between the 18S and 28S nuclear-rDNA coding region. The primers used were *ITS*1 (5′-GTTTCCGTAGGTGAACCTG-3′) and *ITS*2 (5′-CTCGTCTGATCTGAGGTCG-3′), with an expected PCR gene fragment size of 1250 bp. Standard PCR amplifications were carried out in 25-μl reaction mixtures (500-μl thin-walled PCR microtubes,

Gordon Technologies) containing 2 μl DNA template (1:10 dilution), 0.2 mM each of the four deoxyribonucleotide triphosphates (dNTPs) (Sigma), 2.0 mM $MgCl_2$, primers at 0.4 mM, 1 U of *Taq* DNA polymerase (Promega), the manufacturer-supplied PCR buffer and sterile distilled water. The reaction mixtures were overlaid with a drop of mineral oil (Sigma) to prevent evaporation, and were then placed in a programmable thermocycler (MJ Research). The thermocycler protocol consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 20 s, 50°C for 20 s and 72°C for 2 min. Rigorous precautions were taken to prevent template contamination during the PCR procedure: positive displacement pipettes and autoclaved, sterile tips, tubes and reagents were used, and a negative control without the DNA template was run with every batch of samples.

Of each amplified PCR product, 5 μl was digested for 12 h at 37°C with 0.5 U of the restriction enzyme HhaI in a total volume of 15 μl, including 3 μl of buffer supplied by the manufacturer (Pharmacia) and 6.5 μl of ultra-pure distilled water. Enzyme inactivation was accomplished at 65°C for 20 min in a water bath, and the sample was allowed to cool for 20 min at room temperature. The digested products were fractionated electrophoretically in 3% agarose gels (2% Sigma and 1% NuSieve GTG agarose) with 0.5× Tris–borate–EDTA (TBE) buffer for 30 min at 112 V. A negative control with no PCR product was run with every batch of samples. A 1-kb DNA ladder (Gibco BRL) was run on each gel; the DNA fragments were visualized by placing the gels in ethidium bromide (0.5 μg ml^{-1}) and the genotype determined from Polaroid images taken under UV light. A negative control without a DNA template was run with every batch of samples.

A second nuclear-DNA marker, *Glu-5′*, developed by Rawson et al. (1996), targets the gene encoding the mussel polyphenolic adhesive protein produced by the pedal gland. The forward primer used was JH-5 (5′-GTAGGAACAAAGCATGAACCA-3′) and the reverse primer was JH54 (5′-GGGGGGATAAGTTTTCTTAGG-3′). Standard PCR amplifications were carried out in 25-μl reaction mixtures containing approximately 50 ng DNA template, 2.5 nmol dNTPs, 2.0 mM $MgCl_2$, 50 pmol of each primer, 1 U of Tfl DNA polymerase (Promega), the manufacturer-supplied PCR buffer and sterile distilled water. The reaction mixtures were overlaid with a drop of mineral oil (Sigma) to prevent evaporation, and were then placed in the thermocycler. The thermocycler protocol consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 20 s, 53 °C for 20 s and 72°C for 45 s. PCR products were then separated on 3% agarose gels, stained with ethidium bromide and photographed under UV light.

Genetic variation for *ITS* and *Glu-5′* was assessed for larvae sampled in July and August 1996, for juveniles at the BP site in 1996 and 1997 and for adults from all four sites in 1995. For the remaining samples only the *Glu-5′* marker was used to reduce costs, since the *Glu-5′* marker is adequate for classifying individuals into species and hybrids for the analysis of variation in *Mytilus* spp. population genetic structure (Hilbish et al. 2002; Gilg and Hilbish 2003a, 2003b). Although classification based on one or a few markers could be misleading if introgression were extensive, it appears that hybridization in the *M. edulis–M. trossulus* hybrid zone is sufficiently limited that classification based on these diagnostic markers is adequate for comparing groups identified as different species and hybrids (Bates and Innes 1995; Saavedra et al. 1996; Comesaña et al. 1999; Rawson et al. 2001). The nomenclature used in the present study therefore identifies *M. edulis*, *M. trossulus* and hybrids based on the genetic markers but with the caveat that any introgression will reduce the distinction of phenotypic characters among the designated groups.

Statistical analysis

Variation in relative frequency of *M. edulis*, *M. trossulus* and individuals of mixed genotype (hybrids) was tested with R×C *G*-tests (Sokal and Rohlf 1981) or R×C randomization chi-squared tests with the MONTE program (McElroy et al. 1991), based on the algorithm of Roff and Bentzen (1989). Tests of fit to Hardy–Weinberg equilibrium (HWE) for individual loci and tests for linkage disequilibrium between two loci were carried out with GENEPOP (Raymond and Rousset 1995) using a web-based version of the program (http://wbiomed.curtin.edu.au/genepop/). The Analyse program (http://helios.bto.ed.ac.uk/evolgen/Mac/Analyse/index.html) developed by N.H. Barton and S.J.E. Baird was used to estimate the inbreeding coefficient (F_{is}) and the standardized linkage disequilibrium (R) using maximum-likelihood methods. Variation in mussel size was analyzed with ANOVA and Tukey's multiple comparison using the statistics program "R" (Ihaka and Gentleman 1996). Whenever multiple samples involving different sites, years, or months were tested, a sequential Bonferroni procedure (Rice 1989) was used to correct multiple tests for type-I error based on a critical probability value of 0.05.

Results

Larvae

Based on the *Glu-5′* marker, the relative frequency of the *Mytilus edulis*, *M. trossulus* and hybrid larvae (Fig. 1) showed no significant variation among the four sites for five of the six sample dates (19 July 1996 $X^2=12.33$, $df=6$, $P=0.05$; 9 August 1996 $X^2=3.67$, $df=6$, $P=0.726$; 19 September 1996 $X^2=15.84$, $df=6$, $P=0.016$; 2 September 1997 $X^2=12.97$, $df=6$, $P=0.041$; 1 August 1998 $X^2=2.14$, $df=6$, $P=0.909$). Only the 3 September 1996 ($X^2=26.15$, $df=6$,

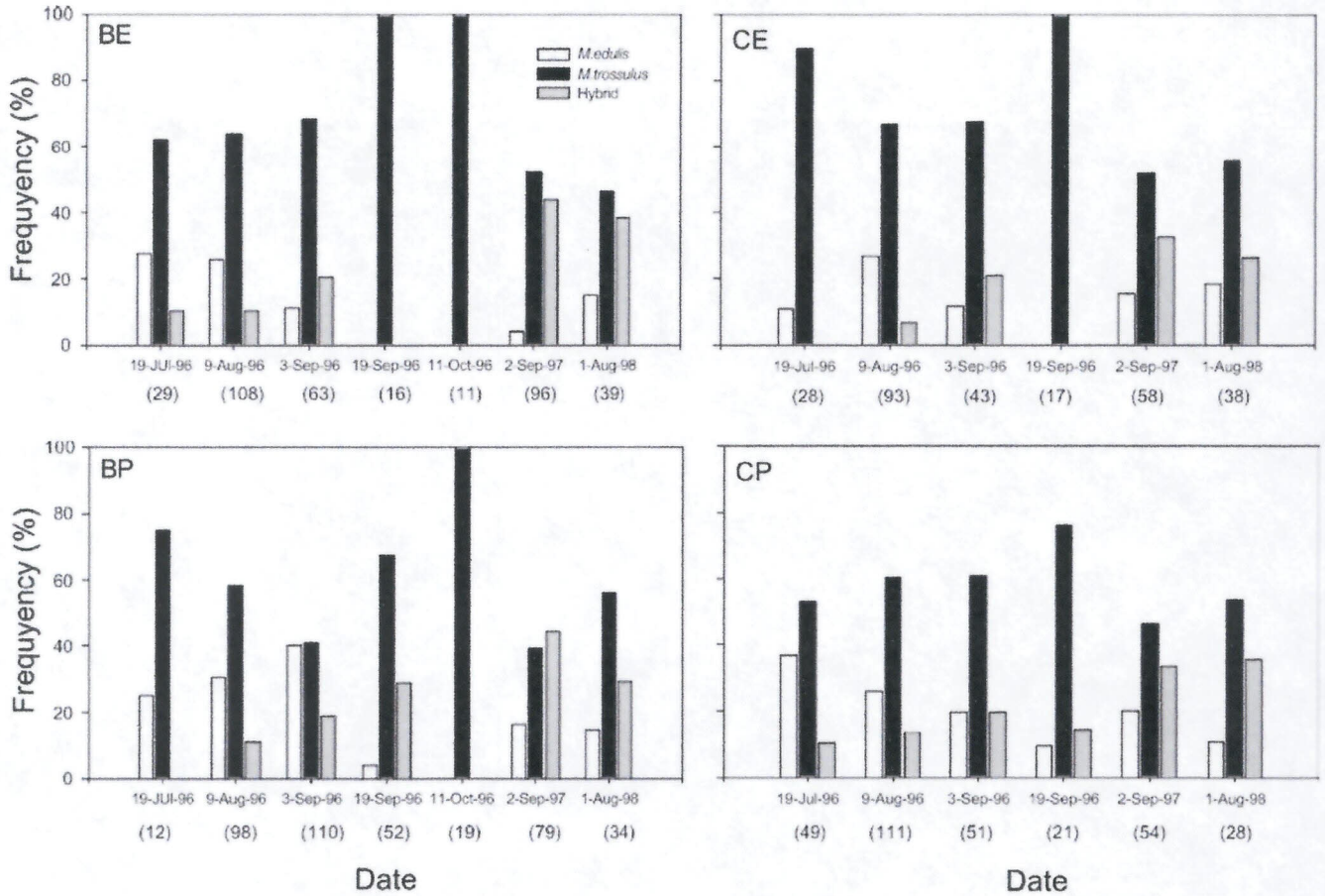

Fig. 1 *Mytilus* spp. Relative frequency (%) of *M. edulis*, *M. trossulus* and hybrid larvae (based on the *Glu-5′* marker) for samples from four sites (*BE*, *BP* Bellevue exposed and protected; *CE*, *CP* Chance Cove exposed and protected) in Trinity Bay, Newfoundland. The CE and CP sites were not sampled on 11 October 1996. Sample size for each date *in parentheses*

$P=0.0001$) sample showed a significantly different relative frequency of the three taxa among the sites following the Bonferroni procedure. The BP site differed from the other three sites on this date due to a lower frequency of *M. trossulus* and a higher frequency of *M. edulis* larvae (Fig. 1). There was, however, significant variation in relative frequency of the three taxa among sample dates within all four sites (BE $X^2=72.01$, $df=12$, $P<0.0001$; BP $X^2=78.36$, $df=12$, $P<0.0001$; CE $X^2=43.02$, $df=10$, $P<0.0001$; CP $X^2=24.89$, $df=10$, $P=0.006$), a similar temporal pattern of variation being observed in each (Fig. 1). *M. trossulus* larvae occurred with the highest frequency in all samples, and only *M. trossulus* was detected in the late season 1996 samples, except for the CP site, where larvae of both species and hybrids were also present. Although *M. edulis* larvae showed a greater frequency than hybrids in July and August 1996, hybrids showed a greater frequency in September 1997 and August 1998 (Fig. 1).

Twenty-two samples of larvae were tested for fit to HWE for the *Glu-5′* marker data given in Fig. 1 (samples containing only *M. trossulus* were excluded). For 11 of the samples the deviation from HWE was significant ($P<0.05$), with a deficiency of heterozygotes (hybrids). Samples from all four sites in July and August 1996 also showed a highly significant ($P<0.001$) deficiency of heterozygotes (hybrids) for the *ITS* marker, and there was highly significant linkage disequilibrium between the two markers in all samples ($P<0.0001$).

There were no significant differences in the frequency of the nine two-locus genotypes between the July and August 1996 samples at each of the four sites (BE $X^2=3.12$, $df=7$, $P>0.05$; BP $X^2=5.56$, $df=8$, $P>0.05$; CE $X^2=12.11$, $df=6$, $P>0.05$; CP $X^2=16.42$, $df=8$, $P=0.03$); therefore, data from the two months were pooled. There was a significant difference in the frequencies of the nine two-locus genotypes among the four sites ($X^2=46.94$, $df=24$, $P=0.003$), but the sites all showed the same general pattern (Table 1). *M. trossulus* larvae occurred with greatest frequency, followed by *M. edulis*, and the seven classes of mixed genotypes were least frequent. Mixed genotypes occurred with low frequency, particularly those consisting of two alleles from one species and two from the other (e/e t/t, t/t e/e). Individuals heterozygous at both loci (F_1-like: e/t e/t) occurred with the lowest frequency (Table 1), and there was a highly significant deviation ($G=46.21$, $df=6$, $P<0.0001$) from equal frequency among the seven mixed genotypes. However, when the F_1-like class was removed, no significant difference ($G=8.29$, $df=5$,

Table 1 *Mytilus* spp. Number of larvae for each of nine two-locus genotypes (*ITS, Glu-5′*) sampled from four Trinity Bay sites (abbreviations see Fig. 1) in 1996. Mean shell length for largest larvae selected from July and August samples. Genotype classes with different *superscripts* (1, 2) have a significantly different mean length within each month based on a multiple comparisons analysis

Genotype		Site						Mean length, μm (SE)	
ITS	*Glu-5′*	BE	BP	CE	CP	Total	Percent	July	August
e/e	e/e	19	21	22	36	98	18.6	139.6 (2.92)	237.4 (1.99)
e/e	e/t	10	3	5	8	26	4.9	150.0 (10.00)	249.6 (3.47)[1]
e/t	e/e	12	8	6	9	35	6.6	134.0 (9.27)	244.0 (2.90)
e/e	t/t	2	5	7	5	19	3.6	140.0 (4.08)	241.3 (5.84)
e/t	e/t	0	2	0	1	3	0.6	140.0 (–)	230.0 (10.00)
t/t	e/e	5	4	0	2	11	2.1	126.7 (3.33)	226.3 (7.78)
e/t	t/t	4	4	4	18	30	5.7	144.0 (8.72)	249.6 (3.29)[1]
t/t	e/t	4	6	1	11	22	4.2	147.5 (4.79)	251.1 (4.84)[1]
t/t	t/t	81	57	76	70	284	53.8	130.4 (1.62)	232.6 (1.41)[2]
Total		137	110	121	160	528			

$P > 0.05$) from equal frequency was detected among the remaining six mixed genotype classes.

Larvae sampled in July were smaller than those sampled in August (Table 1). Although there was significant variation overall ($F = 2.52$, $df = 8, 109$, $P < 0.05$) in larval size among the nine genotype classes in July, a multiple comparisons analysis (Tukey) among all pairs of genotypes did not detect any significant differences. There was also significant ($F = 6.06$, $df = 8, 401$, $P < 0.001$) variation in larval length among the nine genotypic classes in August. A multiple comparisons analysis showed significant differences in shell length only between the smaller t/t t/t genotype and the larger e/e e/t, e/t t/t and t/t e/t genotypes.

Juveniles

The relative frequency of the two species and hybrids (based on the *Glu-5′* marker) showed no significant heterogeneity among seven length classes (2–15 mm SL) for each of three years (1995, 1996, 1997), except for the BP site (1995 $X^2 = 45.09$, $df = 12$, $P < 0.0001$; 1996 $X^2 = 45.00$, $df = 12$, $P < 0.0001$; 1997 $X^2 = 60.00$, $df = 12$, $P < 0.0001$) and the 1995 CP sample ($X^2 = 48.99$, $df = 12$, $P < 0.0001$). In general, *M. trossulus* juveniles were much more frequent than *M. edulis* or hybrid juveniles and for most size classes of juveniles at all of the sites averaged over the three years (Fig. 2). However, samples from the BP and CP sites showed a more complicated pattern, with a decline in relative frequency for *M. trossulus* and an increase in *M. edulis* with increasing size class (Fig. 2).

All 12 samples of juveniles (3 years and 4 sites) showed significant ($P < 0.001$) deviation from HWE, with a deficiency of heterozygotes (hybrids) for the *Glu-5′* marker. Samples from 1996 and 1997 from the BP site also showed a highly significant ($P < 0.001$) deficiency of heterozygotes for *ITS*, and there was a highly significant linkage disequilibrium ($P < 0.001$) between the two loci in both years.

No difference in relative frequency of the nine two-locus genotypes was observed between 1996 and 1997 ($G = 5.29$, $df = 8$, $P > 0.05$), and the two years were therefore pooled (Table 2). As in the larvae, mixed genotypes were much less frequent than pure *M. trossulus* or *M. edulis* genotypes, and F_1-like individuals were rare (Table 2). There was a marginally significant difference ($G = 12.98$, $df = 6$, $P = 0.043$) from equal frequency among the seven mixed genotype classes, and no significant difference ($G = 5.78$, $df = 5$, $P > 0.05$) from equal frequency among the six mixed genotypes after the F_1-like class was removed.

There was significant variation ($F = 7.37$, $df = 8, 321$, $P < 0.001$) in shell length among the nine genotype classes (Table 2). A multiple comparisons analysis revealed that the only significant comparison was that the e/e e/e genotype class was larger than the t/t t/t genotype class.

Adults

The change in frequency of *M. edulis*, *M. trossulus* and hybrids (based on the *Glu-5′* marker) with increasing size (averaged over 1995, 1996, 1997) showed a similar pattern at the four sites (Fig. 2). There was a general decline in frequency of *M. trossulus* and an increase in frequency of *M. edulis* with increasing size. Hybrids showed a low frequency in all size classes at all sites and were absent from the larger size classes. The fit to HWE for the *Glu-5′* marker was tested for the smallest adult size class (15–20 mm SL) for each of the samples from the four sites in each year (1995, 1996, 1997). Only two (CP 1995, CP 1997) of the 12 tests showed a significant deviation from HWE with a deficiency of heterozygotes (hybrids). Tests of the *ITS* locus for fit to HWE for the 15–25 mm size class (1995 data) showed a significant ($P < 0.001$) deficiency of heterozygotes for three (BE, BP, CE) of the four sites. There was also highly significant ($P < 0.001$) linkage disequilibrium between the two loci.

Of the five size classes of adult mussels (Table 3), only the 15–25 mm size class showed a significant difference ($X^2 = 51.6$, $df = 24$, $P < 0.001$) in relative frequency of the nine two-locus genotypes among the four sites. For this size class the sites differed in relative frequency of

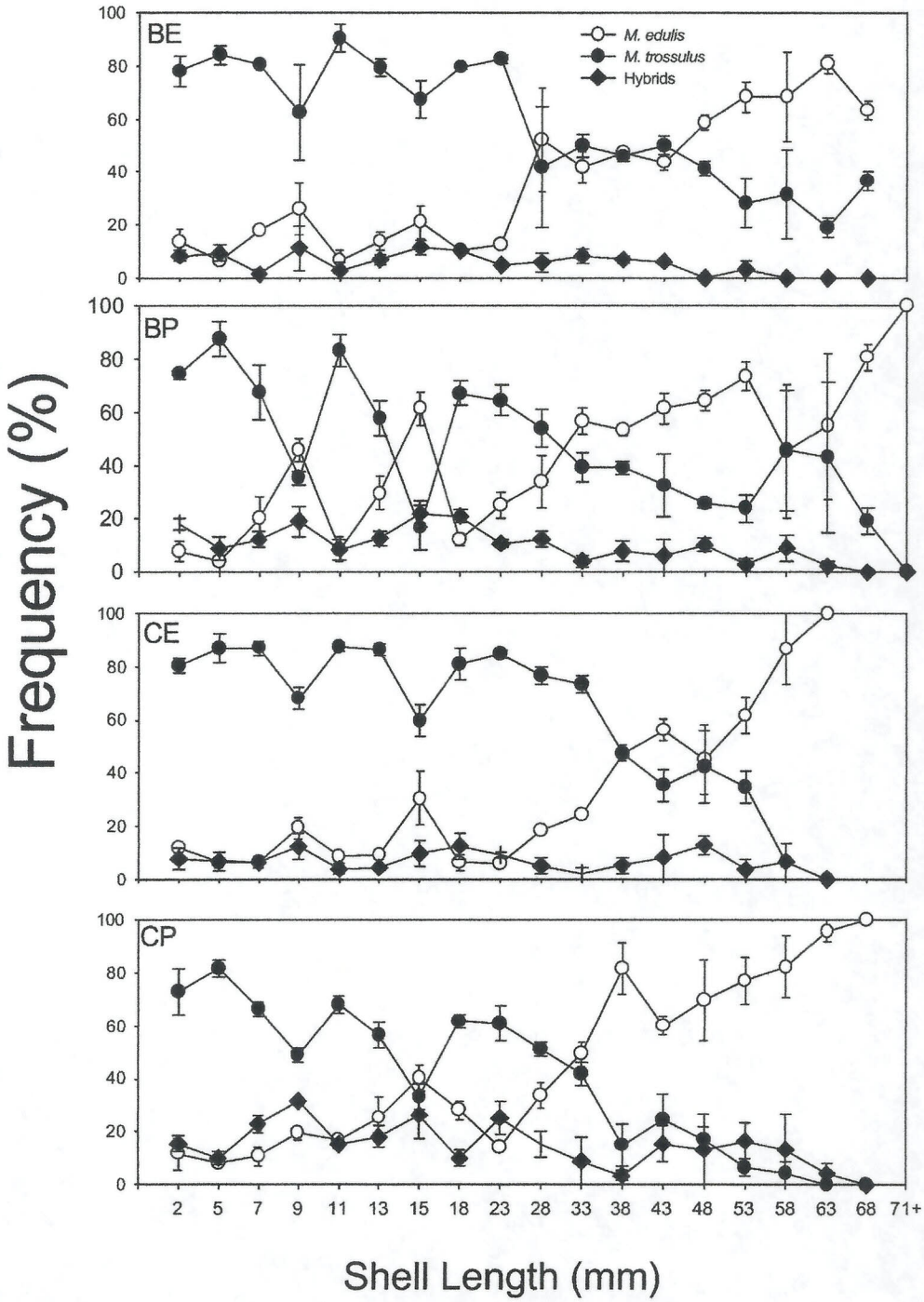

Fig. 2 *Mytilus* spp. Mean (±SE) relative frequency (%) (average of 1995, 1996, 1997 samples) of *M. edulis*, *M. trossulus* and hybrids (based on the *Glu-5′* marker) in different size classes for samples from four sites (abbreviations see Fig. 1) in Trinity Bay, Newfoundland. Juvenile size classes are 2–15 mm SL (2-mm size class intervals), and adult size classes are 18–71+ mm SL (5-mm size class intervals). Average sample size for each year: BE (377), BP (406), CE (358), CP (406)

M. trossulus, but no significant difference ($X^2=22.06$, $df=21$, $P=0.23$) was found among the sites when the *M. trossulus* genotype was removed. Pooling among the four sites, the nine two-locus genotype frequencies showed significant variation ($X^2=155.1$, $df=32$, $P<0.001$) among the five size classes (Table 3). The smallest size class was dominated by *M. trossulus*, and the largest by *M. edulis*. Individuals of mixed genotype occurred at low frequency in all size classes, the F_1-like hybrid occurring with the lowest frequency (Table 3).

Comparison among life-history stages

There was significant heterogeneity ($X^2=277.8$, $df=54$, $P<0.0001$) in the frequency of the nine genotype classes among larvae, juveniles and the five adult size classes. Significant heterogeneity ($X^2=67.9$, $df=36$, $P=0.0014$) was also detected for the frequency of the seven mixed genotype classes among larvae, juveniles and the five adult size classes. However, a comparison of relative frequency of the nine genotypes and the seven mixed

Table 2 *Mytilus* spp. Number of juveniles and mean shell length for nine two-locus genotypes (*ITS*, *Glu-5′*) at the BP site sampled in two years (September, October, December 1996; September, December 1997). Genotype classes with different superscripts (1, 2) have a significantly different mean length based on a multiple comparisons analysis

Genotype		1996	1997	Total	Percent	Mean length (mm)	SE
ITS	*Glu-5′*						
e/e	e/e	21	38	59	17.9	10.3[1]	0.41
e/e	e/t	6	5	11	3.3	9.5	0.90
e/t	e/e	5	5	10	3.0	10.0	1.09
e/e	t/t	5	4	9	2.7	9.5	1.25
e/t	e/t	1	2	3	0.9	10.8	2.22
t/t	e/e	3	4	7	2.1	10.3	0.96
e/t	t/t	10	8	18	5.5	8.8	0.88
t/t	e/t	5	6	11	3.3	9.3	0.91
t/t	t/t	74	128	202	61.2	7.0[2]	0.25
Total		130	200	330			

Table 3 *Mytilis* spp. Number of adults occurring in five shell-length classes for nine two-locus genotypes (*ITS*, *Glu-5′*) pooled from four Trinity Bay sites in October 1995

Genotype		Shell length (mm)					Total	Percent
ITS	*Glu-5′*	15–25	26–35	36–45	46–55	56+		
e/e	e/e	12	34	55	49	52	202	37.2
e/e	e/t	3	4	11	11	6	35	6.5
e/t	e/e	7	8	5	1	1	22	4.0
e/e	t/t	4	5	2	2	1	14	2.6
e/t	e/t	4	0	1	2	0	7	1.3
t/t	e/e	6	5	5	4	0	20	3.7
e/t	t/t	11	5	3	1	0	20	3.7
t/t	e/t	13	10	1	3	1	28	5.2
t/t	t/t	83	47	34	19	12	195	35.9
Total		143	118	117	92	73	543	

Table 4 *Mytilus* spp. Comparison between successive life-history stages for differences in the relative frequency of nine two-locus (*Glu-5′*–ITS) genotypes and seven mixed (hybrid) genotypes. $*P<0.05$

Life-history comparison	Nine two-locus genotypes (X^2,$df=8$)	Seven mixed genotypes (X^2,$df=6$)
Larvae vs. Juveniles	9.4	4.0
Juveniles vs. 15–25 mm	18.9	5.5
15–25 mm vs. 26–35 mm	25.4*	5.7
26–35 mm vs. 36–45 mm	21.2*	7.7
36–45 mm vs. 46–55 mm	6.8	4.8
46–55 mm vs. 56+ mm	9.4	3.8

genotypes between successive life-history stages showed that only two comparisons between intermediate size classes (15–25 vs. 26–35 mm and 26–35 vs. 36–45 mm) were significantly different (Table 4). The relative frequency of the seven mixed genotype classes as a pooled group of hybrids showed no significant variation ($X^2=11.15$, $df=5$, $P=0.05$) among larvae, juveniles, or the first four adult size classes and only significant variation ($X^2=21.96$, $df=6$, $P=0.0016$) when the >56 mm size class was included (Fig. 3A).

Estimates of the overall inbreeding coefficients (F_{is}), based on *ITS* and *Glu-5′*, were significantly different from zero for each life-history stage (Fig. 3B). There was significant heterogeneity (log-likelihood = 14.18, $df=6$, $P=0.013$) among the life-history stages for the estimated inbreeding coefficients due to a decrease observed in F_{is} for the 15–25 mm size class. In addition, linkage disequilibrium, as measured by the standard disequilibrium coefficient R, was also significantly different from zero for the life-history stages (Fig. 3B) and was homogeneous among the different life-history stages (log-likelihood = 5.98, $df=6$, $P=0.425$).

Discussion

The present study extends information on the genetic composition of mussels in the adult stages reported in a previous study (Comesaña et al. 1999) to earlier life-history stages (pre-settlement larvae and juveniles) at the same sites in Trinity Bay, Newfoundland. The high relative frequency of *Mytilus trossulus* and lower frequency of *M. edulis* and hybrids observed in small adult mussels (15–20 mm SL) by Comesaña et al. (1999) was also found for the same size class over three consecutive years (1995–1997) in the present study, and was also recorded for the late-larval stage and juveniles at the same sites. *M. trossulus* larvae were much more frequent than *M. edulis* and hybrid larvae in all samples, and *M. trossulus* was the only species observed in four samples of larvae taken later in the year (September, October). The high relative frequency of *M. trossulus* larvae may be related to differences between the species in population density, reproductive output and spawning time. For example, at many sites

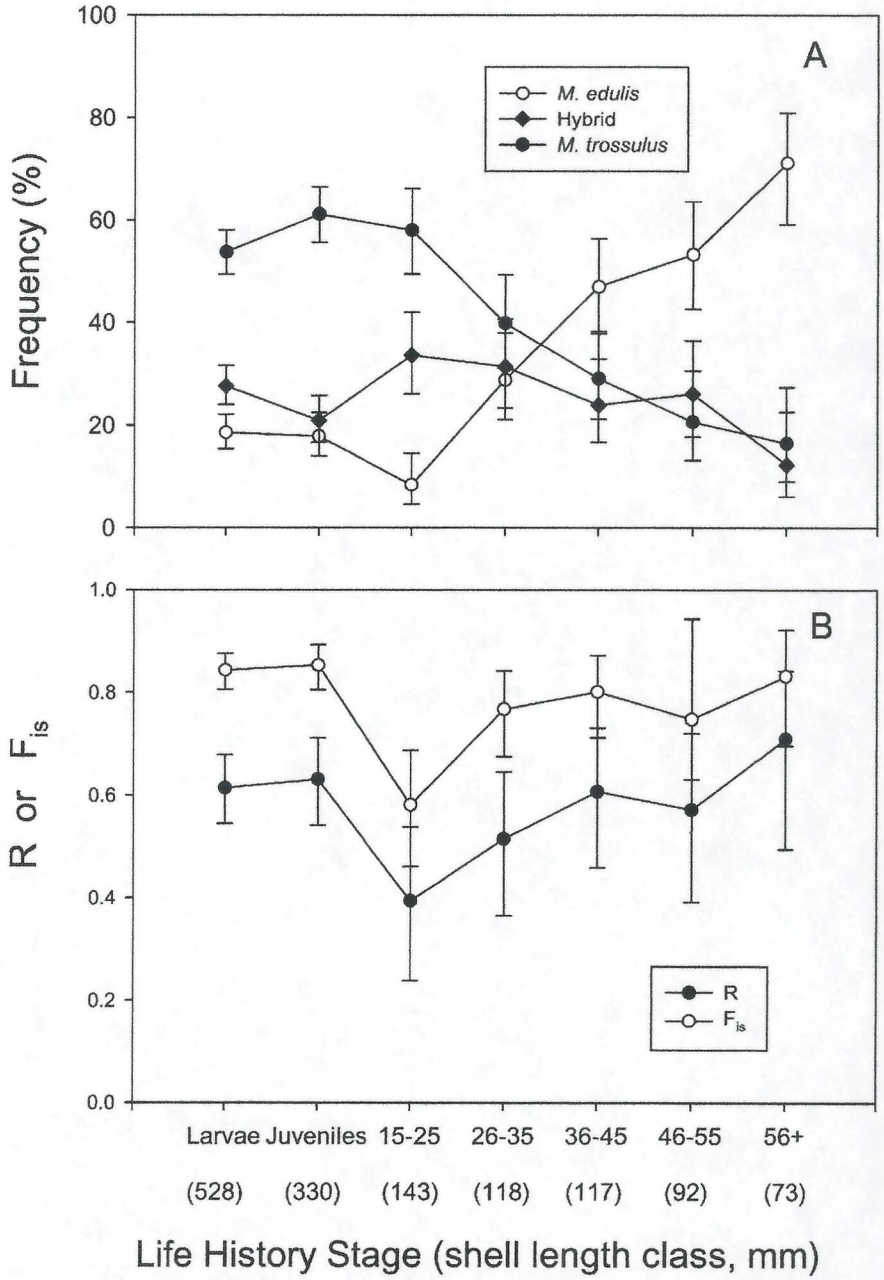

Fig. 3A, B *Mytilus* spp. **A** Frequency (*error bars*: 95% confidence intervals) of *M. edulis*, *M. trossulus* and hybrids for larvae, juvenile and five adult classes of shell length based on two genetic markers (*ITS*, *Glu-5′*). **B** Linkage disequilibrium (*R*) and inbreeding coefficient (F_{is}) (*error bars*: two-unit support limits). Sample size *in parentheses*

M. trossulus dominates the smaller adult size classes (15–30 mm SL) (Comesaña et al. 1999; M. Miranda, unpublished data), which occur at higher density than the larger size classes (Bates 1992). The high density of *M. trossulus*, combined with a greater investment in reproduction than *M. edulis* for individuals of the same size (Toro et al. 2002), probably results in a larger number of *M. trossulus* larvae being produced. The occurrence of only *M. trossulus* larvae in some samples late in the year may be a consequence of the later spawning of *M. trossulus* compared with *M. edulis* at these Trinity Bay sites (Toro et al. 2002). There was a tendency for *M. trossulus* larvae and juveniles to have a smaller shell length than *M. edulis* and hybrids. Although differences in shell length may be due to differences in growth rate, the observed later spawning of *M. trossulus* (Toro et al. 2002) could also explain the smaller size of the larvae.

Juveniles ranging from <2 to 15 mm SL were also predominantly composed of *M. trossulus*, although this pattern differed at two sites such that *M. trossulus* juveniles were less frequent and *M. edulis* more frequent in the larger juvenile size classes. Despite this site difference, there was a consistent pattern in which *M. trossulus* occurred with a high relative frequency and *M. edulis* and hybrid genotypes occurred with much lower frequency among the pre-settlement larvae, juveniles and the 15–30 mm SL adult size class.

Hybrids were uncommon in the present study and, according to the single *Glu-5′* marker, showed little change in relative frequency among the larvae, juveniles and the various size classes of adult mussels (> 15 mm SL). Two markers (*ITS*, *Glu-5′*) identified more hybrid individuals, but the seven hybrid genotype classes thus obtained also showed little change in relative frequency among the larvae, juveniles and the different size classes of juvenile and adult mussels. Furthermore, hybrids for both *ITS* and *Glu-5′* occurred with the lowest frequency for all life-history stages, which is consistent with our previous observations on mussels > 15 mm SL (Comesaña et al. 1999). Although hybrids for both markers may not necessarily be true F_1 hybrids, Comesaña et al. (1999) observed that all F_1-like genotypes, identified using four genetic markers, also had the mtDNA genotype expected for F_1 hybrids. If some of the mussels classified as F_1-like in the present study are actually other variants of mixed genotypes (F_2, etc.), this would support the hypothesis that F_1 hybrids are rare in the *M. edulis–M. trossulus* hybrid zone, as was found by Saavedra et al. (1996) and Comesaña et al. (1999). Hybrid individuals in the larger size classes (> 36 mm) were generally biased towards *M. edulis*.

No change in relative frequency was observed for the hybrid genotypes among the different size classes. This contrasts with the two species, which showed very large shifts in relative frequency with increasing size, as documented previously (Comesaña et al. 1999). The observed pattern of *M. trossulus* declining and *M. edulis* increasing in relative frequency with increasing size class could be due to a greater mortality rate in *M. trossulus* than in *M. edulis*. *M. trossulus* appears to have a thinner shell than *M. edulis*, a weaker adductor muscle and lower byssus production (J. Lowen and M. Miranda, unpublished observations). These differences suggest that *M. trossulus* is more susceptible to predation and has compensated for an increased probability of mortality by increasing investment in reproduction relative to *M. edulis* (Toro et al. 2002). The hypothesis that *M. trossulus* is more susceptible to predation than *M. edulis* is currently being tested.

It cannot be assumed that larvae and juveniles sampled at a particular site were produced by adult mussels at the same site. Based on laboratory studies (Bierne et al 2002a), mussel larvae cultured at 18°C settle after approximately 35 days and therefore have the potential for long-distance dispersal in the plankton. The genetic composition of larvae and juveniles reflects the genetic composition of the source population and may include mixtures of larvae from several different populations (Pedersen et al. 2000). However, recruits may be derived from the local population if the pattern of ocean currents limits dispersal (Gilg and Hilbish 2003a). McQuaid and Phillips (2000) observed that most recruitment of the invasive *M. galloprovincialis* in South African waters occurred within < 5 km of the parent population. It is not known whether local recruitment occurs at the Trinity Bay sites sampled in the present study. The sites lie at the head of a large enclosed bay, so some self-recruitment may be possible. A study of oceanographic conditions in Trinity Bay suggested that weak gyres might retain certain larvae within the bay (Dalley et al. 2002). Regardless of the source population, the genetic composition of larvae and juveniles sampled in three consecutive years consistently showed a high frequency of *M. trossulus* at all four sites sampled. Although there is some variation in the relative frequency of the two species at different sites along the coast of Newfoundland, most sites contain a similar mixture of both species (Bates and Innes 1995; Innes et al. 1999; Penney and Hart 1999). Therefore, the species and hybrid composition of the source population for recruiting larvae and juveniles is not likely to differ very much from that of adult mussels from the Trinity Bay sites. Pedersen et al. (2000) also found that *M. trossulus* dominated newly settled mussels at a site in Nova Scotia. They observed some heterogeneity in the genetic composition of recruits that could be attributed to variation in the relative proportion of the two species. The high frequency of *M. trossulus* in the recruits was probably attributable to the greater frequency of this species in the adult populations in the area (Pedersen et al. 2000).

Our observations of high levels of linkage disequilibrium and a high inbreeding coefficient (heterozygote deficiency) for the genetic markers confirm previous studies showing that *M. edulis* and *M. trossulus* are partially reproductively isolated, with the occurrence of some hybrids in natural populations (Saavedra et al. 1996; Comesaña et al. 1999). A low frequency of hybrids may result from prezygotic factors such as spatial separation of the two species, spawning asynchrony, gamete incompatibility and gamete choice, or postzygotic factors such as genetic incompatibility and mortality at different developmental stages (Palumbi 1994). The relative importance of these factors for reproductive isolation between mussel species has yet to be determined. Metamorphosis from the swimming larva to the settled juveniles represents a major developmental transition in mussels during which any postzygotic genetic incompatibilities in hybrid individuals may be expressed. However, in the present study, no difference in the frequency of hybrids was detected between these two life-history stages for individuals collected from natural populations. Furthermore, laboratory crosses between the two species have produced large numbers of viable F_1 hybrid larvae, juveniles and adults, with little evidence for extensive genetic incompatibility (M. Miranda, unpublished data). A similar low frequency of hybrids observed among larvae and juveniles in nature suggests that factors limiting the production of hybrids operate prior to the late larval stage. Although a degree of spawning asynchrony has been observed between *M. trossulus* and *M. edulis* at the Trinity Bay sites (Toro et al. 2002), it is not clear how much this asynchrony prevents hybrid production, because there is sufficient overlap in the spawning to permit the potential production of some hybrids.

Gamete incompatibility can also be an effective prezygotic reproductive isolating mechanism for coexisting marine invertebrate species that release their gametes into the water column (Levitan 2002). Laboratory fertilization experiments have demonstrated incompatibility between *M. edulis* and *M. trossulus* at the gamete stage (Rawson et al. 2003; M. Miranda, unpublished data), interspecific fertilizations being less frequent than intraspecific fertilizations (Rawson et al. 2003). Furthermore, not all interspecific matings showed the same low fertilization success, and there was evidence for asymmetry in interspecific fertilization success. Fertilization of *M. edulis* eggs by *M. trossulus* sperm was more successful than fertilization of *M. trossulus* eggs by *M. edulis* sperm (Rawson et al. 2003). Nevertheless, the degree of gamete incompatibility is probably sufficient to account for the low frequency of hybrids observed in natural populations of *M. edulis* and *M. trossulus*. Further genetic incompatibility during early embryo and larval development may also contribute to a reduction in the frequency of hybrids (M. Miranda, unpublished data). Our observation of a similar frequency of hybrids in natural populations among the late larval stage and the different adult size classes suggests that hybrids may have a rate of survival intermediate between the two parental species. However, these observations are restricted to samples of the life-history stages and size classes over a short time period (static cohort analysis) rather than following a single cohort (dynamic cohort analysis). Furthermore, changes in relative frequency among genotypes can be explained by a number of possible mortality rates for each genotype. Determination of the survival of a cohort of the two species and hybrids reared in the laboratory as well as deployed to different natural sites is currently in progress (M. Miranda, unpublished data).

The observed higher frequency of mixed hybrid genotypes but lower frequency of F_1-like hybrids suggests that backcross mating between F_1 individuals and one or the other parent species may result in a lower level of gamete and genetic incompatibility. However, the low relative frequency of F_1-like hybrids and higher frequency of other mixed hybrid genotypes could also be explained by spatial and/or temporal segregation of spawning between the two species combined with genetic incompatibility during early development. Subsequent spawning by a small number of F_1 hybrids will generate many more backcross genotypes, because each F_1 individual will release large numbers of gametes that are most likely to encounter gametes from one or the other parent species. Thus, several factors may interact to limit the formation of F_1 hybrids, but once a few viable and fertile F_1 hybrids are produced they can generate more mixed hybrid genotypes. The absence or low frequency of F_1-like individuals in the presence of other hybrid genotypes at higher frequency is commonly seen in hybrid zones (Arnold 1997).

M. edulis and *M. galloprovincialis* hybridize in Europe, and gamete recognition rather than gamete incompatibility may be the important factor limiting this hybridization (Bierne et al. 2002a). Bierne et al. (2002a) carried out fertilization experiments involving mixtures of gametes from the two species, which allowed gamete competition. Mixtures of embryos derived from within and between species crosses were used as controls to test for gamete and genetic incompatibility. A significant deficiency of hybrids was observed only when gamete competition was allowed. The authors concluded that no strong barriers to fertilization exist between the two species, but when gametes of both species are present intra-specific fertilizations occur more frequently than inter-specific fertilizations. There was also some evidence for differential viability among genotypes during the larval and juvenile stages, but the results were equivocal with respect to hybrid viability. Similarly, observations by Beaumont et al. (1993) suggest that the role of post-fertilization incompatibility in the maintenance of the *M. edulis–M. galloprovincialis* hybrid zone requires further study.

A comprehensive understanding of the dynamics and fate of mussel hybrid zones requires information on life-history differences between coexisting species as well as environmental and genetic factors that can explain the observed frequency of hybrids. The structure of the *M. edulis–M. trossulus* and the *M. edulis–M. galloprovincialis* hybrid zones appears to involve two distinct but related processes: those responsible for the production and persistence of hybrids without a merging of the two species and those maintaining the coexistence of two ecologically similar species. *Mytilus* species have a similar habitat, with individuals attached to the substrate, removing food particles from the water column and producing planktonic larvae. We would expect coexisting species to compete for space and food, assuming both factors are limiting. Opportunities for niche diversification to reduce inter-specific competition may include spatial separation through habitat specialization. The *M. edulis–M. galloprovincialis* hybrid zone has been studied more extensively and for a longer period than the *M. edulis–M. trossulus* hybrid zone (Gardner 1994). *M. edulis* and *M. galloprovincialis* differ in habitat distribution as a result of variation in wave exposure and salinity (Bierne et al. 2002b, 2003a). *M. galloprovincialis* has a stronger byssal attachment, and is thus better adapted to habitats with greater wave energy (Gardner and Skibinski 1991; Willis and Skibinski 1992). The consequence is that spatial separation and niche diversification may explain the coexistence of the two species in the same area. The *M. edulis–M. trossulus* hybrid zone exhibits less geographic structure than the *M. edulis–M. galloprovincialis* hybrid zone, and no environmental factors have been identified that favor one species or the other. Similar frequencies of *M. edulis*, *M. trossulus* and hybrids were found at exposed and sheltered sites in Trinity Bay. However, mussels were sampled subtidally, and the habitat differences may not have been great enough to reveal any selective effect of wave exposure. Future sampling should include intertidal sites varying

in wave exposure to test for habitat segregation by *M. edulis* and *M. trossulus*. Life-history differences between *M. edulis* and *M. trossulus*, such as differences in age-specific survival and reproduction, may also explain their coexistence in the absence of any differences in adaptation to specific environmental conditions.

The limited occurrence of hybrids observed in natural populations suggests that *M. edulis* and *M. trossulus* are partially reproductively isolated and that both pre- and postzygotic mechanisms operate. The low frequency of F_1 hybrids observed in natural populations may be due to a combination of spawning asynchrony, gamete incompatibility and genetic incompatibility during early development. Further studies are in progress to investigate the environmental, genetic and life-history factors involved in maintaining the *M. edulis–M. trossulus* hybrid zone.

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Reproductive isolation and reproductive output in two sympatric mussel species (*Mytilus edulis*, *M. trossulus*) and their hybrids from Newfoundland

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Abstract The mussels *Mytilus edulis* L. and *M. trossulus* Gould are found sympatrically in most areas of Newfoundland, with a low frequency of hybrids. To assess the potential for reproductive isolation, we sampled mussels from three sites in an eastern Newfoundland Bay from May–October 1996 to determine if there were differences in the reproductive cycles of the two species and their natural hybrids. In mussels with shell lengths of 38–42 mm, males and females with mature gametes were dominant in June for *M. edulis* and hybrids, while *M. trossulus* showed a lower frequency of individuals with mature gametes. *M. trossulus* and hybrids spawned over a prolonged period (from late spring to early autumn) compared with most *M. edulis* individuals that spawned over a period of 2–3 weeks in July. This asynchrony in spawning activity between the two species may partially explain the low frequency of hybrids found in previous studies of these mussel populations. Female and male hybrids between *M. edulis* and *M. trossulus* showed normal gonad development, ripening and spawning, providing an opportunity for the introgression of genes between the two species. *M. trossulus* had a higher reproductive output than *M. edulis* of similar shell length, while hybrids showed intermediate values of reproductive output. *M. trossulus* females produced smaller eggs than either *M. edulis* or hybrids. Differences in reproductive traits may partially explain the maintenance of the mussel hybrid zone in Newfoundland.

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Introduction

Hybrid zones occur where genetically distinct populations of individuals come into contact, mate, and produce offspring of mixed ancestry. The presence of naturally produced hybrids shows that reproductive isolating mechanisms are not sufficient to prevent hybridization. If hybrids are fertile, the potential exists for introgressive gene flow when hybrids backcross to one or both parental taxa. A major question in evolutionary biology is whether introgression can be an important source of new genetic variation leading to novel genotypes and adaptive evolution (Barton and Hewitt 1989; Harrison 1993; Arnold 1997). Thus it is important to understand factors influencing the generation and fate of hybrids. Depending on the survival and fertility of hybrids, selection may reinforce reproductive isolating mechanisms to maintain distinct taxa or break down barriers to hybridization, resulting in the fusion of taxa (Coyne and Orr 1997; Jiggins and Mallet 2000; Schluter 2001; Turelli et al. 2001). Alternatively, a stable hybrid zone may persist with varying degrees of introgression between the parental taxa (Barton and Hewitt 1989; Arnold and Hodges 1995; Rieseberg 1998).

The degree of reproductive isolation determines the rate of hybridization between closely related species. Reproductive isolation can act pre-zygotically through behavioral mechanisms (mating preference), spatial separation (habitat specialization), temporal separation (asynchronous reproduction), or barriers to fertilization (gamete incompatibility) (Palumbi 1994). Reproductive isolation can also act post-zygotically through reduced viability and fertility of hybrid offspring. Studies on the relative importance of various pre- and post-zygotic reproductive isolating mechanisms can contribute to a

better understanding of the factors responsible for the observed frequency of hybrid individuals in natural populations and the likely evolutionary fate of hybridizing species.

Sedentary invertebrate species that release gametes into the water column increase fertilization success through synchronous spawning of eggs and sperm. Therefore, closely related species may reduce hybridization and become reproductively isolated through asynchronous spawning between species (Palumbi 1994). However, species inhabiting higher latitudes generally have a similar spawning period owing to the strong seasonal occurrence of high water temperature and food availability (McEuan 1988; Gardner and Skibinski 1990; Babcock et al. 1992; Knowlton 1993; Van Veghel 1993). Furthermore, a short reproductive season may limit the role that asynchronous spawning can play in preventing hybridization between closely related species. Some studies have referred to the importance of establishing spawning times for species of hybridizing bivalves to assess the potential for producing hybrids (Ahmad and Beardmore 1976; Skibinski et al. 1980; Seed and Suchanek 1992; Gardner 1994; Eversole 1997; Grant et al. 1998). Although a difference in the spawning period between two species can also reduce the production of hybrids (Babcock et al. 1992; Levitan and Petersen 1995), other factors can reduce hybridization in closely related species, including habitat separation, gametic barriers to hybridization, and post-zygote non-viability (Strathmann 1981; Gardner and Skibinski 1990; Lessios and Cunningham 1990; Uehara et al. 1990; Palumbi and Metz 1991; Gardner 1992; Grant et al. 1998). In many cases the relationship between various reproductive isolating mechanisms and the observed frequency of hybrids is unknown. Despite this potential for reproductive isolation, hybrids are often abundant in populations of some marine invertebrate species. For example, sympatric populations of the quahogs *Mercenaria mercenaria* and *M. campechiensis* (Dillon and Manzi 1989; Bert et al. 1993) and the sea urchins *Echinus esculentus* and *E. acutus* (Hagström and Lönning 1961) are composed of approximately 31–88% and 10–20% hybrid individuals, respectively. Studies on the genetic structure of the *Mytilus edulis*–*M. galloprovincialis* hybrid zone on the coasts of France (Coustau et al. 1991; Viard et al. 1994) and England (Gardner 1996) have also revealed extensive hybridization.

In earlier studies, Lubet (1957) and Hrs-Brenko (1971) noted that spawning of *M. edulis* and *M. galloprovincialis* in France occurred simultaneously, which, together with a high frequency of morphologically intermediate forms along the Atlantic coast of France (Seed 1972), suggests that hybridization and introgression are common between the two species in this area. Studies in SW England have reported asynchrony in spawning between *M. edulis* and *M. galloprovincialis* at certain sites within the hybrid zone (Skibinski et al. 1980; Skibinski 1983; Gardner and Skibinski 1990; Secor et al. 2001). Gardner and Skibinski (1990) found that the asynchrony was more pronounced at the Croyde site than at the Whitsand site, which may explain the greater degree of hybridization and introgression at Whitsand. However, Secor et al. (2001) used more sensitive measures of reproductive condition for Whitsand mussels and established small but significant differences in the spawning times of the two species.

Both *M. trossulus* and *M. edulis* occur on the east coast of North America (Koehn et al. 1984; Bates and Innes 1995; Mallet and Carver 1995; Saavedra et al. 1996; Comesaña et al. 1999; Penny and Hart 1999; Rawson et al. 2001), rather than *M. edulis* alone, as was previously thought (Seed 1976). *M. edulis* and *M. trossulus* are found sympatrically in Maine, Nova Scotia and Newfoundland. Recent studies in Nova Scotia (Saavedra et al. 1996) and eastern Newfoundland (Comesaña et al. 1999) revealed the presence of mussels of hybrid origin, but the frequency of hybrids appeared to be much lower (26% based on four loci) than that found between *M. edulis* and *M. galloprovincialis* in Europe (up to 80%; summarized in Gardner 1996 and Comesaña et al. 1999). Very few F_1 hybrids were observed between *M. edulis* and *M. trossulus*, with most of the hybrid mussels appearing to result from backcrossing to one species or the other (Saavedra et al. 1996; Comesaña et al. 1999). Although hybrids of *M. edulis* and *M. trossulus* have been observed in nature (Saavedra et al. 1996; Comesaña et al. 1999) and produced in the laboratory (Zouros et al. 1994), the role of post-zygotic mechanisms in limiting hybridization has not been examined empirically. Furthermore, asynchronous reproductive cycles may also decrease the frequency with which hybrids are produced in nature. Several studies have been carried out on the reproductive cycle of *M. edulis* (Thompson 1979, 1984a,b; Newell et al. 1982; Hilbish and Zimmerman 1988) and *M. trossulus* (Suchanek 1981; Emmert et al. 1987; Blanchard and Feder 1997) on the east and west coasts of North America, but there have been no studies comparing the reproductive cycles of *M. edulis* and *M. trossulus* in areas where they coexist.

Explaining the coexistence of the two species of *Mytilus* in a zone of hybridization requires information on the factors that limit hybridization and keep the two species distinct. Coexistence of the two species may involve a combination of asynchronous spawning and post-zygotic isolation to maintain genetically distinct species. Most *Mytilus* spp. populations, like those of many temperate bivalves, exhibit a seasonal pattern of reproduction, which starts with a gametogenic phase, is followed by the release of gametes (spawning) in which the reproductive follicles are partially or completely emptied. Gametogenesis occurs mainly in the mantle tissue, but reproductive tissue can also be found in the visceral mass and mesosoma (Bayne et al. 1978; Lowe et al. 1982; Newell et al. 1982). Apart from a few hermaphrodites, the sexes in *Mytilus* spp. are separate

and most populations contain approximately equal numbers of males and females (Seed 1976; Kautsky 1982; Sprung 1983). However, the occurrence of distinct male and female mtDNA genomes raises the possibility that sex ratios may be different in areas of hybridization (Saavedra et al. 1996, 1997).

The objective of the present study was to compare the reproductive cycles of *M. edulis*, *M. trossulus* and their hybrids in eastern Newfoundland, to determine if the observed low rate of hybridization can be explained in part by asynchronous reproductive cycles and to determine if differences in reproductive output play a role in maintaining their coexistence. In addition, sex ratios were tested for any deviation from unity resulting from hybridization and genetic incompatibility, which may disrupt the gender-associated inheritance of mtDNA.

Materials and methods

Study sites and sampling

The study was carried out at two locations in Trinity Bay, Newfoundland, where approximately 34% *Mytilus edulis*, 40% *M. trossulus* and 26% hybrids were observed by Comesaña et al. (1999). At approximately 15-day intervals from May to October 1996, a representative sample of 400–500 mussels was collected sub-tidally by SCUBA divers from an exposed site at Bellevue (BE) and an exposed and a protected site at Chance Cove (CE, CP, respectively) (see Fig. 1 in Comesaña et al. 1999). Mussels were immediately transported to the laboratory, where they were maintained in running, filtered seawater within 0.5°C of the ambient temperature at Bellevue. During the 3 days following each field sampling, an average of 34 mussels (38–42 mm shell length) from each site (BE, CE and CP) were then dissected carefully to separate the mantle (and in some individuals the mesosoma) from other soft tissues. This narrow size range was chosen to increase the

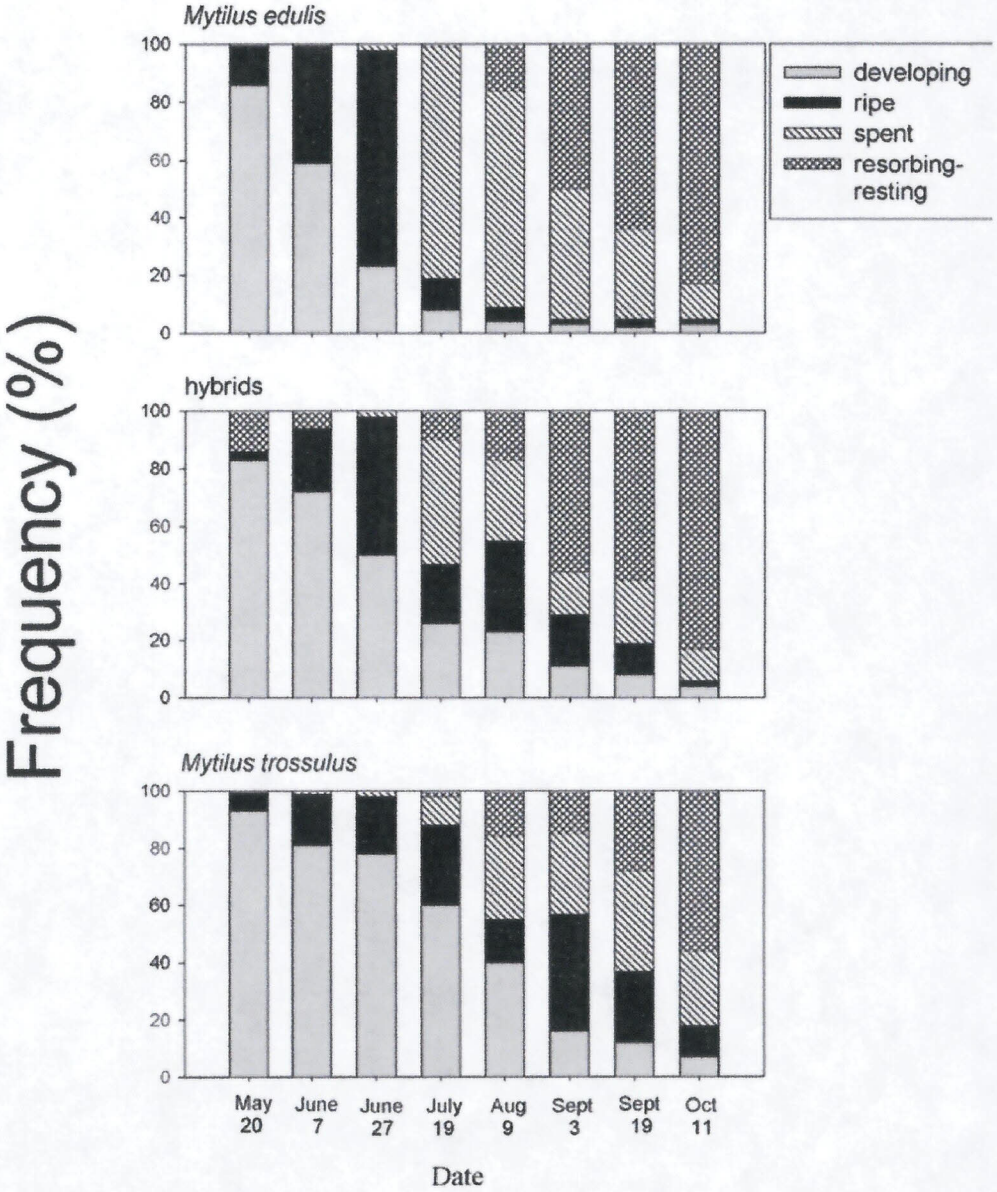

Fig. 1 *Mytilus* spp. Frequency distribution of gonad maturation stages in female and male *M. edulis*, *M. trossulus*, and hybrids during the 1996 reproductive season pooling both sexes and samples from three sites. Average $N = 100$ for each date. For details of each stage see "Materials and methods"

probability of including males and females of both species and some of the hybrids in the sample (Comesaña et al. 1999). A very small piece of mantle border (approximately 20–30 mg) from each individual was stored in 95% ethanol at –20°C to await identification of the two species and their hybrids using two diagnostic genetic markers (ITS and Glu) as described by Comesaña et al. (1999). Both ITS and Glu were used to classify 62% of the individuals sampled, the remaining 38% being classified with only the Glu marker to reduce costs. Using only the Glu marker misclassifies about 8% of the individuals and fails to discriminate between hybrids and the parent species (Comesaña et al. 1999). Additional mussels from a range of shell lengths were also sexed in order to determine the sex ratio and smallest size at maturity for the two species and their hybrids.

Histological analysis

One mantle lobe from each dissected mussel was sub-sampled by cutting a transverse section midway along the anteroposterior axis. This piece of the mantle was weighed, preserved in Bouin's fixative according to Lowe et al. (1982), dehydrated in an ascending alcohol series, cleared in xylene and embedded in paraffin wax. Serial sections (7 μm) were cut, stained with hematoxylin and counterstained with eosin. Only one section of the mantle tissue from each individual was used, since the mantle of *Mytilus* spp. is relatively homogeneous (Lowe et al. 1982; Newell et al. 1982; Bayne et al. 1985).

Terminology for the stages of gametogenesis was modified from King et al. (1989) and Kiyomoto et al. (1996). Each gonad was assigned to one of the following four stages: (1) developing (follicles occupy a large part of the mantle; individuals restoring their gonads after a partial spawning are included in this category), (2) ripe (follicles full of oocytes in female and packed lamellae of ripe spermatozoa in male), (3) spent (follicles begin to collapse and degenerate) and (4) resorbing-resting mussels. An average of 100 individuals was examined on each sampling date, pooling both sexes and samples from the three sites.

Gonadosomatic index (GSI)

Following the removal of the tissue section for histological analysis, the remainder of the mantle was weighed, dried at 80°C to constant weight, cooled in a desiccator and reweighed. The ratio of wet to dry weight for this portion of the mantle was used to correct for the weight of the tissue subsample removed for histological purposes. This adjustment allowed the total mantle dry weight to be estimated. The portion of the body excluding the mantle was also dried and weighed. The sum of body and mantle weights was used to calculate whole-mussel dry weight. The GSI of an individual was then calculated by dividing the mantle dry weight by the whole-mussel dry weight and multiplying by 100. A GSI was calculated separately for males (MGSI) and females (FGSI).

Gamete volume fraction (GVF) and reproductive output

The fractional area of the tissue composed of gametes (GVF) was measured quantitatively with Optimas 6.2 image analysis software and a Nikon stereomicroscope, following standard stereological methods (Lowe et al. 1982). Since the gonad of *Mytilus* is homogenous with respect to the distribution of germinal cells and gametes (Bayne et al. 1985), the tissue sections examined were representative of the whole gonad, an important requirement for stereological analysis (Lowe et al. 1982). The color image acquired was analyzed after adjusting the threshold by sampling area screen objects set by the operator (Heffernan and Walker 1989). A threshold is a set of intensity values that separates pixels of interest from the rest of the image. The percentage of the mantle volume occupied by gametes was calculated from the relationship between the number of pixels occupied by the gametes and the total pixels in the field. Two groups of five sections of 1.3 mm^2 were taken randomly from each individual (histological slide) to estimate the volume of the mantle that is composed of gametes. The average of each group of five sections produced two replicate estimates of the GVF for each mussel. GVF varies between 0% for a reproductively inactive mussel and 100% for a mussel in maximal reproductive condition, and also gives a measure of the relative maturity of the gonad. However, GVF does not estimate total reproductive output because an individual with a high GVF may have very few gametes if the gonad is small (Hilbish and Zimmerman 1988). To correct for this problem, total mantle dry weight was multiplied by the GVF to provide an estimate of the dry weight of gametes for each individual.

A total of 150 individual oocytes with a nucleolus from the histological sections were also examined by image analysis for three individuals from each species and hybrid. For each oocyte, the area and longest axis were recorded. For direct measurements of eggs, mussels were induced to spawn. The mussels were washed in cold seawater, placed in a shallow tray of filtered seawater, and subsequently exposed to rising temperatures (up to 22°C). Once spawning was initiated, the individuals were placed in separate containers with sterile seawater for completion of spawning. All eggs released were collected, put into 10-ml tubes and fixed with 95% ethanol. The mussels from which eggs were collected were then genotyped, and 50 eggs from six *M. edulis*, seven *M. trossulus*, and two hybrids were measured by image analysis to estimate mean egg diameter.

Statistical analysis

Observed sex ratios were tested against a 1:1 ratio using a *G*-test (with Williams' correction) (Sokal and Rohlf 1981). The frequency of individuals in the four developmental stages was tested using a contingency *G*-test among the taxa (*M. edulis*, *M. trossulus* and hybrids) on each sampling date. Normality of reproductive variables (GSI, GVF) was determined by the Lillifors K–S test (Wilkinson 1991). Analysis of the GSI values was performed by two-way, fixed-factor ANOVA (date, taxon) after arcsine-square-root transformation, followed by Tukey's studentized range test (SRT; $\alpha = 0.05$) in cases where the *F*-value exceeded the critical value. Values of GVF were similarly transformed and analyzed by a two-way, fixed-factor ANOVA (date, taxon) for each sex and sampling date. Two estimates of GVF were made for each mussel, i.e. each mussel was nested within date and taxon. Only GVF data for the first five sampling dates (which span the greatest change in GVF) were analyzed. Probability values for both the GSI and GVF ANOVAs were adjusted ($\alpha = 0.05/6$) to reduce the chance of type I errors for each set of six analyses involving the three sites and the two sexes. If there were a difference in the timing of reproductive events among the taxa, the interaction between the two main effects (date, taxon) would be significant. Dry weight of gametes among the taxa was analyzed by a one-way ANOVA for each of the 18 date–sex combinations, with the significance test adjusted to $\alpha = 0.05/18$. Differences in oocyte and egg size among taxa were tested (ANOVA, mussel nested within taxon) following log transformation of the variates. All statistical analyses were carried out with SYSTAT 5.1 (Wilkinson 1991) and SAS v. 6.30.

Results

Sex ratio

For *Mytilus edulis*, 698 mussels were sampled, of which 342 (49.0%) were females, 299 (42.8%) were males, 6 were hermaphrodites (0.86%) and 51 (7.3%) were undifferentiated. The female:male sex ratio (1.14F:1M, $n = 641$) did not differ significantly ($P > 0.05$) from the expected ratio of 1:1. For *M. trossulus*, 782 mussels were sampled, of which 412 (52.7%) were females, 341

(43.6%) were males, 5 were hermaphrodites (0.6%) and 24 (3.1%) were undifferentiated. The sex ratio (1.20F:1M, $n=753$) differed significantly ($P<0.01$) from the expected 1:1 ratio, and females predominated. For hybrids, 280 mussels were sampled, of which 109 (38.93%) were females, 143 (51.1%) were males and 28 (10.0%) were undifferentiated. The sex ratio (1F:1.31M, $n=252$) differed significantly ($P<0.05$) from the expected 1:1 ratio, and males predominated.

Reproductive cycle

Histological sections of male and female gonads of *M. edulis*, *M. trossulus* and their hybrids showed advanced gametogenesis at the first sampling date (20 May). From late May to late June very few mussels had spawned (Fig. 1). Ripe mussels of both sexes were dominant in late June in *M. edulis*, while in *M. trossulus* there were few ripe mussels. Hybrids showed an intermediate proportion of ripe individuals (Fig. 1). A large proportion of spawned *M. edulis* was observed in late July, but there were few spawned *M. trossulus* and an intermediate proportion of spawned hybrids. The frequency of individuals in the four developmental stages was significantly different ($P<0.05$, contingency *G*-tests) among *M. edulis*, *M. trossulus* and hybrids for all dates except the first (20 May) and last (11 October) (Fig. 1). A comparison of the reproductive cycles suggests an abrupt spawning for many *M. edulis* between 27 June and 19 July, compared with a more prolonged period of spawning in *M. trossulus* and an intermediate pattern in hybrids (Fig. 1). Mussels with no gametogenic activity predominated in late September and October in all taxa. These gonad sections contained no follicles at all or only a few very contracted follicles between connective cells, and showed resorption of the undischarged eggs in follicles of the females. By the final sampling in October, >50% of all mussels were in the resorbing-resting stage. Both female and male hybrids showed normal gonad development, ripening and spawning. Insufficient hermaphrodites were collected to determine their reproductive cycle.

Gonadosomatic index

The GSI is primarily determined by the accumulation and release of gonad material, as well as the utilization of stored energy products during the winter. Two-way ANOVA (Table 1) showed a significant interaction ($P<0.001$) between taxon (*M. edulis*, *M. trossulus*, hybrid) and sample date for males (MGSI) and females (FGSI) at each of the three sites, as well for the data pooled across the three sites ($P<0.001$), indicating that GSI differed among the two species and their hybrids according to sampling date. Both the MGSI and FGSI in *M. edulis*, *M. trossulus* and hybrids showed a steady decline after spawning in July (Fig. 2). However, *M. edulis* showed a more abrupt decrease than *M. trossulus*, with an intermediate decline for the hybrids. *M. edulis* also showed a greater recovery of MGSI and FGSI during early autumn than either *M. trossulus* or hybrids (except for FGSI at the CE site), which may indicate an increased storage of nutrients in the gonad (Blanchard and Feder 1997).

Table 1 *Mytilus* spp. Two-way ANOVA testing variation in the gonadosomatic index (arcsine-transformed values) for females and males among *Mytilus* taxa (*M. edulis*, *M. trossulus*, hybrids) at three sites (*BE* Bellevue exposed; *CE* Chance Cove exposed; *CP* Chance Cove protected) in eastern Newfoundland (see Fig. 2)

Site	Sex	Source	*df*	MS	*F*	*P*
BE	Females	Date	7	0.5902	48.71	<0.001
		Taxon	2	0.0239	1.98	>0.05
		Date×Taxon	14	0.0927	7.65	<0.001
		Error	126	0.0121		
BE	Males	Date	7	0.5574	59.47	<0.001
		Taxon	2	0.0108	1.15	>0.05
		Date×Taxon	14	0.0689	7.36	<0.001
		Error	97	0.0094		
CE	Females	Date	7	0.4757	54.23	<0.001
		Taxon	2	0.2688	30.64	<0.001
		Date×Taxon	13	0.0774	8.83	<0.001
		Error	125	0.0088		
CE	Males	Date	7	0.4415	31.98	<0.001
		Taxon	2	0.0190	1.38	>0.05
		Date×Taxon	13	0.0877	6.35	<0.001
		Error	99	0.0138		
CP	Females	Date	7	0.4334	34.06	<0.001
		Taxon	2	0.0140	1.10	>0.05
		Date×Taxon	14	0.0907	7.13	<0.001
		Error	128	0.0127		
CP	Males	Date	7	0.4171	54.59	<0.001
		Taxon	2	0.0368	4.81	>0.05
		Date×Taxon	14	0.0970	12.70	<0.001
		Error	97	0.0076		

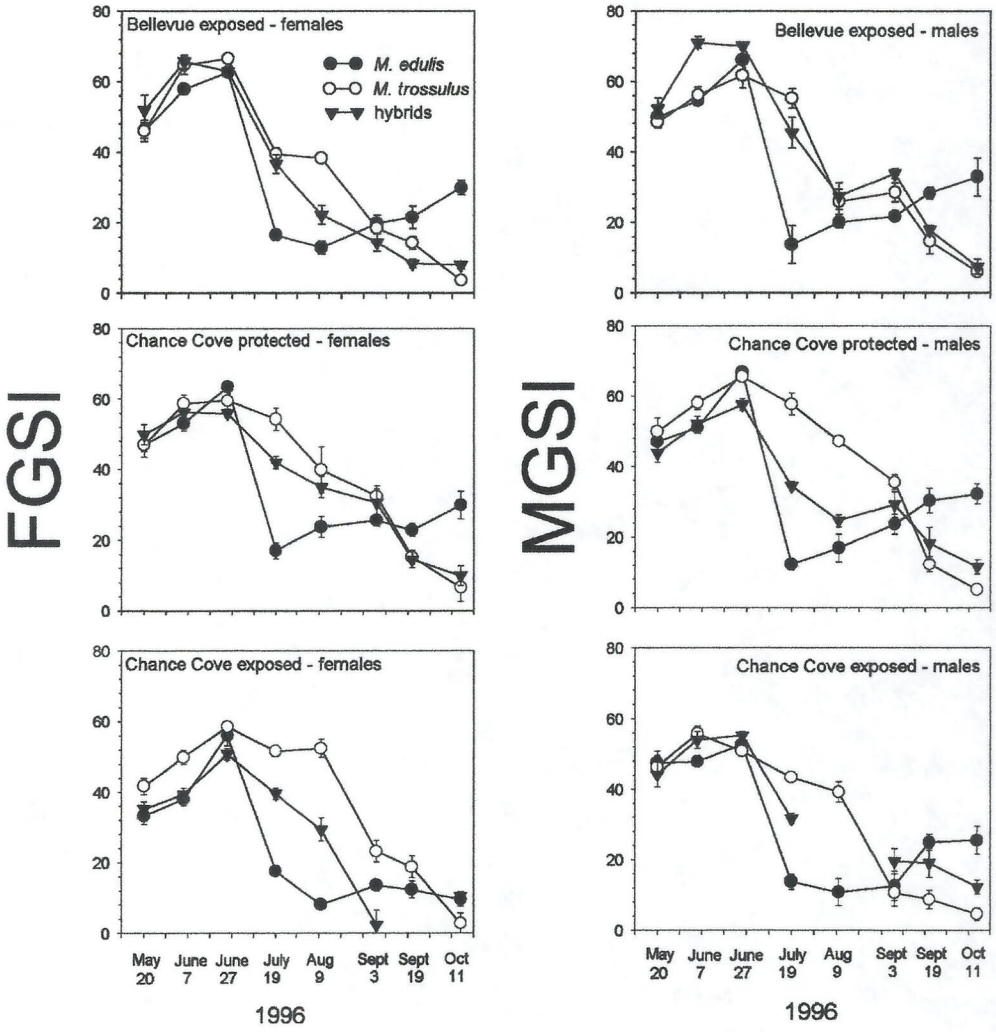

Fig. 2 *Mytilus* spp. Gonadosomatic index (±SE) for female (*FGSI*) and male (*MGSI*) *M. edulis*, *M. trossulus* and hybrids during the 1996 reproductive season at three sites in eastern Newfoundland. Average $N=34$ for each site and date

Gamete volume fraction

The GVF of male and female mussels was analyzed separately at each site to determine reproductive synchrony among the taxa. Two-way ANOVA (Table 2) showed that all date by taxon interactions for females were significant, indicating that the gametogenic cycle, as measured by GVF, differed among the species and hybrids (Fig. 3) and was similar to the pattern observed for GSI (Fig. 2). Although GVF for males showed differences among taxa similar to those observed for females, the date-by-taxon interaction was non-significant (Table 2). *M. trossulus* and the hybrids at all sites released gametes over a 12–15 week period, while *M. edulis* spawned more rapidly for about 6 weeks (primarily between 27 June and 19 July samples; Fig. 3).

Reproductive output

M. trossulus showed the greatest mean GVF throughout most of the sampling period (Fig. 3). A comparison among the taxa for the dry weight of gametes revealed that *M. trossulus* had a significantly greater weight of gametes per gonad than *M. edulis* and hybrids for three of the six sex–site comparisons for each of 7 June, 27 June and 19 July (Fig. 4) as follows: 7 June BE females $F_{(2,18)}=18.39$, $P<0.0001$, CP females $F_{(2,18)}=10.08$, $P<0.01$, CP males $F_{(2,16)}=15.26$, $P<0.001$; 27 June BE males $F_{(2,16)}=16.75$, $P<0.001$, CP females $F_{(2,16)}=17.57$, $P<0.001$, CP males $F_{(2,15)}=9.04$, $P<0.01$; 19 July BE males $F_{(2,14)}=6.87$, $P<0.01$, CP females $F_{(1,17)}=29.10$, $P<0.001$, CE females $F_{(2,15)}=15.86$, $P<0.001$. The significant differences found on 19 July were due to the fact that some *M. trossulus* and hybrids had not yet spawned, whereas most *M. edulis* had done so.

Oocyte dimensions

There were no significant differences in mature oocyte diameter or area among the taxa for histological sections taken from the 7 June sample (Table 3; Fig. 5), but there was significant variation among taxa in the 27 June sample, with *M. edulis* exhibiting the greatest values (Table 3; Fig. 5). There were also significant differences among taxa in the diameter and area of eggs obtained by

Table 2 *Mytilus* spp. Two-way nested ANOVA (mussels nested within date and taxon) testing variation in the gamete volume fraction (arcsine-transformed values) for females and males among *Mytilus* taxa (*M. edulis*, *M. trossulus*, hybrids) at three sites (*BE* Bellevue exposed; *CE* Chance Cove exposed; *CP* Chance Cove protected) in eastern Newfoundland (see Fig. 3)

Site	Sex	Source	*df*	MS	*F*	*P*
BE	Females	Date	4	1.2871	82.43	< 0.001
		Taxon	2	0.9396	60.17	< 0.001
		Date×Taxon	8	0.0689	4.41	< 0.01
		Mussel (Date× Taxon)	72	0.0156	5.09	< 0.001
		Error	85	0.0031		
BE	Males	Date	4	1.5394	33.22	< 0.001
		Taxon	2	0.6391	13.79	< 0.001
		Date×Taxon	8	0.1043	2.25	> 0.05
		Mussel (Date× Taxon)	55	0.0463	50.28	< 0.001
		Error	70	0.0009		
CE	Females	Date	4	0.5765	32.20	< 0.001
		Taxon	2	2.9820	166.56	< 0.001
		Date×Taxon	8	0.1462	8.17	< 0.001
		Mussel (Date× Taxon)	74	0.0179	57.34	< 0.001
		Error	89	0.0003		
CE	Males	Date	4	1.0462	35.33	< 0.001
		Taxon	2	0.5359	18.10	< 0.001
		Date×Taxon	7	0.0570	1.93	> 0.05
		Mussel (Date× Taxon)	59	0.0296	312.25	< 0.001
		Error	73	0.0001		
CP	Females	Date	4	0.6293	19.96	< 0.001
		Taxon	2	1.4083	44.67	< 0.001
		Date×Taxon	8	0.1078	3.42	< 0.05
		Mussel (Date× Taxon)	75	0.0315	38.40	< 0.001
		Error	90	0.0008		
CP	Males	Date	4	1.1721	38.12	< 0.001
		Taxon	2	0.3477	11.31	< 0.001
		Date×Taxon	8	0.0688	2.24	> 0.05
		Mussel (Date× Taxon)	60	0.0307	394.96	< 0.001
		Error	75	0.0001		

spawning in the laboratory. *M. edulis* produced the largest eggs, followed by hybrids and *M. trossulus* (Fig.5; Table 3).

Size at first maturation

Size at maturity was determined by direct observation and measurement of gonad weights for mussels of various sizes. The smallest mussels that could be sexed were *M. trossulus*, a male of 6.9 mm and a female of 8.9 mm shell length, together with several other individuals < 10 mm long that were observed in the June samples. For hybrids and *M. edulis*, earliest gonad development and gamete storage in the mantle tissue occurred at about 12–15 mm shell length. Thus the shell length at first maturation appears to be smaller in *M. trossulus* than in hybrids and *M. edulis*.

Discussion

Sex determination and the unusual doubly uniparental inheritance (DUI) of the mtDNA in *Mytilus* spp. are interrelated, but the details of this relationship have yet to be determined. Recent studies have established that sex determination in *Mytilus* spp. is primarily under maternal control and that the sex ratio of progeny from different mothers can vary widely (Zouros et al. 1994; Saavedra et al. 1997). However, hybridization between *Mytilus* species results in a breakdown in DUI (Zouros et al. 1994) and may also affect sex determination and population sex ratio in a hybrid zone. In the present study, the greatest deviation from a 1:1 sex ratio was observed for individuals classified as hybrids in an area of hybridization between *M. edulis* and *M. trossulus* in eastern Newfoundland. With DUI, males are heteroplasmic, containing both maternally and paternally derived mtDNA, while females contain only maternal mtDNA. Hybridization can generate individual males that contain maternal mtDNA and nuclear DNA from one species, and paternal mtDNA and nuclear DNA from the other species. Any nuclear–cytoplasmic incompatibility may result in a greater mortality of hybrid males, shifting the sex ratio towards a female bias. However, hybrids showed a male-biased sex ratio, the opposite of the pattern predicted for

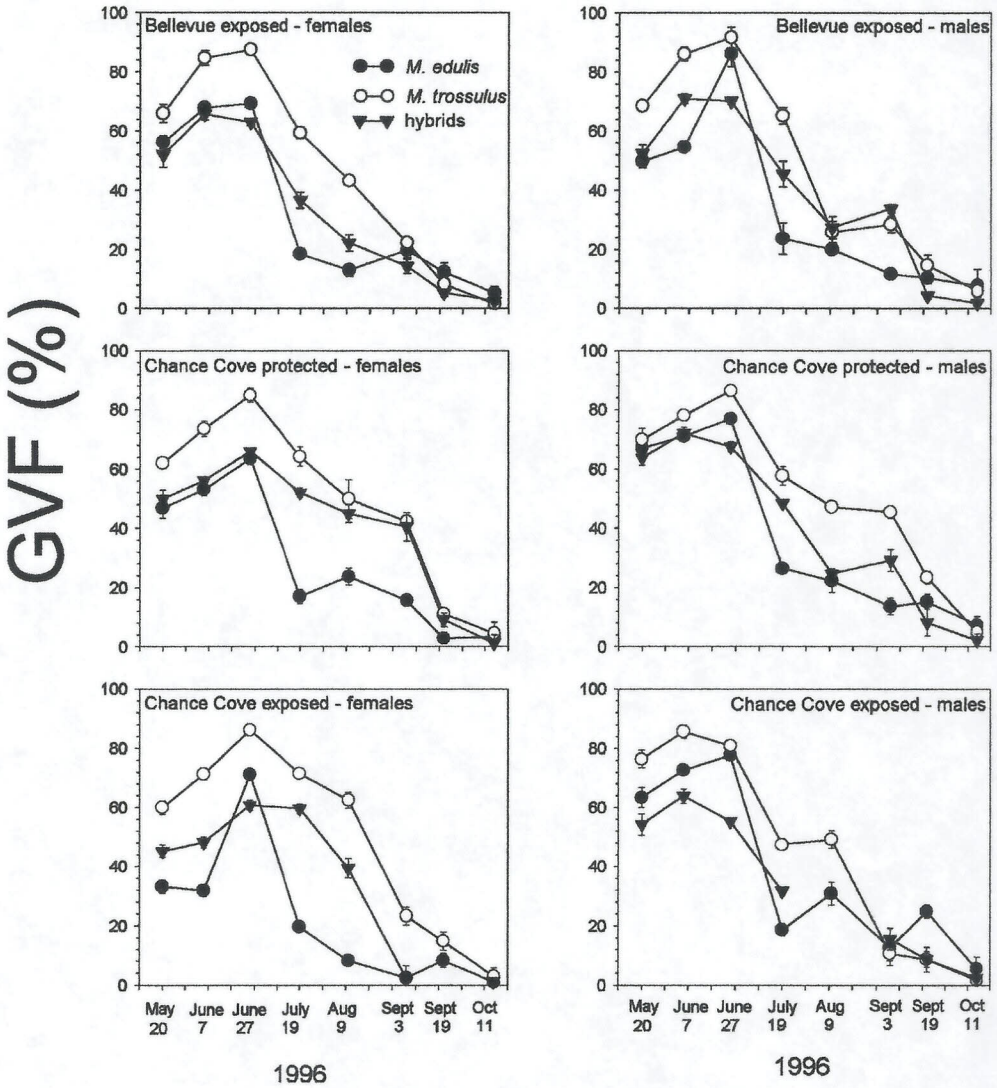

Fig. 3 *Mytilus* spp. Mean gamete volume fraction (*GVF*, ±SE) for female and male *M. edulis*, *M. trossulus* and hybrids at three sites in eastern Newfoundland during the 1996 reproductive season. Average $N = 34$ for each site and date

nuclear–cytoplasmic incompatibility in males. It is unclear if this biased sex ratio is a consequence of nuclear–cytoplasmic incompatibility through hybridization or a deviation simply due to sampling error (Palmer 2000). Furthermore, any bias in the sex ratio would be removed if a large fraction of the undifferentiated individuals were made up of the less common sex.

Mussels from all three sites showed a similar reproductive cycle, with gametogenesis progressing rapidly through spring and early summer and spawning taking place in late July. Similar observations were made on Bellevue mussels at a protected site by Thompson (1984b), who also found that these mussels do not undergo gametogenesis throughout the winter and that reserves accumulated in the previous year do not appear to play a role in gamete development. Some populations of *M. trossulus* also present the same pattern, in which gonad is synthesized in late winter and early spring, e.g. in the Baltic (Kautsky 1982) and in British Columbia (Emmett et al. 1987). Thus subtidal Baltic Sea mussels (Kautsky 1982) and Newfoundland mussels (Thompson 1984b) demonstrate an opportunistic reproductive strategy, which was also observed in mussels (*M. trossulus*) from British Columbia (Emmett et al. 1987), where gametogenesis does not proceed during the winter months. However, Blanchard and Feder (1997) found that mussels (*M. trossulus*) from populations in Port Valdez, Alaska, followed a different pattern, with gametogenic development throughout winter (like *M. edulis* in the North Sea), while the spawning period is similar to that found in the present study.

There have been no previous studies of reproductive cycles of *M. edulis* and *M. trossulus* in the context of hybridization. In this study, *M. trossulus* from eastern Newfoundland spawned over a prolonged period of 12–15 weeks, while *M. edulis* mussels spawned almost completely over a 3-week period in July. Earlier studies also showed that *M. edulis* is a synchronous spawner in Newfoundland (Thompson 1984b). Hybrid mussels in the present study exhibited a spawning cycle that was more similar to *M. trossulus*. Lubet et al. (1984) found asynchronous spawning in hybrids obtained from

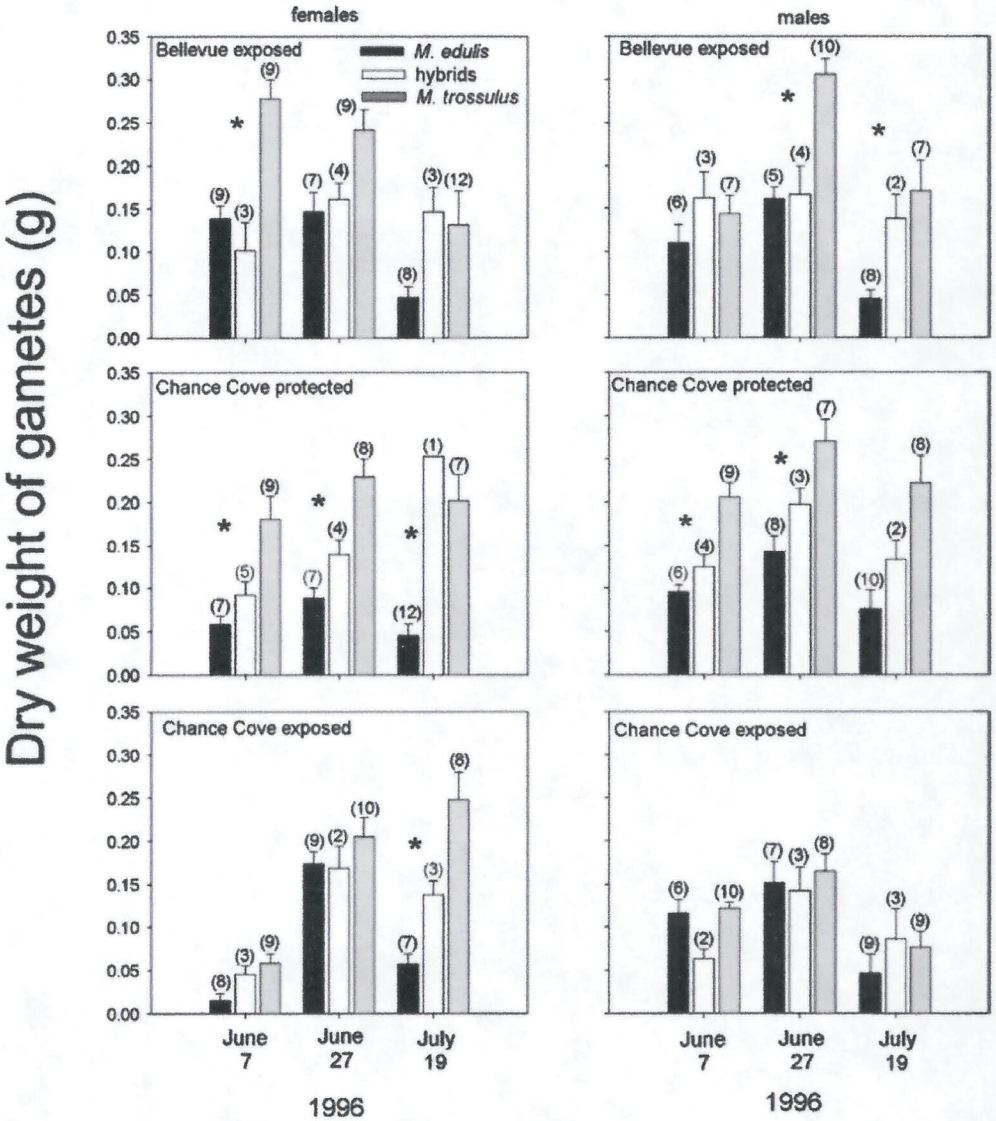

Fig. 4 *Mytilus* spp. Comparison of mean (± SE) dry weight of gametes among *M. edulis*, *M. trossulus* and hybrids on three sampling dates in the pre-spawning and spawning stages at three sites in eastern Newfoundland. Number of mussels analyzed in *parentheses*. *Asterisk* indicates a significant difference among taxa adjusted to 0.0028 (0.05/18) for the 18 tests to reduce the probability of a type I error

intra- and inter-specific crosses of *M. edulis* and *M. galloprovincialis*. *M. edulis* spawning was more restricted in time, while *M. galloprovincialis* showed a more prolonged spawning over several months, and hybrids an intermediate pattern.

There was a strong correlation between the measures of reproductive stages, GSI and GVF, which showed significant differences between the two species and hybrids. In general, *M. edulis* had a shorter spawning period than *M. trossulus*. GSI data also suggested that *M. edulis* began to recover from spawning earlier than *M. trossulus* or hybrids. Asynchronous spawning between *M. trossulus* and *M. edulis* would be expected to result in a low frequency of F_1 hybrids. For example, between 27 June and 19 July *M. edulis* males and females released large quantities of gametes into the water column, whereas *M. trossulus* released fewer gametes, suggesting that the probability of *M. edulis* sperm and eggs encountering each other would be greater than the probability of an encounter between sperm and eggs from the two different species. Comesaña et al. (1999) observed very few F_1 hybrids (based on four nuclear gene markers) at these sites, consistent with reproductive isolation due to a degree of asynchronous spawning between the species. However, the lack of F_1 hybrids could also be a result of other factors such as spatial isolation, assortative fertilization and genetic incompatibility.

Bates and Innes (1995) and Penny and Hart (1999) noted variation in the relative frequencies of *M. edulis* and *M. trossulus* at different sites in Newfoundland. Sites dominated by one species or the other will reduce the frequency of hybridization, owing to spatial separation. However, most sites in Newfoundland appear to have a mixture of both species, allowing opportunity for hybridization if spawning times overlap. Even with a large degree of overlap in spawning, hybridization may be reduced if there is assortative fertilization or a greater success of intra- compared with inter-specific fertilizations. Bierne et al. (2002) have recently demonstrated

Table 3 *Mytilus* spp. Results of nested ANOVA (mussel within taxon) for testing variation in egg area and egg diameter among *Mytilus* taxa (*M. edulis*, *M. trossulus* and hybrids) (see Fig. 5)

Date	Factors	Source	*df*	MS	*F*	*P*
7 June (histological section)	Egg area	Taxon	2	2.7987	1.88	>0.05
		Mussel (Taxon)	6	1.4907	32.41	<0.001
		Error	1341	0.0460		
	Egg diameter	Taxon	2	0.9937	2.45	>0.05
		Mussel (Taxon)	6	0.4060	18.13	<0.001
		Error	1341	0.0224		
27 June (histological section)	Egg area	Taxon	2	11.4297	117.98	<0.001
		Mussel (Taxon)	6	0.0969	2.64	<0.05
		Error	1341	0.3672		
	Egg diameter	Taxon	2	2.3872	72.92	<0.001
		Mussel (Taxon)	6	0.0327	1.85	>0.05
		Error	1341	0.0177		
Spawning (spawned eggs)	Egg area	Taxon	2	6.2151	196.20	<0.001
		Mussel (Taxon)	12	0.0317	1.51	>0.05
		Error	735	0.0209		
	Egg diameter	Taxon	2	1.0908	54.48	<0.001
		Mussel (Taxon)	12	0.0200	2.15	<0.05
		Error	735	0.0093		

that assortative fertilization can play a role in reducing the rate of hybridization between *M. edulis* and *M. galloprovincialis*. Post-fertilization genetic incompatibility can result in early death of hybrids and may also explain their reduced frequency. Both Saavedra et al. (1996) and Comesaña et al. (1999) found that some hybrid genotype combinations between *M. edulis* and *M. trossulus* were less frequent than expected, given equal fertilization and survival. Therefore, a combination of asynchrony in spawning time between the two species, assortative fertilization and genetic incompatibility probably prevents the formation of large numbers of F_1 hybrids between *M. edulis* and *M. trossulus*. Hybrids also appear to be rare at other sites in Newfoundland (authors' unpublished observations) and Maine (Rawson et al. 2001), suggesting that mechanisms reducing hybridization operate over a large area where these species are sympatric. In addition, temporal separation in spawning time between the species may vary among sites, so that the relative importance of various reproductive isolating mechanisms may also vary geographically.

A degree of overlap in the spawning period between hybrids and the two parental species provides the potential for introgression of genes between the two *Mytilus* species. Comesaña et al. (1999) showed that most hybrids of *M. edulis* and *M. trossulus* in the Newfoundland hybrid zone were backcrosses biased towards *M. trossulus*. Although only a few reproductively mature F_1 hybrids may be produced, they can spawn to form a large number of backcrosses with the parental species, especially when hybrids have a spawning period that overlaps with that of the pure species. The high frequency of *M. trossulus*-biased backcrosses appears to be due to the greater gamete output by the *M. trossulus* populations compared with the *M. edulis* populations at the study sites, although variation in viability among different backcross genotypes cannot be ruled out.

According to Bayne et al. (1978), an individual female mussel (*M. edulis*) can produce as many as 8×10^{10} oocytes (70 μm diameter), depending on body size. In our study *M. trossulus* of shell length 38–42 mm exhibited a greater reproductive output than *M. edulis* of the same size, which is consistent with the observations of Mallet and Carver (1995) for two mussel populations in Nova Scotia. However, the present study also suggests that the oocyte is larger in *M. edulis* than in *M. trossulus*, with intermediate values for hybrids, which is in accordance with findings from the east coast of the USA (P. Rawson, personal communication). Furthermore, we found that *M. trossulus* had a greater total gamete output (based on dry weight of gametes). A greater total weight of gametes combined with a smaller egg size suggests that individual *M. trossulus* release more eggs than individual *M. edulis* of equal size. *M. trossulus* is capable of reproduction at a smaller shell length than *M. edulis*, although maturation size depends on rate of growth (Seed 1969) and therefore may differ among species and locations. Nevertheless, this smaller size at first maturation in *M. trossulus* relative to *M. edulis* and hybrids may be a response to a higher rate of mortality for *M. trossulus* (Comesaña et al. 1999). The implications of this higher rate of mortality and greater reproductive output by *M. trossulus* are not clear, but the data suggest that the coexistence of the two species may be linked to life-history differences associated with age-specific reproduction and mortality. Further data are being collected to examine these aspects of the life history in more detail.

Gardner and Skibinski (1990) found that the mean fecundity of *M. galloprovincialis* was 2.8 times that of *M. edulis* at Croyde and 2.2 times greater at Whitsand, because *M. galloprovincialis* had both greater mean length and greater mean fecundity per unit length than *M. edulis*. However, the study also estimated that the total population fecundity of *M. edulis* was 5 and

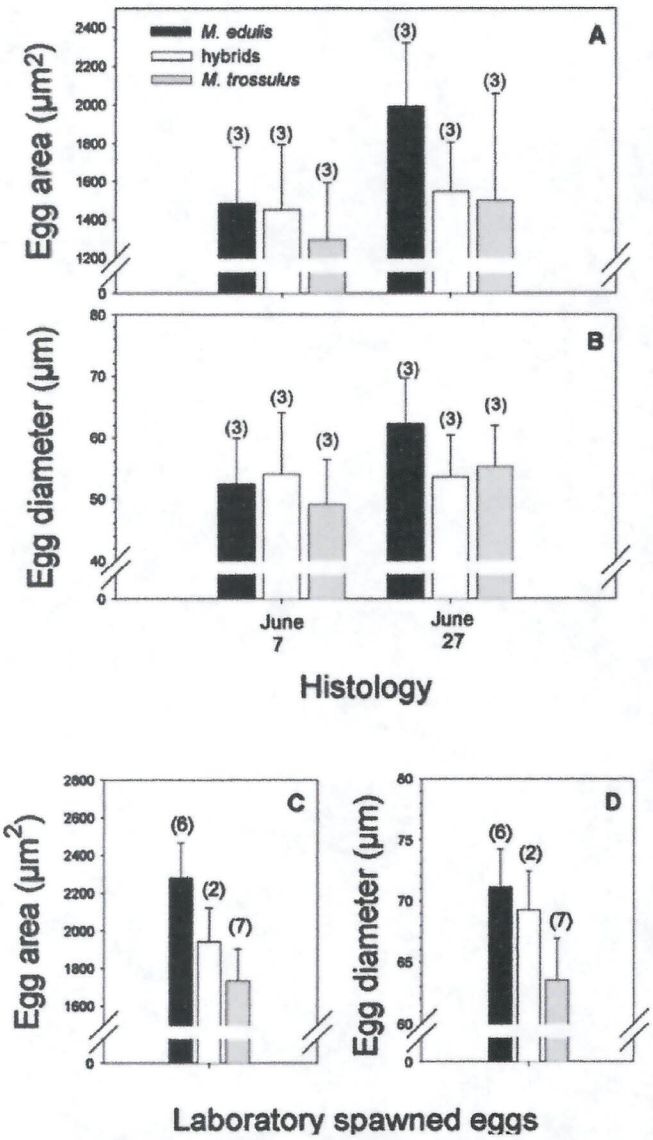

Fig. 5A–D *Mytilus* spp. Comparison of mean (±SD) of egg area (μm^2) (**A**) and egg diameter (µm) (**B**) among *M. edulis*, *M. trossulus* and hybrids determined from histological sections on two dates during the pre-spawning stage and also from laboratory spawned eggs: egg area (**C**) and egg diameter (**D**). Number of mussels used in *parentheses* (50 eggs from each mussel were measured)

17 times that of *M. galloprovincialis* at Croyde and Whitsand, respectively, owing to the presence of larger numbers of small *M. edulis* compared with fewer but larger *M. galloprovincialis*. A similar mussel population structure was found at the present study sites. A higher frequency of *M. trossulus* was observed in the abundant small shell-length classes compared with a higher frequency of *M. edulis* in the much less abundant large shell-length classes (Comesaña et al. 1999; Toro 1999), suggesting a greater reproductive output by the *M. trossulus* population. A higher proportion of *M. trossulus* larval, spat and juvenile stages was observed at the study sites (Toro 1999), which is consistent with a higher proportion of *M. trossulus* contributing to the gamete pool, assuming that the larvae originated from a site with a similar population structure.

Reproductive isolation between *M. edulis* and *M. trossulus* is not complete, because hybrids occur in nature and can also be produced in the laboratory. The lower frequency of hybrids in Newfoundland compared with other mussel hybrid zones suggests that some pre- or post-zygotic reproductive isolating mechanisms may be operating. We observed significant differences in the reproductive cycle and the timing of spawning between the two species that could contribute towards reproductive isolation, but the overlap in spawning suggests that other factors may also play a role. Further studies are in progress to determine the roles played by pre-zygotic gamete and post-zygotic genetic incompatibility in explaining the observed rate of hybridization between *M. edulis* and *M. trossulus* in Newfoundland.

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Respuesta a la selección genética para crecimiento en juveniles de *Ostrea chilensis* Philippi (Bivalvia: Ostreidae), mantenidos en condiciones de laboratorio

Response to genetic selection for growth rate in juveniles of *Ostrea chilensis* Philippi (Bivalvia: Ostreidae), maintained under laboratory conditions

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Abstract.- The oyster parental stock was taken from the 1998 natural spatfall from the wild population of *Ostrea chilensis* at the Quempillen River Estuary, located at Chiloe Island, southern Chile. From a cohort of 34 months of age 5232 oysters were monitored for the traits shell length and live weight. Selection was carried out applying a selection intensity of +1.7 for the trait live weight, using a control group. The conditioning was carried out in 5 tanks of 120 L, 3 of them containing high selected lines and other 2 control lines of oysters. A highly significant correlation (0.76, $p<0.001$, N=5232) was found between the traits live weight and shell length. Spat from the 3 selected subgroups and 2 control groups were obtained. Plastic collectors with the spat attached were placed in a common rearing tank. Shell length was monitored after 6 and 16 weeks of age in 200 juveniles of each selected and control lines. The ANDEVA results show a significant difference ($p<0.05$) between the shell length of the selected and control groups at 6 and 16 weeks of age. Also, a significant response to selection was obtained even when selection was applied for the trait live weight at 34 m-old. These results confirm those obtained in an earlier study using the same natural oyster population from Quempillén.

Key words: Genetic improvement, selection, oyster, molluscs

Resumen.- La población base de *Ostrea chilensis* utilizada en el presente trabajo corresponde a una cohorte obtenida del estuario del río Quempillén, isla de Chiloé, sur de Chile. A los 34 meses de edad y en base a 5232 ostras de la cohorte tomadas al azar, se realizó una curva de distribución para los caracteres peso vivo y longitud de la valva. Como criterio de selección se aplicó una intensidad de selección de +1,7 para el peso vivo, manteniéndose un grupo control. Cada grupo, control y seleccionado (N=400 y N=600 respectivamente), se dividió al azar en subgrupos de 200 ostras, 3 de los cuales contenían líneas seleccionadas y los otros 2, ostras como grupo control. El acondicionamiento se realizó en 5 estanques de 120 L cada uno. El carácter peso vivo mostró tener una alta correlación fenotípica con el carácter longitud de la valva con un valor de 0,76 (N=5.232, $p<0,001$). Se obtuvieron juveniles en los 5 sub-grupos. Los colectores plásticos con los juveniles adheridos marcados se mantuvieron en crecimiento en un estanque común. Se realizaron dos monitoreos de crecimiento de longitud de valva en 200 juveniles tomados al azar en cada uno de los subgrupos seleccionados. El ANDEVA indica una diferencia significativa ($p<0,05$) en la longitud de la valva entre juveniles del grupo control y grupo seleccionado, a las 6 y 16 semanas de edad. Ello, aun cuando la selección se basó en el carácter peso vivo a los 34 meses de edad (talla comercial). Estos resultados confirman los obtenidos en un estudio previo utilizando la misma población natural de ostras de Quempillén.

Palabras clave: Mejoramiento genético, selección, ostra, moluscos

Introducción

La ostra chilena, *Ostrea chilensis* Philippi 1845, se ha cultivado desde hace ya varias décadas (Toro & Chaparro 1990) y es una especie de alto valor comercial. Su cultivo es mas bien extensivo (Lépez 1983) y se ha visto restringido por la falta de juveniles, obtenidos principalmente de captaciones naturales poco confiables en relación con su cantidad y regularidad en el tiempo. Sin embargo, uno de los problemas mas serios en el cultivo de esta especie se refiere a la muy

baja tasa de crecimiento con relación a otros moluscos bivalvos en la región sur-austral de Chile. La talla comercial de 50 mm se alcanza después de los 3 años en cultivo suspendido (Winter *et al.* 1984, Kino & Valencia 1990). La ostricultura en Chile no ha utilizado los beneficios agregados de la manipulación genética para el mejoramiento de la especie, debido a que tradicionalmente no se ha hecho uso de la reproducción artificial para la obtención de "semillas", con la consecuente falta de control sobre el ciclo vital de esta especie, requisito fundamental para cualquier tipo de cambio genético dirigido (Hershberger *et al.* 1984). El mejoramiento genético, a través de programas de selección genética tradicional, posee enormes beneficios para el cultivo de especies marinas (Moav 1976, Newkirk 1980, 1983; Gjedrem 1983, Gjerde 1986, Guiñez 1988, Toro & Newkirk 1990, Bustos *et al.* 1991, Koment *et al.* 2000, Beaumont & Hoare 2003).

El mejoramiento genético de caracteres como tasa de crecimiento se basa en la teoría de la genética cuantitativa, ya que estos caracteres productivos están generalmente determinados por un gran número de genes (poligenes), acompañados de una considerable influencia ambiental (Newkirk 1980, Lande 1982).

Numerosos autores han reportado una alta variabilidad fenotípica para la tasa de crecimiento de esta especie (DiSalvo & Martínez 1985, Toro & Varela 1988, Toro & Newkirk 1991, Bustos *et al.* 1991), sin embargo, la literatura cita muy pocos estudios sobre parámetros genéticos (Guiñez & Galleguillos 1985, Guiñez 1988) ó selección para crecimiento (Toro & Newkirk 1990, Bustos *et al.* 1991, Toro 1992). Toro (1992) llevó a cabo un experimento de selección con *O. chilensis*, en el cual obtuvo una respuesta significativa a la selección para el carácter peso vivo.

El debate sobre el estatus taxonómico de la ostra nativa chilena continúa generando nuevos nombres genéricos para esta especie (Toro 1995, Jeffs & Hickman 1999). En la mayor parte de las referencias científicas esta especie es citada como *Ostrea chilensis* Philippi 1845. Sin embargo, recientemente ha sido asignada al género *Tiostrea* como *Tiostrea chilensis* (Chanley & Dinamani 1980) y seguidamente reasignada al género *Ostrea* como *Ostrea* (*Eostrea*) *puelchana* (Harry 1985) y últimamente, propuesta como *Ostrea puelchana* D'Orbigny 1846 por Carriker *et al.* 1996. Debido a que Carriker *et al.* (1996) proponen a *O. puelchana* como sinónimo de *O. chilensis* y recientemente, Jeff & Creese (1996) y Jeff & Hickman (1999) aceptan como sinónimo taxonómico a *Tiostrea chilensis* y *O. chilensis* dado a las similitudes observadas en su ecología, historia de vida y bioquímica, los autores del presente trabajo continuarán refiriéndose a la ostra nativa chilena como *Ostrea chilensis* Philippi 1845.

El presente estudio se diseñó para evaluar y a su vez validar estudios previos sobre la respuesta a la selección para crecimiento de *O. chilensis,* producto de la aplicación de un diferencial de selección en la generación parental. Este estudio forma parte de un programa a largo plazo para la estimación de parámetros genéticos en esta especie.

Materiales y métodos

Los reproductores se obtuvieron de una cohorte captada como "semilla" mediante fijación natural en diciembre de 1998, desde la población natural del estuario Quempillén, ubicado en la isla de Chiloé (41°51'S; 73°46'O). Los juveniles fueron trasladados para su crecimiento suspendido a la localidad de Calbuco (X Región). Aproximadamente 6.000 ejemplares de ostras, tomados al azar de esta cohorte de 34 meses de edad, se trasladaron al centro de cultivo donde se midieron los caracteres peso vivo y longitud de la valva de 5.232 ostras cuyas distribuciones para ambas variables son normales (prueba Kolmogorov-Smirnov, $p>0,05$). Se aplicó un esquema de selección sobre el carácter peso vivo (por ser una variable tri-dimensional) a los 34 meses de edad. Ello, con el propósito de obtener una línea seleccionada y otra de control para el carácter peso vivo, utilizando como criterio de selección, una intensidad de selección de + 1,755 (Falconer 1981). Los reproductores correspondieron al 10% superior y el grupo control a ostras alrededor del promedio (± 0,5 *s*) de la curva de distribución para el carácter peso vivo. El grupo de ostras seleccionadas se subdividió en tres subgrupos y el grupo control en dos subgrupos para el proceso de acondicionamiento en el laboratorio.

Todos los ejemplares reproductores fueron marcados individualmente (Toro 1992) y el acondicionamiento se llevó a cabo en estanques de 120 L de capacidad con aireación continua (con 200 ostras c/u) instalados en una sala con temperatura controlada. En la Tabla 1 se presentan los principales datos estadísticos para la población (N=5.232), grupo seleccionado y control (n=600 y 400 respectivamente). El proceso de acondicionamiento y sus variables ambientales fueron similares a las llevadas a cabo en el estudio de Toro (1992). El período de acondicionamiento fue de siete semanas, similares a las reportadas en la literatura (Chaparro 1990, Toro & Chaparro 1990). Luego de producido el asentamiento, los colectores se identificaron con la procedencia de los juveniles (grupo

parental) y se mantuvieron en crecimiento en un estanque común de 2000 L. Los cambios de agua se realizaron cada 48 h, manteniéndose una temperatura de 16 ± 1°C y una alta ración (50 L diarios de microalgas con una concentración promedio de 1,8 millones de cél/L) de microalgas durante 4 meses, período durante el cual se llevaron a cabo dos mediciones bajo lupa estereoscópica (a las 6 y 16 semanas de asentados) de la longitud de la valva en 200 individuos tomados al azar de cada sub-grupo seleccionado (Toro *et al.* 1992). Para evaluar el crecimiento en longitud de la valva entre los grupos seleccionados y control se utilizó el análisis de varianza (ANDEVA) (Sokal & Rholf 1981), llevado a cabo con el programa computacional estadístico SYSTAT 5.01. No se determinó la mortalidad en esta fase de crecimiento, ya que aún los juveniles están adheridos a sus colectores.

Resultados

Se obtuvieron correlaciones fenotípicas significativas entre el carácter peso vivo y los caracteres longitud de la valva, ancho de la valva y alto de la valva, con valores de 0,76, 0,77 y 0,82 (N=5.232), respectivamente (p<0,05), lo cual indica que estos caracteres están asociados, permitiendo construir (si mas adelante se obtienen correlaciones genéticas significativas y positivas), un índice de selección que permita incrementar los tres caracteres al mismo tiempo.

Se realizaron dos evaluaciones (6 y 16 semanas) de la longitud de la valva en 200 juveniles tomados al azar en cada una de las diferentes líneas de seleccionadas. Se determinó este carácter ya que en esta etapa los juveniles aún permanecen adheridos a los colectores. En la Tabla 2 se presentan los datos de estas evaluaciones, apreciándose diferencias significativas en los promedios en la longitud de la valva entre los juveniles provenientes de diferentes sub-grupos, tanto del grupo seleccionado como control, a las 6 y 16 semanas de edad (análisis de varianza jerárquico, Tabla 3). Al tomar en consideración los sub-grupos en conjunto para cada grupo (seleccionado y control) se demuestra que ya a las 6 y 16 semanas de edad se obtiene una diferencia significativa (p<0,05) entre ambos grupos, aun cuando la selección de los reproductores se basó en el carácter peso vivo a los 34 meses de edad (talla comercial). Ello podría ser un indicio, por una parte, de la existencia de una correlación genética positiva entre ambos caracteres y, por otra, de la existencia de una alta correlación positiva entre diferentes estados del ciclo de vida de la ostra.

Tabla 1

Estadísticos básicos para una cohorte de *O. chilensis* a los 34 meses de edad, utilizada como población base en el programa de selección. Grupos Control y Seleccionado, para los caracteres peso vivo, longitud, ancho y alto de la valva

Basic statistics from a 34 m-old *O. chilensis* cohort, used as a base population in a genetic selection program. Control and Selected (Seleccionado) groups for the traits live weight, shell length, shell wide and shell height

	Población	Control	Seleccionado
N	5,232	400	600
Peso vivo			
Promedio (g)	30,06	30,26	37,68
Desviación estándar	9,53	4,52	6,31
Coeficiente de variación (%)	31,70	4,93	16,76
Longitud de la valva			
Promedio (mm)	50,65	50,39	62,70
Desviación estándar	7,04	3,10	3,55
Coeficiente de variación (%)	12,00	6,15	5,66
Ancho de la valva			
Promedio (mm)	42,14	39,42	44,18
Desviación estándar	7,28	3,20	3,81
Coeficiente de variación (%)	17,27	8,11	8,62
Alto de la valva			
Promedio (mm)	17,49	18,50	24,19
Desviación estándar	4,11	2,10	2,66
Coeficiente de variación	23,49	11,35	10,99

Tabla 2

Valores promedio ($\overline{X}$) de longitud de la valva (mm) y desviación estándar (s) para los juveniles de *O. chilensis* obtenidos en el programa de selección genética a las 6 y 16 semanas de edad para el grupo control y seleccionado. Se presentan los datos para cada sub-grupo (n= 200) y el promedio para el grupo

Mean values ($\overline{X}$) for shell length (mm) and standard deviation (s) for *O. chilensis* juveniles from the control and selected group, obtained in the genetic selection program after 6 and 16 weeks of growth. Mean data for each replicate (n=200) and group average

GRUPO	6 semanas		16 semanas	
	$\overline{X}$	s	$\overline{X}$	s
Grupo control	3,02	1,53	8,54	4,02
Sub-grupo 1	3,08	1,45	8,38	4,78
Sub-grupo 2	2,96	1,62	8,71	5,99
Grupo seleccionado	3,66	1,03	10,56	2,94
Sub-grupo 1	3,68	1,33	10,17	3,72
Sub-grupo 2	3,72	1,08	11,08	3,88
Sub-grupo 3	3,59	1,14	10,45	3,96

Tabla 3

Análisis de varianza: Cuadrados medios (CM) y valores de p, para el carácter longitud de la valva a las 6 y 16 semanas de edad en juveniles de *O. chilensis*. Efectos: entre subgrupos dentro de cada grupo, entre grupos e interacción, g.l. = grados de libertad

Analysis of variance: Mean squares (CM) and p values for shell length of *O. chilensis* juveniles at 6 and 16 weeks of age. Sources: between sub-groups within group, between groups and interaction, g.l.=degrees of freedom

		6 semanas		16 semanas	
Efecto	**g.l.**	**CM**	p	**CM**	p
Grupo	1	18.779	<0,05	180.324	<0,05
Sub-grupos dentro de grupo	2	2.256	<0,05	22.551	<0,05
Residual	495	0,467		7.619	

En la Tabla 4 se presentan las intensidades de selección calculadas y las respuestas estandarizadas a la selección. Las respuestas estandarizadas fluctuaron entre 0,40 y 0,42 unidades de desviación estándar a partir del grupo control. Las ganancias para incrementar el carácter, expresadas en porcentaje, fluctuaron entre el 19 y el 21% entre las 6 y 16 semanas de edad en relación a los controles (Tabla 4).

Discusión

Se detectó una respuesta significativa a la selección para la tasa de crecimiento en longitud de la valva a partir de la sexta semana de edad (Tablas 3 y 4). La diferencia en el crecimiento de la longitud de valva entre la línea seleccionada y el control, está indicando que existe una componente genética aditiva significativa, la cual prodría ser utilizada en un programa de selección. Estos resultados concuerdan con lo reportado para esta misma especie por Bustos *et al.* (1991) y Toro (1992), quienes obtuvieron una respuesta estadísticamente significativa a la selección para incrementar el carácter longitud de la valva. Toro & Newkirk (1991) reportan también una respuesta significativa a la selección para disminuir el mismo carácter, utilizando un grupo control y una línea para bajo crecimiento. Las respuestas significativas a la selección se observan ya a las 6 y 16 semanas de edad, aun cuando la selección estuvo basada en el carácter peso vivo a los 34 meses de edad. La respuesta entonces pudo ser medida en un carácter correlacionado (Toro & Newkirk 1989, 1990, Toro 1992); sin embargo, esto estaría indicando la presencia de variación genética aditiva para el carácter seleccionado (peso vivo a los 34 meses de edad).

Tabla 4

Intensidades de selección y respuestas estandarizadas de selección, para el carácter longitud de la valva (mm) a las 6 y 16 semanas de edad en *O. chilensis*

Selection intensity and standarized response to selection for the trait shell length (mm) at 6 and 16 weeks of age in *O. chilensis*

	Grupo seleccionado	Grupo control	Población $\overline{X}$	Población s
Intensidad de selección				
Progenitores	62,70	50,39	50,65	7,04
Índice estandarizado	1,75	0,26		
Índice ajustado	1,49	0,00		
Respuesta a la selección				
6 semanas				
Grupos seleccionados	3,66	3,02	3,02	1,53
Índice estandarizado	0,42	0,00		
Porcentaje de ganancia	21,19			
16 semanas				
Grupos seleccionados	10,17	8,54	8,54	4,02
Índice estandarizado	0,40	0,00		
Porcentaje de ganancia	19,08			

A diferencia de otros moluscos bivalvos, la ostra chilena incuba sus larvas hasta que están próximas al asentamiento, es por ello que podría existir un efecto materno como fuente de variación ambiental. Ostras de mayor tamaño (dentro de la cohorte) podrían producir huevos más grandes que resulten con un mayor crecimiento y mejor supervivencia (Chaparro & Paschke 1990). Sin embargo, la fecundidad está correlacionada positivamente con el tamaño de la hembra, lo que podría contrarrestar el efecto del tamaño materno para con los huevos (Walne 1964, 1979). Ryman (1972) reporta que hembras grandes del pez *Lebistes reticulatus* mostraron una mayor fecundidad la cual estaba correlacionada negativamente con la tasa media de crecimiento de la descendencia. Un efecto similar es descrito por Campton & Gall (1988) en el pez *Gambusia affinis.* Sin embargo, el efecto materno en general disminuye al aumentar la edad de la progenie (Monteiro & Falconer 1966, Falconer 1981, Kirpichnikov 1981, Campton & Gall 1988) y por lo tanto podría ser importante sólo en los primeros estadios de desarrollo (Arthur 1984). En estudios previos (Toro *et al.* 1992) y en los resultados obtenidos en el presente trabajo las diferencias entre los grupos control y seleccionados se detecta ya a partir de una temprana edad (algunas semanas), lo que sugiere que el efecto materno tendría una mínima importancia.

En la literatura científica no existen muchos trabajos sobre selección artificial y estimación de parámetros genéticos básicos en la ostra chilena. Uno de los motivos podría ser que sólo hace muy poco se logró el control sobre el ciclo vital de esta especie, a través de la implementación de "hatcheries" (DiSalvo *et al.* 1983, 1984, Chaparro 1990). Por otro lado, dado a que esta especie presenta fertilización interna de sus ovas con un período de incubación que se prolonga por 6 a 9 semanas, impide realizar cruzamientos controlados. La utilización del desove masivo impide el control sobre el número de individuos que contribuye con gametos en cada uno de los grupos, desconociéndose especialmente la contribución de los machos. Este factor es discutido en la literatura por Newkirk (1986), quien reporta un cruzamiento controlado exitoso para *0. edulis.* El diseño de desove masivo se utilizó con el propósito de asegurar la obtención de juveniles de todas las líneas seleccionadas y también debido a limitación de espacio en el laboratorio.

A pesar que cuando se utiliza el desove masivo es difícil estimar cuantos individuos contribuyen con genes para la descendencia que se obtiene, se puede realizar una cierta estimación de la contribución de hembras, a través de la estimación de la cantidad de larvas pedivelígeras con mancha ocular en cada estanque (Toro 1992).

El presente estudio, que forma parte de un programa mucho mas amplio sobre selección artificial para crecimiento en *O. chilensis,* confirma los resultados

obtenidos en la misma especie por Toro *et al.* (1992), validando la presencia de una componente genética aditiva en el carácter longitud de valva a las 6 y 16 semanas de edad, sugiriendo que podría hacerse un efectivo progreso a través de selección para obtener ostras de rápido crecimiento.

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Heritability estimates of larval and spat shell height in the Chilean blue mussel (*Mytilus chilensis* Hupe 1854) produced under controlled laboratory conditions

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Abstract

The heritability of larval size is estimated in *Mytilus chilensis*, based on a nested design, involving 95 full-sib and 19 half-sib families (19 males each mated to 5 separated females) grown under controlled laboratory conditions. The half-sib heritability estimates at 10, 25 and 40 days for larval and spat shell height ranged between 0.38 ± 0.33 and 0.84 ± 0.45, suggesting that selection for shell size would be effective.

Keywords: *Mytilus chilensis*, heritability, larval growth, Chile

Introduction

The Chilean blue mussel *Mytilus chilensis* (Hupe 1854) is an economically important bivalve in southern Chile. Its culture began in 1943 in the area of Quellón located southeast of Chiloé Island (Navarro & Gutierrez 1990; Aiken 1993) and the aquaculture production for this species increased from 3864 t in 1993 to 34 648 t in 2001 (SERNAPESCA 2002). These mussels are marketable at a shell length of about 55 mm, which is attained after 14 to 16 months of growth in suspended culture (Winter, Toro, Navarro, Valenzuela & Chaparro 1984; Kino & Valencia 1990); therefore, a reduction in time attaining the commercial size (12 months) could be an important advance to the culture of this mussel.

Mussel aquaculture has enormous potential in southern Chile mainly because the numerous sheltered bays and estuaries along its coastline are free from industrial pollution. Fertilizers and pesticides are rarely applied in local agriculture, and domestic pollution, owing to the very low human population densities in southern coastal areas, is small (Winter *et al.* 1984; Toro & Chaparro 1990). The culture of *M. chilensis* in Chile has been increasing rapidly in the past few years (Winter *et al.* 1984; Navarro & Gutierrez 1990; Sanchez 2002; SERNAPESCA 2002); because of this potential, estimates of genetic variation (i.e. heritability) are not only important from the purely ecological point of view but also for practical management purposes (Mallet, Carver, Coffen & Freeman 1987; Koment 2002). The type of selection scheme that is appropriate depends upon the relative magnitude of the variation of that trait due to differences in genotype and in environmental factors (Falconer 1989).

Very little has been published on the genetic improvement of the Chilean blue mussel. Control of the complete life cycle, which is required for any type of direct genetic improvement (Hershberger, Perdue & Beattie 1984), has been accomplished only recently (Toro & Sastre 1995). There are several reports in the literature addressing the importance and potential of genetics in aquaculture (Moav 1976; Newkirk 1983; Gjerde 1986; Guiñez 1988; Toro & Newkirk 1990; Koment 2002). Increasing production efficiency, reducing generation time and/or improving survival rate will reduce the negative effects of short-growing seasons in high latitudes (Newkirk 1980).

Growth rate is considered the most economically important trait to be improved in a selection programme. Traits of economic importance are usually determined by a large number of genes each having a small contribution and under considerable environmental influence (Newkirk 1980; Lande 1982). Thus, these kind of traits are best studied through the quantitative genetics theory, which reduces the most

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important features of complex genetic systems to a relatively few variables that can be estimated from phenotypic measurements (Lande 1982; Falconer 1989).

A very high intrapopulation variation of the growth rate of *M. chilensis* has been reported in the literature (Winter *et al.* 1984; Navarro & Gutierrez 1990). However, there have been only a few reports on genetic parameters (Toro & Paredes 1996) or genetic manipulation (Toro & Sastre 1995) in *M. chilensis*. The fact that there is a phenotypic variation in the growth rate suggests that there may be significant genetic differences that could be exploited in a selection programme to produce faster growing mussels. A selection programme requires the estimation of an important genetic parameter such as heritability (h^2) for economically important traits. A review of the literature shows that heritability estimates in blue mussels have been mainly based on full-sib correlation. For example, Innes & Haley (1977) reported a heritability estimate, based on a salinity tolerance experiment, a value of $h^2 = 0.16$ for *M. edulis* (Linnaeus 1758) larvae (Innes & Haley 1977). Low heritability ($h^2_{sire} = 0.11 \pm 0.02$, $h^2_{dam} = 0.19 \pm 0.04$) has been reported for the shell length of *M. edulis* larvae (Mallet, Freeman & Dickie 1986); however, Stromgren & Nielsen (1989) reported very high heritability estimates ($h^2 = 0.5 \pm 0.2$–0.9 ± 0.3) in *M. edulis* based on a nested design involving 27 full-sib families. However, these h^2 values were estimated using a full-sib analysis and may be biased due to maternal or non-additive genetic effects. A high heritability value for a particular trait indicates that a large proportion of the trait is associated with additive gene action, and that genetic progress can therefore be achieved through mass selection (Newkirk, Haley, Waugh & Doyle 1977).

In this study, we consider the influences of h^2 estimates using full- and half-sib analysis to obtain a more accurate estimation of heritability for larval size. To date, there have been no reports on the growth and survival of hatchery-produced seed of *M. chilensis*. The present study represents the first attempt to produce large-scale juveniles under laboratory conditions, and corresponds to a portion of a large-scale breeding programme to improve the growth rate of the Chilean mussel *Mytilus chilensis*.

Material and methods

Mussels and gametes

Adult mussels from a 1999 naturally settled cohort ($N = 1500$; 49.8–77.0 mm) (*Mytilus chilensis* Hupé, 1854) were collected from a mussel farm located in Yaldad Bay, Chiloé Island (43′08′ S; 73′44′ W), southern Chile during October 2001 (before the spawning season). Spawning was conducted over a 2-day period. The mussels were kept out of water for about 4–5 h before attempting spawning by thermal shock. They were rinsed in clean seawater and placed in a 500-L plastic tray with filtered (1-μm) and UV-treated seawater (FSW) at 18 °C. They were then continuously monitored and once a mussel began spawning, it was isolated in a clean bucket containing filtered (1 μm) seawater and allowed to spawn for approximately 60 min. Males and females that had begun spawning within an hour of each other were crossed. The eggs and sperm were held for about 1 h at room temperature (18 °C) before use, to help synchronize egg stage (Scarpa & Allen Jr 1992). A total of 19 male and 95 female mussels were successfully spawned. Sperm from the designated male was added to the oocytes of the designated female in a – 20:1 ratio, and the mixture was gently stirred, maximizing fertilization success while minimizing polyspermy.

Experimental design and larval cultures

Heritability of larval growth rates is based on a balanced nested mating design (Becker 1992). From the spawned parental stock, 95 females and 19 males were chosen at random. Each male was mated to 5 females, involving 95 full-sib families (progeny sharing a common mother and father) and 19 half-sib families (progeny sharing a father but having different mothers) (Falconer 1989). The embryos from each family were placed into a 200 L fibre-glass tank containing 1 μm filtered and UV treated fresh seawater (FSW) 16 ± 1 °C, at a density of 100 individuals per millilitre.

After 24 h, (D-stage larvae) the density was adjusted to five larvae per millilitre. A high cell concentration of the micro algae *Isochrysis galbana* (Parke 1949) and *Chaetoceros gracilis* (Schuett) were used 100 000 cells mL^{-1} as food concentration (Toro & Paredes 1996). Everyday the water in each tank was passed through a 45-micron 'nitex' screen to retain the larvae. Each beaker was rinsed with fresh water followed by seawater. The larvae were then resuspended in FSW and algal food was added daily at the desired cell concentration.

Sampling and data analysis

Samples for analysing larval growth were taken from the larval cultures 10, 25, and from recently settled

spat on NETLON at 40 days after fertilization. At each time, the cultures were well mixed and a sub-sample of 8 mL (approx. 40–60 larvae from each replicate of each full-sub family) was taken from each 200 L tank. Larval samples were preserved in 10% buffered formalin and stored at 4 °C until analysis. Larval samples were videotaped from a plankton decantation chamber in an inverted microscope (ZEISS IM35) fitted with a Pulnex video camera. Selected images were captured for subsequent processing on a computer equipped with an ATI video card. The length (μm) of the larvae was measured, using a Scion Image 3.0b PC Software.

The heritability values and their standard errors were calculated following Becker (1992), using the SYSTAT 5.1 statistical package (Wilkinson 1991). In the model statement: $Y_{ij} = \mu + \text{Sire}_{(i)} + \text{Dam}_{(j/i)} + E_{(ij)}$, log-length was considered to be the dependent variable (Y), μ was the population mean; and the dummy variables were $\text{Sire}_{(i)}$ and $\text{Dam}_{(j)}$, and $i = 1, 2, \ldots, 19$ were the males and $j = 1, 2, \ldots, 95$ were the females nested within each male, and considered to be independent variables, $E_{(ij)}$ was the error term.

The values for larval shell length were normally distributed (Kolmogorov–Smirnov test, $P > 0.05$). However, significant heterogeneity of variances among samples (Bartlett's test, $P < 0.05$) was found. Data transformation did not eliminate heterogeneity of variances. Therefore, all estimates of heritability and their standard errors were calculated from untransformed data. The effect of heteroscedasticity was examined using a non-parametric test (Kruskal–Wallis) to further test for among-family differences in larval shell length (Jones, Bates, Innes & Thompson 1996).

Results and discussion

There was a large quantity of larvae and juveniles from each family, which means that commercial hatchery production of juveniles is possible for the Chilean blue mussel. Table 1 presents the means and the coefficients of variation in shell length of 10-, 25- and 40-day-old mussels. The large coefficients of variation also show that there is a substantial phenotypic variation in shell length among larvae. One-way ANOVA with sampling data as a fixed factor showed that significant growth in shell length occurred during the experiment ($P < 0.05$). Significant differences ($P < 0.001$) were found in the size of the larvae among sires and dams within sires from the 19 half-sib families used (Table 2).

Table 3 shows half- and full-sib heritability estimates in 10-, 25- and 40-day mussels. A heritability estimate indicates the degree to which phenotypic variation is genetically determined. There was significant heritability in shell length at 10, 25 and 40 days, all full-sib heritabilities were large exceeding 1.00, which is theoretically impossible but is commonly recorded in empirical studies owing to the large errors involved in heritability estimation. Furthermore, full-sib analyses inflate the estimate of heritable genetic variation with non-additive genetic, maternal and common environmental components of variation (Falconer 1989). In our study, maternal effects (higher or lower lipid content in the oocyte), dominance variance and common environmental effects may account for much of the difference that inflates our full-sib results of heritability compared with the half-sib heritabilities.

According to Falconer (1989), the heritability is a property not only of the trait but also of the population, of the environment in which the individuals are raised and by how the phenotype is measured. Any change in the above components of variance will thus affect the heritability estimates. In the present study, larvae culture conditions were enhanced using large fibre-glass tanks, which reduces the possibility of temperature and salinity changes in a short period of time; also, according to Jones *et al.* (1996), small buckets used for larval culture affect significantly the larval growth and viability due to the inhibition of normal swimming and swarming behaviour of developing larvae.

Heritability estimates in several bivalve larval stages have been reported. For *Crassostrea gigas* (Lannan 1972), estimates of $h^2 = 0.31$ have been obtained for larval survival, and for various spat traits at 12 months, h^2 ranges between 0.31 and 1.17. Another study (Longwell & Stiles 1973) reports an h^2 of 0.24 for the growth rate of *Crassostrea virginica* (Gmelin) larvae at 14 days. Several other studies also describe heritabilities for larvae growth rates in *C. virginica* by analysing full- and half-sib families; these estimates ranged between 0.25 and 0.71 (Newkirk *et al.* 1977; Losee 1978). Another study based on a nested design reported heritabilities for larval growth rate in *M. edulis* ranging between $h^2 = 0.5 \pm 0.2$ and 0.9 ± 0.3 (Stromgren & Nielsen 1989). These estimates agree with the results obtained in the present study and it seems that a large portion of these traits show additive genetic variation.

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Table 1 *Mytilus chilensis*. Mean shell length (μm) (± SE) and coefficient of variation (CV%) at three different stages of their early life cycle

	Age (days)		
Parameter	**10**	**25**	**40**
Half-sib families	16	12	12
Full-sib families	80	37	40
Number of larvae	2400	914	802
Mean shell length	148 (0.47)	253 (1.99)	1133 (12.31)
CV	15.6	23.7	30.8

Table 2 *Mytilus chilensis*. Analysis of variance for shell length of 10, 25 days larvae and 40-day-old-spat (*df*, degree of freedom; MS, mean square)

Age group/source	***df***	**MS**	***F***	***P***
10-day-old larvae				
Among sires	15	10 218.63	48.09	0.001
Among dams within sires	64	3445.93	16.22	0.001
Error	2320	212.45		
25-day-old larvae				
Among sires	11	99 633.40	75.21	0.001
Among dams within sires	25	51 731.11	39.05	0.001
Error	877	1324.71		
40-day-old spat				
Among sires	11	2 550 423.06	105.82	0.001
Among dams within sires	28	693 163.69	28.76	0.001
Error	762	24 100.29		

Table 3 *Mytilus chilensis*. Heritability estimates of larval growth of *Mytilus chilensis* at two larval stages (10–25 days) and recently settled spat (40 days) using a nested design. Age (10, 25 and 40 days); number of individuals (*N*), heritability estimates for dam (h_d^2) and sire (h_s^2) components and estimated mean change in growth (EMC = $i\ h_s^2$ SD) (i = 10% of selected animals) (Falconer 1989)

Age group	***N***	$h_d^2 \pm$ **SE**	$h_s^2 \pm$ **SE**	**EMC (%)**
10-day-old	2400	1.17 ± 0.25	0.51 ± 0.20	20.06
25-day-old	914	2.02 ± 0.56	0.38 ± 0.33	33.66
40-day-old	802	1.51 ± 0.21	0.84 ± 0.45	18.30

Several studies on bivalves carried out in the laboratory have shown that there is little or no correlation between shell growth of larvae and juveniles (Stromgren & Nielsen 1989; Hilbish, Winn & Rawson 1993), but it is not known if there is a lack of correlation between larval and shell growth in natural populations. However, we expect that heritability values between these two life cycle stages could be disconnected; studies have shown that heterozygotes have a lower viability than homozygotes during the larval stage (Mallet & Haley 1983; Mallet, Zouros, Gartner-Kepkay, Freeman & Dickie 1985) thus changing the allelic frequencies.

Heritability values of 0.20 or larger indicate that genetic progress can easily be achieved through the application of selective breeding programmes (Newkirk *et al.* 1977; Falconer 1989). In the light of the difficulty in selecting individuals that grew fast, as larvae to breed from, the application of family selection instead of individual selection was the option used in the present study. Using the most conservative heritability estimates (sire component) obtained for larval growth in *M. chilensis*, and applying a selection intensity of 1.755 that is equivalent to select the higher 10% of the population for the trait, the estimated mean change in larval shell growth ranges

between 18.3% and 33.6% per generation. Other studies (Mallet *et al.* 1986; Stromgren & Nielsen 1989) report the same conclusion for the blue mussel *M. edulis*. Shell length growth rate is closely correlated with meat weight increase in *M. edulis* juveniles (Nielsen 1985), suggesting that selection for shell growth will at the same time increase the meat growth of mussels.

In common with previous studies of bivalve larvae and spat, our experiments demonstrate a significant genetic variation in shell length, which may have implications for the biology of natural populations of *M. chilensis* as well as for commercial culture.

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The genetic structure of *Mytilus chilensis* (Hupé 1854) populations along the Chilean coast based on RAPDs analysis

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Abstract

The Chilean blue mussel *Mytilus chilensis* is an important commercial species. However, little has been published on the population genetics of this species, despite the need to implement management and conservation policies. Randomly amplified polymorphic DNA-polymerase chain reaction analysis was used to estimate genetic variation within and between eight natural populations along the whole range of its Chilean natural distribution (ca. 1900 km from Arauco (VIII Region) to Punta Arenas (XII Region)). The values of Nei's unbiased genetic distance, D (0.030–0.107), among populations were small, despite the large geographic separation. A mantel test using 50 000 randomizations showed evidence for a significant correlation ($r = 0.74$, $P < 0.05$) between genetic and geographic (coastal) distance. Punta Arenas population was the most genetically differentiated from the others, although the scale of differentiation was not large ($D = 0.076$–0.107). The levels of gene flow ($Nm = 1.55$) found in this study prevent differentiation among populations by genetic drift. This is the result of the long-lived planktotrophic larvae of *M. chilensis*, which provides this species with considerable dispersal ability throughout its range, which is favoured by the ocean currents along the Chilean coast. A restricted larval dispersal towards the north due to the Cape Horn Current derived from the West Wind Drift could be the cause of the higher genetic differentiation of Punta Arenas population from the northern populations. For management purposes of the *M. chilensis* fishery, the results provide no evidence for discrete stocks, with the possible exception of the Punta Arenas population. The present study provides the baseline data in order to continue further characterization of these mussel populations, considering the great increase in aquaculture of this species.

Keywords: genetic structure, *Mytilus chilensis*, RAPDs

Introduction

Chilean aquaculture has increased in production over the last 10 years, from 116 281 t in 1992 to 617 301 t in 2002 (SERNAPESCA 2003). Nearly 78% of aquaculture production in 2002 was finfish (salmon), about 10% was molluscs (62 943 t) and the rest was seaweed. The Chilean blue mussel (*Mytilus chilensis*, Hupe 1854), which is an economically important resource in southern Chile, contributed 66.3% of the total mollusc aquaculture (41 797 t). Its culture began in 1943 in Chiloé Island, southern Chile (Osorio, Atria & Mann 1979) and the aquaculture production, because of the increase in exports, rose from 3864 t in 1993 to 41 797 t in 2002 (SERNAPESCA 2003).

Despite interest from fishers and industry in mussel aquaculture, little is known about the biology and the population genetics of the species. Winter, Toro, Navarro, Valenzuela and Chaparro (1984) determined that there was an annual reproductive cycle, with spawning occurring during spring and summer. Toro and Sastre (1995) and Toro, Alcapán, Vergara and Ojeda (2004) have shown that *M. chilensis* possesses a 45-day planktotrophic larval stage. The species thus has the potential for long-distance dispersal over of hundreds of kilometres along the Chilean coast.

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However, oceanographic features (Strub & Mesías 1998) and human-mediated dispersal may have heavily affected the levels of genetic variation in several southern Chilean stocks. Most of the natural seed supply for the Chilean blue mussel aquaculture is based on the Yaldad Bay natural mussel beds (Winter *et al.* 1984), which may have been used for restocking of several natural populations in southern Chile.

From the scientific literature, population genetic studies involving the Chilean blue mussel have mainly focused on elucidating the distribution of the *M. edulis* species complex (Koehn 1991; McDonald, Seed & Koehn 1991; Toro 1998). The Chilean blue mussel has been recently subjected to a taxonomic revision, using modern molecular techniques; however, at this stage results are still controversial. While some authors describe the Chilean mussel as *M. edulis* related (Koehn 1991; McDonald *et al.* 1991; Toro 1998), other authors report *M. galloprovincialis* in central Chile (Daguin, Bonhomme & Borsa 2000). Meanwhile, until further studies show more evidence we found the use of *M. chilensis* (Hupé 1854) appropriate.

Recently, DNA-based molecular methods have provided new tools for studying the population genetic structure of organisms. Random amplified polymorphic DNA (RAPD) (Williams, Kibelik, Livak, Rafalski & Tingey 1990; Yeh & Boyle 1997) allows the detection of multilocus genetic variation using short primers of arbitrary sequence. In contrast to other molecular techniques, the RAPD technique is very easy to perform and requires no prior knowledge of the genomes under investigation. In this paper, we examine the genetic structure of *M. chilensis* collected from eight widely separated (up to 1900 km) sites along the Chilean coast, using RAPD-PCR (polymerase chain reaction) to establish the extent of gene flow and levels of genetic differentiation.

Material and methods

Samples of mussels were collected by dredging or by diving at eight localities extending over 1900 km along the Chilean coast (covering the whole range of the species natural distribution) from Arauco (VIII Región) to Punta Arenas (XII Región) (Fig. 1). Forty mussels (45–82 mm from each population) were delivered alive to the laboratory where they were immediately dissected, and a small section of tissue (mantle border) was stored in a 1.5-mL Eppendorf tube with 95% ethanol for subsequent DNA extraction and amplification analysis. Approximately

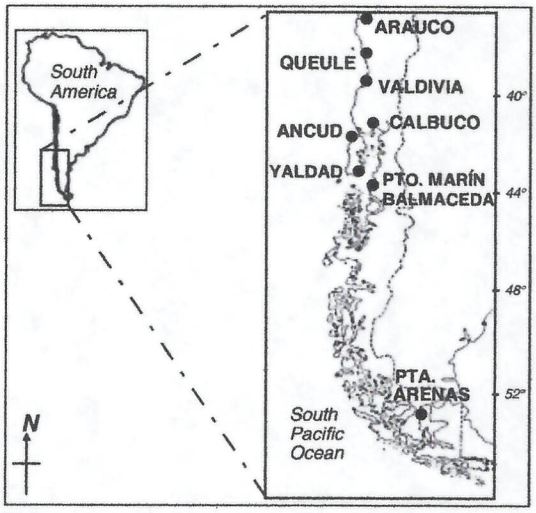

Figure 1 Sampling locations of the eight natural populations of the Chilean blue mussel (*Mytilus chilensis*) (•).

50–100 mg of each fixed mantle-edge tissue was coarsely chopped and digested in 500 μL of lysis buffer (50 mM Tris-HCL (pH 8.0); 1.0% SDS (sodium dodecyl sulphate); 25 mM EDTA (ethylenediaminetetraacetic acid)) with 200 μg proteinase K at 37 °C overnight. The solution was then extracted once with 500 μL of an equal volume of phenol–chloroform–isoamyl alcohol (24:24:1) followed by ethanol precipitation. The extracted DNA was resuspended in 200 μL of ultra-pure sterile distilled water (Toro 1998).

Random amplified polymorphic DNA amplifications using the PCR were carried out in a 25 μL volume containing 50 ng of genomic DNA, 10 × reaction buffer, 25 mM $MgCl_2$, 0.5 mM each of dNTP, 10 mM primer and 1 U of Taq polymerase (Invitrogen, Carlsbad, CA, USA). A total of 49 primers of arbitrary sequence and 10 bp in length were used. The PCR reaction was carried out in a Thermolyne (Amplitron II; Dubuque, IA, USA) thermocycler with the following program: an initial denaturation step of 94 °C for 15 s followed by 36 cycles of denaturation (94 °C, 30 s), annealing (35 °C, 30 s) and extension (72 °C, 2 min) with a terminal extension of 72 °C for 2 min. A negative control with template DNA replaced by water was performed for each set of amplifications in order to verify the absence of contamination. In order to ensure reproducibility, RAPD patterns were tested for the effect of Mg^{2+}, DNA, dNTP and Taq polymerase concentrations, which can affect the size and the number of amplified products. Also, PCR for each DNA sample was replicated at least twice. Visualization of the PCR products was by gel

electrophoresis on 1.5% agarose (Invitrogen High Resolution). The gels were immersed in 0.5× Tris-borate-EDTA (TBE) buffer (44.5 mM tris base, 44.5 mM boric acid, 1 M EDTA pH 8) at 65 V for 150 min. Two lines of 100 bp molecular ladder (Promega, Madison, WI, USA), which provided intervals every 100 bp from 100 to 3000 bp, were included on every gel to allow for fragment comparison. The gels were stained for 20 min in ethidium bromide (0.5 $\mu g\ mL^{-1}$), destained for 10 min and photographed under a ultraviolet (UV) transilluminator with a Sony digital camera (Tokyo, Japan). The number of bands greater than 100 bp but less than 2000 bp produced for each sample for each primer was then scored for as a presence or absence using the molecular ladder as a reference. The phenetic analysis of the data was carried out using the unweighted pair group method with arithmetic mean (UPGMA), using the POPGENE version 2.6.2 program (Hood 2004).

A shell morphology analysis was carried out on three populations, Punta Arenas, Yaldad and Pto. Marín Balmaceda, using eight morphometric variables (Fig. 2) in order to reveal the existence of any shell phenotypic differences caused by distinct environmental selection pressures among these localities. Each character was standardized using $\log_{10}$ and divided by $\log_{10}$ of shell length (Mallet & Carver 1995). A canonical discriminant analysis was used to derive a canonical function that separated the three mussel populations, using Systat V5.1 (Wilkinson 1991).

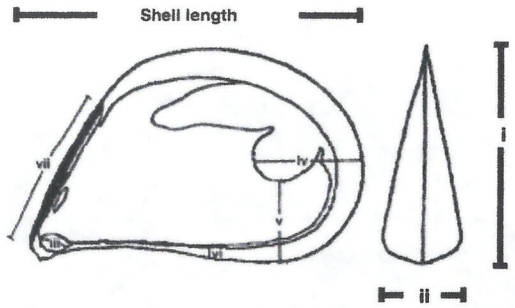

Figure 2 The seven morphometric mussel shell characters used in the canonical discriminant analysis: (i) shell height, (ii) shell width, (iii) length of anterior muscle scar, (iv) distance between the anterior edge of the posterior adductor muscle scar and the posterior margin of the shell, (v) distance between the ventral edge of the posterior adductor muscle scar and the ventral shell margin, (vi) distance between the pallial line and the ventral shell margin midway along the shell and (vii) distance between umbo and posterior end of the ligament. (Modified from McDonald *et al.* 1991.)

Results

Only five primers (Table 1) generated appropriate amplification patterns (clear bands and reproducibility) and were used in the analysis among populations. The number of RAPD bands generated per primer varied between 8 ((MC03 primer) and 14 (MC37 primer). A total of 54 reproducible bands were scored, ranging in size from 100 to 2000 bp. The mean number of alleles per locus ranged from 1.00 to 1.99 among the eight populations of *M. chilensis*. Genetic distances, *D*, among eight populations of *M. chilensis* were on the whole small, ranging from 0.030 to 0.107 (Table 2). A Mantel test using 50 000 randomizations showed evidence for a significant correlation ($r = 0.74$, $P < 0.05$) between genetic and geographic (coastal) distance (Table 2).

The migration rate ($Nm = 0.5\ (1 - Gst)/Gst$) value suggested a theoretical exchange of $Nm = 1.55$ individuals per generation between a pair of samples.

A cluster analysis based on the unweighted pair group method and Nei's co-efficient of unbiased genetic identity (Nei 1978) showed that *M. chilensis* populations were genetically quite similar with similarity values among populations ranging from 0.899 to 0.969 (Fig. 3). Punta Arenas population, located at the Magellanican Channel (Fig. 1), was genetically the most distinct from the other seven northern populations, with similarity values ranging from 0.899 to 0.926 (Fig. 3). Also, the eight shell characters used in the discriminant function were able to distinguish between the three populations of mussels (Wilk's λ, $P < 0.05$). The canonical variates of the shell morphometric analysis (Fig. 4) showed that Punta Arenas population was phenotypically the most distinct among the three mussel populations analysed.

Discussion

Analysis of the RAPD-PCR genetic data revealed very low genetic differentiation among eight populations

Table 1 Sequences of the RAPD primers used in this study

Primer	Sequence 5′ → 3′	Total no. of bands scored
MC03	CCggCCCCAA	08
MC17	gggCAATgAT	10
MC27	ATgTgTTgCg	09
MC29	AgCAgCgTgg	13
MC37	AACgggCAgC	14

RAPD, randomly amplified polymorphic DNA.

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Table 2 Relationship between geographic distances (km) and Nei's unbiased genetic distance *D* (in parentheses) separating eight populations of *Mytilus chilensis*

Population	Arauco	Queule	Valdivia	Calbuco	Ancud	Yaldad	Pto. Marín Balmaceda	Punta Arenas
Arauco	–							
Queule	211 (0.0580)	–						
Valdivia	285 (0.0844)	57 (0.0608)	–					
Calbuco	492 (0.0712)	272 (0.0574)	200 (0.0468)	–				
Ancud	527 (0.0793)	300 (0.0308)	230 (0.0576)	50 (0.0526)	–			
Yaldad	683 (0.0738)	460 (0.0624)	380 (0.0624)	188 (0.0605)	150 (0.0444)	–		
Pto. Marín Balmaceda	761 (0.0571)	537 (0.0733)	450 (0.0685)	241 (0.0803)	231 (0.0715)	110 (0.0558)	–	
Punta Arenas	1787 (0.1065)	1542 (0.0964)	1480 (0.0831)	1280 (0.0811)	1279 (0.0809)	1140 (0.0763)	1040 (0.1050)	–

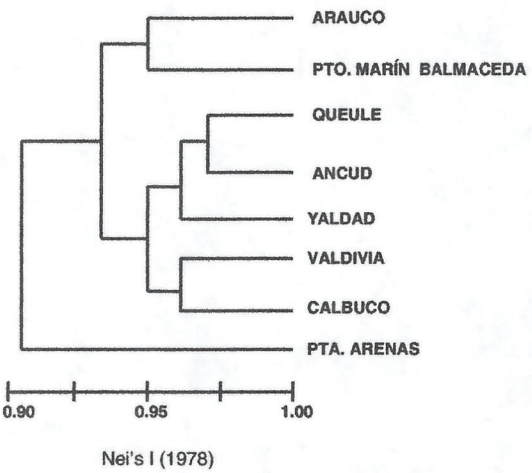

Figure 3 A phenetic dendrogram showing the relationship of eight populations of the Chilean blue mussel (*Mytilus chilensis*), based on randomly amplified polymorphic DNA (RAPD) data, using the computational package Popgene version 1.31.

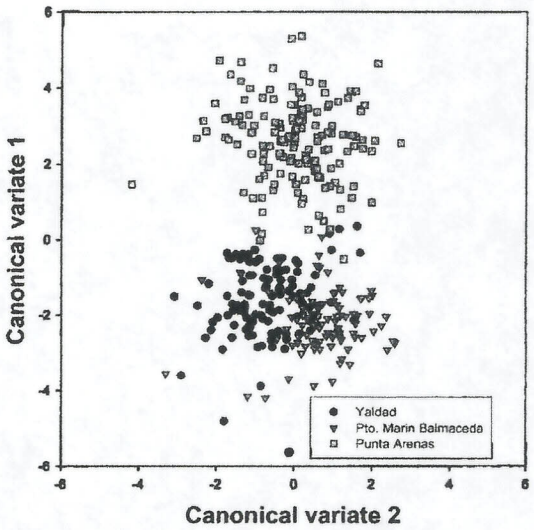

Figure 4 First and second canonical variates of shell morphometric data from mussel populations sampled in southern Chile (Yaldad, Pto. Marín Balmaceda and Punta Arenas.

of *M. chilensis* sampled over distances greater than 1900 km along the Chilean coastline. This indicates levels of gene flow that allow these populations to act as a single interbreeding population. However, one population sampled in the Magellanican Channel, Punta Arenas, was genetically distinct from the others, although the scale of differentiation was not large ($D = 0.032–0.048$) (Fig. 3). At the same time, canonical discriminant analysis of morphometric variation indicates that there is some overlap in canonical variates, but most individuals do provide non-overlapping, discrete clusters, especially the Punta Arenas population, which showed that the shell morphology of this population is the most distinct among the three populations analysed (Fig. 4). The genetic differentiation, even low, could be the result of the West Wind Drift (WWD) and the Cape Horn Current (CHC) (Fig. 5), which precludes larval transport towards the northern mussel populations, while the shell morphology differentiation could be due to a different set of environmental variables that are affecting the most southern mussel population analysed. The morphological distinctness among the three mussel populations is not due to differences in shell size of the populations sampled, because the size ranges of the individuals sampled for the study were restricted to adult size and any further differences in size were corrected by the log-transformed length standardization.

Theory predicts that a global value of $Nm > 1$ prevents random differentiation by genetic drift (Slakin 1987). Thus, the migration rate reported here ($Nm = 1.55$) suggests that some deterministic factors

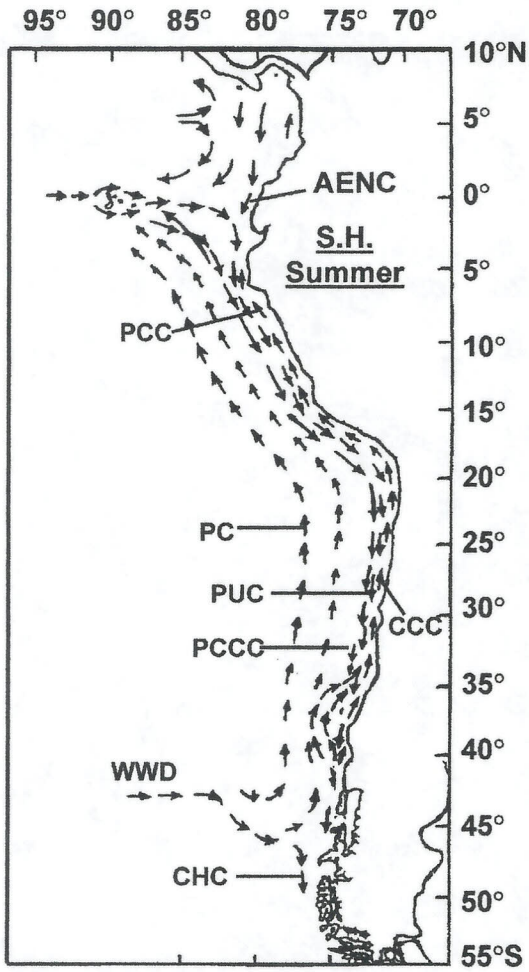

Figure 5 Principal sea currents (superficial and deep) along the Chilean coast during winter and summer. Main currents: West Wind Drift (WWD), Perú Current (PC), Perú-Chile Countercurrent (PCCC), Poleward undercurrent (PUC), Peru Coastal Current (PCC) Chile costal current (CCC) and Cape Horn Current (CHC). (Modified from Strub & Mesías 1998.)

are involved in the among-population differentiation of *M. chilensis*. These results indicate that the long lived (45 days) planktonic larvae of *M. chilensis* (Toro *et al.* 2004) provide the species with considerable dispersal ability, which is not affected but enhanced by oceanographic features such as the WWD and several coastal currents along the Chilean coastline (Strub & Mesías 1998; Camus 2001) (Fig. 5). The main cluster with low genetic differentiation between such populations as Queule, Ancud, Yaldad, Valdivia and Calbuco (Table 2) suggests that larval transport along the coast does take place, perhaps in a stepping-stone fashion, with each generation of larvae dispersing short distances along the coast. Interpopulational genetic homogenity has been described for other marine invertebrates along the Chilean coastline (Gallardo & Carrasco 1996; Toro & Aguila 1996; Gallardo, Peñaloza & Clasing 1998). Mollusc species with extended larval planktonic phases are generally thought to disperse further and have more gene flow, larger geographic ranges, lower levels of genetic differentiation among populations and high levels of genetic variation within populations (Scheltema & Williams 1983; Waples 1987; Williams & Benzie 1993; Palumbi 1995). The results from the present study are consistent with the life-history features of the chilean blue mussel with a very prolonged larval pelagic life span (40–45 days), which suggest a great potential for genetic homogenization over large geographical distances. However, we must keep in mind that this is an economically important resource and also a farming species. Most of the populations within the northern range of the species distribution have suffered strong declines due to overfishing (Sanchez 2002), which caused in some locations the restocking with mussels from other local populations, usually from Yaldad population, which is also the most important source of mussel natural 'seed' (juveniles) for aquaculture activities (Winter *et al.* 1984; Navarro & Gutierrez 1990). This human-mediated dispersal (gene flow) may have affected the levels of genetic variation in several northern stocks.

The management of the *M. chilensis* fishery, according to our results, can be independent of macrogeography, with the only exception of the Punta Arenas population. The management should be focused on a smaller spatial scale, dealing with differentiation of populations caused by environmental factors.

Acknowledgments

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