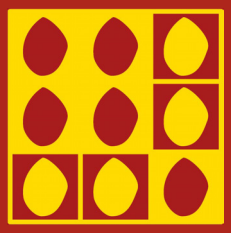
Spatial arrangement of genetic variation in the marine bivalve *Macoma balthica* (L.)



Pieternella C. Luttikhuizen

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voor mijn moeder Christien

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CHAPTER ONE

General introduction

Intraspecific genetic variation forms the raw material for evolution. Without heritable variation, evolution by means of natural selection would not occur. The spatial separation of genetic variation has two sides: it can maintain variation for selection, and it can also be the result of selection. This thesis addresses the question: '*What does a life history of external fertilization and pelagic larval dispersal imply for the spatial arrangement of intraspecific genetic variation?*'

The spatial arrangement of intraspecific genetic variation is partly dependent on the amount of genetic exchange (gene flow) between different spatial units (*e.g.*, populations). This is the case for both neutral genetic variation and genetic variation under the influence of selection. Traditionally, isolation was viewed by many authors, of whom Mayr was the main advocate (*e.g.* Mayr 1942, 1963), as a necessary first step in differentiation. This rather extreme conception was relaxed in later years into one of degree: how *much* gene flow is sufficient to prevent differentiation (*e.g.* Ehrlich and Raven 1969, Slatkin 1987, Lenormand 2002)? If all taxa were linearly arranged along a continuous gradient describing the amount of gene flow, marine broadcast spawning invertebrates would be placed near one extreme end of the gradient, with their high levels of genetic exchange. This makes these invertebrates useful for testing predictions of population genetic models in relation to gene flow. This thesis aims to do this, with particular emphasis on the difference between differentiation in neutral and selective variation in the face of gene flow. The bivalve *Macoma balthica* (L.) or Baltic clam was chosen as the model species for this, as it has been previously well studied in marine benthic ecology, with a focus on its population dynamics, community ecology, population structure and evolutionary ecology (*e.g.* Lammens 1967, Hulscher 1973, Beukema 1993, Hummel *et al.* 1995, Honkoop *et al.* 1998, Edelaar 2000, De Goeij *et al.* 2001, Van der Meer *et al.* 2001, Drent 2002, Hiddink and Wolff 2002).

In this thesis the term 'population' is used throughout. This suggests there is a scale at which a species can be surveyed to reveal a discrete set of populations that have complete mixing within them and some fixed amount of exchange between them. In reality, intraspecific structure tends to be much more complex. The species studied here can be distributed continuously (*e.g.* across large stretches of mudflat such as those in the Wadden Sea) or discontinuously (*e.g.* in river estuaries on a predominantly rocky shore coastline). It is nevertheless insightful to approach population structure with an island-mainland model that assumes discrete populations. Such a model is easier to deal with mathematically than more complex models, and, in the absence of prior knowledge, it can serve well as the simplest possible alternative to a null hypothesis of the absence of population subdivision.

Genetic variation within and among populations

Genetic variation arises due to mutation. In most species, there is abundant spatial differentiation in gene frequencies, and two of the main aims of evolutionary biology are to understand how spatial genetic differentiation originates and how it is maintained. The reverse is also true; spatial separation is one of the forces that helps to maintain genetic variation. Different forces are continually at work, some tend to produce differentiation, while others tend to produce homogenization. The amount of spatial differentation that results depends on the balance between these forces. The nature of the forces involved in local differentiation differs between so-called neutral and selective genetic variation.

The distinction between neutral and selective (non-neutral) variation can be problematic due to the transition zone between them. The crucial difference between the two, as applied in the context of functional biological questions, is that in selective variation, the alternative genetic variants are directly associated with a difference in phenotype and functionality (*e.g.*, biochemically, ecologically). For neutral variation this is not the case. While some authors rank for instance silent substitutions in protein coding DNA and codon bias under neutral variation because it bears no direct relationship to the biological functioning of the individual, others may view these examples as selective variation, *e.g.* when trying to explain GC content of DNA. In this thesis, silent substitutions in mitochondrial protein coding DNA as well as allozyme variants are treated as neutral genetic variation, although the assumed neutrality of the allozymes is discussed in chapter 9. Non-neutral variation studied in this thesis is the globosity of shells.

Neutral variation

If populations were unlimited in size and all individuals reproduced to the same extent, the frequency of alternative, selectively neutral variants would not change (other than by mutation). But because the number of effectively reproducing individuals in a population is finite, gene frequency changes occur. This stochastic process is called genetic drift. The amount of neutral variation contained at equilibrium within populations increases with effective population size (N_e) (Kimura and Ohta 1971). The amount of variation between populations increases with isolation (i.e., reduced gene flow), because isolation creates independent processes of genetic drift within the isolated populations.

Selected variation

The factors mentioned above for neutral variation are also at work on differentiation of selective variation. On top of that, selection can have an effect. Within populations, the amount of selective variation can increase relative to neutral expectation (*e.g.* balancing selection) or it can decrease relative to neutral expectation (*e.g.* stabilizing and purifying selection). The amount of differentiation in selective

genetic variation between populations increases when the selective regimes differ between them.

Neutral vs. non-neutral genetic markers and gene flow

When neutral and selective genetic variation are considered at the same spatial scale, they are subject to the same forces of gene flow and genetic drift, while the latter type of variation is also influenced by selection. Whether selection has a significant effect can be evaluated by studying the distribution of neutral molecular variation as a prediction of what would be expected in the absence of selection (Felsenstein 1986, Lande 1992, Spitze 1993). Standardized measures of the portion of the total genetic variation that is between populations are F_{st} for neutral marker loci (Wright 1951, Nei 1987) and Q_{st} for quantitative genetic variance (Spitze 1993) (see chapter 3 for details). The neutral expectation for Q_{st} is that it should equal F_{st} (Wright 1951, Lande 1992). This thesis uses this prediction to infer whether selection affects the spatial differentiation in globosity of *M. balthica* shells (chapter 3).

A related question is to what extent the degree of neutral marker divergence predicts the degree of divergence in selective genes (Butlin and Tregenza 1998, Merilä and Crnocrak 2001, McKay and Latta 2002). As the two types of variation are partly under the influence of similar forces, with possible added effects of linkage disequilibrium, the amounts of spatial differentiation in neutral and selective genetic variation are expected to be correlated. As a consequence, an absence of neutral marker differentiation between populations is at times translated without further study into the conclusion that phenotypic differentiation at the same scale must be due to environmental (phenotypic plasticity) rather than genetic effects. This has happened both in studies of *M. balthica* (Meehan 1984) and of other bivalves (*e.g. Phacosoma japonicum*: Sato 1996, *Donax serra*: Soares *et al.* 1998, *Placopecten magellanicus*: Herbinger *et al.* 1998). The general discussion (chapter 9) will return to this topic.

Broadcast spawning marine invertebrates

Many marine taxa have the potential for large-scale dispersal. Although in some species adults are mobile (*e.g.* fish, mammals, many polychaetes, even some bivalves), for many taxa movement is restricted to larval stages. The model species studied, the bivalve *Macoma balthica*, is one of a group of marine invertebrates that have pelagic larval dispersal and sessile or semi-sessile adults. The larvae tend to be so small that the viscosity of the water column in the sea should prevent them from having much influence on their position. However, marine invertebrate larvae have more control of their direction of movement and destination than might be expected from their small size. They have evolved structures and sensory systems that enable them to influence their vertical position in the water column and their timing with respect to tidal cycle and time of day. In this way they have been shown to have

some control over dispersal distance and direction, settlement timing and location, and predator avoidance (*e.g.* Bayne 1965, Ahn *et al.* 1993, Snelgrove *et al.* 1999). Because of the small size of pelagic larvae and the nature of the marine habitat, it is hard to study dispersal distance directly (Levin 1990, McEdward 1995).

Broadcast spawning is defined as the reproductive strategy in aquatic organisms in which both males and females spawn their gametes into the water column, where fertilization takes place. This is not to be confused with free spawning, in which only males spawn freely and eggs are fertilized either internally or on some external surface of the female (Giese and Kanatani 1987, Levitan 1998). It is a general assumption in biology that all eggs are fertilized because sperm are superabundant. This assumption is not valid for external fertilization in the sea, where sperm limitation may occur (reviewed in Levitan 1998, Yund 2000). While in taxa with internal fertilization, sperm competition has received predominant attention, in broadcast spawners the relative and absolute abundances of sperm and eggs may also result in egg limitation, sperm limitation or egg competition (Levitan 1998). In contrast to the large distances over which pelagic larval dispersal takes place (kilometers to hundreds of kilometers), external fertilization tends to take place between males and females that are situated near each other (meters apart, *e.g.* Pennington 1985, Levitan 1991, Babcock *et al.* 1992, Levitan and Young 1995).

The high potential for gene flow associated with pelagic larval dispersal predicts populations of broadcast spawning marine organisms to be unstructured in neutral molecular marker loci over large geographic scales. This prediction is confirmed by many studies (*e.g.* Hellberg 1996, Karl *et al.* 1996, McFadden *et al.* 1997, McGormack *et al.* 2000), but several marked exceptions exist (*e.g.* Skibinski *et al.* 1983, Reeb and Avise 1990, Benzie and Williams 1997, Barber *et al.* 2000). Moreover, the level of population subdivision may be expected to be correlated with larval dispersal ability, and to range from complete panmixia for species with extremely long-lived pelagic larvae to fine-grained differentiation for species with limited dispersal abilities. Reviews do observe this relationship to be significant, but in many cases dispersal ability is only a weak predictor of genetic structure (Burton 1983, Hellberg 1994, Bohonak 1999, Grosberg and Cunningham 2001).

The Baltic clam *Macoma balthica*

The Baltic clam *Macoma balthica* (L.) (Bivalvia: Tellinidae) is a burrowing bivalve of intertidal and shallow subtidal soft sediments of the northern hemisphere. It originated in the North Pacific (Meijer 1993), but now has a circumpolar distribution (Fig. 1). The present-day Arctic distribution of *M. balthica* appears to be discontinuous on both the American and the Eurasian sides (Lubinsky 1980, Meehan 1984, 1985, 1989). Distributions on either side of the Atlantic go as far south as Virginia, USA, and the Gironde estuary, in southwest France. In the Pacific Ocean the distribution of *M. balthica* reaches down to Japan and Oregon, USA. The

occurrence of the species in California is thought to result from introduction due to human activity in the late 19th century (Meehan 1989).

The life cycle of NW European *M. balthica* starts with external fertilization in early spring, after which the pelagic larvae remain in the water column for two to five weeks (Caddy 1967). Primary settlement is in the high intertidal zone, and in the winter of their first year, the juveniles may undertake secondary migration using a mucoid thread for support in the water column (Beukema and De Vlas 1989, Beukema 1993). Some individuals can produce gonads and spawn after one year, but most only commence spawning at two years of age (Lammens 1967). Like many bivalves, *M. balthica* has a non-determinate life expectancy and the lifespan varies

Fig. 1 - Geographic distribution of *Macoma balthica* (black areas) (after Meehan 1984, 1985, and Lubinsky 1980).

with location. In the NE Atlantic, *M. balthica* typically reaches 5-8 years (Vogel 1959, Lammens 1967), while extremely long life spans of 25 years and more were recorded at 35 m depth in the Tvärminne area, Finland (Segerstråle 1960). Phenotypically, *M. balthica* shows geographic patterning at a range of different scales, from differences in shell shape between the American and European Atlantic coasts (Beukema and Meehan 1985) to differences in burrowing depth at different tidal levels, less than 600 m apart (Hulscher 1973).

Predation is a hazard to *M. balthica* throughout its life cycle, with predators ranging from ctenophores feeding on pelagic larvae, amphipods eating newly settled recruits (*e.g.* Elmgren *et al.* 1986), probing birds that forage on the entire adult animal (*e.g.* Zwarts *et al.* 1992) to siphon nippers that graze on the part of the siphons that protrude from the sediment surface (*e.g.* De Vlas 1985). As *M. balthica* inhabits a range of coastal zones from the high intertidal zone down into the shallow subtidal (but also some deeper subtidal areas, *e.g.* in the Baltic Sea) the abiotic factors and predators it faces can vary greatly at short spatial scales. Abiotic influences such as sun, rain, air temperature and river outflow increase the temperature and salinity variation with position across the intertidal zone (Sanders *et al.* 1965, Sanders 1968). Predation risk can vary at even smaller spatial scales. For example, *M. balthica* that find themselves in puddles on mudflats have the advantage of being able to feed even when the tide is out, but the disadvantage that the detection mechanism of one of their avian predators (red knots *Calidris canutus*) works better in wet than in dry sediment (Piersma *et al.* 1998).

Unlike its habitat, *M. balthica*'s neutral genetic variation is expected to be undifferentiated over large spatial scales because of its long pelagic larval phase. Genetic variation in *M. balthica* has been studied by several authors since protein electrophoresis became accessible for the study of allele-based enzyme polymorphisms (allozymes) by Harris (1966) and Lewontin and Hubby (1966). Table 1 gives an overview of the allozyme studies that included *Macoma* spp.. The reasons for undertaking these studies differed over time. The first study tried to use allozyme variation to shed more light on taxonomic status within the *Macoma* genus (Reid and Dunhill 1969). Later research studied allozyme variability in relation to habitat characteristics (*e.g.* Levinton 1973, 1975, Green *et al.* 1983, Hummel *et al.* 1996a). The most recent focus has been population subdivision. The overall conclusion was that *M. balthica* is in places more subdivided than was expected considering its pelagic larval dispersal potential. Nevertheless, large regions over which the *M. balthica* populations were unstructured in allozymes were identified, notably the southern parts on either side of the Atlantic Ocean: up to 45ºN on the American side (Meehan 1984, 1985) and up to NW Norway on the European side (Hummel *et al.* 1995, 1997). The most striking examples of significant population differentiation are between the western and eastern Atlantic coasts (Meehan 1989) and between the Baltic Sea and the rest of Europe (Väinölä and Varvio 1989, Hummel *et al.* 2001). The levels of allozyme differentiation are so large that separating *M. balthica* into two

Table 1 - Chronological overview of allozyme studies on *Macoma* spp. and *M. balthica* (L.); n_{loci} = number of loci, n_{poly} = number of polymorphic loci, $n_{samples}$ = number of samples, n_{ind} = number of individuals per sample, het. def. = heterozygote deficiency, intersp. = interspecific comparison, n.m. = not mentioned, n.a. = not addressed. If an indication for selection was observed, the abbreviation listed signifies the concerned locus.

study area	n_{loci}	n_{poly}	n_{samp}	n_{ind}	type of study	het. def.	pop. subd.	indication for selection	reference
NE Pacific	1?	1?	8	5	intersp.	n.m.	n.a.	n.a.	Reid and Dunhill 1969
NW Atlantic (Long Island/ Connecticut)	2	2	6	55-71	intersp.	n.m.	n.a.	yes (overall)	Levinton 1973
NE Pacific (San Juan Islands)	2	2	19	12-105	intersp.	n.m.	n.a.	no	Levinton 1975
Baltic Sea/ Wadden Sea	4	4	20	36-506	pop. subd./ habitat cline	no	yes	no	Nilsson 1987
Baltic Sea	13	9	3	42-184	pop. subd.	n.m.	yes	no	Nilsson 1985
American Arctic (Hudson Bay)	22	6	2	~77	habitat cline	yes	no	no	Green et al. 1983
NE/NW Atlantic	5	5	10	12-64	pop. subd.	yes	yes	no	Meehan 1984, 1985
American Arctic (Hudson Bay)	2	1	2	87-88	habitat cline	n.a.	n.a.	yes (*Akp2*)	Singh and Green 1986
NE Pacific/ NW Atlantic	11	11	3	104-202	pop. subd.	n.m.	yes	no	Meehan 1989
Baltic Sea, Quebec, NE Norway, NE Atlantic	13	13	23	~100	pop. subd.	n.m.	yes	no	Väinölä and Varvio 1989

Table 1 (continued)

study area	n_{loci}	n_{poly}	n_{samp}	n_{ind}	type of study	het. def.	pop. subd.	indication for selection	reference
NE Atlantic (Gironde-Netherlands)	7	4	7	38-40	pop. subd.	yes	no	yes (*Lap*)	Hummel et al. 1995
NE Atlantic (Netherlands)	7	4	8	34-80	habitat cline	yes	no	no	Hummel et al. 1996a
NE Atlantic/ Russian Arctic	7	6	14	40-80	pop. subd.	yes	yes	no	Hummel et al. 1997, 1998a
Russian Arctic (White Sea)	7	5	3	80	habitat cline	yes	yes	yes (*Idh*1)	Hummel et al. 1998b
NE Pacific (Puget Sound)	5	n.m.	3	n.m.	pop. subd.	n.m.	yes	no	Parker 1999
Baltic Sea (Gulf of Gdansk)	7	6	40	40-80	pop. subd./ habitat cline	yes	yes	yes (*Idh*1)	Hummel et al. 2000

or more species seems warranted, with American Atlantic and Baltic *M. balthica* as separate units (Meehan 1989, Väinölä and Varvio 1989).

To the best of my knowledge, there were no estimations of population structuring in quantitative genetic trait variation in broadcast spawning marine invertebrates prior to the work presented here. This is probably due to the difficulties of breeding marine organisms with pelagic larvae in the laboratory. It is, however, not impossible, as testified by the existence of commercial breeders of bivalve larvae and the breeding for research purposes of commercially relevant mussels and oysters (Sophie Launey, formerly at Ifremer, La Tremblade, France, Elefterios Zouros, Institute of Marine Biology of Crete, Greece, and Dennis Hedgecock, Bodega Bay Marine Laboratory, USA, pers. comm.). The technique of breeding *M. balthica* was developed (Honkoop 1998) in the context of the PIONIER programme of which this project was a part. The PIONIER programme focussed on evolutionary interactions between intertidal predators and their prey. The project, that resulted in this thesis, dealt with local adaptation of prey. The *M.balthica* breeding facility enabled us to estimate heritabilities for shell shape during this study. Studying allozyme and mtDNA variation at the same scale as that of shell shape variation provided a no-selection null hypothesis.

This thesis is divided into two parts. The first section (chapters two to four and box I) focusses on the spatial arrangement of genetic variation in *M. balthica* and the role of selection. I propose that one of the keys to understanding local adaptation in high-dispersal marine invertebrates may be that mating is a microscale process. The second part (chapters five to eight and box II) therefore contains observations and models on the process of external fertilization.

Outline of thesis

Chapter two describes the genetic differentiation of *Macoma balthica* populations at the European scale. On the basis of variation in a portion of the cytochrome-*c*-oxidase I gene of mitochondrial DNA within and among populations, the amount of population structuring is estimated. Also, the history of population subdivision is reconstructed using coalescent analysis. The emerging patterns are viewed against geographic history of the area, in particular the Pleistocene ice ages, and the fossil record.

Chapter three contrasts neutral genetic variation with putatively selective genetic variation for a quantitative trait (globosity of the shell) in *Macoma balthica* among populations in the western Dutch Wadden Sea and the adjacent North Sea. It uses population differentiation of molecular marker variation (allozymes and mitochondrial DNA sequences) as a no-selection null hypothesis for the quantitative trait.

Box I addresses one of the selective forces on shell globosity: selective predation by a main predator, the red knot *Calidris canutus*.

Chapter four concerns a common phenomenon associated with the use of allozymes in bivalve population genetic studies: heterozygote deficiencies. The hypothesis is posed that bivalve heterozygote deficiencies are a technical artifact associated with the large numbers of alleles that bivalve populations tend to harbour for allozyme loci. This hypothesis is tested with the use of the allozyme data from chapter 3 and data in the literature on other bivalves.

Chapter five contains data that are consistent with sperm limitation playing an active role in life history decision-making in *M. balthica*: egg size in the field is shown to be strongly correlated with adult density as a proxy for sperm density during fertilization. An analytical model is developed and analysed to test the argument that under increased sperm limitation, females producing larger eggs have increased fitness because larger eggs are a larger target for sperm, even if producing larger eggs also means making fewer of them.

Box II addresses anisogamy, i.e. the fact that male and female gametes (*gamy*) are so often not of the same size (*an-iso*; not the same). Previously, it was claimed that a positive relationship between increased survival rate with increased zygote size has limited potential in explaining the evolutionary origin of anisogamy. It is shown in this box how insights from marine larval biology greatly expand the explanatory power of zygote survival for the origin of anisogamy.

Chapter six describes the timing of spawning activity within and among populations of *M. balthica* in the western Dutch Wadden Sea and the adjacent North Sea. The idea that spawning should be synchronised to avoid eggs remaining unfertilized due to sperm limitation is contradicted by the absence of synchronisation within sites, but corroborated by the observation that subtidal males spawn twice as much sperm upon induction as intertidal males.

Chapter seven shows inferences concerning kinetics of external fertilization in *M. balthica*. It estimates the relationship between sperm concentration and fertilization probability of eggs, the effect of gamete age, and inter-individual induction of spawning. From these relationships a rough indication of what this may mean for the scale of fertilization in the field is derived.

Chapter eight zooms in on the process of fertilization and early development of embryos in *M. balthica*. With the use of cytogenetic observations, it describes the entry of multiple sperm into a single egg (polyspermy). While the chromosomes of one sperm fuse normally with the chromosomes of the egg, one or more additional sperm nuclei form their own haploid cell line within the young embryo.

Chapter nine is a general discussion of the results presented in this thesis in the light of the question posed above: what are the consequences for spatial arrangement of intraspecific genetic variation in a species with external fertilization and pelagic larval dispersal?

PART A

SPATIAL GENETIC VARIATION

CHAPTER TWO

Population divergences predate last glacial maximum in Europe for a marine bivalve with pelagic larval dispersal

P.C. Luttikhuizen, J. Drent and A.J. Baker
(submitted manuscript)

Abstract

Mitochondrial DNA sequence data for 295 individuals were collected from ten sites across the European distribution of the marine bivalve *Macoma balthica* (L.), and from Alaska. The data were used to estimate levels of genetic exchange and infer population subdivision history. The haplotypes encountered represent a deep gene tree, and the geographic distribution of the lineages shows both allopatry and sympatry. Populations in the Baltic Sea contain a clade of haplotypes that is inferred as having diverged from the other haplotypes 9.8-39 million years ago (Mya). This corroborates earlier findings, based on allozyme data, suggesting that Baltic Sea *M. balthica* should be considered a separate species. Pacific *M. balthica* sampled from Cook Inlet, Alaska, were found to be genetically close to the Baltic Sea haplotypes. The remaining haplotypes consist of two also rather divergent clades that share a most recent common ancestor 1.9 to 14 Mya. With the use of coalescent analysis, population divergence times for French vs. other non-Baltic European populations ('Atlantic assemblage') are estimated at a minimum of 110,000 years ago, well before the last glacial maximum (LGM) 18,000 years ago. Some of the populations within the Atlantic assemblage are isolated, while others are connected by gene flow. Sympatry of highly diverged lineages in the Baltic Sea confirms Baltic population divergence prior to French-Atlantic divergences and secondary admixture. The patterns observed only partially fit the existing model for northern European marine biogeographic history, which is thought to consist of recolonization of the area after confinement to France and the south of the British Isles during the LGM. Isolated populations of *M. balthica* must have survived the Pleistocene ice ages and may either all have been present in Europe at the time, or, alternatively, have invaded Europe at a later stage. European populations of this species can be highly subdivided in spite of the potential for high dispersal, meaning that they are numerically and evolutionarily independent.

Introduction

The potential for high dispersal of marine taxa is of great influence for our understanding of how populations in the marine realm function. The large degree of connectivity that would result from high levels of effective dispersal would imply that populations are 'open' in the sense that, numerically, they are not regulated independently. Furthermore, in the absense of effective gene flow, evolutionary genetic processes are independent, facilitating adaptive population divergence. Knowledge on biogeographic history of a species can help formulate hypotheses to explain phenotypic variation across its geographic range (see *e.g.* Drent 2002). Whether or not the potential for high dispersal is realised is of vital importance for marine conservation issues (see *e.g.* Palumbi 2001, Barber *et al.* 2002, Luttikhuizen *et al.* in press).

Macoma balthica (L.) (Bivalvia: Tellinidae), the Baltic tellin, is a common marine bivalve of the northern hemisphere. It inhabits soft sediments in coastal habitats like estuaries, mudflats and the nearshore sea bottom. It has a long history as a model species for marine benthic ecology, with a focus on population dynamics, community ecology and evolutionary ecology (*e.g.* Lammens 1967, Hulscher 1973, Beukema 1993, Honkoop *et al.* 1998, Edelaar 2000, De Goeij *et al.* 2001, Van der Meer *et al.* 2001, Hiddink and Wolff 2002, Drent 2002, Luttikhuizen *et al.* in press). Directly estimating dispersal in marine invertebrates with a free-swimming pelagic phase is extremely difficult (Levin 1990, McEdward 1995). The adults are usually sedentary, and dispersal takes place in the larval phase, when the individuals are typically still very small and mortality is high (Morgan 1995). Dispersal in *M. balthica* takes place during its 2-5 week pelagic larval phase (Caddy 1967, Drent 2002), and during their first winter when they may drift with the use of a byssal thread (Beukema 1993, Hiddink *et al.* 2002). Gene flow, or the lack thereof, and population subdivision leave signatures in the spatial distribution of neutral molecular variation that can be utilized to estimate the amount of gene exchange (Slatkin 1985) and to reconstruct biogeographic history (Avise *et al.* 1987).

Allozyme studies of *M. balthica* have demonstrated more genetic structuring among European populations than was to be expected on the basis of its dispersal potential. For instance, populations inhabiting the Baltic Sea were found to be highly divergent from other European populations (Väinölä and Varvio 1989, Hummel *et al.* 2000). The northeast and northwest Atlantic *M. balthica* were found to differ so much as to warrant separate species status (Meehan 1985). However, large regions over which its populations were unstructured were also identified, for example the eastern Atlantic from France to northwest Norway (Hummel *et al.* 1995, 1997). There are indications that some of the studied allozyme variation may be under the influence of selection, most notably an *Idh* locus (Hummel *et al.* 1995, 1998), which is also the most strongly differentiated locus in Europe. This may hamper population genetic

inference. In addition, allozymes do not lend themselves to coalescent analysis, because the genealogy of their alleles is not known.

Coalescent analysis takes into account the genealogy of alleles or haplotypes observed in population samples (Kingman 1982a,b). Comparing simulations with the data gives an estimate of the probability of particular parameter values, enabling the release of the assumption of equilibrium underlying Wright's (1931, 1951) F-statistics. After initial population subdivision, it takes time until isolation and genetic drift reach an equilibrium level of differentiation in molecular markers, which is a number of generations in the order of the effective population size N_e (Crow and Kimura 1970). This assumption is not reasonable in cases where effective population sizes are large, while population subdivision is relatively recent. Although F_{st} can in such cases provide a useful description of the partitioning of molecular variance over space, it has limited use in answering questions about genetic exchange (Whitlock and McCauley 1999). With the advance of computing power and the speed at which sequence data can be collected, it has in recent years become possible to estimate population genetic parameters using a combination of coalescent-based models and traditional Wright-Fisher models of binomial sampling in successive generations (*e.g.* Bahlo and Griffiths 2000, Nielsen and Wakeley 2001, Beerli and Felsenstein 2001).

Bivalves are a suitable taxonomic group to study molecular ecology and phylogeography, because fossil records of the shells are abundant, enabling calibration of rates of molecular evolution and providing a temporal framework for phylogeographic scenarios. The family Tellinidae appears in the fossil record in the early Cretaceous (which is defined as 144-65 Million years ago, Mya) (Pohlo 1982), and the genus *Macoma* is of North Pacific origin (Meijer 1993). While many *Macoma* species reside in the north Pacific today (see *e.g.* Oldroyd 1924), only two extant species, *M. calcarea* and *M. balthica*, dispersed into the Atlantic across the Bering Strait after its opening 3.5 Mya (Spaink and Norton 1967, Norton and Spaink 1973).

The geography of northern Europe has undergone major transformations during the ice ages of the Pleistocene ~1.8 Mya to ~10,000 years ago (Denton and Hughes 1981, Dawson 1992). The recurrent advance and retreat of land and sea ice and the accompanying climatic changes have been of great influence on the present distribution patterns of organisms inhabiting European terrestrial and aquatic habitats (*e.g.* Van den Hoek *et al.* 1990, Hewitt 1993, 1996). The Pleistocene ice ages and in particular the last glacial maximum 18,000 years ago are expected to have left their signature in the spatial patterns of molecular variation in extant populations of *M. balthica* in Europe.

The questions we address in this paper are the following: first, how much genetic exchange takes place among European populations of *M. balthica*? Second, what is the biogeographic history of current populations of *M. balthica* in Europe? A priori, we expected that historical biogeography would be congruent with colonization of the northeast Atlantic and the Baltic Sea from southern refugia since the last glacial maximum about 18,000 years ago. Mitochondrial DNA sequence data

is used to address the questions of genetic exchange and the history of population subdivision.

Materials and methods

Study populations

Macoma balthica adults were collected between 1998 and 2001 in France (Gironde estuary: a), Wales (UK, Severn River Estuary: b), Scotland (UK, Moray Firth at Inverness: c), Norway (Balsfjord: d), the White Sea (Russia, Mezenskaya Guba: e), The Netherlands (Mok (f), Balgzand (g) and Kimstergat (h) in the western Wadden Sea and Molengat (i), northeast of Texel (j) and north of Terschelling (k) in the adjacent North Sea), Germany (Greifswalder Bodden, Ruegen island: l), Poland (Bay of Gdansk: m), Latvia (Gulf of Riga: n) and Finland (at Tvärminne, Gulf of Finland: o) (Fig. 1). An additional sample of three individuals was obtained in 2002 from Alaska (USA, Cook Inlet: p). This sample was not included in F_{st} or coalescent analyses but used as reference material only. The samples from the Baltic Sea (including the German sample) and the North Sea were taken from the subtidal and all other collections were from the intertidal.

Molecular analysis

Live clams were frozen in liquid nitrogen to prevent DNA from degrading and stored at -80°C until further processing. DNA was extracted from approximately 1 mm^3 tissue using a standard phenol-chloroform extraction method and redissolved in 10 µl water. Using Phase Lock Gel Light (Eppendorf) during centrifugation at the phenol-chloroform stage enabled extraction in spite of the mucoid nature of the tissue. If the individual was a ripe female, the gonad was used for DNA extraction, since this tissue contained relatively little mucus. In all other cases (ripe males and unripe or parasitized individuals) mantle tissue, which does not contain gonadal tissue in this species, was used. Extracted DNA was visualized on 1% TBE agarose gels. PCR amplifications were carried out in 50 µl volumes using a 1:10 dilution of DNA. In rare cases of failed amplification, either diluting the extract further to 1:100 or using the extract undiluted, usually rendered a positive PCR.

Universal primers LCO1490 and HCO2198 (Folmer *et al.* 1994) were used to amplify a 710 bp fragment of the mitochondrial cytochrome-*c*-oxidase I gene. After preliminary sequence results were obtained, new primers internal to the universal primers were designed (forward: 5'-TTAGTGACTTCACACGGTTTGC-3' and reverse: 5'-AGTACAGGTATAGCAACCACCAG-3'). The newly designed primers were used for the remainder of the study. Amplification products were cut out of 1% agarose gels and purified using the manufacturer's protocol (Qiaquick Gel Extraction Kit, Qiagen). Single-stranded DNA was sequenced directly using the BigDye (PE Biosystems) sequencing kit and manufacturer's protocol with the forward primer on an automated sequencer (ABI PRISM 310 Genetic Analyzer). In this way we

sequenced a 393 bp fragment of the mtDNA cytochrome-*c*-oxidase I gene from a total of 295 *Macoma balthica.*

Data analysis

DNA sequences were aligned by hand. A minimum spanning network among the haplotypes was constructed using Arlequin 2.000 (Schneider *et al.* 2000). Analysis of molecular variance (AMOVA) was carried out in Arlequin. As there was no obvious way to *a priori* group the data hierarchically, a single-level AMOVA was used and pairwise F_{st} values were calculated. Exact tests of population differentiation based on haplotype frequencies were executed in Arlequin for all pairwise comparisons. Number of migrants per generation (N_em, where m equals the fraction of effective migrants per generation) were inferred from the pairwise F_{st}'s assuming Wright's (1931) island model of migration. Nucleotide diversities (Tajima 1983) were calculated for each sample.

The coalescent-based analyses of this study employed the infinitely-many-sites model of nucleotide substitution, which assumes that a maximum of one mutation per nucleotide site occurs. For the data to be compatible with the infinitely-many-sites model, only limited pruning was needed, because the haplotype set was still far from mutation saturation. Data pruning consisted of leaving haplotype I and site 143 out of the analysis. The ancestral sequence was inferred using the distance-based Bayesian method of Zhang (1998), Zhang and Nei (1997) and Zhang *et al.* (1998) with *Tellina* cf. *fabula* (Bivalvia: Tellinidae) as outgroup (sequence provided by Judith van Bleijswijk and Tanya Compton, Netherlands Institute for Sea Research).

Nielsen and Wakeley (2001) have implemented a Markov chain Monte Carlo method to model population divergence backwards in time: gene copies coalesce for two populations that derive from a single panmictic population, and may or may not be connected by migration until their time of divergence. Utilizing coalescent theory, samples consisting of DNA sequences from two such populations can be used to obtain joint maximum likelihood estimates for mutation parameter θ ($2N_e\mu$, where N_e is the effective population size and μ is the rate of mutation per sequence per generation), migration parameter M (N_em, where m is the fraction of effective migrants per generation), population divergence time (PDT = divergence time/N_e) and time to most recent common ancestor (TMRCA) for all hapotypes. Bayesian posterior likelihood distributions for all these parameters were jointly obtained using the program MDIV (Nielsen and Wakeley 2001) for all possible population pairs among 12 population samples, starting from uniform priors. The mode of the posterior distributions are the maximum likelihood parameter estimates. The samples containing the higly divergent Baltic haplotypes were excluded from these analyses, because hybridization and introgression are non-neutral processes not covered by this coalescent model. Genetree (Bahlo and Griffiths 1998) is a similar program, which estimates TMRCA's for haplotypes and models the coalescent process in a subdivided population. By running Genetree for 1,000,000 runs with migration

Table 1 - Variable sites in the sixteen cytochrome-*c*-oxidase I haplotypes identified for *Macoma balthica* (L.).

	1111111111111222222233333333	genbank
	1345568001123444478901345580012567 8	accession
	7875926470681036732745087872540684 6	code
A	GGTCCTGTTTCTACAACGACTGCGTTCTTTCTTAA	AF443216
B	.A.....G.....CA.....	AF443217
C	.A.....C..G.....CA.....	AF443218
D	.A.....C....G.....CA.....	AF443219
E	...T.....	AF443220
F	.....T.....	AF443221
G	.....C.....	AF443222
H	.....C.....	AY162255
I	.....C.....T.....	AY162256
J	.....C.....	AY162257
K	.....C.....	AY162258
L	.....C.....T.....	AY162259
M	.A..TCA...T.GT.G.AG..ATAGCTCA.TCCG.	AY162260
N	.A..TCA...T.GT.G.AG..A.AGCTCA.TCCG.	AY162261
O	AA..TCA...T.GT.G.AG..ATAGCTCA.TCCGG	AY162262
P	.AC.TCA...T.GT.G.AG..A..GCTCA.TCCG.	AY162263

matrices obtained from Arlequin and MDIV, we tested whether the data support population growth or not, and a coalescent-based tree of TMRCA's for haplotypes was obtained.

Parameters from the coalescent analyses are in coalescent units that are useful as relative measures of time, but can be placed in a geographical context more readily when transferred to normal time units. To this end, a calibration of molecular divergence rate is necessary. Because of the large degree of difference in molecular clock rates among species and among loci (see *e.g.* Li 1997), we searched for divergence rates in cytochrome-*c*-oxidase I for similar species. Three sets of data for calibration were obtained, all for COI in marine bivalves: (1) geminate species pairs of the family Arcidae on either side of the Isthmus of Panama, (2) the mussel genus *Mytilus*, and (3) the Tellinacea, to which *M. balthica* belongs. Within these groups, we found a total of seven contrasts of sequence data obtained from Genbank and published dates of taxon divergence times, which were fossil record dates in five cases and dated geographic events in the other two cases. The following partial COI

Table 2 - Haplotype frequencies and nucleotide diversity (π) per sampled site; st.dev. = standard deviation.

	A	B	C	D	E	F	H	G	I	J	K	L	M	N	O	P	π (st.dev.)
(a)France	0	1	0	0	0	0	0	0	1	15	2	1	0	0	0	0	0.0028(0.0020)
(b)Wales	2	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0036(0.0026)
(c)Scotland	1	19	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0.0019(0.0016)
(d)Norway	2	15	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0.0029(0.0022)
(e)White Sea	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0000(0.0000)
(f)NL-Mok	15	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0046(0.0030)
(g)NL-Balgzand	8	10	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0.0053(0.0034)
(h)NL-Kimstergat	14	4	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0.0041(0.0028)
(i)NL-Molengat	12	9	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0.0050(0.0032)
(j)NL-Texel	10	13	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0.0048(0.0031)
(k)NL-Terschelling	13	11	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0049(0.0032)
(l)Germany	9	6	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0.0049(0.0032)
(m)Poland	1	6	0	0	0	0	0	0	0	2	0	0	6	0	0	0	0.0264(0.0142)
(n)Latvia	0	2	0	0	0	0	0	0	0	0	0	0	10	2	1	0	0.0123(0.0070)
(o)Finland	0	0	0	0	0	0	0	0	0	0	0	0	19	0	0	0	0.0000(0.0000)
(p)Alaska	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.0000(0.0000)

sequences from Genbank were used: AF253493 (*Arcopsis solida*), AF253482 (*Arcopsis adamsi*), AB076935 (*Arca ventricosa*), AB076931 (*Barbatia lima*), AF242033 and AF242034 (*Mytilus trossulus*), AF241963 (*Mytilus edulis*), U68776 (*Mytilus californianus*), AB040842 (*Donax cuneatus*), AF443221 (*Macoma balthica*), AB040844 (*Donax faba*) and AB076949 (*Sinonovacula constricta*). The Kimura two-parameter (K2P) DNA distance model was used to correct for multiple substitutions per site, using the program *MEGA* version 2.1 (Kumar *et al.* 2001). Whenever more than one sequence was available for a taxon, the smallest distance among all possible pairwise comparisons was used for the calculation of divergence rate.

Results

Sequence data characteristics

Sixteen haplotypes were detected among the samples, which clustered in three main clades. The minimum spanning network among haplotypes is shown in Fig. 1.

Table 1 shows the 35 variable sites among the haplotypes, 33 of which were transitions and two were transversions (TI/TV = 16.5). One variable site was a replacement substitution, and all others were silent substitutions (Ka/Ks = 0.0294). The replacement substitution was observed only once, in an individual sampled from the Netherlands (haplotype E). The predominantly silent nature of the substitutions underlines the neutrality of the variation studied.

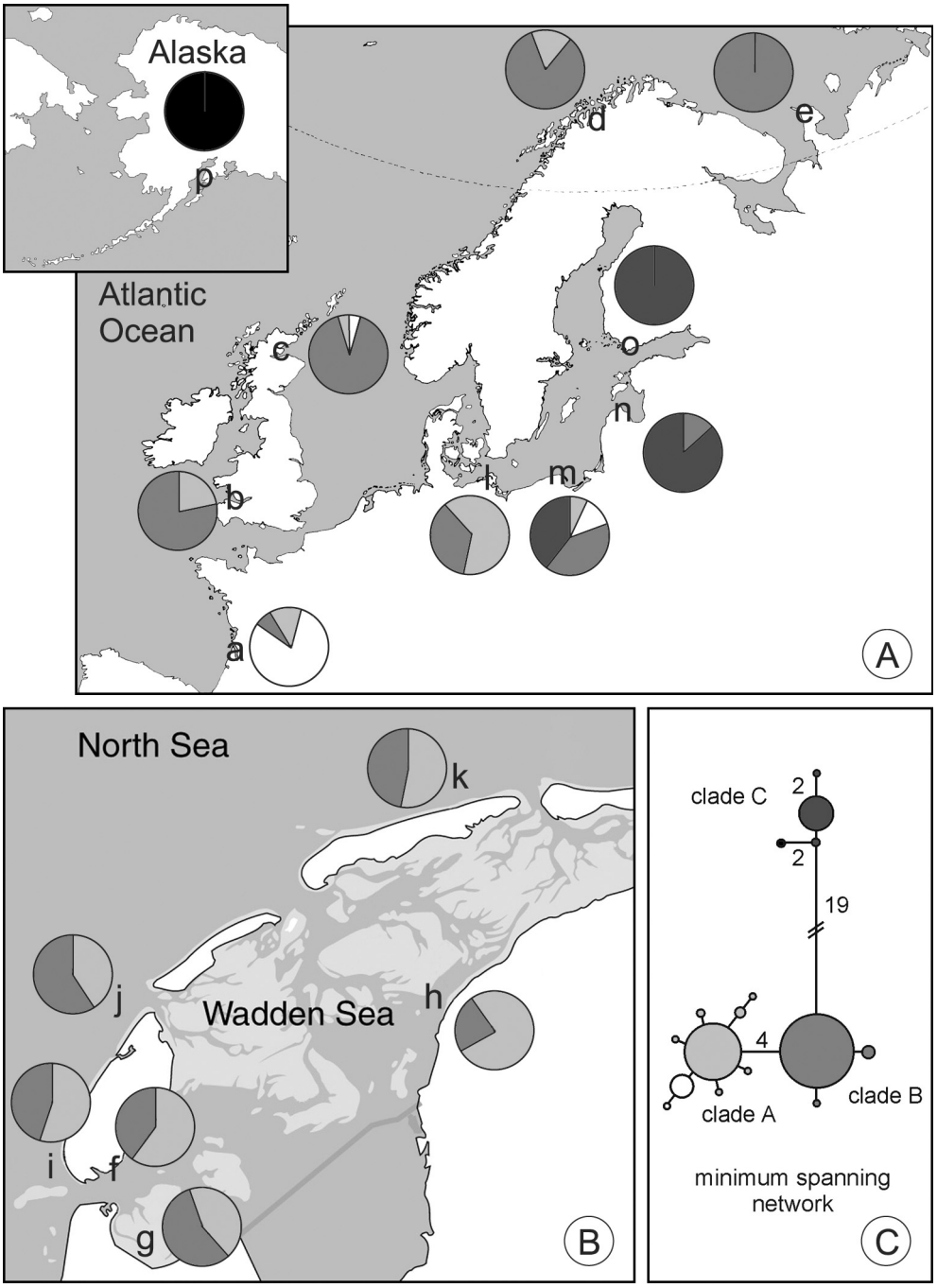
The three clades of haplotypes were not equally different from each other. Genetic distance (K2P model of nucleotide substitution) between clades A and B (Fig. 1) ranged from 0.010-0.018, between clades A and C from 0.056-0.068, and between clades B and C from 0.051-0.062. The three Alaskan individuals were all of the same haplotype, which in the minimum spanning network is situated in clade C (Table 1).

Population genetic analyses

The frequencies with which haplotypes were encountered in the samples as well as nucleotide diversities per sample and their standard errors are shown in Table 2. Population subdivision was found to be highly significant as assessed using AMOVA. The percentage of total molecular variation within samples was 33.1%, and that among samples was 66.9%, amounting to F_{st} = 0.669. Pairwise F_{st} values and inferred number of migrants per generation (N_em) are shown in Table 3. After Bonferroni correction for multiple tests, 58 out of the 105 pairwise population contrasts were significant on the basis of pairwise F_{st}'s, and an additional 6 on the basis of exact testing (Table 3).

Two groups of samples are evident for which there is no support for population subdivision (Table 3). The samples from Wales, Scotland, Norway and the White Sea are highly similar, as well as the seven samples from The Netherlands and Germany, all with very small and nonsignificant pairwise F_{st} values and correspondingly high inferred migration rates. The remaining N_em estimates inferred from the pairwise F_{st}'s ranged from 4.05 for Latvia vs. Finland down to almost zero for all contrasts of Finland vs. non-Baltic samples.

Fig. 1 (opposite page) - Distribution of COI haplotypes for *Macoma balthica* (L.) across Europe and Alaska. Characters indicate sampling site; (a) Gironde, France, (b) Severn River Estuary, Wales, (c) Moray Firth at Inverness, Scotland, (d) Balsfjord, Norway, (e) Mezenskaya Guba, White Sea, Russia, (f-h) Wadden Sea, the Netherlands, (i-k) North Sea, The Netherlands, (l) Greifswalder Bodden, Ruegen island, Germany, (m) Bay of Gdansk, Poland, (n) Gulf of Riga, Latvia, (o) Gulf of Finland at Tvärminne, Finland, (p) Cook Inlet, Alaska. Position of label on map corresponds to location of sampling site in the field. A) Haplotype distribution for Europe and Alaska. B) Haplotype distribution for The Netherlands. C) Minimum spanning network among haplotypes, where each circle signifies a haplotype, size of circle denotes frequency with which haplotype was observed, and distance between haplotypes equals one substitution unless otherwise indicated.


Alaska
p
Atlantic
Ocean
d
e
c
o
n
l
m
b
a
A
North Sea
k
j
Wadden Sea
h
i
f
g
B
clade C
2
2
19
4
clade A
clade B
minimum spanning
network
C

Coalescent analyses

The coalescent tree showing times to most recent common ancestor (TMRCA's) for haplotypes and ages of mutations, obtained from simulations with the program Genetree, is shown in Fig. 2. The coalescent tree is very similar to the minimum spanning network (Fig. 1) in that the split between haplotype clade C versus the rest is estimated to have occurred much longer ago than the split between haplotype clades A and B. Results from prior modelling with Genetree indicated, that it was not necessary to incorporate population growth, as the maximum likelihood value for the population growth rate parameters were always zero and this result was independent of migration matrix or mutation parameter.

Coalescent-based maximum likelihood estimates for population divergence times (PDT's), expected TMRCA's, mutation parameters θ and migration rates N_em (from the program MDIV) are shown in Table 4. In some of the pairwise coalescent comparisons including the monomorphic sample from the White Sea, MDIV did not converge for PDT and N_em (i.e., the likelihood surfaces were very flat and location of the mode of the posterior distribution varied with initial conditions and parameter space searched).

In Table 4, the results are arranged according to the grouping that emerged from the F_{st} analysis, i.e., putting the comparisons among samples from The Netherlands and Germany together, as well as those among the samples from Wales, Scotland, Norway and the White Sea. In addition, the comparisons including the French sample are separated from the rest. Population divergence times are longest for the French sample; up to 4.74 coalescent units for France vs. the White Sea. Migration rates for the French sample are very low. In contrast, divergence times are zero for several comparisons within the groups The Netherlands/Germany and Wales/Scotland/Norway/the White Sea. In the latter groups, coalescent-based migration rates tend to be lower than migration rates inferred from pairwise F_{st}'s (Table 3).

Calibration of divergence rate

The seven sequence contrasts obtained for calibration of the divergence rate of bivalve COI are shown in Table 5. The K2P distances were found to range from 0.000 to 0.472, and the divergence times from 2 to 251 million years ago (Mya). The comparison of *Mytilus edulis* with *Mytilus galloprovincialis* could not be used for calibration, because these species share some of their mitochondrial haplotypes (see also Rawson and Hilbish 1995), meaning that lineage sorting may not yet be complete in this young species pair or that introgression may have removed the result of diverged lineages. Among the remaining six divergence rates, two were an order of magnitude higher than the other ones, namely the rates obtained from dated geographic events. In the absense of an adequate fossil record, geological events are often used to calibrate molecular clocks (*e.g.* Rawson and Hilbish 1995, Knowlton and Weigt 1998). Speciation events may, however, predate the geological event,

Table 3 - Pairwise population comparisons and inferred migration rates. Letters indicate sampling site, see Table 2 and Fig. 1. Below diagonal are pairwise F_{st}'s from analysis of molecular variance (AMOVA). In bold are values significantly different from zero (p-level 0.05, Bonferroni corrected). Results of exact tests of population differentiation are indicated with an asterisk when significant (p-level 0.05, Bonferroni corrected). Above diagonal are values of N_em inferred from the pairwise F_{st}'s (inf = infinity).

site	(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)	(k)	(l)	(m)	(n)	(o)
(a)	-	0.25	0.15	0.20	0.07	0.75	0.54	1.02	0.69	0.50	0.66	0.85	0.56	0.09	0.02
(b)	**0.67***	-	inf	inf	1.42	2.28	49.50	0.85	3.67	inf	5.06	1.67	1.58	0.14	0.01
(c)	**0.77***	-0.01	-	inf	12.00	0.75	2.00	0.38	1.02	2.13	1.17	0.61	0.85	0.10	0.01
(d)	**0.71***	-0.08	-0.03	-	3.67	1.29	5.06	0.59	1.77	5.06	2.13	1.02	1.02	0.11	0.02
(e)	**0.88***	0.26	0.04	0.12	-	0.41	0.89	0.20	0.50	0.89	0.59	0.31	0.75	0.07	0.00
(f)	**0.40***	0.18	**0.40***	**0.28***	**0.55***	-	24.50	49.50	inf	24.50	inf	inf	0.85	0.11	0.03
(g)	**0.48***	0.01	0.20*	0.09	**0.36***	0.02	-	2.83	inf	inf	inf	12.00	1.06	0.13	0.03
(h)	**0.33***	0.37	**0.57***	0.46*	**0.71***	0.01	0.15	-	9.50	2.44	6.64	inf	0.78	0.10	0.02
(i)	**0.42***	0.12	0.33*	0.22*	**0.50***	-0.04	-0.02	0.05	-	inf	inf	inf	0.97	0.12	0.03
(j)	**0.50***	0.00	0.19	0.09	**0.36***	0.02	-0.04	0.17	-0.01	-	inf	9.50	0.97	0.12	0.03
(k)	**0.43***	0.09	0.30	0.19	**0.46***	-0.03	-0.02	0.07	-0.01	-0.02	-	inf	0.93	0.11	0.03
(l)	**0.37***	0.23	**0.45***	**0.33***	**0.62***	-0.04	0.04	-0.02	-0.03	0.05	-0.01	-	1.02	0.12	0.03
(m)	**0.47***	0.24	**0.37***	**0.33***	**0.40***	**0.37***	0.32*	**0.39***	**0.34***	**0.34***	**0.35***	**0.33***	-	1.17	0.38
(n)	**0.85***	**0.78***	**0.84***	**0.82***	**0.87***	**0.82***	**0.80***	**0.83***	**0.81***	**0.81***	**0.82***	**0.81***	0.30	-	4.05
(o)	**0.97***	**0.98***	**0.98***	**0.97***	**1.00***	**0.95***	**0.94***	**0.96***	**0.94***	**0.94***	**0.94***	**0.95***	**0.57***	0.11*	-

which has recently been shown for the geminate species pair *Arca mutabilis* and *A imbricata*. These species were found to be morphologically distinct in the fossil record at least 13 million years before the final closure of the Panama seaway 3 Mya (Peter Marko, pers. comm.). This questions the reliability of the *Arcopsis* and *Mytilus trossulus* calibration points in Table 5, which were consequently left out of the analysis. The inferred rates are rather similar for all five contrasts, and fall between 0.14% and 0.52% divergence per nucleotide site per million years. This translates into a mutation rate μ, as used in the coalescent analyses, between $0.55 \cdot 10^{-6}$ and $2.04 \cdot 10^{-6}$ mutations per sequence per year, assuming a generation time of two years.

Applying these mutation rates to the K2P distances between the three haplotype clades found for *M. balthica*, clades A and B are estimated to have diverged 1.9-7.1 million years ago (Mya), whereas the split between clades A and B versus C has taken place an estimated 9.8-36 Mya. Converting coalescent time units in Fig. 2 into normal time units gives the following result: the time of most recent common ancestor for all haplotypes, coinciding with the splitting off of clade C, was 14-39 Mya, while clades A and B had a most recent common ancestor 3.6-10 Mya. In the same way, the estimates for PDT and expected TMRCA of the coalescent process were converted into standard time ranges (Table 4).

Discussion

The results presented here indicate substantial genetic differentiation among European populations of *Macoma balthica* and that both isolation and migration have contributed to the contemporary mitochondrial DNA distribution. Among the haplotypes encountered, some have diverged a very long time ago, the minimum estimate for the oldest split being about ten million years ago (Mya). Also population subdivision has a long history. Remarkably, some subdivisions appear to be older than the Pleistocene. Most notably, *M. balthica* in the Baltic Sea is quite distinct from other European populations, further substantiating the earlier suggestion on the basis of allozymes (Väinölä and Varvio 1989, Hummel *et al.* 2000) that they represent two different species. Two regions were identified within which there is contemporary genetic exchange. At the pan-European level there is an overall low level of contemporary gene flow and historical biogeography is not congruent with colonization after the last glacial maximum (LGM).

In addition to traditional F_{st} analysis and migration inference, coalescent analysis combined with molecular clock calibration of mutation rate gave useful additional insights with regard to the timing of population subdivision events. Population divergence times (PDT) among the French and Atlantic assemblages were dated to 0.11-2.6 Mya, while populations within the Atlantic assemblage are either still panmictic or have diverged less than 240,000 years ago.

Data and analyses

Both the haplotypes and the populations cluster in three groups referred to as haplotype clades (A, B and C) and population assemblages (French, Atlantic and Baltic), respectively. The French assemblage is represented by the single sample from the Gironde, France. The samples from the British Isles, the Netherlands, Germany, Norway and the White Sea together form the Atlantic assemblage. The three samples from the Baltic-proper (Poland, Latvia and Finland) make up the Baltic assemblage. Within the Atlantic assemblage, two groups can be distinguished that are still connected by gene flow, the first represented by the samples from the British Isles and the sample from Norway, and the second consisting of all samples from The Netherlands and the German sample. While in the Fst analysis, the White Sea sample did not significantly differ from the group Wales/Scotland/Norway (Table 3), the coalescent results suggest lowered migration rates to and from the White Sea (Table 4). The three sequences from Alaska belong to haplotype clade C and to the Baltic population assemblage.

Haplotype clades A and B split a few Mya, while the splitting off of clade C is much older. The timing of these events does not depend much on the method used. For direct molecular clock inference they are 1.9-7.1 Mya and 9.8-36 Mya, respectively, and for coalescent analysis they are 3.6-10 Mya and 14-39 Mya, respectively. The large overlap in these ranges is more important than the difference, as it can be concluded that the clades are rather old (compared to the split between the family Tellinidae, to which *M. balthica* belongs, and the most closely related family, the Donacidae, 90-140 Mya, see Table 5).

Many samples contain haplotypes from two of the three clades (Fig. 1). This could either be because ancestral polymorphism has been maintained since population subdivision, or because of population admixture. Most surveys of mtDNA variation that show this type of pattern (old lineages in sympatry) are interpreted as evidence of secondary admixture between allopatrically evolved populations or species (Avise 2000). In theory, ancestral polymorphism is a possible explanation for species like *M. balthica* which maintain large effective population sizes. However, from the raw data (Fig. 1) it can be seen that the divergent haplotype clade C occurs only in the Baltic and that haplotypes occurring in the rest of Europe reach lower frequencies with increasing distance from the Baltic Sea entrance (at the Kattegat between Denmark and Sweden): 100% at Ruegen, Germany, 47% at the Bay of Gdansk, Poland, 13% at the Gulf of Riga, Latvia, and 0% at Tvärminne, Finland. Furthermore, allozyme studies also point in the direction of secondary admixture (Väinölä and Varvio 1989, Hummel *et al.* 2000), thus making ancestral lineage polymorphism the less parsimonious explanation.

Biogeographic history

The earliest known fossils of *M. balthica* in the northeastern Atlantic region date back to some time into the Pleistocene (Spaink and Norton 1967, Norton and

Table 4 - Coalescent-based joint maximum likelihood estimates for population divergence time (PDT), expected time to most recent common ancestor (TMRCA), mutation rate (theta), and migration rate N_em, which were simultaneously obtained using the method of Nielsen and Wakeley (2001). Letters indicate sampling site, see Table 2 and Fig. 1. Question mark indicates a parameter value for which the likelihood surface was too flat to make an inference. Mya = million years ago.

	PDT	**TMRCA**	**theta**	**N_em**	**PDT(Mya)**	**TMRCA(Mya)**
France vs. Atlantic						
a-b	2.20	5.21	0.64	0.23	0.35 - 1.28	0.82 - 3.04
a-c	4.76	5.46	0.54	0.35	0.63 - 2.34	0.72 - 2.69
a-d	3.60	5.02	0.56	0.37	0.49 - 1.84	0.69 - 2.56
a-e	4.74	5.49	0.61	0.08	0.71 - 2.63	0.82 - 3.05
a-l	1.14	4.42	0.80	0.21	0.22 - 0.83	0.87 - 3.22
a-f	0.82	5.42	0.53	0.21	0.11 - 0.40	0.70 - 2.62
a-g	0.80	4.39	0.75	0.22	0.15 - 0.55	0.81 - 3.00
a-h	0.66	3.83	0.94	0.18	0.15 - 0.57	0.88 - 3.28
a-i	1.04	4.82	0.68	0.18	0.17 - 0.64	0.80 - 2.99
a-j	1.04	4.84	0.67	0.20	0.17 - 0.63	0.80 - 2.95
a-k	1.88	4.93	0.74	0.15	0.34 - 1.27	0.89 - 3.32
Atlantic vs. Atlantic						
b-l	0.39	4.93	0.42	1.89	0.04 - 0.15	0.51 - 1.89
b-f	0.01	5.59	0.36	inf	0.00 - 0.00	0.49 - 1.83
b-g	0.02	4.57	0.55	6.36	0.00 - 0.01	0.62 - 2.29
b-h	0.29	4.06	0.74	1.23	0.05 - 0.20	0.74 - 2.74
b-i	0.04	5.07	0.44	1.96	0.00 - 0.02	0.55 - 2.03
b-j	0.03	4.96	0.47	13.00	0.00 - 0.01	0.57 - 2.12
b-k	0.04	5.07	0.42	4.84	0.00 - 0.02	0.52 - 1.94
c-l	0.71	4.88	0.55	0.34	0.10 - 0.36	0.66 - 2.45
c-f	0.47	5.31	0.39	0.69	0.04 - 0.17	0.51 - 1.89
c-g	0.12	4.34	0.70	1.23	0.02 - 0.08	0.75 - 2.77
c-h	0.56	3.95	0.76	0.55	0.10 - 0.39	0.74 - 2.74
c-i	0.46	4.84	0.50	0.63	0.06 - 0.21	0.59 - 2.20

Table 4 (continued)

	PDT	TMRCA	theta	N_em	PDT(Mya)	TMRCA(Mya)
c-j	0.30	4.85	0.54	1.28	0.04 - 0.15	0.64 - 2.39
c-k	0.17	4.78	0.52	0.74	0.02 - 0.08	0.61 - 2.26
d-l	0.52	4.83	0.52	0.63	0.07 - 0.25	0.62 - 2.29
d-f	0.46	5.38	0.39	1.25	0.04 - 0.16	0.51 - 1.91
d-g	0.13	4.30	0.67	2.95	0.02 - 0.08	0.71 - 2.62
d-h	0.51	3.92	0.86	0.83	0.11 - 0.40	0.83 - 3.07
d-i	0.38	4.83	0.56	1.34	0.05 - 0.19	0.66 - 2.46
d-j	0.15	4.77	0.50	1.81	0.02 - 0.07	0.59 - 2.17
d-k	0.25	4.79	0.51	1.46	0.03 - 0.12	0.60 - 2.23
e-l	?	5.50	0.43	0.12	?	0.58 - 2.15
e-f	?	6.87	0.23	0.11	?	0.39 - 1.44
e-g	?	5.00	0.51	0.16	?	0.63 - 2.32
e-h	?	4.66	0.67	0.11	?	0.77 - 2.84
e-i	?	5.95	0.35	0.11	?	0.51 - 1.90
e-j	?	5.34	0.39	0.11	?	0.51 - 1.90
e-k	?	5.32	0.37	0.15	?	0.48 - 1.79
within the British Isles, Norway and the White Sea						
b-c	0.00	4.87	0.45	3.00	0.00 - 0.00	0.54 - 2.00
b-d	0.02	4.87	0.44	inf	0.00 - 0.01	0.53 - 1.95
c-d	0.01	4.68	0.59	15.92	0.00 - 0.01	0.68 - 2.52
b-e	0.18	5.04	0.34	0.21	0.02 - 0.06	0.42 - 1.56
c-e	0.03	4.95	0.42	0.34	0.00 - 0.01	0.51 - 1.89
d-e	0.65	4.08	0.39	0.25	0.06 - 0.23	0.39 - 1.45
within The Netherlands and Germany						
l-f	0.04	5.35	0.39	inf	0.00 - 0.01	0.51 - 1.90
l-g	0.00	4.25	0.70	3.86	0.00 - 0.00	0.73 - 2.71
l-h	0.27	3.84	0.85	inf	0.06 - 0.21	0.80 - 2.97
l-i	0.05	4.74	0.55	inf	0.01 - 0.03	0.64 - 2.38

Table 4 (continued)

	PDT	TMRCA	theta	N_em	PDT(Mya)	TMRCA(Mya)
l-j	0.01	4.79	0.50	inf	0.00 - 0.00	0.59 - 2.18
l-k	0.03	4.76	0.50	4.96	0.00 - 0.01	0.58 - 2.17
f-g	0.02	4.82	0.58	4.72	0.00 - 0.01	0.69 - 2.55
f-h	0.02	4.37	0.60	2.00	0.00 - 0.01	0.64 - 2.39
f-i	0.00	5.43	0.39	inf	0.00 - 0.00	0.52 - 1.93
f-j	0.03	5.27	0.37	inf	0.00 - 0.01	0.48 - 1.78
f-k	0.02	5.43	0.40	inf	0.00 - 0.01	0.53 - 1.98
g-h	0.00	3.95	0.83	2.64	0.00 - 0.00	0.80 - 2.99
g-i	0.02	4.85	0.51	inf	0.00 - 0.01	0.61 - 2.25
g-j	0.03	4.79	0.54	inf	0.00 - 0.01	0.63 - 2.36
g-k	0.01	4.43	0.67	5.96	0.00 - 0.01	0.73 - 2.70
h-i	0.01	4.42	0.62	inf	0.00 - 0.01	0.67 - 2.50
h-j	0.02	4.42	0.63	3.44	0.00 - 0.01	0.68 - 2.54
h-k	0.01	4.04	0.87	2.16	0.00 - 0.01	0.86 - 3.20
i-j	0.00	5.36	0.42	inf	0.00 - 0.00	0.55 - 2.05
i-k	0.05	4.87	0.48	inf	0.01 - 0.02	0.57 - 2.13
j-k	0.02	4.85	0.51	inf	0.00 - 0.01	0.61 - 2.25

Spaink 1973). There appears to be a time lag between their initial crossing into the Bering Strait, to the north of which early Pleistocene fossils were found, and their arrival in the Atlantic. From this time-lag Meijer (1993) has suggested that *M. balthica* may have taken the long route to migrate to the NE Atlantic, i.e. following the Eurasian and not the American coast.

At the time of the LGM, 18,000 years ago, the terrestrial ice sheets and marine pack ice in Europe reached as far south as the British Isles. An ice-free corridor of dry land in the central North Sea separated the Scandinavian and British ice sheets (Dawson 1992). The habitat of *M. balthica* must have been confined to France and southern parts of the British Isles, where at that time the coastline was further out on to the continental shelf than it is now. Perhaps additional populations may have inhabited the northern North Sea area, and small ice-free refugia along the coasts of Scotland and Norway (Sutherland 1984, Vorren *et al.* 1988, Dawson 1992).

The sampled French population has diverged from the Atlantic assemblage a

minimum of 110,000 and a maximum of 2,630,000 years ago, depending on the population scrutinized and the calibrated COI divergence rate used. The White Sea population split off from France earliest (0.71-2.63 Mya), followed by Norway and the British Isles, while the Dutch and German populations split off from France somewhat later (0.11-1.3 Mya). We may tentatively conclude that the divergence of the French *M. balthica* took place during the Pleistocene but before the LGM. There is little evidence for contemporary migration between the French and Atlantic *M. balthica* assemblages. The migration rates inferred from pairwise F_{st}'s assuming the island model (Table 3) are higher than the coalescent estimates (Table 4). This is probably because the process of genetic drift within the French and Atlantic assemblages has not reached equilibrium yet, which would violate the assumptions of F_{st} analysis but not of coalescent analysis.

There is still genetic exchange among some Atlantic populations. Most notably, *M. balthica* populations from Wales, Scotland and northern Norway (Balsfjord) are connected by high levels of gene flow, and also populations from The Netherlands and Germany (Table 4). Population divergence events for Atlantic assemblage populations have taken place in the last 400,000 years. Coalescent analyses within the Atlantic assemblage suggests that the combined group Wales, Scotland and Norway separated from the White Sea population relatively recently (3,000-230,000 years ago). Older relationships within the Atlantic assemblage exist between the Netherlands and Germany on the one hand and Norway and the British Isles on the other hand.

It would thus appear that estimated population divergence times within and among the French and Atlantic assemblages decrease with increasing latitude. This pattern is consistent with the geographic history of the area: confinement to southern areas during the LGM and subsequent recolonization in a northward direction as the ice retreated. However, the estimated times of population subdivision may be too high. The LGM was 18,000 years ago (Dawson 1992). Even when taking into acount the possibility that PDT estimation may be accompanied by large error, it seems unlikely that the pattern of strong subdivision and differentiation as we see it today is the result of events during the last 18,000 years. A substantial amount of Atlantic population subdivision must have occurred before and survived the LGM in Europe.

However, there were no *M. balthica* in the Baltic basin until 7,500 years ago. Around 14,000 years ago, the water contained within the Baltic basin was entirely frozen. Upon melting, a freshwater ice lake arose initially. The Baltic biota have since changed periodically with changing salinity. About 10,000 years ago the area went through a brackish water period (Yoldia Sea) when salt water entered the basin over what is currently Sweden. Another freshwater period (Ancylus Lake, ~ 9000 years ago) passed when the weight of the melting ice was lifted and the land rose faster than sea levels did. About 7500 years ago, the connection between Denmark and Sweden let in salt water to start the Litorina Sea period (Ignatius *et al.* 1981). Salinity has been gradually declining since. The flora and fauna that today populate the Baltic

Table 5 - Calibration of divergence rate (percent divergence per nucleotide per million years) for bivalve cytochrome-*c*-oxidase-1; T_D = divergence time; Mya = million years ago; K2P = minimum genetic distance according to Kimura-2-parameter model of nucleotide substitution; n.a. = not available.

group	**contrast**	**T_D**	**ref. T_D**	**K2P**	**divergence rate (%)**
Arcidae	*Arcopsis solida* vs. *A. adamsi*	3 Mya	final closure Panama seaway	0.129	4.3
	Arca spp. vs. *Barbatia* spp.	144-206 Mya	fossils, Cox *et al.* 1969	0.322	0.16-0.22
Mytilus spp.	*Mytilus edulis* vs. *M. trossulus*	3.5 Mya	opening Bering Strait, Vermeij 1991	0.153	4.4
	M. edulis vs. *M. galloprovincialis*	2 Mya	fossils, Barsotti and Meluzzi 1968	0.000	n.a.
	M. californianus vs. *M. edulis* species complex	30 Mya	fossils, Coan *et al.* 2000	0.156	0.52
Tellinacea	*Donax* spp. vs. *Macoma* spp.	90-140 Mya	fossils, Pohlo 1982	0.198	0.14-0.22
	Sinonovacula constricta vs. *Donax* spp./*Macoma* spp.	206-251 Mya	fossils, Pohlo 1982	0.472	0.19-0.23

Sea are thought to have entered the Baltic during the Litorina Sea period and to be an extension of eastern Atlantic relatives. In contrast, we have shown here that the Baltic Sea *M. balthica* are more closely related to their Alaskan than to their European conspecifics.

Several biogeographic scenarios may explain the survival of the ice ages by genetically divergent *M. balthica* populations and perhaps even species. First, structured populations may have inhabited a compressed southern distribution range. This possibility seems less unlikely in view of the present result that populations of marine species with pelagic larvae can remain genetically isolated over much smaller distances than generally assumed (see also *e.g.* Karl and Avise 1992, Barber *et al.* 2000, 2002). Second, the units may not have all co-occurred within Europe but may be the result of several subsequent colonization events. Third, distinct and isolated populations ancestral to the present *M. balthica* assemblages may have survived the ice ages in refugia that are believed to have existed along the northern coasts of Scotland and Norway during the LGM (Sutherland 1984, Vorren *et al.* 1988, Dawson 1992).

The close resemblance between Baltic and Alaskan sequences is consistent with multiple colonizations from the Pacific. Further evidence for multiple colonizations stems from the stark, species-level allozyme differences between NE and NW Atlantic *M. balthica* (Meehan 1985). The dating of population subdivisions among NE Atlantic populations conforms to differentiation during the Pleistocene but before the LGM.

Lessons from co-occurring species

Comparisons of genetic marker studies between species in the same region is a potentially powerful tool for evaluating alternative biogeographic scenarios (Avise 2000). The situation presented here for *M. balthica* is highly comparable to that of the

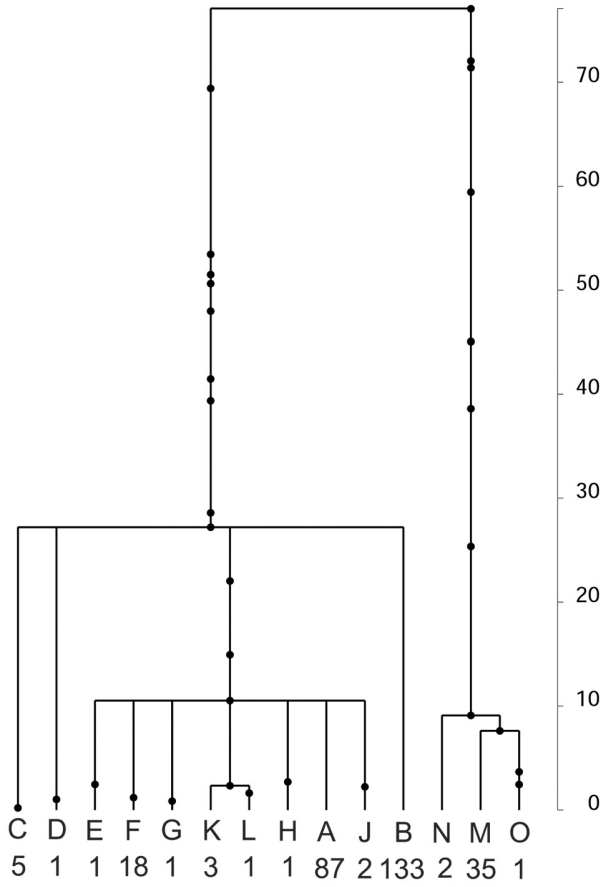


Fig. 2 - Coalescent tree among European COI haplotypes for *Macoma balthica*. Time axis on the right is in coalescent units. Dots indicate substitution events, letters correspond to haplotype (see also Table 1) and numbers to frequency of observation.

Mytilus edulis species complex, though with some pronounced differences, too. The blue mussels in the Baltic Sea, *M. trossulus*, have the species status because of large allozyme differentiation with respect to *M. edulis* and *M. galloprovincialis*, the other two species recognized in the species complex (McDonald *et al.* 1991, Väinölä and Hvilsom 1991). Baltic *M. trossulus* mtDNA lineages, however, do not form a distinct clade within the species complex (Wenne and Skibinksi 1995, Rawson and Hilbish 1998). Pacific *M. trossulus* mtDNA lineages, on the contrary, have diverged substantially (Rawson and Hilbish 1995, Hilbish *et al.* 2000). The blue mussels in the Baltic Sea are seen as belonging to the same species as Pacific *M. trossulus*, but with introgression of *M. edulis* mtDNA (Rawson and Hilbish 1998).

On the basis of allozymes, Väinölä and Hvilsom (1991) estimated genetic distance (Nei 1972) between Baltic blue mussels and *Mytilus edulis* from the North Sea to be 0.28. The allozyme-based genetic distance between Baltic and eastern Atlantic *Macoma balthica* was estimated at larger than 0.30 (Väinölä and Varvio 1989). This raises the question whether *Macoma balthica*, like the *Mytilus edulis* species complex, should be viewed as a complex of separate species. Väinölä and Varvio (1989) argue that it is advisable to await thorough morphological characterisation of Baltic *Macoma balthica* and investigations into the possibility of natural hybridization with eastern Atlantic relatives before changing the taxonomic status.

Baltic fauna and flora are generally seen as recent extensions of their Atlantic relatives. However, neither blue mussel nor Baltic clam genetic data fit this view. Two other studies may shed more light on the scenario of a recent Atlantic origin of Baltic biota. The benthic alga *Phycodrys rubens* (Rhodophyta) occurs in the Atlantic and North Sea, and in those westernmost parts of the Baltic where salinities remain above 15 ppt (i.e., Skagerrak and Kattegat). Baltic and North Sea *P. rubens* are, however, not genetically distinguishable using RAPD's and allozymes (Van Oppen *et al.* 1995), although ecotypes are recognized on the basis of salinity tolerance (Rietema 1992). In contrast, the worm *Hediste diversicolor* (Polychaeta: Nereididae) is strongly differentiated in allozymes between the Baltic Sea and the North Sea, with an F_{st} of 0.892 (Röhner *et al.* 1997). Röhner *et al.* (1997) did not question the hypothesis that the differentiation would have arisen in the time since the LGM. This scenario is more likely to be true for the polychaetes than for *M. balthica* because the worms have limited dispersal capacity, and strong differentiation is also found at short spatial scales (Hateley *et al.* 1992, Fong and Garthwaite 1994, Abbiati and Maltagliati 1996).

Neither the *Phycodrys* nor the *Hediste* study refute the idea that Baltic biota have a recent Atlantic origin. While Baltic *Mytilus trossulus* appear to have lost the mitochondrial lineages initially associated with their nuclear genome, Baltic *Macoma balthica* have not. The data presented here provide further evidence for the possibility of multiple marine colonizations of the eastern Atlantic and the Baltic Sea from the northern Pacific after the opening of the Bering Strait 3.5 Mya.

We conclude that high dispersal potential because of pelagic larvae does not necessarily imply high connectivity among populations for *M. balthica*. In some areas, as for example the Dutch Wadden Sea and North Sea, both inference from F_{st} values and coalescent analysis point to ongoing genetic exchange. Other populations, however, can be highly subdivided and thus independent both numerically and evolutionarily. These inferences should receive careful consideration during marine conservation policy making.

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CHAPTER THREE

Spatially structured genetic variation in a broadcast spawning bivalve: quantitative versus molecular traits

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Abstract

Understanding the origin, maintenance and significance of phenotypic variation is one of the central issues in evolutionary biology. An ongoing discussion focuses on the relative roles of isolation and selection as being at the heart of genetically based spatial variation. We address this issue in a representative of a taxon group in which isolation is unlikely: a marine broadcast spawning invertebrate. During the free-swimming larval phase, dispersal is potentially very large. For such taxa, small-scale population genetic structuring in neutral molecular markers tends to be limited, conform expectations. Small-scale differentiation of selective traits is expected to be hindered by the putatively high gene flow. We determine the geographic distribution of molecular markers and of variation in a shell shape measure, globosity, for the bivalve *Macoma balthica* (L.) in the western Dutch Wadden Sea and adjacent North Sea in three subsequent years, and find that shells of this clam are more globose in the Wadden Sea. By rearing clams in a common garden in the laboratory starting from the gamete phase, we show that the ecotypes are genetically different; heritability is estimated at 23%. The proportion of total genetic variation that is between sites is much larger for the morphological additive genetic variation (Q_{ST} = 0.416) than for allozyme (F_{ST} = 0.000 to 0.022) and mitochondrial DNA cytochrome-*c*-oxidase-1 sequence variation (Φ_{ST} = 0.017). Divergent selection must be involved and intraspecific spatial genetic differentiation in marine broadcast spawners is apparently not constrained by low levels of isolation.

Introduction

The life cycle of many sedentary species in the marine environment contains a free swimming larval phase. This short period early in life in which huge displacements may take place, contrasts strongly with a sedentary (*e.g.* corals, clams, coraline algae) or semi-sedentary (*e.g.* polychaetes, snails) adult stage. Geographically structured intraspecific genetic variation is generally not expected for such large, high dispersal, panmictic populations, as diversification is traditionally assumed to take

place in isolation (Mayr 1942, 1963, 1982). Indeed, intraspecific molecular variation is usually unstructured over large distances in such species (*e.g.* Borsa *et al.* 1991, Koeh, 1991, Palumbi 1996). However, some well known exceptions to this rule exist (*e.g.* oysters: Reeb and Avise 1990, Karl and Avise 1992, giant clams: Benzie and Williams 1997). Also, marine species diversity can be high in the absence of barriers to dispersal (Grassle and Maciolek 1992) and sibling species are more often sympatric or parapatric than allopatric (Knowlton 1993).

Like their terrestrial counterparts, individuals of a single species of marine invertebrate may inhabit very different habitats, *e.g.* either the periodically exposed intertidal or the adjacent subtidal, or either the outside of rocks or the sheltered cracks in between. Here we address the question how marine invertebrates with pelagic larvae deal (in a population genetical sense) with the variation of environments contained within their range. More specifically, we investigate whether local adaptations can occur despite the lack of obvious barriers to gene flow.

Diversification by locally differing selection regimes is traditionally assumed to be prevented already by low levels of gene flow (Slatkin 1987). An extensive body of theoretical work, on the other hand, shows how selective variation can be maintained because of spatial heterogeneity, even if mating and dispersal are random across niches (for reviews see Felsenstein 1976, Hedrick 1976, 1986). Empirical studies in agreement with selection-based spatial diversity are also emerging. Recently, selection, rather than isolation, has been put forward as an important factor in generating and maintaining diversity in birds (little greenbul *Andropadus virens*: Smith *et al.* 1997, blue tit *Parus caeruleus*: Blondel *et al.* 1999) and a lizard (Schneider *et al.* 1999). All three studies contrast the population structuring of neutral genetic variation at the molecular level with that of selective morphological traits and find that, although the former does not, the latter does show marked local divergence. Although in these studies the genetic basis for the morphological structuring is not demonstrated, the observations lead the authors to conclude that selection is a more important factor than geographic isolation in biological diversification.

In high dispersal taxa like marine broadcast spawners with pelagic larvae, however, spatially structured selective genetic diversity is less likely, as it is the combination of partial isolation and selection that makes divergence-with-gene-flow feasible theoretically (Slatkin 1987, Rice and Hostert 1993). Spatial phenotypic differentiation may also be the result of developmental plasticity, driving the same genotypes into different directions in alternative surroundings. Transplantation experiments with adult individuals showed that phenotypic plasticity accounted for at least some of the geographic patterns of shell shape in, *e.g.*, the marine mussel *Mytilus edulis* (Seed 1968) and the marine (but low dispersal) snail *Littorina obtusata* (Trussell and Smith 2000, Trussell 2000).

A quantitative approach which can provide insight into the relative roles of isolation and selection, is a comparison of the degree of differentiation in polymorphic molecular markers versus quantitative traits of which the genetic basis

is estimated. Standardized and equivalent measures for such comparisons are F_{ST} and Q_{ST}, respectively (Spitze, 1993, Merilä and Crnokrak, 2001). The neutral expectation for Q_{ST} is the value of F_{ST} for neutral single-locus genes in the same populations (Felsenstein, 1986, Lande, 1992).

Here we document a geographic, habitat-related pattern in shell shape of the broadcast-spawning bivalve *Macoma balthica* (L.): shells from the Dutch Wadden Sea are more globose than those from the adjacent North Sea. This pattern is contrasted with the distribution of neutral molecular marker variants. Using laboratory reared offspring we then test to what extent additive genetic effects contribute to the shell shape pattern we observed. The null hypothesis that differentiation in quantitative trait genetic variation is similar to that in molecular marker variation is evaluated by comparing F_{ST} and Q_{ST} values.

Materials and methods

Study populations

Macoma balthica (L.) is a tellinid bivalve that lives buried in muddy to sandy sediments in the shallow subtidal to high intertidal along the coasts of Europe and North America. Its pelagic larvae drift in the water column for approximately two to five weeks (Caddy 1969, own observations) before settlement in high intertidal 'nursery areas' (Beukema 1993). There they remain until their first winter, when secondary migration with the use of a mucoid drifting thread brings them to the place where they will spend their sedentary adult life (Beukema and De Vlas 1989, Beukema 1993). The clams do not produce gonads before they are one year old (Lammens 1967).

The Wadden Sea is a soft sediment tidal system enclosed between the mainland of the Netherlands and a chain of barrier islands (Fig. 1). Every low tide water is transported out of the basin into the adjacent North Sea, exposing the mudflats. *Macoma balthica* is found in intertidal and subtidal areas of the Wadden Sea, as well as in relatively shallow subtidal areas of the North Sea outside the chain of islands. The habitats contained within this area differ in averages and fluctuations of water temperature (influenced by *e.g.* wind and solar radiation) and salinity (depending on *e.g.* rain, and mainland freshwater outlets), in sediment coarseness, water current velocities, predation pressure and type (tidal regime rules the presence of *e.g.* birds, predatory snails, crabs and fish), and in food type and abundance (see *e.g.* Cadée and Hegeman 1977).

The populations of *M. balthica* are discontinuous between the North Sea and the Wadden Sea. The tidal currents in the inlets between the islands are as high as 200 cm/s (Postma 1957) and virtually no benthic invertebrates are found there (Van der Veer and Witte 1993, own observations). The distance between populations of *M. balthica* on either side of the inlets is two to four kilometers. The dispersal distance during the pelagic larval phase of *M. balthica* of two to five weeks can be roughly

estimated as tens to hundreds of kilometers as follows: the turnover time of tidal basins in the Western Wadden Sea is 3-24 tidal cycles (Ridderinkhof 1990), *i.e.* less than two weeks. The dispersal potential is therefore certainly large enough to connect all the populations we study here in a single or a few generations.

To map shell shape variation in the field, in 1998, 1999 and 2000, we collected a total of 5342 individuals in 30 samples of *M. balthica* from 17 sites (Fig. 1). We *a priori* distinguished three different habitats: Wadden Sea intertidal, Wadden Sea subtidal and North Sea. Between two and five sites per habitat were sampled per year. (Note, that for this field morphology survey as well as the allozyme survey, we distinguished these three habitats, whereas for the mitochondrial DNA survey and the common garden experiment, by which time we had become aware of a morphological difference between seas only, we focussed on the two seas.)

Common garden experiment

Ripe parents for the common garden experiment were collected in spring 1999 at one site in the Wadden Sea (Mokbaai) and one in the North Sea (Terschelling) (Fig. 1, sites 7 and 4, resp.) and were stored, buried in sediment, in aerated salt water

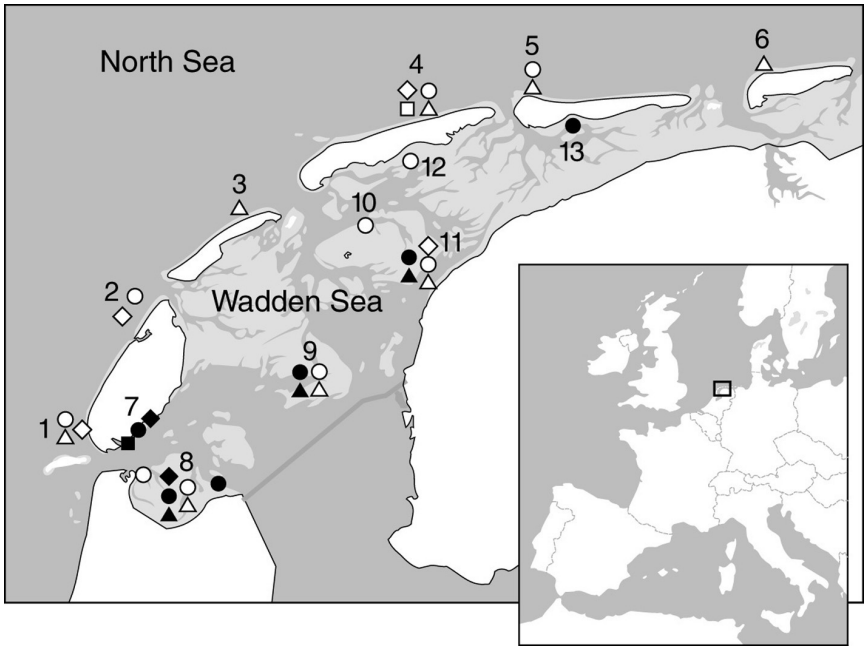

Fig. 1 - *Macoma balthica* sampling sites. Intertidal areas are indicated with light shading. The sampling sites are numbered and circles indicate shell shape sampling sites, triangles allozyme sampling sites, diamonds mitochondrial DNA sampling sites, and squares quantitative genetics parental origins. The locations indicated on the map give approximate positions of the sampling sites. Open symbols represent subtidal sites, solid symbols intertidal sites.

containers below 10°C in the laboratory. The clams were kept for 24 h in ultraviolet-irradiated, filtered (1 μm) seawater (UVFS) of ~0°C prior to induction of spawning. They were then individually put into 120 ml glass beakers with 50 ml aerated UVFS of 15 to 18°C containing Prozac® (Lilly). Details of artificial induction of spawning are described in Honkoop *et al.* (1999). Sperm of a single male was added to eggs of a single female and the suspension left standing overnight at 15°C. The eggs were then viewed under a binocular microscope at 60x magnification to check whether fertilization had been successful and development normal (*i.e.*, embryos were spherical and several cleavages had taken place). During the following three days the embryos were kept in aerated perspex cylinders containing 2 l UVFS with $2.5 \cdot 10^{-5}$ g/l streptomycin sulphate (Sigma) and $1.5 \cdot 10^{-5}$ g/l penicillin (Merck). The larvae were then transferred to aerated plastic bags containing 2 l UVFS containing the same concentrations of antibiotics as in the cylinder phase. The water of each batch was replaced every second day and an equal amount of algae (*Isochrysis galbana* and *Tetraselmis suecica* from continuous cultures) was added at the same time. After 24 days, when most larvae had developed a foot, which is a sign that a bivalve larva is competent to settle, they were transferred to 90 μm mesh carriers submerged in 400 ml UVFS to which the same concentrations of antibiotics and algae were added as in the bag stage. Seventy-five days after fertilization, when most larvae in a batch were larger than ~200 μm, the batch was transferred to an aquarium containing sand (maximum grain size < 300 μm, heated for five hours at 560ºC in order to remove organic material) and 1 l UVFS. In the aquarium phase the offspring were fed algae one to three times per week and the water was replaced once a week. The rearing temperature of the clams was kept constant at 15ºC.

In each of ten separate spawning sessions that took place in May through August 1999, we obtained at least three male and three female spawners. Five spawning sessions were done using North Sea parents, and five with Wadden Sea parents. Of the parents, shell length and width (nomenclature according to Stanley 1970) were measured to the nearest 0.01 mm using calipers. Parents were then frozen alive in liquid nitrogen and stored at -80°C. Three males and three females were crossed in all possible combinations to produce nine fertilized batches. In this way we produced a total of 90 single-pair crosses. Upon reaching the aquarium phase, 76 batches still had at least one survivor. One year after spawning, shell length and width of offspring were measured to the nearest 0.01 mm using electronic calipers. If the offspring were smaller than ~5 mm in length, they were not measured in order to avoid the risk of damaging the shells. Of the 76 surviving batches, 72 contained offspring big enough to be measured. The total number of offspring thus measured was 919.

Molecular analysis

Live clams were frozen in liquid nitrogen to preserve enzyme activity and prevent DNA from degrading, and stored at -80°C until further processing.

For allozyme analysis, a total of 345 adult Baltic clams was collected at 11 sites in spring 1997 (Fig. 1). A piece of tissue (avoiding the trematode parasites *Parvatrema* spp. present in a small percentage of animals in all populations) was ground in water using a porcelain mortar and cup and the lysate subjected to electrophoresis in 12% starch gels. Five enzyme loci were screened: glucosephosphate isomerase (*Gpi*, E.C. 5.3.1.9), isocitrate dehydrogenase I and II (*Idh*, E.C. 1.1.1.43), phosphoglucomutase (*Pgm*, E.C. 5.4.2.2.) and triosephosphate isomerase (*Tpi*, E.C. 5.3.1.1). All samples were run at 200 volts for eight hours on a Tris citrate pH 7.0 buffer. Zymograms were visualized as described in Murphy *et al.* (1996) with some modifications. Gels always contained lysates of individuals from more than one site to enable unbiased scoring of alleles.

A 393 bp fragment of the mtDNA cytochrome-*c*-oxidase I gene was sequenced of a total of 138 adult *Macoma balthica* collected at six sites in spring 2000 (Fig. 1). DNA was extracted from approximately 1 mm^3 tissue using a standard phenol-chloroform extraction method and redissolved in 10 µl water. Phage Lock Gel Light (Eppendorf) during centrifugation at the phenol-chloroform stage enabled extraction in spite of the extreme mucoid nature of the tissue. If the individual was a ripe female, the gonad was used for DNA extraction, since this tissue contained relatively little mucus. In all other cases (ripe males and unripe or parasitized individuals) mantle tissue was used. Extracted DNA was visualized on 1% TBE agarose gels. PCR amplifications were carried out in 50 µl volumes using a 1:10 dilution of DNA. In rare cases of failed amplification, either diluting the extract further to 1:100 or using the extract undiluted, usually rendered a positive PCR.

Universal primers LCO1490 and HCO2198 (Folmer *et al.*, 1994) were used to amplify a 710 bp fragment of the mitochondrial cytochrome-*c*-oxidase I gene. After preliminary sequence results were obtained, new primers internal to the universal primers were designed (forward: 5'-TTAGTGACTTCACACGGTTTGC-3' and reverse: 5'-AGTACAGGTATAGCAACCACCAG-3'). The newly designed primers were used for the remainder of the study. Amplification products were cut out of 1% agarose gels and purified using the manufacturer's protocol (Qiaquick Gel Extraction Kit, Qiagen). Single-stranded DNA was sequenced directly using the BigDye (PE Biosystems) sequencing kit and manufacturer's protocol with the forward primer on an automated sequencer (ABI PRISM 310 Genetic Analyzer).

Statistical analysis

A shell shape measure was determined that can be described as globosity. Maximum shell length and shell width were measured. Shell shape displays positive allometry (larger shells are more globose), for which we corrected by taking the natural log of both shell measures and performing a principal component analysis (PCA) on the two measures (for a discussion of this method and a comparison with an ANCOVA approach, see Sprent 1972). The first principle component is interpreted as size, and the second as size-corrected shape (globosity). In the

Table 1 - Mixed-model ANOVA of shell shape variation of *Macoma balthica* in the field. Habitat (*a*) is treated as a fixed effect and year (*b*) as a random effect; *c* are sites nested within year and habitat; *i*, *j*, *k*, *l* are observed numbers associated with habitats, years, sites and individuals, resp.; s^2_x are estimations of variance components σ^2_a, σ^2_b, σ^2_{ab} and $\sigma^2_{c\subset ab}$, resp. Habitats recognized are Wadden Sea intertidal, Wadden Sea subtidal, and North Sea (always subtidal).

Source of variation	df	Expected Mean Squares	MS	F	*p*	s^2_x
a	(*j*-1) = 2	$\sigma^2+l\sigma^2_{c\subset ab}+kl\sigma^2_{ab}+ikl\sigma^2_a$	172.6	22.1	<0.01	10.3%
b	(*i*-1) = 2	$\sigma^2+l\sigma^2_{c\subset ab}+jkl\sigma^2_b$	2.56	0.18	n.s.	<1%
*a*x*b*	(*i*-1)(*j*-1) = 4	$\sigma^2+l\sigma^2_{c\subset ab}+kl\sigma^2_{ab}$	7.81	0.53	n.s.	<1%
*c(a*x*b)*	*ij*(*k*-1) = 25	$\sigma^2+l\sigma^2_{c\subset ab}$	13.68	18.49	<0.001	8.1%
Error	*ijk*(*l*-1) = 5308	σ^2	0.74			81.7%

remainder of this paper, for the term 'shell shape', read 'the factor loading of the second principal component of the PCA on log(shell length) and log(shell width)'. Separate PCA's were carried out with the data set for the description of shell shape variation in the field and that for the common garden experiment.

We fitted the corrected shell shape field data to the following mixed model:

$z_{ijkl} = a + b_i + c_j + (bc)_{ij} + d_{ijk} + e_{ijkl}$

where z_{ijkl} is an individual's shell shape, a is the population mean, b_i is the fixed effect of habitat i (Wadden Sea intertidal, Wadden Sea subtidal and North Sea), c_j is the random effect of year j, $(bc)_{ij}$ is the interaction effect between habitat and year, d_{ijk} is the nested contribution for the k^{th} site within habitat i of year j, and e_{ijkl} is the error term for the l^{th} shell in site k of habitat i in year j. The expected mean squares of the model are specified in Table 1.

Shell shape data from the common garden experiment were corrected for length using PCA as described earlier. Heritability of shell shape was estimated using a regression of family average offspring shell shape (weighted for family size according to the method described by Kempthorne and Tandon 1953) on midparent shell shape. This gives an estimate of the narrow-sense heritability, *i.e.* additive genetic variance as a fraction of total phenotypic variance. Matings were done within and not between origins, *i.e.* associatively, to maximize the power of estimating heritability (Reeve 1961, Hill 1970), within the constraint of a fixed number of families that we could rear. These analyses were done for the entire data set as well as for North Sea families and Wadden Sea families separately. We tested for maternal effects in a multiple regression containing shell shape of both mother and father as independent variables, a procedure adjusting from Falconer (1981: pp. 151-152).

A measure of quantitative genetic differentiation, Q_{ST}, was calculated from estimated within- and between-population additive genetic variances for shell shape. The narrow-sense heritabilities calculated as described above were used as estimators for the variance components, the within-population component being averaged over the two populations. Q_{ST} is calculated as $\sigma^2_{GB}/(\sigma^2_{GB}+2\sigma^2_{GW})$, where σ^2_{GB} is the among-population component of variance for the quantitative trait and σ^2_{GW} is the within-population component of variance (Wright 1951, Lande 1992).

Allozyme data analyses were done using TFPGA 1.3 (Miller 1997) and Arlequin 2.000 (Schneider *et al.* 2000). Wright's F-statistics for a three-level sampling hierarchy were calculated using the method of Weir and Cockerham (1984) for sample size heterogeneity. 95% confidence intervals for F_{IS} (fixation index within sites), F_{IT} (fixation index in total combined population), F_{ST} (proportionate reduction in heterozygosity of sites and regions relative to total combined population), and F_{RT} (proportionate reduction in heterozygosity of regions relative to total combined population) values were determined by bootstrapping over loci (1000 replications) and significance of individual F-statistics was determined by permuting the data 16,000 times: individuals among sites but within regions (F_{SR}; the proportionate reduction in heterozygosity of sites relative to that within regions), or sites among regions (F_{RT}) (cf. Excoffier *et al.* 1992). Polymorphic loci were examined for Hardy-Weinberg equilibrium. Exact probabilities of conformity to Hardy-Weinberg equilibrium were approximated using a conventional Monte Carlo method.

DNA sequences were aligned by hand. A nested analysis of molecular variance (AMOVA; two populations, North Sea and Wadden Sea, with three subpopulations each) was carried out on the mtDNA sequence data, and a minimum spanning network among the haplotypes constructed, using Arlequin 2.000 (Schneider *et al.* 2000). Significance of the mtDNA $\mathbf{\Phi}_{ST}$, $\mathbf{\Phi}_{SR}$ and $\mathbf{\Phi}_{RT}$, the haploid analogs of F_{ST}, F_{SR} and F_{RT} (Excoffier *et al.* 1992), was calculated by permuting the data for 1000 replicates.

Results

Shell shape variation in the field

Shell shape variation is not randomly distributed over sites within the Wadden Sea and adjacent North Sea. Shells differ significantly between habitats, and also among sites nested within habitats and years (Table 1). The amount of variation explained by differences between habitats is comparable to that explained by differences among sites within habitats within years. Shape does not differ among years. The interaction between habitat and year is not significant, either (Table 1; Fig. 2a). The main difference among habitats is between the North Sea versus the Wadden Sea (Fig. 2a). While the distributions of shell shapes in the Wadden Sea and North Sea display a considerable amount of overlap, Baltic clams are significantly more globose in the Wadden Sea (overall mean shell shape -0.33 ± 0.015 standard

error (s.e.), standard deviation (s.d.) 0.93, n = 3658) and in the North Sea their shells are flatter (mean shape 0.57 ± 0.020 s.e., s.d. 0.83, n = 1684; see Fig. 3).

Common garden experiment

Weighted parent-offspring regression for the entire data set shows that shell shape variation in the field is partly heritable (Fig. 4, Table 2). The slope of the regression, which indicates narrow-sense heritability, is estimated at 0.23 (standard error 0.080) and is significantly different from zero (weighted linear least squares regression, $p<0.01$). The slopes of the parent-offspring regressions for the two experiments separately are also positive (0.20 for the North Sea and 0.12 for the Wadden Sea, Table 2), but do not differ significantly from zero. There is no evidence for maternal effects, as the partial regression coefficient associated with paternal

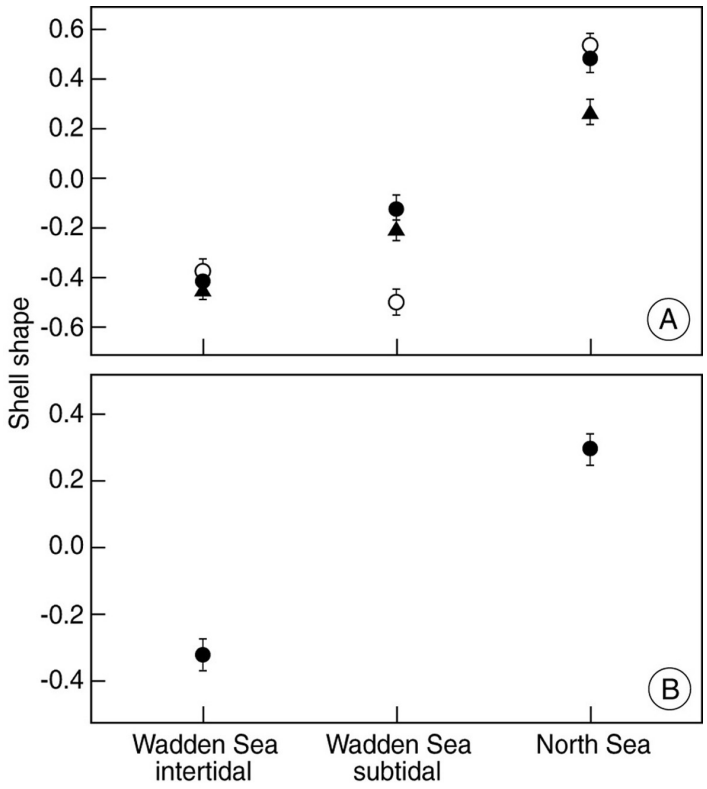

Fig. 2 - (a) Mean shell shapes (± SE) of field collected *Macoma balthica* in three different habitats in three years. Triangles are data for 1998, solid circles for 1999, open circles for 2000. These data are least squares means from mixed-model analysis (Table 1). (b) Shape of offspring in common garden experiment: mean (± SE) for offspring of Wadden Sea (n = 425) and North Sea (n = 496) parents ($p < 0.001$, t-test).

Table 2 - Weighted regressions from common garden experiment with *Macoma balthica*. Weighting according to family size cf. Kempthorne and Tandon (1953). Parent-offspring analyses: fitted linear regression $y = c + b*x$. Maternal effects analysis: $y = c + b_1*x_m + b_2*x_f$; y = average offspring shell shape in a family; c = a constant; b = heritability h^2; b_1, b_2 = maternal resp. paternal components of heritability; x = midparent shell shape; x_1 = shell shape mother; x_2 = shell shape father).

Midparent-offspring analyses

Data set		**df**	**MS**	**F**	*p*	**Parameter estimate (SE)**
all (n=72 families)		1	5.106	7.966	0.006	b = 0.227 (0.080)
	error	70	0.641			
North Sea (n=38)		1	0.877	1.541	n.s.	b = 0.199 (0.160)
	error	36	0.569			
Wadden Sea (n=34)		1	0.223	0.384	n.s.	b = 0.120 (0.194)
	error	32	0.581			

Maternal effects analysis

Data set		**df**	**MS**	**F**	*p*	**Parameter estimate (SE)**
all (n=72)	model	2	2.667	4.122	0.020	
	error	69	0.647			
		1	1.332	2.056	n.s.	b_1 = 0.087 (0.060)
		1	2.813	4.347	0.041	b_2 = 0.148 (0.071)

phenotype in the multiple regression ($0.5h^2 = 0.148 \pm 0.071$ s.e.) is not smaller than the maternal one ($0.5h^2 = 0.087 \pm 0.060$ s.e.) (b_2 and b_1, respectively, in Table 2). The proportion of quantitative additive genetic variance that is among populations is $Q_{ST} = 0.416$.

Molecular variance

The numbers of alleles detected per allozyme locus are 9, 3, 5, 9 and 5, for *Gpi*, *Idh1*, *Idh2*, *Pgm*, and *Tpi*, respectively. The locus *Idh1* is not considered polymorphic on the basis of the criterion that the frequency of the most common allele should not exceed 0.95; estimated overall frequency of its allele b is 0.98. Alleles are named a, b, etc. in order of electrophoretic mobility, such that a is the allele with the shortest electrophoresis distance. Both permuting the data and bootstrapping over loci (Table 3) show that allele frequencies do not differ between habitats (model 1, F_{RT} =

Table 3 - Wright's F-statistics for five polymorphic allozyme loci. Data were examined using two hierarchical models (model 1: regions Wadden Sea subtidal, Wadden Sea intertidal and North Sea, with three, three and five nested sites, respectively; model 2: regions North Sea versus Wadden Sea with five and six nested sites, respectively). Between brackets: 95% confidence intervals obtained from bootstrapping 1000 times over loci.

Model	F_{IS}	F_{IT}	F_{ST}	F_{RT}
1	0.137	0.147	0.0114	0.0015
	(0.070;0.17)	(0.075;0.19)	(0.0045;0.018)	(-0.0062;0.0049)
2	0.137	0.148	0.0123	0.0030
	(0.070;0.17)	(0.076;0.19)	(0.0045;0.020)	(-0.0014;0.0058)

0.0015, n.s.; nested analysis: Wadden Sea subtidal, Wadden Sea intertidal and North Sea, with three, three and five nested sites, respectively) or seas (model 2, F_{RT} = 0.0030, n.s.; nested analysis: North Sea versus Wadden Sea with five and six nested sites, respectively). There is slight allozyme structuring among sites within regions (model 1, F_{SR} = 0.0098, $p < 0.05$; model 2, F_{SR} = 0.0094, $p < 0.05$). Pairwise site comparisons show that this structuring is present only within the Wadden Sea and that the difference is due to the Waardgronden (location 9 in Fig. 1) intertidal sample versus the Waardgronden subtidal and the Balgzand (location 8 in Fig. 1) subtidal samples (permutation tests, $p = 0.018$ and $p = 0.009$, respectively). Inbreeding coefficients F_{IS} and F_{IT} are, however, high and different among loci (Table 4). For three out of the four polymorphic loci, the shortage of heterozygotes is highly significant, also when taking into account multiple testing (exact test, $p<0.01$ for *Gpi*, *Idh2* and *Pgm*). For *Tpi* there is a trend towards heterozygote deficiency. The proportion of total allozyme variation across loci that is between sites and habitats equals F_{ST} = 0.0114 (model 1), and between sites and seas (model 2) F_{ST} = 0.0123. Analyzed per locus, F_{ST}'s for model 1 are 0.007, 0.013, 0.009, 0.019, 0.001 and for model 2, 0.006, 0.015, 0.010, 0.022, 0.000 (for *Gpi*, *Idh1*, *Idh2*, *Pgm* and *Tpi*, respectively).

The number of mitochondrial COI haplotypes detected among the 138 individuals genotyped is seven (Genbank accession numbers AF443216 through AF443222). The haplotype set shows nine polymorphic sites, and the transition:transversion ratio is 8:1. Eight of the substitution polymorphisms are silent, *i.e.* do not correspond to an amino acid substitution, and one is a replacement substitution between alanine and valine (k_a:k_s = 1:8). The haplotype with the replacement polymorphism is haplotype e (Fig. 5, Table 5) and was found only once. Two haplotypes (a and b) are common and observed in roughly equal frequencies. The other five haplotypes are rare. Figure 5 shows an unrooted minimum spanning network among these haplotypes. The two common haplotypes differ from each other by four substitutions. The rare haplotypes are one mutation away from one of

the common ones. Estimated haplotype frequencies per sampling site are listed in Table 5. The populations are genetically homogeneous (Φ_{ST} = 0.017, Φ_{SR} = 0.016, Φ_{RT} = 0.0011, in nested analysis of molecular variance, n = 138, n.s.), indicating that there is neither a difference in haplotype frequencies between the North Sea and the Wadden Sea, nor between sites within seas.

Discussion

Molecular versus quantitative markers of genetic population structure

Shells of *M. balthica* are more globose in the Wadden Sea than in the adjacent North Sea (Fig. 2a and 3). The sites we sampled are very close to each other considering the potential scope for dispersal in this species; the North Sea and the Wadden Sea are separated by a chain of islands and the various connecting tidal channels are merely two to four kilometers long, whereas the larvae remain for two to five weeks in the pelagic phase (Caddy 1969, Drent 2002). The two seas make up different environments with likely associated differential selection pressures, but the general expectation is that gene flow is high and will hinder selective differentiation.

The expectation that gene flow is ongoing at this level was tested and confirmed. Observed F_{ST}'s, that range from 0.000 to 0.022 for allozymes and mitochondrial DNA sequences, correspond to a minimum of M = 14 migrants up to an infinite number of migrants per generation, assuming a classical island model of dispersal. This is much larger than the one migrant per generation which suffices (as a rule of thumb) to offset the diversifying effects of genetic drift (Slatkin 1994). The

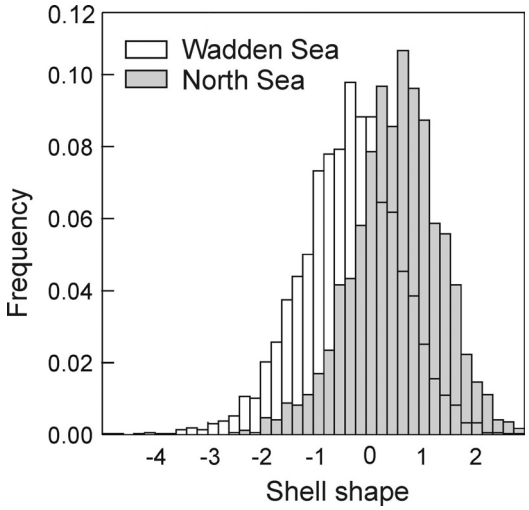

Fig. 3 - Frequency distribution of shell shapes of *Macoma balthica* in the field for the western Dutch Wadden Sea (white bars) and the adjacent North Sea (gray bars). Data are lumped for three years (1998-2000) and sites within seas, n_{total} = 5342.

hypothesis we thus formulate is that the two shell morphs in the two seas are the result of phenotypic plasticity. This hypothesis is rejected because variation in shell shape has a genetic component, and the offspring from one site in the North Sea and one in the Wadden Sea, reared in a common garden, still show the same distinct shell morphs as their parents (Fig. 2b). We can conclude that, despite ongoing gene flow, genetic differences among habitats do exist in this high dispersal marine invertebrate species.

As a measure of spatial structure in additive genetic variance we estimated Q_{ST} = 0.416. The fact that Q_{ST} is much higher than the F_{ST} estimates means that the degree of differentiation in the quantitative genetic trait exceeds that achievable by genetic drift alone, and, consequently, that divergent natural selection favouring different phenotypes in different populations must have been involved to achieve this much differentiation (Whitlock 1999, Merilä and Crnokrak 2001). Recently, Q_{ST}/F_{ST} comparisons were reviewed by Merilä and Crnokrak (2001) and McKay and Latta (2002). When viewed in the context of these comparisons, it follows that the *M. balthica* data point is at the extreme end: high gene flow coupled with strong selection. Certainly, the *M. balthica* data emphasize the weak relationship between F_{ST} and Q_{ST}, which is interesting because of the general assumption of a link between neutral and non-neutral differentiation that is applied to many areas of biology.

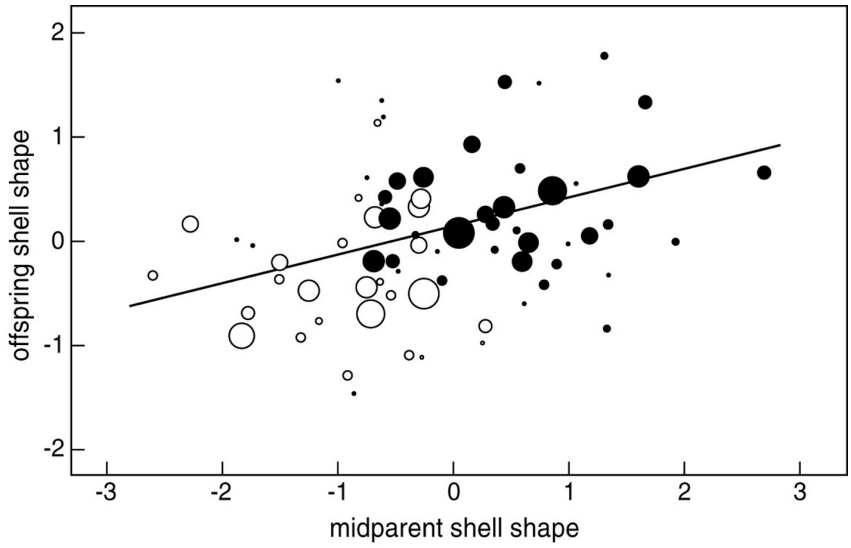

Fig. 4 - Parent-offspring regression of mean offspring shell shape against midparent shell shape. Open symbols are Wadden Sea families, solid symbols North Sea families. Circle area corresponds linearly to family size; the larger the symbol, the larger the family. Value on axes is loading of second PCA factor; higher values indicate less globose shells. Line represents weighted least-squares regression ($y = 0.101 + 0.230*x$; $r^2 = 0.11$; n=72).

Specifics of molecular and morphological data

Allozyme polymorphisms have been shown to be under balancing selection in oysters (Karl and Avise 1992). Other types of selection on particular allozyme alleles have been demonstrated by several authors (*e.g.* Koehn *et al.* 1980b, Burton 1986, Johannesson *et al.* 1995). If, as suggested by Hummel *et al.* (1995, 1998), selection on allozyme polymorphism also plays a role in *M. balthica*, it is possible that our allozyme survey either overestimates or underestimates population subdivision. However, the Φ_{ST} estimated from the mitochondrial haplotype data, which are independent of the nuclear allozymes, is in the same order of the F_{ST}'s from the allozyme data (unlike in the survey of Karl and Avise 1992). The congruence between the nuclear and mitochondrial data sets does not support the idea that selection is an important determinant in spatial allozyme variation in this case.

Allozyme data with locus-specific heterozygote deficiencies form a pattern frequently found in bivalves (*e.g.* Lassen and Turano 1978, Zouros *et al.* 1980, Skibinski *et al.* 1983, Foltz 1986b, Fairbrother and Beaumont 1993). Proposed causes include Wahlund effects (sampling from populations with different allele frequencies), differential selection acting on larvae and/or adults, inbreeding, genomic imprinting, somatic aneuploidy and null alleles. Our allozyme data have been examined in more detail (Luttikhuizen, unpubl.), showing that a technical artifact related to the large number of alleles causes the heterozygote deficiencies, while allele frequency estimates are essentially unaffected. The higher-level statistics F_{SR}, F_{RT} and F_{ST} therefore remain reliable indicators of the extent of allozyme population differentiation.

Both the amount of sequence variation and the absence of population subdivision at this spatial scale are comparable to other studies of mitochondrial DNA haplotypes of high dispersal marine invertebrates (Brown and Paynter 1991, Barber *et al.* 2000). The level of population subdivision here observed is also not larger than in comparable studies at larger geographic scales (Reeb and Avise 1990, Small and Chapman 1997, Wares *et al.* 2001). It is remarkable that the two most abundant haplotypes have a 1% sequence difference of four nucleotide substitutions between them. None of these differences amounts to an amino acid substitution at the protein level, so natural selection can not explain their co-existence. The two haplotypes are distributed evenly across seas (Table 5). An alternative scenario to a sympatric haplotype origin and subsequent lineage sorting in some populations but not the areas studied here, is that the two haplotypes arose in allopatry during an historic split and that the two separate populations have since merged.

The heritabilities estimated from the separate analysis of common garden experiment data within the two origins Wadden Sea and North Sea are positive but do not significantly depart from zero. This can either mean that the power of these analyses is not large enough to detect an existing effect, or, alternatively, that there is no heritable variation within these populations. The positive heritability estimates from the separate analyses, together with the higher power of the between-origin

Table 4 - Inbreeding coefficients F_{IS} and F_{IT} per allozyme locus; p = exact probability of conformity to Hardy-Weinberg equilibrium using conventional Monte Carlo method.

Locus	**F_{IS}**	**F_{IT}**	p
Gpi	0.117	0.122	0.000
Idh1	-0.027	-0.011	1.000
Idh2	0.112	0.122	0.002
Pgm	0.192	0.207	0.000
Tpi	0.050	0.051	0.093

experimental design (larger n, larger parental phenotypic range and mating performed associatively in order ot increase power), argue in favour of the existence of some within-population heritable shell shape variation.

The common garden experiment shows that offspring from the Wadden Sea and the North Sea differ in shell shape from each other in the same way their parents do (Fig. 2a, b). The heritability of shell shape is estimated from parent-offspring regression at 23%. Phenotypic plasticity may therefore also contribute to population differences in the field. For our hypothesis, the crucial information from the experiment is that globosity possesses a significant heritable component, and that therefore the adult populations are genetically different.

Subtle barriers to gene flow and differential selection pressures

Intraspecific covariance between phenotypes and environmental heterogeneities can be due to phenotypic plasticity, to genetic effects, or (most often) to a combination of both. We have shown here that in *M. balthica* genetic effects contribute to shell shape variation that is non-randomly distributed over habitats. Models addressing the issue of how alternative genetic variants may inhabit different habitats within a species' range, when gene flow is ongoing, go back a long way. One of the first attempting to understand the problem was Levene (1953), who showed that under random mating, the distribution of fitnesses across niches must satisfy quite stringent conditions for stable polymorphism to occur. Stable equilibrium becomes more likely when, for example, constant dominance is replaced with reversible dominance, where the heterozygote is always closest in fitness to the fittest homozygote (Gillespie 1976). The scope for stable maintenance of variation is also broadened by the introduction of habitat preference, *i.e.* a preference for niches associated with genotype (Hedrick 1990, Jaenike and Holt 1991), as well as reduced gene flow between niches (Brown and Pavlovic 1992, Meszéna *et al.* 1997). Local mating was originally suggested by Levene (1953) as a relaxing agent, but Strobeck (1974) has shown that in Levene's simple model, the parameter space for stable equilibrium is identical with or without local mating. However, in more complex

Table 5 - Estimated mitochondrial DNA haplotype frequencies for 393 bp fragment of cytochrome-*c*-oxidase I gene in *Macoma balthica*. Number of individuals per site in brackets; for location of sites see Fig. 1.

		North Sea			Wadden Sea		
		site 1	site 2	site 4	site 7	site 8	site 11
Haplotype	**Genbank accession no.**	(22)	(24)	(25)	(25)	(21)	(21)
a	AF443216	0.55	0.42	0.52	0.60	0.38	0.67
b	AF443217	0.41	0.54	0.44	0.40	0.48	0.19
c	AF443218	-	-	0.04	-	-	-
d	AF443219	0.05	0.04	-	-	0.10	0.05
e	AF443220	-	-	-	-	-	0.05
f	AF443221	-	-	-	-	-	0.05
g	AF443222	-	-	-	-	0.05	-

models, *e.g.* one that already incorporates habitat preference, adding local mating does increase robustness (Hoekstra *et al.* 1985). Polygenic models also generally show that reduced gene flow and drift are important facilitators of stable maintenance of polymorphism (*e.g.* Barton 1986, Phillips 1996).

Genetic drift is unlikely to play an important role in this case, as populations of *M. balthica* are very large. The system characteristics for understanding maintenance of geographically structured selective polymorphism here must be one or several of the following: (a) gene flow is reduced, (b) selection is extremely strong, (c) there is some form of habitat preference, or (d) mating is a local event.

There is some recent evidence that, contrary to expectation, marine larvae may be retained near source populations, constituting reduced gene flow (*e.g.* Jones *et al.* 1999, Swearer *et al.* 1999). The levels of gene flow might be high enough to prevent neutral genetic markers from differentiating, but low enough to aid population subdivision if selection plays a role. Interesting in this respect is the fact that the F_{ST} from allozymes for *M. balthica* is, although small, significantly different from zero, as assessed using bootstrapping over loci (Table 3). This may indicate that mixing is not complete, and that therefore a potential for reduced gene flow exists. In addition, neutral marker differentiation may in this case not yet have reached equilibrium between genetic drift and gene flow.

The strength of selection is potentially very large, as in *M. balthica* post-settlement densities decrease from up to 80,000 m^{-2} for newly settled recruits (Günther 1999, Bouma *et al.* 2001), to around 100 m^{-2} for adults (*e.g.* Honkoop *et al.*

1998). Given our inference that the geographic pattern in morphology of *M. balthica* is maintained by selection in the face of gene flow, the question arises what the nature of the selective agents might be. The type of shell shape variation we investigate here has three main effects on mortality. First, being washed out of the sediment poses a great risk for burying bivalves (Yonge 1950). In the more unstable sediments of the North Sea, the chances to become washed out are probably higher than in the finer sediments of the Wadden Sea, because of high current velocities and sand resuspension (Postma 1957). Shell shape also affects predation risk. In the North Sea we find relatively slow predators such as crabs and moon snails Naticidae (Holtmann *et al.* 1996). It may be possible for burying bivalves to escape such slow predators by burying deeper or moving away. Flatter shells enable greater speed and range (Trueman *et al.* 1966, Stanley 1970, Vermeij 1987, McLachlan 1995). Possible selection pressures may be the washing out or selective predation of the more globose shells in the North Sea. Avian predators, that feed on *M. balthica* in the Wadden Sea intertidal, are much faster. Buried prey usually have size windows outside of which they are not profitable (Zwarts and Wanink 1993, Piersma *et al.* 1993, 1994). When the upper limit to ingestibility by an avian predator is set by the minimum diameter of a shell (Zwarts and Blomert 1992), a more globose shell of the same size will be harder to swallow. Another hypothetical advantage of being globose in relation to avian predation is the larger amount of salt water trapped in the space between the valves. Ingestion of this excess of salt water makes it necessary to excrete the salt via the salt glands and this may be energetically very costly to the birds (Visser *et al.* 2000).

Habitat preference at first glance does not seem feasible for drifting pelagic larvae, as the speed of currents is orders of magnitude larger than larval swimming speed. However, many traits have evolved that do enable pelagic larvae to have some control over where they settle, *e.g.* body density for buoyancy, mucus strands for increased drag, and delayed metamorphosis (for a review see Young 1995). Experimental evidence also suggests that pelagic larvae actively select substrate type for settlement (Ahn *et al.* 1993, Snelgrove *et al.* 1999). In *M. balthica*, shell dimensions

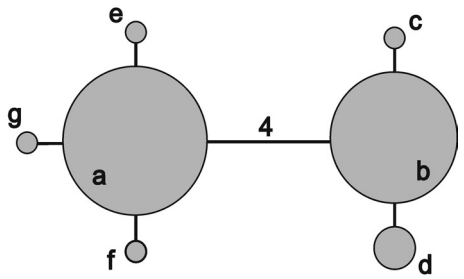

Fig. 5 - Minimum spanning network for the seven detected mitochondrial haplotypes for 393 bp cytochrome-*c*-oxidase I fragment. Branch length is one substitution unless otherwise indicated. Bubble surface area indicates haplotype frequency across entire survey.

have a strong impact during secondary migration, which it undertakes with the use of a mucoid thread in its first winter, taking it away from its high intertidal nursery areas into the entire range of habitats they will inhabit as adults (Beukema 1993). Beukema and De Vlas (1989) found that postmigratory Baltic clams that had settled in the North Sea are smaller as distance to the source (the Wadden Sea) increases. With experiments they demonstrated that this is because smaller shells sink more slowly. In addition to shell length, shell shape might also influence the time it takes for a migrating shell to sink. Baltic clams might thus be sorted non-randomly with respect to shell shape over habitats during secondary migration.

The scale of mating in marine broadcast spawners is extremely small (meters; Pennington 1985, Yund 1990, Levitan 1991, Babcock *et al.* 1992, pers. obs.), relative to the scale of dispersal (up to tens or hundreds of kilometers). This is a consequence of the constraints imposed by the system of external fertilization (for a review see Levitan 1995). Unless no selection whatsoever occurs, on account of the adults being sedentary, phenotypes will mate more often with similar phenotypes than under random mating.

Taking these considerations into account, the important characteristics of broadcast spawners for viewing them in the light of the maintenance of variation in heterogeneous environments are: global larval dispersal with the possibility of larval retention, larval habitat preference, local mating of the sedentary adults, and potential for strong selection early in life.

Genetic diversity of broadcast spawners

Our null hypothesis was that differentiation in quantitative trait genetic variation is not larger than that in molecular marker variation in *M. balthica*. Furthermore, because molecular differentiation was found to be very small, the homogenizing effects of inferred gene flow are expected to hinder selective differentiation. Following similar reasoning, short-distance (short relative to inferred gene flow levels) bivalve phenotypic patterns have been *a priori* assumed to be due to phenotypic plasticity only (*e.g.* Soares *et al.* 1998, Herbinger *et al.* 1998). We have shown in this study that this is not the case for the shell shape variation associated with habitat of *M. balthica* in the Wadden Sea and adjacent North Sea, which does have a significant genetic component.

These observations are important both for evolutionary theory and in the context of nature conservation. First, these data imply that the potential for selective spatial differentiation when isolation is very limited is greater than usually appreciated. In the case of high dispersal marine invertebrates this is probably because of their particular life cycle characteristics: global larval dispersal - possibility of larval retention - juvenile larval selection - local mating of the sedentary adults - potential for strong selection early in life. Second, for commercially exploited marine invertebrates, often particular refuge areas are left undisturbed, so-called 'Marine Protected Areas' (Hall 1999), or 'No-Take Zones' (Horwood 2000). If intraspecific

diversity is geographically structured even though molecular marker studies do not demonstrate it, then harvesting from particular (types of) areas may decrease intraspecific diversity. Also, if the maintenance of intraspecific polymorphism in high dispersal marine invertebrates is a dynamic process in the sense of Levene (1953), changing the relative proportions of particular niches by selective harvesting may disturb the dynamic equilibrium, and thereby indirectly impoverish the genetic make-up of a species.

Acknowledgements

We thank Andy Ruhmaloi, Anneke Bol, Oscar Bos, Jan Hegeman and the crews of NIOZ research vessels 'Griend' and 'Navicula' for practical help, Jaap van der Meer for statistical guidance, Pim Edelaar for helpful discussions, Herman Hummel, Sophie Launey and Dennis Hedgecock for technical advice on rearing bivalve larvae, the 'molecular course' of Steve Palumbi and Andy Martin at Friday Harbor Laboratories in 1998 for kick-starting our DNA work and Pieter Honkoop, without whose pioneering breeding attempts of *M. balthica* this work would not have been possible. The manuscript has benefited greatly from extensive comments on earlier versions by several anonymous reviewers.

BOX I

Differential selection due to bird predation may help maintain marine biodiversity

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(manuscript)

Shells of *Macoma balthica* were shown in chapter 2 of this thesis to differ morphologically between the western Dutch Wadden Sea and the adjacent North Sea; the shells are more globose in the Wadden Sea. From a comparison between spatial differentiation in neutral molecular markers and additive genetic variance for shell globosity, it was inferred that selection must play a role in maintaining the morphological difference. One of the putative selective agents is predation by probing avian predators that ingest *M. balthica* whole and crack the shell in their stomach. As the upper limit to ingestibility by an avian predator is set by the minimum diameter of a shell (Zwarts and Blomert 1992, Zwarts and Wanink 1993, Piersma *et al.* 1993, 1994), a more globose shell of the same size will be harder to swallow. Experiments were carried out to test whether red knots (*Calidris canutus*) prefer to feed on more globose Wadden Sea *M. balthica* or on less globose North Sea *M. balthica* when offered a mixture of both.

In an indoor aviary with an artificial mudflat, red knots were left to feed on 25 North Sea *M. balthica* and 25 Wadden Sea *M. balthica* that were buried in the

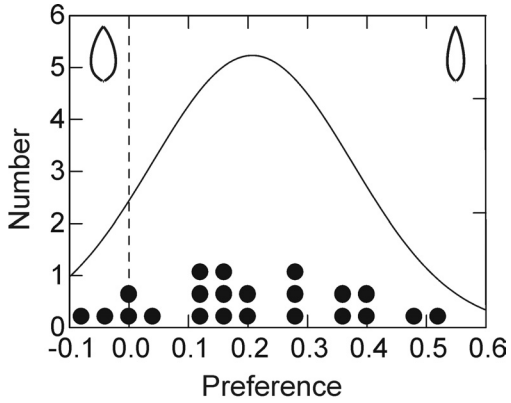

Fig. 1 - Preference of red knots (*Calidris canutus*) for eating flatter North Sea *Macoma balthica* from a mixture of North Sea and Wadden Sea bivalves. Each symbol represents one trial. Positive preference indicates more North Sea *M. balthica* were eaten.

sediment. This experiment was repeated 22 times. The experiments were stopped when approximately 50% of the bivalves had been consumed (approximately five minutes). Preference was calculated as $((n_N-n_W)/N_T)$; n_N = number of North Sea bivalves eaten, n_W = number of Wadden Sea bivalves eaten, n_T = total number of bivalves eaten. The bivalves were collected to the north of Terschelling and in the Mok, the Netherlands. Only the size class 13-18 mm was used. Globosity, calculated as the ratio between log-transformed shell length and shell width, was indeed higher for the Wadden Sea bivalves (ANOVA, $p < 0.001$). The knots displayed a significant preference for North Sea bivalves (Fig. 1, GLM, $F_{1,20} = 32.057$, $p << 0.001$). In all 22 experiments together, the knots had consumed 1.5 times as many North Sea than Wadden Sea *M. balthica*.

The reason for the preference of red knots for less globose *M. balthica* remains unknown. For red knots, the maximum size of the shells they can still swallow depends on the circumference of the bivalve (Zwarts and Blomert 1992, Zwarts and Wanink 1993, Piersma *et al.* 1993, 1994). There were indications in the data presented above to suggest that the preference for North Sea *M. balthica* existed mainly among the larger size bivalves (pers. obs.), which would corroborate the gape size restriction hypothesis. The amount of salt water trapped between the valves may also be related

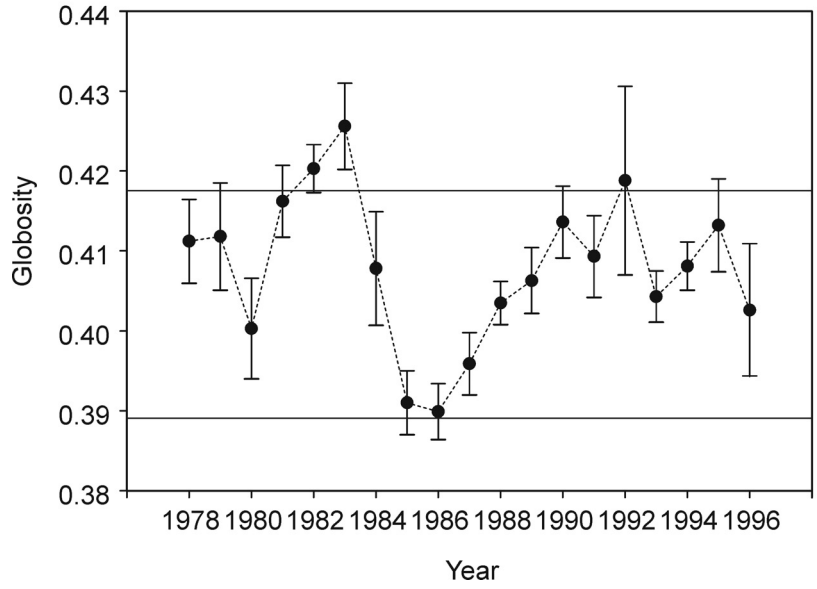

Fig. 2 - Time series of average globosity (+/- standard deviation) of 16-18 mm *Macoma balthica* at the Balgzand in the Dutch Wadden Sea. Horizontal lines indicate average globosity of 16-18 mm *M. balthica* used in the preference experiment shown in Fig. 1; upper line for Wadden Sea and lower line for North Sea bivalves.

to globosity preference; if the soft body parts are equal, a more globose shell will trap more salt water. Ingesting this excess of salt water and having to excrete it via salt glands is energetically very costly to birds (Visser *et al.* 2000).

Predation intensity of red knots on *M. balthica* in the field may be a function of their abundance. Field evidence has shown that the abundance of red knots in part of the Dutch Wadden Sea is highly variable, with an among-year coefficient of variation of 0.55 (Van der Meer *et al.* 2001). The contribution of *M. balthica* to the diet of knots ranges from 100% at high *M. balthica* densities to 0% at extremely low densities (Piersma *et al.* 1993). It may therefore be expected, given the experimental evidence just shown, that survival chances of less globose *M. balthica* in the Wadden Sea shows spatial and temporal variation. Spatial variation in *M. balthica* globosity was indeed documented in chapter 3 of this thesis. To test for temporal variation, the degree of globosity was measured of *M. balthica* shells (size class 16-18 mm) in samples from 1978 through 1996, kept in archive at the Netherlands Institute for Sea Research as part of the long term research programme based on transects at Balgzand, the Netherlands (Van der Meer *et al.* 2001). Length and width of both dried valves was measured and combined after correction for the error associated with the separate measurements of the two valves. Globosity was found to differ significantly among years (Fig. 2, $F_{14,398} = 5.85$, $p < 0.001$). In some years (notably 1985, 1986 and 1987) Baltic clams on the Balgzand were just as flat as typical North Sea *M. balthica*. In the years 1980, 1981 and 1982, on the contrary, Baltic clams at Balgzand were extremely globose.

Differential bird predation helps to maintain spatial differentiation in globosity of *M. balthica* shells in the study area in the north of The Netherlands. The temporal variation in globosity observed is consistent with the idea that gene flow and selection are at work simultaneously. Gene flow from the knot-free North Sea could be at work to homogenize divergence of shells between the two habitats. It is not clear whether it is the influx of genotypes from the North Sea or the selection pressures within the Wadden Sea that fluctuate, or both.

These data suggest that differential predation pressures help to maintain spatially organised biodiversity in marine organisms, in spite of possible homogenizing effects of gene flow. Biological conservation efforts in the marine environment have recently seen a shift from limiting fishery intensity to closing off particular reserve areas (Hall 1999, Horwood 2000). Because of the highly mobile life style of many marine species, it is not immediately obvious that marine reserves will prove to be adequate to sustain the target species (Botsford *et al.* 2001, Palumbi 2001). We like to add to this concern that the design of marine reserves must incorporate the multiplicity of habitats that ultimately provides the selection variety that conserves intraspecific biodiversity.

CHAPTER FOUR

A technical solution to heterozygote deficiencies in the bivalve *Macoma balthica*

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(manuscript)

Abstract

Allozyme data for bivalves often exhibit heterozygote deficiencies. Many hypotheses have been put forward to try and explain this phenomenon, but the problem remains unsolved. Here I formulate and test the hypothesis that a technical problem causes the heterozygote deficiencies, related to the fact that bivalve allozyme loci usually have large numbers of alleles. I propose that heterozygotes for alleles with similar electrophoretic mobilities (which are therefore adjacent on a gel after electrophoresis) are often scored as homozygotes. Existing allozyme data on 345 Baltic tellins *Macoma balthica* from the western Dutch Wadden Sea and nearby North Sea were analyzed to test this hypothesis. The data consist of five allozyme loci with 9, 9, 5, 5 and 3 alleles detected. Heterozygotes are less frequent than expected on the basis of Hardy-Weinberg equilibrium. The deficiency of heterozygotes is present almost exclusively in 'adjacent' heterozygotes. In non-adjacent heterozygotes, a deficiency is present in only one locus. I conclude that the problem of heterozygote deficiencies in the present *M. balthica* data set is primarily of a technical nature. Next, published data from 19 relevant papers are analyzed with respect to the misscoring hypothesis, reconstructing the frequency of adjacent heterozygotes (P^*_{uv}) and of non-adjacent heterozygotes (P^*_{uw}). There is a significant correlation between P^*_{uv} and inbreeding coefficient f, whereas P^*_{uw} and f are not correlated. This supports the misscoring hypothesis, and indicates that this technical problem is (at the very least) part of the solution of the long-standing problem of heterozygote deficiencies in bivalves. The technical solution has implications for the concept of heterozygosity-fitness correlations, the idea that fitness surrogates are associated with multilocus heterozygosities. This concept, which rests heavily on bivalve allozyme studies and is put into practice extensively in conservation biology, may need to be re-examined.

Introduction

Allozyme heterozygote deficiencies are common in bivalves (*e.g.* Lassen and Turano 1978, Zouros *et al.* 1980, Skibinski *et al.* 1983, Foltz 1986a, Fairbrother and Beaumont 1993). The phenomenon has drawn a substantial amount of attention and is still unresolved. Proposed causes include population mixing (the Wahlund effect), differential selection acting on larvae and/or adults, genomic imprinting, somatic aneuploidy, null alleles and a technical artifact.

In bivalves, allozyme heterozygosity has frequently been found to correlate with fitness (Mitton and Grant 1984, Zouros 1987), although the overall relationship is weak at best (Britten 1996). That molecular marker heterozygosity matters is a widely held notion extensively applied in conservation biology (O'Brien and Evermann 1988, Quattro and Vrijenhoek 1989, but see Avise 1994 for a critical review). In this paper I propose, after Ayala *et al.* (1973), that a technical problem is a source of heterozygote deficiencies in bivalves. I discuss the possible implications of this idea for heterozygosity-fitness correlations.

Allozymes are much more variable in bivalves than in other species. Evidence from mussels suggests that not all charge-altering amino acid polymorphisms can be resolved when allozymes are viewed on starch gels. In blue mussels *Mytilus edulis*, the number of alleles detected by polyacrylamide gel electrophoresis for the loci *Pgi* and *Pgm* is about 30 (Hvilsom 1983), much higher than <10 alleles previously detected for the same loci in the same geographical region using starch gel electrophoresis (Theisen 1978). The 30 alleles represent true genetic variation, as inheritance is strictly Mendelian (Hvilsom and Theisen 1984).

In view of the facts that (1) allozymes are much more variable in bivalves than most other species, (2) on starch gels the number of distinguishable alleles is smaller than on the higher-resolution polyacrylamide gels, and (3) most if not all of the original data describing heterozygote deficiencies in bivalves were obtained using starch gels, I put forward the following hypothesis: the missing heterozygotes on bivalve allozyme starch gels are those of which the two alleles have very similar electrophoretic mobilities. The deficiency in those particular classes of heterozygotes may then explain why an overall shortage is often found using standard statistical analysis. To test this hypothesis, I classify heterozygotes into two groups: those of which the two alleles are adjacent on the gel (*e.g.*, genotype 23) and those of which they are not (*e.g.* genotype 24). I then predict, that there should be a deficiency in the observed frequency of adjacent heterozygotes and no shortage in the observed frequency of non-adjacent heterozygotes.

To test the prediction, I re-analyze existing starch gel allozyme data on 345 individuals of the bivalve *Macoma balthica* for five loci, that were collected earlier in the context of a population subdivision study (Luttikhuizen *et al.* in press). Next, an adjusted test is performed on literature data of other bivalve species, because, almost exclusively, only allele frequencies and not genotype frequencies are presented.

Materials and methods

Field sampling

Baltic tellins *Macoma balthica* had been collected in spring 1997 in the Dutch Wadden Sea and the North Sea. More details are described in Luttikhuizen *et al.* (in press), where standard population genetic analyses of these allozyme data can also be found.

Allozyme assays

Live clams were frozen in liquid nitrogen and stored at -80°C until further processing. A piece of tissue (taking great care to avoid the parasites *Parvatrema* spp. present in a small percentage of all populations) was ground in water using a porcelain mortar and cup and the lysate subjected to electrophoresis in 12% starch gels. Five enzyme loci were screened: glucosephosphate isomerase (*Gpi*, E.C. 5.3.1.9), isocitrate dehydrogenase 1 and 2 (*Idh*, E.C. 1.1.1.43), phosphoglucomutase (*Pgm*, E.C. 5.4.2.2.) and triosephosphate isomerase (*Tpi*, E.C. 5.3.1.1). All were run at 200 volts for eight hours on a Tris citrate pH 7.0 buffer system. Zymograms were visualized as described in Murphy *et al.* (1996). Gels always contained lysates of individuals from several sites to enable unbiased scoring of alleles. The numbers of alleles detected were 9, 3, 5, 9 and 5, respectively. Alleles were coded 1, 2, etc., where electrophoretic mobility increases with number.

Exact probabilities of conformity to Hardy-Weinberg equilibrium were approximated with a conventional Monte Carlo method using the program TFPGA (Miller 1997).

Hypothesis testing

Heterozygotes were classified into adjacent (*e.g.* genotype 23) and non-adjacent heterozygotes (*e.g.* genotype 24). Alleles were numbered according to electrophoretic mobility, so that the numbers of the alleles of an adjacent heterozygote differ by one, and those of a non-adjacent heterozygote differ by more than one.

The standard approach to deviations from Hardy-Weinberg equilibrium is by using a model that incorporates within-population inbreeding coefficients f_{uv}. The fully unlimited multi-allelic model is usually parameterized as follows (Weir 1996):

$$P_{uu} = p_u^2 + p_u(1-p_u)\left[\sum_{v \neq u}\left(\frac{p_v}{1-p_u}\right)f_{uv}\right]$$

(1)

$$P_{uv} = 2p_u p_v(1-f_{uv})$$

$$u < v$$

The symbols used are: u, v = subscripts denoting allele classes; P_{uu} = frequency of homozygote genotype class; P_{uv} = frequency of heterozygote genotype class; p_u, p_v = allele frequencies; f_{uv} = inbreeding coefficient, one for each heterozygote class.

From this model, null hypothesis *a* was tested: 'the genotypic data conform to Hardy-Weinberg equilibrium', using an exact test. Null hypothesis *b* was formulated as follows: 'the deviation from Hardy-Weinberg equilibrium does not differ between adjacent and non-adjacent classes of heterozygotes'. Null hypothesis *b* was tested by the formulation of a restricted version of model (1). The restricted model incorporates inbreeding coefficients *f* ' and *q*, representing deviations from Hardy-Weinberg equilibrium in non-adjacent and adjacent classes of heterozygotes, respectively:

$$P_{uu} = p_u^2 + \left((p_{u-1}p_u) + (p_u p_{u+1})\right)q + \left(p_u(1-p_u) - (p_{u-1}p_u) - (p_u p_{u+1})\right)f'$$

(2)
$$P_{uv} = 2p_u p_v(1-q)$$

$$P_{uw} = 2p_u p_w(1-f')$$

$$u < v, u < w, (u-v) = 1, (u-w) > 1$$

where the subscript w denotes an allele class. By sequentially setting parameters f' and q to equal zero, nested models were created that enabled significance testing of f' and q using likelihood ratio tests.

Analysis of published data

Within-population inbreeding coefficients in bivalves were analyzed from published data. To locate relevant studies I searched Biological Abstracts databases from January 1990 to March 2001, using the search algorithm '(bivalve) and (allozyme or isozyme)'. Also, pre-1990 studies were obtained by examining the bibliography sections of the more recent papers. Hardly any papers contain genotype frequency data, so a test was designed that would discriminate between hypotheses using estimated allele frequency data. Those papers that did not report the estimated allele frequencies as well as the per-locus, per-population inbreeding coefficients had to be excluded from analysis. Studies were also excluded when (1) they had used a medium other than starch for their allozyme electrophoresis, (2) data had been pooled for several populations, (3) many of the allele frequencies did not add up to one (one case: De la Rosa-Vélez *et al.* 2000). Some studies used *f* (often called F_{IS}) for measuring heterozygote deficiencies, others used *D*. The parameter *D* was transformed to *f* by multiplying by -1. Some differences in estimation procedures of inbreeding coefficients existed among studies; some of these procedures differ slightly in the way per-locus values are obtained in multi-allelic cases (compare *e.g.*

Table 1 - List of studies used in meta-analysis; n_{loci} = number of loci; n_{pop} = number of populations; n_{ind} = average number of individuals per data point; n_{data} = number of data points for analysis in this paper; figures represent only loci and populations that satisfy criteria specified in text.

Reference	**Species**	$\mathbf{n_{loci}}$	$\mathbf{n_{pop}}$	$\mathbf{n_{ind}}$	$\mathbf{n_{data}}$
Ayala et al. 1973	*Tridacna maxima*	18	1	85.5	18
Bricelj and Krause 1992	*Argopecten irradians*	6	10	219.2	57
Diehl and Koehn 1985	*Mytilus edulis*	5	9	54.1	44
Fairbrother and Beaumont 1993	*Mytilus edulis*	5	1	94.0	5
Foltz 1986b	*Crassostrea virginica*	10	3	54.7	30
Gaffney 1990	*Mytilus edulis*	5	6	447.6	30
Gardner and Kathiravetpillai 1997	*Mytilus galloprovincialis, Perna canaliculus*	1	3	158.0	6
Gardner and Palmer 1998	*Mytilus galloprovincialis*	1	3	126.3	3
Gardner and Thompson 1999	*Astarte boralis, A. elliptica, A. striata*	12	1	76.3	34
Gentili and Beaumont 1988	*Mytilus edulis*	8	3	97.0	21
Hummel et al. 1998	*Macoma balthica*	6	3	80.0	18
Hummel et al. 2001	*Mytilus edulis, M. gallopovincialis, M. trossulus*	7	18	70.7	96
Koehn and Gaffney 1984	*Mytilus edulis*	5	8	81.3	39
Koehn et al. 1976	*Mytilus edulis*	3	3	616.4	9
Michinina and Rebordinos 1997	*Crassostrea angulata*	7	10	53.3	53
Perez et al. 2000	*Euvola ziczac*	1	3	87.0	3
Saavedra et al. 1993	*Ostrea edulis*	9	11	97.3	72
Volckaert and Zouros 1989	*Placopecten magellanicus*	6	6	199.3	29
Zouros et al. 1980	*Crassostrea virginica*	7	1	1699	7

model (1) to model (2)) (Robertson and Hill 1984, Weir and Cockerham 1984). For my rather unrefined type of analysis, these slight differences do not matter. Every locus for every sample (either an unique geographical location or a point in time) was treated as a single data point. Excluding those that were not strictly polymorphic, on

the basis of the criterion that the frequency of the most common allele should not exceed 0.95, a total of 571 data points was assembled. Table 1 lists the 19 studies used in the analysis and details on the data that they report.

Almost all allozyme variation studies provide estimated allele frequencies rather than observed genotype frequencies. From the estimated allele frequencies, expected frequencies of adjacent and non-adjacent classes of heterozygotes were calculated as follows:

$$P^*_{uv} = \sum_u \sum_v 2p_u p_v$$

(3)

$$P^*_{uw} = \sum_u \sum_w 2p_u p_w$$

$$u < v, u < w, (u - v) = 1, (u - w) > 1$$

where P^*_{uv} is the predicted frequency of adjacent heterozygotes, and P^*_{uw} is the predicted frequency of non-adjacent heterozygotes.

An increase in total expected heterozygosity ($P^*_{uv} + P^*_{uw}$) will increase f in the case of biased scoring. Thus, a correlation between expected heterozygosity and f is consistent with my hypothesis. P^*_{uv} is directly related to the likelihood of misscoring heterozygotes as homozygotes. As the true frequency of adjacent heterozygotes on a gel increases, the measured inbreeding coefficient f will increase as well as the frequency of adjacent heterozygotes as reconstructed from estimated allele frequencies. If the parameters P^*_{uv} and P^*_{uw} are independent or negatively correlated, any relationship between P^*_{uv} and f can be contrasted with the relationship between P^*_{uw} and f. An increase in the frequency of non-adjacent heterozygotes on a gel, if independent from an increase in the frequency of adjacent heterozygotes, is not expected to result in an increased f. Given my hypothesis of misscoring adjacent heterozygotes on starch gels as homozygotes, I predict that there will be a positive correlation between P^*_{uv} and f. Similarly, there should be no such correlation between P^*_{uw} and f. If scoring bias is not an issue, neither correlation should exist. Null hypothesis c therefore reads: 'neither P^*_{uv} nor P^*_{uw} is correlated with f'.

Results

Analysis of Macoma balthica *data*

As no population subdivision is present in this data set (Luttikhuizen *et al.* in press), the analyses treat the 345 individuals as a single population. Estimated allele frequencies for the entire data set are listed in Table 2.

Null hypothesis *a*, 'the genotypic data conform to Hardy-Weinberg equilibrium', is rejected for three loci (*Gpi*, *Idh2* and *Pgm*). The positive inbreeding coefficients (ranging from 0.112 to 0.192) indicate that there is a shortage of

Table 2 - Estimated allele frequencies for *Macoma balthica* allozyme loci under two different models. Alleles are numbered consecutively according to electrophoretic mobility (i.e., allele '*1*' has the smallest electrophoretic mobility) For details on models see text.

Allele	**Model**	*Gpi*	*Idh1*	*Idh2*	*Pgm*	*Tpi*
1	(1)	0.057	0.013	0.015	0.027	0.003
	(2)	0.053	-	0.014	0.025	0.003
2	(1)	0.320	0.982	0.098	0.023	0.070
	(2)	0.325	-	0.097	0.023	0.070
3	(1)	0.025	0.005	0.054	0.059	0.010
	(2)	0.023	-	0.055	0.060	0.010
4	(1)	0.057		0.822	0.254	0.915
	(2)	0.056		0.823	0.250	0.915
5	(1)	0.396		0.011	0.107	0.002
	(2)	0.389		0.011	0.116	0.000
6	(1)	0.044			0.109	
	(2)	0.052			0.105	
7	(1)	0.072			0.376	
	(2)	0.071			0.375	
8	(1)	0.019			0.015	
	(2)	0.020			0.017	
9	(1)	0.011			0.029	
	(2)	0.011			0.029	

heterozygotes (Table 3). For *Tpi* there is a trend towards heterozygote deficiency. Genotype frequencies at the nearly monomorphic locus *Idh1* do not depart significantly from Hardy-Weinberg equilibrium.

Null hypothesis *b* ('the deviation from Hardy-Weinberg equilibrium is not larger in adjacent than in non-adjacent classes of heterozygotes') is rejected on the basis of the four pairs of *q* and *f'* values estimated under model (2) (paired t-test, 3 df, $p<0.05$). In all cases, the deficiency of heterozygotes in the adjacent class is significant and large, with *q* ranging from 0.233 to 0.666 (Table 4). The deficiency of heterozygotes in the non-adjacent class does not additionally contribute significantly to the model in three cases; only for the locus *Pgm* it does ($f' = 0.100$, $p<0.001$). The deviation from Hardy-Weinberg equilibrium is always larger in the adjacent than in

Table 3 - Inbreeding coefficient *f* per allozyme locus in 345 *Macoma balthica*; p = exact probability of conformity to Hardy-Weinberg equilibrium using conventional Monte Carlo method.

Locus	***f***	*p*
Gpi	0.117	0.000
Idh1	-0.027	1.000
Idh2	0.112	0.002
Pgm	0.192	0.000
Tpi	0.050	0.093

the non-adjacent heterozygote class, as the maximum likelihood estimate (MLE) for parameter *q* is always larger than the MLE for parameter *f'* (Table 4). Also, estimated *q* in model (2) is always larger than estimated *f* in model (1) (Table 4). The estimated allele frequencies under models (1) and (2) are listed in Table 2.

Analysis of literature data

The reconstructed total heterozygosity ($P^*_{uv} + P^*_{uuvv}$) is positively correlated with inbreeding coefficients *f* (r = 0.116, n = 571, p < 0.01). The reconstructed frequency of adjacent heterozygotes (P^*_{uv}) is significantly correlated with *f* (r = 0.119, n = 571, p < 0.01, Fig. 1a). P^*_{uv} and P^*_{uuvv} are negatively correlated (r = -0.231, n = 571, p < 0.01). The expected frequency of non-adjacent heterozygotes is not correlated with *f* (r = 0.027, n = 571, n.s., Fig. 1b). Hypothesis *c*, 'neither P^*_{uv} nor P^*_{uuvv} is correlated with *f*', is therefore rejected.

Discussion

Biased scoring causes heterozygote deficit in Macoma balthica

The allozyme data on *M. balthica* exhibit heterozygote deficiencies typical for marine bivalves, with *f* ranging from -0.027 to 0.192 (see Britten 1997). Also the fact that all four loci for which the frequency of the most common allele is smaller than 0.95 have an inbreeding coefficient larger than zero, but not all inbreeding coefficients (three out of four in this case) differ significantly from zero, is a common pattern (*e.g.* Volckaert and Zouros 1989, Bricelj and Krause 1992).

The deficiency of heterozygotes in the *M. balthica* is larger among those classes of heterozygote genotypes whose alleles have similar electrophoretic mobilities ('adjacent' heterozygotes) than in other heterozygote classes. It can therefore be concluded that scoring bias is a cause of the overall deficiency of heterozygotes in the data set. The likelihood ratio tests on restrictions of model (2), that first include misscoring of adjacent heterozygotes in the form of parameter *q* and subsequently *f'*

Table 4 - Results of likelihood ratio tests on deviations from Hardy-Weinberg expectations in adjacent versus non-adjacent heterozygote classes, and maximum likelihood estimates of inbreeding coefficients in restricted model. $-2\ln(L_0/L_1)$ = log likelihood ratio of model versus nested model, χ^2 distributed with 1 degree of freedom; *n.s.* = not significant; f, q, f' = maximum likelihood estimates of parameters f, q and f'.

		Model(1)		**Model (2)**		
Locus	$\mathbf{n_{alleles}}$	f	q	$\mathbf{-2\ln(L_0/L_1)}$	$\boldsymbol{f'}$	$\mathbf{-2\ln(L_0/L_1)}$
Pgm	9	0.192***	0.544	48.96***	0.100	7.26***
Gpi	9	0.117***	0.666	55.50***	0.000	0.00 (*n.s.*)
Idh2	5	0.112**	0.233	11.63***	0.092	3.10 (*n.s.*)
Tpi	5	0.050 (*n.s.*)	0.283	5.45*	0.017	0.081 (*n.s.*)

*) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$.

as the shortage of heterozygotes in non-adjacent classes of heterozygotes, show that q is significantly larger than zero in all cases and that adding f' is only significant for one locus (Table 4). Estimation of parameters shows, first, that in all four polymorphic loci, f' decreases relative to f when q is included in the model, and second, that q in model (2) is always larger than f in model (1). The first observation implies that the heterozygote deficiency is always larger in the adjacent than in the non-adjacent heterozygote class. The second fact indicates that the deficiency of heterozygotes due to misscoring was larger than the inbreeding coefficient estimated by standard analysis, presumably because it was an average over heterozygote classes with and without a shortage.

Estimated allele frequencies for *M. balthica* differ only marginally between the two models (Table 2). This is surprising, given the large estimated fraction q of misscored adjacent heterozygotes. Apparently, the misscored heterozygotes are assigned to homozygote classes proportional to those classes, i.e. they are preferentially assigned to the more frequent classes. These marginal differences may nevertheless increase the error in parameter estimation. Thus, biased allozyme scoring in bivalves probably makes the technique slightly less sensitive to detect population subdivision than *e.g.* DNA-based genotyping. Rejecting the null hypothesis of 'no population structuring' is always harder when allele frequencies are estimated with greater error.

Biased scoring partly causes heterozygote deficit in other bivalves

The results of the analysis of published data support the hypothesis of misscoring of adjacent heterozygotes on allozyme gels. A positive correlation was predicted between P^*_{uv} and f, and no correlation between P^*_{uw} and f, both of which are the case. The effect magnitude of the relationship between P^*_{uv} and f coincides

with the trend in the data points for *M. balthica* when the latter are analyzed in the same way as the literature (Fig. 1a). As this paper has presented direct evidence for biased scoring in the *M. balthica* data (Table 4), the correspondence in the two trends is an indication that the observed effect in the literature data does indeed fit the biased scoring hypothesis. The large amount of variation was expected, as many other factors play a role. Given Hardy-Weinberg equilibrium, the strength of the effect of misscoring of adjacent heterozygotes can be expected to differ among loci as well as among studies and perhaps even among populations within studies in certain circumstances. A difference among loci in the strength of the scoring bias must exist as a result of the divergence of physical behavior of enzyme systems during starch gel electrophoresis. For example, a scoring bias will be different for monomeric than for dimeric enzymes, because in the latter a heterozygote produces three bands instead of two, which should increase the probability of misscoring when alleles are similar in electrophoretic mobility. Indeed, heterozygote deficiencies in bivalves are locus-specific (*e.g.* Koehn *et al.* 1976, Gaffney 1990, Gardner and Thompson 1999). This fact has been used previously to discriminate among possible causes of heterozygote deficiencies (Gaffney 1990, Gaffney *et al.* 1990). Also, enzymes differ in the shape of the band produced after staining. Some form clear-cut bands while others produce dots, as a result of smaller or larger diffusion rates of enzyme or reaction product in the starch gel medium (Murphy *et al.* 1996). Differences among studies are to be expected because of laboratory-related differences (*e.g.* electrophoresis system, gel size, starch type) and species-related differences (*e.g.* variation in the amount of tissue used, true number and distribution of alleles). Even differences between populations within a study are possible, for instance, when examination of populations has been done sequentially and a switch in laboratory equipment has taken place.

Variation in scoring bias among loci is exemplified by the data on *M. balthica* presented here. Among the four polymorphic loci there are two with nine alleles (*Gpi* and *Pgm*) and two with five alleles (*Idh2* and *Tpi*). Biased scoring is a larger effect in the more variable loci (compare estimated *q*'s, Table 4). Also, among the more variable loci, the effect is stronger in the dimeric enzyme *Gpi* than in the monomeric enzyme *Pgm*. The latter contrast cannot be made for the other two loci, as both *Idh2* and *Tpi* are dimeric.

In the case of *M. balthica*, at least with regard to the present data set, the problem of heterozygote deficiency is likely to be due to misscoring as homozygotes of adjacent heterozygotes. This does not necessarily mean that the same is true for all other bivalve species and all allozyme loci. There are at least five reasons to believe that causes other than a scoring bias contribute to heterozygote deficits in bivalves. First, cryptic species distribution can be very complicated, with extensive intermixing and interbreeding going on, as demonstrated for *Mytilus* spp. (see *e.g.* Skibinski *et al.* 1983, Gardner and Skibinski 1988), making it highly conceivable that a Wahlund effect does play a role. Second, small-scale temporal or spatial variation in genotypic

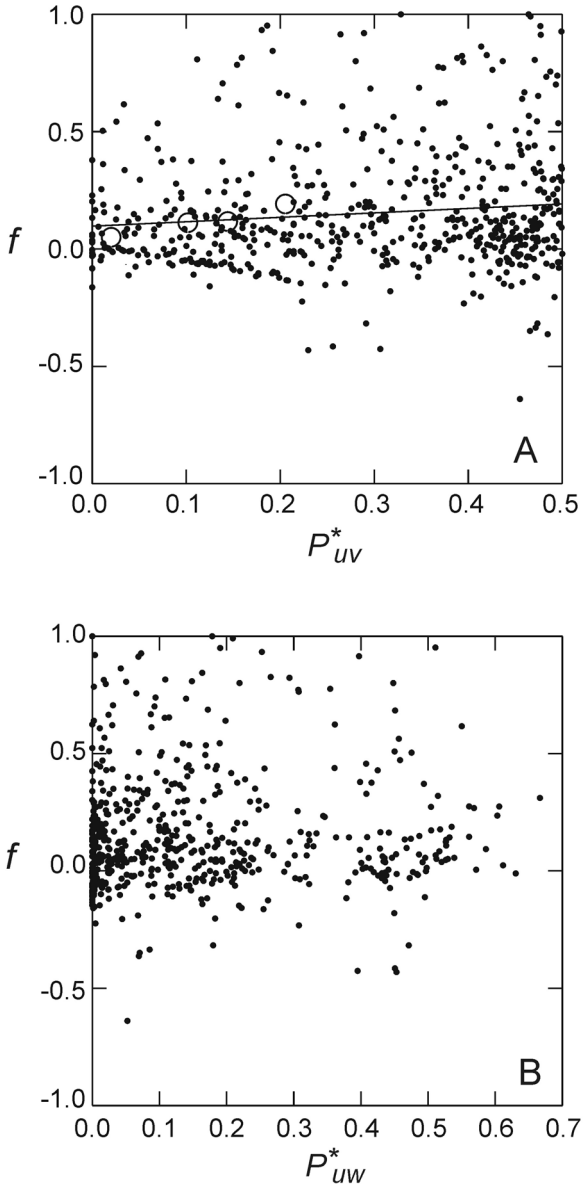

Fig. 1 - Data extracted from 19 published bivalve allozyme studies, analyzed with respect to biased scoring hypothesis. (a) Inbreeding coefficient *f* (for details on variation among studies in estimation see text) versus reconstructed frequency of adjacent heterozygotes P^*_{uv} (solid dots; r = 0.119, n = 571, p < 0.01; plotted line is linear regression). Open circles are the four relevant data points from *Macoma balthica* data analyzed in this paper. (b) Inbreeding coefficient *f* versus reconstructed frequency of non-adjacent heterozygotes P^*_{uw} (r = 0.027, n = 571, n.s.).

frequencies (*e.g.* Hedgecock 1994, David *et al.* 1997), can also constitute a Wahlund effect. Third, selection (direct or through linkage) clearly has been demonstrated to play a role against certain alleles in particular environments (Koehn *et al.* 1980a,b, Hilbish and Koehn 1985). Fourth, heterozygotes for certain loci have been demonstrated to have higher enzyme activities than homozygotes (Pogson 1991, Sarver *et al.* 1992) and in several cases a very strong correlation between multilocus heterozygosity and fitness surrogates has been observed (*e.g.* Koehn *et al.* 1973, Zouros *et al.* 1980, David and Jarne 1997). Both of these may cause heterozygote frequencies to deviate from expectations, although it would seem logical that a surplus should be the result of selection in favor of heterozygotes. Finally, several studies demonstrate the presence of null alleles at elevated frequencies in bivalves (*e.g.* Mallet *et al.* 1985, Foltz 1986b, Gaffney 1994).

I conclude that misscoring of adjacent heterozygotes must always be considered when interpreting allozyme heterozygote deficiencies in bivalves. Existing genotype frequency data can be re-analyzed using the model described in this paper to test for biased scoring.

Implications for heterozygosity-fitness correlations

The supposition that individuals or populations having elevated levels of molecular marker heterozygosity are of superior fitness or at lowered risk of extinction is wide-spread, and although it is heavily debated (see *e.g.* Clarke 1993a, Avise 1994), it has found many practical applications in conservation biology. Heterozygosity-fitness correlations have been observed frequently in bivalves (Mitton and Grant 1984, Zouros 1987), and the discussion on what causes them is still ongoing (David 1998). The evidence for heterozygosity-fitness correlations comes for a large part from bivalves; among a recent meta-analysis of 22 studies (Britten 1996), ten were of bivalves. The same meta-analysis shows that the overall relationship between multilocus heterozygosity and fitness surrogates is weak ($r = 0.133$), also among the bivalves when analyzed separately ($r = 0.162$). After taking into account the low fail-safe numbers associated with these correlations (fail-safe number $N_{fs} = 8$ and 7, respectively; N_{fs} is the number of unpublished zero-effect studies needed to reduce a meta-analytical correlation to some pre-set low value, usually, as in this case, 0.1), Britten (1996) concludes that there is little evidence in literature for strong correlations between allozyme heterozygosity and fitness. Heterozygosity-fitness correlations in bivalves may be confounded by an increased error in establishing heterozygosity, and hence these correlations may turn out to be either stronger or possibly, but less likely, weaker after correcting for biased scoring. Considering the reliance on bivalve data and the evidence presented here that estimates of heterozygote frequencies in bivalves may be biased by a technical scoring problem because of the large numbers of alleles, the heterozygosity-fitness paradigm needs re-examination.

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PART B

EXTERNAL FERTILIZATION

CHAPTER FIVE

Optimal egg size for external fertilization: theory on the role of sperm limitation and field observations for a marine bivalve

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(submitted manuscript)

Abstract

Egg sizes of marine invertebrates vary greatly, both within and between species. The proposed causes of this are still debated, and fall into two main categories. First, a trade-off between size, number and survival of offspring, and second, a selection pressure exerted by sperm limitation during external fertilization. Although larger eggs are indeed a larger target for sperm, producing larger eggs also implies making fewer of them. There has been discussion about whether sperm limitation can (theoretically) and does (in nature) select for larger egg size than under *ad libitum* sperm. In one specific model, based on a particular fertilization kinetics model and an empirically derived mortality function, the theoretical possibility of a negative shift in optimal egg size with sperm concentration was demonstrated and validated with an interspecific correlation. Here we present a generalized analytical model and an intraspecific field data set on the tellinid bivalve *Macoma balthica* (Bivalvia: Tellinidae). Using the model we explore how optimal egg size during external fertilization may or may not depend on level of sperm limitation, and, if it does, whether the dependence is positive or negative. It is shown that, under the condition that an intermediate optimal egg size exists, this qualitative outcome of the model (positive, negative or no relation between optimal egg size and sperm limitation) depends only on the fertilization kinetics part of the model, and is independent of the trade-off between size and fecundity. We show that for two previously published models of fertilization kinetics and our modification of one of them, the relationship between optimal egg size and sperm concentration is always negative. Our intraspecific data are also congruent with a negative relationship between sperm concentration and optimal egg size. Average egg size is strongly correlated (correlation coefficient $r = -0.932$, $p << 0.001$) with adult density as a proxy for sperm concentration.

Introduction

Egg sizes vary extensively between and within species of marine invertebrates (*e.g.* George *et al.* 1990, Clarke 1993b, Jones *et al.* 1996, Bertram and Strathmann 1998, George 1999, Marshall *et al.* 2000), and there has been much debate about the evolutionary causes of this fact. The suggested reasons can be grouped into two different frameworks. The first framework is the trade-off between fecundity and survival of offspring, first proposed by Vance (1973a,b) and Smith and Fretwell (1974). When the amount of resources available for reproduction are fixed, producing larger eggs also means producing fewer and *vice versa*. When eggs produced are not of minimal size, the larger size of the eggs must confer some survival advantage over a smaller size. Any factor that changes the relationship between egg (and thus larval) size and probability of survival may change optimal egg size. Examples of such factors are predation rate, food availability and water temperature (see *e.g.* Bertram and Strathmann 1998, George 1999). Smith and Fretwell (1974) show which types of survival curves predict eggs to be minimal in size, which predict them to be intermediate and which predict that individuals should produce eggs of maximum size. For some intermediate egg size to be optimal, the relationship between egg size (measured in volume) and survival must contain a (continuous or discontinuous) part of disproportionate increase. Because this prerequisite is considered to be too strict to have broad biological validity, it appears that the size-fecundity trade-off more readily predicts a dichotomy in egg sizes than a continuous distribution (*e.g.* Vance 1973*a,b,* Podolsky and Strathmann 1996, Hendry *et al.* 2001). This presents a discrepancy with actual data (*e.g.* McEdward 1997, Levitan 2000, but see Emlet *et al.* 1987). Incorporation of the fact that smaller eggs take longer to develop (*e.g.* George 1996, 1999), however, can indeed lead to the prediction of intermediate egg sizes (*e.g.* when development time is inversely proportional to egg size) and also fits well with empirical data (Levitan 2000).

In 1993, Levitan introduced fertilization kinetics as an alternative framework for explaining egg size variation in marine invertebrates. The eggs of many marine invertebrates are fertilized externally, a reproductive mode that is usually considered ancestral to internal fertilization (Wray 1995). Fertilization kinetics models attempt to describe the process of collision of sperm and eggs during external fertilization (Vogel *et al.* 1982). Because neither sperm nor eggs remain viable forever, it is possible that a portion of the gametes remains unfertilized. Inequalities in fertilization probability between the sexes during external fertilization may be manifest in any of the following ways: sperm limitation, egg limitation, sperm competition, egg competition, or a combination between some of them (Levitan 1998). There is experimental evidence for all of these forces (sperm limitation: *e.g.* Levitan *et al.* 1992, Oliver and Babcock 1992, Marshall *et al.* 2000; egg limitation: *e.g.* Benzie and Dixon 1994; sperm competition: Yund and McCartney 1994, Yund 1998; egg competition: Gruffydd and Beaumont 1970, Vogel *et al.* 1982, Sprung and Bayne

1984), but it remains to be seen which of them is dominant in nature (Levitan 1995, Levitan and Sewell 1998, Yund 2000). Concerning the selection forces on egg size during external fertilization, sperm limitation is by far the most debated one. The basic idea is that, since larger eggs form a larger target for sperm, it might pay to produce larger eggs when sperm are limiting (Levitan 1993). That single eggs are more readily fertilized when they are larger, has been shown experimentally (Levitan 1996*a*). As mentioned earlier, the problem with this idea is that producing larger eggs also means producing fewer eggs. Levitan (1993, 1996*a*, 1996*b*) developed a specific model for echinoderms that includes both a size-fecundity trade-off, a relationship between egg size and survival through development time, and fertilization kinetics that features sperm limitation. This particular model shows that it is theoretically possible that when sperm become more limiting, the optimal size of eggs becomes larger, even though this means that the number of eggs produced decreases (for discussions see Levitan 1996*b,* Podolsky and Strathmann 1996). It is, however, not immediately obvious whether the relationship between optimal egg size and sperm concentration will be negative under more general conditions. Such a general model is necessary to predict the direction of selection pressure exerted on egg size by sperm limitation for a wider range of species. Also, the separate effects of and possible interaction between the fecundity-survival trade-off and fertilization kinetics have not been teased apart. For example, the influence of the shape of the egg size-survival relationship on whether optimal egg size depends on sperm limitation is not yet clear. Also, it has not been explored whether the qualitative result (that optimal egg size increases with sperm limitation) can change with particular parameter values of fertilization kinetics models.

The aims of this study are two-fold. First, we present a general analytical qualitative solution to the question whether optimal egg size depends on sperm concentration. We then substitute fertilization kinetics models into the general solution, using two previously published kinetics models (Vogel *et al.* 1982) and a novel alteration of one of them. Finally, we evaluate whether egg size is correlated with adult density in the field, as predicted on the basis of the outcome of our analytical model, in a marine broadcast spawning invertebrate; the burrowing bivalve *Macoma balthica*. This tellinid is naturally found in a wide range of densities with egg sizes that are known to be quite variable (Honkoop and Van der Meer 1997). Population density has been found to be a strong predictor of fertilization success (Levitan 1991, Levitan *et al.* 1992) and has been used to explain differences among species in gamete traits such as sperm swimming speed, egg size and gamete longevity (Levitan 1993, Coma and Lasker 1997). For the first time we address the question whether egg sizes can be intraspecifically correlated with sperm limitation, values of which may be proximated by the inverse of population density.

Materials and methods

A simplified model optimizing egg size

The starting point of our assessment is the presence of two sexes, one producing microgametes of fixed size in large numbers (males) and the other macrogametes of adjustable size in adjustable numbers (females). We evaluate which female strategy is optimal. The hypothesis (as suggested by Levitan 1993, 1996*b*) is that with increasing sperm limitation, females producing larger eggs have a higher fitness, or in other words, optimal egg size increases.
Assuming that females are in control of the size and the number of their eggs, we use the following equation for female fitness:

(1) $$f(x,y) = s(x)\cdot n(x)\cdot p(x,y)$$

where $x>0$ equals egg size and $y>0$ equals sperm concentration. The probability of embryo survival depends on egg size and is termed s ($0 \le s \le 1$), and the number $n>0$ of eggs a female produces depends also on egg size only. The probability p ($0 \le p \le 1$) for an egg to be fertilized depends on both egg size and sperm concentration. Female fitness is defined as the product of s, n and p and can be interpreted as the number of eggs that are fertilized and survive until reproduction. Equation (1) can be log-transformed into

(2) $$F(x,y) = H(x) + P(x,y)$$

where $F(x,y) = \log[f(x,y)]$, $H(x) = \log[n(x)\cdot s(x)]$ and $P(x,y) = \log[p(x,y)]$.

Field data

The tellinid bivalve *Macoma balthica* (L.) is a circumpolar species of the northern hemisphere that lives in the shallow subtidal to the high intertidal, buried in mud or sand. It is a broadcast spawner (i.e., both sexes release their gametes into the water column for fertilization) with one spawning season a year in most places (Harvey and Vincent 1989).

In early spring of three different years (1995, 1996 and 1999) we collected *M. balthica* using a grab (for subtidal sites) or a sieve (for intertidal sites). In both 1995 and 1996 samples were taken at three intertidal sites, and in 1999 at two intertidal and three subtidal sites (see Table 1). Sampling sites were distributed over the western Dutch Wadden Sea and the adjacent North Sea. The bivalves were individually induced to spawn by administering a temperature shock of about 10°C as described in Honkoop and Van der Meer (1997). Two slides were taken of the batch of eggs of a female, part of which was placed on a flat microscopic slide, under a Zeiss stereo microscope at 63x magnification. Eggs were measured from the projected slides as described in Honkoop and Van der Meer (1997). Two

perpendicular measurements of the diameter of each egg were taken and averaged. Per female, either 30 (1995 and 1996 data) or 15 (1999 data) eggs were measured. The eggs of on average 28 females per site were measured in this way.

Local adult density at the time of gamete production, which for *M. balthica* is in the autumn prior to the spawning season in spring (Caddy 1967, Lammens 1967), was retrieved from the benthos monitoring data base of Jan Beukema and Rob Dekker (Netherlands Institute for Sea Research) for the 1995 and 1996 egg size measurements. For the 1999 sites, we determined local adult density at the time of collection for spawning in early spring. Adult density was regarded as an inverse measure of the potential strength of sperm limitation, because with decreasing adult density, male density decreases similarly, and, all else being the same, the ambient sperm concentration during external fertilization can be expected to be lower.

Results

Model analysis

Given the log-transformed fitness function (2), we can implicitly define

(3) $$\frac{\partial}{\partial x}[F(x^*, y)] = o$$

where optimal egg size x^* is a function of sperm concentration y:

(4) $$x^* = g(y)$$

Note, that the presence of a local maximum for a particular sperm concentration can for instance depend on criteria similar to those outlined by Smith and Fretwell (1974) for their fecundity-survival trade-off. The presence of an intermediate optimal egg size ($0<x^*<\infty$) as defined above is a prerequisite for the following analysis.

The first derivative of (4) is the quantity of interest to the hypothesis:

(5) $$\frac{\partial}{\partial y}[g(y)] = \frac{\partial x^*}{\partial y} = -\frac{\frac{\partial}{\partial y}\left[\frac{\partial}{\partial x}[F(x,y)]\right]}{\frac{\partial}{\partial x}\left[\frac{\partial}{\partial x}[F(x,y)]\right]}$$

(6) $$\Rightarrow \frac{\partial x^*}{\partial y} = -\frac{\frac{\partial^2 F(x,y)}{\partial x \partial y}}{\frac{\partial^2 F(x,y)}{\partial^2 x}}$$

In (6), the denominator is always negative, as we have (because of the condition of an existing intermediate optimum) a differentiable maximum at x^*. The hypothesis, 'optimal egg size decreases with sperm concentration', can then be formalized as follows:

(7) $$\frac{\partial^2 F(x,y)}{\partial x \partial y} < 0$$

Derivation of equation (2) gives:

(8) $$\frac{\partial F(x,y)}{\partial x} = H'(x) + \frac{\partial P(x,y)}{\partial x}$$

(9) $$\Rightarrow \frac{\partial^2 F(x,y)}{\partial x \partial y} = \frac{\partial^2 P(x,y)}{\partial x \partial y}$$

Therefore the hypothesis'optimal egg size decreases with sperm concentration' holds if

(10) $$\frac{\partial^2 P(x,y)}{\partial x \partial y} < 0$$

Interestingly, it becomes clear from (10), that whether the hypothesis holds or not depends only on the fertilization function and not on the fecundity-survival trade-off.

Qualitative testing of existing fertilization kinetics models

Two previously published fertilization kinetics models (Vogel *et al.* 1982) can now be analyzed to see if they are congruent with hypothesis (6), 'optimal egg size decreases with sperm concentration', under all, or a subset, of the ranges of their parameters and variables. Vogel *et al.* (1982) first derive the 'Don Giovanni' model, in which a spermatozoon sticks to an egg for a negligible time only, and then goes on searching (notation slightly modified and log transformed):

(11) $$P(x,y) = \ln(1 - e^{-cxy})$$

Here, c is a positive constant that is the product of the swimming speed and life span of spermatozoa. The second derivative of (11) is

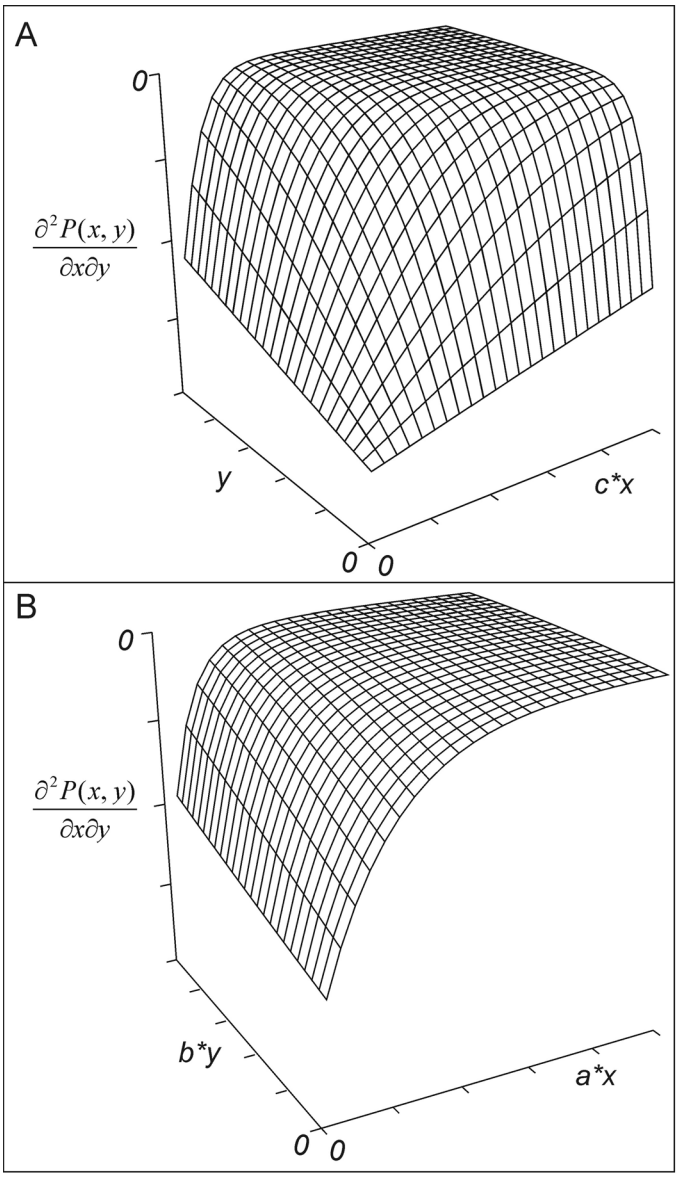

Fig. 1 - Plot of partial derivatives with respect to *x* and *y* of log transformed fertilization kinetics models. *a*) Equation (12) in text: Don Giovanni model; *x* = egg size; *c* = positive constant and *y* = sperm concentration. *b*) Equation (14) in text: Don Ottavio model with constant egg concentration; *x* = egg size; *a,b* = positive constants and *y* = sperm concentration.

(12) $$\frac{\partial^2 P}{\partial x \partial y} = -c \frac{e^{-cxy}(cxy - 1 + e^{-cxy})}{(-1 + e^{-cxy})^2}$$

which can be shown to be always negative (see appendix and Fig. 1a). This agrees with condition (10) and is therefore (given that $0<x^*<\infty$) in accordance with the hypothesis of increasing optimal egg size with increasing sperm limitation.

The second step of Vogel *et al.* (1982) is to derive a more complicated but biologically more realistic model, 'Don Ottavio', in which spermatozoa attach to the first egg they hit, whether fertilization takes place or not. Following Levitan (1996*a*, 1998) in keeping egg concentration constant, the model is the following (notation slightly modified and log transformed):

(13) $$P(x, y) = \ln(1 - e^{-ay(1 - e^{-bx})})$$

where a is a positive constant that equals the sperm swimming speed times the fertilizable egg surface fraction divided by egg concentration, and b is a positive constant that is the product of egg concentration and life span of spermatozoa. The second derivative of (13) is

(14) $$\frac{\partial^2 P}{\partial x \partial y} = -ab \frac{e^{bx} - aye^{ay(1 - e^{-bx})} + (ay - 1)e^{bx + ay(1 - e^{-bx})}}{e^{2bx}(-1 + e^{ay(1 - e^{-bx})})^2}$$

which can be shown to be always negative (see appendix and Fig. 1b).

It may be more realistic to not assume that egg concentration is constant. For instance, when population density is causing variation in sperm concentration, egg concentration can be expected to covary with sperm concentration. The Don Ottavio model can be modified to account for covarying sperm and egg concentrations in the following way (again log transformed with modified notation from Vogel *et al.* 1982):

(15) $$P(x, y) = \ln(1 - e^{-a'(1 - e^{-b'xy})})$$

where a' is a positive constant that equals the sperm swimming speed times the fertilizable egg surface fraction, and b' is a positive constant that equals life span of spermatozoa. The second derivative of (15) is

$$(16)\quad \frac{\partial^2 P}{\partial x \partial y} = \frac{a'b'e^{a'e^{-b'xy}-2b'xy}(-a'b'e^{a'}xy + e^{a'+b'xy}(1-b'xy) + e^{a'e^{-b'xy}+b'xy}(b'xy-1))}{(e^{a'} - e^{a'e^{-b'xy}})^2}$$

which can also be shown to be always negative (see appendix). The conclusion can therefore be drawn, that under both the Don Giovanni and the Don Ottavio models of fertilization kinetics, with the prerequisite that an intermediate egg size exists (i.e., $0<x^*<\infty$), the hypothesis 'optimal egg size decreases with sperm concentration' holds true for all parameter and variable values.

Field data

The average diameter of eggs produced by *Macoma balthica* females per location was negatively related to adult density (Fig. 2, linear regression, n = 11, $r^2 = 0.87$, $p<<0.001$, Pearson correlation coefficient r = -0.932, $p << 0.001$). Data are summarized in Table 1.

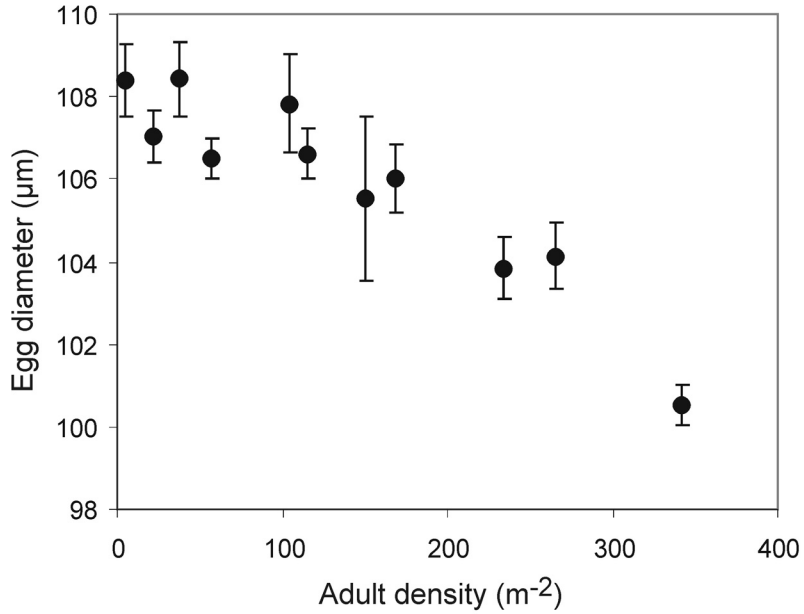

Fig. 2 – *Macoma balthica* (Mollusca: Bivalvia): average egg diameter per female (µm +/- standard error), induced to spawn in early spring of three different years from seven different sites in the Netherlands, plotted against local adult density (number of individuals per m^2). Total number of females = 307, total number of eggs = 7410.

Discussion

Model prediction

Having analytically examined the basic model (1) (after Levitan 1993) that optimizes maternal fitness in relation to egg size and describes fitness as the product of the number, the survival probability, and the fertilization probability of eggs, our analytical solution states that, given an intermediate optimal egg size to start with, the optimum may indeed change with level of sperm limitation. Interestingly, whether a qualitative change exists, and when it does, whether it is positive or negative, depends entirely on the specifics of the fertilization kinetics model and not on details of the fecundity-survival trade-off part of the model. In other words, when two species differ in the way their eggs are fertilized externally, the relationship between optimal egg size and sperm concentration might be different. In contrast, when taxa differ in the relationship between egg size and survival probability but not in the way their eggs are externally fertilized, the relationship between optimal egg size and sperm concentration should show the same trend.

This general analytical solution was applied to two fertilization kinetics models proposed by Vogel *et al.* (1982), which showed that optimal egg size (under the condition that an intermediate optimum exists) increases with sperm limitation under all parameter and variable values. The same result was obtained for a modification of one of these models, where egg concentration covaries with sperm concentration. Given these fertilization kinetics models, the relationship between an intermediate optimal egg size and sperm concentration can never be positive. This is in concordance with the specific example of Levitan (1993, 1996*b*). The intuitive idea that because larger eggs are a larger target for sperm, increased sperm limitation should select for larger eggs, is therefore theoretically correct in a wide range of circumstances.

Fit of field data to model prediction

This is the first demonstration of an intraspecific correlation between egg size and adult density as a putative proxy for strength of sperm limitation. Egg size in *Macoma balthica* is significantly smaller where adult density is higher (Fig. 2). Adult density and average egg size are strongly correlated, with a correlation coefficient of -0.932. The relationship explains 87% of the variation in egg size among sites in a sampling scheme that includes a total of 307 females across 11 sites in three years.

For interpreting this field correlation, a crucial assumption is that adult density scales to sperm concentration. Population density has been directly shown to result in lowered fertilization rates due to lower sperm concentrations in field experiments for other marine invertebrate species (Levitan 1991, Levitan *et al.* 1992). The gonad mass produced and the amount of sperm released upon induction to spawn in the laboratory have been studied for *Macoma balthica* at four of the 1999 sites included in the present study. These comparisons present no evidence for a relationship between

Table 1 - Egg size of *Macoma balthica*: summarized data per year per site.

Year	Site	Position	$n_{females}$	Adult density (ind/m²)	Av. egg diameter (μm) (se)
1995	Balgzand A	52°57.00'N 4°50.39'E	23	104	107.8 (1.19)
	Balgzand B	52°55.30'N 4°48.84'E	20	266	104.1 (0.81)
	Balgzand C	52°54.28'N 4°50.54'E	24	342	100.5 (0.49)
1996	Balgzand A	52°57.00'N 4°50.39'E	46	38	108.4 (0.90)
	Balgzand B	52°55.30'N 4°48.84'E	34	169	106.0 (0.82)
	Balgzand C	52°54.28'N 4°50.54'E	40	234	103.9 (0.76)
1999	Balgzand D	52°55.36'N 4°49.15'E	11	22	107.0 (0.64)
	Mok	53°05.00'N 4°45.80'E	43	56	106.5 (0.47)
	Molengat	53°01.44'N 4°40.08'E	30	5	108.4 (0.88)
	Terschelling	53°27.33'N 5°25.00'E	7	150	105.5 (1.99)
	Wierbalg	52°56.00'N 4°57.05'E	11	115	106.6 (0.59)

local adult density and either gonad mass or amount of sperm released (Drent and Luttikhuizen, in prep.). This suggests that one may indeed assume that during fertilization in the field sperm concentration is lower where adult density is lower.

The field correlation we observed might not be causal but the result of a factor that covaries with adult density. Adult density and maternal condition could be connected through advantageous food quality or amount. Maternal size or condition have been found to correlate with egg size in fish (Baynes and Howell 1996, Kerrigan 1997, Johnston and Leggett 2002) and also in some marine invertebrates (George 1996, 1999, Honkoop and Van der Meer 1998, Marshall *et al.* 2000). However, the direction of the response of marine invertebrate egg size to manipulated food supply is variable, and often such a response is absent, depending on the species and the local conditions at the site of collection of the experimental animals (reviewed in George 1996). In *Macoma balthica*, neither size nor lipid content of eggs could be manipulated experimentally by altering temperature or food availability (Honkoop and Van der Meer 1998, Honkoop *et al.* 1999). It is therefore not likely that maternal nutritional status underlies the correlation between egg size and adult density we observed. Unequivocal establishment of the causality of sperm limitation due to lowered male densities in determining intraspecific egg size variation in *Macoma balthica*, however, awaits an experimental approach.

The possibility of incomplete information availability to the bivalves may be a confounding factor of the field correlation presented in Fig. 2. The females may

measure the density of conspecific males in a number of ways. *Macoma balthica* has been shown to be olfactorily sensitive to the presence of predators (Edelaar 2000), so smell is one possible sensory pathway. Also, as a facultative deposit feeder, neighboring *M. balthica* individuals come into contact with each other indirectly when their grazing areas overlap. Another confounding factor is a possible difference in the census moment of adult density by us and by the bivalves, between which density may have changed. Eggs are produced in autumn and stored until spawning in early spring, which is why the census moment may be in autumn. Our recent investigations have shown that changes in gonad mass, both positive and negative, may occur (own unpublished data), implying that adjustments to egg number and/or size could take place during winter.

An interspecific egg size comparison that includes *M. balthica* has been made earlier in the light of the egg size-fecundity trade-off and the conditions for larval survival (Honkoop and Van der Meer 1998). The pelagic larvae of the three co-occurring species *M. balthica*, cockles *Cerastoderma edule*, and mussels *Mytilus edulis* inhabit the same water column but at different times of the year. These species produce large, medium and small eggs, respectively, and the numbers of eggs they release follow the same trend: small, intermediate and large, respectively. Honkoop and Van der Meer (1998) suggest that water temperature may be a causal factor. In *M. balthica*, whose larvae are pelagic in early spring as opposed to in late spring/early summer for the other two species, larval development is relatively slow. With increased larval development time and unaltered larval survival probability per unit time it would pay parents to produce fewer but larger offspring (Smith and Fretwell 1974, Levitan 2000).

We can now examine the interspecific variation in egg sizes in the light of sperm limitation theory. The three species mentioned before live in very different types of population conglomerations. *M. edulis* lives in banks, the individuals being attached to one another and to the substrate by the use of byssus threads. *C. edule* is a shallow burrowing bivalve that lives in dense populations (cockle-beds), whereas *M. balthica* is a deep burrowing bivalve living in low densities. The emerging pattern (Fig. 3) fits very well with predictions from sperm limitation theory and is qualitatively comparable to similar interspecific comparisons that have been made previously (gorgonians: Coma and Lasker 1997; echinoderms: Levitan 1993).

Further discussion of model

The prerequisite for our analytical model solution is that an intermediate optimal egg size exists. In order to specify this condition more clearly, it is clarifying to go back to the way Smith and Fretwell (1974) found the optimum in any plot of survival against egg volume. The presence of a local maximum for a particular sperm concentration depends on criteria very similar to theirs. They show that, because the number of eggs is a function of the form c/d^3 (where c is a positive constant denoting the amount of resources available for egg production and d equals egg diameter),

relative fitness in the survival plot is calculated as s/d^3 (where s equals volume-dependent survival probability). Of all points on the survival curve, the point with the largest $s(d^*)$ confers the highest fitness to the female. For $0<d^*<\infty$ it is necessary that the survival curve contains a part of disproportionate increase (which may also be discontinuous, see *e.g.* Levitan 2000). When we evaluate the product of survival function $s(d)$ and fertilization function $p(d)$, it becomes clear that Smith and Fretwell's (1974) conditions for intermediate optimal egg size are relaxed. If, for example, survival is of the form $(1-\exp(-ad^3))$ and fertilization of the form $(1-\exp(-bd^2))$, where a and b are positive constants, their product predicts an intermediate rather than extreme optimal egg size. In contrast, such a survival function on its own would predict optimal egg size to be the smallest possible size.

A secondary outcome of our simple model is, therefore, that the prerequisites for an optimal egg size *per se* are relaxed compared with the conclusions of Smith and Fretwell (1974). Our model may be used to show that when fertilization kinetics are taken into account, an intermediate optimal egg size exists for a wider range of survival functions. The outcome depends on the precise interplay between the fecundity-survival trade-off and the fertilization kinetics function. This effect may help further solve the apparent inadequacy of the fecundity-survival trade-off framework for explaining egg sizes in externally fertilizing species (see *e.g.* McEdward 1997, Levitan 2000). It is beyond the scope of this paper to go into more detail on this, but the issue should certainly be studied more closely.

When returning to the dichotomy presented in the introduction, where fecundity-survival trade-off and fertilization kinetics were depicted as alternative

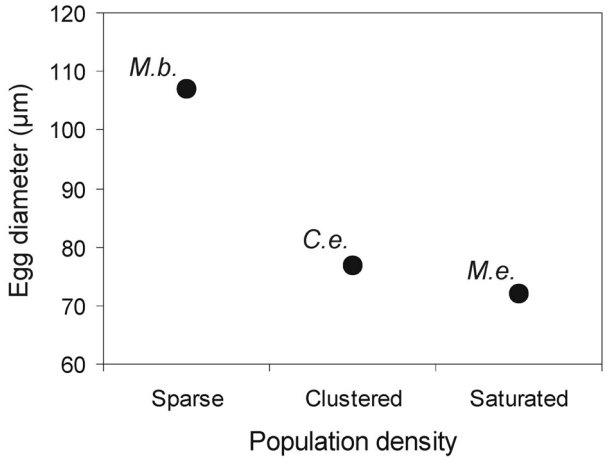

Fig. 3 - Interspecific comparison between egg diameter (without jelly coat present in *C. e.*) and population density for *Macoma balthica* (*M.b.*), *Cerastodema edule* (*C.e.*) and *Mytilus edulis* (*M.e.*) in the Dutch Wadden Sea.

hypotheses, the analysis in this paper has shown that they can act and interact together in the process of selection on egg size. This is first of all evident from the fact that any relationship of optimal egg size with sperm limitation, positive or negative, depends on the presence of an intermediate optimum, which is under the influence of the fecundity-survival trade-off. Also, the two frameworks act together in determining the existence and value of intermediate optimal egg size. Examples of selection factors ranking under fecundity-survival are food quality (Parker and Begon 1986, George 1994), larval competition (Parker and Begon 1986), temperature (Clarke 1993b) and maternal age or size (Marshall *et al.* 2000). Fertilization kinetics-type selection factors are population size and density (Levitan 1991, Levitan *et al.* 1992, Atkinson and Yund 1996), male body size (Levitan 1991), egg competition (Gruffydd and Beaumont 1970, Vogel *et al.* 1982, Sprung and Bayne 1984), sperm competition (Yund and McCartney 1994, Yund 1998), polyspermy (Styan and Butler 2000) and water turbulence (Serrão *et al.* 1996). The one area in which fertilization kinetics acts independently of the fecundity-survival trade-off is in determining whether the relationship between optimal egg size and sperm limitation is negative or positive.

To determine the sign of the relationship between optimal egg size and sperm limitation, we have shown that it suffices to evaluate the partial derivative of the fertilization model with respect to egg size x and sperm concentration y. The fact that the second partial derivative, evaluated for x and y, of two log-transformed fertilization kinetics models, Don Giovanni and Don Ottavio, is always negative (Fig. 1), does not, however, imply that this conclusion is universally valid. The Don Giovanni model, as the authors themselves already note (Vogel *et al.* 1982), is biologically irrelevant. It assumes that sperm remain with an egg an infinitely small amount of time and then go on searching. The second model, Don Ottavio, is more realistic (sperm stick with the first egg they chance upon), but may not always be applicable. It is well known that external fertilization often does not follow the very minimalistic Don Ottavio model. For example, the assumption that external fertilization happens in a well mixed, three-dimensional space, is often wrong, because the gametes of many marine invertebrates sink or float or stay in clumps or strings in the water column (McEuen 1988, Thomas 1994a,b). A final important factor is polyspermy, which is common in *M. balthica* (Luttikhuizen and Pijnacker 2002). Adverse effects of more than one sperm entering an egg cell may set an upper limit to egg size when the block to polyspermy is not instantaneous (Styan 1998, Styan and Butler 2000). Addition of a jelly layer to the outside of egg cells can increase collision rate with sperm, but in a different way than increasing egg cell size does (Farley and Levitan 2001). Also, the jelly layer may decrease in size due to shear stress during the trajectory through the oviduct as well as externally as a result of water movements (Thomas and Bolton 1999, Farley and Levitan 2001). Such confounding effects might cause the qualitative outcome of egg size-fertilization kinetics models to shift. Some of these effects can be modeled and the sensitivity of

optimal egg size can then be evaluated with respect to sperm limitation using the analytical model presented in this paper. Other, more complicated factors such as the occurrence of gametes in clumps and strings (McEuen 1988, Thomas 1994a,b) are best addressed in experiments.

In externally fertilizing species, the trade-off concerning the production of eggs is not accurately described as being between size, fecundity and survival, but rather as a fecundity- survival-fertilization trade-off. Even though producing eggs of minimal size means producing the largest possible number of eggs, theory helps to understand how benefits in the form of increased survival and fertilization probabilities can interact to make many species produce eggs that are larger than that, and to formulate hypotheses on why and how the eggs of many species vary in size intraspecifically.

Acknowledgements

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Appendix

The Don Giovanni model

The denominator of the right-hand side of equation (12) and the term ce^{-cxy} are always positive. Let $x'=cxy$. We can then evaluate

(17) $$f(x') = x' - 1 + e^{-x'}$$

The first derivative of $f(x')$ is

(18) $$\frac{df(x')}{dx} = 1 - e^{-x'}$$

which is always positive. Because of this and because $f(0)=0$, $f(x')$ is always positive and therefore the second derivative (12) is always negative.

The Don Ottavio model with constant egg concentration

The denominator of the right-hand side of equation (14) and the term ab are always positive. Let $x'=bx$ and $y'=ay$ so that we can evaluate

(19) $$f(x', y') = e^{x'} - y' e^{y'(1-e^{-x'})} + (y'-1)e^{x'+y'(1-e^{-x'})}$$

which can be rewritten into

$$f(x',y') = e^{x'} + e^{x'}e^{y'}e^{-y'e^{-x'}} + y'e^{y'}e^{-y'e^{-x'}}(e^{x'}-1) \quad (20)$$

Because $e^{x'}>1$ for all $x'>0$, function (20) is always positive. Therefore, the second derivative (14) is always negative.

The Don Ottavio model with variable egg concentration

The denominator of the right-hand side of equation (16) and the term $a'b'e^{a'e^{(-b'xy)-2b'xy}}$ are always positive. Let $x'=b'xy$ so that

$$f(x') = -a'x'e^{a'} + e^{a'+x'}(1-x') + e^{a'e^{-x'}+x'}(x'-1) \quad (21)$$

Rewriting (21) gives:

$$f(x') = -\left(a'x'e^{a'} + e^{x'}(x'-1)\left(e^{a'} - e^{a'e^{-x'}}\right)\right) \quad (22)$$

which is negative for all a' when $x'>1$ because $e^{-x'}<1$ and therefore $e^{a'}>e^{a'e^{(-x')}}$. Rewriting (21) once more gives:

$$f(x') = -e^{a'}\left(a'x'-e^{x'}\right) - x'e^{x'}e^{a'} - x'e^{x'}\left(e^{a'} - e^{a'e^{-x'}}\right) \quad (23)$$

which is negative for all a' when $0<x'\leq 1$, because under those conditions $(a'x'-e^{x'})$ is a positive term. Thus, the second derivative (16) is always negative.

BOX II

Survival and anisogamy

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Randerson and Hurst (2001) claim that the disruptive selection theory for the evolution of anisogamy detailed by Parker, Baker and Smith (1972) (PBS) requires 'an unusual and unverified assumption regarding the relationship between zygote size and fitness'. We disagree that the form of relationship between the fitness f of a zygote and its size, *S*, required by PBS is unusual, and show that if two mistakes are corrected in Randerson and Hurst's interpretation of the work of Levitan (2000), we in fact obtain the kind of f(S) relation that is required by PBS.

Levitan (2000) suggests that the relationship between the time, *T*, taken for a facultative planktotroph larva to develop to maturity and the size of the zygote, *S*, is of the form:

(1) $T = a + b/S$

From this, Randerson and Hurst assume the fitness of the zygote to be given by its probability of survival to maturity, and claim that if there is a constant mortality, p, the fitness will be

(2) $f(S) = 1-pT, \quad T < 1/p$

$f(S) = 0, \quad T > 1/p$

This fitness function is shown in Fig. 1 with the parameter values used by Randerson and Hurst (2001). They observe that, above the cutoff point $S = 0.00012$, fitness is a decelerating function of zygote size, contrary to the requirement of PBS, and conclude that 'it seems unlikely that the relationship between egg size and developmental time will rescue the untested assumption of the PBS model'.

There are two mistakes in this analysis. First, the correct form of the fitness function should be

(3) $f(S) = e^{-pT}$

as Levitan proposes, after Vance (1973a). This is approximated by Eqn 2 when pT is small, but not otherwise. It has the correct form to generate anisogamy with any parameter values, because it is an accelerating function of zygote size near the origin.

Although Randerson and Hurst (2001) have used an incorrect formula in Eqn 2, it is interesting to consider whether anisogamy will evolve with this type of fitness function, which might sometimes be appropriate (*e.g.* if a minimum amount of yolk were needed to permit embryonic development). Using the formulation of PBS in Randerson and Hurst (2001, Box 1), it can be shown that there is an anisogamous equilibrium with microgametes having zero size (in practice, the minimum size δ for viability as gametes) and macrogametes having size S satisfying:

(4) $\quad f'(S) = f(S)/S$

(The fitness of a female producing M/S eggs each of size S is $Mf(S)/S$, whose maximum value satisfies Eqn 4.) This is a version of the Smith and Fretwell (1974) result for the optimal balance between number and size of offspring. It can be represented graphically by plotting the tangent from the origin to the curve $f(S)$ and finding the value where they meet (Fig. 1, which is similar to the graphical derivation of Charnov's (1976) marginal value theorem). In this example, the optimal egg size is $S^* = 0.00024$. (We have ignored the fact that $\delta > 0$, because $S^* >> \delta$.)

This anisogamous strategy is an ESS, because sperm of minimal size are the optimal response to eggs of size 0.00024 and vice versa. There is also an isogamous strategy with both eggs and sperm of size $S/2$, where S satisfies:

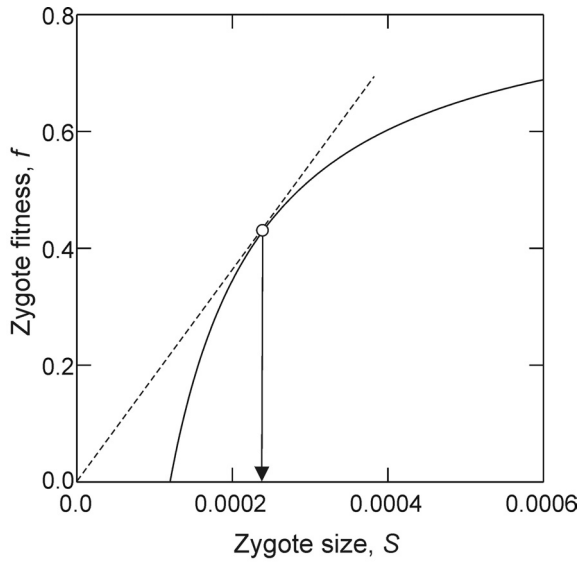

Fig. 1 - Plot of $f(S)$ from equation (2) with $a = 13$, $b = 0.0103$, $p = 0.01$, as used by Randerson and Hurst (2001). The tangent from origin to curve gives the optimal egg size when sperm make no contribution.

(5) $f'(S) = 2f(S)/S$

In the example, both eggs and sperm are of size 0.000089, giving a zygote size less than that under anisogamy. This strategy is an ESS, but it is continuously unstable (Eshel 1983) in the sense that the system will diverge from it after a perturbation. If both eggs and sperm are exactly of size 0.000089, they will stay there, balanced on a knife-edge. But if eggs are of size 0.0001, sperm will decrease in size to 0.000065, in response to which eggs will increase to 0.000133, which allows sperm to decrease to their minimal possible size, followed by an increase in egg size to 0.00024. Thus, the anisogamous strategy is the only continuously stable ESS.

Isogamy, with both types of gamete having their minimum size δ, is only expected when *f(S)* has the kind of shape shown in Maynard Smith (1978, Fig. 17a), which might have been primitive for eukaryotes. The shape of *f(S)* must have changed considerably during evolution, and conditions generating anisogamy under PBS are argued to have become more probable during the transition from uni- to multicellularity (see references cited in Randerson and Hurst (2001)).

We conclude that anisogamy is expected under the model of Levitan (2000), whether we use the correct form of the fitness function in Eqn 3 or the incorrect form in Eqn 2. Although we fully agree that other factors probably also played a part in the evolution of anisogamy, and that a more pluralistic approach is merited, we must stress that Randerson and Hurst's claim that the PBS theory relies on unusual assumptions and lacks empirical support is unfounded.

CHAPTER SIX

Temporal and spatial variation in spawning of *Macoma balthica*

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(manuscript)

Abstract

Broadcast spawners are expected to locally synchronise their spawning activity to minimise the adverse effects of sperm limitation and to match with the optimal environmental conditions for the development of the larvae. When an environmental cue for spawning is timed differently between habitats, this will lead to asynchronous reproduction between these habitats. We observed the timing of reproduction in the broadcast spawning bivalve *Macoma balthica* (Tellinidae) at two tidal levels, subtidal and intertidal, in the western Dutch Wadden Sea and adjacent North Sea. We measured seasonal changes in gonad mass, the likelihood of inducing spawning after a sudden temperature rise of 5°C, and the amount of sperm released by spawning males upon induction. In the subtidal as well as in the intertidal habitats the spawning season lasted two months, during March and April, when the mean sea surface temperature reached 8.3°C. Individuals spawned multiple times per season. Upon induction, males from the subtidal released twice as much sperm as intertidal males, likely a result of more severe sperm limitation in deeper water. We hypothesise that in a stochastic environment with highly variable larval survival, spawning multiple times will have an advantage over spawning all gametes at once, and that the number of occasions over which an individual will spend its gametes is traded off against the number of gametes released per event. Increasing the number of occasions will increase the chance that surviving larvae will be produced but per event gamete concentrations must reach levels ensuring effective fertilisations.

Introduction

Broadcast spawning is a common reproductive strategy among benthic marine invertebrates in which both males and females release their gametes into the water column, where fertilisation takes place. In many species, the larvae subsequently live a planktotrophic life, during which they drift in the water column for a few days to several months. Timing of reproduction in broadcast spawners is of great importance for at least two reasons (Olive 1992). First, a certain amount of synchronisation in spawning among neighbouring individuals must exist to ensure successful fertilisation of eggs (Levitan 1995, Yund 2000). Second, it is necessary to match

reproduction with the most optimal environmental conditions for the first vulnerable life stages (Sastry 1975, Todd and Doyle 1981, Olive 1992).

Reproduction and spawning can be synchronised with season and conspecifics via several mechanisms. Water temperature reaching a certain threshold is often mentioned as a cue (Orton 1920, Giese 1959), but also the seasonal cycle of the photoperiod or changes in the food availability (Starr *et al.* 1990) may steer the timing of reproduction. A number of these cues are liable to differ spatially. The thermal environment, for instance, is much more buffered in the subtidal than in the intertidal habitat (De Wilde and Berghuis 1979). These differences may lead to spatial variation in reproductive timing.

The Baltic tellin, *Macoma balthica* (Tellinidae) is a common broadcast spawning bivalve of boreal intertidal and shallow subtidal soft sediments. Spawning is thought to be triggered by water temperatures reaching a threshold of about 10°C (Caddy 1967, Lammens 1967, De Wilde and Berghuis 1978). Because this threshold value is reached at an earlier date in the thermally more variable intertidal than in the buffered subtidal (De Wilde and Berghuis 1979), it is hypothesised that timing of spawning of *M. balthica* will differ between these two habitats. Battle (1933) already suggested a relationship between tidal height and moment of spawning. In 1967, Lammens inferred from field data on spawning and the settlement of recruits in the intertidal that subtidal populations should have delivered a second, later cohort of larvae. Recently, Günther *et al.* (1998) reported on the occurrence of two separate peaks in the abundance on pelagic *M. balthica* larvae, which they suggest represent an intertidal and a subtidal spawning event, respectively.

In this study we directly investigate the issue of timing of spawning related to habitat. We report on the course of spawning of *M. balthica* in spring 1999 at four stations in the Netherlands, two of which were subtidal and two intertidal. We address two questions. First, is spawning within stations synchronised (as it should be to ensure successful fertilisation)? And second, is timing of spawning indeed later in the subtidal than in the intertidal? We approach these questions by examining in a time series of field collected *M. balthica* the decline of gonad mass, the change in inducibility of spawning by administering a temperature shock, and the relative amount of sperm released by spawning males.

Materials and methods

The four sampling sites are located in the western Dutch Wadden Sea and the adjacent North Sea (Fig. 1). The two intertidal sites, Mokbaai and Balgzand, are both elevated 1.1 m above mean lower lowwaterspring. They are exposed approximately six to seven hours every tidal cycle. The subtidal sites Molengat and Wierbalg are continuously submerged. Station Molengat lays just outside the island chain in the North Sea. The Wierbalg station is located in the Dutch Wadden Sea, well outside the tidal gullies (for more details on stations see Table 1).

From the beginning of March 1999 until halfway June 1999 samples were collected with weekly or fortnightly intervals. The subtidal stations were sampled with a Van Veen grab employed from an anchored vessel. The area sampled had a radius of at least 50 m, accounting for DGPS accuracy and movements of the ship with respect to the anchoring position. Animals were collected at the intertidal sites during low tide by sieving the sediment through a mesh of 1 mm². Positions of the intertidal sampling sites were marked with a pole driven into the sediment. The sampled area at the intertidal stations had a radius of 10 m. To ensure maturity only specimens of > 10 mm length were collected.

The shell lengths of 20 freshly collected specimens were measured to the nearest 0.01 mm with electronic calipers. By opening the shell and visually inspecting the gonadal tissue under a dissecting microscope the spawning status was estimated. Totally full was scored as 0 (unspawned), and partly empty (partly spent) or completely emptied gonads (completely spent) as 1. The gonadal tissue was then separated from the somatic tissue using tweezers under a dissecting microscope. Tissue was dried for three days at 60°C, after which samples were weighed and

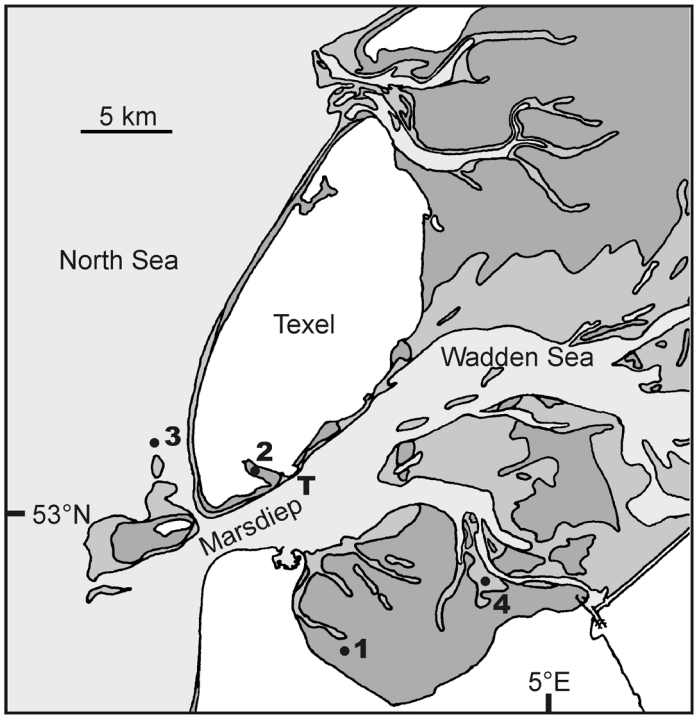

Fig. 1 - Map showing the sampling stations in the Western Wadden Sea and adjacent North Sea, numbers refer to intertidal stations 1) Balgzand, 2) Mokbaai and the subtidal stations 3) Molengat and 4)Wierbalg. The site where sea surface temperature measurements were taken is marked with T. The 0 m and –2 m depth contour lines reduced to mean lower lowwaterspring are drawn.

Table 1 - Geographic position, tidal height reduced to mean lower lowwaterspring, and the numerical density of *Macoma balthica* at the four sampling stations.

station	latitude (°N)	longitude (°E)	depth (m)	number *M. balthica* (m^{-2})
Balgzand	52°55.38'	4°49.09'	1.1	71
Mokbaai	53°00.50'	4°45.40'	1.1	60
Molengat	53°01.44'	4°40.08'	-3.3	5
Wierbalg	52°57.03'	4°57.63'	-1.0	106

incinerated at 560°C and reweighed, to generate the Ash Free Dry Mass (AFDM) of gonadal and somatic tissue. Masses were standardised for shell size by dividing by the cubic shell length and expressed as a mass index (mg/cm^3).

Inducibility of spawning was tested with 100 individuals of each sample. Directly after collection the animals were stored in a thin layer of stagnant seawater at 10°C. The next day the clams were induced to spawn by a rapid temperature rise of 5°C. For this purpose they were placed individually in 100 cm^3 glass jars containing well aerated and filtered (1 μm) seawater of 15°C. For the first two hours the jars were emptied every half hour and new well aerated water of 15°C was added. After three hours the experiment was terminated and the total numbers of individuals that had spawned were noted. Animals were opened and checked for infection by the trematode *Parvatrema affinis* (Swennen and Ching 1974). Infected animals were excluded from the analysis.
The amount of sperm released was quantified by measuring the optical density of the sperm suspension resulting from a male spawning in 100 cm^3 of sea water. To this end the light extinction was measured with a photospectometer at a wave length of 660 nm and the sperm quantity expressed as the optical density at 660 nm (OD660nm).

Sea surface temperatures (SST) where taken from the NIOZ long term measuring series from the Marsdiep inlet (Fig. 1). Monthly means were calculated from measurements taken at 8 a.m. every day (Hendrik van Aaken, pers comm).

Statistical analysis

Proportional data resulting from the scoring of the spent status and the spawning trials were analysed with a logistic model $p= \exp(a-bx)/(1+\exp(a-bx))$ (Crawley 1993), which was fitted to the data by iteration. Factors were added separately to the model and with a χ^2 test it was estimated whether the addition caused a significant reduction of the deviance. Data on gonad mass and spawning volume were analysed with analysis of variance ANOVA. Time is presented as day numbers, counting from 1 January onwards.

Table 2 - Logistic analysis of the number of *Macoma balthica* spent during the spawning season at four different sites. Six models were fit to the data by iteration. Log likelihood values are given for the different models. Each model is tested against a simpler version whether it gives a significantly better fit with the data, using a χ^2 test.

No	Model	Para-meters	log likeli-hood	tested against model	2*[LL(N) -LL(0)]	df	*p*
1	constant	1	-465.5				
2	constant + day	2	-337.9	1	255.2	1	<0.001
3	constant + station	4	-463.9	1	3.2	3	0.359
4	constant + day + station	5	-328.3	2	19.1	3	<0.001
5	constant + day*station	5	-323.8	2	28.1	3	<0.001
6	constant + day + station + day*station	8	-315.3	5	17.0	3	<0.001

Results

The time course of spawning in the field, depicted as the fraction of animals with partly or completely emptied gonads, is given in figure 2A. A logistic model including day, station and the interaction day*station fitted significantly best to the data (Table 2). Largest reduction in the deviance was realised by entering day in the model but the significant effects of station and the day*station interaction indicate significant differences in timing and time course of spawning at the four stations. In figure 2A the curves were fitted for the four stations separately, with the parameter values given in Table 3. The day at which half of the individuals had reached the spent status was two weeks later for Mokbaai and Molengat than for Wierbalg and Balgzand (Table 3). The period over which spawning took place in Molengat was twice as long as in the three other stations, accounting for the significant day*station interaction.

Inducibility of spawning by administering a standardised sudden temperature rise of 5°C varied through time and between stations (Fig. 2B). Day, day^2, station, day*station and (day*station)^2 all contributed significantly to the logistic model (Table 4). Separate fits for the four stations are depicted in Figure 2B and the parameter values given in Table 5. Wierbalg and Balgzand showed large similarities in timing shape and height of the spawning peak. In Mokbaai the period over which *M. balthica* could be induced to spawn was longer than at Balgzand and Wierbalg. The fractions that could be induced to spawn were twice as high. The period in which

Table 3 - Parameter values of the logistic model fitted for each station separately on the number of animals spent. The day at which half of the population has reached the status spent is given by -b/a.

Station	a (SE)	b (SE)	-b/a
Balgzand	0.06904 (0.01145)	-6.52996 (1.11931)	95
Mokbaai	0.09115 (0.01218)	-9.83293 (1.32811)	108
Molengat	0.03282 (0.00628)	-3.53978 (0.74671)	108
Wierbalg	0.07612 (0.01244)	-7.24502 (1.20534)	95

induction was successful was two weeks earlier and longer. Molengat showed the smallest number of spawners extended over a long period of time, latest in the season.

Mean total body mass indexes with standard deviations for Balgzand, Mokbaai, Molengat, and Wierbalg were 10.3 ± 2.7, 13.2 ± 2.8, 9.0 ± 2.6 and 10.0 ± 5.5 respectively. The mass of the gonad, expressed as gonad mass index decreased during the spawning season (Fig. 2C). Analysis with a general linear model over the period of decrease from day 50 to day 150, revealed date, station and the date*station interaction as significant effects (Table 6). Individuals from the Mokbaai had by far the heaviest gonads in the onset of the spawning season. Wierbalg and Balgzand gave similar results, while *M. balthica* from Molengat did not show a decrease in gonad mass index at all. At the end of the spawning season gonad mass index converged to the same values for all four stations.

Males from the subtidal stations Molengat and Wierbalg released twice as many gametes as males from the intertidal stations Balgzand and Mokbaai, when induced to spawn (Fig. 3). In a nested ANOVA, difference in sperm release is significant between tidal levels but not between stations at the same level (Table 7).

Discussion

Synchronisation of spawning across habitats

Spawning activity in *Macoma balthica* at four stations, in three western Dutch Wadden Sea sites and one North Sea site, in 1999 took place during March and April. This finding is in close agreement with earlier studies in the Wadden Sea all reporting

Fig. 2 (opposite page) - Reproductive parameters of *Macoma balthica* monitored at two intertidal stations, Balgzand and Mokbaai and two subtidal stations, Molengat and Wierbalg, from March until June. (A) Fraction of 20 individuals showing loss of gametes as assessed via visual inspection. A logistic curve is fitted for each station. (B) The fraction of 100 individuals that were succesfully induced to spawn by a sudden temperature rise of 5°C, with logistic curves fitted for each station. (C) Mean gonad mass index with standard errors from samples of twenty dissected individuals. Curves are running means.

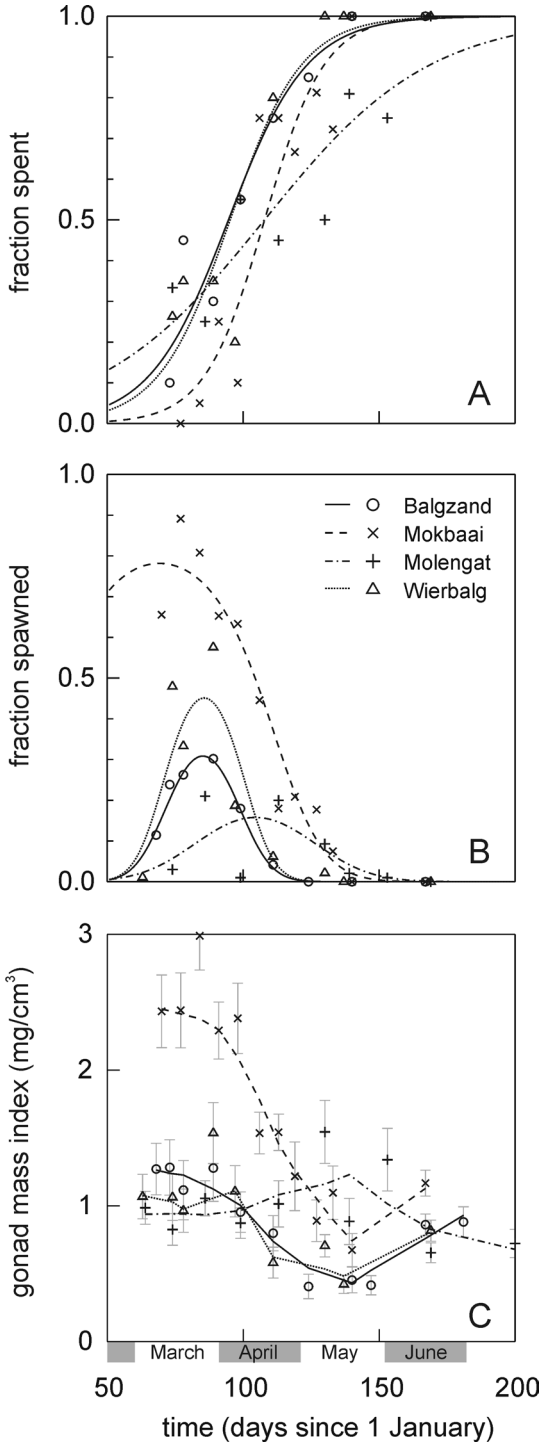
1.0
0.5
0.0
fraction spent
A
fraction spawned
Balgzand
Mokbaai
Molengat
Wierbalg
B
gonad mass index (mg/cm3)
3
2
1
0
C
March
April
May
June
50
100
150
200
time (days since 1 January)

Table 4 - Logistic analysis of the number of *Macoma balthica* succesfully induced to spawn within three hours after administering a temperature shock.

No	Model	para-meters	log likeli-hood	tested against model	2*[LL(N) -LL(0)]	df	*p*
1	constant	1	-1880.1				
2	constant + day + day^2	3	-1523.1	1	714.1	2	<0.001
3	constant + day + day^2 + station	6	-1321.0	2	404.0	3	<0.001
4	constant + day + day^2 + station + station*day	9	-1281.0	3	80.0	3	<0.001
5	constant + day + day^2 + station + station*day + (station*day)^2	12	-1257.9	4	46.2	3	<0.001

March and April as the spawning season in *M. balthica* (Lammens 1967, De Wilde and Berghuis 1978, Madsen and Jensen 1987). The spawning periods of the four stations overlapped to a large extent (Fig. 2A,B,C). The contribution of the effect 'station' to the complete logistic model of data on visual inspection of gonad status, as full or spent, through time is nevertheless significant (Table 2). Spawning at the stations Balgzand and Wierbalg was two weeks earlier than at the other two sites (Table 3). It is possible that the estimate for Mokbaai was too late due to a scoring bias related to the much larger gonad volumes of the animals at this station (Fig. 2C); after a partial release of gametes, the decrease in gonad volume would be less apparent to visual inspection if the initial amount had been larger. Of particular interest regarding these observations is the fact that, incongruent with previous indirect inferences (Battle 1933, Lammens 1967, Günther *et al.* 1998), the variation was not related to tidal level. We therefore have to reject the hypothesis that *M. balthica* in the intertidal and the subtidal have different spawning seasons.

Inducibility of spawning in the lab fell within the same period for all sites (Fig. 2B). In all four stations the peak of inducibility was reached before 50% of the field population showed spawning activity in the field (Fig. 2A,B, Table 3, 5). Analogous to the data on the fraction of animals spent in the field, the patterns of inducible spawning at the stations Wierbalg and Balgzand were very similar. Both the location of the peak and its height were almost identical for these two stations (Fig. 2B, Table 5), illustrating that there were no tidal level related differences in induction of spawning, by administering a temperature shock. These data, too, are in accordance with a rejection of the hypothesis that the intertidal and the subtidal *M. balthica* have different spawning seasons.

Table 5 - Parameter values of the logistic models fit separately for each station to the spawning data. The max value of the curve is reached at day=-b/2a.

Station	a (SE)	b (SE)	c (SE)	-b/2a	max
Balgzand	-0.00381 (0.0007)	0.6490 (0.1272)	-28.444 (5.4516)	85	0.32
Mokbaai	-0.00103 (0.0002)	0.1426 (0.0460)	-3.660 (2.2535)	69	0.78
Molengat	-0.00122 (0.0003)	0.2549 (0.0665)	-14.985 (3.5434)	104	0.15
Wierbalg	-0.00412 (0.0006)	0.7059 (0.0982)	-30.435 (4.1810)	86	0.46

If a final environmental trigger for spawning were later at one tidal level than at the other, an extended period of high spawning inducibility before actual spawning in the field would be expected. However, spawning inducibility (Fig. 2b) and spawning in the field (Fig. 2a) followed each other closely. There was no long delay between the maturation of gametes (assessed via spawning inducibility) and final spawning. Timing of the spawning season is thus tightly linked with the rest of the reproductive cycle (Giese 1959, Giese and Kanatani 1987, Olive 1995). The daily fluctuations in temperature, which are much stronger in the intertidal than in the subtidal (De Wilde and Berghuis 1979), thus do not seem an important determinant

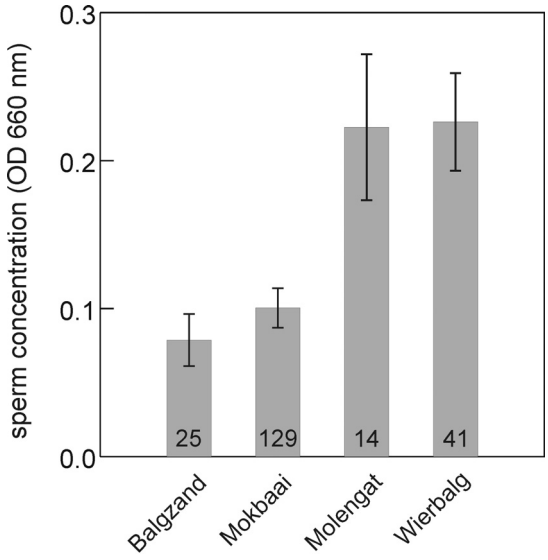

Fig. 3 - The average, standard error and number of samples of the amount of sperm produced per male for the two intertidal stations Balgzand and Mokbaai and the two subtidal sites Molengat and Wierbalg. Sperm amount is expressed as the optical density of a 100 cm^3 sperm suspension at 660 nm.

Table 6 - Analysis of variance of the gonad mass index of *Macoma balthica* from March through May 1999, at the four sampling stations. Squared multiple R = 0.87.

Factor	Sum of squares	df	Mean square	F	*p*
day	2.769	1	2.769	44.923	<0.001
station	4.785	3	1.595	25.876	<0.001
date*station	3.161	3	1.054	17.092	<0.001
error	1.664	27	0.062		

of the spawning season of *M. balthica*. Timing of the reproductive cycle might be timed by an internal rhythm or cued by more gradual environmental signals as average sea surface temperature, day length or food availability (Giese 1959, Bayne 1975). By combining present data from Balgzand with spent data from the same site in 1974, 1975 and 1976 (De Wilde and Berghuis 1978 Fig. 3c) considerable between year variation in timing of reproduction becomes obvious (day 94, 113, 116 are the estimates when half of the individuals are spent, for the years 1974 until 1976). It is unlikely that this can be explained by day length variation, and thus temperature seems a more likely cue. To further explore this we used monthly means of the sea surface temperature (SST) from the Marsdiep inlet. During spring, the months of March until June, the SST rises in an almost perfect linear manner. By linear regression equations for the different years the SST in the middle of the spawning season was estimated to be 8.3°C (n=4, SD=1.6). This temperature is logically somewhat lower than the 10°C reported by Lammens and De Wilde for the same area, because they refer to maxima of SST measurements. The validity of this estimate can be tested using data on the occurrence of *M. balthica* larvae in the northern Wadden Sea for three consecutive years (1996-1998) (Strasser and Günther 2001). In all three years the peaks of larval abundance fall within the four-week period (length of the larval period at 10°C, Drent 2002) after the mean sea surface temperature had reached 8.3°C.

The argument that a difference in thermal environment between the intertidal and subtidal habitats would lead to a difference in spawning season by means of a possible temperature threshold cue for spawning reached at different moments, is redundant because different spawning seasons do not exist. Not only the present data but also the results of Harvey and Vincent (1989) support this conclusion. They also did not find a relationship between timing of spawning of *M. balthica* with tidal height in the St. Lawrence estuary. Reports have been made on the effect of shore level on reproduction in the bivalve species *Cerastoderma edule C. glaucum*, *Modiolus modiolus*, *Geukensia demissa*, *Mercenaria mercenaria* (Boyden 1971, Seed and Brown 1977, Borrero 1987, Walker and Heffernan 1994). In all cases reproduction is retarded at the higher tidal levels and moreover reproductive output is less than at the lower

Table 7 - Nested analysis of variance of the amount of sperm released by *Macoma balthica* males, from the two intertidal stations and two subtidal stations, after induced spawning in the lab.

Source	Sum of squares	df	Mean square	F	*p*
tidal level	0.66679	1	0.66679	25.52	<0.05
station(tidal level)	0.01000	2	0.00500	0.191	n.s.
error	5.35612	205	0.02613		

levels. As these species are all suspension feeders, feeding opportunities are lower at the higher tidal levels, energetically constraining reproduction. In *M. balthica*, energetic conditions also have been shown to result in asynchronous timing of the reproductive cycle at different sites (Wenne and Styczynska-Jurewicz 1985, Thompson and Nichols 1988). Similarly, Newell *et al.* (1982) attributed local spatial variation in reproduction of *Mytilus edulis* to differences in food conditions. Although body mass differences were found between the stations this did not lead to spawning asynchronisation in this study.

When considering the optimal pelagic conditions for larval development and survival, it seems logical that the spawning period is the same for all stations. Although the four stations are situated at different sites and tidal elavations, they are all distributed in the same tidal basin (Ridderinkhof 1991). The larvae originating from these four sites are released into the same body of water, so they should encounter the same pelagic conditions, with most optimal circumstances at the same moment. On top of this, these larvae all settle together in the high intertidal, and distribution to other lower tidal levels only takes place in their first winter (Beukema 1993). Thus, the environment for the offspring from all different habitats in a single tidal basin is the same during not only the pelagic period but also at the first months of the start of their benthic life. Thus, in agreement with a settlement-timing hypothesis (Todd and Doyle 1981), the reproductive period is the same at all stations.

Spawning within sites

Whereas the spawning season is synchronised between stations, it is surprisingly spread out in time within sites. Rather than spawning locally in one single outburst as for example in corals (Babcock 1983), which might be expected from the perspective of fertilisation success, the spawning season within a site takes about two months (Fig. 2). It has been suggested earlier that *M. balthica* is a partial spawner (Caddy 1967, Lammens 1967, Pekkarinen 1983), i.e. that individuals release their gametes in several episodes rather than spawning them all at once. An alternative explanation for a protracted spawning season is that individuals spawn

entirely in one event but do so at different times. This, however, is unlikely, as during the spawning season ripe individuals are clearly half filled as assessed by visual inspection.

Numerous marine invertebrate species spawn multiple times over protracted time periods. There must be an advantage of multiple spawning, as partial spawning increases the risk of eggs remaining unfertilised due to sperm limitation. Reproductive success in *M. balthica* is very variable between years (Honkoop and Van der Meer 1998, Beukema *et al.* 2001). Also within year and population a large variance in reproductive success is expected, because of a sweepstakes-chance of matching reproductive activity with environmental conditions conducive to spawning, fertilisation, larval survival and successful recruitment (Hedgecock 1984). Contrary to the first life stages, adult survival is high and constant in *M. balthica* (Van der Meer *et al.* 2001). Analogous to the reasoning that in a variable environment iteroparity is favoured above semelparity (Murphy 1968, Roff 2002), multiple spawnings per season will increase the chance that any successful reproducing offspring will be produced at all. The advantage of producing numerous batches of larvae through time is supposedly to increase the chance of matching an unpredictable optimum for larval survival: the match/mismatch hypothesis (Cushing and Dickson 1976). When 'putting all one's eggs in one basket', the chance of a complete failure is too large in relation to the number of seasons an individual will be able to contribute.

More insight into the process of partial spawning comes from the observation that male clams from the subtidal release twice as much sperm upon induction by a temperature shock than those from intertidal sites (Fig. 3, Table 7). This pattern cannot be explained by differences in amounts of gametes stored between sites, as the amount of sperm released is not related to the gonad mass index (Pearson correlation coefficient = -0.409, $p=0.59$, $n=4$). In the subtidal, sperm will need to be effective in a larger volume of water. The concentration of sperm spawned into the intertidal will therefore decrease less quickly than that of subtidally spawned sperm, which may result in stronger effects of sperm limitation in the subtidal. Indeed, fertilisation success of the broadcast spawning sea star *Asterias vulgaris* decreases with increasing water depth (Levitan 1998). In terms of a trade-off between the amount of gametes released per spawning event and the number of events, this trade-off appears to result in a smaller number of events for subtidal males because of the stronger effect of sperm limitation in that habitat.

Population dynamical spin-offs

Although there are no clear differences between the stations in timing of the spawning season, there are remarkable deviations in factors affecting chances for reproductive success. Molengat *M. balthica* had the lowest gonad mass, showing no decline during the spawning season (Fig. 2c), had a longer spawning period with a less distinct peak (Fig. 2a, b) and the numerical density was very low (Table 1). Under these circumstances chances for reproductive success are poor because, apart from

low gamete production, successful fertilisation is also unlikely. Local synchronisation with neighbouring individuals is obtained by waterborne cues (Miller 1989, Zeeck *et al.* 1996, Hardege and Bentley 1997, own unpublished observations). Unless clams living in very low densities undertake active clustering activities, distances between them are likely too large to enable this type of pheromone communication. In the broadcast spawning sea urchin *Diadema antillarum*, spawning is asynchronous when it lives in low densities, and the author concludes that fertilisation success must be low under such circumstances (Levitan 1988). We suspect that the effective contribution of the Molengat population to the next generations must be very limited, thus constituting a 'sink'.

On the other hand the Mokbaai station had by far the highest gonad masses (Fig. 2c) and large fractions of the population spawned at the same moment (Fig. 2b). Chances for successful production of fertilised eggs were by far the best for this station. Among the four sites studied it is most likely that Mokbaai individuals have contributed a disproportinately large fraction of the 1999 cohort of recruits.

Acknowledgements

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CHAPTER SEVEN

Fertilization in broadcast spawning marine bivalves is a small scale process: the Baltic clam *Macoma balthica*

P.C. Luttikhuizen and J. Drent
(manuscript)

Abstract

Here we argue that fertilization in marine broadcast spawners is a small scale process, especially when compared to the scales of pelagic larval dispersal and many selection gradients. Small-scale fertilization is important with respect to the likelihood that genetic polymorphisms can be maintained by spatially differentiated selection. For the marine bivalve *Macoma balthica* (L.), we demonstrate the relationship between sperm concentration and fertilization probability of eggs. Also, the effect of gamete age on egg fertilization probability is addressed and it is shown how spawning individuals, particularly females, induce spawning in nearby conspecifics. Determining the number of sperm released by males during one spawning bout, the results are translated into a qualitative field prediction.

Introduction

A species is defined as a broadcast spawner when both sperm and eggs are released into the water column and fertilization takes place externally. Many marine invertebrates are broadcast spawners but lead a sessile or semi-sessile life as an adult. The larvae remain for hours up to months in the pelagic, where they drift with water currents and may travel great distances. The habitat of the adults may vary at a range of scales (*e.g.* with latitude, with water depth, or precise position in puddles, on rocks etc.). Different selective regimes may be associated with a change in habitat.

If a species' range contains various environments with different selective regimes, this may result in the evolution of stable genetic polymorphism (Levene 1953, Felsenstein 1976, Hedrick *et al.* 1976, Hedrick 1986, Phillips 1996). A difference between the spatial scales of fertilization and selection may be one of the factors that influence the likelihood of the stable maintenance of genetic polymorphisms (Hoekstra *et al.* 1985, Goldstein and Holsinger 1992). If adults generally mate with adults that have spent their entire mature life at the same location, couples that end up siring offspring together will on average have been

exposed to more similar selective forces than two randomly drawn individuals. This would effectively result in positive assortative mating. While the dispersal, that takes place in the pelagic larval phase, is a force reducing the likelihood of the stable maintenance of a genetic polymorphism, assortative mating may increase that likelihood.

Macoma balthica (L.) is a marine bivalve with a pelagic phase of 2-5 weeks (Caddy 1967, Drent 2002). The relationship between sperm concentration and fertilization rate in this species was measured as an approximation of the effect of distance in the field; the larger the distance between two individuals, the more diluted their gametes will be when they meet. An additional factor important in kinetics of external fertilization is gamete mortality rate. The effect of sperm age on fertilization probability of eggs was determined by using ageing sperm suspensions for fertilization assays on freshly spawned eggs. The same was done for ageing egg batches. We further tested whether spawning *M. balthica* can induce spawning in conspecifics by exposing ripe individuals to spawned products (eggs or sperm plus the water the spawning individuals had been contained in). Finally, the number of spermatozoa released by males upon induction of spawning was counted. From these results, we deduce a qualitative prediction of typical sex partner distance in the field for *M. balthica*.

Materials and methods

Sperm concentration experiments

Fertilization trials consisted of letting a batch of eggs from a single spawned female be fertilized by a sperm suspension from a single spawned male. The suspension was gently mixed, left standing overnight (i.e., more than 12 and less than 24 hours) and approximately 150 eggs were then scored as fertilized or unfertilized. By that time, fertilized eggs have typically developed into well-recognizable unhatched multicellular embryos (Luttikhuizen and Pijnacker 2002), while unfertilized eggs have not changed in morphology. Thirteen females and six males were combined for a total of 78 trials. Sperm concentration was measured as the optical density of the well mixed sperm suspension at 660 nm. The relationship between optical density and sperm concentration was calibrated by counting the number of sperm using a flowcytometer or a hemocytometer ($S = 2.57 \cdot 10^7 D$, n = 10, $r^2 = 0.969$, S = number of sperm per ml, D = optical density at 660 nm).

Gamete age experiments

Trials consisted of adding a batch of eggs from a single female to a sperm suspension from a single male. Mixture was left standing overnight and approximately 150 eggs were scored as fertilized or unfertilized. For sperm age experiments, freshly spawned eggs (<52 min. elapsed since spawning) from a total of 26 females were used for fertilization. For egg age experiments, freshly spawned

sperm (<55 min. elapsed since spawning) from a total of eight males was used for fertilization at seven points in time (two males were used at the first census moment).

Spawning induction experiments

Ripe clams were collected in early spring and kept overnight in a thin layer of stagnant sea water at 7ºC. The next day, a starter group was induced to spawn individually in jars containing 50 ml aerated sea water of 15ºC. The spawned gametes plus surrounding water from several males and females was used as treatment in the experiments, which consisted of putting a total of 177 (experiment 1) or 288 clams (experiment 2) individually in jars containing 50 ml aerated sea water plus treatment water. Treatment water was either 5 ml aerated sea water ('control'), 5 ml water with spawned sperm from starter group males ('sperm'), 5 ml water with eggs from starter group females ('eggs'), or 5 ml starter group sperm suspension plus 5 ml starter group eggs suspension ('both'). The latter treatment was not carried out in experiment 2. The number of spawning individuals was recorded as well as the time of initiation of spawning.

Amount of sperm per male

Ripe Baltic clams were collected in early spring and individually induced to spawn similarly to the control group of the spawning induction experiments but in 100 ml aerated sea water. Once males had begun to spawn, they were left until they ceased spawning of their own accord. Afterwards, 76 animals were opened and inspected visually for spent status of their gonads. Shell length was measured along the longest shell axis with electronic calipers.

Results and discussion

Effect of sperm concentration on fertilization

Percentage of eggs fertilized was strongly dependent on sperm concentration (Fig. 1). The transition from no fertilization to all eggs fertilized took place between 10^5 and 10^7 sperm/ml. The concentration at which half of the eggs are fertilized is estimated to be $3.5 \cdot 10^5$ sperm/ml. This is similar to the concentration range of 2-$9 \cdot 10^5$ sperm/ml at which half of *Cerastoderma edule* eggs were fertilized in comparable experiments (André and Lindegarth 1995). Much lower concentrations of sperm necessary to obtain 50% fertilization were observed for *Mytilus edulis* (Sprung and Bayne 1984), *Pecten maximus* (Gruffydd and Beaumont 1970), and the scallops *Chlamys bifrons* and *C. asperrima* (Styan and Butler 2000). André and Lindegarth (1995) actually suggest their concentration experiments may have overestimated the dilution effect, because in their gamete age experiments much higher fertilized fractions are reached at low concentrations; perhaps their initial result was related to the small number of observations (three males).

Effect of gamete age on fertilization

Age of sperm (time since spawning) had a strong effect on fertilization rate of eggs within a few hours after spawning (Fig. 2a). For one male (squares in Fig. 2a) no decrease was observed, which may be due to the limited length of the time series for that individual, or to a larger viability of the sperm suspension of that particular male. The differences in the rate of ageing among the other males also appear to suggest variation among males. However, larger viability of a sperm suspension does not necessarily mean larger viability per spermatozoon; one distinct effect is larger sperm concentration (Fig. 1, André and Lindegarth 1995, Williams and Bentley 2002). To test for inter-individual variation in spermatozoon viability, future experiments should take into account sperm concentration and sperm age at the same time.

No effect of egg age on fertilization rate was detected over the time range observed (Fig. 2b). The two females were tested on the same day and against sperm of the same eight males (the small time lag between the two females in Fig. 2 is due to a difference in the onset of their spawning). As can be seen readily from the graph, the large variation among observed fertilization rates is correlated mainly with identity of the male. This indicates that any ageing effect of eggs arising before seven

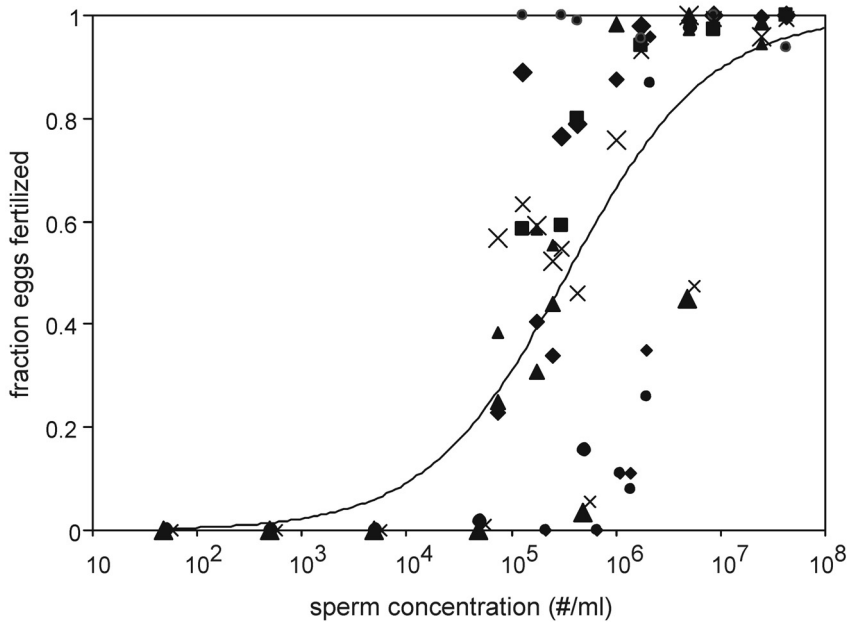

Fig. 1 - Effect of sperm concentration on fertilized fraction of eggs in *Macoma balthica*. Each dot shows the results of a single trial, the symbols indicate specific females. The curve is the fitted relationship between sperm concentration and fraction of eggs fertilized (logit regression, $p << 0.001$, estimated parameters 1.484 and -8.221); 50% fertilization is predicted at $3.5 \cdot 10^5$ sperm/ml.

hours are swamped by differences in performance among sperm suspensions, at least in these experiments. The results are in contrast to the situation in cockles, *Cerastoderma edule*, where eggs had lost the ability to be fertilized within four hours after spawning (André and Lindegarth 1995). There is, however, large variation

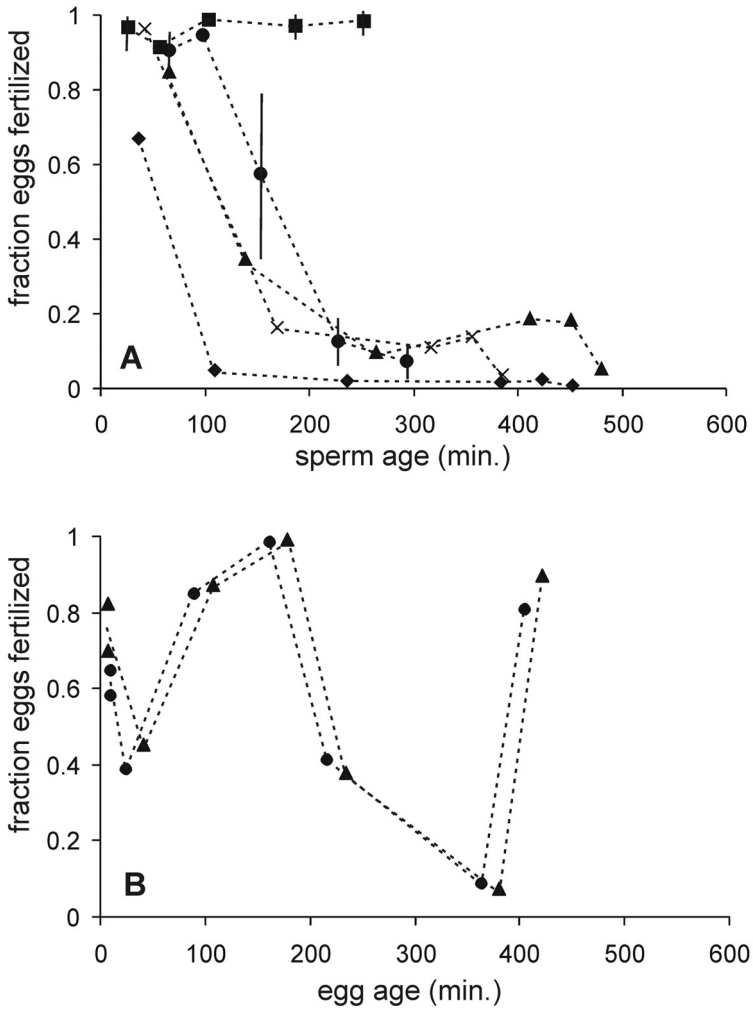

Fig. 2 - Effect of gamete age on fertilized fraction of eggs. a) Time series of the effect of sperm age for five males. Individual males are indicated by different symbols. Vertical bar is displayed when more than one female was used at one sensus moment, the range indicating largest and smallest value observed. Line connects averages for each male. b) Time series of the effect of egg age for two females. Individual females are indicated by different symbols. Line connects averages for both females.

among marine invertebrates with external fertilization in egg longevity. Williams and Bentley (2002) report on life times of 60-120 hours for eggs of the polychaetes *Arenicola marina* and *Nereis virens* and 4-24 hours for the asteroid echinoderm *Asterias rubens*. Eggs of the sea urchin *Strongylocentrotus droebachiensis* remain viable for up to 48-72 hours (Meidel and Yund 2001). Future experiments addressing *M. balthica* egg longevity should extend over longer periods of time and use standardized sperm concentrations to lower the contribution of male-associated variation in fertilization rate.

Effect of spawned products on spawning

Spawning activity was induced by spawning activity of conspecific individuals, especially dramatically so when female spawned products were used as a cue (Fig. 3). Fraction of individuals spawning differed significantly between treatments, both in experiment 1 (test for goodness-of-fit, $G = 33.18$, $df = 2$, $p < 0.005$) and in experiment 2 ($G = 22.54$, $df = 1$, $p < 0.005$). The observed increase in fraction of spawners in the treatment with male spawned products was only small. The effect of adding female products, on the contrary, was very large. Earlier experiments with addition of gametes from conspecifics failed to demonstrate increased spawning activity in *M. balthica* (Caddy 1967, De Wilde and Berghuis 1978). In the oyster *Ostrea virginica* both males and females can be induced to spawn by exposing them to sperm of conspecifics, and males also respond to conspecific eggs (Galtsoff 1938, 1940).

Other cues known previously to induce spawning in *M. balthica* are a temperature shock (De Wilde and Berghuis 1978) and the serotonin reuptake inhibitor (SRI) fluoxetine (Honkoop *et al.* 1999), which is the active substance of the medical drug Prozac. Serotonin acts as a neurotransmitter in synaptic clefts. In other bivalves, serotonin as well as SRIs have been demonstrated to induce spawning (Van Citter 1984, Gibbons and Castagna 1984, Fong 1998).

Amount of sperm released per male

Among 209 males that were induced to spawn in the laboratory by a temperature shock, the total amount of sperm averaged $3.36 \cdot 10^8$ spermatozoa and varied between a minimum of $2.57 \cdot 10^6$ and a maximum of $2.72 \cdot 10^9$ spermatozoa (Fig. 4). The distribution of the among-male variation in number of spawned sperm was very skewed, with only few males producing large amounts. This predicts that not all males will perform equally well in the field. Larger males released more sperm (Pearson rank correlation between shell length and number of sperm released, $r = 0.372$, $n = 76$, $p < 0.01$). Approximately 40% of the induced males were completely spent after induced spawning, while the rest had not entirely emptied their gonads. There was no difference in number of sperm produced between completely versus partially spent males (Kruskal-Wallis analysis of variance, $n = 76$, n.s.).

The amounts of sperm produced per male (Fig. 4) can be combined with the data on the effect of sperm concentration (Fig. 1) to obtain an estimate of the

volume within which a male would be able to fertilize 50% of eggs present ($V_{0.5}$), assuming a homogenous concentration within that volume. The average sperm production per male of $3.36 \cdot 10^8$ would translate to a $V_{0.5}$ of 965 ml or approximately

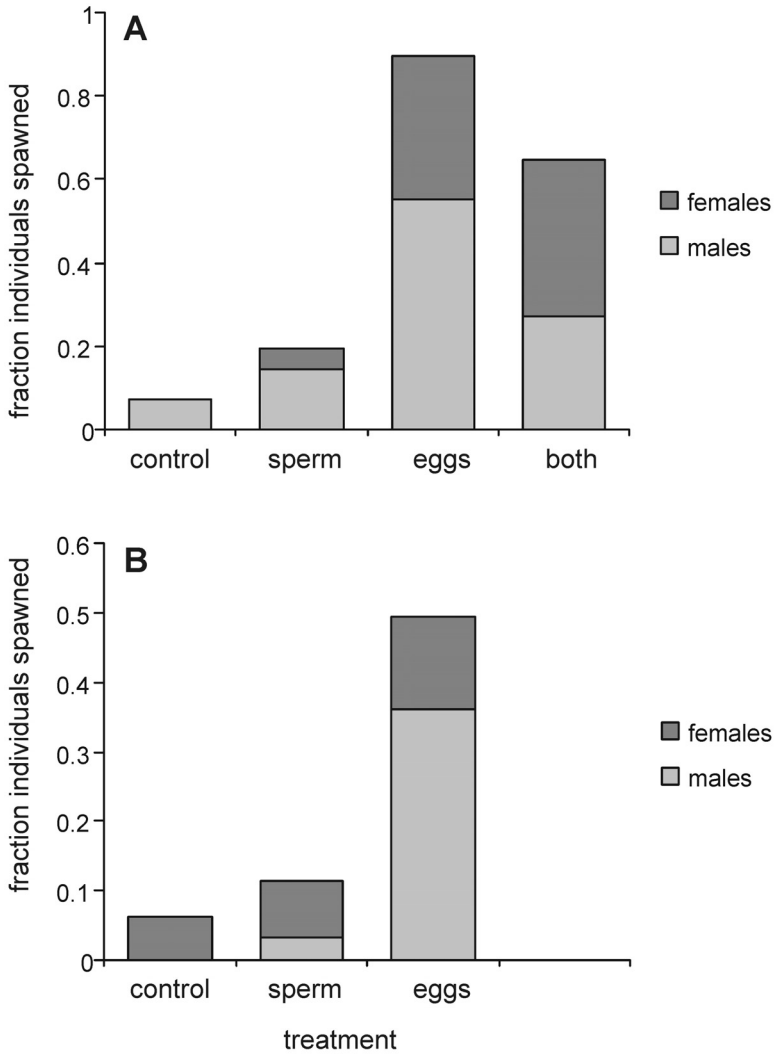

Fig. 3 - Effect of presence of gametes from conspecifics on fraction of readiness of *M. balthica* individuals to spawn. a) Results of experiment 1. Fraction of individuals spawning differs significantly between treatments (test for goodness-of-fit, $G = 33.18$, $df = 2$, $p < 0.005$). b) Results of experiment 2. Fraction of individuals spawning differs significantly between treatments (test for goodness-of-fit, $G = 22.54$, $df = 1$, $p < 0.005$).

1 liter. The range among the 209 males tested runs from 7 ml to 8 l, with 70 % of males having a $V_{0.5}$ smaller than 1 l and 6% reaching a $V_{0.5}$ larger than 3 l.

Qualitative translation to field situation

Although laboratory observations on spawning and fertilization kinetics are hard to translate to quantitative field predictions (because of for example the difference in speed between diffusion and current transport), they may enable qualitative inferences. Such qualitative inferences are informative as it is hard to study these issues in the field (for reviews see Levitan 1995, Levitan and Petersen 1995, Yund 2000), especially in murky and turbulent environments. From the results presented here, we can make two tentative qualitive predictions. First, sperm limitation is most likely to play a role in fertilization of *M. balthica* eggs in the field, and second, fertilization is probably a small scale process, i.e., most zygotes will be sired by parents that were situated not far from each other.

It can be deduced from the experiments described here that some females, perhaps together with some males, initiate spawning. The presence of female

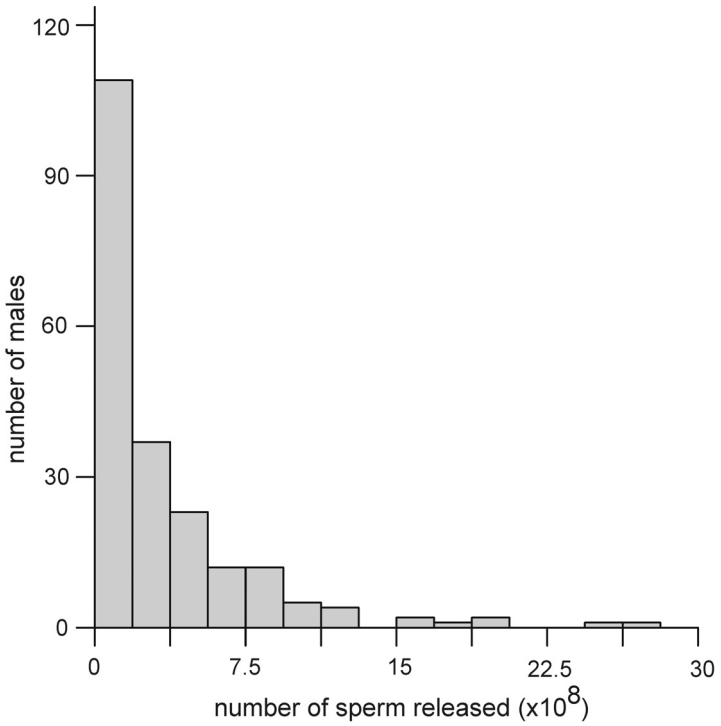

Fig. 4 - Distribution of amount of sperm released per male among 209 *M. balthica* during induced spawning.

spawned products in the water column induces spawning activity in males and in other females (Fig. 3). The synchronized spawning effort will lead to fertilization of a portion of the eggs, not far from the location of the female that produced them. Sperm will not move far from the male that produced them before they lose their viability (Fig. 2a). Most eggs will be fertilized by a few males (assuming no relationship between amount and quality of sperm produced) (Fig. 4), particularly the larger ones, that produce larger numbers of sperm. The fraction of eggs that can be fertilized locally may not be 100%, judging from the small inferred $V_{0.5}$ volumes within which single males are able to fertilize 50% of eggs: on average 1 liter. The eggs that remain unfertilized locally may be transported away by diffusion and/or currents and end up in (or even induce) sperm suspensions somewhat further removed from their place of origin. The latter possibility is likely to extinguish rapidly with distance, considering that much of *M. balthica* habitat is subtidal and potential dilution volumes are therefore huge. Evidence for this comes from field studies on asteroid echinoderms (Babcock *et al.* 1994) and ascidians (Grosberg 1991), where the largest scale of fertilization observed was still less than 100 m downstream. Underlining the possibility of an effect of sperm limitation in *M. balthica* field spawning is the fact that the spawning season in this species extends over two months, during which individuals may spawn more than once (Lammens 1967, Drent and Luttikhuizen, in prep.).

Fertilization in *M. balthica* and many other marine broadcast spawning invertebrates is of a small scale relative to the scale of larval dispersal and of many selection gradients. This difference in scale is critical for assumptions on random mating, because sessile individuals who are situated in close proximity will have survived similar selection pressures. Their genomes may therefore be expected to be more similar in those regions that contain the selective variation than a randomly drawn pair of adults. Even if settlement is entirely random, local selection and local fertilization ensure that individuals with similar genotypes will have an increased probability of producing offspring.

It is a general observation that neutral molecular markers are unstructured over thousands of kilometers in marine species with pelagic larvae (reviews by Palumbi 1996, Grosberg and Cunningham 2001). In contrast, quantitative genetic variation for shell shape in *M. balthica* was found to be differentiated with habitat at a spatial scale of kilometers (Luttikhuizen *et al.*, in press). The stable maintenance of spatially structured genetic polymorphisms in marine broadcast spawners, in spite of high dispersal capacity during the pelagic larval phase, may be easier to understand in the light of local fertilization.

CHAPTER EIGHT

Mosaic haploid-diploid embryos and polyspermy in the tellinid bivalve *Macoma balthica*

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Abstract

We investigated the meiosis, fertilization, and early development in eggs of the tellinid bivalve *Macoma balthica* (L.), which has external fertilization. Meiosis is standard but polyspermy is found to be very common. In all eight crosses examined, mosaic embryos, consisting of a mixture of diploid (2n=38) and haploid cells occur at a frequency ranging from 2.7 to 29.1%. The earliest mosaic found is in the two-cell stage. We propose that an androgenic haploid cell lineage can originate from one supernumerary sperm that decondenses into a functional haploid nucleus, starts mitosis, and is incorporated in the developing embryo.

Introduction

Polyspermy has been observed in a number of bivalves with external fertilization (*Mytilus galloprovincialis* (Dufresne-Dubé *et al.* 1983), *Mytilus edulis* (Togo *et al.* 1995), *Crassostrea virginica* (Alliegro and Wright 1983), *C. gigas* (Stephano and Gould 1988, Togo and Morisawa 1999), *Spisula solidissima* (Clotteau and Dubé 1993), and *Dreissena polymorpha* (Misamore *et al.* 1996)). Generally, these studies on polyspermy in externally fertilizing species focus on mechanisms that block polyspermy, and on when and how the block fails. However, the fate of supernumerary sperm cells in bivalve eggs has not, to the best of our knowledge, been investigated.

We investigated for the first time the meiosis, fertilization and early development in eggs of the tellinid bivalve *Macoma balthica* (L.) (Bivalvia: Tellinidae) and report on the common occurrence of polyspermy and on the presence of haploid cell lineages among the normal diploid cells.

Materials and methods

Baltic clams *M. balthica* (L.) with mature gonads were collected in spring of 1998 at intertidal and subtidal sites close to the island of Texel, The Netherlands, and

on an intertidal mudflat in the Ythan Estuary, Scotland. They were transported to the laboratory and kept in a thin layer of natural seawater at 0°C for at least 24 h.

The clams were individually induced to spawn by transferring them to 100-mL glass beakers filled with oxygen-rich filtered sea water at temperatures ranging from

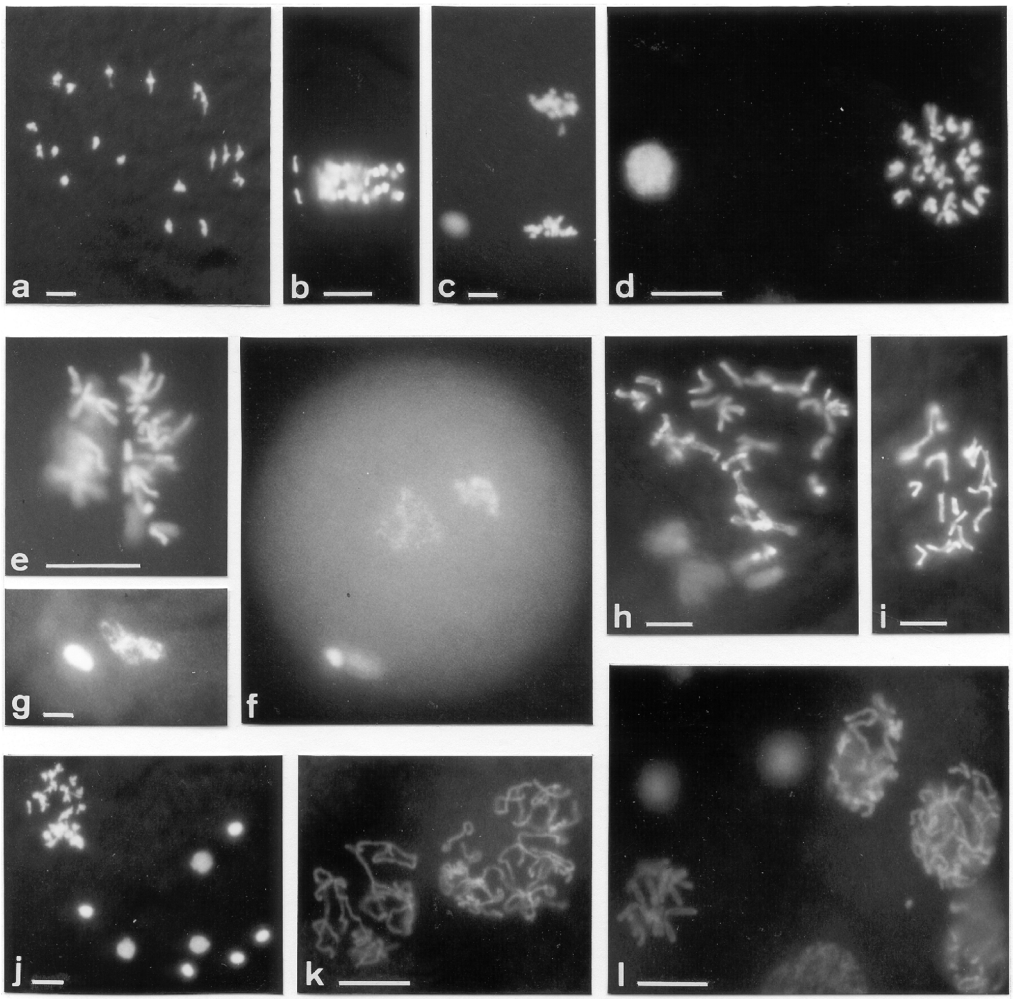

Fig. 1 - Micrographs of DAPI-stained eggs of *Macoma balthica* (L.). Bar represents 10 µm. (*a*) First meiotic metaphase with 19 bivalents. (*b*) First anaphase. (*c*) First telophase with sperm nucleus (latter not in focus). (*d*) Second metaphase with decondensed sperm nucleus (first polar body outside view). (*e*) Fusion of male (left) and female chromosome sets. (*f-i*) Two-cell embryo (*f*; at low magnification) with polar bodies (*g*); diploid (*h*; not all chromosomes in focus) and haploid (*i*) prometaphase. (*j*) Second metaphase with overlapping first polar body and three larger decondensed and five compact sperm nuclei. (*k*) Haploid and diploid prophase in 40-cell embryo. (*l*) Haploid metaphase and haploid and diploid prophase in 50-cell embryo.

15 to 17°C. For four hours, the water was refreshed every half hour. Those clams that had not spawned were put back in the 0°C storage container. Eggs (diameter about 100 μm) were transferred to 10-mL glass vials filled with 5-10-mL sea water at 15 to 17°C and fertilized within 2.5 h with a few drops of a sperm suspension between 15- and 150-min-old from one of the males.

Artificial crosses were always made between a single female and a single male. The eggs were fixed for chromosome preparation at different time intervals after adding sperm, i.e., every 10 min during the first two hours and thereafter irregularly until 14 h (total embryonic development lasts at least 24 h). In addition, dissected, unspawned eggs from two females, as well as spawned but unfertilized eggs from one female, were fixed. Each fixed batch contained 100 or more eggs.

Ovarial eggs and spawned eggs were fixed in cold (-20ºC) Carnoy's solution (glacial acetic acid : absolute ethanol, 1:3) and stored at 4°C. A subset of the eggs with embryos were pre-treated with 1% colchicine for 30 min for a better spreading of the chromosomes. Eggs were embedded in drops of 2 μg 4,6-diamino-2-phenylindole (DAPI) in 1 mL Vectashield (Vector Laboratories, Burlingame, Calif.) on a microscope slide and covered with a cover slip. After one day and, if necessary, after squashing they were examined under a fluorescence microscope (Zeiss, Germany). Phase-contrast was used to ascertain cytokinesis or multicellular development. Photos were taken on Kodak Elite chrome 400 colour slide film (Kodak, U.S.A.) from which black and white photos were processed.

Results

Mature dissected and early spawned eggs were at first metaphase of meiosis (Fig. 1a). About two percent were at diplotene, even for a period of 24 h after spawning and such eggs were not penetrated by sperm. The number of bivalents was always 19 (2n=38) and univalents were not observed. Spawned eggs remained at first metaphase as long as sperm suspension was not added. In unfertilised eggs, bivalents either started to degenerate or decondensed first and polyploidised by endomitosis from 3 h onwards.

In fertilised eggs the reduction division (Fig. 1b) and second division (Figs. 1c, 1d, and 1j) were normal and meiosis finished about 40 min after the addition of sperm. The spindles of the first and of the second meiotic division were perpendicular and in close proximity to the egg periphery. A first polar body with 19 two-chromatid chromosomes and a second polar body with 19 one-chromatid chromosomes were expelled and degenerated (Fig. 1g). Remnants were sometimes still present after 24 h. The first polar body may have entered an abnormal, often sticky, division, but a normal equational division was never observed. The fine-structured interphase female pronucleus moved to the centre of the egg and started mitosis quickly (no prophase found) with 19 prometaphase chromosomes at about 50 min.

The compact ovoid sperm, after entering the egg cell, always became round and started to increase in size within 10 min (Figs. 1c, 1d, and 1j). The enlarged sperm nucleus contained finely structured chromatin (Fig. 1d) and would change quickly (no prophase found) into 19 chromosomes close to the haploid set of female chromosomes at about 50 min. They could be distinguished because they take less space than the female chromosomes (Fig. 1e). The two haploid sets of chromosomes would fuse and share a single, bipolar spindle and proceed with the first diploid cleavage division. Amphimixis with more than one male set of chromosomes in a triploid or higher polyploid fusion configuration was not observed. Sperm remaining on the outside of the egg did not change in morphology.

Eggs apparently can be triggered to continue with their meiosis in the absence of a decondensing sperm. In these rare cases (<1%) even a compact sperm could not be detected inside the egg cell. Meiosis then stopped in the metaphase of the second meiotic division, and chromosomes began their degeneration.

Polyspermy was very common (Fig. 1j). All sperm nuclei within the egg cell enlarge and become structured as described above, and can be distinguished easily from the sperm remaining at the surface of the egg. Figure 2 shows the frequency distribution of sperm nuclei found inside the eggs of one of the crosses, fixed 30 min after adding sperm suspension; the maximum number scored is 12. The nuclei were not found in clusters and are apparently distributed randomly in the egg cell.

Cell multiplication is asynchronous and it may already be so at the two-cell stage at prophase. The chromosomes of one cell then spiralize earlier than those of the other cell. After 9 h the embryo may consist of more than 50 cells. However, with phase contrast, cleavage could no longer be established reliably for all cells beyond the four cell stage. The number of mitoses of which the chromosome

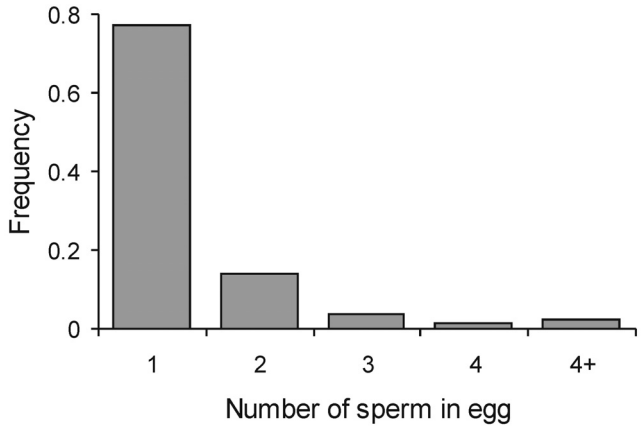

Fig. 2 - Polyspermy in eggs (n = 78) of one single-pair cross of *Macoma balthica* (L.). Frequency distribution of numbers of decondensing sperm nuclei in egg cytoplasm 30 min after adding sperm suspension to eggs.

Table 1 - Mitoses in embryos of *Macoma balthica* (L.).

Cross	No. of embryos	No. of cells/embryos	% of cells with Mitoses	One mitosis	Haploid and diploid mitoses
a	72	8	87.5	50.0	2.8
b	150	8	93.3	72.0	9.3
c	81	8-16	55.6	27.2	9.9
d	185	8-16	24.9	8.1	2.7
e	39	~40	97.4	17.9	5.1
f	79	~40	98.7	17.7	29.1
g	61	~50	96.7	23.0	3.3
h	33	~50	87.9	18.2	9.1

number could be examined, among 2-cell to 50-cell embryos, ranged from zero to nine per embryo. Two types of embryos were encountered in all crosses: one in which all mitoses are diploid and another in which haploid and diploid mitoses are present (Figs. 1f-1i, 1k, and 1l). The maximum observed number in one embryo was four haploid plus five diploid mitoses. The proportion of mosaic embryos, calculated for eight crosses and shown in Table 1, varied from 2.7 to 29.1%. Mosaics could not be scored in multicellular embryos with all cells in interphase or with one mitosis. Considerable variation in nuclear size and the absence of distinct heterochromatic structures did not allow us to distinguish haploid and diploid interphase nuclei. Consequently, the observed percentages are an underestimation. The youngest mosaic haploid-diploid embryos, observed twice, were at the two-cell stage, with one haploid and one diploid metaphase and two normally shaped polar bodies (Figs. 1f-1i).

The mosaics developed normally and cell degeneration did not occur in regions with (a) haploid cell(s). Supernumerary sperm in decondensed state may still be found in cells of 16-cell embryos. Older embryos displayed small pycnotic bodies which may have indicated degenerating sperm.

Discussion

The fertilisation and meiotic divisions in the eggs of *M. balthica* (L.) follow the standard type (Wilson 1928), but the occurrence of polyspermy and mosaic haploid-diploid embryos are exceptional. As to the origin of the haploid mitoses in mosaic embryos, their occurrence at the two-cell stage is of crucial importance. The haploid metaphase cannot have originated from the polar bodies because the latter were

expelled and degenerated, nor could it be one of the haploid metaphases of a haploid embryo at the two-cell stage (in which one of the metaphases has fused with the sperm chromosomes), because a haploid embryo does not develop beyond the second meiotic metaphase. For the same reason, endomitosis of one of the haploid cells of a two-cell haploid embryo can not have taken place. Moreover, the diploid metaphase should have demonstrated pairs of identical chromosomes (ski-pairs or diplochromosomes, cf. Pijnacker and Ferwerda 1990), which were not observed. This means that the haploid set of chromosomes may result from a supernumerary sperm.

Additional sperm would thus be able to enter mitosis and the haploid male set of chromosomes should be able to generate a normal bipolar spindle and induce cytokinesis. Because haploid mitoses are formed very early in development, and the number of cells does not increase progressively, it could not be established whether supernumerary sperm can also enter mitosis later during development, which would imply that the haploid cells would be genetically different. Also, it is not known if haploid cells multiply faster or slower than diploid ones, and so have a selective advantage or disadvantage, respectively. The ultimate fate of the haploid cells is at present unknown, nor is it known if they form complete embryonic tissues or organs. Future research should concentrate on this issue.

The onset of the process we report on may have been observed in other bivalve species. Supernumerary sperm progressing towards the prophase condition were reported for both *Pecten maximus* (Gruffydd and Beaumont 1970) and *Crassostrea virginica* (Stiles and Longwell 1973). Ahmed and Sparks (1970) briefly mention the presence of haploid metaphases in early embryos of the two mussel species *Mytilus edulis* and *M. californianus*, but the authors do not discuss their origins. This indicates that the occurrence of haploid cell lineages stemming from supernumerary sperm amidst the normal diploid cells in early bivalve embryos may not be restricted to the species we studied.

Acknowledgements

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CHAPTER NINE

General discussion

What does a life history involving external fertilization and pelagic larvae mean for intraspecific genetic variation? This type of life cycle is on one extreme end of a continuum. Marine broadcast spawning invertebrates are one of the taxa with the highest potential for dispersal. For highly dispersing taxa, both neutral and selected variation are predicted to have a largely random spatial distribution. The large amount of genetic exchange between sites is expected to keep neutral variation from differentiating (i.e., allele and haplotype frequencies remain similar) and to hamper local adaptation (i.e., non-neutral variation with a genetic basis remains spatially similar despite local environmental differences).

These predictions are not confirmed by the results presented in this thesis. Neutral variation in molecular markers is highly differentiated among European populations of *M. balthica*. While many comparable studies of broadcast spawning marine invertebrates conform to the expectation of large scale genetic uniformity (*e.g.* Hellberg 1996, Karl *et al.* 1996, McFadden *et al.* 1997, McGormack *et al.* 2000), the results presented here are congruent with a growing number of examples of unexpected amounts of molecular marker differentiation in these taxa (*e.g.* Reeb and Avise 1990, Benzie and Williams 1997, Barber *et al.* 2000). Furthermore, the present study is the first to demonstrate that quantitative genetic variation (for shell shape) can be strongly spatially differentiated despite gene flow in marine broadcast spawning invertebrates.

Because of the importance of dispersal for the spatial organisation of genetic variation, more knowledge of the spawning and fertilization processes was required. Spawning and fertilization, unlike larval dispersal, generally occur locally. The investigations related to spawning and fertilization in this thesis suggest that behaviour (timing of spawning, induction of spawning by conspecifics, the amount of sperm released) and phenotype (egg size) are tuned to local fertilization conditions and vary within the species. Mating partners are expected to be relatively close to each other in space (within meters).

Neutral genetic variation

Neutral genetic variation in Macoma balthica*: a review of previous studies*

The first allozyme studies on *Macoma* spp. were interspecific comparisons. Reid and Dunhill (1969) determined variation in esterase for eight *Macoma* species (not including *M. balthica*) and found that there was too much variation within the species for the locus to be taxonomically informative. Levinton (1973, 1975) contributed to the discussion on whether allozyme polymorphisms are maintained by

selection or by neutral processes like mutation and genetic drift (for a review see Avise 1994). The selectionist hypothesis that environmental variability regulates allozyme variability was supported as among six distantly related bivalve species (one of them *M. balthica* and all sampled from the northwestern Atlantic) the number of alleles per locus was strongly correlated with burrowing depth (Levinton 1973). However, his 1975 paper described the absence of such a difference between two intertidal and eight subtidal species within the *Macoma* genus (all sampled from the San Juan Islands in the northeastern Pacific).

In later years, allozymes were used to study intrapopulation allozyme variability in relation to habitat and environmental stress in *M. balthica*. Green *et al.* (1983) studied 22 allozyme loci in *M. balthica* from Hudson Bay, Canada, at two tidal levels and found that six loci were polymorphic. They observed a significant positive correlation between growth rate and number of polymorphic loci at the 1.1 m tidal level, but not at the mean low water level, and an overall trend towards heterozygote deficiency. Trends towards heterozygote deficiencies in *M. balthica* have also been reported by other authors (Meehan 1985, Hummel *et al.* 1995 to 2000), and Hummel *et al.* (1996a) observed decreased heterozygosity lower in the intertidal zone. Two studies demonstrated a cline of *Idh*1 genotype with tidal level (White Sea: Hummel *et al.* 1998b, Gulf of Gdansk: Hummel *et al.* 2000).

From the collective studies on population subdivision using allozymes in *M. balthica* it must be concluded that *M. balthica* is not a single species. The most divergent group consists of undifferentiated populations along the NW Atlantic coast, between Virginia (37ºN) and the Bay of Fundy (45ºN) (Meehan 1984, 1985). Apart from many allele frequency differences, the NW Atlantic group expresses an extra *Mdh* (malate dehydrogenase) locus that has not been found in any other *M. balthica* population. Väinölä and Varvio (1989) suggested that units within the remaining *M. balthica* distribution range be given the status of semi-species. Many allozyme studies have been carried out to define the identities and distribution limits of the putative subspecies, but it can be difficult to discern global patterns from them, as all of the studies focus on part of the range and do not all employ the same set of loci. NE Pacific *M. balthica* are more closely related to NE Atlantic than to NW Atlantic relatives (Meehan 1989). Populations all along the western European coasts, from the south of France up to northern Norway are undifferentiated (Hummel *et al.* 1995, 1997). Populations across the Eurasian Arctic, on the contrary, are subdivided to some extent: one sample form NE Norway could be distinguished from NE Atlantic samples (Väinölä and Varvio 1989), as well as some samples from the White Sea, and Pechora Sea samples clearly differ from the NE Atlantic (Hummel *et al.* 1997, 2000).

Samples from the Baltic Sea are unusual (Väinölä and Varvio 1989) as they are more closely related to a Quebec sample than to any of the geographically closer samples (representatives of both the NE Atlantic and Aurasian Arctic groups; *e.g.* the British Isles and NE Norway). The distinctiveness of Baltic Sea *M. balthica* within the

European Atlantic and Eurasian Arctic samples was confirmed by Hummel *et al.* (2000, but see Meehan 1985). Moreover, Väinölä and Varvio (1989) report on evidence consistent with limited interbreeding in the transition zone between the Baltic Sea and North Sea, which suggests that the two groups are distinct species. Interestingly, Hummel *et al.* (2000) documented a time series in allozyme constitution within the Gulf of Gdansk, Poland, for 1995, 1997 and 1998. This series shows that, while in 1995 populations within the Gulf contained Baltic-specific allele frequencies, they had been replaced by North Sea populations by 1997. The authors suggest that this event may have been due to influx of saline North Sea water. These data are consistent with a hybridization zone in the southwestern Baltic Sea with limited interbreeding or introgression.

Biogeographic history of Macoma balthica

The family Tellinidae first appears in the fossil record in the early Cretaceous (Fig. 1, Pohlo 1982). *Macoma balthica* is of North Pacific origin, and was already present there during the Miocene (Meijer 1993). Many *Macoma* species still occur in the North Pacific (see *e.g.* Oldroyd 1924). The only *Macoma* spp. to make it through the Bering Strait opening approximately 3.5 million years ago (Mya) and across the Arctic into the Atlantic basin were *M. balthica*, *M. calcarea*, *M. obliqua* (probably conspecific with Pacific *M. incongrua*, Coan 1969) and *M. praetenuis*. The latter two species are now extinct in the Atlantic. It took a long time for *M. balthica* to disperse after first crossing the Bering Strait (to the north of which early Pleistocene fossils were found) to arrive in the North Sea basin some time into the Pleistocene (Spaink and Norton 1967, Norton and Spaink 1973). Because of this time-lag Meijer (1993) has suggested that *M. balthica* may have taken the long route to migrate to the NE Atlantic, i.e. following the Eurasian and not the North American coast. This is also substantiated by the fact that fossils from the time of dispersal are absent from Islandic records (Gladenkov *et al.* 1980).

Macoma balthica is one of many marine species that invaded the North Atlantic from the North Pacific after the Bering Strait opened 3.5 Mya (Vermeij 1991, Cunningham and Collins 1998). This event was followed by the Pleistocene glaciations, which probably influenced regional extinction and recolonization patterns. The latter events are thought to have had strong effects on extant patterns of molecular variation in marine taxa of the northern hemisphere (Cunningham and Collins 1998) and may also account for the situation in *M. balthica*.

Chapter 2 of this study confirms the suggestion of Väinölä and Varvio (1989) that Baltic clams in the Baltic Sea form a separate species. The NE Atlantic mitochondrial DNA haplotypes introgress into the Baltic Sea at least as far as Latvia. Haplotypes very similar to the Baltic types were identified from Cook Inlet, Alaska. One might hypothesize that the putative species complex diversified entirely since the Bering Strait opening, possibly in isolation during the glacial maxima of the Pleistocene. However, the estimated time to the most recent common ancestor

of all haplotypes predates the Bering Strait opening, which suggests that the putative species had started to diverge much earlier than that (see also discussion of chapter 2).

A difference between the mitochondrial DNA sequence data presented here and earlier allozyme studies on *M. balthica* is that a sample from the south of France is genetically distinct from more northerly samples (the British Isles, the Netherlands and Norway). In contrast, samples across this entire range proved to be

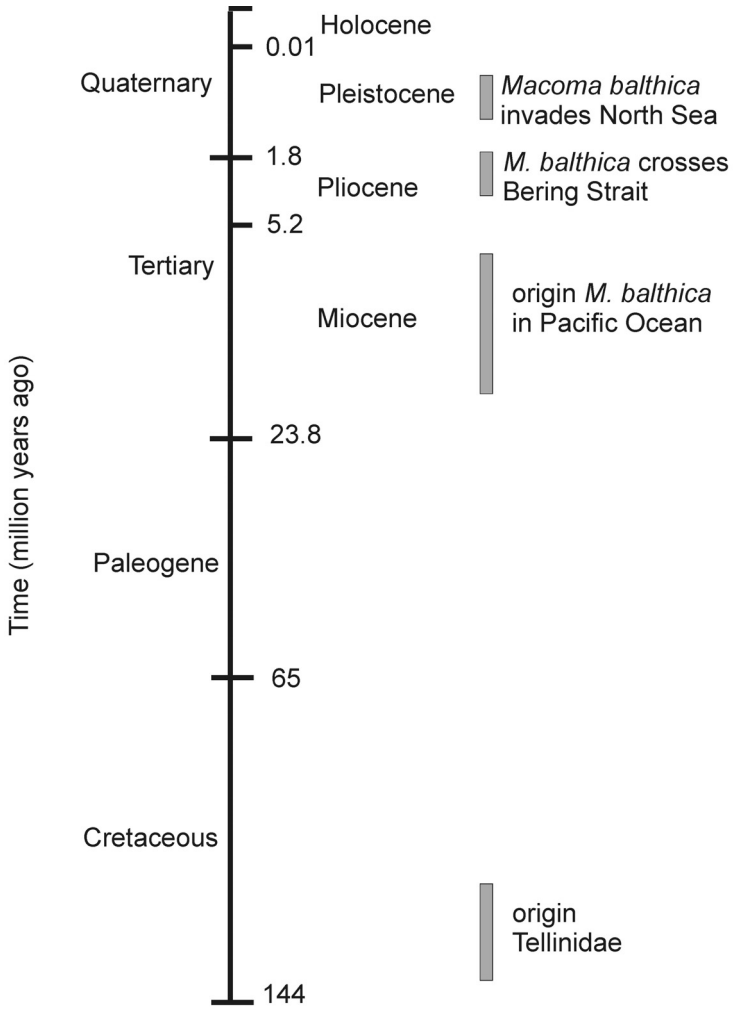

Fig. 1 - Geological time scale (in million years before present) and major biogeographic events in the evolution of *Macoma balthica* as inferred from the fossil record.

undifferentiated on the basis of allozymes (Hummel *et al.* 1995, 1997). This contrast between undifferentiated allozymes and a sharp break in mitochondrial haplotypes is reminiscent of the situation in American oysters *Crassostrea virginica* at the Atlantic coast of Florida and the Gulf of Mexico (Reeb and Avise 1990, Karl and Avise 1992). The case of the American oyster is only one example of a myriad of maritime species that demonstrate an abrupt genetic break between the Atlantic coast of Florida and the Gulf of Mexico (reviewed in Avise 2000). The reason for the phylogeographical similarity across multiple species has not been entirely unraveled, but is thought to result from a combination of historical influences and contemporary ecological effects on gene flow. While there is little evidence for dramatic historical-genetic partitions within the Florida-Gulf of Mexico region, the European history of advance and retreat of sea ice during the Pleistocene ice ages may have enabled historically isolated differentiation. This is corroborated by the coalescent-based timing of population subdivision for the splitting off of the French *M. balthica* population (chapter 2).

Neutrality of allozymes

With regard to the selective neutrality of allozyme polymorphisms, the general consensus is currently that while most alleles are neutral with respect to phenotype (*e.g.*, morphology, physiology), a few specific alternative alleles have direct functional consequences and associated fitness effects. Some thoroughly worked out examples are an *Adh* (alcohol dehydrogenase) polymorphism in *Drosophila* (*e.g.* Van Delden 1982, Aquadro *et al.* 1986), *Ldh* (lactate dehydrogenase) in the fish *Fundulus* (*e.g.* Place and Powers 1979, 1984) and *Lap* (leucine aminopeptidase) in the mussels *Mytilus* (*e.g.* Hilbish *et al.* 1982, Hilbish and Koehn 1985).

In *M. balthica*, there are indications that alleles of the loci *Idh*1 (isocitrate dehydrogenase) and *Lap* are under the influence of selection. Strong tidal level related clines for the locus *Idh*1 were observed at a White Sea location (Hummel *et al.* 1998b) and in the Gulf of Gdansk, Poland (Hummel *et al.* 2000). Although these clines were interpreted as evidence of selection in relation to temperature, they are also consistent with population subdivision, as *Idh*1 is the most strongly differentiated locus throughout the NE Atlantic and Eurasian Arctic region. Differential enzyme activity of *Akp*2 (alkaline phosphatase) genotypes was associated with tidal level and survival at high temperature in a population in Hudson Bay (Singh and Green 1986). Alleles at the *Lap* locus follow a latitudinal cline along the NE Atlantic coast and their non-neutrality was demonstrated by selection experiments with exposure to environmental polution (copper) (Hummel *et al.* 1995).

The non-neutrality of some allozyme alleles can be a drawback in their applicability to population genetic studies. If, for instance, the *Idh*1, *Akp*2 and *Lap* loci in *M. balthica* are under the influence of selection, they may overestimate subdivision among some populations and underestimate it among others. A more important disadvantage, however, is that the genealogy among the alleles is

unknown. This impedes phylogeographic studies, which are rapidly accumulating since DNA sequence data are gaining momentum (*e.g.* Avise 2000, chapter 2 of this thesis). An advantage of allozymes over DNA markers is the relative ease with which multilocus genetic data can be gathered. The large numbers of alleles found in bivalve allozymes are an added advantage as few loci will give much information (of the same level as microsatellites in other taxa), but the numbers of alleles can also be so high that it becomes increasingly hard to resolve all alleles to an adequate resolution (chapter 4 of this thesis). In conclusion, the usefulness of allozymes for population subdivision studies should not be underestimated but may become redundant once collecting multilocus DNA sequence data becomes equally cost-effective, and has the added advantage that gene genealogies can be estimated.

Selected genetic variation

Geographic patterns in phenotype of Macoma balthica

Studies of geographic variation in *M. balthica* have pointed out that phenotypic variation exists at a wide range of different spatial scales. The most obvious phenotypic polymorphism in *M. balthica* is its shell colour, which can be white, yellow or red with some variation in intensity (Cain 1988). The fraction of the shells that is white is relatively high (larger than about 70%) in the St Lawrence estuary, Quebec, and the Baltic Sea (Beukema and Meehan 1985, Väinölä and Varvio 1989). Among non-white shells, the fraction of yellow shells is elevated in France and The Netherlands, while coloured shells are more often red in northern Europe (both Atlantic and Baltic sites) (Beukema and Meehan 1985, Väinölä and Varvio 1989). Shell shape also varies with geographic location. The height/length and width/length ratios of the shell are on average lower in NW Atlantic *M. balthica*, i.e. shells are more oblong and less globose in America. Within the NW Atlantic and the NE Atlantic a trend of more oblong shells with increasing latitude occurs, and globosity of shells within the NE Atlantic region shows geographic structure but without a clear trend with latitude (Beukema and Meehan 1985). Burrowing depth of *M. balthica* was found to differ with tidal height (deeper burrowing at lower tidal levels, Hulscher 1973), between locations within the Dutch Wadden Sea (Zwarts and Wanink 1993, De Goeij 2001) and between the Wadden Sea and Wash, UK (Reading and McGrorty 1978). Sensitivity to stress from copper exposure is elevated near the southern limit of the European distribution range (Hummel *et al.* 1996b). Growth and development of *M. balthica* larvae are faster for offspring of French parents than for those of Norwegian parents (Drent 2002).

Some of the previously observed geographic patterns in phenotype have been shown to be plastic, for example translocated shells will burrow to depths typical of the new translocation site (Hulscher 1973). Other phenotypic patterns have a genetic basis, such as larval traits (Drent 2002) and shell colour polymorphism (Luttikhuizen and Drent, unpublished data).

The globosity of shells examined earlier by Beukema and Meehan (1985) was studied during my research for assessing spatial organisation of genetic variation (chapter 3 and box I of this thesis). Globosity of *M. balthica* shells at multiple locations in the western Dutch Wadden Sea and the adjacent North Sea was determined for three years. It was found that shells were more globose at the Wadden Sea sites in all years. In addition, there was significant variation between sites within each sea. In a common garden experiment using laboratory-reared *M. balthica* (chapter 3), the globosity difference between seas was found to have a heritable component, meaning that there is spatially organised genetic variation for this quantitative trait.

Quantifying the difference in spatial organisation of neutral molecular markers (measured as F_{st}) and of additive genetic variance for shell globosity (measured as Q_{st}) in *M. balthica* in the same geographic region showed that Q_{st} was much larger than F_{st} (chapter 3). This implies that neutral differentiation processes alone cannot account for the observed patterns, and that selection must play a role. One of the selection factors involved is selective predation by red knots *Calidris canutus*, who preferentially feed on less globose *M. balthica* but do not have access to bivalves in the (entirely subtidal) North Sea coastal zone (Box I).

One of the goals of the PIONIER pogramme, of which this project was a part, was to analyze evolutionary aspects of the trophic interactions between shorebirds and intertidal invertebrate organisms. A critical factor in understanding the effects predators may have on their prey's phenotype and *vice versa* is whether the involved traits of prey and/or predator have a genetic basis or are phenotypically plastic or flexible. The present study has shown that spatial variation of phenotypes in intertidal benthic communities can have a heritable genetic component in spite of considerable amounts of gene flow. Pelagic larval dispersal could be expected to randomly distribute prey genotypes over a large scale, but the influences of shorebird selection can affect this distribution on a small scale. This is noteworthy, as many intertidal invertebrates typically have high dispersal potential because of their pelagic larvae.

The combination of spatial organisation of non-neutral genetic variation through selection and the potential for high dispersal suggests large scope for non-equilibrium evolutionary interactions between predators and prey in intertidal foodwebs. This is corroborated by the large fluctuations in mean shell globosity of *M. balthica* at one site in the Wadden Sea (the Balgzand) over nineteen years (Box I). Future investigations should determine whether there is a direct link between the large fluctuations in shorebird numbers, their shifting diet preferences and the spatially and temporally varying morphology of their prey.

Molecular marker vs. quantitative trait differentiation; F_{st} vs. Q_{st}

Before further discussion of the mechanisms involved in the maintenance of shell globosity spatial differentiation in *M. balthica*, we should consider the general

issue of spatial organisation of non-neutral genetic variation. First, the link with neutral variation will be briefly touched upon, and subsequently, some theoretical aspects of the conditions for stable maintenance of non-neutral genetic polymorphism will be addressed.

Recently, reservations about the correlation between quantitative and molecular marker differentiation have arisen (*e.g.* Butlin and Tregenza 1998). The two might be expected to be correlated, because they share some of the forces that influence them; gene flow and genetic drift. The assumed correlation is an important issue, because of, for example, its applications in conservation biology. On the basis of molecular marker studies, taxonomic units are selected that are sufficiently distinct to justify biological conservation (*e.g.* Ryder 1986, Moritz 1994, Crandall *et al.* 2000). The same kind of studies are also used to guide decisions regarding translocations of individuals in attempts to rescue endangered populations or re-establish extinct ones (Storfer 1999).

This issue can be quantified by using F_{st} as a null hypothesis of no selection for Q_{st} (Wright 1951, Spitze 1993, see also chapters 1 and 3). Two recent reviews compiled published F_{st}/Q_{st} contrasts and observed that Q_{st} is typically larger than F_{st}, which suggests that selection plays an important role in population differentiation of quantitative traits. While the first study found a significant positive relationship between F_{st} and Q_{st} using data for 17 species (Merilä and Crnocrak 2001), the second (McKay and Latta 2002) found the correlation to be poor among 29 species.

In my view we must be cautious when interpreting these relationships between Q_{st} and F_{st}, as special care was not always taken to randomly select quantitative traits. As in the present study, it is possible that some traits were chosen on the basis of their phenotypic differentiation. As most quantitative traits tend to have a positive heritability, this could produce first of all the observation that Q_{st}'s are larger than F_{st}'s and, second, the poor correlation between Q_{st} and F_{st}. The F_{st}/Q_{st} contrast presented in this thesis (chapter 3) forms a good example of a comparison that was not chosen randomly: first, the morphological differentiation in shell shape was noted, and subsequently, Q_{st} was estimated.

These reservations notwithstanding, the large discrepancy between F_{st} and Q_{st} indicates that selection plays a role in maintaining the morphological pattern of more globose *M. balthica* in the Dutch Wadden Sea compared to the adjacent North Sea. The F_{st}/Q_{st} contrast for *M. balthica* contributed by the present study has a lower F_{st} than any of the species featured in McKay and Latta's (2002) review (Fig. 2).

The issue of the maintenance of genetic variation by differential selection in the face of gene flow has been studied theoretically, both for the single locus case (*e.g.* Levene 1953, Dempster 1955, reviews in Felsenstein 1976, Hedrick *et al.* 1976, Hedrick 1986) and for multilocus trait expansions (*e.g.* Slatkin 1978, Phillips 1996). One of the main conclusions from theory has been that stable maintenance of polymorphism in a random mating population inhabiting niches with different selection pressures is not very robust. The difference in selection pressures needs to

be rather high and the relative sizes of the niches need to be rather similar for stable equilibrium to occur (*e.g.* Levene 1953, Maynard Smith and Hoekstra 1980). Factors that can expand robustness of these models include reduced gene flow (Brown and Pavlovic 1992, Meszéna *et al.* 1997), habitat preference (Templeton and Rothman 1981, Rauscher 1984, Hedrick 1990) and local mating (Hoekstra *et al.* 1985, Goldstein and Holsinger 1992, Lenormand 2002). All of these can influence shell shape differentiation in *M. balthica*, and the spatial arrangement of selective genetic variation in marine invertebrates with pelagic larvae in general. Although reduced gene flow was not detected at the smaller scale studied in this thesis (North Sea - Wadden Sea, chapter 3), it certainly plays a role at the larger, European scale (chapter 2). The possible selection pressures involved in globosity of *M. balthica* shells have been dealt with in the previous parapraph and in chapter 3 and box I. In the following section, local mating and habitat preference are considered in more detail.

Possible roles of habitat preference and local mating for the spatial maintenance of variation

Including genotype-specific habitat preference in models of the maintenance of genetic variation by spatially varying selection may increase the scope for stable maintenance of polymorphism (*e.g.* Templeton and Rothman 1981, Rauscher 1984, Hedrick 1990).

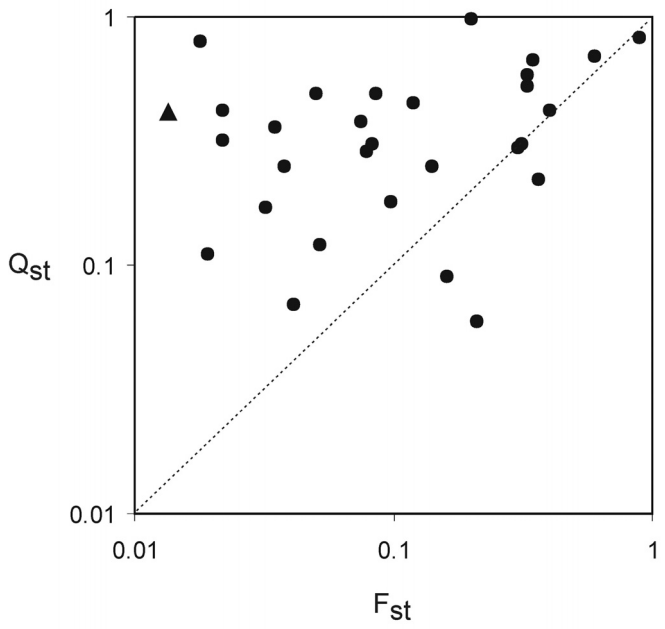

Fig. 2 - Spatial differentiation in genetic variation for quantitative traits (Q_{st}) versus neutral molecular markers (F_{st}) for a range of species. Circles are from the review by McKay and Latta (2002), the triangle is from *M. balthica* data presented in this thesis.

Habitat preference initially does not seem a likely phenomenon in species with drifting pelagic larvae, as the speed of currents is orders of magnitude larger than larval swimming speed. However, many traits have evolved that enable pelagic larvae to have some control over where they settle, for example body density for buoyancy, mucus strands for increased drag, and delayed metamorphosis (for a review see Young 1995). Experimental evidence also suggests that pelagic larvae actively select substrate type for settlement (Ahn *et al.* 1993, Snelgrove *et al.* 1999).

The habitat used by *M. balthica* for settlement is likely to be non-random. Genotype specific settlement in *M. balthica* may take place during for instance secondary migration, which is accomplished with the use of a mucoid thread in its first winter, taking the individuals away from their high intertidal nursery areas into the range of habitats they will inhabit as adults (Beukema 1993). Beukema and De Vlas (1989) found that Baltic clams of smaller length take longer to sink to the bottom. They predicted that, because of this, smaller shells will migrate further and validated their prediction by showing that postmigratory Baltic clams which settled in

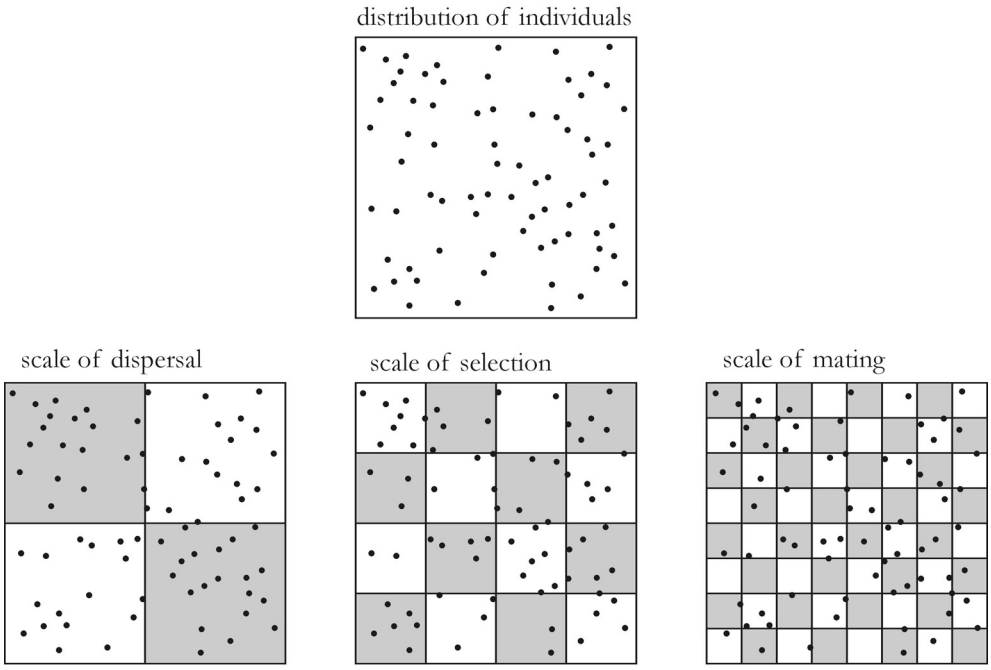

Fig. 3 - How the spatial scales of selection, dispersal and fertilization may interact in marine broadcast spawning invertebrates to result in local differentation of non-neutral genetic traits. Individuals situated in the same square are part of the same dispersal, selection or mating group. Individuals will disperse over large scales, selection factors will vary over a smaller scale, and mating takes place at the smallest scale. Spatially varying selection and local mating may aid in differentiating genetic variation, while large-scale dispersal may homogenize it.

the North Sea off the coast of Terschelling, are smaller as distance from the source (the Wadden Sea) increases. It is conceivable that, in addition to shell length, shell shape also influences the time it takes for a migrating shell to sink. A difference in shell density is associated with *M. balthica*'s globosity differences (Yohannes Afeworki, pers. comm.), which could influence its sinking speed. In this way, shape classes of Baltic clams might be sorted non-randomly over habitats during their secondary migration.

In most of the models addressing the maintenance of genetic variation by spatially varying selection, mating is globally random, and if not, its non-randomness is often a side-effect of restricted gene flow (see *e.g.* Levene 1953, Maynard Smith and Hoekstra 1980, Bush 1993). Levene (1953) was one of the first to study this topic and stated that: 'if [...] there is a tendency for mating to occur within a niche rather than at random over the whole population, conditions will be more favourable for equilibrium'. However, Strobeck (1974) proved this supposition wrong, at least for the Levene model (one locus, two alleles, two niches). The fact that the scales of dispersal and mating are not usually considered separately probably results from most of the models being inspired by studies on animal species in which these scales are indeed similar, such as vertebrates or the small invertebrates that motivated Bush (1993). A few later studies did find that local mating increases the robustness of the amount of genetic variation that can be maintained (Hoekstra *et al.* 1985, Goldstein and Holsinger 1992). Local mating after selection will result in positive assortative mating because individuals who survive to reproduce have relatively similar genotypes. This will increase the variance in fitness and therefore enhance the efficiency of selection (Lenormand 2002). In this way, local mating can contribute to the maintenance of genetic variation. The level to which it does will most likely depend on the type of selection (*e.g.* hard or soft, truncation selection) and the genetic architecture considered (*e.g.* single-locus versus multilocus) and requires additional theoretical consideration.

It can be concluded from this thesis (chapters 5 and 7) and other studies (*e.g.* Pennington 1985, Levitan 1991, Babcock *et al.* 1994, Levitan and Young 1995, Levitan 1998) that mating in marine sessile invertebrates with external fertilization is a small-scale process (in the order of meters). The idea that sperm limitation may be an active selection force in the field for *M. balthica* is corroborated by the negative correlation between egg size and adult density, suggesting adaptive tuning of egg size to sperm concentration (chapter 5), and by the fact that polyspermy is not efficiently blocked (chapter 8). The probability of two individuals becoming sexual partners will decrease rapidly with increasing distance between them. Pelagic larval dispersal, on the other hand, is of a larger scale. As mating takes place during the adult stage, i.e. after selection, mates will have a higher genetic correlation than random pairs of individuals. This is effectively positive assortative mating, which may contribute to the maintenance of genetic variation by spatially differential selection (Fig. 3).

Questions and future directions of research

The outcomes of my work on *M. balthica* raise many questions and directions for further research.

- Do multiple marine invertebrate species display similar geographic distribution patterns of DNA sequence variation? What do these codistribution patterns tell us about the influence of historical biogeographic events on contemporary genetic differentiation?
- Why are historical signatures of biogeographic events on population differentiation in marine broadcast spawning invertebrates not erased by contemporary gene flow?
- Is spatial differentiation of quantitative traits even more independent of spatial differentiation of neutral genetic traits in marine broadcast spawners than in other taxa? More studies are needed that estimate additive genetic variances for quantitative traits within and between populations of marine broadcast spawners.
- Is selection pressure due to sperm limitation a cause of the correlation between egg size and adult density in *M. balthica*? Translocation experiments in the field could answer this question.
- To what extent can local mating theoretically contribute to the stable maintenance of genetic variation with spatially differential selection under various selection models and genetic architectures?

Summary of main conclusions

- *Macoma balthica* from the Baltic Sea and from the North Pacific are best considered as a separate species. There is substantial introgression of mitochondrial DNA from the NE Atlantic into the Baltic Sea, but less so than in mussels (*Mytilus* spp.).
- There is substantial population subdivision within the European Atlantic part of the distribution range of *M. balthica*, as inferred on the basis of cytochrome-*c*-oxidase I mtDNA sequences, which contrasts with its high potential for pelagic larval dispersal.
- There is little evidence for population subdivision within *M. balthica* populations from the western Dutch Wadden Sea and adjacent North Sea, as inferred from allozymes and cytochrome-*c*-oxidase I mtDNA sequences.
- Additive genetic variance for a morphological trait (globosity of shells) is strongly differentiated between the western Dutch Wadden Sea and the adjacent North Sea.
- Shells of *M. balthica* in the western Dutch Wadden Sea are more globose than those in the adjacent North Sea. Selection contributes to this geographic pattern, and one of the selection factors involved is preferential predation on less globose shells by red knots *Calidris canutus*.

- The large numbers of allozyme alleles in marine bivalves hamper their resolution, particularly in heterozygotes for alleles with similar electrophoretic mobilities. The often reported heterozygote deficiencies in allozyme studies of marine bivalves may partly result from biased scoring of homozygotes on starch gels.
- The validity of the hypothesis that under increased sperm limitation with external fertilization, females which produce larger eggs have increased fitness, depends only on the shape of the fertilization kinetics model and not on details of the trade-off between size and number of offspring. The hypothesis is valid for two previously published fertilization models.
- Egg size in *M. balthica* displays significant spatial variation in the western Dutch Wadden Sea and adjacent North Sea, and is strongly correlated with local adult density, which may be interpreted as a measure of sperm limitation.
- Restrictions to the relationship between zygote size and survival, which are necessary to explain the origin of anisogamy, are not biologically unreasonable.
- Spawning of *M. balthica* in the field is not highly synchronized; the spawning season lasts about two months and happens over the same time period in the western Dutch Wadden Sea and in the adjacent North Sea. This bet-hedging strategy is consistent with the idea of sweepstakes chances of larval survival.
- Successful external fertilization in *M. balthica* depends on sperm concentration and sperm age. Spawning is induced by the presence of female spawned products. Fertilization in *M. balhica* is a small-scale process.
- More than one spermatozoon can fertilize one egg in *M. balthica*. One sperm fuses with the egg pronucleus to form a diploid cell line. Additional sperm can form haploid cells that survive and multiply in young embryos.

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SUMMARY

Not all organisms are the same; some are more similar than others. A fruitfly is definitely more like a housefly than an elephant. A fruitfly has arguably more in common with another fruitfly than a housefly. A great dane is perhaps more similar to a chihuahua than to a wolf. How do Mendel's wrinkly peas compare to the wrinkly bacterial colonies of Rainey and Travisano?

Organisms that are considered to belong to the same species, are not all the same and they are not even equally different from each other. In addition, the way in which they are grouped based on similarities changes depending on the traits examined. Two main origins of similarity can be distinguished: shared ancestry and shared circumstances. In most species, the two co-vary. Individuals that are closer to each other in space, usually have elevated coancestry and also live under more similar circumstances. This is because the distance between parents and offspring tends to be smaller than the distance between randomly chosen individuals, and because similar conditions tend to be closer in proximity than different conditions. It is therefore difficult to tease apart shared ancestry and shared circumstances.

Phenotypic similarities come in two kinds: those that are partially based on genetic differences and those that are not. Genetic variation is inherently heritable and without it, evolution by means of natural selection could not occur. It is therefore important to understand the origin and maintenance of heritable genetic variation. The main theme of the research presented here is non-random spatial arrangement of genetic variation. Spatially differentiated genetic variation has two sides, it can maintain variation for selection and it can also be the result of selection.

This thesis focuses on broadcast spawning marine invertebrates as it is generally assumed that in these animals, shared coancestry and shared circumstances are correlated to the smallest extent possible. The adults are sedentary or semi-sedentary and release their eggs or sperm freely into the water, so that fertilization is external. The ensuing larvae live a mobile life, drifting with the water from hours to months, depending on the species. By the time they settle down for the sedentary phase of their lives, they are thought to have been shuffled so thoroughly that most of the correlation between distance and coancestry will have been lost. The spatial autocorrelation in circumstances is, however, essentially not different for marine broadcast spawners than for other organisms. Given that there truly is little distance-coancestry correlation, one may propose that most locally elevated phenotypic similarities are due to shared circumstances.

Levels of genetic differentiation tend to decrease with increasing amounts of genetic exchange. This is true for both selectively neutral and non-neutral genetic variation. Theory predicts that even low levels of exchange will prevent diversification by genetic drift, with one migrant per generation as a rule-of-thumb. Spatial heterogeneity in selection pressure is regarded as one of the determinants of non-neutral genetic differentiation. This process, too, however, is hindered by

genetic exchange and, theoretically, the scope for non-neutral genetic differentiation in the face of moderate to high gene flow is deemed low.

Recently, empirical data on divergence-with-gene-flow have begun to accumulate. The goal of the present study was to test the prediction of little divergence under high gene flow in a marine broadcast spawner, as they are regarded as being on the extreme end of dispersal potential. The Baltic clam, *Macoma balthica* (L.) (Bivalvia: Tellinidae), was chosen as the model species. It is a common burrowing bivalve of northern hemisphere shores, where it inhabits intertidal and shallow subtidal sandy to muddy sediments. The larvae spend two to five weeks drifting in the pelagic zone. After initial settlement and secondary migration as juveniles, the adults are sedentary.

Spatial genetic variation

The spatial arrangement of neutral and non-neutral genetic variation in *M. balthica* was studied at several scales. The main conclusions were that the realized gene flow is lower than expected and that non-neutral genetic variation can be much more strongly differentiated than neutral variation at the same scale. One of the spatially heterogeneous selection pressures involved in the latter pattern appears to be selective predation.

Neutral genetic variation, in the form of mitochondrial DNA (mtDNA) sequences, was compared for ten sites across the European distribution of *M. balthica*, as well as for reference individuals from Alaska. The level of population structuring was found to be very strong. Effective levels of genetic exchange between populations a few hunderds of kilometers apart can be virtually nonexisting in the absense of obvious barriers to dispersal. Fossil records have shown that the genus *Macoma* originated in the Miocene at the latest in the Pacific Ocean and spread to the Atlantic Ocean after the opening of the Bering Strait 3.5 million years ago. Earlier suggestions that the Baltic clams in the Atlantic actually comprise more than one species were corroborated by the mtDNA data. With the use of coalescent theory it was inferred that extensive population divergence has taken place among eastern Atlantic *M. balthica* during the Pleistocene and that isolated populations of *M. balthica* have survived the last glacial maximum (approximately 18,000 years ago).

At the smaller spatial scale of the western Dutch Wadden Sea and adjacent areas of the North Sea, spatial arrangement of neutral and non-neutral genetic variation was contrasted. At this scale, neutral genetic variation, in the form of mtDNA sequences and five allozyme loci, was essentially undifferentiated, with F_{ST} estimates ranging from 0.000 to 0.022. Phenotypic variation in shell morphology, presumably non-neutral variation, was not randomly distributed over the area. Shell globosity, *i.e.* width relative to length, was on average higher in the Wadden Sea than in the North Sea and consistently so for three years (1998-2000). The laboratory-reared offspring of Wadden Sea parents were still more globose than those of North Sea parents in a common garden experiment. Heritability of globosity across

populations was estimated to be 0.23. Q_{ST}, a quantitative measure of differentiation of additive genetic variance, was estimated as 0.42. These data imply that, first, there is a role for selection in maintaining globosity variation, and second, that non-neutral differentiation can be considerable even when neutral differentiation is virtually absent.

During the allozyme data analyses it became apparent that fewer heterozygotes were found than expected on the basis of Hardy-Weinberg equilibrium. This is a common feature of bivalve allozyme studies. Bivalve allozyme loci tend to be more variable than they are in other species, with numbers of alleles in the order which is typical of microsattellite loci in other taxa. The hypothesis was raised that perhaps misscoring of individuals that are heterozygous for alleles with very similar electrophoretic mobilities contributes to observed heterozygote deficits. The *M. balthica* allozyme data support this hypothesis. A literature survey showed that it might be worthwhile to re-analyze published data because there are indications that misscoring may not only be restricted to the present data set.

One of the spatially heterogeneous selection pressures that may be responsible for the globosity differences is selective predation by shorebirds. Red knots, *Calidris canutus*, were offered a mixture of *M. balthica* from the North Sea and the Wadden Sea in experiments in an indoor aviary with an artificial mudflat. The birds displayed a strong preference for the flatter bivalves, possibly because they are easier to swallow. Wild red knots can only feed on *M. balthica* in the Wadden Sea, where the bivalves are relatively globose.

External fertilization

Theoretically, the scope for the stable maintenance of genetic variation by spatially heterogeneous selection is not large. Especially when genetic exchange is ongoing, as in the case of the globosity example, the selection pressures that need to be invoked are high. One of the factors that may facilitate such stable maintenance is local mating, although the issue is controversial and not well studied. Post-selection local mating may increase overall homozygosity and the efficacy of selection in the next generation. While effective dispersal of larvae was already found to be of a smaller scale than expected, experiments with *M. balthica* and other broadcast spawners show that fertilization is a micro-scale process.

Species with external fertilization, especially those that are sessile and are not capable of actively seeking out sexual partners, run the risk of spawned gametes remaining unfertilized. Sperm limitation has been demonstrated in many field studies. While its numerical effects are generally not thought to be high enough in the field to influence population dynamics, it may result in selection pressures that impact evolutionary dynamics.

If sperm is limiting during external fertilization, female fitness may depend on egg size because larger eggs are a larger target for sperm. On the other hand, zygote survival probability depends on egg size as well and producing larger eggs also

implies making fewer of them. Natural egg size variation of *M. balthica* was assessed at sites in the northeast of The Netherlands and was found to be strongly negatively correlated with adult density as a proxy for sperm concentration. This is in accordance with theoretical predictions that were generalized with respect to earlier, more specific, models. Thus it would seem that *M. balthica* tunes the size of its eggs to expected levels of sperm limitation, a hypothesis that can be tested with transplantation experiments.

Survival chances for a zygote depend on its size, and zygote size depends on egg size. It is generally believed that larger zygotes are more likely to survive. Sperm in general do not contribute to zygote size. In fact, there are many differences between the sexes but none is so universal as the size difference between the two types of gametes ('anisogamy'). The question whether disruptive selection on gamete size could explain the transition from isogamy to anisogamy, has been the subject of some controversy. It is shown in this thesis that biologically realistic survival curves are well capable of explaining anisogamy.

If gametes lived forever, gamete limitation would not be possible. However, spawned sperm suspensions of *M. balthica* lose their viability after a few hours. Eggs appear to remain viable for longer times, but more experiments are needed to confirm this. Laboratory tests of the relationship between sperm concentration and fraction of eggs fertilized showed that below 10^5-10^6 sperm/ml, more than 50% of *M. balthica* eggs remain unfertilized. On average, males in the laboratory spawned $3.4 \cdot 10^8$ spermatozoa. The well-mixed volume in which males are able to fertilize 50% of the eggs would thus be 1 liter, suggesting that sperm limitation may indeed be prevalent in the field.

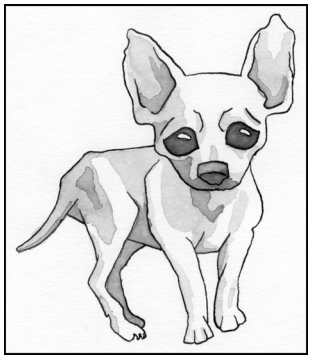
Polyspermy was found to be very common in laboratory produced embryos of *M. balthica*. A possible block to polyspermy, that works so efficiently in many species, is certainly not fast-acting, which fits the idea that sperm superabundance is not the rule. Surprisingly, young embryos do not appear to experience adverse effects from polyspermy. The superabundant sperm can despiralize into a functional haploid nucleus (n=19) and start to divide normally. The fate of the haploid cells in later stages of development remains open for future investigations.

If gamete encounter rate is important, it may be expected that local spawning happens in a short timeframe. Because the crossing of a threshold temperature has been suggested as the cue to spawning for *M. balthica*, the hypothesis was tested that, within sites, there is a single spawning event per year, and that individuals in subtidal sites spawn later in spring than those in intertidal sites. Neither of these predictions was confirmed, as in a field study, within-site spawning at two intertidal and two subtidal sites in The Netherlands was spread over two months and no difference was detected between tidal levels. These results suggest that bet-hedging in relation to variable and unpredictable larval survival prospects are important enough factors in this species for the animals to take the risk of gamete limitation.

The results presented in this thesis demonstrate how neutral differentiation is not necessarily a good predictor of non-neutral genetic differentiation. Estimating neutral molecular marker differentiation is therefore not a good test of hypotheses concerning the genetic basis of phenotypic patterns in the field. Theoretical understanding of how neutral and non-neutral differentiation can deviate to such an extent is limited. The fact that fertilization is a small scale process may be a contributing factor. Finally, these data imply that harvesting from particular (types of) areas might decrease intraspecific marine biodiversity.

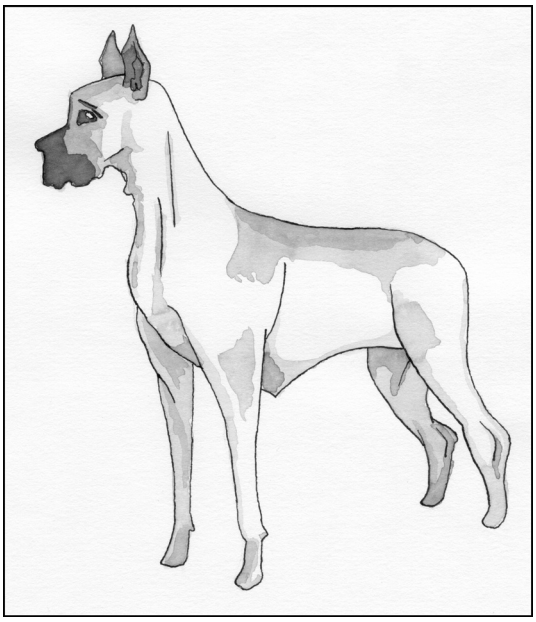
SAMENVATTING

Niet alle organismen zijn hetzelfde. Sommige lijken meer op elkaar dan andere. Zo zal iedereen het erover eens zijn dat een fruitvliegje meer op een huisvlieg lijkt dan op een olifant. Ook zal de stelling dat het ene fruitvliegje meer op een ander fruitvliegje lijkt dan op een huisvlieg, weinig stof doen opwaaien. Maar heeft een Deense Dog meer gemeen met een Chihuahua dan met een wolf, of andersom? Het antwoord is afhankelijk van waar je naar kijkt. Uiterlijk lijkt een Deense Dog misschien meer op een wolf, maar uitspraken over genetische kenmerken of gedragseigenschappen zijn minder eenvoudig.



Chihuahua

Laten we gelijkenissen indelen in twee groepen, gebaseerd op de oorsprong van de gelijkenis: gelijke afstamming en gelijke omstandigheden. De meeste van de genetische gelijkenissen onder honden en wolven zijn het resultaat van gelijke afstamming. Honden en wolven stammen af van iets wat niet veel van de moderne wolven verschilde. Voor de uiterlijke kenmerken en de gedragseigenschappen ligt het ingewikkelder. Ook daar speelt gelijke afstamming mee, maar bovendien een geschiedenis van gelijke omstandigheden. Het gedrag van volwassen honden bevat adolescente elementen die wolven kwijtraken bij het ouder worden, zoals tolerantie ten opzichte van het toelaten van nieuwe groepsleden. Zowel Deense Doggen als Chihuahua's zijn hierop door mensen geselecteerd - gelijke omstandigheden dus.

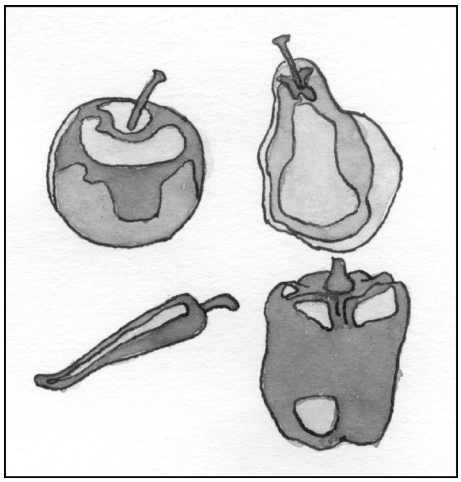


Deense Dog

Het is niet altijd eenvoudig om gelijke afstamming en gelijke omstandigheden achteraf van elkaar te onderscheiden. Dat komt omdat ze in de historie vaak samen opgaan; ze covariëren. Het is niet bekend of wolven één keer of meerdere keren tot honden gedomesticeerd zijn. Het is goed mogelijk dat een gezamenlijke voorouder van de Deense Dog en de Chihuahua zijn tolerantie tegenover vreemden al had. In dat geval is de afstamming en de omstandigheid dezelfde. In de natuur veroorzaakt de

factor ruimte vaak een covariatie in gelijke afstamming en gelijke omstandigheden. De afstand tussen ouders en nakomelingen is vaak kleiner dan die tussen willekeurige individuen, en ook de overeenkomst in omstandigheden is vaak groter wanneer plekken zich dichter bij elkaar bevinden.

Laten we vervolgens ook verschillen tussen organismen indelen in twee groepen: verschillen die gedeeltelijk op genetische verschillen berusten, en verschillen die dat niet doen. Dit is een belangrijk onderscheid, omdat genetische eigenschappen erfelijk zijn en niet-genetische eigenschappen niet. Zonder genetische verschillen zou evolutie door middel van natuurlijke selectie niet mogelijk zijn. Nog een keer het honden-voorbeeld: je kunt wel selecteren op vriendelijkheid, maar als vriendelijkheid niet erfelijk is, dan wordt je ras nooit vriendelijker.



Zonder genetische variatie zou plantenveredeling onmogelijk zijn

Het is dus van belang om te begrijpen hoe genetisch variatie ontstaat en hoe deze behouden blijft. Eén van de factoren die hierbij een rol speelt is ruimte. Dit proefschrift gaat over de verspreiding van genetische variatie in de ruimte. Het samenspel tussen ruimte en genetische variatie kan van twee kanten bekeken worden. Aan de ene kant kan genetische variatie het gevolg zijn van ruimtelijke patronen in natuurlijke selectie, en aan de andere kant kan ruimtelijke struktuur in genetische variatie ervoor zorgen dat er erfelijke variatie blijft voor natuurlijke selectie.

Zeedieren als model

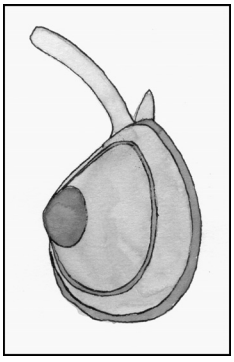
In mijn onderzoek heb ik ongewervelde zeedieren als model gebruikt. De meest interessante onder de ongewervelde zeedieren zijn diegene waarbij de bevruchting buiten het lichaam, in het water, gebeurt. Ze laten het sperma en de eieren het water in stromen en hebben er verder geen omkijken meer naar. Mocht het tot een bevruchting komen, dan ontstaat er een larve, die enkele uren tot soms meer dan een jaar in het water blijft zweven. Wanneer ze zich uiteindelijk ergens op een vaste plek vestigen, zouden ze, als een hand goed geschudde kaarten, willekeurig over de ruimte verdeeld zijn. Deze levenswijze is voor de evolutiebiologie zo interessant, omdat die er, in theorie, toe leidt dat gelijke afstamming en gelijke omstandigheden zo min mogelijk covariëren. Dat zou het makkelijker moeten maken de twee los van elkaar aan het werk te zien.

De hoeveelheid aan genetische verschillen die binnen een zich goed mengende groep individuen behouden kan blijven, is beperkt. Door mutaties ontstaan nieuwe

verschillen, die door toevalsprocessen, genetische drift, algemener kunnen worden of uitsterven. Ruimtelijke patronen in de verspreiding van individuen zorgen ervoor dat er meer genetische variatie behouden kan blijven. Dit komt doordat de toevalsprocessen in gescheiden populaties dan onafhankelijk van elkaar zijn. Bovendien geldt voor genetische variatie die onder invloed van selectie staat, dat door ruimtelijke patronen in selectieomstandigheden extra grote hoeveelheden variatie kunnen blijven bestaan. Nu wordt gedacht, dat al een heel kleine mate van genetische uitwisseling tussen populaties de behoudende werking van ruimtelijke selectiepatronen opheft. De selectie zou buitengewoon sterk moeten zijn om dit te voorkomen. Eerder vroegen onderzoekers zich al af, hoe het dan mogelijk is, dat er toch zoveel verschillende soorten zeedieren bestaan. Ik vroeg mij af, of het zo is dat in zeedieren genetische variatie, die onder invloed van ruimtelijke selectiepatronen staat, in dezelfde mate willekeurig verdeeld is als genetische variatie die selectief neutraal is.

Het nonnetje

De soort waar ik mee gewerkt heb is het tweekleppige schelpdier *Macoma balthica*, het nonnetje. Op het NIOZ (Koninklijk Nederlands Instituut voor Onderzoek der Zee), aan de RUG (Rijksuniversiteit Groningen), en elders wordt deze soort al decennia als modelsoort gebruikt voor marien-biologisch en evolutionair-biologisch onderzoek. Het is daarom niet verwonderlijk dat het NWO-PIONIER project (Nederlandse Organisatie voor Wetenschappelijk Onderzoek; Persoons-gerichte Impuls voor Onderzoeksgroepen met Nieuwe Ideeën voor Excellente Research) van Theunis Piersma (NIOZ/RUG) over evolutionaire wapenwedlopen tussen predatoren en pooidieren op het wad, waar mijn onderzoek een deel van was, het nonnetje als één van de modelsoorten koos.



Het nonnetje

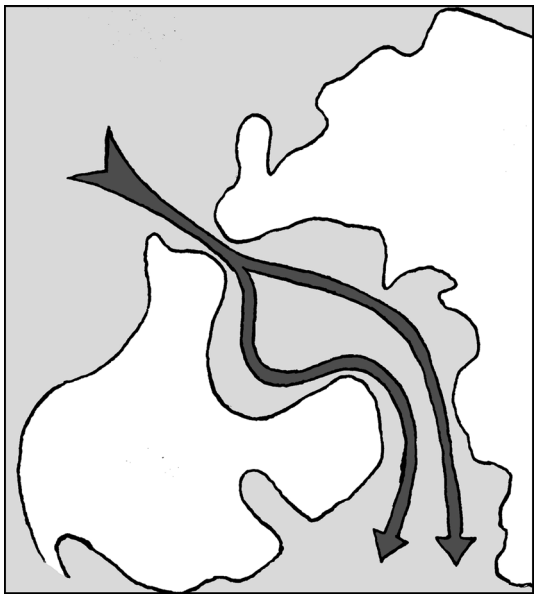
Volwassen nonnetjes leven ingegraven in modder of zand. Ze paaien in het voorjaar, en na de externe bevruchting zweven de larven drie tot vijf weken in het water, waarna ze zich vrij hoog op wadplaten vestigen. In hun eerste winter migreert een deel van hen nog een keer, naar zand- en modder-bodems dieper in het water. Ze worden in hun geheel gegeten door bijvoorbeeld wadvogels, zoals kanoeten *Calidris canutus*, en vissen. Krabben kraken nonnetjes eerst en eten dan de zachte lichaamsdelen op. Slakken als tepelhoorns raspen een gaatje in de schelp om bij de inhoud te komen. En ook zijn er predatoren, bijvoorbeeld jonge schollen, die grazen op de siphonen die nonnetjes uit het wad steken.

Nonnetjes zijn rond het hele noordelijk halfrond te vinden. De soort is ontstaan in het Mioceen (5 tot 24 miljoen jaar geleden) in de Stille Oceaan. Toen de Bering Straat 3,5 miljoen jaar geleden doorbrak verspreidden ze zich samen met veel andere mariene organismen via het noordpoolgebied naar de Atlantische Oceaan.

Fossielen getuigen dat ze in het Pleistoceen (vanaf 1,8 miljoen jaar geleden) in West-Europa aangekomen zijn.

Ruimte en genetische variatie

De verspreiding van neutrale genetische variatie in nonnetjes vertelt maar voor een deel hetzelfde verhaal als de fossielen. Uit eerder onderzoek bleek al, dat de soort uit verschillende groepen bestaat, die misschien wel als aparte soorten gezien moeten worden. Door naar DNA uit mitochondriën (onderdelen van de cel die voor de energiehuishouding zorgen en hun eigen DNA hebben) te kijken, vond ik dat nonnetjes in de Oostzee sterk verschillen van hun soortgenoten in de rest van Europa. Hun gemeenschappelijke voorouder leefde meer dan 10 miljoen jaar geleden. Dat is vreemd, want de Oostzee is in de laatste ijstijd helemaal dichtgevroren en bestaat in zijn huidige vorm pas ongeveer 8000 jaar. Opvallender nog is dat de Oostzeenonnetjes genetisch veel meer lijken op nonnetjes uit de Stille Oceaan (het zuiden van Alaska) dan op de andere Europese nonnetjes. Alles wijst erop dat de Atlantische Oceaan niet één maar meerdere keren gekoloniseerd is door nonnetjes vanuit de Stille Oceaan. Hoewel er ook aanwijzingen zijn, dat de verschillende groepen genetische uitwisseling met elkaar hebben, is die uitwisseling blijkbaar niet groot genoeg om de ruimtelijke structuur teniet te doen.



Na de opening van de Bering Straat, 3,5 miljoen jaar geleden, verspreidden veel mariene organismen zich van de Stille naar de Atlantische Oceaan

Iets soortgelijks zien we als we op een iets kleinere schaal kijken, binnen Europa zonder de Oostzee. Ook daar is de genetische variatie sterk gestructureerd. Frankrijk, bijvoorbeeld, herbergt een populatie nonnetjes die zich minstens 110.000 jaar geleden afsplitste van de rest. Dit strookt niet met het heersende beeld over de geschiedenis van het leven in de ondiepe kustzone in Europa. De laatste ijstijd had zijn maximum 18.000 jaar geleden, en alles wat in de kustzone leefde zou zijn samengedrukt rond de Franse en Zuid-Engelse kust. Het is onduidelijk hoe de Franse populatie van nu zich toen als eenheid heeft kunnen handhaven terwijl er heel veel genetische uitwisseling via de larven kon plaatsvinden. Misschien waren er geïsoleerde refugia in het noorden of ergens anders, of misschien leiden migrerende

larven toch niet tot veel genetische uitwisseling. Per slot van rekening valt er ook een goede boterham te verdienen met het gestructureerd schudden van speelkaarten.

Pas op de kleinste schaal die ik bekeken heb bleek de mitochondriale genetische variatie vrijwel willekeurig verspreid te zijn: in de Waddenzee en de aanliggende gedeelten van de Noordzee. Enzymvariatie liet hetzelfde beeld zien. De mitochondriale varianten en de enzymvarianten hebben echter geen relatie met het functioneren van de dieren. Genetische varianten die dat wel hebben, en waarop selectie in kan grijpen, bleken wel degelijk een ruimtelijk patroon te vertonen.

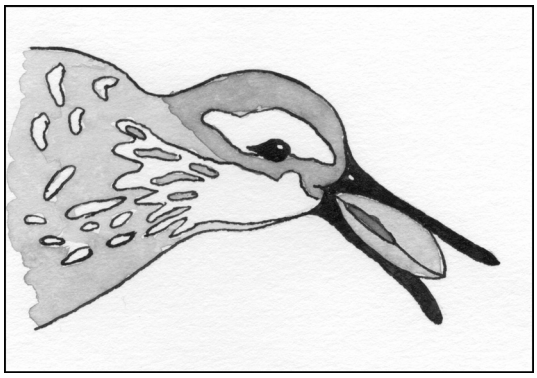
Oostzeenonnetjes lijken meer op nonnetjes in zuidelijk Alaska dan op hun Europese verwanten

Bolle en platte schelpen

In de Waddenzee zijn de schelpen van nonnetjes boller dan in de Noordzee. Dit gegeven op zich hoeft niet te betekenen dat ook genetische variatie voor schelpvormen ruimtelijk gestructureerd is. Het verschil tussen de twee zeeën zou ook kunnen komen door verschillen in omstandigheden. Om erachter te komen of er genetica in het spel is, moesten nonnetjes in het laboratorium gekruist en opgekweekt worden. Dit is geen sinecure, omdat de minuscule zwevende larven erg kwetsbaar zijn en vatbaar voor infecties. Met hulp van mede-onderzoekers Pieter Honkoop en Jan Drent is het gelukt om uit sperma en eieren van nonnetjes larven te krijgen, de larven te laten groeien tot ze gingen metamorfoseren, en de juvenielen in aquaria tot wasdom te laten komen. Dit alles gebeurde voor nakomelingen uit de twee zeeën onder identieke omstandigheden, om effecten van verschillende omstandigheden uit te sluiten.

De nakomelingen van de bollere Waddenzeenonnetjes waren boller dan de nakomelingen van de plattere Noordzeenonnetjes. Dit toont aan dat genetische variatie voor de eigenschap schelpvorm ruimtelijk gestructureerd is. Het contrast met de ongestructureerde moleculaire, selectief neutrale, variatie kon gekwantificeerd worden en bleek erg sterk te zijn. Het verschil kan niet anders dan in het effect van selectie zitten. Er moeten ruimtelijke verschillen in selectieomstandigheden zijn, die in de Noordzee bolle nonnetjes een nadeel bezorgen en in de Waddenzee platte nonnetjes.

Eén van die nadelen is selectieve predatie door kanoeten. Het verspreidingsgebied van nonnetjes in de Noordzee staat altijd onder water. Een deel van de Waddenzee komt bij laag water droog te staan, en alleen daar kunnen kanoeten nonnetjes eten. Piet van den Hout en Yohannes Afeworki voerden in de NIOZ-wadvogelunit experimenten uit met kanoeten en Noordzee- en Waddenzeenonnetjes. De kanoeten vertoonden een sterke voorkeur voor de plattere Noordzeeschelpen. Waarschijnlijk komt dit doordat ze meer moeite hebben bollere schelpen door te slikken, want onder de kleinere nonnetjes leek de voorkeur minder uitgesproken.



Kanoeten eten liever platte nonnetjes dan bolle

De vraag die ik mij gesteld had, was: 'is in zeedieren genetische variatie, die onder invloed van ruimtelijke selectiepatronen staat, in dezelfde mate willekeurig verdeeld als genetische variatie die selectief neutraal is?' Het antwoord op deze vraag is 'nee'; genetische variatie onder invloed van selectie kan veel sterker ruimtelijk gestructureerd zijn dan neutrale genetische variatie. Ruimtelijk gestructureerde patronen in selectieomstandigheden kunnen bijdragen aan het behoud van genetische variatie, zelfs wanneer alles erop wijst dat er veel genetische uitwisseling tussen gebieden is. Gelijke afstamming (moleculaire variatie) en gelijke omstandigheden (wel of geen kanoetenpredatie) zijn in dit voorbeeld inderdaad losgekoppeld, en toch is selectieve genetische variatie ruimtelijk georganiseerd (bolle of platte schelpen).

De volgende vraag is dan natuurlijk: 'hoe is dit nu mogelijk?' We zouden dit gegeven graag ook theoretisch willen begrijpen. Wiskundige modellen op dit terrein geven aan, dat de bandbreedte voor stabiel behoud van genetische variatie door ruimtelijke selectiepatronen smal is. Vooral wanneer er genetische uitwisseling plaatsvindt.

Een factor die deze bandbreedte mogelijk vergroot, is een kleine afstand tussen seksuele partners. Bij ongewervelden in de zee is de verwantschap tussen individuen vlak bij elkaar misschien niet verhoogd, maar de overlevers hebben wel genetische eigenschappen met elkaar gemeen. In het geval van nonnetjes komen genen voor platte schelpen in verhoogde mate voor in de Noordzee. Als paring, in tegenstelling tot larvale migratie, op kleine afstand gebeurt, lijken seksuele partners genetisch meer op elkaar dan willekeurige individuen. De selectiekrachten zouden hierdoor efficienter kunnen worden en de bandbreedte voor behoud van genetische varaitie groter. Dit is een theoretisch nog weinig bestudeerd en omstreden onderwerp, maar voor ongewervelden in de zee een veelbelovende mogelijkheid. Bij

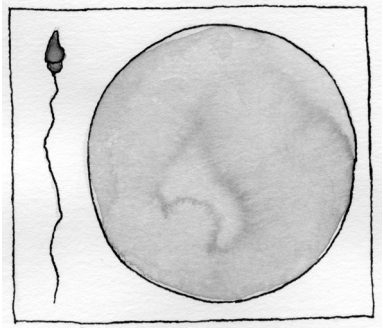
nonnetjes heb ik daarom gekeken naar aspecten van de externe bevruchting, die hoogstwaarschijnlijk op ruimtelijk kleine schaal plaatsvindt.

Bevruchting buiten het lichaam

Organismen waarbij de bevruchting buiten het lichaam plaatsvindt, lopen het risico dat hun eieren onbevrucht blijven. Sperma kan hierbij een beperkende factor zijn in de watermassa. Als de concentraties aan spermacellen en eicellen laag is, is de kans aanwezig dat ze elkaar niet tegenkomen voordat een van de twee zijn vitaliteit verloren heeft. In veldstudies bij andere soorten is aangetoond dat dit inderdaad voorkomt. Bij nonnetjes zijn zulke veldstudies slecht mogelijk, vanwege de troebelheid en onrustigheid van het water boven wadplaten. We konden wel indirecte aanwijzingen verzamelen.

Als sperma een beperkende factor is, zouden vrouwtjes de grootte van hun eieren hier mogelijk aan aanpassen. Nonnetjes komen van nature voor in verschillende dichtheden. In hoge dichtheden (bijvoorbeeld 400 dieren per m^2) mag verwacht worden dat spermalimitatie minder snel op zal treden dan in lage dichtheden (bijvoorbeeld minder dan 10 dieren per m^2). Als er weinig sperma is, zal tegen een groter ei eerder een spermacel opbotsen dan tegen een klein ei. Als er genoeg sperma is, botst elk ei wel tegen een aantal spermacellen op, en zal het voor vrouwtjes voordeliger zijn kleinere eieren te maken, want dan kunnen het er meer zijn. In het veld was een duidelijke samenhang te zien tussen dichtheid en eigrootte: waar nonnetjes in hoge dichtheden voorkomen maken nonnetjes kleinere eieren dan waar ze in lage dichtheden leven.

Een veel basalere vraag is waarom eieren überhaupt zoveel groter zijn dan spermacellen. De Britse onderzoekers Randerson en Hurst verwoordden dit fenomeen mooi: er zijn veel verschillen tussen de geslachten, maar er is er niet één zo universeel als het grootteverschil tussen mannelijke en vrouwelijke geslachtscellen (*anisogamie*). In dit proefschrift laat ik, samen met Michael Bulmer en Geoff Parker, nog twee Britse onderzoekers, zien dat een theoretisch waarschijnlijke oorzaak van dit fenomeen ligt in de positieve relatie tussen de kans op overleven en de afmeting van een bevruchte eicel. Geoff Parker en anderen suggereerden dit al in 1972, maar toen leek het erop dat de voorwaarden voor die relatie biologisch onrealistisch waren. Het is nu, onder meer dankzij nieuwe inzichten in overlevingskansen van bevruchte eicellen, duidelijk dat de benodigde relatie, om het ontstaan van anisogamie te verklaren, biologisch volkomen realistisch is.



Anisogamie is het meest universele verschil tussen de geslachten

Als bevruchting gewoonlijk op ruimtelijk kleine schaal gebeurt, omdat anders verdunningseffecten problemen veroorzaken, mag je verwachten dat alle nonnetjes

tegelijk en in één keer paaien. Deze laatste voorspelling hebben Jan Drent en ik getest door in het veld op vier plaatsen te kijken hoe lang het paaiseizoen is. Dit bleek onverwacht lang te zijn: ongeveer twee maanden. Wanneer je ze in het laboratorium laat paaien, produceren nonnetjes afkomstig van wadplaten wel slechts half zoveel sperma dan nonnetjes uit dieper gelegen gebieden. Onze hypothese is, dat dit laatste duidt op een aanpassing aan de grotere watermassa's die in dieper water overmeesterd moeten worden. Verder vermoeden we dat het optimale moment om als larve te overleven heel kort is en slecht te voorspellen. In de hoop toch enkele succesvolle nakomelingen voort te brengen, zou het beter kunnen zijn het risico van onbevuchte eieren te nemen dan het risico dat alle eieren weliswaar bevrucht worden, maar verloren raken op zee.

Als geslachtscellen het eeuwige leven hadden, waren ze geen beperkende factor. Spermacellen van nonnetjes verliezen echter hun vitaliteit al een paar uur na het paaien. Eieren lijken het langer vol te houden, maar meer experimenten zijn nodig om dit te bevestigen. Bevruchtingsexperimenten in het laboratorium lieten zien, dat spermacellen van nonnetjes slechts minder dan de helft van de eieren in een glas zeewater kunnen bevruchten, wanneer de spermaconcentratie onder de 100.000 tot 1.000.000 per ml blijft. Een paaiend mannetje produceert gemiddeld 340 miljoen spermacellen. Dat betekent, dat elk mannetje gemiddeld de helft van de eieren in 1 liter kan bevruchten. Dat is verbazingwekkend weinig. Het is natuurlijk mogelijk dat het bevruchtingsproces in de natuur efficiënter verloopt dan in het laboratorium, maar het lijkt erop dat er sterke ruimtelijke beperkingen zitten aan de externe bevruchting bij nonnetjes.

Uit andere laboratoriumexperimenten, die ik uitvoerde samen met Laas Pijnacker (RUG), bleek, dat eieren van nonnetjes geen snelle barrière hebben tegen het binnendringen van meer dan één spermacel, en dat dit ook niet direkt grote problemen oplevert voor de jonge embryo's. Veel andere soorten hebben dit wel. Ook dit is een indirecte aanwijzing dat nonnetjes in de natuur niet vaak te maken hebben met hoge spermaconcentraties.

Tot slot beginnen meer nonnetjes te paaien wanneer er een paaiend vrouwtje in de buurt is. Deze en de voorgaande waarnemingen bij elkaar doen sterk vermoeden dat seksuele partners bij deze soort zich inderdaad op niet al te grote afstand van elkaar zullen bevinden.

Conclusies

Nonnetjes, die model stonden voor ongewervelde zeedieren met vrijzwevende larven, hebben niet zo'n grote mate van genetische uitwisseling tussen populaties als verwacht. Alleen op de relatief kleine schaal van bijvoorbeeld de Waddenzee bleek neutrale genetische variatie vrijwel geen ruimtelijke structuur te vertonen. Bovendien kan zelfs op deze kleinste schaal genetische variatie, wanneer die onder invloed van selectie staat, ruimtelijk georganiseerd zijn, zoals bleek in het geval van de schelpvormen. Selectieve predatie is één van de factoren die hieraan bijdraagt.

Larvale migratie gebeurt blijkbaar op een schaal van de Waddenzee en aansluitende delen van de Noordzee, maar de afstand tussen seksuele partners is vrijwel zeker kleiner dan dat. Dit laatste is mogelijk een handvat voor de toekomst om de ruimtelijke organisatie van genetische verschillen in de zee theoretisch te kunnen begrijpen.

Deze conclusies hebben ook relevantie voor de manier waarop we omgaan met de mogelijkheden die de zee ons biedt. Het natuurbeleid ten aanzien van visserij gaan er vanuit dat een leeggevist gedeelte in de zee weer bevolkt zal worden door nakomelingen uit de met rust gelaten gedeelten. Vooral wanneer het gaat om soorten met vrijzwemmende larven, zoals veel schelpdieren. Men neemt aan dat de herbevolkers het even goed zullen doen als de oorspronkelijke dieren. Uit mijn onderzoek blijkt, dat de herbevolkers wel degelijk genetisch anders kunnen zijn, en bovendien vooral in die genetische eigenschappen die relevant zijn voor het functioneren van de dieren.

Een voorbeeld is het specifiek beschermen van schelpdieren in getijdegebieden als voedselreservering voor vogels. Het is denkbaar dat we daar met genetische selectie bezig zijn. Nu is het naar mijn mening niet goed om terug naar af te gaan en gewoon alles te bevissen, omdat pure aantalsregulatie en het bewaken van plekken met hoge dichtheden van groot belang zijn. Wel is het cruciaal dat het selectief-genetische argument in de toekomst meegenomen wordt in het besluitvormings-proces rond mariene reservaten.

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