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Role of spawning synchrony on hybridization barriers in sympatric intertidal fucoid algae – when and why

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TESE PARA A OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS DO MAR DA TERRA E DO AMBIENTE, RAMO DE CIÊNCIAS DO MAR, ESPECIALIDADE EM ECOLOGIA MARINHA

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluídas.

Carla Alexandra da Silva Monteiro

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"O valor das coisas não está no tempo que elas
duram, mas na intensidade com que acontecem.
Por isso existem momentos inesquecíveis, coisas
inexplicáveis e pessoas incomparáveis."
Maria Júlia Paes em "Qual é o tempo do cuidado"

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Dedico esta tese ao meu filho e ao futuro

Abstract

In sympatric assemblages of congeners with incomplete reproductive barriers, the synchrony within species and asynchrony between species may be under strong selection, reinforcing prezygotic reproductive isolation and reducing hybridization. Interspecific asynchrony and gametic incompatibility are considered the two most important prezygotic mechanisms reinforcing assortative mating and acting against hybridization. However, divergence of reproductive strategies in closely related species might also contribute to reproductive isolation. The main goal of this thesis was to investigate hypotheses concerning the mechanisms that act against hybridization in sympatric species with external fertilization, including the asynchrony of gamete release, mating system variations and gametic incompatibility.

As a model we used sympatric *Fucus* species with different reproductive modes, hermaphroditic (selfing) and dioecious (obligate outcrossing). The results show a clear relationship between patterns of gamete release and tidal/circadian cycles that contributes to interspecific variation in spawning time between hermaphroditic and dioecious species. The data support the hypothesis that asynchronous egg release acts as a prezygotic barrier to hybridization between hermaphroditic and dioecious species, and therefore, that mating system variation between congeners plays important role against hybridization. We also provide evidence that gametic incompatibility is an incomplete prezygotic barrier between *Fucus* species. Results show the potential of interspecific fertilization, and the potential growth and survival of hybrids relative to the parental lineage.

Keywords: External fertilization; Hybridization; Mating system; Pre- and postzygotic barriers; Sympatric species; Synchrony of release

Resumo

A libertação síncrona dos gâmetas entre indivíduos da mesma espécie e a libertação assíncrona entre indivíduos de espécies diferentes, pode estar sob uma selecção forte, de forma a reforçar o isolamento reprodutivo pré-zigótico e reduzir a hibridação entre populações congéneres que vivem em simpatria e possuem barreiras reprodutoras incompletas. Os mecanismos pré-zigóticos mais importantes contra a hibridação são a libertação assíncrona entre espécies e a incompatibilidade entre os gâmetas. No entanto, estratégias reprodutoras divergentes entre congéneres podem também contribuir para o isolamento reprodutor. O objectivo principal desta tese foi testar as hipóteses relacionadas com os mecanismos que actuam contra a hibridação entre espécies congéneres, que vivem em simpatria e apresentam fertilização externa. Os mecanismos estudados foram a libertação assíncrona dos gâmetas, a variação dos sistemas de acasalamento e a incompatibilidade entre gâmetas de espécies diferentes. Como modelo de estudo usamos espécies simpátricas do género *Fucus*, e com estratégias reprodutoras diferentes, hermafroditas (autofertilização) e dióicas (fertilização cruzada). Os resultados sugerem claramente uma forte relação entre os padrões de libertação dos gâmetas e os ciclos de marés e os circadianos, que contribuem para a libertação assíncrona entre espécies hermafroditas e dióicas. Estas observações suportam também, a hipótese da importância dos diferentes modos de acasalamento contra a hibridação. No entanto, os resultados demonstram que a incompatibilidade gamética ente espécies de *Fucus* não é um mecanismo pré-zigótico contra a hibridação e que existe um potencial de fertilização interespecífica. E por fim, os resultados indicam um potencial crescimento e sobrevivência dos híbridos relativamente à linhagem parental.

Palavras-Chave: Barreiras pré- e pós-zigóticas; Espécies simpátricas; Fertilização externa; Hibridação; Libertação síncrona; Sistemas de acasalamento

Resumo Alargado

A libertação síncrona dos gâmetas em organismos com fertilização externa tem implicações importantes no sucesso de fertilização e recrutamento, e consequentemente no sucesso reprodutor. No entanto, em espécies simpátricas e com barreiras reprodutoras incompletas, a libertação síncrona dos gâmetas entre indivíduos da mesma espécie (sincronia intra-específica), e a libertação assíncrona entre indivíduos de espécies diferentes (asincronia interespecífica), pode estar sob uma selecção forte, de forma a reforçar o isolamento reprodutivo pré-zigótico e reduzir a hibridação. Várias observações demonstraram que a libertação dos gâmetas entre congéneres é usualmente assíncrona, podendo ocorrer em escalas temporais mais ou menos restritas (e.g. intervalos mais restritos de horas), mas que podem evitar a hibridação. Para além disso, a libertação assíncrona entre espécies e a incompatibilidade gamética são considerados os mecanismos pré-zigóticos mais importantes contra a hibridação. No entanto, a evolução de estratégias reprodutoras divergentes em espécies estritamente relacionadas, podem também contribuir para o isolamento reprodutor, i.e., os sistemas de acasalamento diferentes podem actuar como um mecanismo ecológico contra a hibridação. Enquanto, nas espécies dióicas, o cruzamento obrigatório entre indivíduos aumenta a variação genética intra-populacional, e o tamanho efectivo da população (em comparação com as espécies que se auto-fertilizam), nas espécies hermafroditas, a autofertilização pode contribuir para o desenvolvimento do isolamento reprodutivo e especiação.

Os objectivos principais desta tese foram testar as hipóteses relacionadas com os mecanismos ecológicos que actuam contra a hibridação entre espécies congéneres, que vivem em simpatria e apresentam fertilização externa. Os mecanismos estudados foram 1) a libertação assíncrona dos gâmetas entre espécies; 2) a variação dos sistemas de acasalamento e 3) incompatibilidade gamética. Para testar a importância dos dois primeiros mecanismos contra a hibridação, foram comparados os níveis de (a)sincronia entre indivíduos da mesma espécie e entre congéneres, com sistemas de acasalamento divergentes e que vivem em simpatria, através do estudo da periodicidade reprodutora, a diferentes intervalos de tempo (a nível sazonal até intervalos mais restritos de horas). Para determinar a importância dos ciclos de maré e circadianos sobre a sincronia de libertação dos gâmetas, os padrões reprodutores foram também estudados em condições laboratoriais. O efeito da interacção entre as condições ambientais e os sistemas de acasalamento sobre

os padrões de reprodução e recrutamento também foram analisados numa costa rochosa exposta e num ambiente estuarino, entre espécies hermafroditas e dióicas. Por fim, foi estudado o potencial de hibridação (compatibilidade gamética) através de cruzamentos laboratoriais (cruzamentos intra- e interespecíficos) entre espécies hermafroditas e dióicas, e o potencial de sobrevivência e de crescimento dos híbridos relativamente aos indivíduos da linhagem parental.

As espécies do género *Fucus* foram utilizadas como modelo nesta tese, porque a fertilização é externa, têm modos de reprodução contrastantes (hermafroditas e dióicas), assim como diferentes sistemas de acasalamento (autofertilização e fertilização cruzada). Para além disso, muitas espécies coexistem na zona entre marés e apresentam uma distribuição vertical distinta, apesar de muitas vezes existir uma sobreposição das populações nos limites de distribuição vertical. Nessas zonas de sobreposição, podem ocorrer híbridos, mas as espécies têm mantido a sua integridade ao longe de uma vasta distribuição geográfica. Para testar diferenças entre sistemas de acasalamento, quatro espécies de *Fucus* foram estudadas, duas espécies hermafroditas (que apresentam autofertilização, *Fucus spiralis* and *Fucus guiryi*) e duas espécies dióicas (com fertilização obrigatoriamente cruzada, *Fucus vesiculosus* and *Fucus serratus*). Portugal é uma região marginal para as populações grandes e contínuas de *Fucus*, e é o limite de distribuição sul de co-ocorrência (distribuição simpátrica) destas quatro espécies. A sul de Viana do Castelo, *F. guiryi* habita somente a costa aberta, enquanto a espécie, *F. vesiculosus* ocorre exclusivamente em estuários e lagoas costeiras abrigadas. A espécie *F. serratus* está completamente ausente a sul de Viana do Castelo e a espécie *F. spiralis* é observada apenas nas praias a norte do Porto. O trabalho de campo foi realizado em Viana do Castelo (norte de Portugal), numa costa rochosa (Praia Norte) e no estuário do rio Lima (localizado a cerca de 1 Km a sul da Praia Norte). A Praia Norte é caracterizada por afloramentos rochosos bastante elevados, que reduzem drasticamente os efeitos da ondulação predominantemente de NW. As condições calmas e abrigadas, o clima temperado frio e o regime de marés semidiurno favorecem o desenvolvimento de grandes populações de muitas espécies de *Fucus*. A coexistência de populações de *Fucus* num ambiente estuarino proporciona uma excelente oportunidade para estudar os padrões de reprodução em espécies com estratégias de acasalamento diferentes, em dois habitats distintos.

Os resultados mostraram claramente que a libertação dos gâmetas nas espécies de *Fucus* é altamente síncrona à escala sazonal e semilunar. No entanto, a nível diário, a libertação natural é controlada pelos ciclos de marés e os circadianos que contribuem para uma potencial variação interespecífica na libertação dos gâmetas entres espécies hermafroditas e dióicas. Os resultados suportam a hipótese que a libertação dos gâmetas assíncrona actua como uma barreira pré-zigótica contra a hibridação entre espécies hermafroditas e dióicas. Desta forma, é possível também concluir que a variação dos sistemas de acasalamento entre congéneres desempenha um papel importante contra a hibridação. Os resultados também indicaram que tanto na natureza como em condições laboratoriais, a libertação dos gâmetas nas espécies dióicas é síncrona e controlada fortemente pelos ciclos circadianos. No entanto, as espécies hermafroditas são menos síncronas que as espécies dióicas. Esta libertação assíncrona é mais evidente em condições laboratoriais do que na natureza, reforçando a hipótese que as espécies hermafroditas, com a capacidade de autofertilização estão sob uma selecção menos vigorosa para reforçar o isolamento reprodutivo ao nível da libertação dos gâmetas do que as espécies dióicas, visto que a autofertilização pode ser o mecanismo pré-zigótico mais importante contra a hibridação. Contrariamente, os cruzamentos laboratoriais, claramente demostraram que a incompatibilidade entre gâmetas não é uma barreira pré-zigótica eficaz contra a hibridação entre espécies de *Fucus*. Inclusivamente, alguns híbridos possuem as mesmas capacidades para sobreviver e crescer, que os indivíduos da linhagem parental.

Esta tese é uma contribuição importante para compreender os mecanismos pré-zigóticos que actuam contra a hibridação entre espécies congéneres. Fornece umas das descrições mais completas e detalhadas sobre os padrões de reprodução em organismos com fertilização externa e com diferentes sistemas de acasalamento, à escala anual até a intervalos mais restritos, de horas.

Structure of the Thesis

This thesis is organized in seven distinct chapters. The first chapter (Chapter 1) provides an introduction to the subject and presents the general aims of the thesis and the last chapter (Chapter 7) provides a general discussion of the main findings of this thesis and synthesizes the overall contribution and considers some future remarks.

Chapters 2 to 6 constitute independent studies that seek to address the proposed aims. These were written in a style appropriate to be published in scientific journals and can be read separately:

Chapter 2. Prezygotic barriers to hybridization in marine broadcast spawners: reproductive timing and mating system variation. Monteiro CA, Serrão EA, Pearson GA (2012) PLoS ONE, 7(4): e35978

Chapter 3. Temporal windows of reproductive opportunity reinforce species barriers in a marine broadcast spawning assemblage.

Chapter 4. Reproductive investment, synchrony and recruitment success in marine broadcast spawners: effects of mating system and habitat (exposed shore *versus* estuary). Monteiro CA, Serrão EA, Pearson GA (2015) Marine Environmental Research, 112:33-39

Chapter 5. Circadian cycles (light:dark) control the time of spawning.

Chapter 6. Hybridization between marine broadcast spawners: incomplete barriers to reproductive isolation.

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Chapter 1

Introduction

1. Introduction

1.1. The challenges of broadcast spawning

External fertilization is a widespread mode of reproduction in the sea, and usually involves a motile sperm and non motile eggs. The marine organisms that retained external fertilization, i.e., broadcast spawning, as a means of reproduction, depend crucially on processes that increase gamete encounters in the water column. This is particularly challenging for closely related species for which sympatric speciation has been a controversial and highly debated issue for decades (Coyne and Orr 2004). Several ecological and biological mechanisms influence fertilization success in broadcast spawners. Sperm limitation may decrease egg fertilization success (reviewed by Brawley and Johnson 1992, Levitan and Petersen 1995, Yund 2000), however, high concentration of sperm may increase polyspermy, i.e., egg is fertilized by more than one sperm and is usually lethal (Brawley 1992, Pearson and Brawley 1996, Serrão et al. 1996, 1999, Berndt et al. 2002, Levitan 2004). The occurrence of polyspermy in natural populations and the mechanisms that block or decrease polyspermy are considered indirect evidence that the success of fertilization in broadcast spawners is high (Brawley and Johnson 1992, Yund 2000, Serrão and Havenhand 2009). Polyspermy has been considered a problem causing loss of reproductive effort (Brawley and Johnson 1992), and as a mechanism for sexual conflict (i.e., the inverse relationship between male and female reproductive success, reviewed by Serrão and Havenhand 2009). However, it can also be considered an advantage since it promotes the specificity of sperm:egg binding compatibility in broadcasting marine species (Gavrilets 2000, Levitan and Ferrel 2006). Furthermore, the distance between males and females may decrease the probabilities of gamete encounters (Levitan et al. 1992, Levitan and Petersen 1995, Serrão et al. 1996), although this could be balanced to a limited extent by higher sperm velocity and/or longevity (Rothschild and Swann 1951, Babcock and Mundy 1992, Babcock et al. 1994) as well as by egg longevity. In broadcast spawners gamete longevity is usually greater in eggs than in sperm, indicating that rapid fertilization is required before gamete dilution limits further gamete encounters (Serrão and Havenhand 2009). Differences between male and female longevity may also block polyspermy or act as a prezygotic mechanism against hybridization.

Synchronous spawning is a common phenomenon amongst marine organisms and its important role in reproductive success suggests that the traits involved are strongly favoured by selection. However, where closely related species reproduce in sympatry, and where hybrids are less fit than the parental lineages, selection may also strongly favour interspecific asynchrony in reproductive timing. The evolution of mechanisms that minimize heterospecific crossing is crucial to preserve species identities, as well as a key factor favouring assortative mating during sympatric or ecological speciation. Ecological mechanisms such as asynchronous spawning (temporal isolation) between closely related species and/or gametic incompatibility may be the major mechanisms or barriers to hybridization (Palumbi 1994, Gardner 1997, Coyne and Orr 1998, Fukami et al. 2003, Levitan et al. 2004). Postzygotic barriers such as low hybrid fitness triggered by genetic incompatibilities between species and/or intermediate adaptive success of hybrid phenotypes in parental environments (Hatfield and Schluter 1999) may prevent hybridization, but have been studied much less. In contrast, the importance of hybridization as a speciation mechanism in plant evolution is widely accepted (e.g., Rieseberg and Carney 1998, Arnold et al. 2003, Rieseberg et al. 2003). One of the consequences is the evolution of new lineages through recombinant homoploid or polyploid speciation, with the formation of hybrid zones resulting from gene flow between incompletely isolated species (reviewed in Mallet 2005). In marginal habitats the growth and survival of juvenile hybrids of corals is greater than pure species, a result suggesting that hybrids may adapt to new or rapidly changing environments, as well as increasing genomic flexibility to novel disease challenges (Willis et al. 2006).

Closely related species often have contrasting reproductive modes (e.g., hermaphroditic and dioecious), as well as different mating systems (selfing and outcrossing), which suggest that differences in reproductive strategies between taxa may promote the development of reproductive isolation and speciation (Barrett 1998, Dorken et al. 2002, Engel et al. 2005). Many hermaphrodite species are capable of self-fertilization, which increases the rate of gamete encounters (and therefore fertilization success) and consequently increases reproductive assurance and colonizing capacity (Pannell and Barrett 1998, 2001), while reducing the chances of hybridization. However, such an inbreeding strategy decreases intra-population genetic variation and effective population sizes in comparison with obligatory outcrossing dioecious species (Hamrick and Godt 1997, Holsinger 2000). In dioecious species the maintenance of genetic diversity and avoidance of inbreeding depression are important advantages of outcrossing (Maynard Smith 1978). The evolution of mechanisms such as mating system variation between closed related species may preserve species barriers (Perrin et al. 2007) and may contribute to our understanding of the role of reproductive barriers.

1.2. (A)synchronous spawning: speciation *vs.* hybridization

The synchrony of gamete release is a critical mechanism that increases fertilization success by maximizing the number of sperm and eggs in space and time. The most famous mass spawning events are those observed in corals (Harrison et al. 1984, Babcock et al. 1986, Slattery et al. 1999, Levitan et al. 2004). However, many other marine organisms also show synchronous release, e.g., sea stars (Babcock and Mundy 1992, Mundy et al. 1994), sea urchins (Pennington 1985, Levitan 2005), polychaetes (Watson et al. 2003), kelps (Lüning and Müller 1978), green algae (Harrison et al. 1984, Clifton 1997, Clifton and Clifton 1999) and fucoid algae (Brawley 1992, Brawley and Johnson 1992, Pearson and Brawley 1996, Serrão et al. 1996, Pearson et al. 1998, Brawley et al. 1999, Berndt et al. 2002, Santelices 2002). Synchronized spawning is usually correlated with lunar and/or tidal cycles (Lüning 1981, Babcock et al. 1986, Brawley 1992, Pearson and Brawley 1996, Berndt et al. 2002, Levitan et al. 2004, Monteiro et al. 2009) or is triggered by specific temperature (Bacon and Vadas 1991), and/or photoperiod (Smith 1947, Jaffe 1954, Lüning 1981, Brawley and Johnson 1992, Pearson and Brawley 1996, Serrão et al. 1996, Clifton 1997, Pearson et al. 1998, Clifton and Clifton 1999). A relationship between hydrodynamic conditions and synchronous release has been also reported, and low hydrodynamic conditions may stimulate gamete release (Denny and Shibata 1989, Denny et al. 1992, 2002, Brawley 1992, Brawley and Johnson 1992, Levitan et al. 1992, Benzie et al. 1994, Levitan 1995, Levitan and Petersen 1995, Pearson and Brawley 1996, Serrão et al. 1996, Pearson et al. 1998, Pearson and Brawley 1998, Yund 2000, Berndt et al. 2002). Despite that water motion may be less important for self-fertilizing hermaphrodites than for dioecious (obligate outcrossing) species, since the latter are under stronger selective constraint to ensure successful gamete encounters for reproduction (Pearson and Serrão 2006). Effective water motion may be further reduced by releasing gametes in mucilage in order to decrease their dilution rate (e.g., Brawley et al. 1999; further advantages of mucilage will be discussed below).

Synchronous spawning is considered to play an important role in reproductive success and the traits involved are strongly favoured by selection. However, where closely related species reproduce in sympatry, and where a common sexual pheromone is shared across taxa (Müller and Gassmann 1985, Maier and Müller 1986, Bolton and Havenhand 1996), hybridization may occur. In such a situation, and particularly when hybrids are less fit than the parental lineages, strong counterbalancing selection should act to favour interspecific asynchrony in reproductive timing. In support of this idea, despite overall synchrony, small temporal differences (on an hourly scale) in spawning between related taxa have been observed in nature (Harrison et al. 1984, Hayashibara et al. 1993, Clifton and Clifton 1999, Fukami et al. 2003, Levitan et al. 2004, Wolstenholme 2004), which can be interpreted as a mechanism for avoiding hybridization. Together with gametic incompatibility, asynchronous spawning is considered the major prezygotic mechanism against hybridization between closely related species (Palumbi 1994, Gardner 1997, Coyne and Orr 1998, Fukami et al. 2003, Levitan et al. 2004). Further, sympatric and closely related species often have different reproductive modes (hermaphroditic and dioecious) and different mating systems (selfing and outcrossing) (see Pearson and Serrão 2006). Although in hermaphroditic species the selfing strategy decreases intra-population genetic variation and effective population size in comparison with obligatory outcrossing dioecious species (Hamrick and Godt 1997, Holsinger 2000), self-fertilization may promote the development of reproductive isolation and speciation, particularly within hermaphroditic species, where individuals release male and female gametes in close proximity with minimal sperm limitation (Barrett 1998, Dorken et al. 2002, Engel et al. 2005).

Despite all mechanisms that species may develop to ensure the success of reproduction, hybridization does occur in several marine organisms, e.g., corals (Vollmer and Palumbi 2002, Levitan et al. 2004), sea urchins (Levitan 2002), bivalves (Bierne et al. 2002, Beaumont et al. 2004) and fucoid algae (Coyer et al. 2002ab, 2006, 2007, Engel et al. 2005). Generally, low hybrid fitness triggered by genetic incompatibilities between species is reported, although intermediate adaptive success of hybrid phenotypes in parental environments (Hatfield and Schluter 1999, Rundle and Whitlock 2001) may also occur. The role of hybridization as a speciation mechanism in plant evolution is widely accepted (Rieseberg and Carney 1998, Arnold et al. 2003, Rieseberg et al. 2003), contributing to the evolution of new lineages through recombinant speciation and polyploidy, or the formation of hybrid zones resulting from gene flow between incompletely isolated species (reviewed in Mallet 2005). In certain marine organisms such as corals, the growth and survival of juvenile hybrids can be greater than pure species (Willis et al. 2006). This could be positive for rapidly changing environments, as well as increasing genomic flexibility in response to novel disease challenges (Willis et al. 2006).

1.3. Model organisms

1.3.1. Ecology and distribution

Fucoids species are major components of the benthic biomass at temperate latitudes and are an excellent model to study reproductive ecology and physiology in marine algae (Brawley et al. 1999). Together with several invertebrates and fish, *Fucus* species are one of the most well-studied groups of broadcast spawners (Pearson and Serrão 2006) and over the past decades 1500 publications about the genus *Fucus* have been produced (excluding purely taxonomic contributions; Wahl et al. 2011). The use of *Fucus* species as model organisms has several advantages, e.g., fertilization is external, sexual reproduction is oogamous and it is possible to obtain large numbers of uniform eggs and sperm (Evans et al. 1982, Motomura 1994). Furthermore, the incomplete reproductive barriers (Billard et al. 2010) and allopatric and sympatric distribution of these closely related species, together with variability in breeding systems, makes *Fucus* one of the best and most challenging model genera to study the nature and role of speciation and reproductive barriers, i.e., coexistence despite hybridization and introgression.

Fucus is one of the most common genera on temperate to Arctic northern hemisphere shorelines (Fritch 1959), while being completely absent from the southern hemisphere (Bold and Wynne 1985). It has a wide latitudinal gradient, ranging from the Arctic Ocean and the Baltic Sea (Lüning 1990) in the north, to the Adriatic Sea in the south (Wahl et al. 2011). However, the highest species diversity and abundance is observed in the eastern North Atlantic Ocean (Lüning 1990), especially in southwestern Ireland and the Brittany peninsula of France or NW Iberia. Molecular data has identified hotspots of genetic diversity in these regions, predicting that these areas were glacial refugia during the Last Glacial Maximum (Coyer et al. 2003, 2011b; Hoarau et al. 2007, Neiva et al. 2010). *Fucus* species occupy distinct, but often overlapping, niches on protected to moderately exposed rocky intertidal shores, shallow subtidal shores, estuaries (tidal marshes) and lagoons.

Fucus spiralis occurs in the North Atlantic, ranging between northern Canada and northern Norway (White Sea) to Portugal. It also occurs in the North Pacific, but is a recent introduction here (for further review see Coyer et al. 2011a). *Fucus vesiculosus* ranges from the Arctic Ocean (Northern Russia, Spitsbergen) to the Northern Atlantic from Canada to Northern Norway (including the brackish Baltic Sea), south to Morocco. *Fucus serratus* occurs in the Arctic, from Spitzbergen and Iceland to Northern Norway (White Sea), Faroe Islands, the Baltic Sea, United Kingdom, Channel Islands, to the southern limit of distribution in Northern Portugal. It also occurs in North America (Maritime Provinces; Lüning 1990). *Fucus guiryi* occurs between United Kingdom (Ireland, England and Scotland) and France, further south to Portugal, and occurs in the Canary Islands, Azores and northern Morocco (Zardi et al. 2011). Northern Portugal is the southern limit of sympatric occurrence of these four species, as well as the southern limit of distribution for *F. serratus* and *F. spiralis*, while further south *F. guiryi* inhabits only the open coast and *F. vesiculosus* occurs exclusively in estuaries and sheltered coastal lagoons (Ladah et al. 2003, Araújo et al. 2009, 2012, Pearson et al. 2009).

The vertical distribution of these four *Fucus* species in sympatry is usually distinct although with overlapping zones (Lüning 1990, Serrão et al. 1997, Dudgeon et al. 2001, Dudgeon and Petraitis 2001, Billard et al. 2005b, 2010, Coleman and Brawley 2005ab, Zardi et al. 2011) and the common vertical distribution is: *F. spiralis* in the high intertidal zone (usually below *Pelvetia canaliculata*), *F. guiryi* in the high intertidal below *F. spiralis*. *Fucus vesiculosus* occurs between mid- and low intertidal and *F. serratus* in the low intertidal to subtidal (Fig. 1.1). In the eastern Atlantic ocean these species coexist as distinct entities (Billard et al. 2005a, Engel et al. 2005, Zardi et al. 2011) but multilocus genotypes (Engel et al. 2005) and variation in sexual phenotype (Billard et al. 2005b) suggests that hybridization with introgression occurs, and therefore that hybrids are fit (i.e., reproduction is successful), and appear as vigorous as their parental species (Billard et al. 2005b, 2010, Engel et al. 2005). A considerable number of studies have reported hybridization between *F. vesiculosus* and other *Fucus* species, such as *F. spiralis* (Wallace et al. 2004, Billard et al. 2005ab, 2010, Engel et al. 2005, Coyer et al. 2006, 2011a) and *F. ceranoides* (Neiva et al. 2010). Hybridization is also reported to occur between *F. serratus* and *F. distichus* (Coyer et al. 2002ab, 2007), but offspring are less fit than either parental species (Coyer et al. 2007). Putative natural hybrids between *F. vesiculosus* and *F. spiralis* (determined based on intermediate genotypes) were shown to be fit in terms of reproductive investment (Billard et al. 2005b). Where novel morphological traits in hybrids such as dwarfism and vegetative propagation coincide with expansion into novel environments, e.g., in certain populations of the non-monophyletic entity *Fucus cottonii* which might in some cases have a hybrid origin, an adaptive role for hybridization is suggested (Wallace et al. 2004, Coyer et al. 2006, Neiva et al. 2012).

Figure 1.1. Vertical distribution of *Fucus* species in Viana do Castelo, Northern Portugal.

The number of *Fucus* species and their different morphological adaptations influenced by environmental conditions, combined with complex processes of hybridization and ecotypic differentiation have raised taxonomic confusion. Wahl et al. (2011) suggest to genetically identify *Fucus* entities at the outset of any proposed study, except for *F. vesiculosus* (Fig. 1.2), which is clearly distinguishable by the presence of air-bladders (or air vesicles), *F. serratus* (Fig. 1.3), which is clearly distinguishable by receptacle morphology, and a flat and serrated thallus, and *F. spiralis* (Fig. 1.4), which can be identified by shorter thallus and receptacle length and by the high ratio of receptacles to vegetative apices. In contrast, the identification of *F. guiryi* (Fig. 1.5) is more controversial, although the receptacles are longer and wider, with a sterile rim and fewer receptacles per apical frond. In addition the thallus of *F. guiryi* is longer than *F. spiralis* and the branching pattern is different (for further descriptions see Zardi et al. 2011).

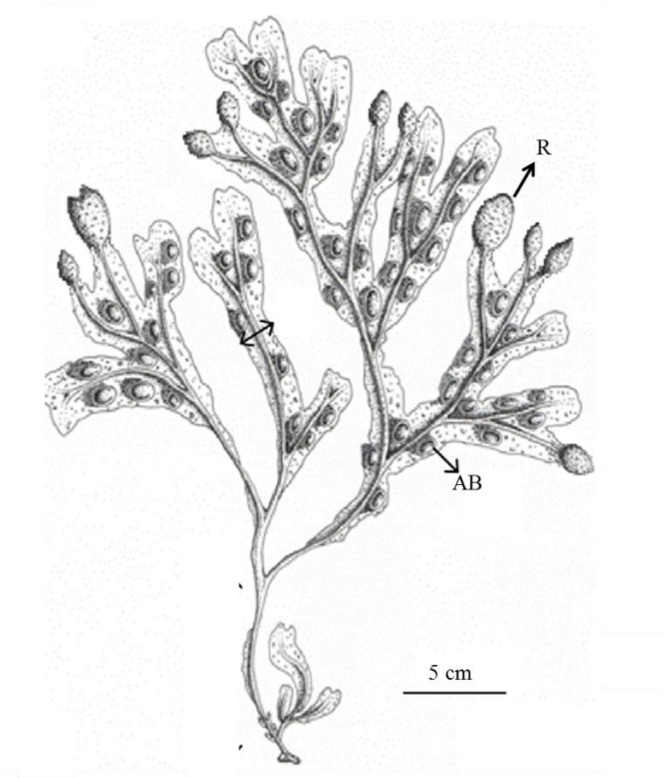

Figure 1.2. *Fucus vesiculosus* morphology. R: receptacle; AB: air-bladders (Zardi et al. 2011).

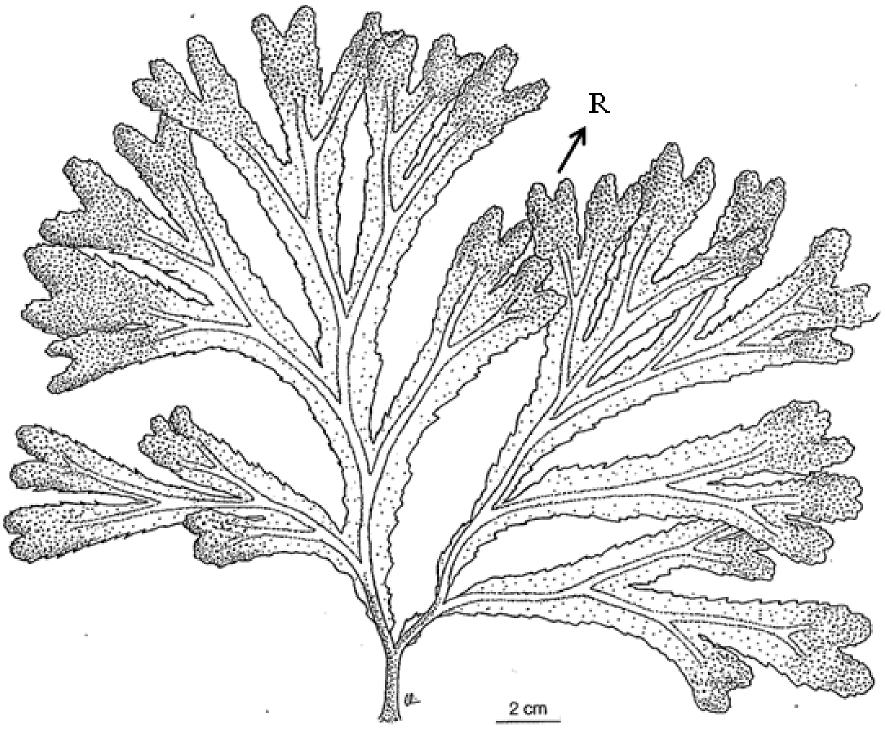

Figure 1.3. *Fucus serratus* morphology. R: receptacle (Pérez-Ruzafa 2001).

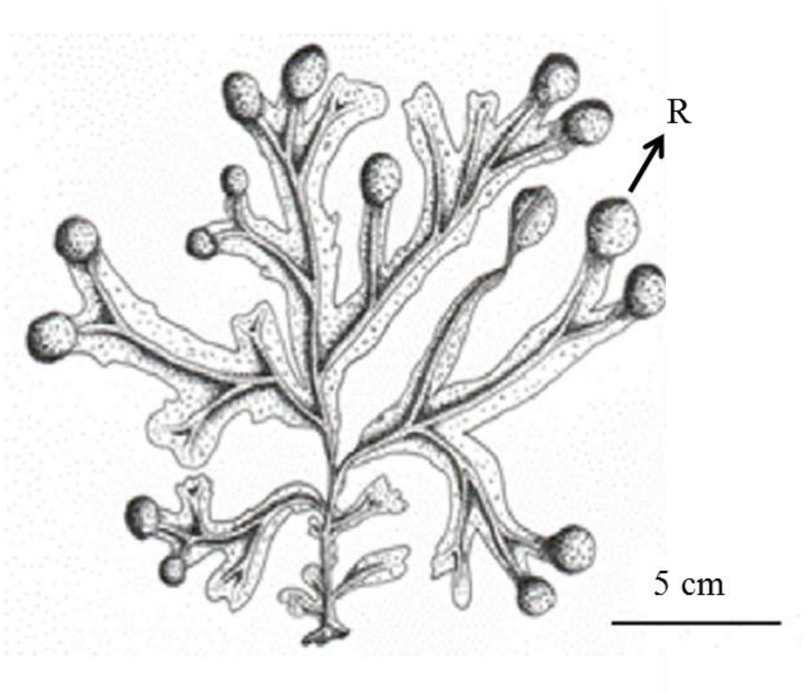

Figure 1.4. *Fucus spiralis* morphology. R: receptacle (Zardi et al. 2011).

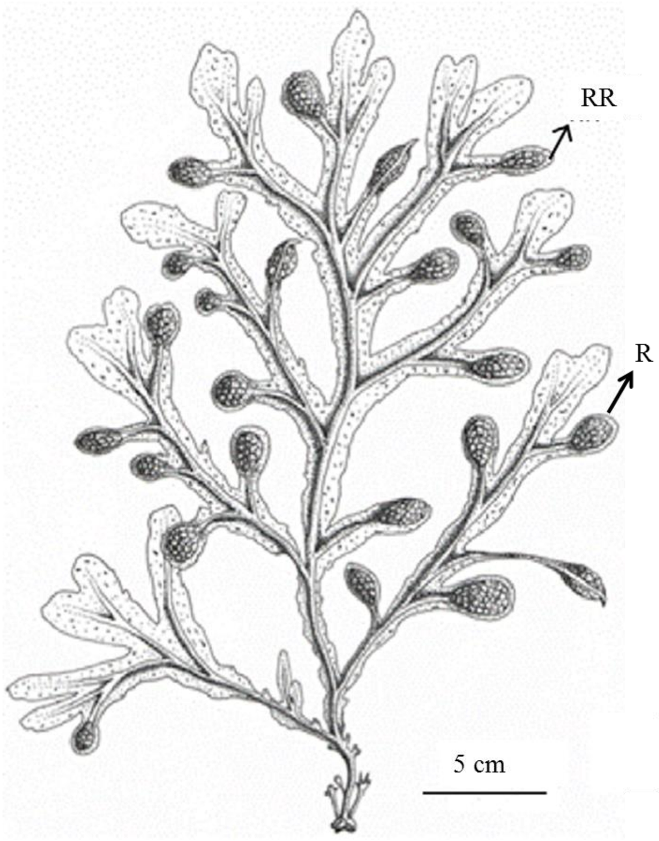

Figure 1.5. *Fucus guiryi* morphology. R: receptacle; RR: presence of receptacle sterile rim (Zardi et al. 2011).

1.3.2. Life cycle

In *Fucus* species fertilization is external and the reproductive structures, called receptacles, develop apically. Within the receptacles several spherical conceptacles with numerous antheridia (each containing 64 sperm) and/or oogonia (each with 8 eggs) develop. Sperm are biflagellate and reach ca. 5 µm in length, while eggs are non motile and reach ca. 80 µm in diameter. In hermaphroditic species the conceptacles contain both antheridia and oogonia (with sperm and eggs, respectively), but in dioecious species conceptacles of separate male and female individuals develop antheridia and oogonia, respectively (Callow et al. 1985).

Antheridia and oogonia are released through ostioles from within each conceptacle. Both are negatively buoyant (i.e., they sink), and sperm are in addition negatively phototactic. Therefore, settlement in *F. vesiculosus* occurs immediately after release in calm hydrodynamic conditions as reported (Serrão et al. 1996, Pearson et al. 1998). Shortly after release the antheridia and oogonia open, liberating the eggs and sperm and fertilization occurs externally (Fig. 1.6, life cycle in *Fucus*), most likely at or near the bottom of the water column. Sperm are chemically attracted to eggs by a sexual pheromone (Müller and Gassmann 1985). Fertilization success is usually close to 100% (Brawley 1992, Pearson and Brawley 1996, Serrão et al. 1996, Brawley et al. 1999, Berndt et al. 2002, Ladah et al. 2003). However, fertilization success may decrease at low salinities due to increased polyspermy (Serrão et al. 1999). In *Fucus* species polyspermy may occur in both hermaphroditic and dioecious species (Vadas et al. 1992) but usually at rates below 10 % (Brawley 1992, Pearson and Brawley 1996, Serrão et al. 1999). In contrast, in low salinity environments polyspermy may reach levels as high as 30% (Serrão et al. 1999). In estuarine populations (e.g., *F. ceranoides*) high success of fertilization is due to gamete release at high tide, when salinity is higher (Brawley 1992). An important mechanism that blocks polyspermy is the depolarization of the egg plasma membrane after fertilization (the electrical “fast block”). This response occurs between one to three minutes after fertilization. Sperm remaining outside the egg have been observed to swim away following egg depolarization (Brawley 1991). At the same time, alginic acid secretion occurs in the cell wall immediately after the entry of one sperm into the egg (constituting a “slow block” to polyspermy; Evans et al. 1982, Bold and Wynne 1985, Brawley 1991).

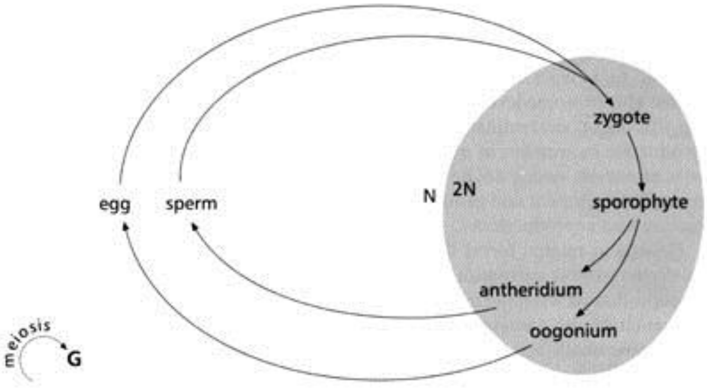

Figure 1.6. The life cycle of *Fucus* species (Graham and Wilcox 2000).

The timing at which fertilization occurs relative to tidal cycles may predict dispersal patterns, however several data suggest that eggs tend to fall immediately below the releasing individual, and therefore that dispersal is highly restricted (Serrão et al. 1997, Dudgeon and Petraitis 2001, Coleman and Brawley 2005ab, Engel et al. 2005, Perrin et al. 2007). Attachment of fertilized zygotes to the substratum occurs 4-6 hours after the fertilization (Brawley and Johnson 1992, Brawley et al. 1999). The secretion of mucilage (containing fucoidan and alginic acid) present in receptacles may play an important role in low dispersal patterns in hermaphroditic species, e.g., *F. spiralis* is able to secure the oogonia to the receptacle until the egg are fertilized and drop off, and thereby enhance the success of fertilization (Müller and Gassmann 1985). Furthermore, mucilage is an important multi-functional secretion, e.g., gamete discharge, lubrification, sperm entrapment, desiccation resistance, increased buoyancy, adhesion to the substratum and defense (reviewed by Clayton 1992).

After adhesion to the substratum, the development of rhizoids is the first step in germination and is the most critical period in the life-cycle after fertilization, where highest mortality is reported (for further review see Vadas et al. 1990, 1992, Jenkins et al. 1999, Dudgeon and Petraitis 2001, Ladah et al. 2003). Recruitment in fucoid species is considered a bottleneck stage (Gunnill 1980, Vadas et al. 1990, Brawley and Johnson 1991, Johnson and Brawley 1998) and is dependent on several biotic and abiotic factors. For example, canopy presence is reported to have both beneficial (e.g., Brawley and Johnson 1991, Johnson and Brawley 1998, Dudgeon and Petraitis 2001, Ladah et al. 2003) and detrimental effects on recruitment (Chapman 1989, 1990, Vadas et al. 1990, 1992, Åberg and Pavia 1997, Jenkins et al. 1999). Besides, canopy may protect the early stages against physiological damage due to desiccation from exposure during emersion, as well as from herbivory (Hay 1981, Brawley and Johnson 1991, Vadas et al. 1992, Dudgeon and Petraitis 2001), thereby increasing recruitment success. Ecological and molecular works predicts that dispersal in *Fucus* species is low (Serrão et al. 1997, Dudgeon and Petraitis 2001, Coleman and Brawley 2005ab, Engel et al. 2005, Perrin et al. 2007), suggesting that canopy has an important role for early recruits. However, this also may vary between species with different mating systems.

1.3.3. Reproductive patterns

Reproductive periodicity is one of the major factors contributing to the successful distribution and abundance of many marine organisms (Menge 1991, 2000, Roberts 1991). It is usually correlated with rhythmical environmental factors such as circannual, lunar, semilunar, tidal and circadian cycles, especially important in broadcast spawners that inhabit the intertidal zone. It can be triggered by specific temperature, photoperiod, or is controlled by natural rhythms – the biological clock (Dring 1982).

Several species of intertidal *Fucus* have been shown to release gametes synchronously on a semilunar cycle (≈ 2 week cycle), usually coinciding with full and new moons (spring tides) (Brawley 1992, Berndt et al. 2002), although timing varies in other species and/or habitats (reviewed by Pearson and Serrão 2006). Fucoids release gametes at periods of low water motion in natural populations, either during low tide in pools, at slack high tide, or during low tide (Brawley et al. 1999, Pearson and Serrão 2006), and periods of high environmental water motion can override semilunar periodicity. Water motion is sensed via photosynthetic carbon limitation due to the diffusion barrier across unmixed boundary layers (Serrão et al. 1996, Pearson et al. 1998, Pearson and Brawley 1998). Together, synchronous gamete release and water motion sensitivity result in very high fertilization success in *Fucus* spp. (reviewed in Pearson and Serrão 2006). The diurnal timing of high and low tides during the semilunar cycle varies from location to location, e.g., between the western and eastern Atlantic, and in particular shows a strong latitudinal gradient in timing along eastern Atlantic shores (Schmidt et al. 2008). This, together with different exposure (hydrodynamic) conditions may account for some of the variations in semilunar and tidal phase (diurnal) timing observed in the literature (e.g., Berndt et al. 2002, Ladah et al. 2008). Although reproductive ecology in dioecious species has been the subject of several studies, especially in *F. vesiculosus*, less attention has been given to hermaphroditic species (but see Pearson and Brawley 1996). There is in particular, a lack of detailed information about gamete release at tidal and diurnal timescales in sympatric species assemblages.

Within the genus *Fucus*, the sister species *F. spiralis* and *F. guiryi*, *F. vesiculosus* and *F. serratus* often co-occur on the same shores in mixed stands. These species differ in reproductive mode (selfing hermaphrodite and dioecious), and form hybrids (Wallace et al. 2004, Billard et al. 2005ab, 2010, Engel et al. 2005, Coyer et al. 2006, 2011ab, Neiva et al. 2012) at low frequency (Engel et al. 2005). The *Fucus* species are a useful model system that raises important questions concerning the maintenance of species integrity in sympatry; i.e., is mating system variation sufficient for reproductive isolation, or are other factors involved? In addition to mating system effects, prezygotic isolation might involve evolution of both molecular/biochemical (e.g., sperm-egg recognition, lowering the frequency of hybrid gamete fusions) and ecological mechanisms such as differences in the timing of gamete release (Levitan et al. 2004).

In summary, the incomplete reproductive isolation between *Fucus* species, and the occurrence of different mating systems and differentiated allopatric and sympatric populations over a large latitudinal distribution, raises very interesting questions and implications concerning spawning (a)synchrony, reproductive isolation, and the mechanisms that act against hybridization in sympatric species with external fertilization (broadcast spawners).

1.4. Thesis outline

The timing of egg release, as well as the synchrony of release, have important implications not only for fertilization success, but also for the success of recruitment and early survival in organisms with external fertilization. However, synchrony within species and asynchrony between species may be under strong selection to reinforce prezygotic reproductive isolation and reduce hybridization (Gardner 1997, Fukami et al. 2003, Levitan et al. 2004). Besides, the divergence of reproductive strategies might contribute to reproductive isolation in sister species with similar geographical ranges (sympatric species), with important ecological and evolutionary consequences. Reproductive synchrony also affects the distribution of genetic diversity and gene flow between and within populations. Since interspecific asynchrony and gametic incompatibility are considered the most important prezygotic mechanisms that may act against hybridization, the main objectives of this thesis are to test hypotheses concerning the (a)synchrony of egg release, the role of egg release and mating system variation, including the prediction that selfing hermaphrodites should show less synchrony than obligate out-crossing species. Finally, to test the gametic (in)compatibility between closely-related species with external fertilization. Specifically, this thesis aims to:

1. Identify the degree of intraspecific synchrony and interspecific asynchrony in sympatric assemblages of congeners that differ in reproductive modes. By examining the reproductive periodicity from seasonal to hourly timescales (Chapters 2 and 3).
2. Test whether reproductive success is affected by the interaction between environmental conditions (exposed rocky shore and estuarine habitats) and mating systems (selfing and obligate outcrossers). Identify the importance of spawning synchrony for reproductive assurance *versus* recruitment assurance at the southern limit of sympatric distribution (Chapter 4).
3. Predict the role of circadian cycles on spawning patterns and test synchrony of gamete release in dioecious and hermaphroditic species under shifting timing of light:dark cycles (Chapter 5).
4. Test the hybridization potential (gametic compatibility) in laboratory controlled crosses (intra- and inter-species), and study hybrid survival and growth relative to parental lineages (Chapter 6).
5. Concluding remarks: where to go from here (Chapter 7).

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Chapter 2

Prezygotic barriers to hybridization in marine broadcast spawners: reproductive timing and mating system variation

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2. Prezygotic barriers to hybridization in marine broadcast spawners: reproductive timing and mating system variation

2.1. Abstract

Sympatric assemblages of congeners with incomplete reproductive barriers offer the opportunity to study the roles that ecological and non-ecological factors play in reproductive isolation. While interspecific asynchrony in gamete release and gametic incompatibility are known prezygotic barriers to hybridization, the role of mating system variation has been emphasized in plants. Reproductive isolation between the sibling brown algal species *Fucus spiralis*, *Fucus guiryi* (selfing hermaphrodite) and *Fucus vesiculosus* (dioecious) was studied because they form hybrids in parapatry in the rocky intertidal zone, maintain species integrity over a broad geographic range, and have contrasting mating systems. We compared reproductive synchrony (spawning overlap) between the three species at several temporal scales (yearly/seasonal, semilunar/tidal, and hourly during single tides). Interspecific patterns of egg release were coincident at seasonal (single peak in spring to early summer) to semilunar timescales. Synthesis of available data indicated that spawning is controlled by semidiurnal tidal and daily light:dark cues, and not directly by semilunar cycles. Importantly, interspecific shifts in timing detected at the hourly scale during single tides were consistent with a partial ecological prezygotic hybridization barrier. The species displayed patterns of gamete release consistent with a power law distribution, indicating a high degree of reproductive synchrony, while the hypothesis of weaker selective constraints for synchrony in selfing *versus* outcrossing species was supported by observed spawning in hermaphrodites over a broader range of tidal phase than in outcrossers. Synchronous gamete release is critical to the success of external fertilization, while high-energy intertidal environments may offer only limited windows of reproductive opportunity. Within these windows, however, subtle variations in reproductive timing have evolved with the potential to form ecological barriers to hybridization.

Keywords Dioecious; Egg release; External fertilization; Hermaphrodite; Mating system; Prezygotic barriers; Semilunar and tidal timescales; Sympatric species

2. 2. Introduction

Speciation occurs by the evolution of reproductive barriers that ultimately prevent genetic exchange between previously interbreeding populations (Mayr 1963). Prezygotic isolating mechanisms range from ecological barriers to gametic incompatibility, while postzygotic isolation may arise through hybrid inviability, sterility, or reduced hybrid fitness. Ecological barriers are commonly the first to arise, and are more important than intrinsic postzygotic factors (reviewed for plants by Rieseberg and Willis 2007). In the sea, where reproduction by external fertilization is common, it may be more difficult for extrinsic ecological barriers to arise, resulting in more opportunities for hybridization (Palumbi 1994). Asynchronous gamete release between related taxa, and/or gametic incompatibility are considered the major prezygotic barriers to hybridization between externally fertilizing sympatric animal species (Levitan et al. 2004). In contrast to terrestrial plants, where mating system (particularly selfing) can maintain almost complete reproductive isolation between sympatric species in the presence of gene flow (Martin and Willis 2007), outcrossing, albeit at varying rates, appears to dominate in hermaphroditic broadcast spawners (Heyward and Babcock 1986).

The intertidal zone imposes unique constraints on external fertilization synchrony because during tidal emersion efficient gamete mixing is prevented and abiotic stresses can be intense, while during immersion hydrodynamic conditions may result in rapid gamete dilution and/or shear stresses on gametes that may severely reduce fertilization success (Denny et al. 1992, Mead and Denny 1995). Despite this, broadcast spawning is a common reproductive mode in diverse marine intertidal taxa, from invertebrates to macroalgae (Brawley 1992, Pearson and Brawley 1996, Berndt et al. 2002, Marshall 2002, Ladah et al. 2003, 2008, Marshall et al. 2004).

Synchronous gamete release is critical to reproductive success in broadcast spawners, but the cyclical fluctuation of environmental conditions arising from interacting cycles at diurnal, tidal, semilunar and seasonal temporal scales suggests that external fertilization in intertidal species may be constrained within narrow windows of opportunity. We predict that selective pressures to restrict gamete release within these temporal windows will be strong. Indeed, some intertidal taxa possess sophisticated mechanisms to sense the environment and restrict gamete release, particularly to periods of low environmental water motion (e.g., in fucoid algae, Serrão et al. 1996, Pearson et al. 1998, Pearson and Brawley

1998, Pearson and Serrão 2006), maximising the probability of gamete encounters. However, some species seen to be stimulated to spawn by storms (e.g. *Patella* sp.; Orton et al. 1956).

A major question in the ecology of broadcast spawning taxa is whether patterns of gamete release synchrony at any particular temporal scale may act as a prezygotic barrier, a process identified at very fine scale in corals (see review by Serrão and Havenhand 2009 for details). When hybrids display reduced fitness relative to parental taxa, selective constraints for maximizing reproductive success should be counterbalanced by selection to reinforce prezygotic barriers to hybridization. This may be achieved by interspecific variation in spawning time (Levitan et al. 2004), such that congeneric gamete encounters are minimized. A second prezygotic mechanism may be mating system variation, particularly self-fertilization within hermaphroditic species, which is expected to dramatically reduce hybridization where individuals release male and female gametes in close proximity with minimal sperm limitation. An alternative to prezygotic barriers maintaining species coherence in the face of hybridization, is that hybrid fitness is conditional on the environment (Rundle and Whitlock 2001), and that hybrids are either favoured, or selectively neutral, under certain conditions or in particular microhabitats along the intense gradients of selection in the intertidal.

Species capable of coexistence despite hybridization and introgression remain one of the best and most challenging models to study the nature and role of reproductive barriers. This is the case of three sister species with different reproductive modes and mating systems; hermaphroditic *Fucus spiralis* and *Fucus guiryi*, in which selfing is the dominant form of reproduction throughout the studied range (Engel et al. 2005, Perrin et al. 2007), and dioecious, outcrossing *Fucus vesiculosus*. These species coexist as distinct entities (Billard et al. 2005a, Engel et al. 2005ab, Zardi et al. 2011) in the mid to high shore throughout most of their range in the eastern Atlantic (Lüning 1990). Analyses of both multilocus genotypes (Engel et al. 2005) and variation in sexual phenotype (Billard et al. 2005b) indicate that hybridization with introgression occurs, and therefore that hybrids are reproductively successful.

Investment into male function is an order of magnitude lower in *F. spiralis* compared with *F. vesiculosus*, suggesting that sperm limitation is not an important factor in the hermaphrodite (Billard et al. 2005b). If synchrony is selectively maintained primarily to ensure gamete encounters, then selfing hermaphrodites should display a relaxed synchrony relative to dioecious, obligately outcrossing species.

In this study we examined reproductive periodicity from seasonal to hourly timescales for the hermaphroditic fucoids *F. spiralis* and *F. guiryi*, and dioecious *F. vesiculosus*. This allowed us to test two predictions: 1) Reproductive synchrony is more important and therefore under stronger selective constraint in obligate outcrossers (*F. vesiculosus*) than in selfing species (*F. spiralis* and *F. guiryi*); 2) The timing of gamete release should vary between potentially hybridizing species in order to reinforce prezygotic (ecological) barriers to fertilization. Our data support both predictions, and also emphasize the ecological, developmental, and/or physiological constraints that operate to restrict reproduction to narrow temporal windows of opportunity in the intertidal zone.

2.3. Materials and methods

2.3.1. Model organisms

The genus *Fucus* develops specialized reproductive tissue called receptacles on some apical tips of the thallus, and inside the receptacles are numerous spherical conceptacles. Depending on the species, sperm and egg develop inside the same conceptacle (i.e., hermaphroditic species, such as *F. spiralis* and *F. guiryi*); or in separate conceptacles from male and female individuals (i.e., dioecious species, as in *F. vesiculosus*). The three model species can be distinguished morphologically as described in Zardi et al. (2011). In all species, sperm and eggs develop inside gametangia called antheridia and oogonia, respectively, and both are released unfertilized (see review Pearson and Serrão 2006 for details). Fertilization is external and *Fucus* eggs are large (ca. 70 mm diameter), negatively buoyant and settle immediately as they are released. Sperm are negatively phototactic. Empirical studies (Serrão et al. 1997, Dudgeon et al. 2001, Dudgeon and Petraitis 2001), as well as local patterns of genetic structure (Coleman and Brawley 2005ab, Engel et al. 2005, Perrin et al. 2007) all indicate that gamete and zygote dispersal is highly restricted. Eggs tend to fall immediately below the releasing alga (Serrão et al. 1997) allowing, in settlement studies conducted in mono-specific patches, the assignment of egg origin to species with a high degree of confidence. The vertical distribution of these species on the shore is distinct although with overlapping zones (Billard et al. 2010, Zardi et al. 2011); *F.*

spiralis occurring mainly above *F. guiryi*, and these above the dioecious species *F. vesiculosus*. Hybrids between these species are uncommon and can be found mainly in the intertidal ranges where their parental species overlap (Billard et al. 2010); they are fertile and appear as vigorous as their parental species (Billard et al. 2005b, Engel et al. 2005). The restriction of hybrids to vertical contact zones is additional support for the low dispersal of gametes.

2.3.2. Study site and sampling

The study was conducted on a rocky shore at Viana do Castelo (Northern Portugal), at Praia Norte (Fig. 2.1). This location is protected from severe wave action by offshore rocky bluffs. The region is the southernmost limit for the sympatric occurrence of *F. vesiculosus*, *F. spiralis* and *F. guiryi* in Europe, and hybridization is more frequent in this contact range (Moalic et al. 2011), possibly as a consequence of lack of reinforcement in southern allopatric populations. The distribution of these species continues to the south, but *F. vesiculosus* is confined to estuaries and sheltered coastal lagoons, while *F. spiralis* and *F. guiryi* continues to occur on the open coast where suitable rocky substrate is available.

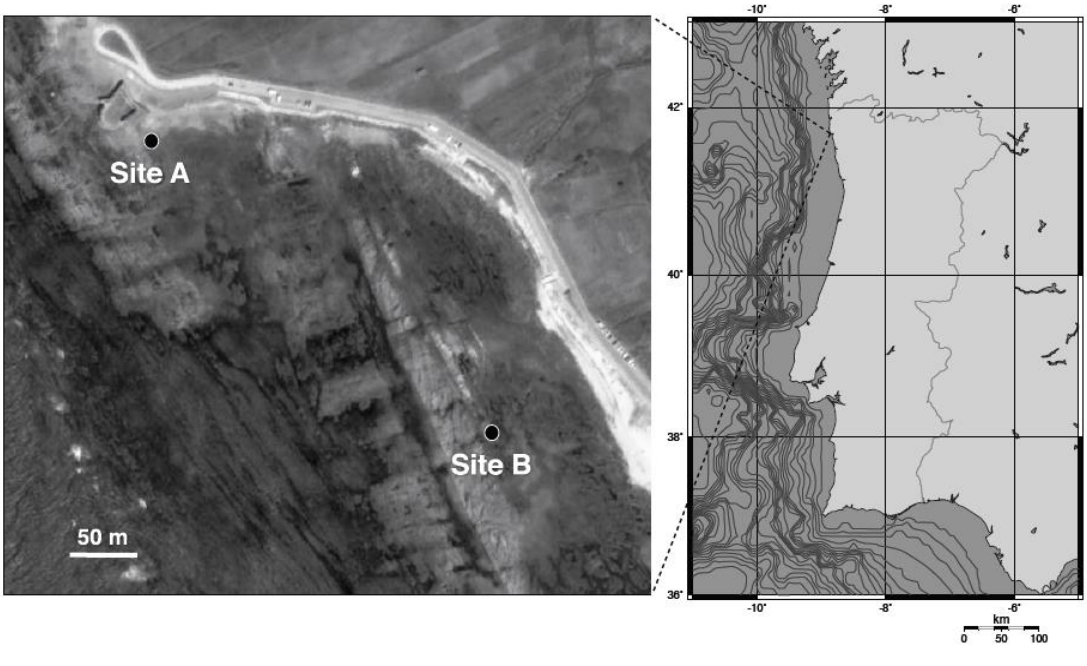

Figure 2.1. Map showing the location of the study site at Viana do Castelo, Portugal, and detailed view of the shore indicating the positions of the sampling sites A and B.

2.3.3. Periodicity of egg release – semilunar timescales

The periodicity of eggs settled was monitored daily from February 2002 to July 2003, in two sites for *F. vesiculosus* and one site for *F. spiralis* and *F. guiryi*. The eggs were collected on artificial substrates (each with 5.96 cm^{-2}) with a complex surface to promote egg retention (substrate preparation, fixation and sampling were as described in Ladah et al. 2003). Ten disks (or fifteen at sites where due to strong wave action disk loss appeared more likely) were fixed under the algal canopy in Site A and B, respectively. Disks were replaced daily during low tide and were returned to the laboratory where egg release was quantified under a dissecting microscope. Eggs of both species are morphologically similar; all eggs, which settled on disks, were assumed to be from the species that cover the disks, since *Fucus* eggs tend to fall within less than 0.5 m of the releasing alga (Serrão et al. 1997) and at each site the nearest individuals of any other *Fucus* species were more than 5 m away.

2.3.4. Periodicity of receptacle maturation – semilunar timescales

In order to compare the temporal variability of gamete maturation in both species, total and mature oogonia were quantified every two days during 2002 and once a week during 2003. In five haphazardly chosen individuals within each site and species, one receptacle was collected at low tide. The total number of oogonia and the mature oogonia were quantified under a microscope in 3 conceptacles of each receptacle. Mature oogonia were defined as those in which cleavage furrows were evident (as in Pearson et al. 1998).

2.3.5. Sampling at hourly – tidal timescales

The timing of egg release during the day was studied in June to July 2009, in natural populations of *F. vesiculosus*, *F. spiralis* and *F. guiryi*. Along the vertical direction, the distance between species/ entities was approximately 10 m, and at each height female gamete release by 5 individuals (females only for *F. vesiculosus*) was monitored in each of two replicate sites separated by approximately 5 m. Nylon mesh bags (40 µm) were used to enclose 2-3 receptacles per individual. A mesh size of 40 µm was chosen in order to retain eggs, while allowing water to circulate as freely as possible. The bags were attached to individuals with plastic clips with neoprene seals to provide a full seal around the thalli. During neap tide days, eggs were sampled every 2 h between 6:00 and 20:00, with a final sample taken at 21:30, prior to darkness. Bags were replaced carefully to prevent any egg loss in the field and were immediately taken to the laboratory where eggs were counted under a dissecting microscope. The bags were collected underwater or out of water, according to the tidal levels at each sampling time. At maximum high tide it was not possible to sample *F. vesiculosus* and the results presented are for 2 h periods before and after the high tide.

2.3.6. Statistical analyses

We tested the hypothesis that relaxed constraint on reproductive assurance due to selfing in *F. guiryi* has led to a reduction in reproductive synchrony, relative to the obligate outcrossing species *F. vesiculosus*. To do this, we used two-tailed *F*-tests (degrees of freedom are the number of observations-1 for each of the distributions) to compare the variance in the distribution of spawning times (defined as the time of the first high tide after sunrise) for spawning events of a particular threshold magnitude (e.g. ≥10 % or ≥20 % of the maximum value). These data were obtained from the complete seasonal – semilunar settlement dataset from Feb 2002–Jul 2003.

The cumulative frequency distribution of egg release during neap tide cycles (2 h sampling intervals; see above) was plotted against ranks (i.e., Pareto or rank/frequency plots). For this, egg counts for each bag (n =10 bags per 2 h sampling interval) were used to generate cumulative ranks, starting with the largest number of eggs released and adding sequentially the next largest observation until all observations have been summed. Linear regression lines were then fitted to such cumulated frequencies plotted in rank order and using logarithmic scales on both axes. Pairwise comparisons of linear regression slopes between species/ entities fitted to double logarithmic plots were performed by standard techniques using dummy (or indicator) variables to compare linear models using the R statistical package and a custom script. In addition, the distributions were also compared using more robust non-parametric Kolmogorov-Smirnoff tests.

2.4. Results

2.4.1. Seasonal periodicity of reproductive output

Gamete release assessed by egg settlement in the field over 2 years was strongly seasonal in all study species (Figs. 2.2a,b). At a monthly temporal scale the patterns were indistinguishable between the three species. Although some egg settlement was detected throughout the year (see Figs. 2.2a,b insets plotted on Log scale), major settlement was detected only in May to September in 2002, and also only after May in 2003, until at least July 2003, when the survey was completed.

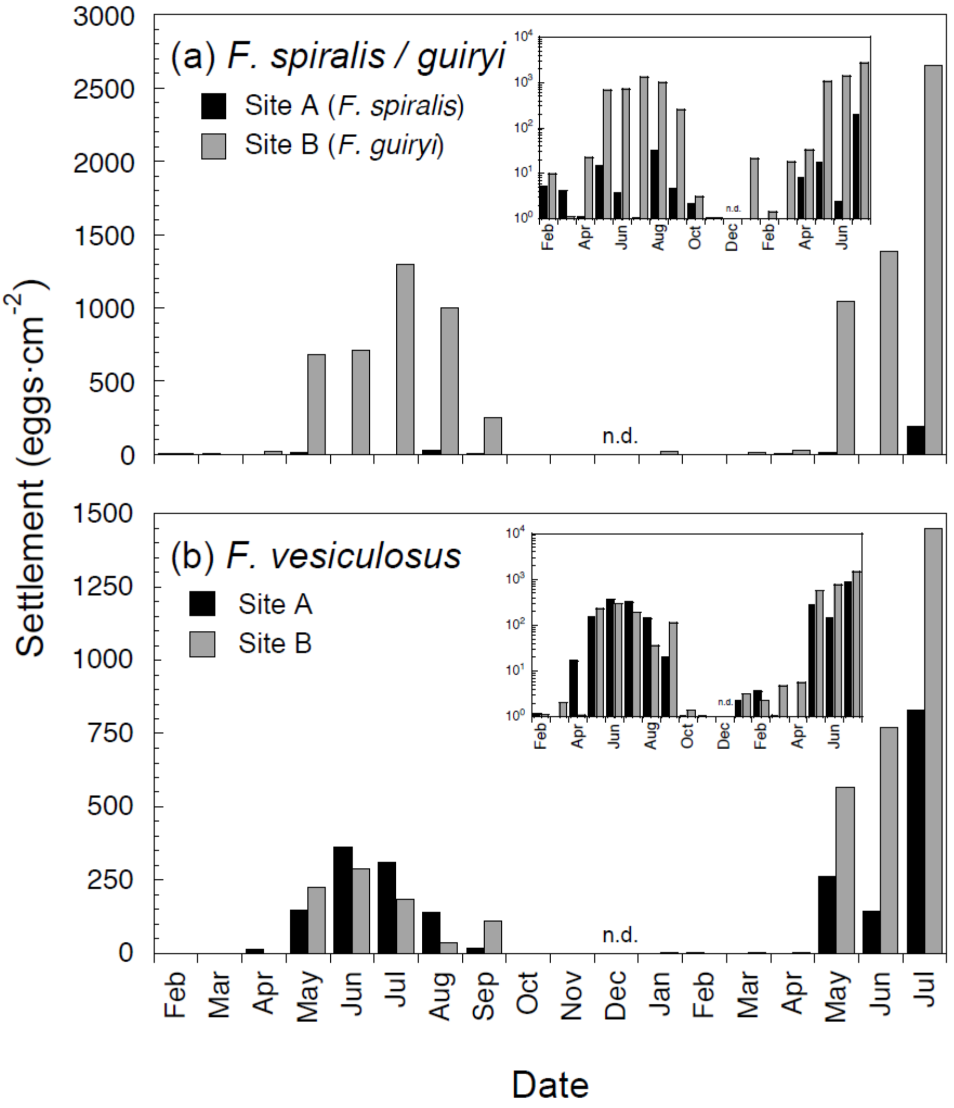

Figure 2.2. Monthly egg settlement from *Fucus spiralis* and *Fucus guiryi* (a) and *Fucus vesiculosus* (b) in site A (dark bars) and site B (grey bars) between February 2002 and July 2003. Periods when sampling was not carried out are represented by n.d. (no data). Insets show the data plotted on a Logarithmic scale.

Both total and mature oogonia were observed throughout the year, peaking in April–May, immediately prior to the onset of major settlement events (Figs. 2.3a,b). During the main reproductive season there was a general decline in oogonia, although numbers were similar in periods when settlement was still observed (in August) and when settlement was near zero (October–November). Abundance of mature receptacles declined following the main reproductive period, coinciding with declining reproductive output (personal observations). However, the continued presence of mature oogonia following the end of the main reproductive period in October suggests that gamete release may not be directly related to the availability of gametangia.

Consistently lower settlement occurred in *F. spiralis* (site A), which had low density of reproductive individuals in the immediate vicinity of settlement disks. We included these data in Fig. 2.2a to illustrate that settled eggs originated from reproductive individuals immediately adjacent to sampling disks. Egg settlement quantification at the study site at various distances from the nearest females also consistently demonstrated low dispersal (Monteiro, Pearson, Serrão, unpubl. data). In addition to supporting the absence of interspecific contamination between sites monitored for each species, the ca. 2-fold lower settlement observed in *F. vesiculosus* compared with *F. guiryi* (site B, Fig. 2.2) may also be related to the low egg dispersal, since all individuals of hermaphroditic species produce eggs, compared with only half of dioecious *F. vesiculosus* (assuming equal sex-ratios); randomly placed settlement substrates closer to males than females may therefore account for the reduced overall egg count for *F. vesiculosus*.

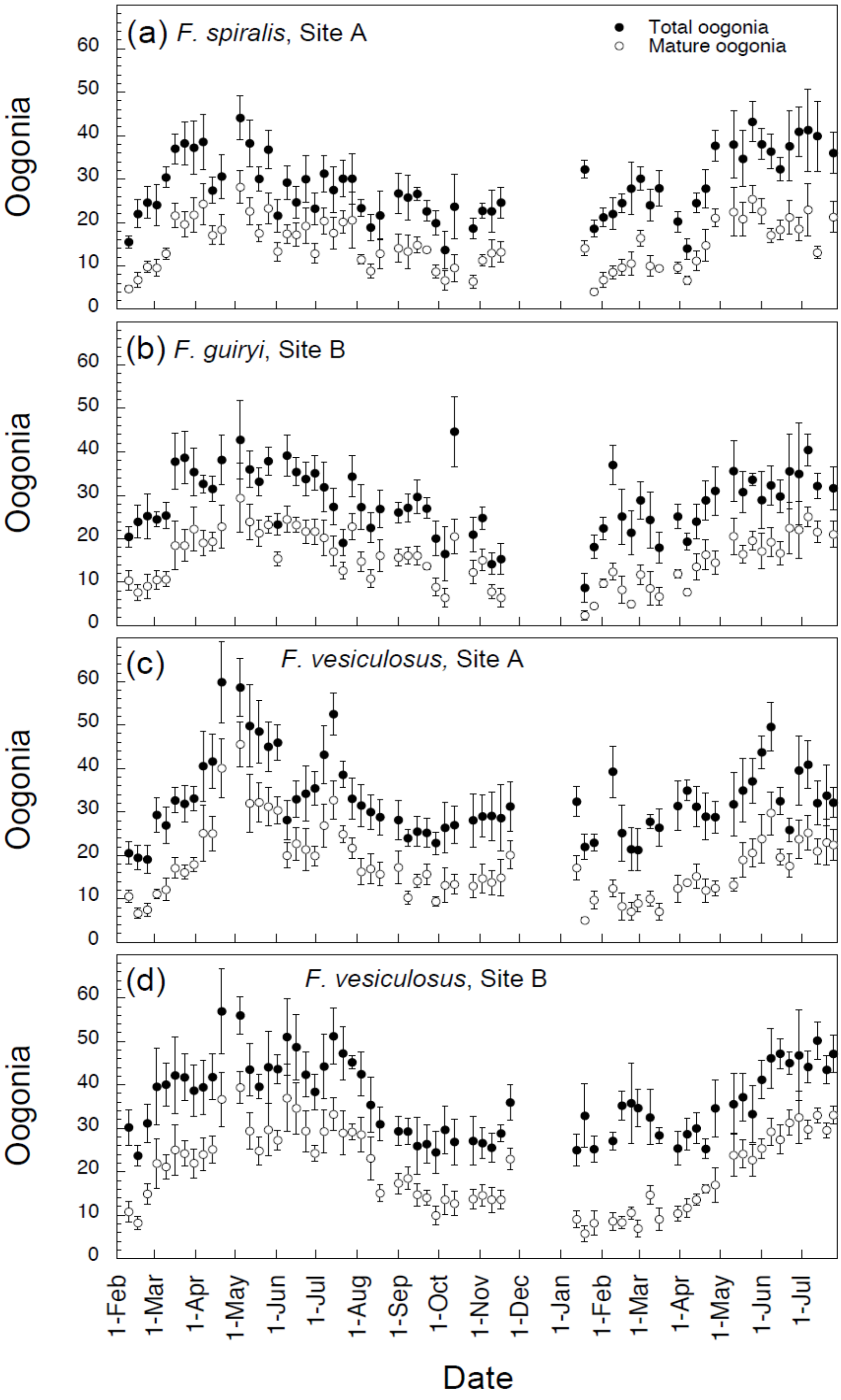

Figure 2.3. Total number of oogonia (closed symbols) and number of mature oogonia (open symbols) per conceptacle (n = 5 receptacles, ± SE) from *Fucus spiralis* (plot a), *Fucus guiryi* (plot b), and *Fucus vesiculosus* (plots c and d) between February 2002 and July 2003.

2.4.2. Periodicity of gamete release on a daily scale within the semilunar cycle

On a finer daily scale during the semilunar cycle, gamete release was highly discrete, with peaks of release coinciding with neap (minimum amplitude) tides in both 2002 and 2003 (Fig. 2.4). In 2002, 9 major settlement events, and in 2003 a further 6 were recorded over a total of 311 days. In most cases, release occurred over 1–2 days, more rarely with an additional 1–2 days of low release before or after the main peak. In 2003, release events were somewhat broader in time (5–6 days, compared with 1–2 days in 2002) and peak settlement was higher than in 2002. Greater gamete release in 2003 may have contributed to the temporal broadening of release events as a result of sampling efficiency (i.e., increased detection efficiency of larger release events may have contributed to apparent peak broadening). Overall, the data show that the large majority of days on which release occurred were shared between species, and reveal no temporal barriers to interspecific gamete encounters in the water column at a semilunar time scale.

We compared the daily tidal phase on days of major gamete release in this study with those of Berndt et al. (2002) for *F. vesiculosus* on western Atlantic shores in Maine, USA, because these authors observed release during spring tides, while we observed release exclusively during neap tides. However, the tidal phase (i.e., the timing of the low and high tides during the day) when gamete release occurred was very similar in both studies (Fig. 2.5a). Gamete release in *F. vesiculosus*, both in our study and in Berndt et al. (2002), happens mainly on days when the first diurnal high tide occurs in the late morning. In figure 2.5b we added tidal data from Brawley (1992) for days with maximum gamete release in the estuarine dioecious fucoid, *F. ceranoides*. In this case peak gamete release appears shifted forwards by about 2 h relative to *F. vesiculosus*.

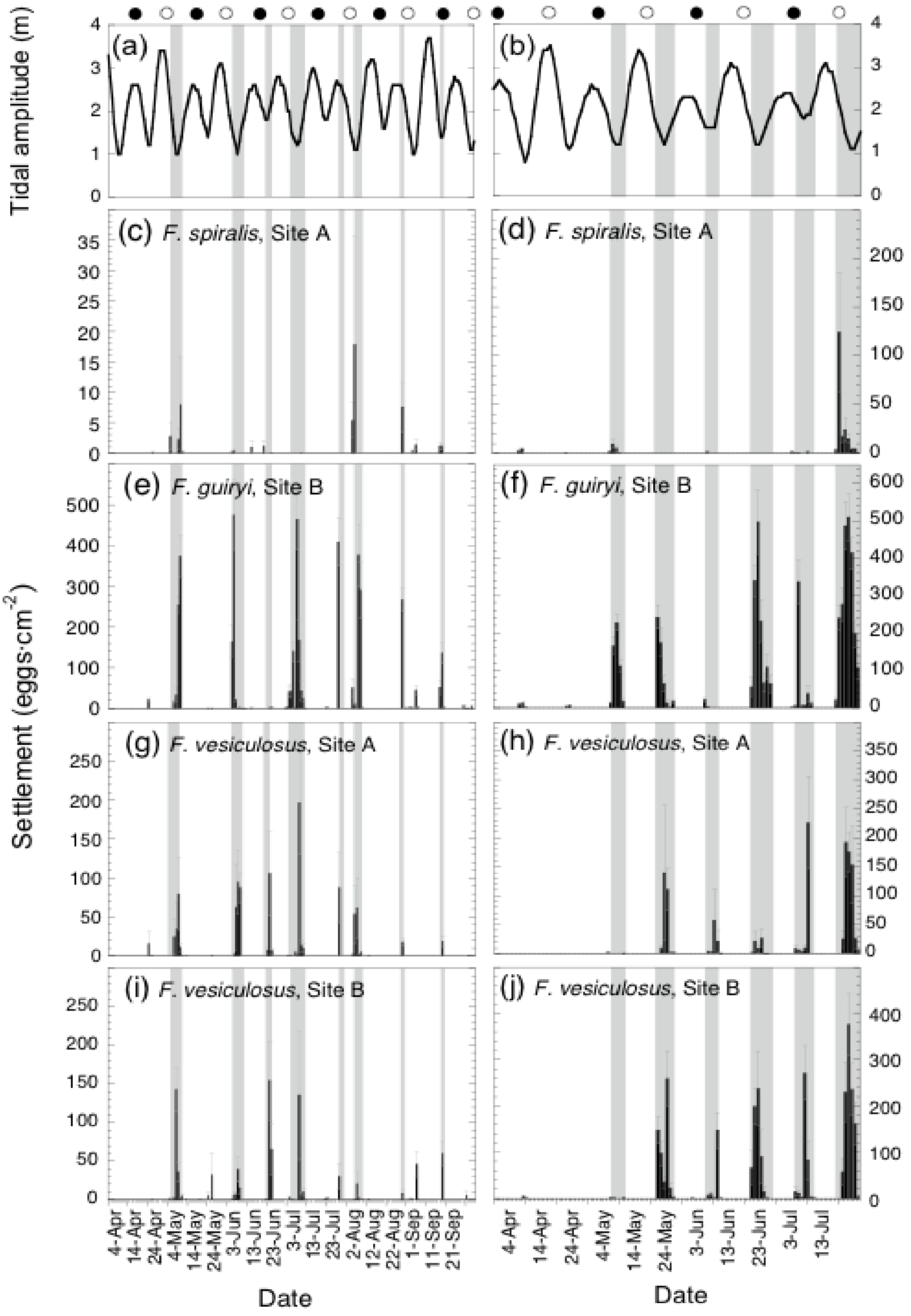

Figure 2.4. Daily egg settlement from *Fucus spiralis* (panels c and d), *Fucus guiryi* (panels e and f) and *Fucus vesiculosus* (panels g–j), between 1 April and 31 September 2002 (left hand panels) and 1 April and 31 July, 2003 (right hand panels). Values are means ± SE from n = 10 (Site A) or n = 15 (site B) sampling disks. Tidal amplitude (black line) and lunar phase for the sampling period are shown in a) and b); grey shading indicates the timing and duration of major settlement events from both species/sites.

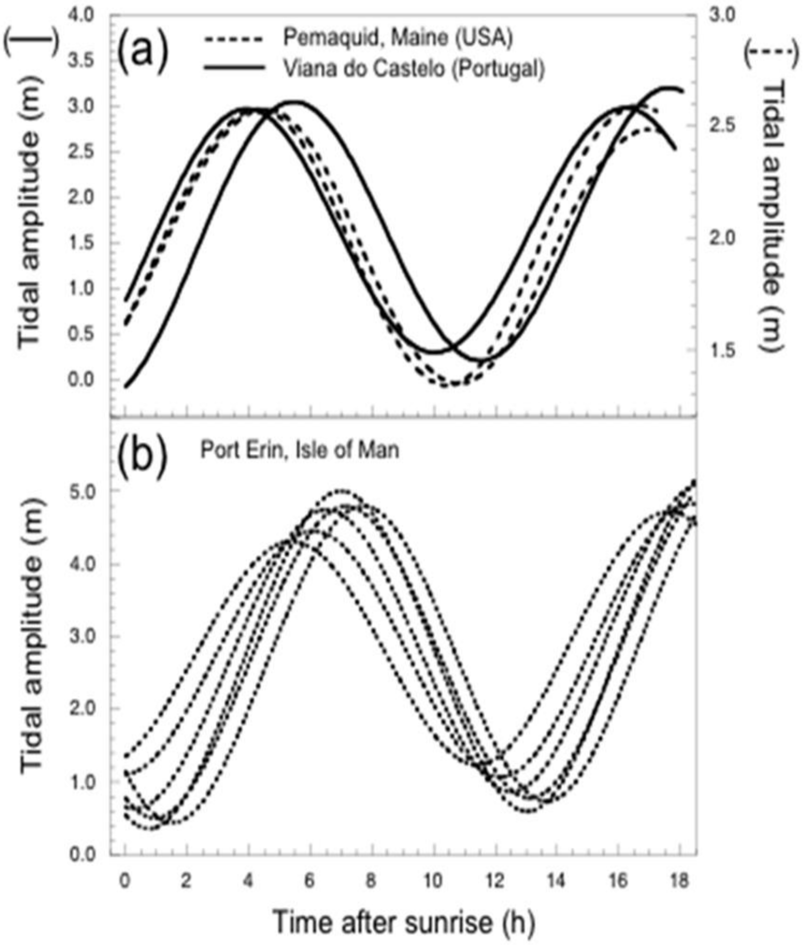

Figure 2.5. Relationship between tidal amplitude and time after sunrise on days with peak gamete release from a) *Fucus vesiculosus*, and b) *Fucus ceranoides*. Data are from Pemaquid, Maine, USA (Oct 8 and Nov 8, 1999; from Berndt et al. 2002), Viana do Castelo, Portugal (Jun 23, Jul 22, 2003; this study), and Port Erin, Isle of Man (Jul 17, Aug 1–3, 1989, and Jul 22, Aug 4, 1990; from Brawley 2002).

2.4.3. Interspecific comparison of synchrony on a daily scale within the semilunar cycle

To compare interspecific reproductive synchrony within semilunar cycles, we used the daily egg settlement data from 2002–2003 to compare the variance in the timing of settlement events above certain threshold values. The data were normalized as a percentage of the maximum value observed, and plotted against the time of the first daylight high tide (i.e., phase in the semilunar cycle) on the day of release (Fig. 2.6). Gamete release from hermaphroditic *F. guiryi* had a higher variance (i.e., was less synchronous) with respect to semilunar phase than that from *F. vesiculosus*. Mean settlement values ≥ 10% of the maximum, occurred on days when the maximum level of the day-time high tide (defined as the first high tide after sunrise) fell within the time interval 07:41–14:27 h in *F. guiryi* (a range > 6 h), but were restricted to days with peak high tides within the time interval 08:27–13:13 h in *F. vesiculosus* (ranging ca. 4:46 h) (two-tailed F-test; $F_{(32, 41)} = 2.083$, $p = 0.027$). For settlement events of ≥ 20% of the maximum the values were 07:41–13:49 h in *F. guiryi* (06:08 h) and 09:01–12:31 h in *F. vesiculosus* (03:30 h) (two-tailed F-test; $F_{(26, 30)} = 2.772$, $p = 0.008$).

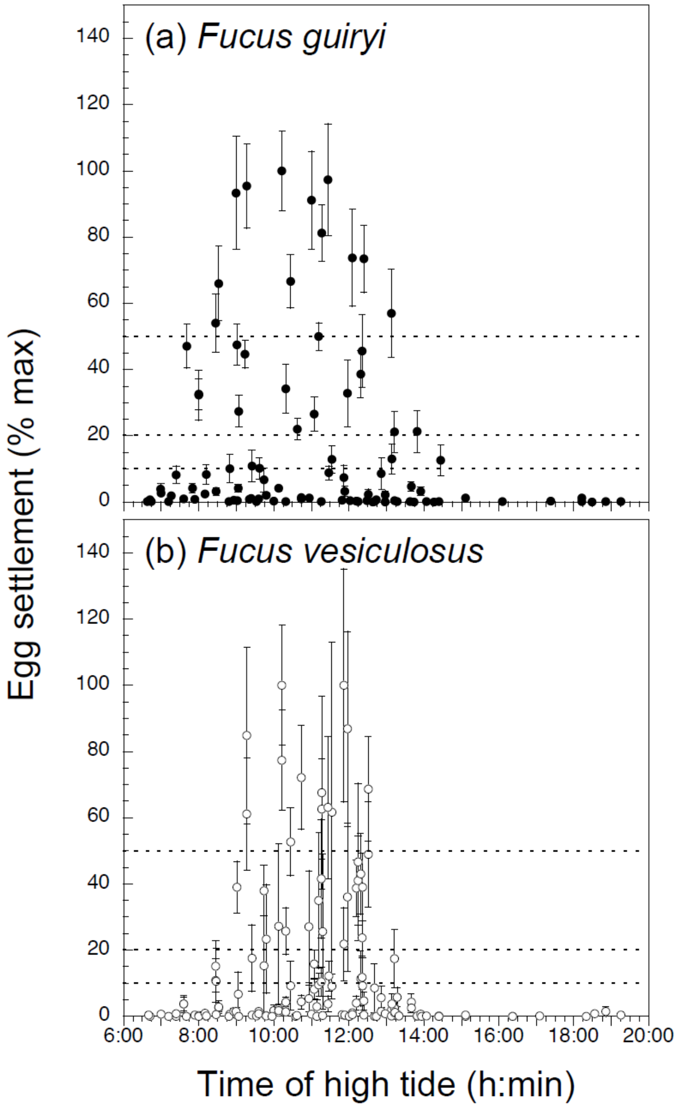

Figure 2.6. Relationship between the timing of high tide and egg settlement (shown as a percentage of the maximum) in a) *Fucus guiryi*, and b) *Fucus vesiculosus* at site A and B. Data are daily samples taken between February 2002 and July 2003 (n = 10 and 15 disks at site A and B, respectively, ± SE).

2.4.4. Intraspecific timing and synchrony of gamete release: hourly scale during the tidal cycle

During the 4 neap tide periods studied, major egg release events (defined as > 1000 eggs per bag in a 2 h sampling period) were observed on 8 days in *F. vesiculosus* (June 2–3, June 15–17, and July 17–19); 4 days in *F. guiryi* (June 3, 14, 17 and 18); and 6 days in *F. spiralis* (June 3, 5, 14, 17, 18 and July 3). While the three species showed co-occurring gamete release on several days, the largest events were shared mainly by the two hermaphrodites, *F. spiralis* and *F. guiryi* (Figs. 2.7a,b), to the exclusion of *F. vesiculosus* (Fig. 2.7c). Moreover, the timing of gamete release within the tidal cycle was divergent between *F. vesiculosus* and the two hermaphrodite species. While *F. vesiculosus* released gametes almost exclusively immediately prior to, and especially following, the high tide (mid-morning to early afternoon), *F. spiralis* and *F. guiryi* released gametes consistently at the earliest sampling time of 6:00. Although we were unable to measure gamete release during peak high tide in *F. vesiculosus* due to the difficulty of sampling immersed individuals *in situ*, our data are consistent with this being the case, as reported by Berndt et al. (2002) who observed the onset of release occurring prior to immersion. The precise timing of release in *F. spiralis* and *F. guiryi* remains uncertain, with both the dark to dawn transition, or release in the dark after the last sampling bags were attached (i.e., after 21:30 on the previous day) being possible.

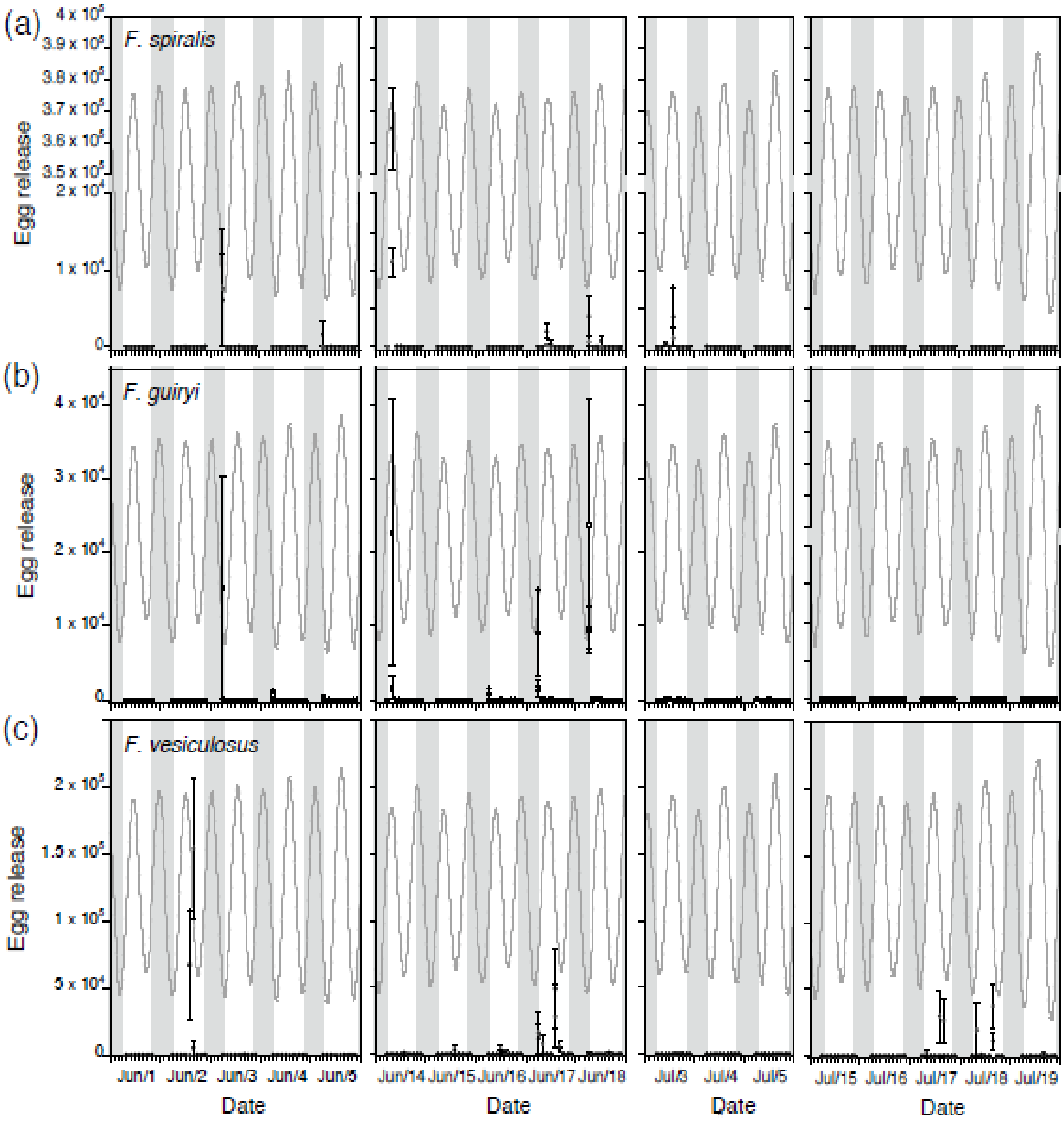

Figure 2.7. Egg release during 2 h sampling intervals (n = 5 ± SE) by a) *Fucus spiralis*, b), *Fucus guiryi*, and c) *Fucus vesiculosus* at two replicate sites/species (open and closed symbols) during four neap tide periods in June and July 2009. Grey lines are tidal amplitude and grey bars the dark periods during the daily cycle.

Double logarithmic plots of cumulative frequency against ranked egg release magnitude for 2 hourly intervals (Pareto plots) revealed power-law distributions for all three entities (*F. spiralis*, *F. guiryi* and *F. vesiculosus*; Fig. 2.8). The relationship deviated from linearity only at values of egg release >ca. 10^5 eggs per sampling bag (2–3 receptacles), which probably approaches the upper limit of eggs contained in the sample receptacles. The regression fit to the *F. vesiculosus* data was improved (r^2= 0.987 *versus* 0.952) by removing values $\geq 10^5$, so this was chosen as a cut-off value for number of eggs sampled. The fit of the regressions were also high for hermaphrodite species (r^2 = 0.9813 and 0.9701 for *F. guiryi* and *F. spiralis*, respectively), and were highly significant in all cases ($p < 0.0001$). Pairwise comparisons of the regression slopes showed no significant difference between the two hermaphrodite species (p = 0.208), but that both were significantly different from *F. vesiculosus* ($p < 0.0001$ in both cases). The same was found using Kolmogorov-Smirnoff tests; the distributions of egg release data did not differ between the hermaphroditic species (*F. spiralis versus F. guiryi*, p = 0.800) but differed between these and the dioecious *F. vesiculosus* (*F. guiryi versus F. vesiculosus*, p = 0.000; *F. spiralis versus F. vesiculosus*, p = 0.000). It follows that the exponent or scaling factor, α, for the power law relationships calculated from the regression slopes varied little between the two hermaphrodite species (0.4760 and 0.4883 for the *F. spiralis* and *F. guiryi*, respectively), but was lower for *F. vesiculosus* (0.3454).

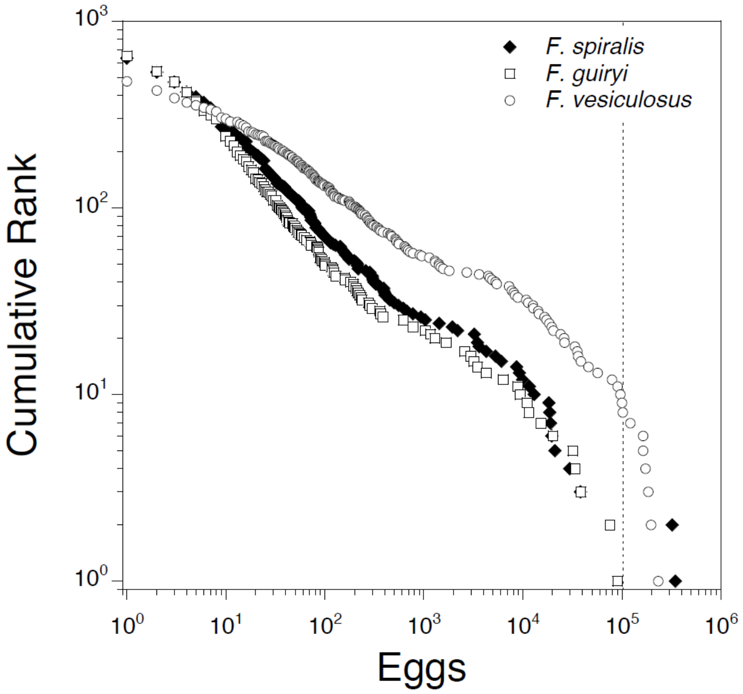

Figure 2.8. Pareto double Log_{10} plot of cumulative rank distribution of egg release. Data were collected during 2 h sampling intervals over four neap tide periods in June and July 2009 (see text and Fig. 2.7). Summary results of the regressions are 1) *Fucus spiralis*: Log(y) = 2.853–0.476*Log(x), R^2= 0.981; 2) *Fucus guiryi*: Log(y) = 2.767–0.488*Log(x), R^2= 0.970, and 3) *Fucus vesiculosus*: Log(y) = 2.812–0.345*Log(x), R^2 = 0.987.

2.5. Discussion

This study provides one of the most comprehensive descriptions to date of reproductive timing (egg release) at annual to hourly scales in the marine environment. We had two main goals; firstly to investigate the potential for interspecific variation in spawning time to act as a prezygotic ecological barrier to hybridization between sympatric, externally-fertilizing congeners. Our data show that, while coincident at seasonal and semilunar scales, differences in the timing of gamete release during single tides are consistent with a partial ecological barrier to hybridization. Secondly, we addressed the hypothesis that selfing species are under reduced selective constraint for spawning synchrony relative to obligately outcrossing species. We discovered that while reproductive synchrony remains a feature in selfing hermaphroditic species, it is reduced relative to sister species with obligate outcrossing. Reduced synchrony was identified on daily time scales as increased variance of major spawning periods during the semilunar reproductive cycle, and from a relative excess of small spawning events during single favourable tides. Finally, we provide evidence from these data and previously published studies on both sides of the Atlantic (Brawley 1992, Berndt et al. 2002) that gamete release in natural fucoid populations is controlled by environmental cues arising from the interaction of tidal and diurnal cycles, rather than semilunar cycles.

Spawning patterns at seasonal to daily temporal scales were highly coincident for *F. vesiculosus*, *F. spiralis* and *F. guiryi*. Reproductive output, estimated as daily egg settlement, a close proxy for gamete release (Brawley 1992, Pearson and Brawley 1996, Serrão et al. 1996), or oogonial maturation, followed essentially the same temporal pattern for the two hermaphrodite species *F. spiralis* and *F. guiryi*, and for dioecious *F. vesiculosus*. Maximum reproductive output occurred from late spring to summer (May–Sep). We are not aware of other comparative long term spawning datasets for *Fucus* spp. or other intertidal organisms. The restriction of most gamete release to massive spawning events over a few days during the reproductive season is typical, and parallels reports of mass reproductive events in broadcast spawning corals or green algae in tropical reef systems (e.g., Harrison et al. 1984, Clifton 1997, see also review by Serrão and Havenhand 2009).

Gamete release in all study species followed a ≈14 day period, synchronized with neap tides during semilunar cycles. Synchronous spawning with the same periodicity has been reported for both dioecious and hermaphrodite *Fucus* species (Brawley 1992, Pearson and Brawley 1996, Serrão 1996, Ladah et al. 2003, 2008). However, the timing within the semilunar cycle we observed (i.e., neap tide periods) is out of phase with data for *F. vesiculosus* from the eastern Atlantic (Maine coast, USA; Serrão 1996, Berndt et al. 2002), as well as those for an estuarine fucoid, *F. ceranoides* in the Irish Sea, UK (Brawley 1992), both of which show peak gamete release during spring tides around full and new moon. This apparent conflict is resolved by considering tidal, rather than semilunar cycles; tidal and diurnal cycles are similar between Maine and the Irish Sea, where mid– late morning high tides occur near full and new moons, whereas in northern Portugal, similar timing of high tides occurs during the neap period (i.e., around quarter moons). These results strongly suggest that gamete release in intertidal fucoids at the daily scale is entrained by the interaction between semidiurnal cycles of high and low tides and daily light:dark cues, rather than by cues arising from semilunar (spring – neap tide) cycles (Fig. 2.5). Similarly, a model combining fitness components associated with environmental cycles of differing periodicity was shown to underlie the apparent semilunar spawning patterns of a puffer fish (Yamahira 2004). While our data are compelling, independent confirmation could be obtained by comparisons of spawning times in natural populations in the eastern Atlantic, subject to a north – south gradient in the timing of tides (Schmidt et al. 2008) or in areas with striking tidal phase shifts along short distances, *versus* the western Atlantic, where there are no such tidal gradients.

At hourly time scales, spawning was largely synchronous between hermaphroditic *F. spiralis* and *F. guiryi*, but asynchronous between hermaphroditic and dioecious species, at daily and particularly hourly time scales (Fig. 2.7). Major spawning events in hermaphrodites occurred mainly on days when spawning by *F. vesiculosus* was very low or did not occur. Temporal reproductive isolation is a potentially effective means of reinforcing reproductive isolation, and a key ecological factor in sympatric speciation. Temporal isolation in sympatry has been considered unlikely (Coyne and Orr 2004), although empirical data from conspecific broadcast spawning algae and corals (Clifton 1997, Knowlton et al. 1997, Levitan et al. 2004), as well as theoretical models (Tomaiuolo et al. 2007) indicate that it does occur, with important evolutionary consequences for reproductive isolation and sympatric speciation in natural communities. These changes in synchrony and timing of reproduction in *Fucus* have evolved recently, since we now know that *F. spiralis* and *F. guiryi* most likely arose during the Pleistocene glacial cycles, and that their evolution coincided with a switch in reproductive mode from the ancestral dioecious state shared with *F. vesiculosus* (Cánovas et al. 2011). Our data for *F. vesiculosus* broadly agree with those of Berndt et al. (2002) in suggesting that major spawning events occur during daytime high tide immersion, since we observed that release began prior to, and continued after, the high tide (see also Ladah et al. 2008 for details). In contrast, major spawning events in hermaphrodite species were observed by the first sampling interval (06:00, around dawn), implying either that spawning occurred during the light to dark transition, during the night (the last sampling bags were placed at 21:30), or at the dark to light transition at dawn (Clifton 1997). In this respect it is interesting to consider that a more distantly related member of the family Fucaceae (*Silvetia compressa*) releases gametes in the laboratory on a light to dark transition after a period under favourable conditions in the light (Pearson and Brawley 1998, reviewed by Pearson and Serrão 2006), and can release gametes at low tide in the field (Johnson and Brawley 1998). Whatever the exact timing in hermaphrodite species, the interspecific shift in spawning of congeneric hermaphroditic and dioecious species observed here is consistent with selection favouring ecological reproductive isolation, since in congeneric corals a shift in spawning of only 2 h was inferred to reinforce reproductive isolation (Levitan et al. 2004).

Two hypothetical selective mechanisms could have originated the temporal reproductive isolation between the dioecious (*F. vesiculosus*) and hermaphroditic (*F. spiralis* and *F. guiryi*) lineages, sympatric speciation or reinforcement against hybridization. Before the split of the hermaphroditic and dioecious lineages (Cánovas et al. 2011) mutations leading to spawning timing difference could have created the reproductive isolation that originated, or contributed to, speciation. Alternatively, after lineage split, reinforcement might have occurred if hybrids were less fit than their parental species along each specific vertical zone, in agreement with the observations that these species vertical distribution is correlated with different stress resilience (Zardi et al. 2011) and that hybrids are found mainly in the contact zones along the vertical zonation (Billard et al. 2010). In such a scenario, populations that remained non-introgressed due to different spawning times would have been positively selected.

The species *F. spiralis* and *F. guiryi* were only recently detected as distinct genetic entities with limited gene flow (Cánovas et al. 2011, Zardi et al. 2011). We found no evidence for ecological prezygotic barriers to gene flow between these species, suggesting that either mating system is a sufficient hybridization barrier in these compatible and selfing entities (Perrin et al. 2007), or that other pre- or postzygotic barriers exist. Mating system variation can reinforce speciation processes, e.g. a shift from outcrossing to selfing resulted in almost complete reproductive isolation between potentially hybridizing plant species (Martin and Willis 2007). However, while flowering plants rely on pollen vectors (e.g., insects) for cross-fertilization, or have developed effective selfing mechanisms like cleistogamous (non-opening) flowers, it is less clear how effective mating system variation can be in broadcast spawning marine external fertilizers. Temporal differences in spawning times occur also in sympatric and inter-fertile marine invertebrates, and may play a role in prezygotic reproductive isolation, reinforcing some degree of gametic incompatibility (e.g., Forgarty et al. 2012). Since syngamy occurs shortly after spawning, selfing rates presumably depend on the spatial proximity of the eggs and sperm released from the same individual. Eggs from different fucoid species share the same pheromonal sperm attractant (Müller and Seferiadis 1977), and dioecious male conceptacles produce an order of magnitude more sperm than hermaphrodites (Billard et al. 2005b). Therefore, as a hybridization barrier in mixed stands of broadcast spawning congeneric species, mating system alone is expected to be an imperfect isolating mechanism.

Theoretically, the selection pressure to maintain spawning synchrony in dioecious (i.e., obligately outcrossing) broadcast spawning species should be stronger than for self-fertilizing hermaphrodites. Separate male and female individuals are selectively constrained to ensure reproductive success by spawning into the water column at the same time, while individuals of selfing hermaphrodites can assure reproductive success by releasing male and female gametangia from the same reproductive tissue (conceptacles), and are not therefore constrained in the same way. Our finding of larger temporal variance in spawning time during semilunar cycles in *F. spiralis* and *F. guiryi* than in *F. vesiculosus* supports the hypothesis of a reduced constraint on synchrony in hermaphroditic species. Despite this, at a smaller temporal scale, power law-like distributions of spawning intensity during reproductive periods (neap tide cycles) were observed in all three taxa/ genetic entities, indicating very few large release events and many small events. This indicates that, for both dioecious and hermaphroditic species, reproduction (and therefore recruitment, as the two processes are closely linked in fucoids) depend for their success on very limited temporal ‘‘windows of opportunity’’. During these rare events of synchronous spawning, the predominant form of crossing may still be between neighbouring individuals due to the limited gamete dispersal (Serrão et al. 1997), which may explain high levels of biparental inbreeding observed in several *F. vesiculosus* populations (Perrin et al. 2007). Although our data support a reduced constraint on synchrony in hermaphroditic species at semilunar scales, at smaller hourly scales within peak release days, the opposite trend is observed, possibly resulting from their use of different environmental triggers for circadian release timing. The shallower slope (significantly lower α) of the power law relationship in dioecious *F. vesiculosus* (i.e., greater ‘‘evenness’’), indicates less synchrony at hourly scales during the neap tide release periods. A possible reason for this contrast could be use of different cues for gamete release timing within circadian scales. Spawning in *F. vesiculosus* was observed to coincide with immersion at high tide, a more extended interval including the immediately prior and following emersion periods. In contrast, the shift to low tide (emersed) release in *F. spiralis* and *F. guiryi*, possibly in response to putative dark/light shift signals, appears to result in more synchronous release at this smaller temporal scale.

On a semi-lunar scale, our data support the prediction that constraints on reproductive synchrony are reduced in selfing species in the intertidal. However, the broadly coincident semilunar spawning patterns, as well the power-law relationships for egg release, showing high synchrony for all entities independent of mating system, both indicate that any synchrony differences are quite small. Why is reproductive synchrony maintained in hermaphrodite species at all? Possible reasons include evolutionary contingency, given the very recent divergence of the two species (Müller and Seferiadis 1977, Cánovas et al. 2011), maintenance of adaptive potential by some degree of continued outcrossing (Stebbins 1957, Serrão et al. 1999), and/or ecological factors unrelated to reproduction directly. If *F. spiralis* and *F. guiryi* arose recently from a dioecious ancestral lineage (Cánovas et al. 2011), then synchrony may simply be a retained ancestral trait. Populations of *F. spiralis* and *F. guiryi* show evidence of high levels of inbreeding (Engel et al.2005, Perrin et al. 2007), which may be favoured in marginal habitats (such as the upper intertidal shoreline where *F. spiralis* and *F. guiryi* occur), and where maintaining locally adaptive gene combinations and/or purging of deleterious alleles may be particularly advantageous (reviewed by Takebayashi and Morrell 2001). Nevertheless, the benefits of occasional outcrossing may be maintained via continued selection for reproductive synchrony. A third possible reason may relate to other life-history traits such as limiting dispersal to favourable potential habitats. Since in fucoids zygotes settle and develop immediately post-fertilization, the timing of spawning may also be selected for optimal recruit survival in the steep vertical abiotic gradients of the intertidal zone, as suggested earlier (Pearson and Serrão 2006). Releasing gametes at low tide by these hermaphroditic taxa might favour retention of propagules at their higher intertidal range as the upwards moving tide washes pre-released gametes onto local safe micro-sites. Thus gamete release at slack high tide (Berndt et al. 2002), or at low tide in selfing species, besides maximising fertilization success, might simultaneously limit zygote wastage from dispersal out of their narrow favourable intertidal range.

Fucoid algae are a fascinating system in which to address, from an ecological viewpoint, some of the problems associated with maintaining reproductive barriers and more broadly with speciation/ hybridization processes, in broadcast spawning marine species. The timing of reproduction in the intertidal zone may be particularly constrained for broadcast spawners, with very limited temporal windows for reproductive assurance imposed by immersion-emersion cycles and the dilution effects of surf zone hydrodynamism (Denny and Shibata 1989). Therefore species with incomplete reproductive isolation must balance reproductive assurance (e.g., fertilization success under optimal hydrodynamic conditions) while minimizing hybridization with sympatric congeners. The species studied here have incomplete reproductive isolation, and low proportions of hybrids are commonly observed, mainly at contact zones (Billard et al. 2005ab, 2010, Engel et al. 2005), but are nevertheless maintained as genetically distinct entities in sympatry.

While the seasonal, tidal and diurnal cues entraining gamete release are shared by sister taxa, a temporal shift in reproductive timing within single tides constitutes an ecological barrier to gene flow between the selfing hermaphrodite, *F. spiralis* and *F. guiryi* and dioecious *F. vesiculosus*. Mating system variation plays two important roles; while dioecious species require high tide immersion to mix gametes and a high degree of reproductive synchrony, selfing hermaphrodites are less constrained, possibly including reproduction during emersion at low tide (Brawley et al. 1999). The mechanism(s) preventing gene flow between the two hermaphroditic species *F. spiralis* and *F. guiryi* remains to be seen, but subtle differences in reproductive timing (Levitan et al. 2004), gamete release during low tide emersion, and selfing (Martin and Willis 2007) may, separately or in concert, be sufficient prezygotic barriers to maintain them as distinct genetic entities in sympatry.

2.6. Acknowledgments

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Chapter 3

Temporal windows of reproductive opportunity reinforce species barriers in a marine broadcast spawning assemblage

3. Temporal windows of reproductive opportunity reinforce species barriers in a marine broadcast spawning assemblage

3.1. Abstract

Prezygotic isolating mechanisms act to limit hybridization and maintain the genetic identity of closely-related species. While synchronous intraspecific spawning is a common phenomenon amongst marine organisms and plays an important role in reproductive success, asynchronous spawning between potentially hybridizing lineages may also be important in maintaining species boundaries. We tested this hypothesis by comparing reproductive synchrony over daily to hourly timescales in a sympatric assemblage of intertidal fucoid algae containing selfing hermaphroditic (*Fucus spiralis* and *Fucus guiryi*) and dioecious (*Fucus vesiculosus* and *Fucus serratus*) species. Our results confirm that gametes are released on semi-lunar cycles in all species. However, sister species with different mating systems showed asynchronous spawning at finer circadian timescales, thus providing evidence for a partial reproductive barrier between hermaphroditic and dioecious species. Finally, our data also emphasize the ecological, developmental, and/or physiological constraints that operate to restrict reproduction to narrow temporal windows of opportunity in the intertidal zone and more generally the role of ecological factors in marine speciation.

Keywords: External fertilization; Mating system; Prezygotic reproductive barriers; Semilunar and tidal timescales; Sympatric species

3.2. Introduction

Reproductive success in organisms with external fertilization is highly dependent on gamete encounter rates. Mechanisms such as spawning synchrony, optimal spawning conditions, morphological and physiological adaptations, and chemical signals (e.g., pheromone systems) all increase fertilization rates, particularly in sessile organisms (Harrison et al. 1984, Levitan and Petersen 1995, Pearson and Brawley 1996, Serrão et al. 1996, Clifton 1997, Brawley et al. 1999, Yund 2000, Santelices 2002, Pearson and Serrão 2006, Serrão and Havenhand 2009). The widespread occurrence of synchronous spawning amongst marine organisms suggests that the traits involved are strongly favoured by natural selection. However, where closely related species reproduce in sympatry, and where hybrids are less fit than the parental lineages, selection may also strongly favour asynchrony in reproductive timing between potentially hybridizing lineages. The evolution of such ecological mechanisms to minimize hybridization is crucial to preserve species identities, and may be key factors driving assortative mating during sympatric or ecological speciation (Palumbi 1994, Gardner 1997, Coyne and Orr 1998, Fukami et al. 2003, Levitan et al. 2004).

The brown algal genus *Fucus* (Phaeophyceae, Heterokontophyta) is a useful model for reproductive ecologists. Several species co-exist on North Atlantic rocky intertidal shores, occupying distinct but overlapping vertical niches with respect to tidal level and emersion stress intensity. Two major clades have been identified in *Fucus,* in both of which dioecious (outcrossing) and hermaphrodite (selfing) sister species have arisen (Cánovas et al 2011). Similar evolutionary patterns are observed in other groups of closely related plant and algal species, and is thought to promote reproductive isolation and divergence leading to speciation (Barrett 1998, Dorken et al. 2002, Engel et al. 2005, Perrin et al. 2007), and to maintain species barriers (Maynard Smith 1978). Selfing increases reproductive assurance and colonizing capacity (Pannell and Barrett 1998, 2001), while reducing the chances of hybridization. However, the costs of inbreeding include lower genetic variation and effective population size compared with obligatory outcrossing dioecious species (Hamrick and Godt 1996, Holsinger 2000). Hybridization in *Fucus* has been reported in several studies (Coyer et al. 2002, 2006, Wallace et al. 2004, Billard et al. 2005ab, 2010, Engel et al. 2005, 2011, Neiva et al. 2010), with hybrids reaching reproductive maturity in some cases (Billard et al. 2005b). Indeed, historical introgression has left clear evolutionary signals in extant lineages (Neiva et al. 2010). Despite this, contemporary levels of hybridization are apparently insufficient to blur the boundaries of distinct genetic entities in sympatry (Billard et al. 2010, Zardi et al. 2011).

The reproductive ecology of *Fucus* has been studied quite extensively; gametes are released with a semilunar periodicity and under calm water motion (i.e., following intervals of several hours under low current velocity, estimated as $< 0.2\ m \cdot s^{-1}$) (Brawley 1992, Pearson and Brawley 1996, Serrão et al. 1996, Pearson et al. 1998, Berndt et al. 2002, Monteiro et al. 2012). However, comparative studies of spawning over fine circadian timescales are lacking for sympatric species assemblages. Such an approach might identify potential sources of reproductive isolation and improve our understanding of the evolution of reproductive isolation in marine broadcast spawners.

Here we present results of field and laboratory studies focusing on fine-scale temporal variation in gamete release during daily tidal cycles between two hermaphroditic and two dioecious *Fucus* congeners, to test whether differences in spawning time may act as a prezygotic barrier to hybridization between closely related species.

3.3. Material and methods

3.3.1. Study site and species

The study site was Viana do Castelo, northern Portugal (41º41’47N 8º51’10W), which is the southernmost sympatric distributional limit of the four species of *Fucus* studied. There, *Fucus spiralis* is found in the high intertidal zone; *Fucus guiryi* and *Fucus vesiculosus* in mid-intertidal zone; *Fucus serratus* in the low-intertidal zone.

The gametes in all species of the genus *Fucus* develop inside gametangia in specialized apical structures called receptacles. In dioecious species, the sperm and eggs develop in different individuals (male and female) whereas in hermaphrodites both egg and sperm occur in same individual. Spawning consists in the release of gametangia that are negatively buoyant (i.e., they sink). Each female gametangium (oogonium) contains 8 eggs (non motile, ca. 80 µm in diameter) and each male gametangium (antheridium) contains 64 sperm (motile, ca. 5 µm in length). The gametangia open shortly upon release in seawater liberating negatively bouyant eggs and negatively phototactic sperm (which therefore swim towards the bottom). Fertilization then occurs externally, and most likely near the substrate. Fertilization success in *Fucus* species has been shown to be high (Brawley 1992, Pearson and Brawley 1996, Serrão et al. 1996, Pearson et al. 1998, Berndt et al. 2002, Monteiro et al. 2012). Egg dispersal is highly restricted since eggs tend to fall immediately below the releasing individual (Serrão et al. 1997, Teixeira et al. 2016). The occurrence of fertilization shortly after synchronous egg and sperm release together with low gamete dispersal might function as partial prezygotic barriers preventing hybridization between species occupying different tidal zones. Putative hybrids (identified as intermediate genotypes) were found mainly in the contact zones where species overlap, however they are rare (Engel et al. 2005, Billard et al. 2010).

Mature reproductive individuals of *F. guiryi* (hermaphroditic) and female *F. vesiculosus* (dioecious) were collected from the same site for tidal and circadian laboratory experiments. Species were identified as described previously (Zardi et al. 2011). Sampling of eggs (for natural spawning patterns) and mature individuals (for experimental manipulation of spawning conditions) took place in the middle of their respective intertidal range, to avoid hybrids that are mainly found at overlapping range edges (Engel et al. 2005, Billard et al. 2010).

3.3.2. Natural spawning patterns – semilunar and tidal timescales

Egg release at semilunar timescales was estimated using rugose artificial substrates (5.96 cm^{-2}) to retain settled eggs. Egg settlement for the four species was monitored daily at two sites between Jun 7 and Aug 3, using five disks per site per species fixed under the algal canopy, as described previously (Ladah et al. 2003, Monteiro et al. 2012).

Egg release during tidal cycles was monitored during four periods, consisting of a few days before and after the neap tides (lower tidal amplitude), when spawning peaks take place (Monteiro et al. 2012). These were the days when minimal low tide level was higher than ca. 1 m and the maximal high tide level was lower than ca. 3 m, in Jun (9–12 and 22–27) and Jul (9–13 and 23–28). Nylon mesh bags (40 µm) were used to retain eggs; *Fucus* eggs are all larger than 60 µm (Serrão et al. 1996). Each bag contained 2-3 receptacles per individual (females for dioecious species). During each sampling period, for each species, 5 individuals (1 bag per individual) were monitored for egg release at each of 2 sites (separated by approximately 5 m; Monteiro et al. 2012). The bags were collected and replaced every 2 h between 5:00 and 22:00 h in June 9–12 and 22–27 and July 9 and 23 (the first and last samples were taken in darkness). To complement the data with detailed patterns of night release, in July the sampling period was extended over the night, i.e., over 24 h per day (sampling was performed every 2 h during 88 and 94 consecutive hours in Jul 10–13 and 24–28, respectively).

3.3.3. Experimental manipulation of spawning conditions

The effects of light and tidal cycles on the timing of gamete (egg) release were studied in *F. guiryi* and *F. vesiculosus* in a laboratory experiment. *Fucus vesiculosus* (dioecious) was sexed in the laboratory to select females; hermaphroditic receptacles (*F. guiryi*) contain both oogonia and antheridia. Mature receptacles were excised and acclimated in individual 50 mL tubes (Falcon) containing 40 mL filtered seawater (SW; 35 psu) for 2 days prior to quantification of egg release, and SW was replaced daily.

In a culture chamber (14 ºC; 12:12 h light:dark cycle; 100 µmol photons $m^{-2}s^{-1}$), tidal regimes were simulated in tanks for 24 days as follows: Tank 1 – timing of high and low tide coincident with that at Viana do Castelo. Tank 2 – opposite phase to tank 1, i.e., peak low tide in tank 2 corresponded to peak high tide in tank 1. Tank 3 – no tides, receptacles were constantly immersed. Tides were programmed by timers controlling the pumping and draining of SW in the tanks (complete pumping and draining each took ca. 5 min). Receptacles were submerged for 4 h per high tide, corresponding to 2 h on either side of the natural timing of high tide (tank 1) or of low tide (tank 2) in the field. Immersion time was within the range seen by both species on the shore. Eight individuals were used as replicates for each species. For each species and tank, two receptacles of similar size were placed in each of n = 8 tubes. To allow SW to drain at low tide a small hole was made in the base of the tubes, protected by nylon mesh (40 µm) to retain the eggs. Egg release was quantified for 24 days, receptacles were transferred to tubes with fresh SW at 8:30 h, 12:30 h, 16:30 h and 20:30 h (no collection was performed at night). The eggs present in each tube were counted under a dissecting microscope. The numbers of eggs released were comparable across replicates within species on the basis of equal amounts of reproductive tissue (2 receptacles) being used per replicate. However, fecundity was not tested in this study because the variable of interest was the timing of maximum gamete release, rather than absolute numbers of gametes released, to assess our hypothesis (i.e., whether differences in spawning time may act as a prezygotic barrier to hybridization between closely related species). Previous studies (Billard et al. 2005b, Monteiro et al. 2012) have shown that the variability in the amounts of eggs produced is orders of magnitude lower than the variability between the numbers of eggs released on a peak spawning day *versus* the amounts released on other days.

3.3.4. Statistical analyses

Analyses aimed to test both effects of circadian and tidal regimes. Cumulative egg release in tidal shift treatments (tidal conditions; tank 1 and 2) was analyzed under the following design: species (2 levels: *F. guiryi* and *F. vesiculosus*, orthogonal and fixed), tanks (2 levels, orthogonal and fixed), daytime high tide interval (3 levels: between 8:00–12:00 h, 12:01–16:00 h and 16:01–20:00 h, nested within tanks) and sampling time (4 levels: 8:30 h, 12:30 h, 16:30 h and 20:30 h, orthogonal and fixed).

To assess the effects of circadian light:dark intervals on egg release by *Fucus* in the absence of tides, cumulative egg release in the tank without tides (atidal condition) was analyzed under the following design: species (2 levels: *F. guiryi* and *F. vesiculosus*, orthogonal and fixed) and sampling timing (4 levels: 8:30 h, 12:30 h, 16:30 h and 20:30 h, orthogonal and fixed).

To test for differences in cumulative egg release between light and dark periods (circadian cycles) the following design was analyzed: species (2 levels: *F. guiryi* and *F. vesiculosus*, orthogonal and fixed), tide conditions (4 levels: 8:00–12:00 h, 12:01–16:00 h, 16:01–20:00 h and no tide, orthogonal and fixed) and circadian cycles (2 levels: day and night, orthogonal and fixed).

In all analyses the number of replicates was eight and cumulative egg release for each sampling interval was summed over 24 days. Means were compared using PERMANOVA (Anderson 2001). The permuted p-value was the number of times the p-value was equal to or outside the 95% confidence interval divided by the total number of permutations (9999).

3.4. Results

3.4.1. Natural spawning patterns – semilunar and tidal timescales

The peak of egg release in both hermaphroditic and dioecious species occurred with a periodicity of 2 weeks coincident with neap tides (Appendix S3.1 in Supplementary Information). Peaks of egg release were observed during all four neap tide periods monitored over 2 months, and the majority of days on which release occurred were coincident across all species.

During the 4 neap tide periods studied in 2011 (Fig. 3.1), major egg release events (defined as > 1000 eggs per bag in a 2 h sampling period) were observed on 7 days in *F. spiralis* (June 10–12, 27 and July 13, 22–23); 9 days in *F. guiryi* (June 10, 12, 25, 26 and July 9,12,13,23,26), 15 days in *F. vesiculosus* (June 9, 13, 25–27 and July 9, 25, 27) and 7 days in *F. serratus* (June 9,11, 27 and July 9,11,12, 27). While gamete release co-occurred in all four species on several days and mostly when individuals were immersed, the timing of spawning within the tidal cycle was clearly divergent between dioecious (*F. vesiculosus* and *F. serratus*) and hermaphroditic (*F. spiralis* and *F. guiryi*) species (Fig. 3.1).

Since hermaphroditic species consistently released eggs within the period 22:00–05:00 h in June (Figs. 3.1A,B), in July we sampled egg release during the night between Jul 10–13 and 24–28 (Figs. 3.1C,D). This sampling confirmed that spawning was coincident with the nightly high tide (Figs. 3.1C,D). In *F. spiralis* the major egg release event was observed at 03:00 h on 13 July (Fig. 3.1C and Fig. 3.2C) and in *F. guiryi* maximum release was recorded at 01:00 and 03:00 h on July 12–13 and 28 (Figs. 3.1C,D and Fig. 3.2D). Some early morning release was also seen coinciding with the early high tides (Figs. 3.1A–D). In both species peaks of egg release (≥ 200 eggs per bag) frequently occurred when individuals were immersed (Figs. 3.3A,B).

In contrast to hermaphrodites, both dioecious species released eggs exclusively during the daytime (Figs. 3.1A–D and Figs. 3.2E,F). The majority of release in *F. serratus* took place between 11:00 and 15:00 h. Timing appeared somewhat less tightly constrained in *F. vesiculosus* (c.f. Figs. 3.2E,F), but major egg release events clustered mainly around peak daytime high tides for both species (Fig. 3.1 and Figs. 3.3C,D).

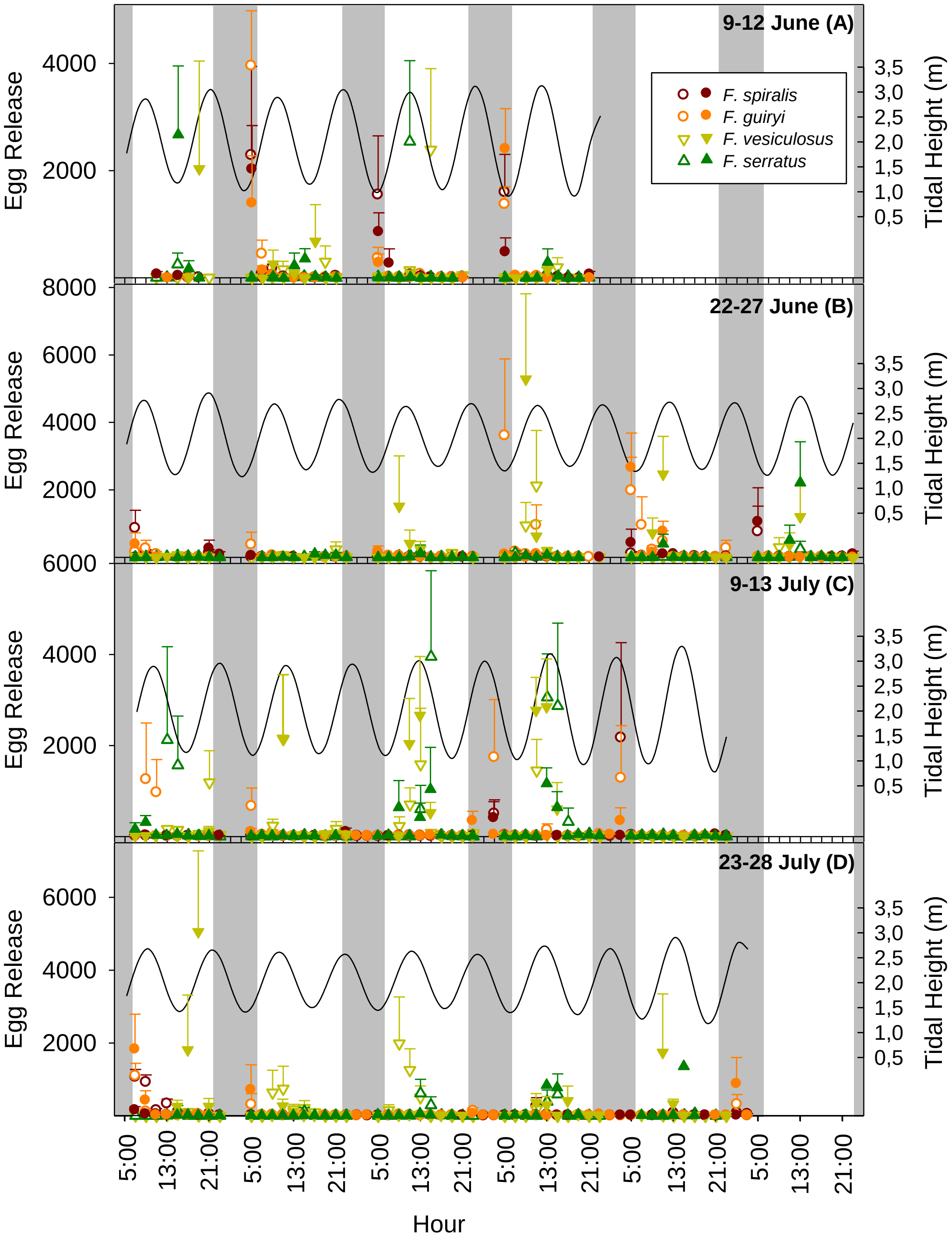

Figure 3.1. Egg release during 2 h sampling intervals (n = 5 ± SE) by hermaphroditic (circles) *Fucus spiralis*, *Fucus guiryi*, and dioecious (triangles) *Fucus vesiculosus* and *Fucus serratus* at two replicate sites (open and closed symbols) during four neap tide periods. Black lines show tidal heights and grey bars the night periods.

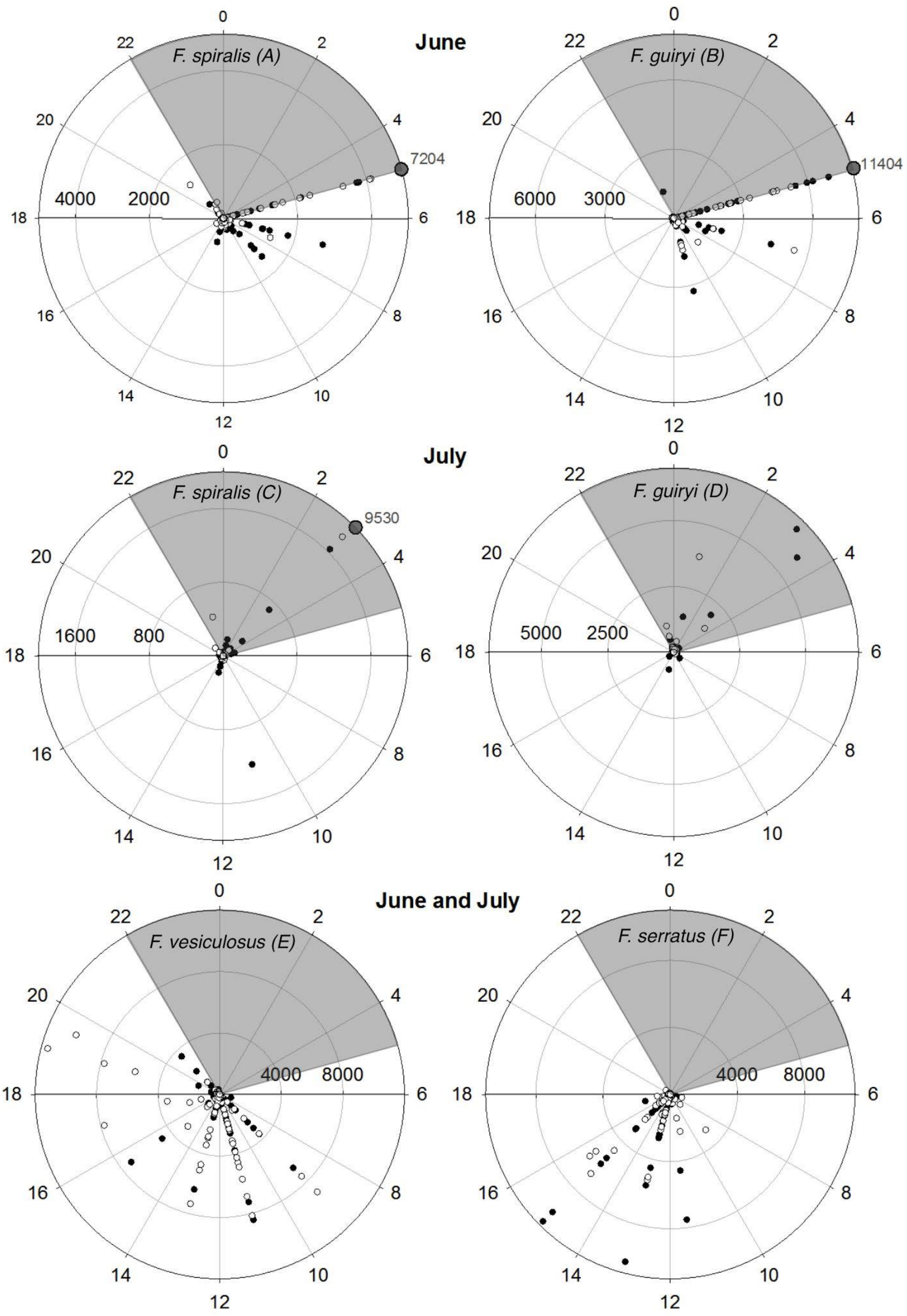

Figure 3.2. Egg release (radial data) relative to timing of day (angular data) by (A) *Fucus spiralis*, (B) *Fucus guiryi*, in June (sampling between 05:00 and 22:00 h); (C) *Fucus spiralis*, (D) *Fucus guiryi* in July; (E) *Fucus vesiculosus* and (F) *Fucus serratus* in June and July at two replicate sites (open and closed symbols). Grey boxes are the dark periods during daily cycle.

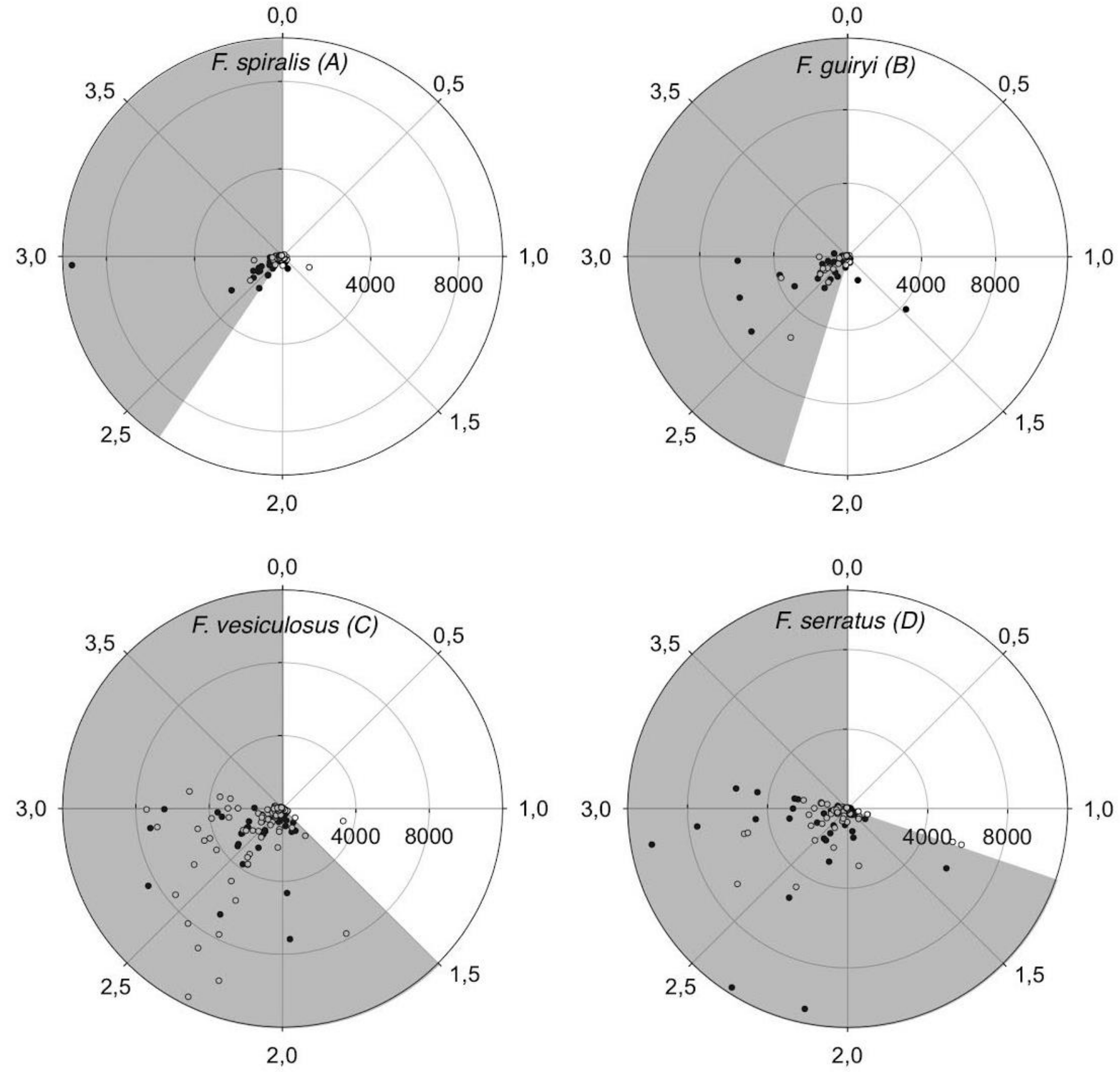

Figure 3.3. Egg release (radial data) relative to tidal level (angular data) by (A) *Fucus spiralis*, (B) *Fucus guiryi*, (C) *Fucus vesiculosus* and (D) *Fucus serratus* at two replicate sites (open and closed symbols). Grey boxes indicate periods of immersion during the tidal cycles.

3.4.2. Experimental manipulation of spawning conditions

The comparison of egg release by *F. guiryi* (hermaphroditic) and *F. vesiculosus* (female dioecious) under experimentally manipulated tidal (high *versus* low tide) and circadian (light *versus* dark) regimes showed that these two species differed in their patterns of cumulative egg release over superimposed circadian and tidal cycles (Table 3.1A; Sp x Ti(Ta) x Sa interaction). However, tidal cycle phase (tank 1 *versus* tank 2) had no effect, indicating that egg release was entrained more by environmental conditions rather than intrinsic rhythms.

In hermaphroditic *F. guiryi*, egg release consistently occurred during the night (20:31–08:30 h) in all tanks, and extended into the morning period (08:30–12:30) when the high tide occurred in the morning (08:31–12:30 h), or early afternoon (12:31–16:30 h) (Figs. 3.4A,B). During earlier high tides (08:31–12:30 h) a second late afternoon peak of egg release was observed (16:30–20:30 h; Fig. 3.4A). When the high tide was later (16:31–20:30 h) egg release was more restricted to the dark period (Fig. 3.4C). Egg release was lowest in the middle of the day (12:31–16:30 h), irrespective of the tidal cycle.

In contrast, egg release was very low during the night in dioecious *F. vesiculosus*, irrespective of the tidal cycle conditions (Figs. 3.4E–G). We observed significant peaks of release corresponding with high tide (08:31–12:30 h; Fig. 3.4E), and prior to and during high tide when high tide is later (12:31–16:00 h; Fig. 3.4F). In contrast, when the high tide was in the late afternoon, very little egg release was observed (16:31–20:30 h; Fig. 3.4G), although significantly more eggs were counted at 16:30 and 20:30 h than at earlier sampling times.

In the absence of tides, a significant interaction (Table 3.1B) was observed between species and sampling interval. While under constant immersion the greater amount of egg release in *F. guiryi* occurred during the dark period, in *F. vesiculosus* egg release occurred throughout the day, with no significant difference between daytime sampling intervals (Figs. 3.4D,H).

Table 3.1. Results of PERMANOVAs testing the cumulative egg release in tidal shifts, in constant immersion (atidal conditions) and at circadian cycles. Sp; species (*Fucus guiryi* and *Fucus vesiculosus*), Ta; tanks (tidal phase coincident or opposite that at the collection site in the field); Ti; tides (high tide between 8:00–12:00 h, 12:01–16:00 h and 16:01–20:00 h) and Sa; sampling time (8:30 h, 12:30 h, 16:30 h and 20:30 h), Ti^; tides (high tide between 8:00–12:00 h, 12:01–16:00 h, 16:01–20:00 h and no tidal) and Ci; circadian cycles (night and day sampling). Significant differences ($p < 0.05$) are noted with *.

	Source of variance	df	F	*p*
Tidal condition (A)	Sp	1	0.0014	0.9738
	Ta	1	0.1088	0.8951
	Ti(Ta)	4	22.5528	0.0001*
	Sa	3	1.8161	0.1964
	Sp x Ta	1	0.7522	0.4269
	Sp x Ti(Ta)	4	10.6207	0.0001*
	Sp x Sa	3	14.1226	0.0004*
	Ta x Sa	3	0.4804	0.6953
	Ti(Ta) x Sa	12	14.4957	0.0001*
	Sp x Ta x Sa	3	0.4235	0.7485
	Sp x Ti(Ta) x Sa	12	7.0750	0.0001*
	Residual	336		
Atidal condition (B)	Sp	1	9.4194	0.0023*
	Sa	3	1.4363	0.2346
	Sp x Sa	3	16.484	0.0001*
	Residual	56		
Circadian Cycles (C)	Sp	1	22.4222	0.0001*
	Ti^	3	10.2701	0.0001*
	Ci	1	23.8428	0.0001*
	Sp x Ti^	3	16.1779	0.0001*
	Sp x Ci	1	75.0505	0.0001*
	Ti^ x Ci	3	19.4517	0.0001*
	Sp x T^ x Ci	3	4.3833	0.0040*
	Residual	112		

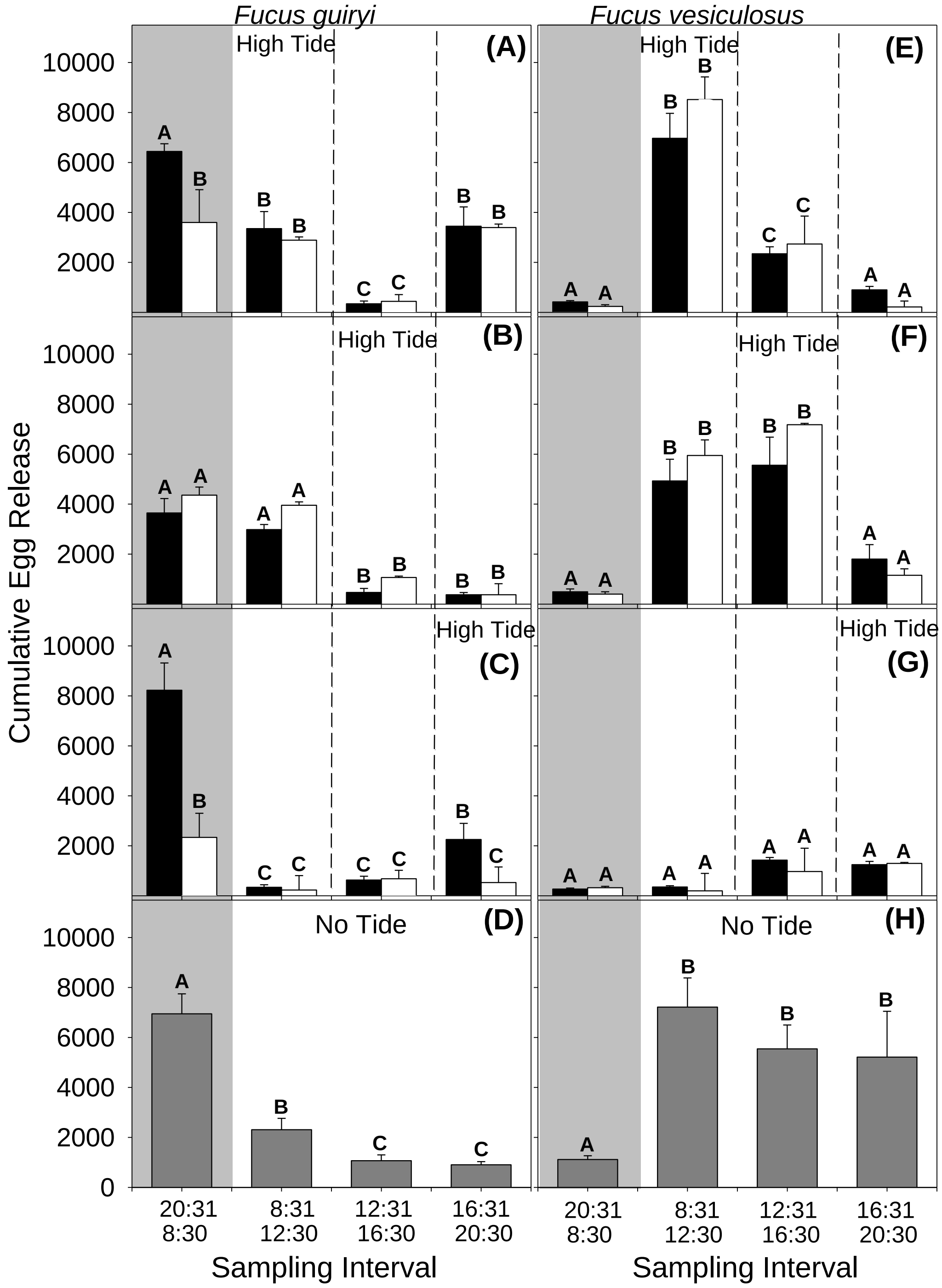

Figure 3.4. Effects of tidal shifts and circadian cycle on egg release by *Fucus guiryi* and *Fucus vesiculosus*. Cumulative egg release (n=8 ± SE) under (A and E) high tide between 8:00 and 12:00 h; (B and F) high tide between 12:01 and 16:00 h; (C and G) high tide between 16:01 and 20:00 h; and (D and H) constant immersion (no tidal regime). Black and white bars represent the means in each tank. Dark grey bars represent the means in tank with no tidal regime. Different letters above bars indicate significant differences (PERMANOVA, $p < 0.05$). Light grey shading indicates the night periods and longitudinal lines (black dotted lines) separate the timing of high and low tide periods.

For circadian cycles (day and night), no significant differences were observed between the numbers of eggs released by *F. guiryi* between night and day when the high tide was between 8:00–12:00 h and 12:01–16:00 h (Fig. 3.5A and Table 3.1C). However, significant differences were observed for high tides later in the day (16:01–20:00 h) and for atidal conditions; in both cases the amount of egg release in *F. guiryi* was higher at night than during the day. In contrast, egg release in *F. vesiculosus* was always significantly higher during the day than at night (Fig. 3.5B and Table 3.1C).

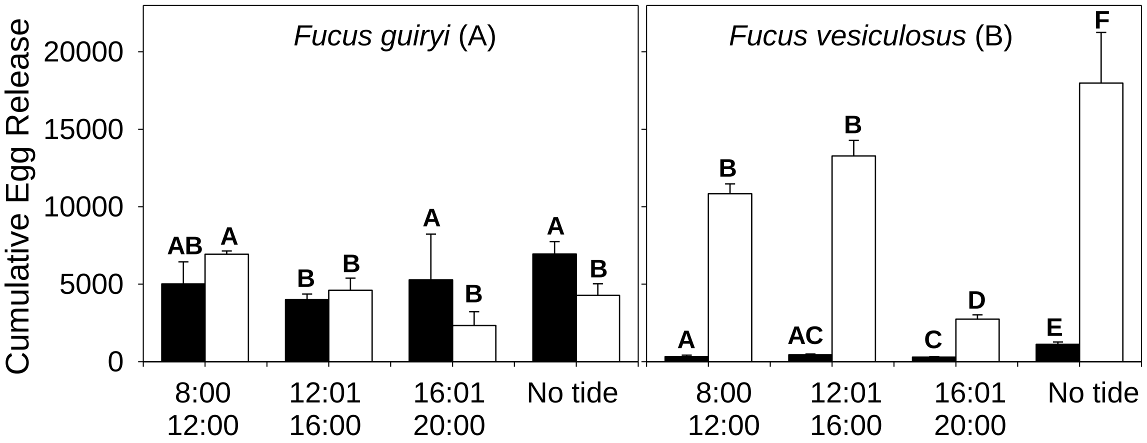

Figure 3.5. Effects of tidal shifts and circadian cycle on egg release by *Fucus guiryi* and *Fucus vesiculosus*. Cumulative egg release (n=8 ± SE) under high tide between 8:00–12:00 h, 12:01–16:00 h, 16:01–20:00 h and constant immersion (no tidal). Black and white bars represent the means of egg release at night and day, respectively. Different letters above bars indicate significant differences (PERMANOVA, $p < 0.05$).

3.5. Discussion

The experimental and field data presented in this study provides clear evidence for divergent reproductive timing between congeners in an assemblage of intertidal fucoid algae. The differences we found in spawning time have evolved recently, alongside variation in reproductive mode and mating system (Cánovas et al. 2011). While at semilunar timescales the four congeners studied share a common spawning pattern in northern Portugal, the previously unrecognised divergence in spawning times during circadian cycles supports the hypothesis that temporal (partial) reproductive isolation has evolved. At least under these ecological conditions, this timing divergence might constitute an ecological barrier to hybridization within the most closely related members of the *F. vesiculosus* subclade.

Reproduction is highly constrained by environmental cycles in the intertidal; the interaction of tidal (immersion-emersion) and circadian light:dark cycles are crucial cues that regulate spawning in fucoids (Pearson and Brawley 1996, Pearson et al. 1998, Berndt et al. 2002, Ladah et al. 2003, Monteiro et al. 2012). Our data confirm previous reports (Berndt et al. 2002) that natural gamete release occurs preferentially during high tide immersion. However, we found that while dioecious *F. vesiculosus* and *F. serratus* spawned during daytime neap high tides, two hermaphroditic species sister to *F. vesiculosus* spawned mainly during night-time high tides during the same phase of the semilunar cycle, a pattern that has not been observed previously (Pearson and Brawley 1996). The divergence in circadian patterns of spawning between hermaphroditic (*F. spiralis* and *F. guiryi*) and dioecious (*F. vesiculosus*) sister species is striking given their divergence time may be less than 1 MYA (Cánovas et al. 2011). Earlier-diverging dioecious members of the genus all share a pattern of daytime high-tide spawning (Brawley 1992, Berndt et al. 2002, Monteiro et al. 2012), which therefore appears to be the ancestral state within *Fucus*, while nocturnal/early morning spawning in the hermaphrodites *F. guiryi* and *F. spiralis* indicates a recent change to a modified or alternate signal – response pathway. Gamete release in fucoids involves a water-motion sensing system based on photosynthetic carbon acquisition (Serrão et al. 1996, Pearson et al. 1998), linked by downstream signalling to turgor changes that are presumed to directly trigger the expulsion of gametes (Pearson and Brawley 1998, Speransky et al. 2001). Nocturnal spawning has presumably arisen either by bypassing the photosynthesis dependent part of the process, or to modifications in timing of subsequent parts of the pathway.

Whatever the mechanism(s) involved, the potential ecological drivers of nocturnal spawning patterns may be linked with habitat, as both *F. guiryi* and especially *F. spiralis* are stress-tolerant species with vertical ranges that extend higher than either low-mid intertidal *F. serratus* or mid-intertidal *F. vesiculosus. Fucus spiralis* inhabits the upper intertidal zone, and even during high tide this species may be under water for less than 30 min, while during extreme neap tides individuals remain uncovered at high tide. Therefore, escape from desiccation, thermal and/or irradiance stress on eggs, sperm and embryos and selection for recruitment success may be a driver of nocturnal/early morning spawning.

Laboratory experiments in which only tidal and circadian cycles were manipulated were able to capture much of the complexity of natural spawning rhythms (Fig. 3.4), confirming differential spawning patterns between *F. guiryi* (hermaphroditic) and *F. vesiculosus* (dioecious), in broad agreement with field observations. Indeed, simple light:dark cycling without tidal treatment was sufficient to produce hermaphrodite – dioecious (nocturnal – diurnal) spawning patterns (Fig. 3.4 and Fig. 3.5). Spawning was also qualitatively unaffected by changing the tidal phase in experimental tanks, indicating that any potential intrinsic rhythms are secondary to the proximal environmental cues that trigger gamete release. Spawning was suppressed during darkness in *F. vesiculosus*, and was dependent on timing of high tides in the light. In contrast, cumulative spawning in cultured *F. guiryi* was similar or greater in darkness than in the light, independent of the timing or presence of tides (Fig. 3.4 and Fig. 3.5). The main difference between field and culture conditions was the tendency for *F. guiryi* to spawn late in the day in culture prior to the night-time high tide (Fig. 3.4A), perhaps a consequence of relaxed stress regime with no desiccation and moderate temperature (14ºC).

Some early morning spawning events in natural stands of *F. spiralis* and *F. guiryi* occasionally overlapped with dioecious species, particularly earlier in the reproductive season (June; Figs. 3.1A,B). Thus, temporal segregation of spawning at the interspecific level within the assemblage is incomplete, and the ecological conditions for hybridization exist between all four species, which coexist within a few meters of each other on the shore. Despite early reports of high levels of hybrid fertility between *F. vesiculosus* and *F. serratus* (Burrows and Lodge 1951), compelling experimental evidence for strong (although incomplete) prezygotic barriers were later reported (Bolwell et al. 1977). In contrast, early reports as well as more recent molecular evidence support the occurrence of hybridization within both the *F. vesiculosus* (Billard et al. 2005b, 2010, Engel et al. 2005, Zardi et al. 2011), and *F. serratus* subclades (Coyer et al. 2002). In potentially hybridizing lineages, ecological barriers such as temporal variation in reproduction may be strongly selected traits, as seems to be the case here. The main examples for marine broadcast spawners have been reported for corals: small temporal differences in gamete release of ca. one hour were observed between corals within the genus *Montastraea* (Levitan et al. 2004), and small variations have been observed in other sympatric coral species (Harrison et al. 1984, Hayashibara et al. 1993, Levitan et al. 2004, Wolstenholme 2004, Forgarty et al. 2012). Interestingly, as we observed here in *Fucus*, an inverse relationship between interspecific spawning synchrony and phylogenetic distance has been seen in *Montastraea* (Levitan et al. 2004).

Phylogenetic divergence and build-up of gametic incompatibility can explain why spawning times can overlap in sympatric populations of dioecious *Fucus* species without risk of excessive hybridization. Other ecological mechanisms, such as release of eggs in high concentrations of mucilage (personal observations) may also play a role in limiting the dispersal of gametes (Johnson and Brawley 1998, Brawley et al. 1999). However, given the highly coincident spawning between *F. spiralis* and *F. guiryi*, what prevents hybridization between these sister species? The answer appears to be that a shift in reproductive mode to hermaphroditism, together with a predominantly selfing mating system is sufficient (Coleman and Brawley 2005, Engel et al. 2005, Perrin et al. 2007). It may help that hermaphrodites produce relatively little sperm (Billard et al. 2005b), which is released simultaneously from the same reproductive structures (receptacles) as the eggs.

The relative contributions of pre- and post-zygotic barriers to the evolutionary history of the genus *Fucus* are unknown. Several sources of evidence support both hypotheses of pre- and post-zygotic barriers as important in our study species. First, the occurrence of a range of intermediate genotypes in the field (Engel et al. 2005, Billard et al. 2010), indicates that hybrids and introgressed individuals can be reproductively viable, lacking intrinsic complete post-zygotic barriers. However, comparative hybrid fitness studies are lacking. Second, the rarity of such hybrids in the field (see references above) and the persistence of each species as cohesive genetic entities, indicates that although hybrids can be viable, they are either not commonly produced (prezygotic barriers), or less fit (post-zygotic barriers), or likely both of them. The observation that hybrids are rare outside of contact zones matches both of the previous hypotheses. Our study demonstrates that reproductive ecology effectively acts as a prezygotic barrier for some species, but does not claim that it is the only barrier, and indeed it cannot be for species with similar mating systems. In addition, there might also be a role of partial gamete compatibility in mediating such barriers, allowing only some rare hybrid mattings, but further work is necessary to assess this hypothesis.

Our study shows that spawning synchrony (constraints) on semilunar timescales within an intertidal assemblage masks spawning asynchrony on smaller time scales (circadian and tidal cycles) in interfertile sister species of fucoid seaweeds. This likely represents an early-evolving and critical ecological mechanism that reinforces prezygotic isolation and maintains species boundaries between sister taxa of these externally-fertilizing broadcast spawners. Where interspecific spawning is synchronous, evidence from the literature suggest that phylogenetic distance is sufficient to prevent frequent crossing (Bolwell et al. 1977), while genetic data suggest that mating system is an additional prezygotic mechanism against hybridization by minimizing gene flow between selfing hermaphrodites (Perrin et al. 2007). The cues that trigger spawning during tidal immersion in all species are generated by the combined effects of circadian and tidal cycles. However, further studies, perhaps genome-enabled analyses, will be required to understand the mechanisms underlying the recent evolutionary shift between diurnal and nocturnal spawning patterns described here.

3.6. Acknowledgments

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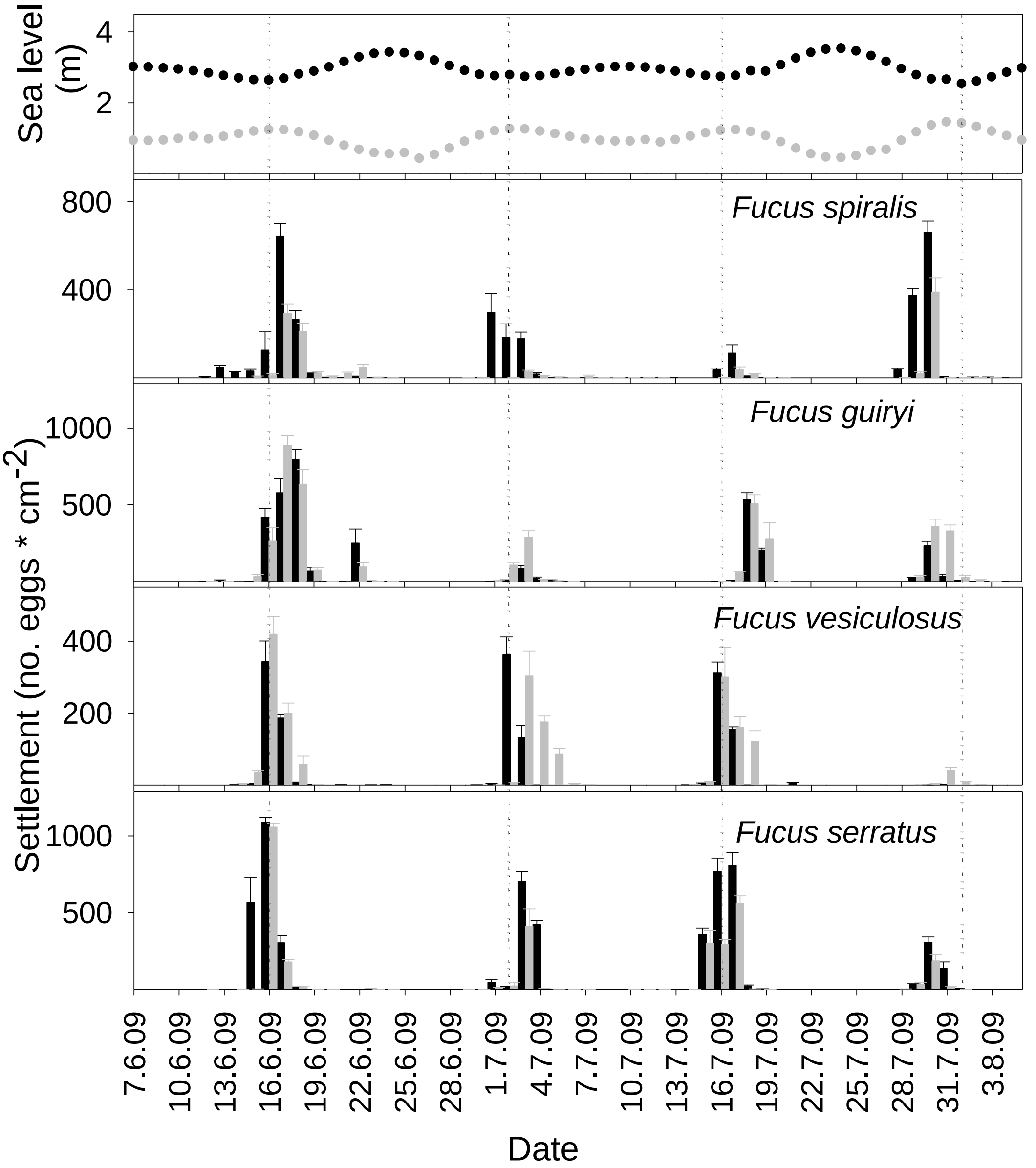
Supplementary Information

Appendix S3.1. Daily egg settlement (n = 5 ± SE) from *Fucus spiralis*, *Fucus guiryi*, *Fucus vesiculosus* and *Fucus serratus*, at two replicate sites (dark bars and grey bars) between 7 June and 5 August 2009. The low tide (open symbols) and high tide (closed symbols) levels are shown in the first panel. Vertical black dotted lines indicate the peak of neap tides. Settlement data for *Fucus guiryi* and *Fucus vesiculosus* was published in Monteiro et al. (2015).



Chapter 4

Reproductive investment, synchrony and recruitment success in marine broadcast spawners: effects of mating system and habitat (exposed shore *versus* estuary)

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4. Reproductive investment, synchrony and recruitment success in marine broadcast spawners: effects of mating system and habitat (exposed shore *versus* estuary)

4. 1. Abstract

The timing and synchrony of gamete release in broadcast spawners have important implications for fertilization success, recruitment and to explain differences in reproductive success under distinct reproductive modes in sympatry. Our objective was to compare the reproductive timing and investment for sister species with contrasting mating systems; *Fucus guiryi* (selfing hermaphroditic) and *Fucus vesiculosus* (dioecious) in habitats with different wave exposures (exposed shore and estuary). Over two months, daily gamete release, recruitment and population structure were recorded. Our results show spawning synchrony between species and habitats, but release events in hermaphrodites occupied broader temporal windows in estuarine than exposed shore habitats. On the exposed shore both species increased the synchrony of release and amount of eggs. In the estuary, hermaphrodites relied on broader temporal spawning windows and a larger canopy, and the dioecious species had higher recruitment success, important factors determining persistence.

Keywords: Dioecious; Estuary; Exposed shore; Hermaphrodite; Mating system; Recruitment; Reproductive periodicity; Sympatric species; Synchrony

4.2. Introduction

The timing of reproduction plays a key role in the success, distribution and abundance of many marine organisms (Menge 1991, 2000, Roberts 1991). This is particularly important for broadcast spawners, for which environmental cues can regulate spawning synchrony and timing in ways that maximize reproductive success (Serrão and Havenhand 2009). Furthermore, such effects might explain the divergence of reproductive modes in closely related species in sympatry (Pearson and Serrão 2006). Environmental cycles, such as daily, tidal, lunar and seasonal cyclic changes, are able to set the timing of reproduction in many marine organisms. The fucoid algae that structure ecosystems along cold and temperate shorelines worldwide use cues from tidal and/or lunar cycles to synchronise reproduction (reviewed in Pearson and Serrão 2006, see also Monteiro et al. 2009, 2012). If the tidal cycle is the most important factor in timing reproduction, then periodicity of gamete release within a species would be expected to vary across the geographic range (spatial scale) according to different tidal patterns. Tidal patterns are also expected to cause variation at a range of temporal scales (Connell 1985, Jenkins et al. 2000). Different habitats have different selective pressures and these might affect the timing of gamete release, increasing/decreasing the optimal windows of opportunity. On exposed shores (open coast), tidal cycles cause differences in hydrodynamic conditions and timing of tidal phases relative to the neighbouring sheltered estuarine habitats. Exposed rocky shore *versus* estuarine habitats can therefore provide useful case studies of natural populations under contrasting wave exposure regimes, allowing predictions derived from hypotheses of wave action effects on reproductive success to be tested.

Many marine organisms retain external fertilization as a means of reproduction; a process that depends crucially for its success on gamete encounters in the water column. The timing, as well as the synchrony of gamete release have important implications not only for fertilization success, but also for the success of recruitment and early survival in organisms with external fertilization. The divergence of reproductive strategies might contribute to reproductive isolation in sister species with similar geographical ranges (sympatric species) and has important ecological and evolutionary consequences. It also affects the distribution of genetic diversity and gene flow between and within populations. Different reproductive modes between closely related *taxa* also provide good models to understand the implications of spawning synchrony, hybridization and reproductive isolation in species with external fertilization (broadcast spawners).

Together with marine invertebrates and fish, the brown algal genus *Fucus* is one of the most well-studied groups of broadcast spawners (Pearson and Serrão 2006). *Fucus* species occupy distinct, but often overlapping, niches on exposed shores, estuaries and lagoons. Several species of *Fucus* are sympatric throughout much of their ranges. Mating systems can vary between species from obligatory outcrossing to predominant self-fertilization (e.g., Engel et al. 2005, Perrin et al. 2007), potentially influencing evolutionary responses to habitat differences and reproductive isolation. Such variability in breeding systems is therefore interesting to assess the relationships between mating systems, reproductive ecology and reproductive isolation in sympatric closely related species with incomplete reproductive barriers (e.g., Billard et al. 2010).

The timing of spawning by *Fucus* species has been studied on several distinct Atlantic shorelines, with contrasting tidal regimes, along the European and American coasts (reviewed Pearson and Serrão 2006). These include estuaries (Brawley 1992), intertidal exposed rocky shores (Berndt et al. 2002, Ladah et al. 2003, Monteiro et al. 2012), tide pools (Pearson and Brawley 1996), and the non-tidal Baltic Sea (Serrão et al. 1996). All these studies showed synchronous semilunar cycles of reproduction. Additionally, calm hydrodynamic conditions were required for gamete release (Serrão et al. 1996, Pearson et al. 1998). To date, most studies have focused on the reproductive periodicity of a single species and habitat, but have not addressed habitat-related variation within a species and/or the role of mating system on reproductive success. This study aims to fill this gap, by comparing reproductive timing, investment and success in sympatric species with divergent mating systems in different habitats, and assessing the hypothesis of stronger selection for synchronous spawning in obligate outcrossers relative to selfing species under similar environmental conditions (Pearson and Serrão 2006).

Our objective was to test the hypothesis that the reproductive success of marine broadcast spawners is affected by interactions between habitat conditions (exposed rocky shore *versus* sheltered estuarine habitats) and reproductive modes (selfing hermaphrodites *versus* dioecious obligate outcrossers). We compared reproductive investment, spawning synchrony and recruitment success in hermaphroditic and dioecious species of the genus *Fucus*. The models were two co-occurring sister species that coexist as distinct entities despite potential hybridization (Billard et al. 2005, Engel et al. 2005, Zardi et al. 2011) in northern Portugal, *Fucus guiryi* (hermaphrodite) and *Fucus vesiculosus* (dioecious).

4.3. Materials and methods

4.3.1. Study habitats

The study was carried out at Viana do Castelo, northern Portugal, on estuarine (Lima estuary) and exposed shore (Praia Norte) habitats (41º41'47 N 8º51'10 W), which differed strongly in wave exposure. This region is the southernmost distribution limit where *F. guiryi* and *F. vesiculosus* co-occur in sympatry. Further south, *F. guiryi* inhabits only the open coast, while *F. vesiculosus* occurs exclusively in estuaries and sheltered coastal lagoons (Ladah et al. 2003). Where these species co-occur, *F. guiryi* is found predominantly higher in the intertidal zone than *F. vesiculosus*, despite overlapping at their vertical distributional edges (Billard et al. 2010, Zardi et al. 2011). Viana do Castelo has a cool temperate climate and a semidiurnal tidal regime. The west-facing shore (Praia Norte) contains rocky outcrops that reduce the predominant NW wave action (see Ladah et al. 2003, Monteiro et al. 2009, Araújo et al. 2012). The Lima river estuary is located ≈ 1 km south of Praia Norte. The study sites were located on the northern margin and ≈3 Km from the mouth of the estuary.

4.3.2. Model organisms

Fucus guiryi is a self-compatible hermaphrodite and *F. vesiculosus* is dioecious and therefore an obligatory outcrosser. In addition to different reproductive structures, these species are clearly distinguishable by the presence of air vesicles in *F. vesiculosus*. Both species were identified as described by Zardi et al. (2011). In the genus *Fucus* the reproductive structures (receptacles) develop apically. Each receptacle contains spherical conceptacles with numerous antheridia (each containing 64 sperm) and/or oogonia (each with 8 eggs). Sperm are biflagellate and reach ca. 5 μm in length whereas eggs are non motile and reach ca. 80 μm in diameter. In hermaphrodites all conceptacles contain both sperm and eggs, whereas in dioecious species the sperm and egg develop in separate conceptacles from different male and female individuals. Antheridia and oogonia are released through the ostiole from each conceptacle. Both are negatively buoyant (i.e., they sink), therefore settlement occurs immediately after release in the calm water conditions under which gamete release takes place (Serrão et al. 1996, Pearson et al. 1998). Shortly after release the antheridia and oogonia open, liberating the eggs and sperm and fertilization occurs externally, most likely at the bottom. Most eggs become fertilized (Brawley 1992, Pearson and Brawley 1996, Serrão et al. 1996, Berndt et al. 2002, Ladah et al. 2003; see also Pearson and Serrão 2006 for a review of conditions influencing fertilization success), therefore a sample of settled eggs most likely consists almost entirely of zygotes. Egg tends to fall immediately below the releasing individual (Serrão et al. 1997). The highly restricted dispersal inferred in several studies (Serrão et al. 1997, Dudgeon and Petraitis 2001, Coleman and Brawley 2005, Engel et al. 2005, Perrin et al. 2007) might function as one ecological mechanism that prevents hybridization. Fertile hybrids do occur but are rare and are found mainly in the contact zone within the intertidal where the vertical species distributions overlap (Billard et al. 2005, 2010, Engel et al. 2005).

4.3.3. Daily spawning periodicity

The periodicity of egg settlement (which occurs immediately after spawning) was monitored daily from June 7 to August 5 2009, for *F. guiryi* and *F. vesiculosus* in two habitats (exposed shore and estuary). The eggs were collected on artificial substrates (5.96 cm^2, as described in Ladah et al. 2003) with a roughened surface to promote zygote settlement and adhesion. Two sites per habitat were haphazardly selected in the centre of the intertidal range of each species. Sites within species were ca. 15 m apart, and more than 5 m from the nearest individual of the other species. In *F. guiryi*, the study sites were at heights of 2.4 and 2.3 m on the exposed shore and in the estuary, respectively. In *F. vesiculosus* the sites were at 1.7 and 1.9 m on the exposed shore, and 1.7 and 1.8 m in the estuary. Five disks per site were fixed to the rocks with bolts, under different algae ca. 10 cm apart. Disks were collected and replaced daily during the diurnal low tide and the number of eggs was counted in the laboratory under a dissecting microscope (as in Monteiro et al. 2012).

4.3.4. Recruitment

Recruitment was estimated during two months (June 7 to August 5, 2009) for the same species and habitats, using a second set of disks as described above for the settlement estimates, except that these were left permanently at the sites during the study period. Each recruitment disk was ca. 5 cm from a settlement disk. All disks were collected on August 5 and the number of individuals per disk was counted under a dissecting microscope. The ratio between recruitment and settlement was estimated by dividing the total recruits found at the end of the study in each disk by the sum of all settlement recorded on the respective settlement disk, for every pair of recruitment/settlement disks.

4.3.5. Demographic traits

To examine population structure, all individuals that were within ca. 1 m^2 around the sampling discs (used for settlement and recruitment studies) were measured for several parameters: thallus length (L), maximum circumference (C) (if ≤ 1 cm it could not be measured accurately and was defined as 1 cm), the number of receptacles (= reproductive structures), and the sex (in *F. vesiculosus*). Three stage structures were identified for each individual: 1) mature, when receptacles were present; 2) adult but without receptacles, when the principal thallus was broken; and 3) juveniles, all individuals without reproductive structures, usually with only one or two vegetative apices. From these data we calculated at each site within each habitat, and for both species, the total number of individuals (total density), the mature individuals (both female and male, in *F. vesiculosus*), the number of receptacles (both female and male, in *F. vesiculosus*) and the density of juveniles per area. Sex-ratios were estimated in *F. vesiculosus* to test for differences between habitats. In all individuals the maximum length and maximum circumference were measured to estimate the size of individuals (as a population volume index, LC^2), which can be used as a proxy of the biomass per area (Åberg 1990).

4.3.6. Statistical analyses

Settlement, recruitment and recruitment/settlement ratio were analysed according to their sampling design with 3 factors: habitats (2 levels: exposed shore and estuary, orthogonal and fixed), species (2 levels: *F. guiryi* and *F. vesiculosus*, orthogonal and fixed) and sites (2 levels: site 1 and site 2, random and nested within habitat), with N=5 replicates (5 disks per site per species).

Juvenile density, thallus length, total density (data were square-root transformed), population volume, mature density and density of receptacles were analysed under a 2-factor design: habitats (2 levels: exposed shore and estuary, orthogonal and fixed) and species (2 levels: *F. guiryi* and *F. vesiculosus*).

Means were compared using ANOVA (GMAV5 software, Institute of Marine Biology, University of Sydney, Australia) after testing for homoscedasticity using Cochran's C-test. Post-hoc multiple comparisons were done with Student-Newman-Keuls (SNK) tests at $\alpha = 0.05$. The recruitment and recruitment/settlement ratio data failed the homoscedasticity test, therefore they were analysed with PERMANOVA (v.1.6 software, Department of Statistics, University of Auckland, New Zealand), which does not assume normality nor homoscedasticity (Anderson 2001, McArdle and Anderson 2001). The number of times the permuted *p*-value was equal to or lying outside the 95% confidence interval was divided by the total number of permutations (9999) and the resulting number was taken as the permuted *p*-value. To test for differences in the sex-ratio of mature individuals and the sex-ratio of receptacles between habitats (exposed shore and estuary), chi-square tests were performed.

4.4. Results

4.4.1. Daily spawning periodicity

Four settlement events were recorded for both species and habitats, with a periodicity of ca. two weeks (semi-lunar pattern) at neap tides. The large majority of days with release were coincident across species and habitats (Fig. 4.1). However, the settlement peak differed by one or two days between habitats in both species. In estuarine *F. vesiculosus*, the peak of settlement occurred always one day later than on the rocky shore. In a total of

60 sampling days, longer periods of egg release were observed for *F. guiryi* in the estuary (almost every day) than on the exposed shore (85 % and 52 % of sampling days, respectively). In contrast, in *F. vesiculosus* the days with egg release on the exposed shore a

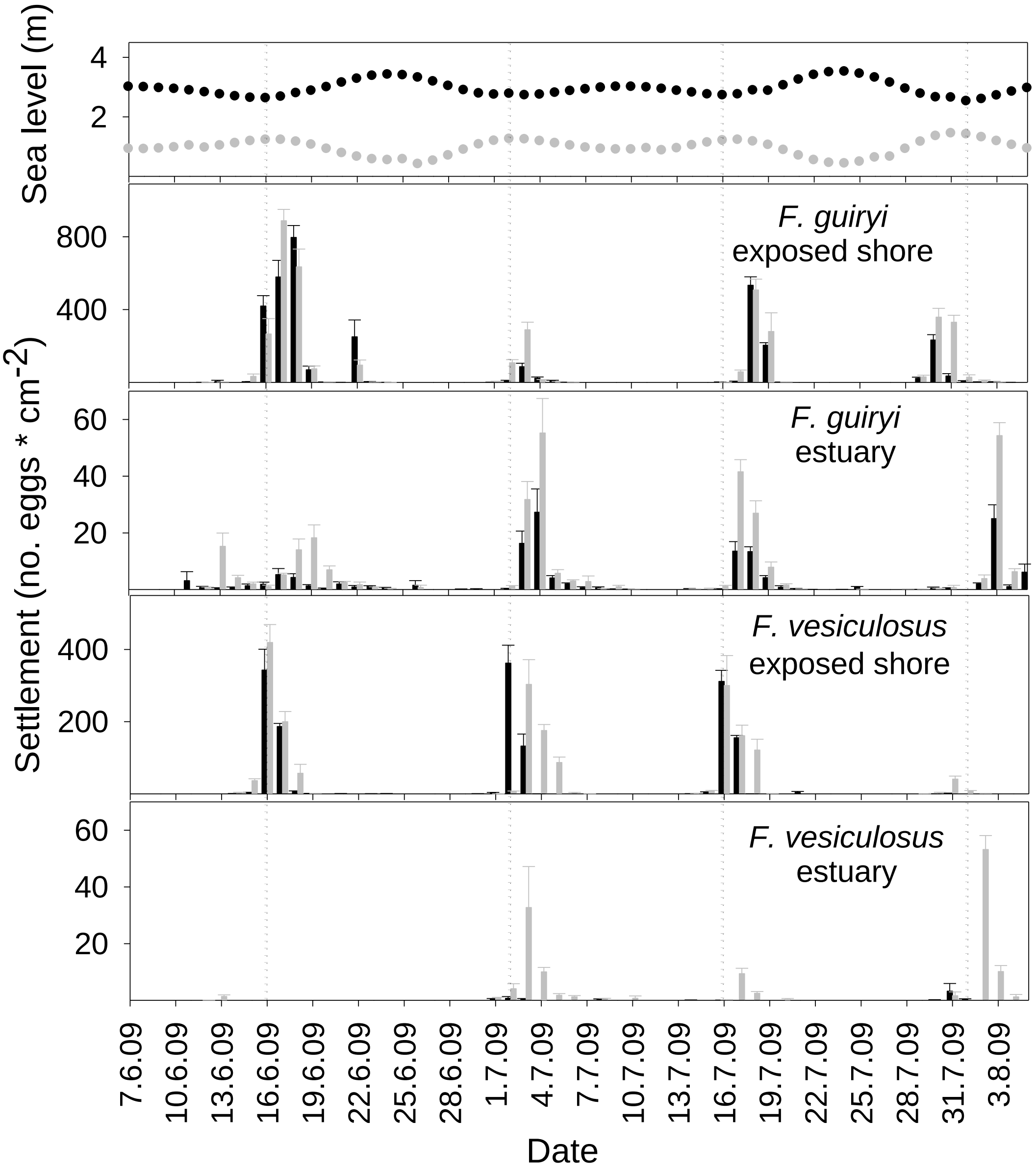

Figure 4.1. Daily egg settlement (mean ± SE, n = 5) by *Fucus guiryi* and *Fucus vesiculosus*, at exposed shore and estuary (two replicate sites: black bars and grey bars). Sampling was performed daily from June 7 to August 5 2009. Sea level (black and grey dotted lines are the high and low tide, respectively). Vertical black dotted lines indicate the peak of neap tides.

4.4.2. Settlement and recruitment

Settlement was significantly different between species, higher in *F. guiryi* than in *F. vesiculosus*, and between habitats, higher on the exposed shore than in the estuary (Table 4.1, Fig. 4.2). Recruitment in both species was higher on the exposed shore than in the estuary. In contrast, the recruitment/settlement ratio (i.e., the proportion of settlers that recruited, or recruitment success) was higher in the estuary than on the exposed shore (PERMANOVA, p = 0.04 and p = 0.01 for *F. guiryi* and *F. vesiculosus*, respectively), especially in *F. vesiculosus* (58.6 % and 5 % in the estuary and in the exposed shore, respectively). In *F. guiryi,* the number of recruits per mature individual was significantly higher on the exposed shore than in the estuary, and although a similar tendency was observed in *F. vesiculosus*, however it was not significant. No significant differences were detected in the density of juveniles between habitats or species, although in total there were more juveniles in the estuary than on the exposed shore in both species (Table 4.2, Fig. 4.2).

Table 4.1. Analysis of variance of the effects of habitat and species on settlement (m^{-2}), recruitment (m^{-2}) and recruitment and settlement ratio (recruitment/settlement). ① Results of the ANOVA applied to settlement and ② results of the PERMANOVA applied to recruitment and recruitment/settlement ratio (p-value by Monte Carlo test). Ha; habitats (exposed shore and estuary), Sp; species (*Fucus guiryi* and *Fucus vesiculosus*) and Si (2 s

		Settlement①		**Recruitment②**		**Recruitment/Settlement②**	
Effect	df	F	*p*	F	*p*	F	*p*
Ha	1	74.46	0.01*	8.90	0.10	59.23	0.00*
Sp	1	186.67	0.01*	1.45	0.35	0.94	0.46
Si(Ha)	2	14.95	0.00*	4.96	0.01*	0.88	0.48
Hab x Sp	1	132.68	0.01*	0.21	0.70	1.75	0.26
Sp x Si(Ha)	2	1.01	0.37	9.72	0.00*	2.52	0.05
Residual	32						

Table 4.2. Analysis of variance of the effects of habitat and species on juveniles (m^{-2}), recruitment per mature individual (recruitment/mature, m^{-2}), thallus length (cm), total density per m^2, volume (LC^2), density of mature individuals (mature individuals, m^{-2}) and density of receptacles (receptacles, m^{-2}). ① data were square-root transformed. Ha; habitats (exposed shore and estuary) and Sp; species (*Fucus guiryi* and *Fucus vesiculosus*). Significant differences ($p < 0.05$) are noted with *.

		Juveniles		**Recruitment/mature**		**Thallus length**		**Total density ①**		**Volume (LC^2)**		**Mature individuals**		**Receptacles**	
Effect	***df***	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
Ha	**1**	5.81	0.74	36.77	0.004*	39.65	0.003*	9.76	0.04*	12.86	0.02*	9.92	0.03*	50.96	0.00*
Sp	**1**	0.01	0.94	20.39	0.011*	53.77	0.002*	0.11	0.76	51.58	0.00*	0.00	0.96	23.04	0.01*
Ha x Sp	**1**	0.08	0.79	14.54	0.019*	8.06	0.047*	4.28	0.11	4.5	0.10	3.54	0.13	1.58	0.28
Residual	**36**														

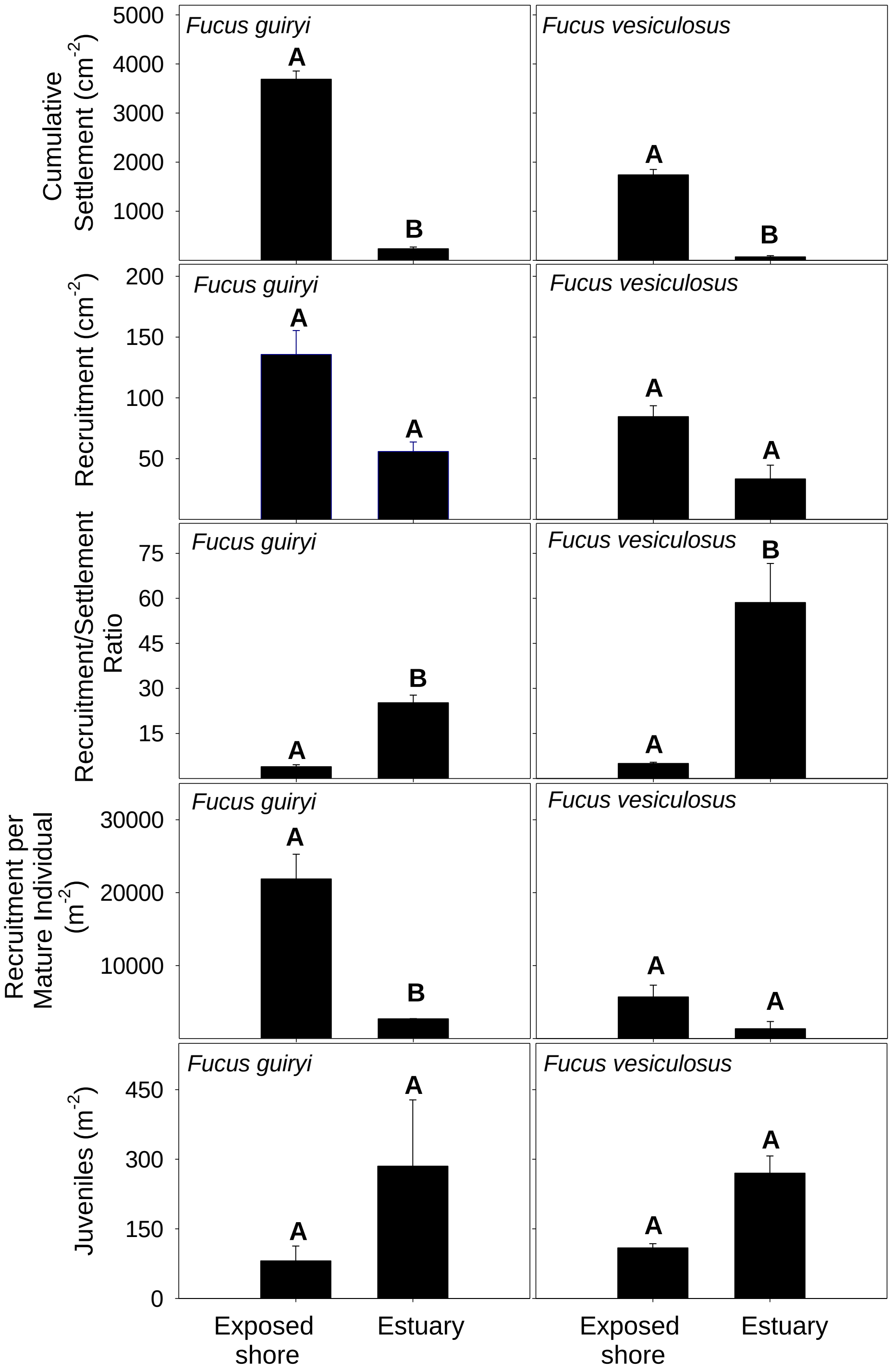

Figure 4.2. Two-months of cumulative egg settlement, recruitment, recruitment and settlement ratio (mean ± SE, n=5), recruitment per mature individual and density of juveniles (mean ± SE, n=2) at exposed shore and estuary. Different letters above bars indicate significant differences ($p < 0.05$).

4.4.3. Demographic traits

Estuarine populations of *F. guiryi* had significantly more individuals (total density), more mature individuals and with greater length and volume index (LC^2) than the exposed shore populations. No significant differences were observed for *F. vesiculosus* in these parameters, except for length, which was shorter in the exposed populations (Table 4.2, Fig. 4.3). In both species, the density of reproductive structures (number of receptacles per m^2) was significantly higher in the estuary than on the exposed shore (3.4 and 1.6 times in *F. guiryi* and *F. vesiculosus*, respectively). Sex-ratio in the dioecious species, *F. vesiculosus*, was slightly biased towards males in both habitats (male:female ratio: 1:0.85 and 1:0.92 on the exposed shore and in the estuary, respectively). Despite the higher proportion of females in the estuary than on the exposed shore, the habitats did not significantly differ in sex-ratio ($\chi^2 = 0.622$; $p = 0.81$; df = 3). The proportion of male and female receptacles (sex-ratio of receptacles) was also male-biased (male:female receptacles ratio: 1:0.86; $\chi^2 = 10.7$; $p = 0.01$; df = 3), but did not differ between habitats.

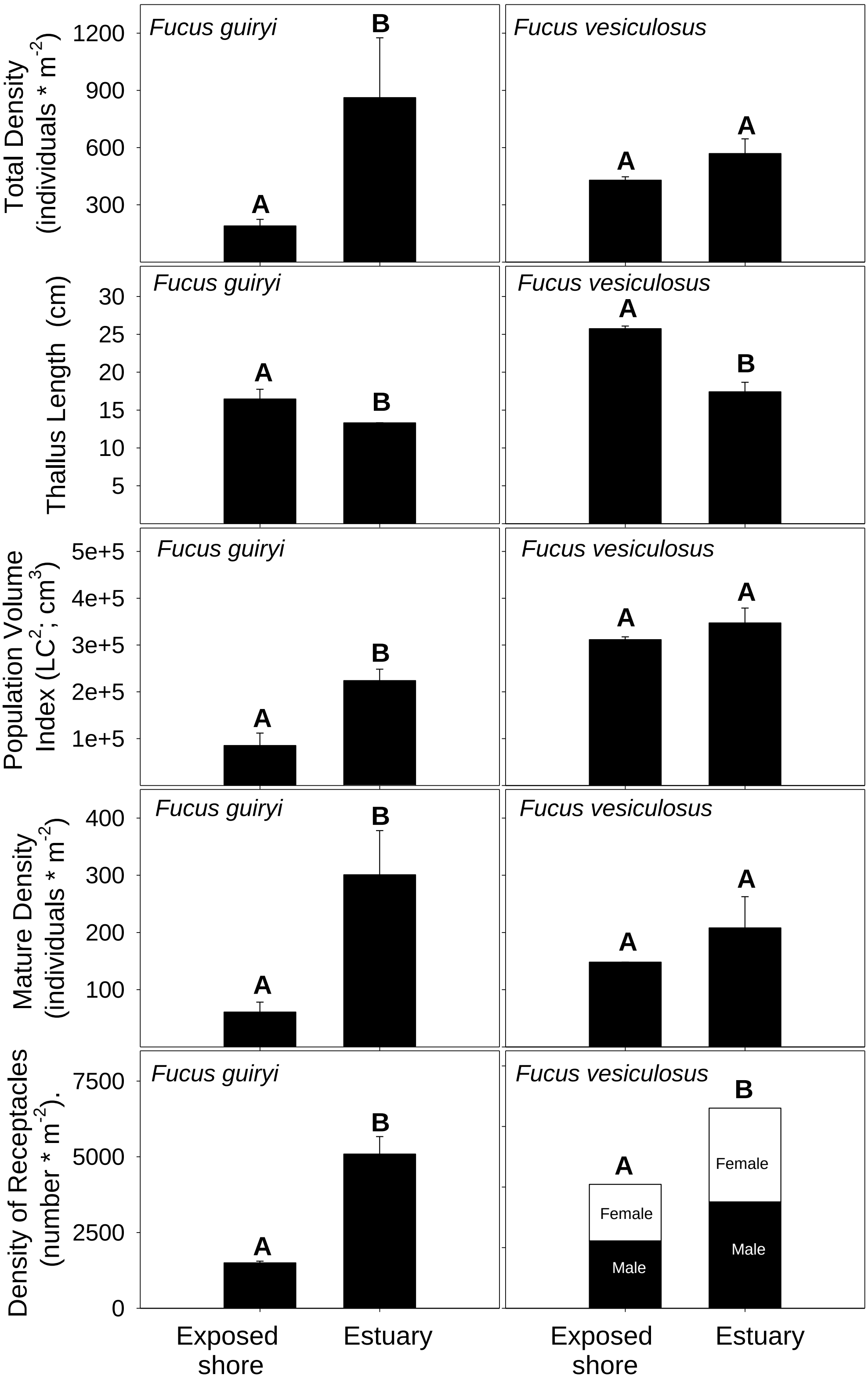

Figure 4.3. Total density (individuals * m^{-2}), thallus length (cm), population volume index (LC^2), density of mature individuals, density of receptacles (male and female), all in *Fucus guiryi* and *Fucus vesiculosus* at an exposed shore and an estuary (mean ± SE, n=2). Different letters above bars indicate significant differences ($p < 0.05$).

4.5. Discussion

The present study indicates a remarkable effect of environmental conditions on reproductive investment of *Fucus* species. Our results show habitat-related and species-related differences in settlement, recruitment success, reproductive potential and canopy effect/protection. Reproductive success of *F. guiryi* and *F. vesiculosus* on the exposed shore seems to be more dependent on the magnitude of settlement and, therefore, on higher recruitment. In contrast, in estuarine populations, the reproductive success of *F. guiryi* was more dependent on longer periods of settlement, high reproductive potential and canopy structure, whereas recruitment success (survival from settlement to recruitment) was more important for the success of *F. vesiculosus*.

During two sampling months, four settlement events were recorded and the peaks of gamete release were almost coincident between species. Both species showed a clear semilunar pattern of gamete release, in agreement with previous studies (Brawley 1992, Pearson and Brawley 1996, Berndt et al. 2002, Monteiro et al. 2012), coincident with neap tides in both habitats (exposed shore and estuary). Nevertheless, the settlement events of *F. guiryi* were longer in the estuary than on the exposed shore, whereas they were similar (slightly shorter) in *F. vesiculosus*. Although on the exposed shore settlement frequency did not differ between species (around 50 % of the days for both), in the estuary settlement was much more frequent in *F. guiryi* (almost every day) than in *F. vesiculosus* (< 50 % of the sampling days). These longer settlement events indicate a broader temporal window of opportunity in self-fertile species, which are predicted to be less constrained by reproductive synchrony, since gamete encounters between distinct individuals are not required for reproductive success (Pearson and Serrão 2006). Reduced hydrodynamic exposure provides a powerful proximal signal controlling fucoid gamete release (Serrão et al. 1996, Pearson et al. 1998) and reducing pheromone dilution (Gordon and Brawley 2004), besides increasing fertilization success in broadcast spawners in general (reviewed in Serrão and Havenhand 2009). The low hydrodynamic conditions in the estuary seem to favour longer settlement events in the hermaphrodites. Contrarily, the settlement events of the dioecious species were smaller in the estuary than on the exposed shore and smaller than in *F. guiryi* in both habitats. In dioecious species, reproductive opportunities on exposed coasts are more limited due to the mating system constraints on successful gamete encounters. Higher selection for synchronous spawning is expected in obligate outcrossers than in hermaphrodites (Pearson and Serrão 2006).

Settlement and recruitment were greater on the exposed shore than in the estuary. They were also greater in the hermaphrodite *F. guiryi* than in dioecious *F. vesiculosus* (as in Monteiro et al. 2012). This might be expected if weight density is similar, because hermaphroditic species of *Fucus* produce more eggs per weight of tissue than dioecious species (Vernet and Harper 1980). On the exposed shore, settlement and recruitment were higher in *F. guiryi* than in *F. vesiculosus*, even though recruitment success (recruitment/settlement ratio) was similar between species, suggesting a higher mortality of early post-settlement recruits of *F. guiryi*. This species inhabits a high position in the shore, where desiccation is stronger than in *F. vesiculosus*. The higher investment in eggs (Vernet and Harper 1980) and consequent high settlement, might offset the mortality of recruits in the higher shore habitat of *F. guiryi*, and thereby increase recruitment. In the estuary, recruitment success may be positively affected by canopy density and low hydrodynamic conditions. Besides, the lower settlement numbers and the higher settlement duration may also have an important role in recruitment success by decreasing the competition between recruits (see below). We hypothesize that settlement might be the most important step for reproductive investment in *F. guiryi*, as suggested for many others marine species (Menge 1991, 2000, Roberts 1991). Reproductive potential (the number of receptacles) was higher in *F. vesiculosus* than in *F. guiryi* but settlement and recruitment were lower in *F. vesiculosus* in both habitats. The higher number of receptacles in *F. vesiculosus* than in *F. guiryi* may be a consequence of their different reproductive strategies. While in *F. guiryi* the egg and sperm are in the same receptacles, allowing self-fertilization and increasing the success of fertilization, in *F. vesiculosus* eggs and sperm are formed in separate individuals and must find each other externally. By increasing the numbers of eggs and sperm, the higher number of receptacles in the dioecious species may consequently increase the probabilities of fertilization, counteracting the inability to self-fertilize. Moreover, in *F. vesiculosus* the reproductive potential was higher in the estuary than on the exposed shore, but the settlement and recruitment were higher on the exposed shore, while no differences in canopy effect were observed between habitats. However, recruitment success and the number of juveniles were higher in the estuary. These data indicate that the survival of recruits in *F. vesiculosus* may not be very dependent on reproductive potential or population structure (see below).

While settlement for both species was higher on the exposed shore than in the estuary, recruitment success (recruitment/settlement ratio) and the number of juveniles were lower. These data suggest a negative effect of high settlement density on survival and growth of recruits, i.e., density-dependent mortality (see Kendrick 1994, Creed et al. 1996, 1997, Choi 2003). Contrarily, the adult density may have a positive effect on recruitment success for *F. guiryi*. Adult density has also been reported to have both beneficial (Brawley and Johnson 1991, Johnson and Brawley 1998, Dudgeon and Petraitis 2001, Ladah et al. 2003) and detrimental (Black 1974, Chapman 1989, 1990, Vadas et al. 1990, 1992, Åberg and Pavia 1997, Jenkins et al. 1999) effects on survival of early post-settlement stages. Recruits under the adult algae are usually protected from exposure during emersion and suffer less physiological damage due to desiccation, wave action, and also are more protected from herbivory (Hay 1981, Brawley and Johnson 1991, Vadas et al. 1992, Dudgeon and Petraitis 2001). Furthermore, morphological differences (in LC^2) observed between habitats in *F. guiryi* are consistent with previous studies showing high morphological variability in *Fucus* species along their geographical distribution. In environments with low salinity, the length of *F. vesiculosus* decreases, but the number of branches increases (Kalvas and Kautsky 1993, Ruuskanen and Bäck 1999). In our study we observed the same pattern of length reduction in the estuary for both species, i.e., the individuals from the exposed rocky shore were higher than the estuarine populations. Although no data was collected for the number of branches, the LC^2 data indicates that the individuals had more branches in the estuary than in the exposed shore, a canopy structural difference that may affect recruitment survival.

Viana do Castelo is the southernmost distribution limit where *F. guiryi* and *F. vesiculosus* co-occur; further south *F. guiryi* inhabits only open coast habitats, while *F. vesiculosus* is restricted to estuaries and coastal lagoons. Ladah et al. (2003) raised the question of whether reproductive system might determine the contrasting distribution of these two *Fucus* species. In southwest Portugal, where these species live in such contrasting habitats, the success of fertilization of the dioecious species on the exposed shore was higher than expected, similar to natural stands of the hermaphrodites. Nevertheless, recruitment and survivorship on the exposed shore were lower in *F. vesiculosus* than in *F. spiralis* (Ladah et al. 2003). The absence of *F. vesiculosus* from exposed shores was thus not caused by fertilization failure but by reduced or absent recruitment and the low survivorship of outplanted recruits. The open coast (exposed shore) in Viana do Castelo is characterized by calm hydrodynamic conditions (compared with others open coast habitats in Portugal), due the offshore rocky bluffs that reduce (predominantly NW) wave action. The geographic position and the geomorphology make this coastline a unique and exceptional open coast environment for *F. vesiculosus* in Portugal. Considering this scenario, our results showing higher recruitment success and juvenile density for *F. vesiculosus* in the estuary, where wave action is considerable lower than on open shores, support the hypothesis that wave action strongly influences the success of *F. vesiculosus,* and that south of Viana do Castelo this species fails to establish on open coasts due to greater hydrodynamic forces that negatively affect the survivorship of recruits (Ladah et al. 2003).

The present study is the first to compare the reproductive patterns (timing, investment, reproductive potential, population effects, recruitment and survival) in sympatric sister species with divergent mating systems in estuarine and exposed shore habitats. Our results show habitat-related variation, as well as species-related variation. On the exposed shore, populations of *F. guiryi* rely more on high settlement that might offset abiotic stress-induced mortality of recruits (e.g., high temperatures and/or desiccation), thereby increasing recruitment. In the estuary, the longer settlement events (broader temporal window of opportunity) and population structure might contribute to the success of this species. Furthermore, low hydrodynamic conditions and low salinity in estuaries can explain the larger volume of individuals, and all these may protect the recruits from abiotic stressors, increasing survivorship of recruits, especially in *F. guiryi*. High recruitment success, reproductive potential and the positive effects of population density for *F. guiryi* in the estuary raises an important unanswered question - why is this species absent from estuarine habitats further south of Viana do Castelo? Contrarily, in *F. vesiculosus*, we expected settlement and recruitment to be greater in estuarine than in exposed shore populations (and greater than in estuarine populations of *F. guiryi*) - however we observed the opposite. Additionally, in *F. vesiculosus* no significant differences were observed in population density between habitats, but recruitment success and juvenile density were higher in the estuary and higher than in *F. guiryi*. These data may indicate that in *F. vesiculosus*, survivorship of recruits is more affected by wave action than by canopy protection. Recruitment has been reported to be a bottleneck stage for fucoid algae (Vadas et al. 1990, Brawley and Johnson 1991, Johnson and Brawley 1998, Ladah et al. 2003).

Our data suggests that recruitment of *F. vesiculosus* may indeed be the main bottleneck stage, at least for the geographic distribution south of this southernmost sympatric region.

Our research provides a better understanding of the population dynamics, reproductive ecology and the early life history of two important species that, together with other fucoid algae, dominate the northern European coast line, from exposed shores to estuaries and coastal lagoons. *Fucus* species occur exclusively in the northern hemisphere on (eastern and western) Atlantic and (western) Pacific coasts, but the highest diversity occurs on European coastlines, where species with distinct reproductive modes frequently occur in sympatry. Additional work contrasting distinct habitats in other regions of their distribution will be needed to further understand their adaptation and phenotypic plasticity to the contrasting environments found in different habitats.

4.6. Acknowledgments

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Chapter 5

Circadian cycles (light:dark) control the time of spawning

5. Circadian cycles (light:dark) control the time of spawning

5.1. Abstract

Circadian cycles of light:dark are important regulators of many biological activities, providing predictable cues for processes that require strict timing. In this study we ask whether circadian cycles (light:dark) might be used as cues for timing of spawning patterns within daily intervals. We also analyse the relationship between mating systems and synchrony of egg release, to assess the prediction that dioecious species need to be more synchronized in spawning than selfing hermaphroditic species. We tested these hypotheses by compared egg release between selfing hermaphroditic (*Fucus spiralis* and *Fucus guiryi*) and dioecious (*Fucus vesiculosus* and *Fucus serratus*) species by shifting the time of light:dark cycles in experimental conditions. Results demonstrate that egg release in dioecious species is very synchronous, taking place after some hours in the light, and that it is controlled by light:dark cues which when shifted also change the timing of spawning. In contrast, hermaphroditic species spawned in the dark and light intervals, and were less synchronous than dioecious species (larger variance in spawning time within circadian cycles). Constant conditions (of light or darkness) inhibited the amount of release in all species, but not their synchrony, indicating a role for the alternation of light and dark in mediating gamete release.

Keywords: Dioecious; Egg release; Hermaphrodite; Light:dark cycles; Mating system; Spawning; Synchrony

5. 2. Introduction

Sessile marine organisms that inhabit the intertidal zone (e.g., seaweeds and invertebrates), experience a large variation in physical conditions, mainly due to the tidal and diurnal cycles that rapidly change environmental conditions in a small scale of time. Biological adaptation to these environmental extremes makes such organisms a good model to understand the relative roles of endogenous rhythms *versus* environmental signals associated with changing tidal amplitude and/or light cycles in determining egg release and synchrony of externally fertilizing marine species (see Serrão and Havenhand 2009), and therefore the reproductive success. Synchronized egg release at short time scales is usually correlated with circadian and tidal cycles (Lüning 1981, Babcock et al. 1986, Brawley 1992, Pearson and Brawley 1996, Berndt et al. 2002, Monteiro et al. 2009, 2012, Chapter 3) or is triggered by specific temperature (Bacon and Vadas 1991) and photoperiod (reviewed by Pearson and Brawley 1998). The importance of photoperiod to stimulate gamete release in algae has been reported in laboratory experiments with *Pelvetia fastigiata* (Jaffe 1954), *Laminaria* spp. (Lüning 1981) and in field work with *Ulva lobata* (see Smith 1947) and synchronously in many species of green algae on coral reefs (Clifton 1997, Clifton and Clifton 1999). Despite widespread correlations between spawning and environmental cues, the physiological mechanisms behind such patterns are poorly understood (see reviews by Pearson and Serrão 2006, Serrão and Havenhand 2009).

Spawning in *Fucus* species occurs exclusively in the light (Pearson and Brawley 1996, Serrão et al. 1996). In dioecious species of *Fucus*, it has been recorded to take place in the afternoon or late afternoon in non-tidal populations of *Fucus vesiculosus* (Serrão et al. 1996) and near slack high tide at daytime in intertidal population of *F. vesiculosus* (Berndt et al. 2002) and *Fucus ceranoides* (Brawley 1992). In hermaphroditic *Fucus distichus* (tide-pools populations) release occurs when low tide is around midday, thus in calm conditions in the light (Pearson and Brawley 1996). A similar requirement for a period in the light was observed in intertidal shores in northern Portugal for the dioecious species *F. vesiculosus* and *Fucus serratus* (Monteiro et al. 2012, Chapter 3). These observations suggest that the inhibition of egg release at night reflects a requirement for photosynthesis (Pearson and Brawley 1996, Serrão et al. 1996) and indeed gamete release is impaired by inhibition of photosynthesis (Serrão et al. 1996). However in hermaphroditic species (*Fucus spiralis* and *Fucus guiryi*), egg release occurs at night or early morning (Chapter 3), suggesting that egg release is not inhibited by darkness in all species of *Fucus*, and therefore that light is not required at the time of spawning. The significant divergence observed between hermaphroditic and dioecious species of *Fucus* in circadian cycles of egg release in natural conditions can act as a prezygotic mechanism against hybridization between species with different mating systems (Chapter 3). However, the environmental signal used to synchronize gamete release to distinct times of day in different species still remains to be understood.

Within the genus *Fucus*, the species *F. spiralis, F. guiryi*, *F. vesiculosus* and *F. serratus* often co-occur on the same shores in mixed stands. These species differ in reproductive mode (the former two are selfing hermaphrodites and the latter are dioecious), and form hybrids at low frequency (Engel et al. 2005). This is thus a useful model system that raises important questions concerning the maintenance of species integrity in sympatry; i.e., is mating system variation sufficient for reproductive isolation, or are other factors involved? In addition to mating system effects, prezygotic isolation might involve evolution of both molecular/biochemical (e.g., sperm-egg recognition, lowering the frequency of hybrid gamete fusions) and ecological mechanisms such as differences in the timing of gamete release (Levitan et al. 2004). In summary, the incomplete reproductive isolation between *Fucus* species, and the occurrence of different mating systems and differentiated allopatric and sympatric populations over a large latitudinal distribution, raises very interesting questions and implications concerning spawning (a)synchrony, reproductive isolation, and the mechanisms that act against hybridization in sympatric species with external fertilization (broadcast spawners).

If the inhibition of egg release in darkness reflects the requirement for photosynthesis in *F. vesiculosus* (Pearson and Brawley 1996, Serrão et al. 1996) and probably in *F. serratus*, how and why do hermaphroditic species synchronize their egg release with the absence, or very low, light intensity? The timing of spawning might potentially influence the levels of stress experienced by the released gametes, thereby having fitness implications. The dark:light morning shift appears to acts as a signal for synchronous release in hermaphroditic species (discussed by Monteiro et al. 2012 and in Chapter 3). The alternation of light:darkness is predictable but light intensity is one of the most variable abiotic factors in the intertidal and subtidal zone (Schubert et al. 2001) and it may be associated to high levels of ultra violet radiation (UVR), especially during emersion. Species that inhabit the high intertidal, as the hermaphroditic ones in this study, are less sensitive to UVR than those that occur in the low intertidal, as the dioecious ones, probably due to the higher density of phlorotannin containing physodes in the former (Schoenwaelder et al. 2003). However to reduce UV stress some mechanisms to repair or shield compounds are involved, which could potentially reduce physiological performance due the metabolic cost (Wahl et al. 2011). Such mechanisms might compromise the timing of gamete release in upper intertidal species. The circadian timing of egg release in hermaphrodites could thus be influenced by processes involving inhibition of egg release when light intensity, UVR and temperatures are highest.

Since circadian light:dark cycles overlap with tidal cycles in natural conditions, our objectives were to test in laboratory cultures without tidal cycles: 1) the role of circadian (light:dark) cycles on spawning patterns (egg release) and; 2) if dioecious species are more synchronized than hermaphroditic species, even after shifting the time of the light:dark cycles, in four species with different mating systems (hermaphrodites *vs.* dioecious). Besides, the experimental laboratory work also allowed us to test the hypothesis of whether there is an endogenous circadian rhythm of egg release (*sensu* Sweeney 1987). As a model, we used four species of *Fucus*: self-compatible hermaphroditic *F. spiralis* and *F. guiryi* and dioecious *F. vesiculosus* and *F. serratus*. Our work provides important information concerning the sensing of light to time egg release and the divergence in spawning patterns between species with different mating systems. Finally our data also emphasize the importance of exogenous signals over endogenous circadian rhythms of egg release.

5. 3. Material and methods

5.3.1. Model organisms and sampling in the field

To study the effects of circadian (light:dark) cycles on the timing of egg release in the four target species we used mature individuals from Viana do Castelo (Northern Portugal, for further description see Ladah et al. 2003, Araújo et al. 2012, Monteiro et al. 2012). The reproductive structures in *Fucus* sp. are called receptacles, where numerous spherical conceptacles with antheridia (each with 64 sperm, 5 µm long) and/or oogonia (each with 8 eggs, ca. 80 µm in diameter) develop. Two different reproductive modes are described in *Fucus* sp.; while some species are hermaphroditic and the conceptacles contain both sperm and eggs (e.g., *F. spiralis* and *F. guiryi*), others are dioecious, where the sperm and egg develop in separate conceptacles from different male and female individuals (e.g., *F. vesiculosus* and *F. serratus*). For further description of egg and sperm release, settlement, external fertilization and reproductive patterns see reviews by Callow et al. (1985) and Pearson and Serrão (2006). The species *F. serratus* was identified by its serrated edges and *F. vesiculosus* by its air bladders. The hermaphroditic species were identified as described by Zardi et al. (2011).

5.3.2. Laboratory preparation and acclimation of the receptacles

In all experiments we used only female gametes (eggs) to compare the timing of gamete release across species. In the laboratory, the dioecious species (*F. vesiculosus* and *F. serratus*) were sexed and only the receptacles of females were used. For the hermaphroditic species (*F. spiralis* and *F. guiryi*) this was not necessary since all receptacles contain eggs (in addition to sperm). For all experiments, we chose large mature receptacles from different individuals. During two days, prior to the quantification of egg release, the receptacles were acclimated to culture conditions inside large test tubes with natural filtered seawater in culture chambers (ARALAB, Portugal) at 14 ºC (natural seawater temperature during upwelling periods that are common in the study region), and at 100 μmol photons $m^{-2}s^{-1}$ (in order to conduct the study below photoinhibitory light levels, to detect signalling effects of light presence/absence rather than stress). During these days all receptacles were changed to clean tubes with new seawater once a day but no quantification of eggs was performed. The acclimation was implemented for receptacles to adjust to the culture conditions after the transport from the field.

5.3.3. Photoperiod timing shifts

The timing of the light:dark cycles was tested on cultures of reproductive material from the four species. For each species, two receptacles were placed in each of 6 replicate glass tubes with ≈ 60 ml seawater (35 psu) in culture chambers under controlled light, temperature and photoperiod (14 ºC, 100 μmol photons $m^{-2}s^{-1}$). Seven treatments were used. In five treatments, photoperiod was kept at 8 h darkness:16 h light, either at the 1) approximate natural photoperiod of Viana do Castelo in summer (22:00-6:00 h) or by shifting it, to make darkness occur at 2) 3:00-11:00 h; 3) 8:00-16:00 h; 4) 13:00-21:00 h;

and 5) 18:00-2:00 h. Two more treatments consisted of 6) constant light and 7) constant darkness. For each treatment, six tubes (replicates) were used for each species (24 tubes per treatment). During six days, all receptacles were moved to new tubes with seawater every 3 hours, at 00:00, 3:00, 6:00, 9:00, 12:00, 15:00, 18:00 and 21:00 hours. The eggs present in each tube, corresponding to the release that occurred in each 3 h period, were counted under a dissecting microscope. Due the high amount of tubes to be counted per day, some samples were fixed in acetic acid:ethanol (1:3) and counted later. To replace the receptacles in chambers with dark conditions, the collections were performed in almost dark conditions to prevent any light stimulus yet with sufficient dim light to see the contours of the tubes to enable the manipulation of receptacles. This work was performed twice, in August and in October.

5.4. Results

In the hermaphroditic species, *F. spiralis* and *F. guiryi*, regular peaks of gamete release occurred in all treatments that had alternation of light and darkness conditions, and gamete release occurred during the light and dark periods (Figs. 5.1A-E, 5.2A-E and 5.3A,B). However, in *F. spiralis* the highest release in the light occurred within the 4 hours subsequent to the onset of light, while in *F. guiryi* peaks of gamete release were observed at different times of the day (Fig. 5.3A,B).

Under constant conditions, without a light:dark alternation, gamete release was inhibited (Figs. 5.1F,G, 5.2F,G) after the first day. Under constant light, *F. guiryi* had no peaks of egg release (besides the first day when still influenced by the previous night) (Figs. 5.1F and 5.2F). Under constant darkness, there was often no release, but several peaks of egg release were observed in *F. spiralis* (only in August; Fig. 5.1G) and in *F. guiryi* (Fig. 5.2G). Among the few gamete release peaks in constant conditions, several follow the same timing of egg release as observed in the treatment with natural conditions. The constant conditions treatments mainly resulted in inhibition after the first day but some release happened after some days of inhibition, and was synchronous between the different receptacles.

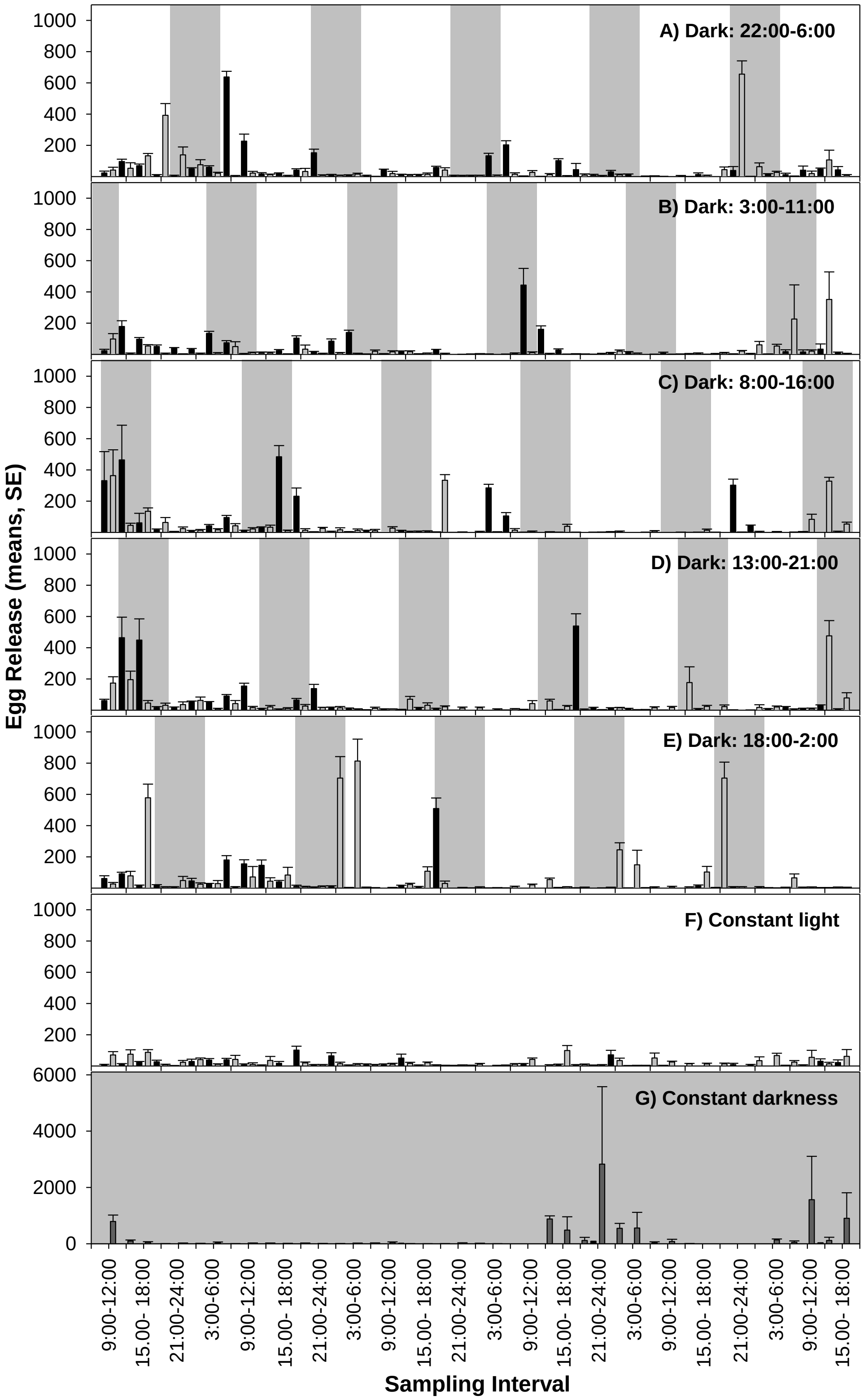

Figure 5.1. Effects of photoperiod shifts (16:8 L:D) and constant light and darkness on egg release by *Fucus spiralis*. Egg release (n=6 ± SE) under (A) photoperiod coincident with natural conditions in northern Portugal (dark period: 22:00-6:00 h); photoperiod in which the dark period occurred at (B) 3:00-11:00 h; (C) 8:00-16:00 h; (D) 13:00-21:00 h and (E) 18:00-2:00 h. Constant conditions: (F) constant light and (G) constant darkness. Grey boxes are the dark periods. Dark and grey bars represent two experiments (in August and in October, respectively).

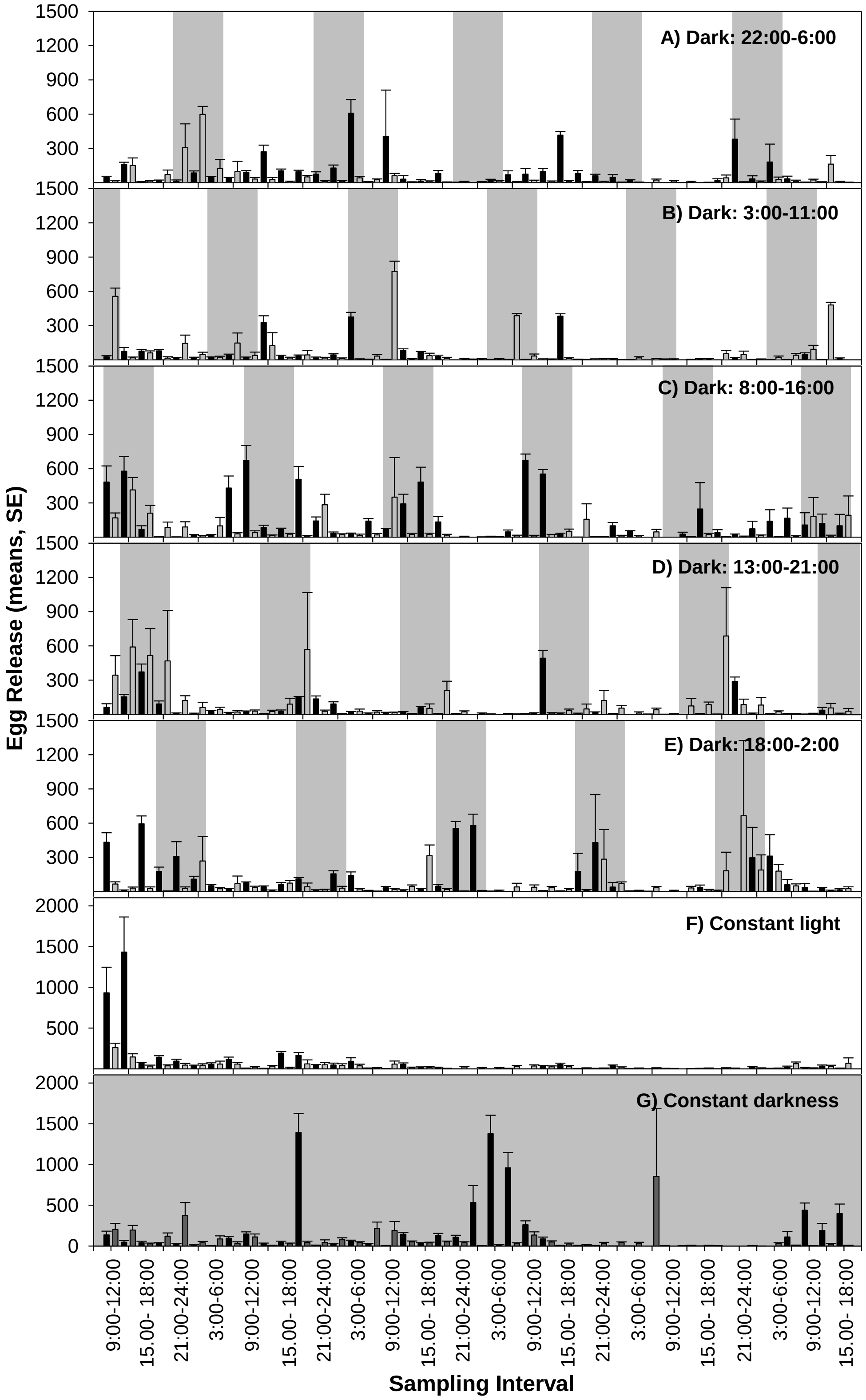

Figure 5.2. Effects of photoperiod shifts (16:8 L:D) and constant light and darkness on egg release by *Fucus guiryi*. Egg release (n=6 ± SE) under (A) photoperiod coincident with natural conditions in northern Portugal (dark period: 22:00-6:00 h); photoperiod in which the dark period occurred at (B) 3:00-11:00 h; (C) 8:00-16:00 h; (D) 13:00-21:00 h and (E) 18:00-2:00 h. Constant conditions: (F) constant light and (G) constant darkness. Grey boxes are the dark periods. Dark and grey bars represent two experiments (in August and in October, respectively).

The dioecious species *F. vesiculosus* and *F. serratus* showed very different spawning patterns in comparison with the hermaphroditic species. Gametes were never released in the dark, spawning peaks took place towards the end of the light period (Figs. 5.4A-E and 5.5A-E). Again, under constant conditions (constant light or constant darkness; Figs 5.4F,G and 5.5F,G) the gamete release pattern was disrupted or inhibited. The onset of release in *F. vesiculosus* and *F. serratus* occurred around 8 hours after the onset of light in all treatments with day:night cycles and in both experiments. Under simulated natural conditions (Figs. 5.4A, 5.5A) the peaks of egg release occurred between 12:00-18:00 h. Thus, at the natural circadian cycle the egg release occurs between midday and late afternoon, and this is maintained but shifted when the time of the night is changed (Figs. 5.4B-E and 5.5B-E), resulting in a spawning interval always around 8-12 hours for *F. vesiculosus* and around 8-14 hours for *F. serratus* after the onset of light (Figs. 5.3C,D).

A similar study to detect the circadian timing of gamete release by shifting the time of the onset of light and darkness conditions was performed with *F. vesiculosus* from the Baltic (Askö), where it is subtidal (Appendix S5.1 in Supplementary Informartion). Results show that the gamete release occurs 12-13 hours after the onset of light in the treatment simulating natural conditions (in Baltic Sea) and also in treatments where the onset of light and darkness was shifted. Little or no gamete release was observed at night and during the first hours after the onset of light. Under constant light or constant darkness few gametes were released, and only a few small events of release were observed (more under constant light than in constant darkness). Although the amount of gametes released in constant conditions was much smaller than in the treatments with circadian cycles, when release happened, the same periodicity was kept as in the circadian cycles.

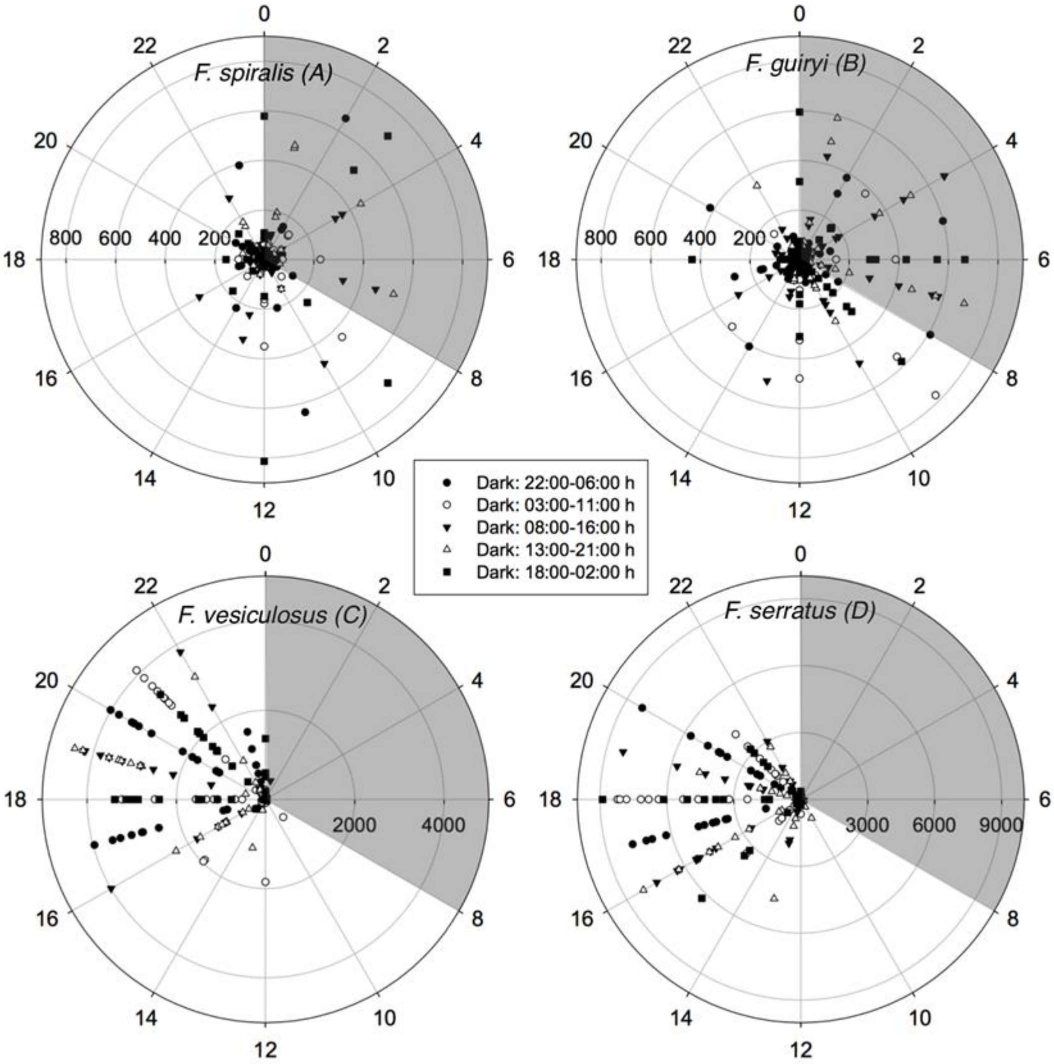

Figure 5.3. Egg release (radial data) by (A) *Fucus spiralis*, (B) *Fucus guiryi*; (C) *Fucus vesiculosus* and (D) *Fucus serratus* in August and in October relative to the timing of start of dark period (angular data). The time shown in angular data is not real time, i.e., it is relative to the time for the start of the dark period (time 0). Grey boxes are the dark periods during a circadian cycle (light and dark conditions; 16:8 L:D). Time shown in the legends is the real time for the start of the dark period in each chamber: dark circles correspond to the photoperiod coincident with natural condition in northern Portugal (dark period: 22:00-6:00 h) and the other symbols correspond to the chambers in which the dark period occurred at 3:00-11:00 h, 8:00-16:00 h, 13:00-21:00 h and 18:00-2:00 h.

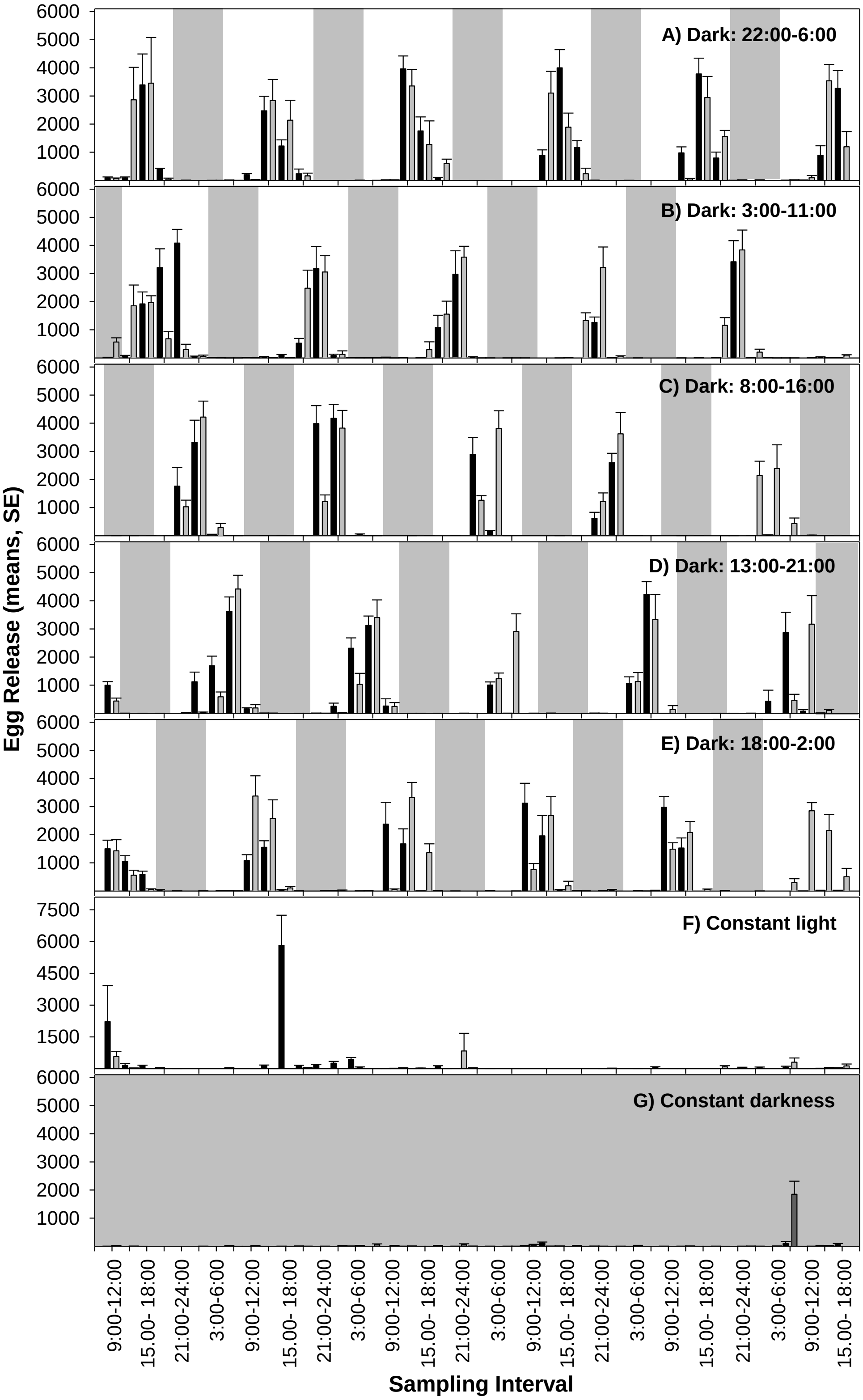

Figure 5.4. Effects of photoperiod shifts (16:8 L:D) and constant light and darkness on egg release by *Fucus vesiculosus*. Egg release (n=6 ± SE) under (A) photoperiod coincident with natural conditions in northern Portugal (dark period: 22:00-6:00 h); photoperiod in which the dark period occurred at (B) 3:00-11:00 h; (C) 8:00-16:00 h; (D) 13:00-21:00 h and (E) 18:00-2:00 h. Constant conditions: (F) constant light and (G) constant darkness. Grey boxes are the dark periods. Dark and grey bars represent two experiments (in August and in October, respectively).

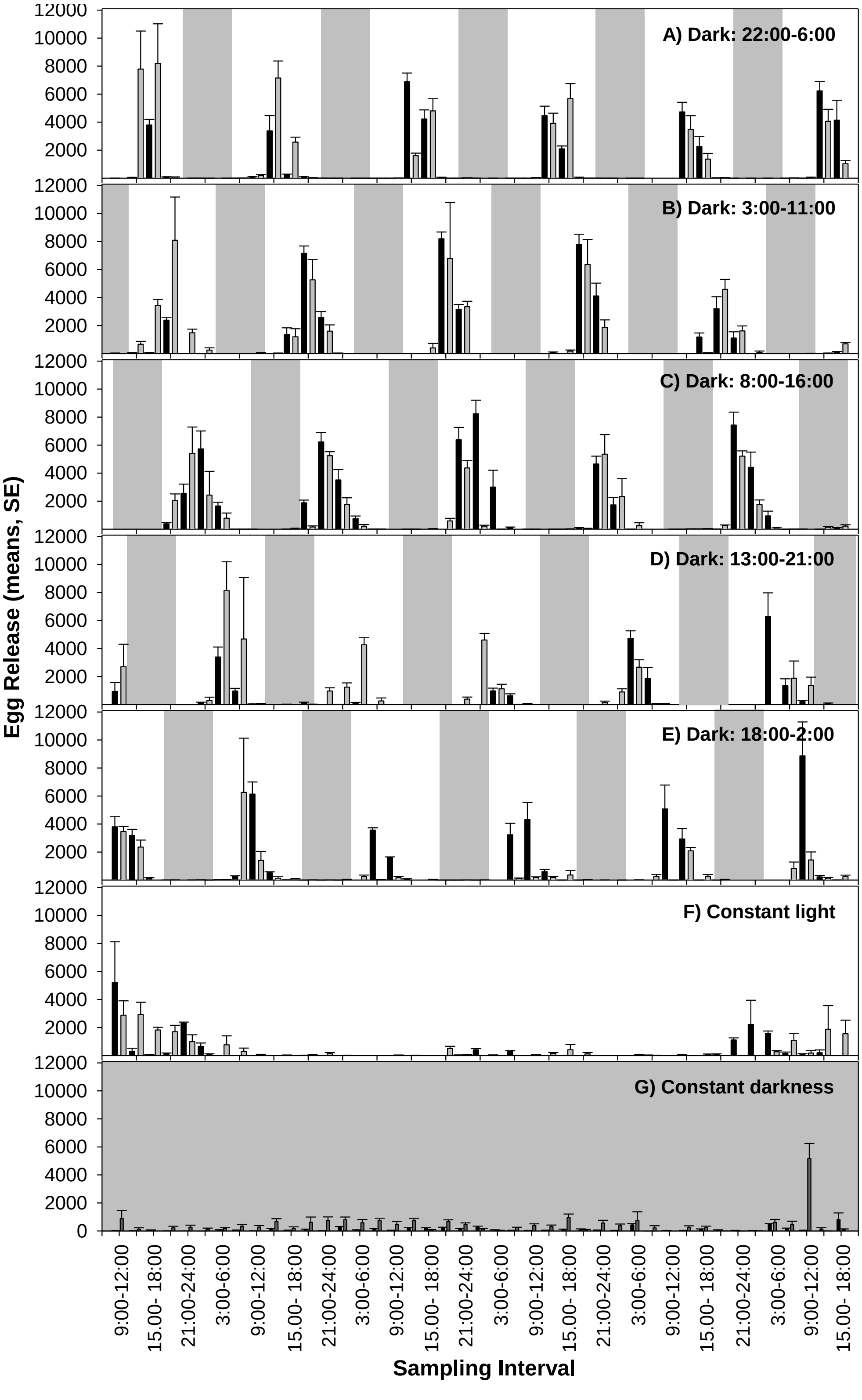

Figure 5.5. Effects of photoperiod shifts (16:8 L:D) and constant light and darkness on egg release by *Fucus serratus*. Egg release (n=6 ± SE) under (A) photoperiod coincident with natural conditions in northern Portugal (dark period: 22:00-6:00 h); photoperiod in which the dark period occurred at (B) 3:00-11:00 h; (C) 8:00-16:00 h; (D) 13:00-21:00 h and (E) 18:00-2:00 h. Constant conditions: (F) constant light and (G) constant darkness. Grey boxes are the dark periods. Dark and grey bars represent two experiments (in August and in October, respectively).

5.5. Discussion

This study reveals an effect of circadian cycles of light:dark in the control of gamete release, contributing to a better understanding of signals triggering mechanisms that control spawning synchrony in marine broadcast spawners. By shifting the timing of the daily photoperiod phases we discovered that gamete release by fucoid algae is responsive to timing of light:dark phases and that this response is different in species with different mating systems. Our data show that dioecious species release gametes in synchrony after several hours (ca. 8-14 hours) in the light phase, in contrast with hermaphroditic ones that have major spawning events the dark but also release during the light phase. However, while in *F. spiralis* the peaks of release in the light occur in the first hours of light immediately after the end of darkness, in *F. guiryi* gamete release may also occur later in the day. We also demonstrate that the patterns of release are strongly inhibited in constant light or constant darkness, indicating that the alternation between light and dark is a requirement for gamete release. Remarkably, the synchronization of gamete release with circadian cycles in dioecious intertidal species from northern Portugal is similar to that recorded in subtidal Baltic *F. vesiculosus*, supporting the hypothesis that circadian cycles of light:darkness tightly control the timing of gamete release in this species.

The level of synchrony was distinct between dioecious and hermaphroditic species. We predicted that might be the case if hermaphrodites are mainly selfing and therefore their reproductive success is not so dependent on synchronous release for maximizing gamete encounters. The hermaphroditic species had a large variance in spawning time (egg release), and this variability was larger in *F. guiryi* than in *F. spiralis*. Many release events occurred at different times of the day, including many small events, in the hermaphrodites, but especially in *F. guiryi*. One possible hypothesis to explain the higher variability in gamete release timing in this hermaphroditic species is related to the populations of *F. guiryi* in Viana do Castelo being introgressed with the dioecious species *F. vesiculosus* (Cánovas et al. 2011, Zardi et al. 2011).

In both dioecious and hermaphroditic species, constant light and darkness inhibited gamete release. This demonstrates that at least the magnitude of release is strongly inhibited in constant light and darkness, especially in constant light. The occasional gamete release events observed in darkness, especially in *F. guiryi*, may represent a lower degree of inhibition by constant darkness than by constant light, but more data would be necessary to understand this. In general, after the first day, fewer gametes were released in constant conditions especially during the first days, and in some cases after the initial inhibition for a few days under constant conditions a gamete release event or more were observed. This suggests that in the absence of a stimulus for gamete release the receptacles retain the gametes only up to a certain time when they finally spawn. Interestingly, the spawning events at the end of a constant period of several days still occur synchronously across the different receptacles submitted to the same conditions, despite the apparent absence of a light:dark signal.

Endogenous biological rhythms (Sweeney 1987) require an external signal to set the periodicity, and the immediate response of the algae to the timing of dark:light intervals in setting their spawning time, would support the hypothesis that the transition between light:dark or dark:light phases might function as such an external pulse. In contrast, the inhibition of gamete release in constant conditions, does not support the hypothesis of an endogenous circadian rhythm of egg release, as such a hypothetical biological circadian clock would have been expected to persist in constant conditions. However, the spawning control processes may be more complex, as the absence of dark:light transitions inhibited the magnitude of gamete release, but when they did release it was nevertheless following a synchronous pattern across receptacles from the same species. We can therefore conclude that there is an external light:dark signal triggering the periodicity of gamete release and although our data do not provide unequivocal evidence to support an endogenous biological rhythm, this remains an open question (see also Serrão 1996).

The patterns of egg release in our experimental conditions follow a similar pattern of egg release as observed in natural populations in northern Portugal (Chapter 3). In dioecious species, natural egg release events in *F. vesiculosus* were observed always a few or several hours after sunrise, between 9:00 h and 20:00 h and in *F. serratus* natural egg release events were observed between 10:00 h and 16:00 h, and no significant egg release was ever observed at night (Chapter 3). In both species, this diurnal pattern of egg release in natural populations occurred always when the individuals were immersed at high tide (Chapter 3), and gamete release in *F. vesiculosus* has been shown to take place at high tide also on the western Atlantic (Berndt et al. 2002). We can therefore conclude based on these experimental treatments and on the natural field observations (Chapter 3), that both dioecious species release their gametes during the day-time under the light, but only under water, therefore the exact time of release during the day is also influenced by the timing of the high tide. The time of high tide is an important environmental factor that coordinates the timing of egg release in dioecious species (Monteiro et al. 2012). While in natural population of *F. vesiculosus* in northern Portugal gamete release is coincident with neap tides (Monteiro et al. 2012), and this is maintained in populations transplanted to different habitats (Ladah et al. 2003), in Maine, USA, it occurs during spring tides (Berndt et al. 2002). However, what is common between these apparently distinct patterns in both locations is that release events occur in both cases during the days when the high tide is in the middle of the day, suggesting a dependence of egg release on the timing of the first diurnal (i.e., under the light) high tide (Monteiro et al. 2012). While these studies in natural populations determine that the timing of gamete release is dependent on both tidal cycles and day:night light cycles, the present study in experimentally controlled conditions was able to unravel the effects of light independently of the tidal influences. In the absence of a tidal regime we demonstrated that light:dark switches are an important environmental factor that sets the timing of gamete release. This is also in agreement with experimental work conducted on subtidal populations of *F. vesiculosus* (Baltic Sea), which always releases the gametes in the afternoon (Serrão et al. 1996), therefore also submerged and after a period of several hours in the light.

In the hermaphroditic species, gamete release in our experimental conditions took place during the dark and light phases, indicating that these do not use the same exact signal for gamete release as in dioecious species. Furthermore, the large variance in the timing of egg release along the circadian cycles suggests a reduced constraint on synchrony in hermaphroditic than in dioecious species. The reduced circadian synchrony in both hermaphroditic species observed in the present study, and the larger windows of spawning timing along semilunar cycles in *F. guiryi* (Monteiro et al. 2015) and along tidal cycles in hermaphroditic *F. spiralis* (Ladah et al. 2008) and *Silvetia compressa* (Johnson and Brawley 1998) in which egg release may occur in both high and low tide periods, suggest a generalized pattern of lower spawning synchrony in hermaphroditic relative to dioecious (discussed above) fucoid algae. This hypothesis matches the evidence for hermaphroditic species having greater reproductive assurance by relying on high selfing (e.g., Engel et al. 2005, Perrin et al. 2007), as a mechanism for recruitment assurance (as proposed by Pearson and Serrão 2006). These sources of evidence are in agreement with the hypothesis that the selfing mating system in hermaphroditic fucoids results in less selective pressure for synchronous gamete release events, thereby explaining the variance of egg release in relation to environmental conditions such as tidal phase and light:dark signals.

The differences in the timing of gamete release found between hermaphroditic (nocturnal and diurnal spawning) and dioecious (diurnal spawning) species may have important consequences, and could even be selected for, in natural conditions. In addition to the differences in mating systems, the intertidal ranges of these hermaphroditic and dioecious species are also very distinct. While these hermaphrodites are found predominantly along the high intertidal (*F. spiralis*) and mid to high intertidal (*F. guiryi*), the dioecious species occur along the mid- and low-intertidal (*F. vesiculosus* above *F. serratus*). These habitats differ in emersion-related stress factors, such as desiccation, time spent under extreme air temperatures, photoinhibitory light levels and ultra-violet radiation (UVR), and the survival of early life stages can be particularly affected by such emersion-related stress factors. Although the aim of this study was not to assess any possible effects of environmental stress, it is interesting to note that the predominantly nocturnal or early morning release pattern of the hermaphroditic species may be advantageous at the southernmost distributions of these species, where heat, light and desiccation stress can be very intense, and where the low survival of settlers explain the presence of hermaphroditic and the absence of dioecious species on the open coast (Ladah et al. 2003). In natural populations, the hermaphroditic species released the gametes at night and early morning and no significant egg release was observed between 12:00-22:00 hours (Chapter 3), i.e., during the peak of high light intensity and temperature. In contrast, in dioecious species, the egg release in both natural and laboratory conditions were relatively similar, occurring in the middle of the day, although they were not exposed to desiccation and heat stress because they were immersed during spawning intervals. The hermaphroditic species in this study without tidal effects did not follow strictly the natural pattern of night or early morning gamete release, and had some spawning events also at mid-day and in the afternoon. One possible hypothesis to explain this difference between the experimental and natural conditions is the absence of tidal effects in this study, which was aimed at studying the effects of light:dark cycles independently of the tides.

The comparison of this controlled light experiment with natural field data suggests that in the field, the tidal cycles may have a particularly important effect in restricting gamete release. Several tidal patterns have been identified for spawning timing, such as the immersion requirement for spawning in the dioecious *F. vesiculosus* (Berndt et al. 2002, Monteiro et al. 2012, Chapter 3). In high intertidal tide-pool populations, egg release by hermaphroditic *F. distichus* occurs at low tide, but individuals are hydrated (Pearson and Brawley 1996). Moreover, in the low intertidal, the species *F. serratus* may also release at the peak of low tide, but during neap tides, when individuals are still covered by seawater at the minimum tidal level (Chapter 3). The hermaphroditic species here studied occur predominantly along the higher tidal levels along the shore, therefore spending most of the day exposed to emersion stresses, and these are therefore likely to affect the daily time for spawning. We raise this hypothesis but our study does not provide evidence for nor against it, as the question of emersion effects was not approached. In our experimental conditions, permanence under light did not inhibit diurnal egg release in hermaphroditic species, but our experimental light levels were not photoinhibitory and did not include UVR, which could potentially affect the survival of gametes if spawning took place at low tide near midday. Furthermore, the experimental temperatures (14 ºC) reflected natural seawater levels, during the common spring upwelling conditions, which are much lower than maximal air temperatures in natural populations, thus not reflecting possible air temperature effects. Nevertheless, it is possible that there are selective pressures and trade-offs affecting the timing of spawning due to its consequences for the survival of early life stages in natural populations. Further studies are required to confirm the last statement.

Light (presence/absence) was clearly shown in this study as triggering the timing of spawning in the four species studied. Light has a very wide spectrum of effects on seaweeds. It is an obvious important resource for photo-autotrophic organisms (Wahl et al. 2011). Photoperiod may trigger the development of reproductive structures in fucoid algae (Bird and McLachlan 1976) and gametogenesis in red algae (Guiry and Cunningham 1984). The release of gametes occurs as a response to changes of light properties in several species (e.g., Smith 1947, Jaffe 1954, Lüning 1981, Pearson et al. 2004). The restriction of gamete release to periods when irradiance is blue-shifted in an intertidal fucoid has been proposed to act as depth sensing mechanism to restrict spawning to favourable high tide periods (Pearson et al. 2004). Furthermore, UVR might be a major factor determining the upper distribution limit of several species (e.g., Laminariales: Wiencke et al. 2000, 2006; Fucales: Schoenwaelder et al. 2003), probably due to effects on the early life stages (Schoenwaelder et al. 2003, Wiencke et al. 2006). Intertidal populations of *Fucus* exhibit regular photoinhibition, and although photodamage and reduction in physiological performance seem rare (Huppertz et al. 1990, Hanelt et al. 1997, Michler et al. 2002) these could potentially compromise the physiological mechanisms involved in spawning.

Several effects of light on spawning by fucoid algae have been previously demonstrated; in particular, a photosynthesis-dependent signaling pathway and the roles of light quality during potentiation have been described on several fucoid algae (Pearson et al. 1998, 2004, Pearson and Brawley 1998, reviewed by Pearson and Serrão 2006). This study identifies a distinct requirement for spawning in fucoid species; a requirement for alternation of light:dark phases during the daily cycle as a signal to trigger the circadian timing of gamete release. Such a triggering mechanism by shifts between light:dark or vice-versa is needed for both the dioecious and hermaphroditic species here studied. However, these species had opposite responses, timing the spawning mainly during the night (hermaphroditic) or restricting it to the day (dioecious), raising novel questions for further studies to clarify these differences. In addition, the hermaphroditic species showed lower spawning synchrony, supporting the hypothesis that a selfing mating system provides reproductive assurance thereby relaxing the selective constraints for synchrony of gamete release.

5.6. Acknowledgments

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5.7. References

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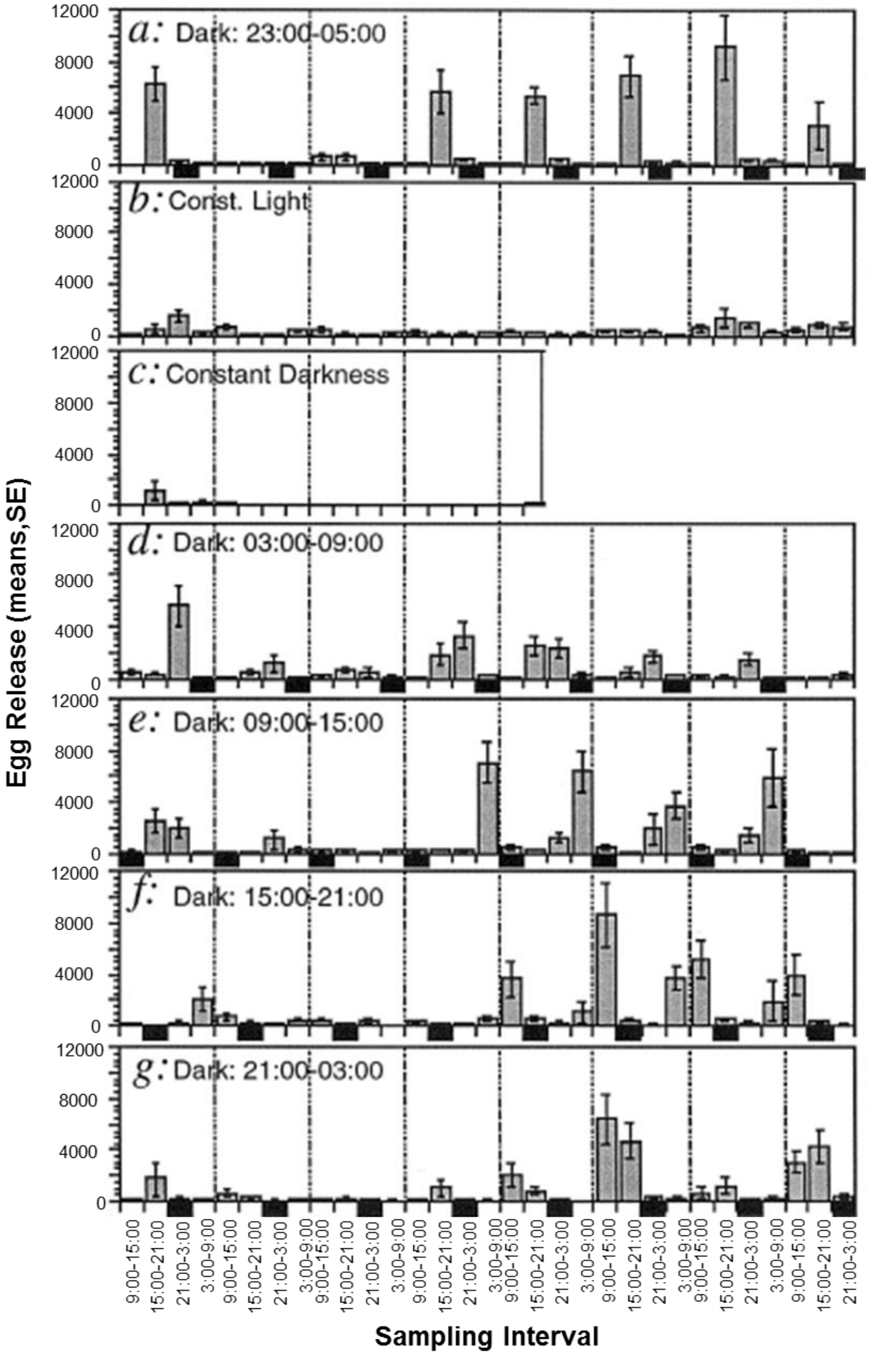
Supplementary Information

Appendix S5.1. Effects of photoperiod shifts and constant light and darkness (240 µmol photons $m^{-2}s^{-1}$; 18:6 L:D) on egg release by Baltic (Askö) *Fucus vesiculosus*. Egg release (n=10 ± SE) under (a) photoperiod coincident with natural condition in Askö (dark period: 23:00-5:00 h); (b) constant light, (c) constant darkness; photoperiod in which the dark period occurred at (d) 3:00-9:00 h; (e) 9:00-15:00 h; (f) 15:00-21:00 h and (g) 21:00-3:00 h. Black bars under the x-axis represent the dark intervals of the photoperiod. During 7 days (4 days for constant dark conditions) all receptacles (one receptacle per replica) were moved to new tubes with seawater (13 ºC) at 3:00, 9:00, 15.00 and 21:00 hours (from Serrão 1996).



Chapter 6

Hybridization between marine broadcast spawners: incomplete barriers to reproductive isolation

6. Hybridization between marine broadcast spawners: incomplete barriers to reproductive isolation

6.1. Abstract

The development of reproductive isolation between genetic entities with sympatric (overlapping) geographic ranges is an important ecological and evolutionary process in speciation. The evolution of contrasting reproductive strategies in sister species with external fertilization (=broadcast spawning) provides an opportunity to understand hybridization and the evolution of reproductive isolation. Here, we investigated reproductive barriers to inter-breeding between sympatric sister species, using two self-compatible hermaphroditic (*Fucus spiralis* and *Fucus guiryi*) and two dioecious (*Fucus vesiculosus* and *Fucus serratus*) species. To test for gametic (in)compatibility, i.e., the potential for hybridization between these species, we performed laboratory crosses between all combinations (male and female gametes) of intra-specific (conspecific or positive control) and inter-specific (hetero-specific) crosses. The paternity and maternity of resulting F1 individuals were identified using multilocus microsatellite genotyping, nuclear markers and mitochondrial DNA. Other F1 individuals were maintained in culture conditions to compare hybrid growth and survival rates. Our data revealed the existence of gamete compatibility and hybridization between sister species. The results show variable growth and survival across treatments, in some cases higher in hybrids than in intra-specific crosses, suggesting a role for hybridization in providing a source of variation for adaptation. While with microsatellite loci it was very difficult to identify maternal and paternal inherence, and nuclear markers only prove hybridization between species from Clade 2 (*F. spiralis*, *F. guiryi* and *F. vesiculosus*), mitochondrial DNA shows that the hybrids may have maternal, paternal and biparental inherence. This work demonstrates gametic compatibility in inter-specific crosses, and supports the hypothesis that gametic incompatibility is not a strict prezygotic mechanism against hybridization between sympatric *Fucus* species.

Keywords: Broadcast spawning; Dioecious; Gametic (in)compatibility; Hermaphrodite; Hybridization; Intra- and inter-specific crosses; Mating system; Pre- and postzygotic barriers; Speciation; Sympatric species

6.2. Introduction

Reproductive isolation is the first step towards speciation, and in sister species with similar geographic range (sympatric species) the degree of reproductive isolation has important ecological and evolutionary consequences for the distribution of genetic diversity and gene flow between and within populations. Understanding the potential of hybridization and the consequent production of fertile or sterile hybrids is important to understand the fitness of closely related species and the factors that influence the evolutionary pathway between different reproductive systems (e.g., Billard et al. 2005ab). The consequences of hybridization between species may be the evolution of new lineages (through recombination, speciation and polyploidy) or the formation of hybrid zones resulting from gene flow between species with incomplete reproductive isolation (Coyer et al. 2007). During the last 30 years, hybridization has been reported mainly in terrestrial species, however hybridization in marine environments occurs at a rate similar to that of terrestrial environments (Coyer et al. 2007). The *Mytilus edulis* species-complex is one of the best examples of hybridization in European marine environments, where two blue mussel species may hybridize in sympatry, creating a mosaic of hybrids (Daguin et al. 2001), but other examples around the world have been described, such as corals (Vollmer and Palumbi 2002, Levitan et al. 2004), sea urchins (Levitan 2002), bivalves (Bierne et al. 2002, Beaumont et al. 2004) and fucoid algae (Coyer et al. 2002ab, 2006, 2007, Engel et al. 2005). Broadcast spawning (external fertilization) potentially increases hybridization and gene flow, and there is evidence that hybridization between marine algae can occur between species with different mating systems, namely hermaphroditic and dioecious species (Bold and Wynne 1985, Coyer et al. 2002a, Engel et al. 2005). Since the variability in the mating system can affect the movement of genes among closely related hybridizing species (Sweigart and Willis 2003) it is important to understand the implications of mating systems on the potential for hybridization as it will affect the ecology and evolution of species.

The strict dependence on external fertilization, occurrence of sympatric and allopatric distributions and the different reproductive modes within *Fucus* (Fucales) provide an opportunity to understand hybridization and reproductive isolation between sister species. *Fucus* species is one of the most well study macroalgal genus. The character dioecy/hermaphroditism, the wide distribution over northern Atlantic and the overlapping but distinct vertical distribution between many species of this genus make *Fucus* a good model for studies of reproductive ecology. Typically, the vertical distribution of these species is vertically segregated (parapatric) however it is possible observe mixed stands of these species (sympatric), especially in the transition zone and shores with complex topology (Billard 2005). One of the main differences between species is their reproductive mode: self-compatible hermaphroditic (e.g., *Fucus spiralis* and *Fucus guiryi*) *versus* dioecious (e.g., *Fucus vesiculosus* and *Fucus serratus*). Genetic studies (e.g., Engel et al. 2005) suggest that the hermaphrodites reproduce primarily through selfing while the dioecious species mainly show endogamous breeding. This divergence in breeding systems increases premating isolation and creates a barrier to complete mixing and consequently may play an important role for species integrity in these taxa (Engel et al. 2005). Furthermore, the development of contrasting reproductive strategies in sympatric species provides an opportunity to understand causes and consequences of hybridization and reproductive isolation.

Hybridization has been documented for many terrestrial and marine organisms but mostly relying on putative hybrids being recognized in the field by the intermediate morphologies between two co-occurring species. In *Fucus* species, field observations of intermediate morphologies have been reported in several studies, particularly as hybrids between *F. vesiculosus* and other species (e.g., with *F. spiralis* and *F. serratus* and others, Scott and Hardy 1994, but see also review in Coyer et al. 2002a). However, these suggestions are not supported with molecular approaches and the exact status of “hybrids” must be assessed on somewhat circumstantial evidence. More recently, molecular studies provided evidence for *Fucus* hybridization and introgression in natural populations (Coyer et al. 2002ab, Wallace et al. 2004, Billard et al. 2005ab, 2007, Engel et al. 2005) and for the past role of hybridization in shaping the present spatial distribution of genetic variation (e.g., Neiva et al. 2010, Zardi et al. 2011). For example Engel et al. (2005) estimated that 10% of study individuals were genetically intermediate between the two genetics entities (*F. spiralis* and *F. vesiculosus*), especially in the vertical overlap zones, as also shown by Billard et al. (2010). The spatial proximity between these species on local scales could favour and increase hybridization. Most studies suggest that hybridization occurs only at a very short distances (Wallace et al. 2004, Engel et al. 2005, Billard 2007). Hybridization was shown particularly in the zones of contact (sympatry) between species, e.g., *F. serratus* and *F. evanescens* (Coyer et al. 2002ab, 2007), *F. vesiculosus* and *F. spiralis*

(Wallace et al. 2004, Billard et al. 2005ab, 2010, Engel et al. 2005, Coyer et al. 2006, 2011a, Moalic et al. 2011) and between *F. vesiculosus* and *F. ceranoides* (Neiva et al. 2010). There is evidence that contact zones where expanding allopatric populations first encounter other species in sympatry are particularly susceptible to hybridization and introgression, e.g., when *F. ceranoides* (Neiva et al. 2010) or *F. guiryi* (previously named *F. spiralis* var. *platycarpus*; Moalic et al. 2011, Zardi et al. 2011) contact *F. vesiculosus*. This is likely due to the absence of reinforcement of barriers against hybridization in allopatric populations that have not previously encountered potentially hybridizing species (Moalic et al. 2011).

The species distribution among lineages of *Fucus* suggests that hybridization can occur between hermaphroditic and dioecious species (e.g., Coyer et al. 2007) because species with low phylogenetic distance often have contrasting mating systems. The genus *Fucus* is characterized by two lineages, with hermaphroditic and dioecious mating systems represented in both. While *F. spiralis, F. guiryi* and *F. vesiculosus* are part of a recent evolutionary radiation within *Fucus* (clade 2), *F. serratus* and *F. evanescens* are in a divergent lineage (Clade 1; Cánovas et al. 2011). Previous studies with corals having gamete incompatibility between distantly-related species, show asymmetrical fertilization in which sperm of the more ancestral species are able to fertilize eggs of the more recently diverged species, but not the opposite. Furthermore, egg release in corals is more synchronous in species that are more distantly related than in sister species (Fukami et al. 2004, Levitan et al. 2004, 2011, Willis et al. 2006, Fogarty et al. 2012ab).

Phylogenetic distance is a hypothetical explanation for the lack of experimental hybrids reported between the two main *Fucus* lineages, particularly between the often sympatric species *F. vesiculosus* and *F. serratus* (although a few natural reports occur, e.g. Billard et al. 2010). Phylogenetic distance might potentially increase gametic incompatibility as a prezygotic mechanism against hybridization. In *Fucus*, the parental lineages of the unambiguously demonstrated hybrids are part of the same clade and no hybrids have experimentally been demonstrated between species of different clades. Previous studies suggest incomplete reproductive barriers to hybridization within the clade of *F. spiralis, F. guiryi* and *F. vesiculosus* (Billard et al. 2005b, 2010, Engel et al. 2005) as shown within the clade of *F. serratus* and *F. evanescens* (Coyer et al. 2007), but the level of reproductive barriers to hybridization between clades is even less understood.

The spawning pattern of egg release between *F. vesiculosus* and *F. serratus* in Northern Portugal is highly coincident with the same phases of the daily tidal cycles, suggesting that the time of egg release is not a prezygotic mechanism against hybridization between these two dioecious species, and also suggesting that other mechanisms are involved to prevent hybridization (Chapter 3). Until now such mechanisms are unknown. The main objective of this work was to test if gametic incompatibilities act as a prezygotic mechanism (reproductive barrier) to inter-breeding between sympatric and related species and test the influence of phylogenetic distance on gametic (in)compatibility. As a model, we used four species of *Fucus*; *F. spiralis*, *F. guiryi*, *F. vesiculosus* and *F. serratus* from the southern limit of their sympatric distribution (Viana do Castelo, Northern Portugal). Laboratory crosses were used to test the potential of hybridization of *Fucus* sp. between all combinations (male and female gametes) of conspecific (positive controls) and heterospecific crosses. The relative hybrid survival and growth (postzygotic mechanisms) were also estimated and compared with conspecific controls. Finally, we verified hybridization in inter-specific crosses using microsatellites and nuclear sequence markers, and assessed the inheritance patterns of mitochondria in putative hybrids.

6.3. Materials and methods

6.3.1. Gametic (in)compatibility – prezygotic processes

In the genus *Fucus*, the development of gametangia (antheridia and oogonia) occurs in numerous spherical conceptacles, which are inside reproductive structures called receptacles (for details see Pearson and Serrão 2006). In hermaphroditic species (like *F. spiralis* and *F. guiryi*), the antheridia and oogonia develop inside the same conceptacle and in dioecious species (like *F. vesiculosus* and *F. serratus*) they are formed in distinct male and female individuals. Each antheridium contains 64 biflagellate and motile sperm and each oogonium contain 8 non motile eggs. To produce artificial hybrids in the laboratory we used fertile individuals collected from Viana do Castelo (northern Portugal) and transported to the laboratory in cooler boxes.

The number of replicated individuals per species was pre-defined as three for these crosses but it became unbalanced due to taxonomic changes that took place in the course of the experimental work. Prior to 2011, the species *F. guiryi* was identified as *F. spiralis* low (see Billard et al. 2010), and the early design of this study aimed to use three individuals of *F. spiralis* from the high shore and three individuals of *F. spiralis* from the low shore. However, when Zardi et al. (2011) published the genetic differences between these two identities and determined that *F. spiralis* low is a new species (i.e., *F. guiryi*) then the diagnostic microsatellite loci (L20 and L78) showed that two of the three individuals of "*F. spiralis* high" were in fact *F. guiryi*. For this reason, only one individual of *F. spiralis* was used in the present crosses.

Obtaining unfertilized gametes from the hermaphroditic species, *F. spiralis* and *F. guiryi*, was challenging because the conceptacles contain both oogonia and antheridia that are released together. Therefore, collecting oogonia after release from conceptacles would have been inappropriate as it would imply a high probability of eggs being fertilized by conspecific sperm upon release. Oogonia and antheridia were therefore carefully separated from conceptacles by hand and oogonia were rinsed in seawater over a nylon mesh (40 μm), to remove any eventually remaining conspecific sperm. In this approach, the oogonia and eggs are retained on the nylon mesh (eggs reach 70-80 μm in diameter, Callow et al. 1985, Brawley et al. 1999) and the antheridia settle through the small holes of the nylon mesh (sperm reach 5 μm in length, Callow et al. 1985). Furthermore, the mature oogonia were again carefully selected one by one and placed in glass dishes (the selection of oogonia was performed 2 times).

In the dioecious species, *F. vesiculosus* and *F. serratus*, the sex of each individual was determined using a microscope. Antheridia and oogonia were dissected out of the reproductive structures (conceptacles) and separated before they opened and released the gametes. To prevent differences in manipulative approaches between hermaphroditic and dioecious species, the antheridia and oogonia were also similarly washed in seawater over a nylon mesh (40 μm).

While the number of oogonia (40 oogonia = 320 eggs) was exactly quantified for all female individuals under a dissecting microscope, the sperm was not quantified, but the sperm concentrations used from the same "father" (sperm donor) were equal for all treatments. Therefore, an equal relationship between eggs and sperm was used per male individual. A reciprocal crossing design was used in all species, e.g., *F. spiralis* eggs were crossed separately with *F. guiryi*, *F. vesiculosus* and *F. serratus* sperm and the sperm of the same *F. spiralis* individuals were crossed separately with eggs from *F. guiryi*, *F.*

vesiculosus and *F. serratus*. All possibilities of crosses were performed between all species used in each experiment. To control for the success on sex separation a negative control for fertilization was performed, consisting of oogonia and no antheridia added, and the opposite. To control for the success of fertilization of each species a positive control of fertilization viability was performed, consisting of conspecific crosses between sperm and eggs within each species.

Crosses were performed in glasses containing seawater, and after 2 hours the biological material was moved to petri dishes and stored inside a culture chamber (ARALAB, Portugal) at 14 ºC under controlled salinity (33-35 psu) and constant photoperiod 12/12 light/dark. Fertilized eggs manifest polarization 12-24 hours after fertilization, when induced by unilateral light (other stimuli also induce polarity, for further review see Callow et al. 1985), i.e., the rhizoid grows towards the dark (or shading) side of the zygote. Fertilization was determined by rhizoid development. To facilitate microscope counts of fertilization proportion as soon as possible, all petri dishes were cultured in a a light gradient (in a black box with a single lateral opening for the light to induce horizontal rhizoid growth). The number of embryos (individuals with rhizoids) were quantify after 2 days and the immature oogonia (where the eggs were kept inside the cell wall of oogonia were not considered for estimating fertilization success).

6.3.2. Percentage of survival and growth rate – postzygotic processes

In order to compare the success of survival between intra- and inter-specific crosses under controlled conditions, recruits were kept in a culture chamber (ARALAB, Portugal) at 14 ºC, 60-70 μmol photons $m^{-2}s^{-1}$ (12/12 hours darkness/ light) and controlled salinity (33-35 psu). The recruits were counted every year along five years. The percentage of survival was estimated in relation to the total number of embryos when they were 2-days-old. To study the growth rate, a maximum of ten individuals from each cross (intra and inter specific crosses) were used (this study started 15 months after fertilization). The individuals were monitored in petri dishes (9 cm diam.) in the first two years, and subsequently they were moved to plastic bottles with 1 liter of seawater. To measure growth, each individual was carefully dried with paper and weighed to the nearest milligram. All treatments were maintained under the same experimental conditions and were run at the same time.

6.3.3. DNA extraction

To attest the laboratory hybridization success we excised 3-4 vegetative tips from each individual that contributed for the crosses (parental individuals). For the DNA extraction of early stages, the cultures were maintained in artificial conditions for eight weeks, and a random selection of a maximum of thirty individuals (recruits) from each of the crosses were stored in a freezer. DNA was extracted using the NucleoSpin Plant II kit (MACHEREY-NAGEL) and extraction buffer PL1 with some modifications to the protocol for DNA extraction of small recruits. Each recruit was introduced in a tube with extraction buffer PL1 plus RNAse a tungsten sphere and grinded for 1 min at 30G (Mixer Mill MM300, Retsch) instead of grinding 5 min at 30G without the extraction buffer as was done for adult tissue. After this, the procedure was as described in the kit protocol. Tubes were shortly centrifuged with recruits after each step until the first incubation; recruits where eluted in 50 µL instead of 100 µL of PE as it was done for the adults. DNA was stored at -20ºC after extraction.

6.3.4. PCR reaction and genotyping

Six polymorphic microsatellite loci F9, F12, F19, F49 (Coyer et al. 2009), L20 and L78 (Engel et al. 2003) were used to determine the parental origin. We also used four more polymorphic microsatellite loci, F21, F42, F60 and Fsp1, but maternal and paternal inheritance were not detected in the putative hybrids for those markers, so they were discontinued. A mitochondrial intergenic spacer, mtIGS (Neiva et al. 2010), was used to to assess organelle inheritance. Adapted conditions for all microsatellite loci are described in Table 6.1 The mtIGS were amplified as described in (Neiva et al. 2010), with template DNA diluted ten times for of adult individuals but not for recruits. All adult and recruit mtIGS fragments were genotyped (primer F labelled) to estimate fragment size. Sometimes more than one size for the same individual was detected so we cloned some of them (pGem-T Easy Vector System, Promega) to verify the sequences.

Amplification results were loaded into an ABI 3130 XL automated DNA sequencer (Applied Biosystems) for genotyping and sequencing. All loci and mtIGS were genotyped with LIZ500 and ROX1000 size standards (Applied Biosystems), respectively.

The genotyping chromatograms were analysed using STRand 2.3.79 (Davis 2007) and MsatAllele (Alberto 2009). All mtIGS sequences were aligned and analysed using GENEIOUS 3.04 (Drummond et al. 2006).

Table 6.1. PCR conditions in simplex adapted from Coyer et al. (2009) (F9, F12, F19 and F49) and Engel et al. (2003) (L20 and L78) including locus name, $MgCl_2$, dNTP, primer Forward (F) and Reverse (R) concentrations and annealing temperature (Ta) used to amplify adult of *Fucus spiralis, F. guiryi*, *F. vesiculosus* and *F. serratus* and the recruits resulting from their crosses. PCR amplifications were performed in 10 µL and 15 µL of reaction mixture, containing 1 or 4 µL (respectively for loci F9, F12, F19, F49 or loci L20, L78) of template DNA from the adults (1:10), 1×GoTaq polymerase buffer (Promega) and 0.5U GoTaq polymerase (Promega). For recruits, 4 µL of template DNA were used in all PCR reactions.

Locus	$MgCl_2$ (mM)	dNTP (mM)	Primer F (µM)	Primer R (µM)	Ta (ºC)
F9[1]	2	0.2	0.3	0.3	55 *
F12[2]					
F19[1]					
F49[3]					
L20[1]	2	0.12	0.17	0.33	54 ▼
L78[1]					55 ▼

Primer Forward labelled: [1] FAM; [2] HEX; [3] NED

PCR reactions were carried out in a GeneAmp 2720 (Applied Biosystems) with the following cycling parameters: * Initial denaturation step of 2 min at 94ºC,"touchdown" consisting in 25 cycles of 20 s at 94ºC, 10 s at 60ºC reduced by 0.2ºC each subsequent cycle, and 35 s at 72ºC, then the following step are 10 cycles of 20 s at 94ºC, 10 s at the annealing temperature, and 35 s at 72ºC, to finish exists a final extension of 10 min at 72ºC; ▼ Initial denaturation step of 5 min at 94ºC, 30 cycles of 30 s at 94ºC, 35 s at the annealing temperature, and 40 s at 72ºC, to finish exists a final extension of 20 min at 72ºC.

6.3.5. Nuclear sequence markers

Nuclear markers were developed from several cDNA derived contigs from *F. serratus/F. vesiculosus* EST libraries (Canóvas et al. 2011, Martins et al. 2013). Primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and tested for amplification in genomic DNA. Primers successfully amplifying a single intron-rich genomic DNA fragment across four *Fucus* species (*F. spiralis, F. guiryi, F. vesiculosus* and *F. serratus*) were used for cloning the fragment (pGEM-T plamid, Promega). Amplification products were purified with ExoSAP-It (USB, Cleveland, OH, USA) prior to sequencing (Macrogene, Europe). The resulting chromatograms were analysed using CodonCode Aligner v3.7.1 (CodonCode Corp., Dedham, Massachusetts, USA) and sequences showing the highest inter-specific polymorphism were selected; eight new primers were designed using Primer3 (Appendix S6.3 in Supplementary Information), with three used in final analysis.

PCR reactions were performed in a GeneAmp 9700 thermocycler (Applied Biosystems). Amplification was performed by polymerase chain reactions (PCR) using GoTaq in 20 L total volume containing 4µl GoTaq Flexi buffer, 1.5 – 3.0 mM $MgCl_2$, 0.1 mM each dNTP, 0.05 – 0.5 µM each primer, 1 U GoTaq Flexi DNA Polymerase (Promega). Cycling conditions consisted of an initial denaturing step of 5’ at 94°C, followed by 35 cycles of 30’’ at 94°C, 25’’ – 35’’ at 58º – 69ºC, 40’’ at 72°C, and a final elongation step at 72°C for 10’. Standard DNA dilution was 1:10 for six-months-old individuals (Gotaq) and 1:1 and/or 1:10 when using Phire (except in PCR tests; see below). PCR products were genotyped on an ABI PRISM 3130xl (Applied Biosystems), with Rox 1000 size standard at CCMAR, Portugal (labelled FAM, NED or HEX forward). Raw allele sizes were scored with STRand (Toonen and Hughes 2001).

6.4. Results

6.4.1. Gametic (in)compatibility – prezygotic processes

The proportion of eggs that became fertilized (Table 6.2) in intra-specific crosses (positive controls) was higher in the dioecious species, *F. vesiculosus* and *F. serratus* (92.6 and 96.5 %, respectively) than in the hermaphroditic species, *F. spiralis* and *F. guiryi* (73.4 and 71.4 %, respectively). In putative hybrids, higher fertilization success always involves one female of dioecious species (*F. vesiculosus* or *F. serratus*). No fertilization was observed in negative controls of dioecious (male and female) species. However, in hermaphrodites, a small percentage of fertilization was observed in negative controls with eggs of *F. spiralis* and *F. guiryi* (0.33 and 0.67 %, respectively, Appendix S6.1 in Supplentary Information). Fertilizations between dioecious species (inter-specific crosses) were more successful between the eggs of *F. vesiculosus* and the sperm of *F. serratus* than in the reciprocal crosses (96.2 and 77.4 %, respectively). Between hermaphroditic species, higher fertilization was observed between the eggs of *F. guiryi* and the sperm of *F. spiralis* than in the reciprocal crosses (33.1 and 20.4%, respectively).

Table 6.2. Fertilization success (means of percentages; 2-days-old) for all combination between 1 individual of *Fucus spiralis*, 3 individuals of *Fucus guiryi*, 6 individuals of *Fucus vesiculosus* (3 females and 3 males) and 6 individuals of *Fucus serratus* (3 females and 3 males). Dark boxes: percentage of fertilization in intra-specific crosses.

Male Female	*F. spiralis*	*F. guiryi*	*F. vesiculosus*	*F. serratus*
F. spiralis	**73.4**	**20.4**	**21.7**	**29.7**
F. guiryi	**33.1**	**71.4**	**28.1**	**37.3**
F. vesiculosus	**91.4**	**82.9**	**92.6**	**96.2**
F. serratus	**90.8**	**86.5**	**77.4**	**96.5**

6.4.2. Percentage of survival and growth rate – postzygotic processes

Survival in intra-specific crosses (positive control) was higher in *F. serratus* than in the other species (Fig. 6.1). In inter-specific crosses, survival was higher in putative hybrids from the eggs of *F. vesiculosus* and the sperm of *F. serratus* (*Fv F+ Fse M*) than in the other crosses. All putative hybrids from the crosses between hermaphroditic species died before the first and second year of age (*Fg F+ Fs M*, *Fs F+ Fsg M*, respectively) in laboratory conditions. Only three individuals from the inter-specific crosses between the eggs of *F. guiryi* and the sperm of *F. vesiculosus* had survived at the end of the first year in laboratory conditions. Moreover, these three individuals still survived in May 2015, when approximately 5 years old.

In the intra-specific crosses (positive controls), the highest weight (i.e., largest growth) was observed in *F. serratus* and the smallest in *F. guiryi* (Fig. 6.2). The weight of putative hybrids from inter-specific crosses between the eggs of *F. spiralis* and the sperm of *F. vesiculosus* (*Fs F+ Fv M*) or of *F. serratus* (*Fs F+ Fse M*) were higher than in any other crosses.

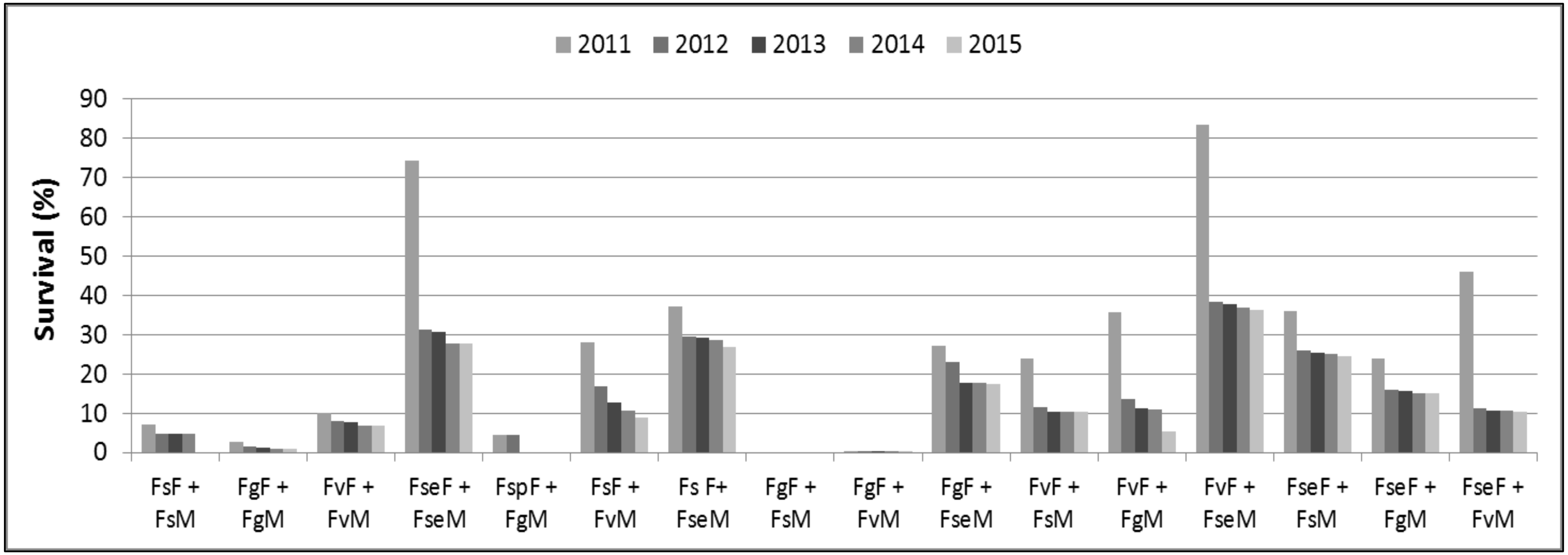


Figure. 6.1. Survival success (%) along five years. Data for all combinations of crosses between 1 individual of *Fucus spiralis* (*Fs F* and *Fs M*), 3 individuals of *Fucus guiryi* (*Fs F* and *Fs M*), 6 individuals of *Fucus vesiculosus* (3 females and 3 males: *Fv F* and *Fv M*, respectively) and 6 individuals of *Fucus serratus* (3 females and 3 males: *Fser* F and *Fser M*, respectively).

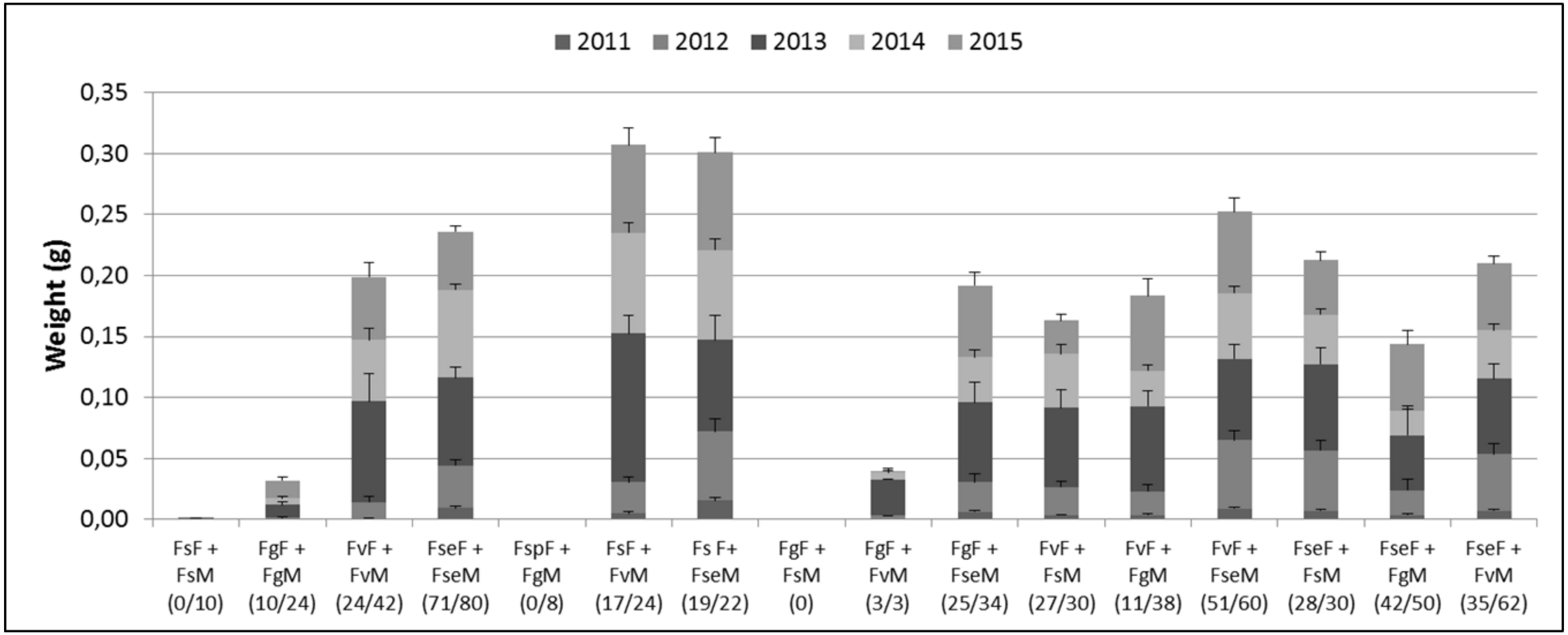


Figure. 6.2. The growth (weight, g) along five years of the recruits from intra- and inter-specific between 1 individual of *Fucus spiralis* (*Fs F* and *Fs M*), 3 individuals of *Fucus guiryi* (*Fs F* and *Fs M*), 6 individuals of *Fucus vesiculosus* (3 females and 3 males: *Fv F* and *Fv M*, respectively) and 6 individuals of *Fucus serratus* (3 females and 3 males: *Fser* F and *Fser M*, respectively).

6.4.3. Molecular identification of hybrids

Molecular data, from microsatellite loci, mtIGS (mitochondrial) and nuclear sequence markers reveal hybrids between the inter-specific crosses (Table 6.2). Microsatellite analyses reveal hybridization in some inter-specific crosses between *Fucus* species (Table 6.2). However the high amount of recruits used in genotyping and the low amount of confirmed hybrids suggest that the microsatellite markers, used in present work, are not the most appropriate genetic tools for detecting maternal and paternal inherence in *Fucus* hybrids.

From 44 recruits (two-years-old) genotyped with nuclear markers, maternal and paternal inheritance was confirmed in 29 recruits from inter-specific crosses between species of clade 2 (*F. spiralis*, *F. guiryi* and *F. vesiculosus*). In the inter-specific crosses between the species of clade 2 and *F. serratus* (clade 1), the nuclear markers always revealed only *F. serratus* inheritance in a total of 44 recruits analyzed. However, in 5 individuals (3 from eggs of *F. spiralis* and the sperm of *F. serratus* and 2 from eggs of *F. vesiculosus* and sperm of *F. serratus*) the microsatellite loci revealed both maternal and paternal inheritance, in agreement with the mitochondrial DNA (see above).

In mitochondrial DNA, length variants were detected in inter-specific crosses between species from clade 2 (*F. spiralis*: 569 bp, *F. guiryi*: 551 bp), *F. guiryi**: 576 bp and *F. vesiculosus*: 575 bp) and clade 1 (*F. serratus*: 699 bp) (see Appendix S6.2 in Supplementary Information). From 48 recruits (8-weeks-old) genotyped for the mtDNA IGS, 10 revealed maternal inheritance, 22 reveal paternal inheritance and 4 reveal maternal and paternal inheritance, however 12 were heteroplasmic but one of the lengths was not from the mother nor the father. This occurs in crosses between *F. spiralis* or *F. guiryi* and *F. serratus*, and never between *F. vesiculosus* and *F. serratus* (Table 6.2 and Appendix S6.4 in Supplementary Information). And the length size was 576 bp, equal no “pure *F. vesiculosus*”.

From 49 recruits that were two-years-old, genotyped for the mtDNA IGS, 5 revealed maternal inheritance, 16 revealed paternal inheritance and 7 reveal maternal and paternal inheritance, however 21 were heteroplasmic but one of the lengths were not from the mother or the father. This occurred in crosses between *F. spiralis* or *F. guiryi* or *F. vesiculosus* and *F. serratus* (Table 6.2 and Appendix S6.4 in Supplementary Information).

Table 6.2. Genotypes (microsatellites and nuclear markers) and maternal and paternal inheritance of mitochondria, for young F1 individuals resulting from all combinations of inter-specific crosses between: Fs (*Fucus spiralis*), Fg (*Fucus guiryi*), Fv (*Fucus vesiculosus*) and Fse (*Fucus serratus*). The letters F and M indicate female and male gametes, respectively. * no data (no diagnostic loci found between parents or no PCR amplification). Values represent the number of F1 individuals confirmed with those loci (only PCRs that amplified at least one allele are counted) and in brackets the total number analysed (not confirmed means that only one of the parents alleles was present).

	Microsatellite markers maternal and paternal	Nuclear markers maternal and paternal	Mitochondrial (IGS)		
			Maternal inheritance	Paternal inheritance	Maternal and paternal inheritance
Fs F + Fg M	*	*	*	*	*
Fs F + Fv M	*	5 (9)	*	5 (9)	*
Fs F + Fse M	1	*	*	7 (14)	*
Fg F + Fs M	*	*	*	*	*
Fg F + Fv M	1	*	*	*	*
Fg F + Fse M	*	*	*	12 (20)	5 (20)
Fv F + Fs M	*	*	*	*	*
Fv F + Fg M	4	12 (15)	*	*	*
Fv F + Fse M	10	*	*	5 (10)	1 (10)
Fse F + Fs M	3	*	5 (9)	*	*
Fse F + Fg M	2	*	4 (10)	*	3 (10)
Fse F + Fv M	1	*	6 (20)	2 (20)	2 (20)
Fg* F + Fv	*	12 (14)	*	*	*
Fv F + Fg*	3	5 (10)	*	*	*
Fg* F + Fse	*	*	*	3 (5)	*
Fse F + Fg*	2	*	*	*	*

6.5. Discussion

Young individuals from laboratory crosses for all combinations of egg and sperm origins showed a potential for hybridization between *Fucus* species, i.e., there is gametic compatibility. The rates of gamete compatibility based on fertilization rates indicate that the chances of fertilization between individuals of different species (inter-specific crosses) could in some of our experimental combinations and conditions be higher than within species (intra-specific crosses or positive controls). This suggests that other mechanisms against hybridization are effective, allowing the persistence of sympatric distinct species without total genetic admixture. For the first time, the DNA of early recruits of *Fucus* sp. (8 weeks-old) was extracted and hybrids were determined by microsatellite loci, nuclear markers and mtIGS. Finally, the laboratory crosses indicate that gametic incompatibility is not a strict prezygotic mechanism against hybridization between *Fucus* species.

Reproductive success in the positive controls of dioecious species was near 100 %, as estimated in natural egg release events (Brawley 1992, Brawley et al. 1999, Pearson and Brawley 1996, Serrão et al. 1996, Berndt et al. 2002, Ladah et al. 2003). However, in hermaphroditic species, lower fertilization success was observed, indicating that the manipulative conditions used for hermaphrodites might decrease the viability of sperm, since sperm are more susceptible to manipulation than eggs. However, the percentage of fertilization between dioecious mothers and the hermaphroditic fathers was higher than in the intra-species crosses of hermaphroditic species, suggesting that the viability of sperm was not responsible for low fertilization in intra-specific crosses of hermaphroditic species. Furthermore, the percentage of fertilization between dioecious fathers and the hermaphroditic mothers was lower than reciprocal crosses. These results suggest another hypothesis to explain our results, low viability (or low maturity) of the hermaphroditic eggs used in the experiments, resulting in low percentage of fertilization. Low maturity could be a result of having collected the eggs (oogonia) from the receptacles instead of waiting for their natural release.

The higher fertilization success between dioecious mothers (*F. vesiculosus* and *F. serratus*, clade 2 and clade 1, respectively) and the hermaphroditic fathers (clade 2) than between reciprocal crosses, suggests asymmetrical fertilization. Natural hybridization between species from different clades is not common (Coyer et al. 2002a, 2006ab, Wallace et al. 2004, Billard et al. 2005b, Mathieson et al. 2006). Furthermore, a previous study reported that natural hybridization between *Fucus* species involves hermaphroditic mothers and dioecious fathers, probably due to differences in sperm-egg recognition proteins (Coyer et al. 2006a) and/or to the low sperm production in hermaphroditic species (Vernet and Harper 1980, Billard et al. 2005b). Coyer et al. (2002a) also reported an absence of natural hybrids between a dioecious mother (*F. serratus*) and a hermaphroditic father (*F. evanescens*), both species from clade 1. However, laboratory crosses show that fertilization could be lower than reciprocal crosses (*F. evanescens* mother and *F. serratus* father) but still occurred in laboratory conditions, suggesting no barriers formed by sperm-egg recognition proteins (Coyer et al. 2002a). Laboratory crosses have been performed between species from different clades, *F. serratus* and *F. vesiculosus* (Burrows and Lodge 1953) and between species from clade 1, *F. serratus* and *F. evanescens* (Coyer et al. 2002a). In both studies, the percentage of fertilization between intra- and inter specific crosses was not determined. While Coyer et al. (2002a) determined the reproductive success at the end of four weeks and detected asymmetrical reproductive success between reciprocal crosses, Burrows and Lodge (1953), only reported that the development of hybrids (between species from different clades) that were four-months-old was similar to the parental species, and no differences between reciprocal crosses were mentioned. Furthermore, previous laboratory crosses performed between *Fucus* species (Burrows and Lodge 1951) suggest that the reproductive success is highly variable between studies, even in crosses between the same species.

The asymmetrical mate choice hypothesis, proposed by Kaneshiro (1976), predicts that females of a newly evolved or derived taxon readily will accept males of the ancestral taxon because all of the male characteristics required by the females are present. Derived males, however, would be unable to fertilize females of an ancestral taxon if these males have ‘lost’ necessary stimuli in the mate recognition system. Thus, females from a derived taxon would be expected to mate with males from an ancestral taxon, but not vice versa. Asymmetrical fertilization successes have been reported in several species of plants and animals (Kaneshiro 1976, Levin 1978, Arnold et al. 1996, Coyne and Orr 1998, Funk 1998). However, in the present study, there is no evidence of asymmetrical fertilization success between any pairs. Asymmetrical hybridization was observed in natural hybrids of *Fucus* where crosses between *F. evanescens* egg and *F. serratus* sperm were successful but not the reciprocal cross (Coyer et al. 2002a). However, laboratory crosses show the potential of hybridization in both reciprocal crosses suggesting that gametic compatibility was not responsible for asymmetrical differences observed in natural hybrids. In natural conditions, the larger productions of sperm from dioecious than hermaphroditic species (e.g., Billard et al. 2005b) and/or postzygotic selection against hybrids with *F. serratus* chloroplast DNA could be the mechanisms responsible for the natural asymmetrical hybridization (see also Coyer et al. 2007).

Ten polymorphic loci were used to identify hybrids, however only six microsatellite loci identified both maternal and paternal inherence, and only in some of the individuals analysed. This is suggesting that the microsatellite loci used in this study are not useful to detect maternal and paternal inheritance in young *Fucus* hybrids. We hypothesize that the very small amount of DNA obtained from young individuals kept in culture, resulting in very diluted DNA solution, favours preferential or random amplification of one of the alleles. The nuclear sequence markers had higher success of molecular identification than microsatellite loci, but only confirmed hybridization between species from clade 2, where maternal and paternal inheritance were detected in 29 of 44 recruits. However, in hybrids from both clades (inter-specific crosses between *F. serratus* and species from clade 2), only nuclear sequences of *F. serratus* were detected, suggesting that no interspecific crosses were achieved (but see below).

We also used mitochondrial DNA (mtDNA) to detect the maternal inheritance, given earlier evidence that mtDNA is maternally inherited (reviewed by Motomura et al. 2010). Recent studies however, revealed mitochondrial heteroplasmy (transmission of paternal and maternal mtDNA into the egg and its presence at the adult stage) in a diversity of taxa including animals, fungi, green plants and brown algae (e.g., Hoarau et al. 2009). Our results show maternal, paternal and biparental (heteroplasmy) inheritance in the present hybrids. Confirmation was obtained by sequencing the mtDNA locus, revealing biparental inheritance in a cross between *F. spiralis* and *F. serratus* (female and male, respectively), however the nuclear sequences contained only *F. serratus* alleles.

Biparental and paternal leakage were recently reported in marine algae (e.g., *Fucus* sp. between *F. serratus* and *F. evanescens* (Coyer et al. 2004, Hoarau et al. 2009) and in *Ectocarpus* sp. (Peters et al. 2004). However, Hoarau et al. (2009) only detected biparental leakage in individuals resulting from backcrossing between F1 hybrids (first generation) and *F. serratus*. In pure individuals of *Fucus* the sperm mitochondria are present in the egg (perinuclear region) 16 h after fertilization, and are then less distinct than in early stages but they do not divide and eventually degenerate (Brawley et al. 1976). However, no observations have been reported for *Fucus* hybrids concerning the mechanisms involved in mtDNA targeting of paternal degradation or recombination are still unknown. Since recent studies show that maternal mtDNA inheritance does not always occur, and paternal and biparental mtDNA transmission seems more common than expected, especially in hybrids, it is necessary to consider such paternal (or biparental) leakage in evolutionary studies. This is especially relevant in sympatric distributions of related species, which may form hybrid zones, and where the backcrossing after several generations, might lead individuals to be misclassified as a "pure" non introgressed lineage. Paternal and biparental mtDNA leakage occurs in hybrids (Coyer et al. 2004, Peters et al. 2004, Hoarau et al. 2009), although the link between biparental and paternal leakage and hybridization is not fully understood, it may involve failure in tagging the paternal mtDNA for degradation (Sutovsky et al. 1999). The presence of natural hybrids with unknown morphological characteristics, together with problems of molecular identification is a potential dilemma to test gametic (in)compatibility in laboratory crosses, i.e., the probability to use hybrids instead of "pure" individuals in laboratory crosses could be higher than expected.

Our experimental crosses suggest that some putative hybrids have similar or higher hypothesis to growth and survive in culture as the "pure" individuals. The survival and growth of intra-specific crosses was lower in the hermaphrodites than in the dioecious species. Lower survival was also observed in inter-specific crosses between hermaphrodites (between *F. spiralis* and *F. guiryi*). Furthermore, all recruits from the inter-specific crosses between hermaphrodite species did not survive at the end of one year (parental lineage: female of *F. guiryi* and the male of *F. spiralis*) and two years (parental lineage: female of *F. spiralis* and the male of *F. guiryi*). In contrast, survival was higher in inter-specific crosses between hermaphrodites and dioecious species. The low success of survival in intra- and inter-specific crosses from both hermaphrodite species may be due to the absence of tidal regime (absence of air exposure) in cultures, in contrast with natural conditions. The percentage of survival in "pure" *F. vesiculosus* was higher than in hermaphrodites, but lower than in *F. serratus*, however growth was similar between *F. vesiculosus* and *F. serratus*. These results suggest that absence of a tidal regime could be unfavorable for the growth and survival in hermaphroditic species that naturally inhabit the upper intertidal and spend more time out the water than underwater. This may also restrict the survival in *F. vesiculosus*. The species *F. serratus* occurs in low intertidal and subtidal shores, and may be more able to survive in no tidal regime conditions than the other sister species. This suggests that another hypothesis to explain the biased genetic markers in *F. serratus* crosses being only from *F. serratus* could be differential selection by the atidal regimes of culture. This hypothesis is supported by the predominance of *F. serratus* specific markers in older embryos but not in very young ones from the same hybrid crosses. There are several populations of *F. vesiculosus* inhabiting the subtidal, such as in the Baltic Sea, however some genetic variation for subtidal adaptation should be considered. There is some evidence that the development of early stages, in experimental conditions, of *F. vesiculosus* and *F. serratus* is not affected by the absence of tidal conditions at the end of two months (Burrows and Lodge 1953). But long-term data of growth and survival in laboratory conditions are not available in the absence of a tidal regime in *F. spiralis* and *F. guiryi*. The surprising growth and survival of putative hybrids from the crosses between hermaphrodite (high intertidal species) and dioecious (mid- and low species), suggest that there is a potential role for hybrids in providing a source of variation for adaptation to no tidal condition, when one of the parental lineages is more able to survive without a tidal regime. There is some evidence of habitat related differences in survival and growth of coral hybrids relative to parental lineage, results that suggest that hybrids grew and survive in a wide range of habitats, i.e., hybrids may survive in habitats where parental lineage is present, as well as in habitats where parental lineage is absent (for further review see Willis et al. 2006). The higher growth and survival of putative hybrids (inter-specific crosses between hermaphrodites and dioecious species) in atidal regimes relative to the “pure” hermaphrodites, suggests a potential role for hybrids in providing a source of variation for adaptation to new or extreme environments has been reported in corals (Willis et al. 2006).

The present laboratory crosses demonstrate that gametes are compatible between *Fucus* species, when each egg only has the opportunity to mate with inter-specific sperm. However, in the natural environment the spawning events may evolve intra-specific and inter-specific sperm competing when the release between species occurs at same time. Testing intra- and inter-specific choice between gametes could be useful to test prezygotic isolation under laboratory conditions that more closely resemble *in situ* spawning events. Intra- and inter-specific crosses between eggs and sperm were tested in coral species, and results suggest gametic compatibility between inter-specific sperm in no choice crosses, but in multi-choice crosses results show species-specificity in gamete recognition (Willis et al. 2006). Furthermore, species specificity in gamete recognition and binding proteins were reported in abalone (Vacquier et al. 1990) and sea urchins (Metz and Palumbi 1996). Besides, in abalone the success of fertilization in inter-specific crosses is highly dependent on sperm concentration, i.e., the fertilization in inter-specific crosses only occurs in high sperm concentration (Vacquier et al. 1990). Multi-choice crosses would be useful for further research to understand the effects mating systems and synchrony of spawning in *Fucus* and other broadcast spawners.

The success observed in the methodology for separating the sexes (oogonia and antheridia) in hermaphroditic species is shown by the low percentage of fertilization present in the negative control (less than 1 % in hermaphroditic eggs, and 0 % for dioecious species). This supports the result that the embryos resulting from pairing gametes from hermaphrodites with the other species are truly hybrids. However, paternal and maternal molecular identification of hybrids revealed several technical problems that may be related with PCR artifacts. Furthermore hybridization is not fully understood, and hybrids might undergo processes different from the “pure” individuals, such as biparental or paternal mtDNA inherence, possibly associated with impaired paternal mtDNA tagging for degradation (Sutovsky et al. 1999). Understanding the ecology and fitness of hybrids and the interactions between the hybrid and the “pure” individuals seems an important challenge in marine science, but first more efficient tools for molecular identification of hybrids are required.

6.6. Acknowledgments

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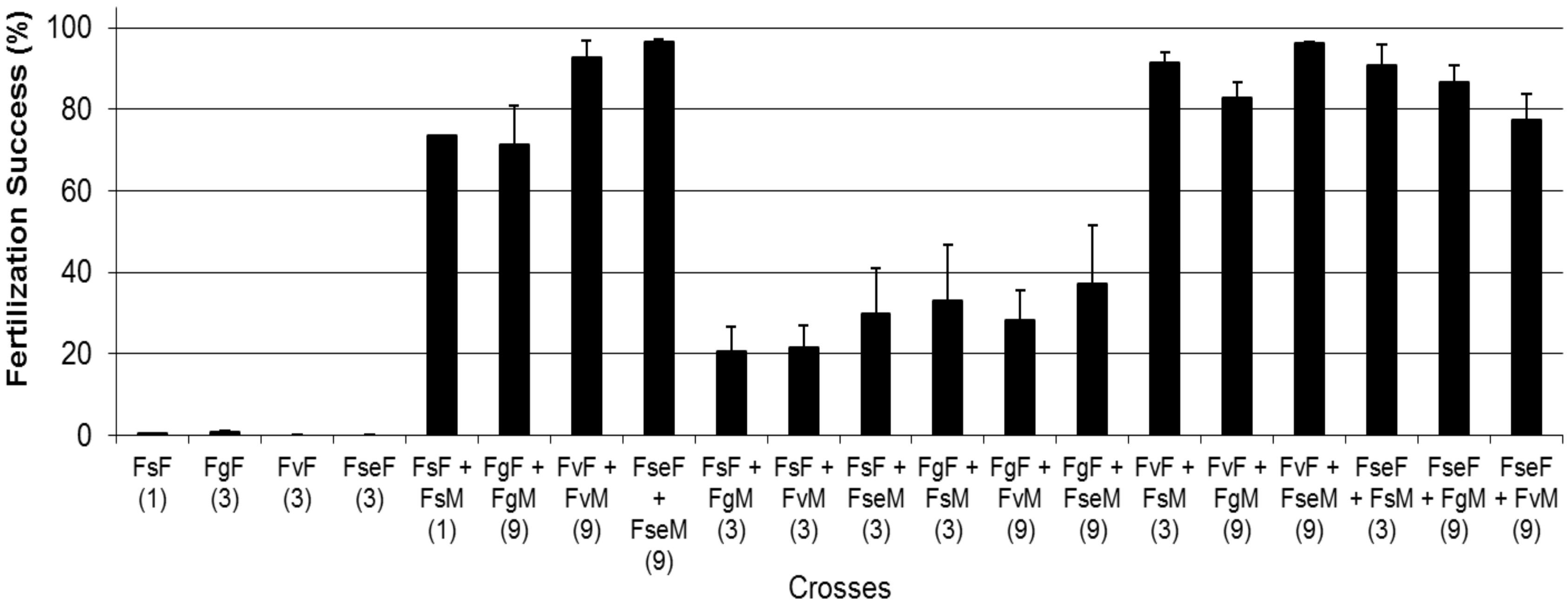
Supplementary Information

Appendix S6.1. Fertilization success (mean %). Data for all combinations of crosses between 1 individual of *Fucus spiralis* (*Fs F* and *Fs M*), 3 individuals of *Fucus guiryi* (*Fs F* and *Fs M*), 6 individuals of *Fucus vesiculosus* (3 females and 3 males: *Fv F* and *Fv M*, respectively) and 6 individuals of *Fucus serratus* (3 females and 3 males: *Fser* F and *Fser M*, respectively). Error bars show standard error (the sample sizes are shown under the name of each treatment).



Appendix S6.2. Microsatellite alleles for each locus and mtIGS alleles for parental individuals. The allele size at six microsatellite locus and mtIGS (mitochondria) for the adults: *Fucus spiralis* (Fs), *Fucus guiryi* (Fg), *Fucus vesiculosus* (Fv) and *Fucus serratus* (Fse). F = female; M = male; Fg*, Fg ** = *F. guiryi*. The values 75 and 76 and 77 are parental-specific identification codes.

Adults	**Microsatellite locus**												**Mitochondria**	
	F9		**F12**		**F19**		**L20**		**L78**		**F49**		**mtIGS**	
Fs75 = Fg*	182	182	199	199	191	203	203	134	154	154	203	203	576	576
Fs76 = Fg**	182	182	199	199	191	203	203	134	154	154	203	203	576	576
Fs77	177	177	199	199	191	203	203	164	136	136	203	203	569	569
Fg75	182	182	199	199	156			134	154	154			551	551
Fg76	182	182	199	199	191	203	203	134	154	154	203	203	551	551
Fg77	182	182	199	199	191	203	203	134	154	154	203	203	551	551
Fv75 F	180	182	273	273	188	203	203	163	168	171	203	203	575	575
Fv76 F	180	180	201	250	188			166	168	171			575	575
Fv77 F	182	182	197	197	188			166	168	174				
Fv75 M	180	180	267	267	191	201	201	175	171	171	201	201		
Fv76 M	180	180	267	267	194	203	203	163	168	171	203	203	575	575
Fv77 M	180	180	197	197	156	201	203	147	168	171	201	203	575	575
Fser75 F	197	197	338	338	201	197	197	161	156	156	197	197		
Fse76 F	197	197	338	338	203	197	197	157	156	156	197	197		
Fse77 F	197	197	338	338	188	197	197	157	156	156	197	197	699	699
Fse75 M	197	197	338	338	194	197	197	157	156	156	197	197	699	699
Fse76 M	197	197	338	338	201	197	197	157	156	156	197	197		
Fse77 M	197	197	338	338	201	197	197	157	156	156	197	197		

Appendix S6.3. Nuclear primer sequences, observed parental size range and offspring, and detail information on PCR amplification conditions (Go taq).

Primer	abv	Fw	Rv	Ta/Time	primer (µM)	MgCl (mM)
Ser116_1	Ser116	AAGGCGCTTAAGATGGTGACGGTCG	ACGTGCCGGACACCATAGCGG	66ºC/35''	0.05	2
Ves136_2	Ves136	AAAGGACGCCGTGGCAGACTACG	TGAGGCGGGCGCTCAACGTG	66ºC/35''	0.1	1.5
ClpB_2	ClpB	CGACGATGGACGCGTTACCGA	CGGAATCCTTCCCGCATCGCA	69ºC/35''	0.1	2.5
HS870_1_1	HS870	GACCTCGGCACAGACGACAGCG	GCCCAGCTGTTCCTGCAGCC	66ºC/30''	0.5	1.5
Fv_rc1805_B	1805	GGGAGCCCGTACCAGCAGTG	AAGATGGGTGTCTCCGGGTTGT	63ºC/25''	0.05	3
Fv_rc3387	3387	ATAATACGGGCTGTGCCAAGAGTT	GATGCGGATGGTGAATGCAG	58º/35''	0.05	2.5
Fv_rc11042_A	11042	GGATTTACTGCACCGAGACATCAAG	CTTCTCCAGCTACACCAACCCGTAT	62ºC/35''	0.1	2
Fv_rc6719_A	6719	CTTTCCCTCGTCTCCACTTCTTCAT	CATACTTTTCCCTCCCTCGGTCCTA	62ºC/35''	0.05	3

Appendix S6.4. Mitochondrial (mtIGS) inheritance patterns in reciprocal crosses between species of clade 2 (*Fucus spiralis*: *Fs*, *Fucus guiryi*: *Fg* and *Fucus vesiculosus*: *Fv*) and clade 1 (*Fucus serratus*: *Fse*). F = female; M = male; the values 75, 76 and 77 are parental-specific identification and the number are individual-specific identification codes (bold and not bold numbers are recruits with 8-weeks-old and with 2-years-old, respectively). Maternal and parental mtIGS are in grey boxes. Heteroplasmy are represented at bold.

Individuals	**mtIGS**	
Fs 77 F	569	
Fv 77 M	575	
11	575	
12	575	
13	575	
15	575	
91	575	
92	575	
93	575	
94	575	
95	575	
Fs 77 F	569	
Fse 75 M	699	
1	699	
3	**576**	**699**
4	699	
5	**576**	**699**
81	699	
82	**551**	**699**
83	699	
84	**551**	**699**
85	**551**	**699**
Fs 77 F	569	
Fse 77 M	699	
6	576	699
7	699	
8	699	
9	576	699
10	699	

Individuals	**mtIGS**	
Fg* 76 F	576	
Fse 75 M	699	
86	**551**	**699**
87	699	
88	**551**	**699**
89	699	
90	699	
Fg 76 F	551	
Fse 75 M	699	
21	699	
22	699	
23	699	
24	**576**	**699**
25	699	
96	**551**	**699**
97	**551**	**699**
98	699	
99	**551**	**699**
100	**551**	**699**
Fg 76 F	551	
Fse 77 M	699	
26	699	
27	699	
28	**576**	**699**
29	**576**	**699**
30	699	
Fg 75 F	551	
Fse 75 M	699	
101	699	
102	699	
103	**551**	**699**
104	699	
105	699	

Individuals	**mtIGS**	
Fv 75 F	575	
Fse 75 M	699	
51	699	
52	**575**	**699**
53	699	
54	699	
55	699	
121	**551**	**699**
122	**551**	**699**
123	**551**	**699**
124	**551**	**699**
125	699	
Fse 75 F	699	
Fs 77 F	569	
61	699	
62	699	
63	699	
64	**576**	**699**
65	**576**	**699**
131	**551**	**699**
132	699	
133	**551**	**699**
134	699	
Fse 75 F	699	
Fg 76 F	551	
66	**576**	**699**
67	699	
68	**576**	**699**
69	**551**	**576**
70	**576**	**699**
136	**551**	**699**
137	**551**	**699**
138	699	
139	699	
140	699	

Individuals	**mtIGS**	
Fse 75 F	699	
Fv 77 M	575	
71	699	
72	**575**	**699**
73	699	
74	699	
75	699	
Fse 76 F	699	
Fv 76 M	575	
76	699	
77	575	
78	575	
79	699	
80	**575**	**699**
Fse 77 F	699	
Fve 77 M	575	
141	**551**	**699**
142	**551**	**699**
143	**551**	**699**
144	**551**	**699**
145	**551**	**699**
Fse 76 F	699	
Fve 77 M	575	
146	**551**	**699**
147	**551**	**699**
148	**551**	**699**
149	**551**	**699**
150	**551**	**699**

Chapter 7

Concluding remarks

7. Concluding remarks

This thesis is a major contribution to the controversial and poorly understood question of sympatric speciation in broadcast spawners, a common and ancestral reproductive mode in the sea, with unique challenges for the evolution of reproductive barriers. It also provides one of the most comprehensive descriptions to date of reproductive timing at annual to hourly scales of sympatric *Fucus* species with different mating systems. In general, the studies conducted along this thesis infer mechanisms that act as barriers to hybridization, by investigating the roles of intraspecific synchrony and interspecific asynchrony of egg release in species with different mating systems. In addition, results are presented showing a surprisingly high degree of gametic compatibility in no choice crosses between *Fucus* species. Moreover, high rates of hybrid viability, survival and growth relative to parental lineages demonstrate incomplete genomic barriers to reproductive isolation between *Fucus* species. A point by point summary of the major results follows:

1. Egg release in *Fucus* species is highly synchronous within a species at seasonal and semilunar scales. Both hermaphroditic and dioecious species require high tide immersion for spawning and both display a high degree of reproductive synchrony within each species.
2. At the monthly scale, the egg release in natural fucoid populations is controlled by environmental cues arising from the interaction of tidal (timing of high and low tides) and circadian (light:dark) cycles, rather than semilunar cycles (spring – neap tide).
3. Species-specific spawning responses to circadian and tidal cycle cues have resulted in a temporal shift in reproductive timing between selfing hermaphroditic and dioecious species on hourly scales (within single tides). This forms the basis of an ecological barrier to gene flow that potentially constitutes a prezygotic barrier to hybridization between hermaphroditic and dioecious species.
4. The switch in spawning timing from daytime to nighttime high tides in hermaphroditic species, living in the high intertidal, suggests an adaptation to escape abiotic stressors such as high light or temperature.
5. Mating system variation plays an important role: the hypothesis that selfing species are under reduced selective constraint for spawning synchrony relative to obligate outcrossing species was supported, especially in low hydrodynamic environments (estuaries).

6. High levels of fertilizations were observed in inter-specific crosses, suggesting that gametic incompatibility is not a strict barrier against hybridization between *Fucus* species.
7. Hybrid survival and growth were high, except in intra-specific and inter-specific crosses of high shore hermaphroditic species, possibly due to the absence of tidal cycles in laboratory conditions. The lower survival in culture of hermaphroditic (upper-intertidal) compared with dioecious (mid- and low intertidal) crosses suggests that the existence of tidal cycles (particularly emersion periods) is more critical for survival of upper shore species and that this is maintained even in hybrid progeny.

This thesis shows that the maintenance of species boundaries in *Fucus* species on intertidal shores can be explained by asynchronous egg release between species and mating systems variations. The finding that egg release was strongly synchronous within species and asynchronous between mating systems at tidal and circadian cycles, suggests that a small variation in the timing of spawning is an important prezygotic mechanism against hybridization between hermaphrodites and dioecious species. However, the synchrony of egg release between hermaphroditic (*F. spiralis* and *F. guiryi*) and dioecious species (*F. vesiculosus* and *F. serratus*) and the gametic compatibility in laboratory no choice crosses, indicates that others mechanism are involved.

I propose that future studies should focus on studying the following hypotheses: 1) in dioecious species the high concentration of mucilage (especially in *F. serratus*, personal observations), might increase the concentration of egg and sperm in space and time (low dispersion), and consequently increase fertilization success within *F. serratus* individuals, thereby reducing/preventing hybridization with *F. vesiculosus*; 2) Phylogenetic distance is expected to increase gametic incompatibility and consequently reduce/prevent hybridization between *F. vesiculosus* (clade 2) and *F. serratus* (clade 1). These conclusions are supported by previous studies with coral species complexes that show low interspecific gamete compatibility between species that have overlapping spawning times. Multi-choice crosses were never performed with *Fucus* and further research in this area would be useful; and 3) Inter-specific crosses between both hermaphroditic species (*F. spiralis* and *F. guryi)* resulted in a lower percentage of fertilization than intra-specific crosses (either within hermaphrodites or dioecious species), suggesting that low interspecific gamete compatibility between species with overlapping spawning times, such as *F. spiralis* and *F.*

guiryi is an important hypothesis to be tested in hermaphroditic species of *Fucus*. We also suggest that in hermaphroditic species, self-fertilization (i.e., the mating system) could be considered the most important mechanism preventing hybridization between *F. spiralis* and *F. guiryi*.

The surprising levels of gametic compatibility between hermaphrodites and dioecious species, especially between species with higher phylogenetic distance (between *F. serratus* and the others species), raises another important unanswered question - do hybrids become reproductively viable? To answer this question, putative hybrids are being maintained in controlled conditions with the aim of studying their survival and fitness once they reach reproductive maturity (adult stage). Despite the asynchrony between mating systems and the synchrony within mating systems, other mechanisms are likely involved to prevent hybridization and contribute to species maintenance. Further research on both hermaphroditic and dioecious species is necessary to determine the timing of fertilization after egg release, multi-choice crosses (to test egg and sperm preference), dispersal and sex ratio (egg: sperm). Understanding the reproductive features in early life history (egg release, settlement and early recruitment), and comparison of reproductive strategies between closely related species is an important step to understand the evolution of the reproductive strategies and speciation. In recent years, research on hybridization has made important advances, but much remains to be discovered.