Molecular ecology and fitness of striped (Stenella coeruleoalba, Meyen, 1833) and common (Delphinus delphis, Linnaeus, 1758) dolphins

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by

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ABSTRACT

In this study the molecular ecology and fitness of two Delphinidae species, the striped dolphin (*Stenella coeruleoalba*) and the common dolphin (*Delphinus delphis*), were investigated in the Mediterranean Sea and Atlantic Ocean. This thesis provides a comparative assessment of the striped and the common dolphin, using molecular methods regarding the genomic diversity and parasite resistance.

The striped and the common dolphin have a world-wide distribution, inhabiting pelagic waters and differ with respect to population structure within the study area. The aim is to reveal different patterns of genetic diversity and fitness in the species that shows greater populations structure. In order to test this, my analyses structure was a) to analyse the population structure of the striped dolphin in the Mediterranean Sea and Atlantic Ocean and compare structuring patterns with previous published studies, b) to examine the heterozygosity fitness correlation for both species, using neutral and non-neutral markers and specific parasites that are important of animal's health. In this context this study tests that local populations show stronger relationship between genetic diversity and fitness.

This study suggested that different methods regarding to power and studied subareas show a fine-scale structure beyond that reported previously in striped dolphin populations. A key new finding is the structuring pattern in the Atlantic Ocean, where populations from Scotland and the Biscay Gulf were isolated from the one in Ireland. Also, the Ionian Sea samples grouped with the western Mediterranean, which could either be an effect of the small sample size from the Ionian Sea, or reflect a boundary closer to Greece, dividing the basins of the Mediterranean for this species.

In this study I found differences between the two hosts with respect their genetic diversity and parasite loads for both nuclear and functional loci. I also found that evidence for a heterozygosity fitness correlation was strongest for females, and this was true both for the correlation with genomic diversity as assessed using neutral markers, and for the functional immune system gene. This observable association suggests that parasites may act as an energetic stress, and may reflect the non-identical pathogenesis of parasites and their ability to inflict damage through the hormone profiles. Results illustrate potentially important interactions between genetic drift and selection, and provide specific information that will be valuable towards the conservation and management of diversity in these species.

To my family

...When you set out for Ithaca

hope the voyage is a long one..

C.P. Cavafis

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This thesis is dedicated to my family. I wish to thank my parents for all their patience during the last 4 years as they have given me invaluable support, as financially as psychologically. I couldn't have done this without them. Thank you!

DECLARATION

The material contained in this thesis has not previously been submitted for a degree at the Durham University or any other university. The research reported within this thesis has been conducted by the author unless otherwise indicated

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CHAPTER 1

GENERAL INTRODUCTION

1. General Introduction

1.1 Population structure in delphinid species

Delphinid species consist of many different morphological and ecological types, and many of them are distributed over a wide geographic range in tropical and warm-temperature latitudes (see review in Martin and Reeves, 2002). Some have very limited distributions, such as the Chilean dolphin (*Cephalorhynchus eutropia*) off South America and Hector's dolphin (*C. hectori*) off New Zealand, while others are intermediate, such as *Stenella frontalis* found only in the lower latitudes of the Atlantic Ocean, and *Tursiops aduncus* inhabiting the western Pacific and Indian Oceans. Some other species have highly cosmopolitan distributions, such as the killer whale (*Orcinus orca*), which is found throughout the world from the Arctic to the Antarctic Ocean.

These distributions can often be classified according to the physical and biological characteristics of their habitat. Cetaceans can be grouped into two main categories; the coastal and the pelagic. For example, the pan-tropical spotted dolphin (*Stenella attenuata*) and typically common dolphins (*Delphinus delphis*) are pelagic species (with some exceptions), whereas bottlenose dolphins (*Tursiops truncatus*) and *Cephalorhynchus sp.* are mostly coastal species. However, populations of a given delphinid species can be found in multiple habitats. One possible factor could be the local food resources, and foraging specializations have been proposed to help explain population distributions and structure in Cetaceans (e.g. Hoelzel, 1998).

A well documented example is the foraging strategy of the killer whale. Killer whales live in highly social groups and this social formation is specialized on prey resources, representing two different ecotypes in the North Pacific, one focussing on fish and the other on marine mammal prey (e.g. Ford *et al.*, 2000). These ecotypes are

genetically differentiated even in sympatry, but regional populations of the same ecotype are also differentiated (Hoelzel *et al.*, 2007). There is social phylopatry in both sexes, possibly related to prey expoitation, and the social cohesion in kin-groups is strong enough to largely define regional population structure (Hoelzel *et al.*, 2007, Pilot *et al.*, 2010).

Delphinid species are highly mobile species capable of movements within and between large geographical areas and in, apparently, homogeneous environments. Although it would be expected that this may lead to panmixia, fine-scale structure has been reported for a variety of delphinid species (see review in Hoelzel, 2009). For example, Hayano *et al.* (2004) investigated the genetic diversity of 5 microsatellite loci and mtDNA control region of the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) for Japanese coastal and offshore populations in the North Pacific. Genetic differentiation was found between these two populations, with the coastal population showing reduced diversity suggesting a lower population size.

Cassens *et al.* (2005) using 9 microsatellite loci and two mtDNA gene fragments (cyt-b and control region) found that a dusky dolphin (*Lagenorhynchus obscurus*) population from Peru was differentiated from Argentina and southern African populations. Moreover, an isolated population from New Zealand showed low genetic diversity. Mendez *et al.* (2010) found a genetically isolated population of 275 franciscana dolphins (*Pontoporia blainvillei*) within Argentina using genetic data from mtDNA and 12 microsatellite loci. Andrews *et al.* (2010) studied whether environmental and social differences influence the population genetic structure of spinner dolphins (*Stenella longirostris*) throughout the Hawaiian Archipelago using mtDNA control region and 10 microsatellite loci. These analyses revealed population genetic differentiation between most of the islands, with less genetic structuring among the northwest atolls than among the southeast high islands.

Dawson *et al.* (2001) found a population subdivision between the North Island, the west coast of the South Island and the east coast of the South Island along the New Zealand coast in Hector's dolphin (*Cephalorhynchus hectori*) using mtDNA. Each of these local populations showed low genetic diversity. Van Vuuren *et al.* (2002) found weak differentiation between South African and Namibian populations of Heaviside's dolphin (*Cephalorhynchus heavisidii*) at the mtDNA control region. They proposed that the low level of genetic differentiation reflected an overall small effective population size for the species.

As expected by theory (see Nei *et al.*, 1975), population isolation in delphinid species does show a reduction in genetic variation within local populations from empirical studies (as shown in the previous examples). According to Wright (1931) when population size is reduced, the average level of heterozygosity per locus is expected to be decreased at a rate that depends on the effective population size (inversely proportional to 2Ne). The relevant process is genetic drift. This reduction in genetic variation is caused by the loss or fixation of alleles (see Nei *et al.*, 1975).

In small populations inbreeding is an unavoidable process as a result of mating between genetically related individuals, and inbreeding depression will be particularly intense following a sharp population decline (because deleterious alleles can be maintained in the heterozygous condition in large populations). Valsecchi *et al.* (2004) found that striped dolphins that died early in a morbillivirus outbreak were significantly more inbred than those dying later. Inbreeding has a potentially detrimental effect on lifetime fitness (e.g. Charlesworth and Charlesworth, 1987). Inbreeding can impact fitness either through genome-wide patterns, exposing deleterious recessive alleles (inbreeding depression), or through single locus effects (such as heterosis). In order to study the fitness effects of inbreeding, studies in the literature often use indirect estimates of fitness (such

as fluctuating asymmetry or susceptibility to disease) and estimate inbreeding using molecular markers. These studies focus on the relationships between genetic diversity and fitness related traits, known as heterozygosity-fitness correlations.

1.2 Heterozygosity fitness correlation

The definition of a heterozygosity-fitness correlation (after David, 1998) is 'the *empirical observation of a correlation between heterozygosity measured at a marker locus, or at a set of marker loci, and a fitness-related trait*.' Three main hypotheses have been proposed to explain the Heterozygosity-fitness correlation (David, 1998; and see Chapters 3&4 for further discussion);

a) the *direct effect* hypothesis: heterozygote advantage due to overdominance at the specific locus scored

b) the *local effect* hypothesis: heterozygote advantage detected at marker loci that are closely linked to fitness loci

c) the *general effect* hypothesis: heterozygote advantage due to genome-wide effects (high level of heterozygosity at the marker loci reflecting a high level of heterozygosity in the genome as a whole).

Microsatellite and allozyme markers are the most commonly used markers in studies of correlation with fitness. Many authors have proposed other measures of variation instead of mean level of heterozygosity, all of which aim to produce a measure that correlates most strongly with the inbreeding coefficient *F*. Those estimates are the mean d^2 (Coulson *et al.*, 1998), the *Standardized Heterozygosity* (Coltman *et al.*, 1999) the *Internal Relatedness* (Amos *et al.*, 2001) and *HL* (Aparicio *et al.*, 2006) (see Chapter 3). However,

most studies focus on Multilocus Heterozygosity and d^2 measurements for fitness correlations (Hedrick *et al.*, 2001; Hansson *et al.*, 2001).

Published data have shown that heterozygosity is often correlated with indirect fitness measures such as fluctuating asymmetry (an unbiased asymmetry in bilateral traits, reflecting developmental instability; van Valen 1962). Borrel *et al.* (2004) studied the relationship between heterozygosity of six allozyme and eight microsatellite loci and fluctuating asymmetry in two samples of Atlantic salmon (*Salmon salar*) with different timings of first active feeding (early (EA) and late (LA) salmon). The first active feeding was considered as a trait related to higher fitness, and it was found that EA fish showed smaller values of fluctuating asymmetry and were more heterozygous than LA fish. Hoelzel *et al.* (2002) studied the fluctuating asymmetry of pre- and post-bottleneck populations of the northern elephant seal (*Mirounga angustirostris*) and the genetic diversity of mtDNA and five microsatellite DNA loci. The authors suggest that increased fluctuating asymmetry in the post-bottleneck population was related to the loss of genetic diversity, and reflected lower fitness. Moreover, Neff (2003) used genetic (10 microsatellite loci) and phenotypic measures (fluctuating asymmetry) in a wild population of bluegill sunfish (*Lepomis macrochirus*) to investigate the possible impact of stabilizing selection on genetic divergence. Analyses showed that fish with either low or high genetic diversity (mean d^2) were more asymmetrical than individuals of intermediate levels of divergence.

Heterozygosity–fitness correlations have been studied for a variety of different fitness traits measures. Coltman *et al.* (1999) studied the heterozygosity fitness correlation of the Soay sheep (*Ovis aries*) and 14 microsatellite loci. Analyses showed that less inbred individuals had a significant relationship with longevity and that mediated selection acts to maintain genetic variation by removing less heterozygous individuals. Slate *et al.* (2000)

using nine highly polymorphic microsatellite loci tested the heterozygosity fitness correlation of a wild population of red deer (*Cervus elaphus*) and found that heterozygosity is positively associated with long breeding success in this species on the Isle of Rum in Scotland. Marshall *et al.* (2003) examined the heterozygosity fitness correlation between 7 microsatellite loci and song complexity, as a sexually selected trait related to fitness, in the colour-ringed sedge warbler (*Acrocephalus schoenobaenus*) at Wraysbury (England). The calculated individual mean d^2 was found to be strong correlated with the song complexity suggesting that species' mating preferences may be subject to fine tuning aimed at increasing offspring genetic variability. Moreover, Seddon *et al.* (2004) using nine polymorphic microsatellite loci found that heterozygosity was associated with territory size, and also with the structure of songs used to defend those areas in the sub-desert mesite bird (*Monias benschi*) in Madagascar. In particular, more heterozygous groups had larger territories and more heterozygous males used longer lower-pitched trills in their songs.

1.3 Host – parasite interactions

Another important consideration at the individual level is fitness in the context of parasite load. There are resultant community consequences from the interaction between parasite and host such that parasites may regulate host population size (Anderson and May, 1979), and host demographic structure (Freeland, 1976) driving host population cycles (Dosbon and Hudson, 1992) and thereby mediating host community structure (Minchella and Scott 1991). Minchella and Scott's (1991) review considers evidence for direct effects of parasites on host age and sex structure. In the study of Gunn (1990) two species, the Dolphin-Union caribou (*Rangifer tarandus*) and musk ox (*Ovibos moschatus*)

coexist and the author suggests that parasites negatively impact on the condition and fecundity of the caribou. Furthermore, he proposes that the increasing musk ox population has led to increased abundance of the shared gastro-intestinal nematodes in the caribou. More broadly, it is important to understand the consequences of host/ parasite interactions in the context of other regulatory mechanisms such as nutrition, predation, competition, behavioural factors, as well as ecological/environmental factors such as habitat use and climate changes (Irvine, 2006). In general, fitness impacts due to parasite load can also impact on demographics, and therefore indirectly on factors that can determine genetic diversity.

For marine mammals, studies on host parasite relationships are relatively scarce (see Balbuena *et al.*, 1995) even though parasites are known to cause major health problems in marine mammals (Dierauf and Gulland, 2001). The interaction between populations of parasites and marine mammals can be approached from either the parasite's or the host's prespective (Evans and Raga, 2001). In the first case, emphasis is on the dynamics of the parasite populations and on how the host or other factors influence the parasite population (Smith, 1994; Blair and Hudson, 1994; Aznar *et al.* 1997; Faulkner *et al.* 1998). The second approach is host-based, aiming either to ascertain the effect of parasites on the host population, or to gain information on the host population by analyzing parasite data (Evans and Raga, 2001). Raga *et al.* (1997) suggest that parasites can play an important role in marine mammal populations not only at the ecological scale, but at the evolutionary one as well. The authors argue that parasite-induced mass mortalities may be an important driver of marine mammal population dynamics, using as documented cases the PDV virus which decimated the European common seal (*Phoca vitulina*) populations in 1988 and the Mediterranean striped dolphin (*Stenella coeruleoalba*) morbillivirus infection of 1990-

1992. The infectious diseases of the Delphinidae family can be grouped as Viral Disease, Bacterial Disease, Mycotic Disease and Parasite Disease.

Viral Diseases

The last decade of the 20th century saw an increased recognition and characterization of viruses in Delphinidae species. This increase can be partially attributed to the heightened public concern about repeated morbillivirus (DMV) epizootics in dolphins throughout the waters of the world (Dierauf and Gulland, 2001). According to the summary of morbillivirus events in Reidarson *et al.* (1998), the first established marine mammal morbilliviral epizootic began in June 1987 and involved bottlenose dolphins (*Tursiops truncatus*) along the Atlantic coast of the United States (Lipscomb *et al.*, 1996; Schulman *et al.*, 1997). During 1988, morbillivirus infections occurred epizootically in harbour seals (*Phoca vitulina*) and gray seals (*Halichoerus grypus*) in northwestern Europe and continued in Atlantic bottlenose dolphins (Kennedy *et al.*, 1989; de Swart *et al.*, 1995). Since then, epizootics have occurred in striped dolphins (*Stenella coeruleoalba*) along the western Mediterranean Sea (Duignan *et al.*, 1992; Domingo *et al.*, 1995), and Atlantic bottlenose dolphins in the Gulf of Mexico (Lipscomb *et al.*, 1996; Taubenberger *et al.*, 1996). The authors also noted that from August 1995 to August 1997, six of 18 common dolphins (*Delphinus delphis*) that stranded along the beaches of southern California (USA) tested antibody positive for dolphin morbillivirus (DMV).

Bacterial Disease

Bacterial diseases can be secondary infections after infection with morbillivirus or phytotoxins. Increasingly, there are reports of altered immune response and a decrease in natural resistance to bacterial and viral infection in marine mammals exposed to high levels of anthropogenic substances, such as organohalogens (Thompson and Hall, 1993; de

Guise *et al.*, 1995a; Parsons and Jefferson, 2000). Brucellosis is a globally distributed zoonotic disease of mammals that causes inter alia diseases of the reproductive system and abortion. It is caused by Gram-negative, facultative intracellular bacteria of the genus *Brucella* (Bricker *et al.*, 2000). In the 1990s, the previously unknown strains of *Brucella* were detected in captive bottlenose dolphins (Ewalt *et al.*, 1994; Miller *et al.*, 1999; Van Bressem *et al.*, 2001b). On the basis of host preference and molecular characteristics, it was proposed that these brucellae belong to at least two new species: *Brucella cetaceae* for cetacean isolates and *Brucella pinnipediae* for pinniped isolates (Cloeckaert *et al.*, 2003).

Mycotic diseases

In humans and animals mycoses represent only a small, but often critically significant, fraction of infectious diseases (Nicholls *et al.*, 1993). A recent survey on mycotic infections in captive and wild marine mammals reports 168 cases, of which 27 species of marine mammals were affected by 22 species of fungi (Reidarson *et al.*, 1999). The greater number of cases has occurred in stranded bottlenose dolphin (*Tursiops truncatus*) also infected by dolphin morbillivirus (Bossart, 2007).

Parasitic diseases

Some common parasites and commensals of cetaceans are flukes (Platyhelminthes, Trematoda), tapeworms (Platyhelminthes, Cestoda), roundworms (phylum Nematoda) (Dailey and Otto, 1982; Walker *et al.*, 1984), amphipods, particularly cyamid whale lice (Balbuena and Raga, 1989; Kaliszewska *et al.*, 2005), copepods and cirripeds (phylum Arthropoda) (Bushuev, 1990; Mackintosh and Wheeler, 1929; van Waerebeek *et al.*, 1993), spiny headed worms (phylum Acanthocephala) (Dailey and Otto, 1982) and several species of remora (phylum Chordata) (Fertl and Landry, 1999). Currently, there are

relatively few published studies on the relationship between genetic diversity at the individual level and individual load for pathogens in marine mammals. These are reviewed in Chapter 3.

1.4 Heterozygosity fitness correlations at functional genes

Neutral markers are shown to be an informative source for genetic diversity correlations with fitness (see above). However, an investigation of such correlations with pathogen infestation as an indicator of fitness (the focus of this study, see below), allows an assessment of the interaction with functional loci known to play a key role in fighting these infections. Many of the relevant genes are in the Major Histocompatibility Complex (MHC). The MHC is a large genomic region, or gene family, found in most vertebrates. It is the most gene-dense region of the mammalian genome and it plays an important role in the immune system, autoimmunity, and in reproductive success (e.g. in mate choice, see Jordan & Bruford 1998). Thus far, most of the empirical evidence related to the above derives from human studies.

According to Bernatcez and Landry (2003), studies in free-ranging wild animal populations are still very limited. The authors argue that two main types of mechanisms may operate to maintain the unusually high level of MHC polymorphism: the disease-based and reproductive mechanisms (the latter not reviewed here but see Jordan & Bruford 1998). The disease-based models infer that genetic diversity at the MHC is maintained by balancing selection stemming from the co-evolution of host with their pathogens and parasites.

The MHC contains some of the most polymorphic functional loci in vertebrates (Hedrick, 1994). One of the postulated mechanisms for maintaining this diversity is heterozygote superiority, or overdominance. In population studies, it is used in the general sense to imply that the mean fitness of heterozygotes is higher than the mean fitness of all homozygotes (Carrington *et al.*, 1999; Thursz *et al.*, 1997). Most explanations invoke balancing selection, a broad term that identifies any kind of natural selection for which no single allele is most fit (Bernatchez and Landry, 2003). Frequency-dependent selection and heterozygote advantage are the two main types of balancing selection, and both have been suggested to explain MHC allelic diversity (Hughes and Nei, 1988). There are three main theories that have been proposed concerning the role of MHC – parasite load interactions in this context.

Heterozygote advantage Hypothesis

The heterozygote advantage hypothesis presumes that heterozygous individuals are favoured because they process more different alleles than homozygous individuals do, and therefore, are able to recognize a broader spectrum of pathogens (Doherty and Zinkernagel, 1975; Hughes and Nei 1988). Heterozygote advantage was suggested by Thursz *et al.* (1997) to result in a slower progression to AIDS, and by Carrigton *et al.* (1999) to promote the effective clearance of hepatits B viral infections.

Rare allele advantage hypothesis

The rare allele advantage hypothesis assumes that MHC diversity is maintained through frequency – dependent co-evolutionary processes between hosts and parasites (Takahata and Nei, 1990). The most resistant allele will be favoured and spread through the population. However, it will not go into fixation because when the resistant allele

becomes common this increases selection on parasites to evade the recognition by this common allele (Jeffery and Bangham, 2000).

Frequency – dependent Selection

Under frequency-dependent selection, the fitness of an allele is determined by its relative frequency in the population. Selection under this hypothesis may vary such that the fitness of the allele is affected by spatial or temporal factors. There are a few studies which support the hypothesis that MHC polymorphism is maintained through pathogen–driven selection acting by means of frequency-dependent selection rather that heterozygous advantage (Langefors *et al.*, 2001; Froeschke and Sommer, 2005; Harf and Sommer, 2005; Schad *et al.*, 2005). For example, Hedrick (2002) suggested that the resistance conferred by specific alleles to temporally variable pathogens may contribute to the observed polymorphism at MHC genes and other similar host defence loci. Further discussion is provided in Chapter 4.

1.5 AIM of the study

Studies investigating the relationship between genetic diversity and fitness have the potential to show the effects of evolutionary process and demographic history by documenting patterns of genetic differentiation and levels of genetic diversity using neutral and functional markers. Small populations often suffer from the loss of genetic diversity due to genetic drift and inbreeding effects. This loss of genetic variation can lead to a short-term reduction in fitness. Fitness can be measured directly, for example based on lifetime reproductive success, but is more typically (and readily) assessed using indirect measures (see above). In this study I use pathogen load as a measure of individual fitness,

because this can be easily quantified from dolphins found dead, it has been found to be associated with genetic diversity in other studies, and because this measure facilitates the inclusion of correlation studies with functional, immune system genetic markers. The most well known pathogen parasites in dolphins are found in the lungs and stomachs. Diseases related to these parasites are directly related to the animal's health. Therefore, this study uses burdens of various organisms that parasitize in these internal organs. Of course, the choice of this specific system means that the correlation studies will be blind to other factors that may be relevant to the evolution of disease resistance in these species (see review above), but any positive relationships found should be informative about this system in particular. The principal aim of this study is to better understand the evolutionary processes of host – parasite association for two delpinid species; the striped dolphin and the common dolphin.

The striped and the common dolphin are closely related species that differ with respect to population structure within the study area (see Chapter 2 for detailed discussion). Striped dolphins show relatively fine-scale population genetic structure while common dolphins have a continuous distribution in the Mediterranean Sea and Atlantic Ocean. To this extent, this study constructs a population genetic model for the striped dolphin inhabiting its geographical range in the Mediterranean Sea and the eastern North Atlantic Ocean, providing context for the interpretation of genetic diversity.

As an extension of previous studies I test the hypothesis, using higher resolution, that the striped dolphin has a fine-scale pattern of population structure within the Mediterranean Sea and the northeastern Atlantic Ocean, where relatively thorough studies have already shown little structure for the common dolphin. Given evidence for greater structure and consequently smaller population effective size in local populations of the striped dolphin, I test the hypothesis that the comparison between the common and the

striped dolphin will reveal a stronger relationship between genetic diversity and fitness in the species that shows greater population structure. The 'general effect' hypothesis (see Chapter 3) contends that a diverse genome will be reflected in diversity at neutral markers, and that these markers will therefore correlate to measures of fitness. However, it may be expected that a direct effect for specific functional markers may show a stronger correlation. Here I test the hypothesis that using the same set of samples, the genetic diversity and functional patterns of the exon-2 MCH Class II DQB1 locus will show a clearer association directly involved in pathogen resistance.

CHAPTER 2

Population genetic structure of striped dolphin (*Stenella coeruleoalba*) in the Mediterranean Sea and Atlantic Ocean

2.1 Introduction

Delphinid cetaceans are highly mobile species, and this characteristic allows them to move within and between large geographical areas. This would suggest a mechanism for genetic panmixia over broad geographic ranges, and this is seen in some cases. For example, the common dolphin (*Delphinus delphis*) shows little or no genetic differentiation among populations inhabiting the same side of an ocean basin (Natoli *et al.*, 2006; Mirimin *et al.*, 2009). Natoli *et al.* (2006) used nine microsatellite DNA loci to investigate population structure among eight regions in the Atlantic and North Pacific Ocean for both types of common dolphin; the short-beaked and the long-beaked form. Bayesian analysis based on individual genotypes suggests a single population in the North East Atlantic and showed no genetic structure among these regions. Structure was seen only at a much broader geographic scale among ocean basins and either side of the Atlantic, but also including differentiation between long and short-beacked morphotypes in the Pacific (Rosel *et al.*, 1994) and off South Africa (Natoli *et al.*, 2006). However, population genetic structure over much smaller geographic scales is more typical for dolphin species, in spite of their high mobility (see review in Hoelzel, 2009).

Understanding the mechanisms that generate population structure in mobile marine species is critical to the understanding of evolutionary process, and to the development of effective conservation policy. This is especially true since the nature of boundaries to gene flow in marine systems is often poorly understood, and genetic structure therefore cryptic. Across the geographic range extending from the Black Sea through the Mediterranean Sea and through the eastern North Atlantic to Scotland, a number of studies have investigated population genetic structure for delphinid species. Berube *et al.* (1998) suggest the existence of several divergence populations of fin whale in the North Atlantic and Mediterranean Sea using both nuclear and mtDNA markers. However, MtDNA loci detect

higher heterogeneity relative to nuclear loci probably due to male-mediated gene flow among populations. Another example is the fission-fusion groups of bottlenose dolphins where show a strong genetic structure worldwide (Hoelzel *et al.*, 1998a; Natoli *et al.*, 2004). In the case of North Atlantic and Mediterranean Sea Natoli *et al.* (2005) found a strong population structure between the Black sea, the Eastern Mediterranean and the Western Mediterranean showing 3 distinct populations respectively. Natoli *et al.* (2005) in the Atlantic found a population in Scotland that is separated from populations further south and the MtDNA analysis suggest a high rate of female emigration for the Scottish population. This fine genetic structure of local bottlenose dolphin populations could be based on local habitat dependence for both males and females and reflects the demographic history of the species (Natoli *et al.*, 2005). Genetic structure over this range appears to correlate with distinct habitat regions, though the specific characteristics that may be affecting gene flow are not known. Similar structure among apparent habitat regions has been seen elsewhere, for example Escorza-Trevino *et al.* (2005) using seven microsatellite loci found statistically significant differentiation between coastal (N=91) and offshore (N=50) populations of spotted dolphins in the Eastern Tropical Pacific.

In the Mediterranean and eastern North Atlantic some of the apparent boundaries seen for dolphin species are reinforced by differentiation for other species such as the sperm whale (Berube *et al.*, 1998) and various fish species such is the *Solea vulgaris* (Guarniero *et al.*, 2002) and the *Dicentrarchus labrax* (Bahri-Sfar *et al.*, 2000). Here we investigate population genetic structure across the same geographic range for the striped dolphin, but using higher resolution genetic analyses than had been previously applied.

Social coherence within local populations may be an ancillary mechanism promoting philopatry and habitat dependence. In one extreme case, that of the killer whale (*Orcinus orca*), social cohesion in kin-groups is strong enough to largely define regional population

structure (Hoelzel *et al.*, 2007, Pilot *et al.*, 2010). Social kinship associations have been investigated in other delphinid species including the striped dolphin (Gaspari *et al.*, 2007a). Gaspari *et al.* (2007a) used eight microsatellite DNA loci to investigate kinship within and among social groups of striped dolphin, and found a significant association among adult female kin in small social groups. However, these associations accounted for a relatively small amount of substructure within local populations (F_{ST}=0.0217), and this is typical of other dolphin species, apart from the killer whale.

The striped dolphin is distributed word-wide in tropical and temperate waters (see Archer and Perrin, 1999; Hammond *et al.*, 2008; Figure 2.1). In the northern hemisphere it inhabits in the Atlantic Ocean from Newfoundland to northern Scotland and Denmark. In the Mediterranean Sea the striped dolphin is the most common and abundant species (Notarbartolo di Sciara *et al.*, 1993).

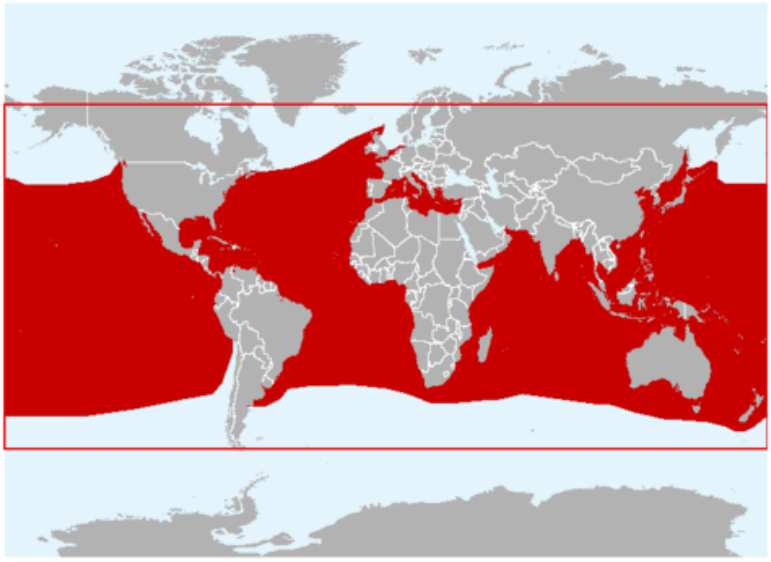


Figure 2.1. Worldwide distribution map of the striped dolphin.

However, it is not uniformly distributed in the Mediterranean, for example, Galov *et al.* (2009) report that striped dolphins are not resident in the Croatian part of the Adriatic Sea. The authors, reporting a lack of genetic differentiation for striped dolphin mtDNA

control region haplotypes between the Croatian part of the Adriatic Sea and the rest of Mediterranean Sea, and together with the scarce reports of striped dolphin strandings in the area, suggest that those animals may be transient individuals and part of a larger, more diverse source population. Bourret *et al.* (2007) suggest that there is significant differentiation based on five microsatellites between the Mediterranean and Atlantic Ocean populations, and between the Mediterranean and Pacific Ocean as well. Authors propose that the difference between the Mediterranean and Atlantic basins may be explained by the higher effective population size of the Atlantic population in relation to its large geographical range. However, potential inference is limited due to the small panel of microsatellites used in the study.

Gaspari *et al.* (2007a) also reported differentiation between the Mediterranean populations and the North Sea, between samples from either side of Italy, and showed a weak isolation by distance pattern between nearshore and offshore samples in the Ligurean Sea (to the west of Italy) based on eight microsatellite DNA loci. On the other hand, Garcia-Martinez *et al* (1995) found no subdivision within the Mediterranean Sea using mtDNA markers using 26 samples from the western Mediterranean (Balaeric Sea), 43 samples from the central Mediterranean (Italy) and 3 samples from eastern Mediterranean (Greece and Israel).

This study provides a further assessment of the population genetic structure of striped dolphins in the Mediterranean and eastern North Atlantic. The difference between this study and the previous analyses is the use of a much larger panel of microsatellite DNA loci (providing higher resolution for fine-scale geographic comparisons) and the inclusion of some geographic comparisons not previously assessed. According to the Agreement of the Conservation of Cetaceans of the Black Sea, Mediterranean Sea and Contiguous Atlantic Sea (ACCOBAMS) which came into effect in 2001, the identification of dolphin

stock structure is a particular priority to facilitate effective conservation and management. However, further comparative data on the pattern and range of population connectivity over the shared geographic distribution recognised by ACCOBAMS will also help determine the evolutionary mechanisms responsible for the partitioning of diversity in these species. For the striped dolphin in particular I test specific hypotheses based on data from earlier studies.

The molecular ecology of the striped dolphin in the Mediterranean Sea, revealed structuring patterns as has been previously reported (Gaspari *et al.*, 2007a). In this study I use a large panel of microsatellite DNA loci and sample sites that are located further to the eastern Mediterranean. Therefore, I test the hypothesis that the different methods with respect to power and the putative populations sampled provide sufficient power to detect small differences and beyond that reported previously.

A sample from Korinthiakos Gulf is included in the analysis. Frantzis and Herzing (2002) in a study of Delphinidae species sightings and abundance in the Korinthiakos Gulf point out the different pigmentation observed in the local striped dolphin population. Published studies in other marine species have been reported genetic differentiation between groups typically favouring morphological variations of bottlenose dolphins (Natoli *et al.*, 2004) and common dolphins (Natoli *et al.*, 2006). Therefore, I test the importance of the morphology that distinguishes this local population

Towards this end I greatly extend the representation of regional populations in the Atlantic Ocean, and include a comparison of previous studies in the same geographic range. My further objective is to address the question of how population structure may have evolved in a highly mobile marine species given the pattern of the differentiation observed between the two areas.

2.2 Materials and Methods

2.2.1 Samples collection and Study area

Samples were collected from stranded, bycatch and free-range striped dolphins from the Mediterranean Sea and the North East Atlantic Ocean. Sampling sites are shown in Figure 2.2. The total sample size was 258: 6 individuals from Israel (IS), 25 individuals from Central Greece (CGR), 8 individuals from Eastern Greece (EGR), 3 individuals from Strait of Sicily (SS), 94 individuals from Valencia (WM), 6 individuals from Gibraltar Strait (GS), 16 individuals from Biscay Gulf (BG), 49 individuals from Ireland (IR) and 51 individuals from Scotland (SC). Samples from Central Greece, Eastern Greece ,Strait of Sicily and Strait of Gibraltar obtained from biopsy sampling (total: 42). The rest samples were obtained from stranded animals (204) and 16 out of the 49 individuals from Ireland were obtained from bycatch animals. Samples from Israel, Central and Eastern Greece and samples from Strait of Sicily (total: 42 individuals) were considered as Eastern Mediterranean site whereas samples from Valencia are considered as Western Mediterranean site.

Biopsy sampling was carried out using a dart system, which contains a cross-bow and a lightweight dart with a steel biopsy tip at the end. The biopsy tip, a cylinder shape, has a length of 1.5 cm, a diameter of 0.5cm, and penetrates the skin and blubber of the animal. For the biopsy sampling, a 13m catamaran boat was used with two diesel engines having 6 knots velocity and surveys were carried out in cross lines. The samples were stored in 20% DMSO NaCl 5M. Stranded animals' samples were collected and stored either in 20% DMSO NaCl 5M buffer or in 70% ethanol.

The majority of the stranded individuals from Western Mediterranean died due to the high mortality of the morbillivirus between 1990 and 2007. However, further stranded

samples from the same region were collected from 1989 and 2008, and the cause of death is unknown. Similar, samples from Israel, Biscay Gulf, Ireland and Scotland were from stranded animals and the cause of death is unknown. For all stranded animals a necropsy procedure was carried out and sex was determined whenever possible.

Figure 2.2. Sampling sites of striped dolphin (*Stenella coeruleoalba*) individuals in the Mediterranean Sea and North East Atlantic Ocean

2.2.2 DNA extraction and PCR amplification

Skin and muscle tissue were used for the DNA extractions. DNA was extracted following either the standard phenol/chloroform extraction protocol (Sambrook *et al.*, 1989) or a standard salt extraction protocol (see Aljanabi and Martinez, 1997). The quality

of DNA was better when the phenol/chloroform protocol was used for those samples that were preserved in 70% ethanol. DNA was preserved in 10mM TE and stored in -20^{o}C.

A total panel of 29 universal DNA microsatellites markers were tested and optimized for the genetic analyses (Table 2.1). A multiplex PCR Kit (Qiagen) with a hot start Taq was used for the DNA amplifications. The 29 pairs of primers were divided into 4 multiplex Groups (Table 2.1) according to size range and florescent primer's pigment. The PCR cycling profile was: 95^{o}C for 15'; 30 cycles of 95^{o}C for 1', annealing temperature for 30'' and 72^{o}C for 30''; 72^{o}C for 15'. PCR products were verified by agarose gel electrophoresis. Amplified DNA products were screened on an ABI 3730 DNA Analyser (Applied Biosystems). Each specimen's alleles were scored by the STRand software v.2.0 (Toonen and Hughes, 2001) and the 10% of genotypes were redone for error checking. Sex was determined using the primers P15EZ, P23EZ for the *Zfx*/*Zfy* gene (Aasen and Medrano, 1990) and Y53-3c, Y53-3d for the *SRY* gene (Gilson *et al.*, 1998). The thermo cycling profile was an initial 15 minutes denaturation step at 95^{o}C, following by 35 cycles of 1 minute denaturation at 95^{o}C, 30 seconds annealing at 60^{o}C, 30 seconds elongation at 72^{o}C, and a final 10 minutes elongation at 72^{o}C. PCR products were screened by agarose gel electrophoresis and single bands or double bands indicated females and males respectively.

2.2.3 Statistical analysis

All loci were tested for the presence of null alleles or allelic dropout using the software Micro-Checker v. 2.2.3 (Van Oosterhout *et al.*, 2004) where a Monte Carlo simulation method is used to generate expected homozygote and heterozygote allele size difference frequencies. Exact tests for Hardy-Weinberg equilibrium, Linkage disequilibrium (using Fisher's Exact Test), expected Heterozygosity (H_{EXP}) and observed

Heterozygosity (H_{OBS}) was carried out using the software Genepop v. 3.4 (Raymond and Rousset, 1995). Fixation index F_{ST} (using the formulations described by Weir and Cockerham, 1984), number of alleles per locus were calculated using the FSTAT v. 2.9.3.2 software (Goudet, 2001). FSTAT v. 2.9.3.2 was also used to assess evidence for sex-biased dispersal (using sex-specific diversity estimates and assignment indices after Goudet, 2001). This was assessed for all populations and the significance was based on 1,000 randomizations.

Isolation By Distance software (IBD) was used to assess whether the association between genetic similarity ($F_{ST}/(1-F_{ST})$ – Rousset, 1997) and geographic distance is statistically significant using a Mantel Test based on 1,000 randomization (Bohonak, 2002). The software uses partial correlation coefficients between genetic and geographical distance.

Evidence of recent bottlenecks event was tested in the software BOTTLENECK v. 1.2.02 (Piry *et al.*, 1999). Two different approaches were used. In the first approach assumed that a recently bottlenecked population the gene diversity will be higher than the expected. Gene diversity was estimated under the infinite allele model (IAM), the stepwise mutation model (SMM) and the two-phase model (TPM). TPM used with 95% single step mutation and 5% multiple step mutations with a variance among steps of 12 (see Piry *et al.*, 1999). 10,000 iterations were used for each model. One-tailed Wilcoxon singed rank test was used to determine numbers of loci in heterozygosity excess. The second approach, the mode shift indicator, tested the allele frequency distribution which discriminates bottlenecked populations from stable populations (Luikart *et al.*, 1997).

Factorial Correspondence Analysis was performed using the software Genetix v. 4.05.2 (Belkhir *et al.*, 2002). The analyzed putative populations were visualised as groups

of dots in a ruled surface in two dimensions. Each dot represents the individual's genotypic data by its score for each term of each variable (alleles at different loci), that is 0 for the absence, 1 for the presence of the allele with the heterozygote state and 2 for the homozygote state. The inertia values determine where the dots lay by consistency between themselves in the data.

Population structure was further assessed using the software STRUCTURE v. 2.3 (Pritchard *et al.*, 2000a) where identified migrants and admixed individuals were assigned. The Correlated allele Frequency Model (Falush *et al.*, 2003a) was used, which records the allele frequencies in a hypothetical "ancestral" population. Two different models were run; at the first one the geographic area was specified as a prior, whereas at the second model was run without specifying geographic are as a prior. To test the convergence of the priors and the appropriateness of the chosen burn-in length and simulation length 3 independent repeats were run for each value of K ($5 \leq K \leq 10$). Burn-in length and length of simulation were set at 500,000 and 1,000,000 repetitions respectively.

The migrants estimation using a likelihood-ratio test was identified using the frequency-based method of Paetkau *et al.* (1995) and the probability-based method of Paetkau *et al.* (2004). The likelihood computation was $L = Lhome/Lmax$, which is the ratio of the likelihood computed from the population where the individual was sampled (*Lhome*) over the highest likelihood value among all population samples including the population where the individual was sampled (*Lmax*). The analysis was performed in Geneclass v. 2.0 (Piry *et al.*, 2004). The probability method is based on Monte Carlo resampling algorithm where the minimum number of simulated individuals and Type-I error (alpha) were set to 1,000 and 0.01 respectively.

The software Barrier v. 2.2 (Manni *et al.*, 2004) was used to identify locations and the directions of barriers using a computational geometry approach. It was used the Monmonier (1973) maximum difference algorithm to provide a more realistic representation of the barriers in a genetic landscape and a significance test was implemented by means of bootstrap matrices analysis. In order to obtain a geometrically satisfactory map from a list of geographic X/Y coordinates a Voronoi tessellation (Voronoi, 1908) calculator was used. Out of this tessellation a Delaunay triangulation (Brassel and Reif, 1979) was obtained.

The software Bayescan v. 1.0 (Foll and Gaggioti, 2008) was used to identify candidate loci under natural selection. This software uses a Bayesian method to estimate directly the probability that each locus is subject to selection. The software uses differences in allele frequencies between populations. One of the scenarios covered consists of an island model, in which subpopulation allele frequencies are correlated through a common migrant gene pool from which they differ in vaying degrees. The difference in allele frequency between this common gene pool and each subpopulation is measured by a suppopulation specific F_{ST} coefficient. The number of iterations and burn-in length were 5,000 and 50,000 respectively. To reduce the autocorrelation of the data generated from a Markov chain, iterations between two samples were set to 20.

Table 2.1. Multiplex Groups of the 29 microsatellites and annealing temperature.

		Annealing Temperature	References
Multiplex Group1	KWM1b		Hoelzel *et al.* 1998
	Dde84		Coughlan et al. 2006
	Sco28		Mirimin et al. 2006
	MK3		Krützen et al. 2001
	D08		Rooney et al. 1999
	KWM9b	55 ^{o}C	Hoelzel *et al.* 1998
	MK5		Krützen et al. 2001
	Ev37		Valsecchi and Amos 1996
	Dde72		Coughlan et al. 2006
	Dde59		Coughlan et al. 2006
	Dde09		Coughlan et al. 2006
	MK8		Krützen et al. 2001
Multiplex Group2	D18		Rooney et al. 1999
	Sco65		Mirimin et al. 2006
	D28	53 ^{o}C	Rooney et al. 1999
	KWm12a		Hoelzel *et al.* 1998
	Dde65		Coughlan et al. 2006
	Sco55		Mirimin et al. 2006
Multiplex Group3	Dde70		Coughlan et al. 2006
	Sco66		Mirimin et al. 2006
	KWM2a	55 ^{o}C	Hoelzel *et al.* 1998
	Dde69		Coughlan et al. 2006
	TexVet5		Shinohara et al. 1997
	Dde66		Coughlan et al. 2006
Multiplex Group4	D22		Rooney et al. 1999
	KWM2b	53 ^{o}C	Hoelzel *et al.* 1998
	Sco11		Mirimin et al. 2006
	D14		Rooney et al. 1999
	Ev14		Valsecchi and Amos 1996

2.3 Results

Among the 29 loci screened, one locus showed evidence of null alleles and two couldn't be amplified for the entire sample-set, therefore 26 loci were used for the analysis (are shown in Figure 2.3). Those 26 loci were tested for Hardy-Weinberg equilibrium (HWE) where no significant departures were detected (after Bonferroni correction), thus they were used for further analysis. A high level of polymorphism was detected, and the range of alleles varied from 28 at locus MK5 to 7 at locus Sco28. The numbers of alleles per locus are shown in Figure 2.2

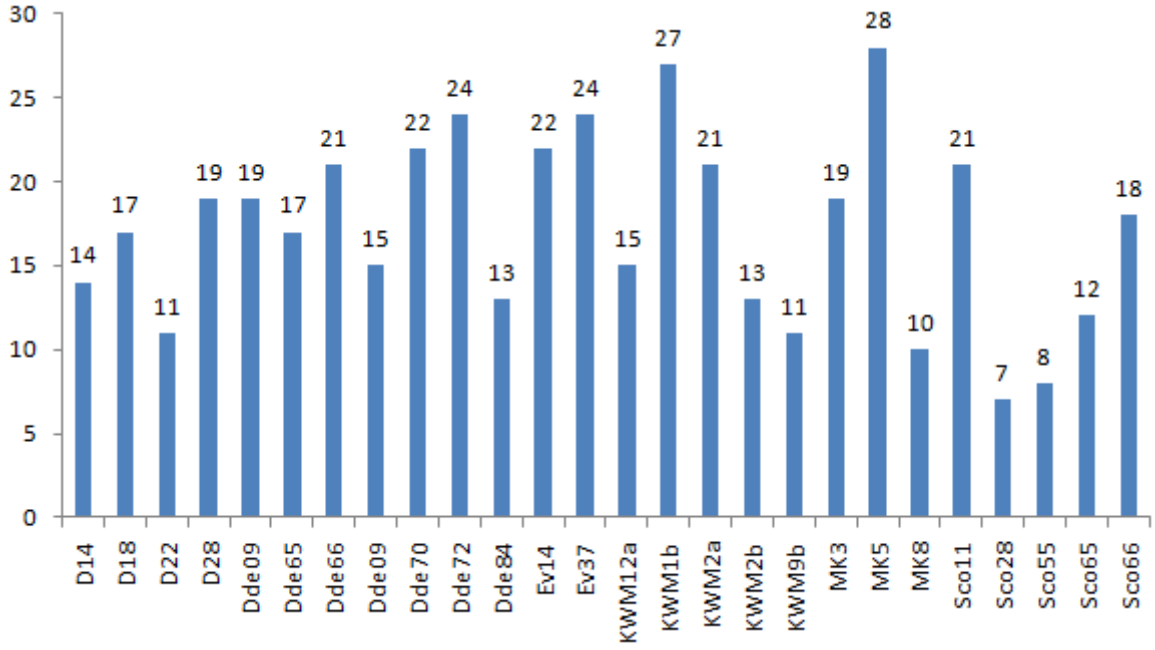


Figure 2.3. Number of alleles per locus in striped dolphin populations

F_{ST} values between putative populations from six different geographical areas ranged from 0.0135 between Biscay Gulf and Scotland to 0.0565 between the Eastern Mediterranean and Ireland (Table 2.2). All values were significant apart from the

comparison between the Biscay Gulf and Scotland. The F_{ST} values were remained significant after Bonferroni correction.

Table 2.2. Fst values between 6 geographical areas of striped dolphin populations (NS:non significant, *:p<0.05. **p<0.01, ***p<0.001)

	Scotland	Ireland	Biscay Gulf	Gibraltar Strait	Valencia	Eastern Mediterranean
Scotland	0					
Ireland	0.0238^{***}	0				
Biscay Gulf	0.0135^{NS}	0.0197^{*}	0			
Gibraltar Strait	0.0549^{***}	0.0431^{*}	0.063^{**}	0		
Valencia	0.0487^{***}	0.0533^{***}	0.0548^{***}	0.0328^{**}	0	
Eastern Mediterranean	0.0375^{***}	0.0565^{***}	0.0433^{***}	0.0521^{**}	0.0246^{***}	0

A further genetic differentiation was calculated among pairwise populations of Ionian Sea, Korinthiakos Gulf and Israel (Table 2.3). The Ionian Sea was significant differentiated from the Korinthiakos Gulf (after Bonferroni, p<0.05).

Table 2.3. Fst values between Ionian Sea, Korinthiakos Gulf and Israel areas of striped dolphin populations (NS:non significant, *:p<0.05)

	Ionian Sea	Korinthiakos Gulf	Israel
Ionian Sea	0		
Korinthiakos Gulf	0.0436^{*}	0	
Israel	0.0443^{NS}	0.0241^{NS}	0

Observed Heterozygosity values were relative high across all loci for all populations (mean H_{OBS} = 0.7768) and higher than overall Expected Heterozygosity (mean H_{EXP} = 0.7716). Heterozygosity and gene diversity for all 26 microsatellite DNA loci are shown in Table 2.4. Average Gene Diversity was lowest for the Ionian Sea (0.715) and the highest for the Ireland population (0.8115).

Table 2.4. Genetic variation at each locus for each population. The number of individuals analysed for each population is indicated below the population name. Gene diversity, F_{IS} values, heterozygosity observed (Ho) and heterozygosity expected (He) are reported.

		Scotland	Ireland	Biscay Gulf	Gibraltar Strait	Valencia	Sicily Strait	Ionian Sea	Korinthiakos Gulf	Israel
D14										
	He	0.8687	0.843	0.8418	0.8194	0.8486	0.7778	0.8203	0.8498	0.74
	Ho	0.9412	0.9388	0.9375	1	1	1	0.875	0.9583	0.8
	Gene Diversity	0.877	0.851	0.867	0.883	0.852	0.917	0.875	0.866	0.825
	F_{IS}	-0.074	-0.103	-0.082	-0.132	-0.173	-0.091	-0.000	-0.107	0.030
D18										
	He	0.89	0.9034	0.8457	0.6806	0.7488	0.7222	0.6406	0.5773	0.64
	Ho	0.94	1	0.875	1	0.883	1	1	0.875	1
	Gene Diversity	0.899	0.912	0.873	0.717	0.752	0.833	0.661	0.583	0.675
	F_{IS}	-0.046	-0.097	-0.002	-0.395	-0.174	-0.200	-0.514	-0.500	-0.481
D22										
	He	0.8499	0.8605	0.8047	0.7222	0.8125	0.2778	0.7813	0.7109	0.86
	Ho	1	1	0.9375	0.6667	1	0.3333	1	0.75	1
	Gene Diversity	0.857	0.868	0.827	0.8	0.816	0.333	0.821	0.726	0.95
	F_{IS}	-0.167	-0.152	-0.134	0.167	-0.226	0.000	-0.217	-0.034	-0.053
D28										
	He	0.9014	0.9002	0.8911	0.8472	0.9052	0.7778	0.8594	0.7995	0.86
	Ho	1	1	1	1	1	1	1	0.9583	1
	Gene Diversity	0.909	0.909	0.919	0.917	0.91	0.917	0.911	0.813	0.95
	F_{IS}	-0.100	-0.101	-0.088	-0.091	-0.099	-0.091	-0.098	-0.178	-0.053
Dde09										
	He	0.7797	0.8221	0.8555	0.7222	0.7627	0.5	0.75	0.796	0.68
	Ho	0.8039	0.7609	0.4375	1	0.7527	0.6667	1	1	1
	Gene Diversity	0.787	0.832	0.898	0.767	0.767	0.583	0.786	0.809	0.725
	F_{IS}	-0.021	0.085	0.513	-0.304	0.019	-0.143	-0.273	-0.236	-0.379

		Scotland	Ireland	Biscay Gulf	Gibraltar Strait	Valencia	Sicily Strait	Ionian Sea	Korinthiakos Gulf	Israel
Dde65										
	He	0.8201	0.8192	0.8311	0.5694	0.7425	0.2778	0.7188	0.8264	0.72
	Ho	0.7843	0.7347	0.8667	0.8333	0.7553	0.3333	0.875	0.7083	0.4
	Gene Diversity	0.829	0.829	0.86	0.6	0.746	0.333	0.759	0.847	0.85
	F_{IS}	0.053	0.113	-0.008	-0.389	-0.012	0.000	-0.153	0.164	0.529
Dde66										
	He	0.8922	0.8889	0.8958	0.6111	0.823	0.6667	0.7653	0.7474	0.84
	Ho	0.8431	0.8936	0.75	0.3333	0.8	0	0.7143	0.6667	0.8
	Gene Diversity	0.902	0.898	0.943	0.7	0.828	1	0.833	0.765	0.95
	F_{IS}	0.065	0.005	0.205	0.524	0.033	1.000	0.143	0.129	0.158
Dde69										
	He	0.7766	0.7953	0.7883	0.6528	0.7448	0.6111	0.7344	0.7995	0.64
	Ho	0.7451	0.7959	0.8571	0.5	0.7111	0.6667	1	0.875	0.4
	Gene Diversity	0.785	0.804	0.816	0.733	0.749	0.75	0.768	0.815	0.75
	F_{IS}	0.050	0.010	-0.051	0.318	0.051	0.111	-0.302	-0.073	0.467
Dde70										
	He	0.9104	0.9284	0.8733	0.7917	0.8581	0.7778	0.5078	0.7891	0.72
	Ho	0.9804	0.9796	1	0.8333	0.8191	1	0.5	0.75	0.8
	Gene Diversity	0.919	0.938	0.9	0.867	0.863	0.917	0.545	0.807	0.8
	F_{IS}	-0.067	-0.045	-0.111	0.038	0.051	-0.091	0.082	0.071	-0.000
Dde72										
	He	0.875	0.91	0.8594	0.7778	0.9043	0.7222	0.8516	0.862	0.7813
	Ho	0.8627	0.8261	0.75	0.8333	0.9111	0.3333	0.875	0.7083	0.25
	Gene Diversity	0.884	0.921	0.892	0.85	0.909	1	0.911	0.884	1
	F_{IS}	0.024	0.103	0.159	0.020	-0.002	0.667	0.039	0.199	0.750
Dde84										
	He	0.8576	0.883	0.8105	0.8194	0.8429	0.6667	0.8516	0.8229	0.78
	Ho	0.9412	0.8333	0.75	0.6667	0.7872	1	0.875	0.75	0.8
	Gene Diversity	0.865	0.893	0.84	0.917	0.848	0.75	0.911	0.842	0.875
	F_{IS}	-0.088	0.067	0.107	0.273	0.071	-0.333	0.039	0.110	0.086

		Scotland	Ireland	Biscay Gulf	Gibraltar Strait	Valencia	Sicily Strait	Ionian Sea	Korinthiakos Gulf	Israel
Ev14										
	He	0.8985	0.9259	0.8965	0.8472	0.8549	0.8333	0.8047	0.7847	0.82
	Ho	0.902	1	0.75	1	0.8817	1	1	0.7917	0.8
	Gene Diversity	0.907	0.935	0.931	0.917	0.859	1	0.848	0.802	0.925
	F_{IS}	0.006	-0.070	0.195	-0.091	-0.026	-0.000	-0.179	0.012	0.135
Ev37										
	He	0.8223	0.9343	0.7813	0.6667	0.7252	0.8333	0.6429	0.5894	0.64
	Ho	0.7447	0.9535	0.75	1	0.8085	1	1	0.625	0.8
	Gene Diversity	0.832	0.945	0.808	0.7	0.729	1	0.667	0.601	0.7
	F_{IS}	0.105	-0.009	0.072	-0.429	-0.110	-0.000	-0.500	-0.039	-0.143
KWM12a										
	He	0.8908	0.8842	0.832	0.75	0.857	0.7778	0.7969	0.8663	0.84
	Ho	0.98	0.898	0.9375	1	0.9149	1	1	0.875	0.8
	Gene Diversity	0.899	0.893	0.856	0.8	0.861	0.917	0.839	0.885	0.95
	F_{IS}	-0.090	-0.005	-0.095	-0.250	-0.062	-0.091	-0.191	0.011	0.158
KWM1b										
	He	0.8796	0.6007	0.7773	0.5	0.9084	------	0.5547	0.8602	0.8
	Ho	0.86	0.7083	0.875	1	0.7312	0	1	0.9583	0.8
	Gene Diversity	0.889	0.606	0.8	0.5	0.914	NA	0.563	0.877	0.9
	F_{IS}	0.032	-0.169	-0.094	-1.000	0.200	NA	-0.788	-0.093	0.111
KWM2a										
	He	0.9258	0.9279	0.918	0.6944	0.7708	0.8333	0.7188	0.7717	0.8
	Ho	0.9804	0.9796	0.9375	1	0.9355	1	0.875	1	1
	Gene Diversity	0.935	0.937	0.948	0.733	0.774	1	0.759	0.784	0.875
	F_{IS}	-0.049	-0.045	0.011	-0.364	-0.208	-0.000	-0.153	-0.276	-0.143
KWM2b										
	He	0.7689	0.8615	0.7988	0.7361	0.7797	0.7778	0.8125	0.809	0.68
	Ho	0.7843	0.898	0.6875	0.6667	0.8298	1	0.75	0.7083	0.6
	Gene Diversity	0.776	0.87	0.829	0.817	0.784	0.917	0.875	0.829	0.775
	F_{IS}	-0.010	-0.032	0.171	0.184	-0.059	-0.091	0.143	0.145	0.226

		Scotland	Ireland	Biscay Gulf	Gibraltar Strait	Valencia	Sicily Strait	Ionian Sea	Korinthiakos Gulf	Israel
KWM9b										
	He	0.6978	0.499	0.2266	0.4861	0.5768	0.2778	0.5078	0.625	0.58
	Ho	0.9804	0.7143	0.125	0.8333	0.8511	0.3333	0.75	0.9583	1
	Gene Diversity	0.702	0.502	0.238	0.5	0.578	0.333	0.527	0.631	0.6
	F_{IS}	-0.397	-0.423	0.474	-0.667	-0.471	0.000	-0.424	-0.518	-0.667
MK3										
	He	0.9125	0.9138	0.9102	0.8194	0.9148	0.8333	0.84	0.8906	0.75
	Ho	0.8163	0.8958	0.9375	0.6667	0.4222	1	0.8	0.6667	0.75
	Gene Diversity	0.923	0.924	0.94	0.917	0.923	1	0.95	0.915	0.875
	F_{IS}	0.116	0.030	0.002	0.273	0.542	-0.000	0.158	0.271	0.143
MK5										
	He	0.9048	0.8963	0.9	0.75	0.9028	0.7778	0.8438	0.7903	0.82
	Ho	0.9608	0.9796	0.8	0.8333	0.9247	1	0.875	0.8182	1
	Gene Diversity	0.913	0.905	0.936	0.817	0.908	0.917	0.902	0.808	0.9
	F_{IS}	-0.052	-0.083	0.145	-0.020	-0.019	-0.091	0.030	-0.012	-0.111
MK8										
	He	0.5815	0.6789	0.6348	0.6806	0.6287	0.6111	0.5	0.6571	0.42
	Ho	0.8824	0.9592	0.875	1	0.9894	1	1	0.9583	0.6
	Gene Diversity	0.584	0.683	0.648	0.717	0.63	0.667	0.5	0.665	0.45
	F_{IS}	-0.510	-0.404	-0.350	-0.395	-0.570	-0.500	-1.000	-0.441	-0.333
Sco11										
	He	0.8218	0.8867	0.8333	0.5	0.6582	0.7222	0.4844	0.75	0.82
	Ho	0.7255	0.898	0.8	0.3333	0.6383	0.3333	0.5	0.6667	0.6
	Gene Diversity	0.831	0.896	0.864	0.567	0.662	1	0.518	0.768	0.95
	F_{IS}	0.127	-0.002	0.074	0.412	0.036	0.667	0.034	0.132	0.368
Sco28										
	He	0.2907	0.3728	0.2813	0.1528	0.0618	0.4444	0.2266	0.3924	0
	Ho	0.098	0.3061	0.1875	0.1667	0	0	0.25	0	0
	Gene Diversity	0.295	0.377	0.294	0.167	0.062	0.667	0.241	0.409	0
	F_{IS}	0.668	0.189	0.362	-0.000	1.000	1.000	-0.037	1.0000	NA

		Scotland	Ireland	Biscay Gulf	Gibraltar Strait	Valencia	Sicily Strait	Ionian Sea	Korinthiakos Gulf	Israel
Sco55										
	He	0.2457	0.4142	0.1172	0.5417	0.4056	0.5	0.4063	0.487	0.32
	Ho	0.2549	0.1429	0.125	0.5	0.3936	0.6667	0.5	0.4167	0.4
	Gene Diversity	0.248	0.421	0.121	0.6	0.408	0.583	0.429	0.499	0.35
	F_{IS}	-0.028	0.661	-0.034	0.167	0.035	-0.143	-0.167	0.165	-0.143
Sco65										
	He	0.5502	0.6826	0.4219	0.5833	0.6343	0.7222	0.4063	0.4731	0.46
	Ho	0.5686	0.5102	0.375	0.5	0.6489	0.3333	0.375	0.4167	0.2
	Gene Diversity	0.555	0.692	0.438	0.65	0.638	1	0.438	0.485	0.55
	F_{IS}	-0.024	0.262	0.143	0.231	-0.018	0.667	0.143	0.140	0.636
Sco66										
	He	0.831	0.8584	0.8262	0.7222	0.7443	0.7222	0.6875	0.6684	0.78
	Ho	0.8627	0.8163	0.5625	0.8333	0.75	0.3333	0.375	0.7083	0.4
	Gene Diversity	0.839	0.868	0.863	0.783	0.748	1	0.759	0.682	0.925
	F_{IS}	-0.028	0.059	0.348	-0.064	-0.002	0.667	0.506	-0.039	0.56

Bayesian individual assignment implemented in Structure v. 2.3 is shown in Figure 2.4, using geographical area a prior. The highest posterior probability was for K=7 and Ln= -26586.1. (Figure 2.4 and Table 2.5)

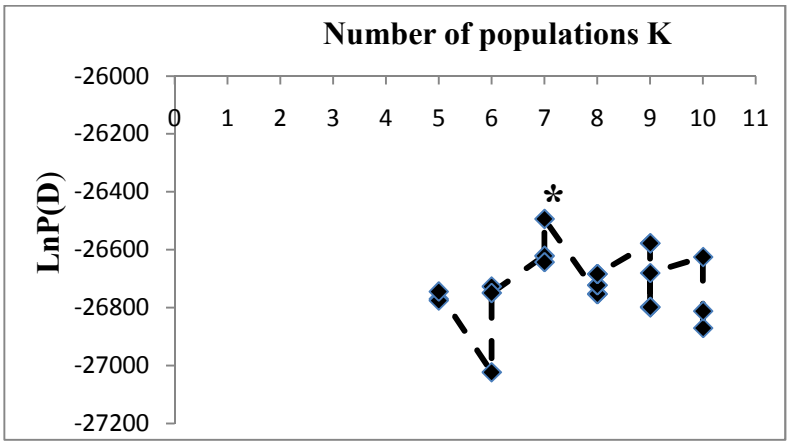


Figure 2.4. Determination of the number of clusters (K) including all 3 repetitions for each K (rhomb shape) using geographical area as a prior. A star denotes the most likely number of clusters according to the Pritchard Bayes Formula.

Table 2.5. Determination of the numger of clusters (K) including all 3 repetitions for each K, specifying geographical area as a prior.

K	5	6	7	8	9	10
	-26744.4	-26749.4	-26493.9	-26683.9	-26680.6	-26625.4
Ln(PD)	-26775.7	-26726.8	-26643	-26722.6	-26798.2	-26870.7
	-26769.7	-27023.1	-26621.3	-26753	-26577.9	-26811.8

Running an alternative bayesian individual assignment implemented in Structure v. 2.3 (Figure 2.5), without using geographical area a prior, results remained the same. The highest posterior probability was for K=7 and Ln = -26586.1. (Figure 2.4 and Table 2.6).

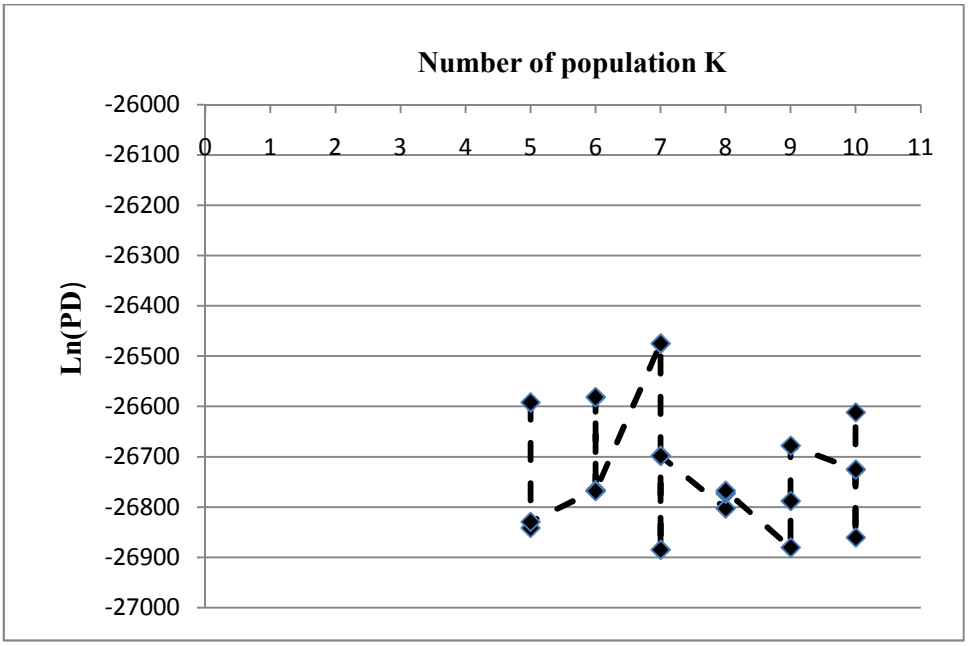


Figure 2.5. Determination of the number of clusters (K) including all 3 repetitions for each K (rhomb shape) without geographical area as a prior. A star denotes the most likely number of clusters according to the Pritchard Bayes Formula.

Table 2.6. Determination of the numger of clusters (K) including all 3 repetitions for each K, without specifying geographical area as a prior.

K	5	6	7	8	9	10
	-26592.3	-26766.8	-26475.1	-26802.8	-26880.6	-26725.4
Ln(PD)	-26841.6	-26581.6	-26884.7	-26773	-26788.2	-26860.7
	-26829.5	-26768.1	-26698.1	-26767.7	-26677.9	-26611.8

According to Figure 2.9 and Figure 2.10, in the Northeast Atlantic Ocean there are 4 different populations represented among the samples from Scotland and Ireland, where both regions are subdivided in multiple clusters, but in each case dominated by two. The Biscay Gulf shares assignments with both Scotland and Ireland but is more homogenous with Scotland's populations. In the Mediterranean Sea populations from Gibraltar Straight and Valencia are assigned as one single population. The Eastern Mediterranean is subdivided into 3 main different populations, where populations from Sicily and Western Greece are homogenous with the populations from Gibraltar Straight and Valencia. There is a unique population in Eastern Greece and a different one in Israel (though the latter is

based on very few samples). Performing an analysis in GENECLASS v.2.0 of possible migrants detected 3 putative migrants from Scotland to Biscay Gulf ($p<0.01$) and one from Ireland to Biscay Gulf ($p<0.01$).

The Isolation By Distance (IBD) analysis did not reveal any significant isolation by distance ($Z = 1203.5300$, $r^2 = 0.00087$, $p=0.4670$) of the 9 putative populations (Figure 2.6). However, a significant correlation ($Z=53.5059$, $r^2=0.898$, $p<0.001$) was detected in the Atlantic Ocean populations (Figure 2.7).

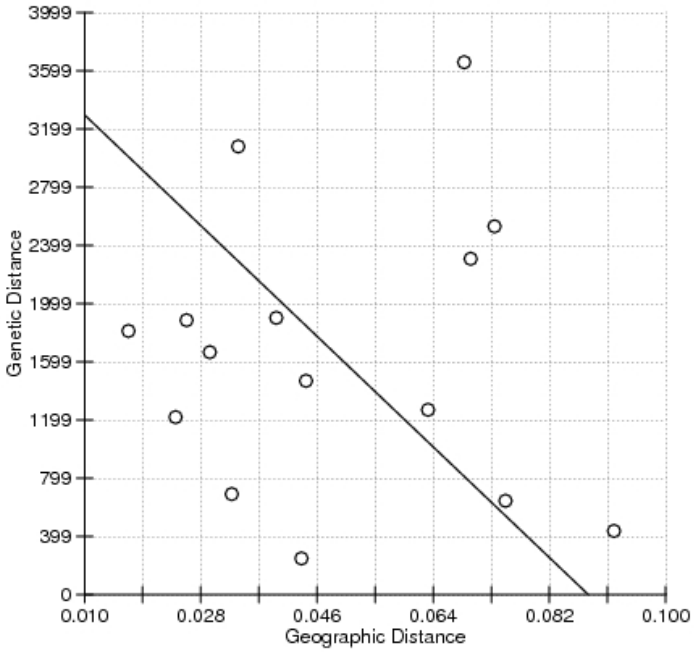


Figure 2.6. Isolation By Distance for the nine putative populations in the Mediterranean Sea and Atlantic Ocean.

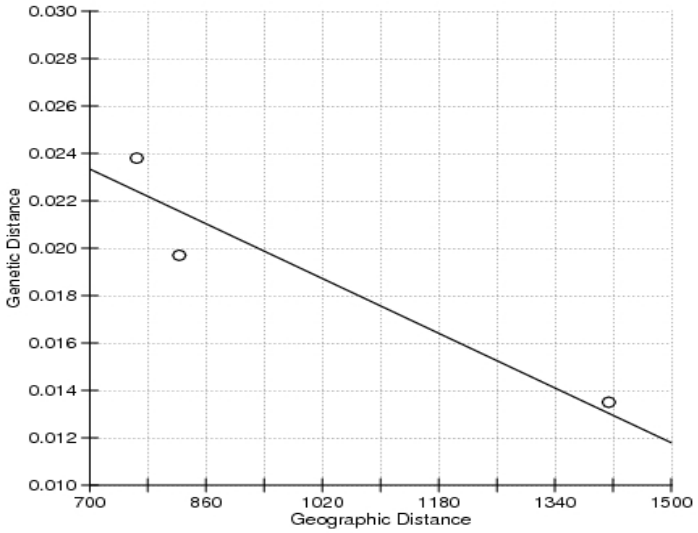


Figure 2.7. Isolation By Distance for the Atlantic Ocean populations.

The geometrically map, using the Barrier software, showed two barriers (Figure 2.8). The first barrier seperates the Mediterranean Sea and the Atlantic Ocean populations, and the second one, between Ionian Sea and Korinthiakos Gulf populations, determine a barrier that devides the Mediterranean basin.

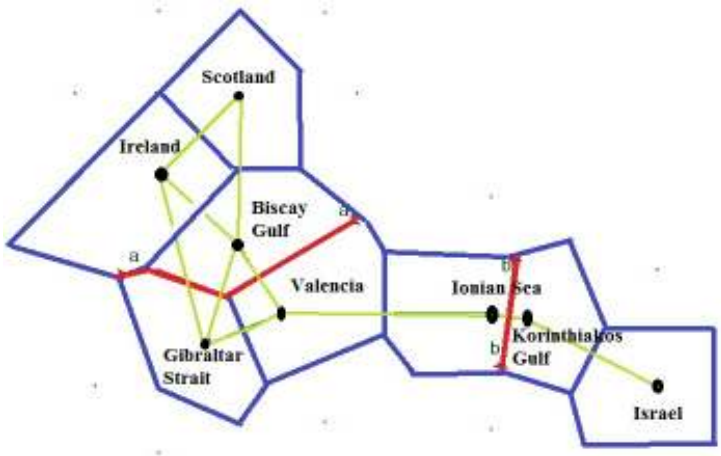


Figure 2.8. Voronoi tessellation (in blue) of the points (populations) according to geographical locations (black spots) and the corresponding Delaunay triangulation (in green).

Figure 2.9. Bayesian individual assignment implement for K=7, using a prior geographical areas; Length of burning period: 500000, No of MCMC Reps after burning: 1000000. 1: Scotland (SC), 2: Ireland (IR), 3: Biscay Gulf (BG), 4: Gibraltar Straight (GS), 5: Western Mediterranean (WM – Valencia), 6: East Mediterranean (EM - first 3 individuals from Sicily, second 6 individuals from Western Greece, the following 28 from Eastern Greece, last 5 individuals from Israel)

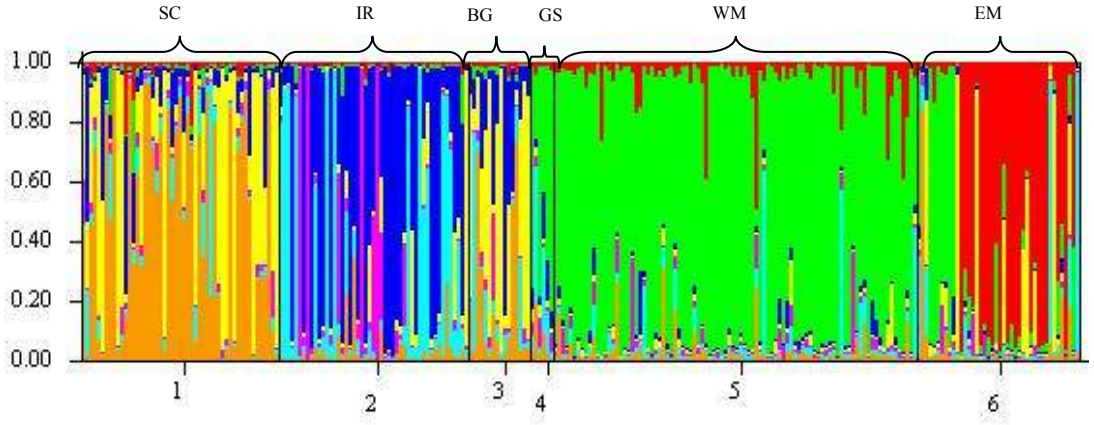
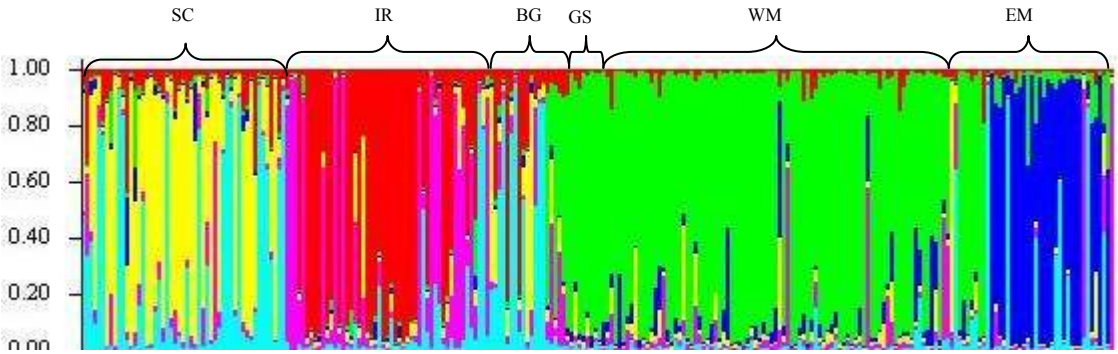


Figure 2.10. Bayesian individual assignment implement for K=7, without using a prior geographical areas; Length of burning period: 500000, No of MCMC Reps after burning: 1000000. 1: Scotland (SC), 2: Ireland (IR), 3: Biscay Gulf (BG), 4: Gibraltar Straight (GS), 5: Western Mediterranean (WM – Valencia), 6: East Mediterranean (EM - first 3 individuals from Sicily, second 6 individuals from Western Greece, the following 28 from Eastern Greece, last 5 individuals from Israel)



When the FCA multidimensional analysis (Figure 2.11) performed, it showed similar patterns with STRUCTURE analysis. The Atlantic Ocean was differentiated from the Mediterranean Sea. The Scotland and Biscay Gulf populations were clustered together and both populations were different with the Ireland one. Korinthiakos Gulf was differentiated from Ionian Sea and Gibraltar Strait belonged to the same cluster as Valencia and Sicily Strait. FCA analysis was consistent with STRUCTURE analysis regarding the subdivision between eastern and western Mediterranean Sea further east than the boundary across Sicily.

Performing the Bayesian method to estimate directly the probability that each locus is subject to selection, three loci showed evidence of natural selection; Ev37 and Sco11 under positive selection and KWM12a under balancing selection (Figure 2.12). There was a weak but not significant evidence of a third locus under positive selection (Sco28).

Sex-biased dispersal was tested and there was a small but significant F_{ST} and Relatedness differences between males and females (Table 2.7). However, the assignment index was not significant (p=0.23, assignment variance: p=0.58). When the analysis was based on the 23 neutral loci, the F_{ST} and Relatedness values were remained significant.

Table 2.7. Sex-biased analysis for striped dolphins individuals. (**: p<0.01, *: p<0.05). In parenthesis values based on 23 neutral loci.

	F_{IS}	F_{ST}	Relatedness
Females	-0.024 (-0.022)	0.052^{**} (0.053^{*})	0.107^{**} (0.102^{*})
Males	-0.016 (-0.020)	0.036^{**} (0.037^{*})	0.073^{**} (0.072^{*})
Overall	0.017 -0.019	0.043 (0.042)	0.083 (0.082)

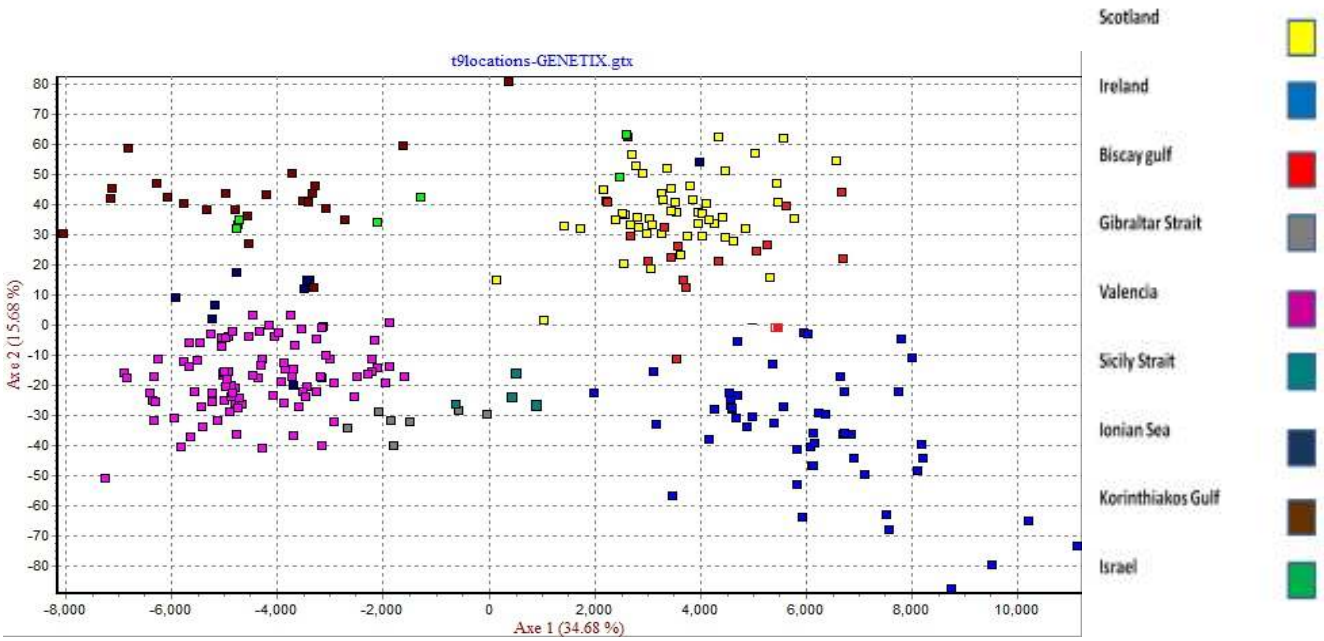


Figure 2.11. Factorial correspondence analysis for the nine putative populations of striped dolphin.

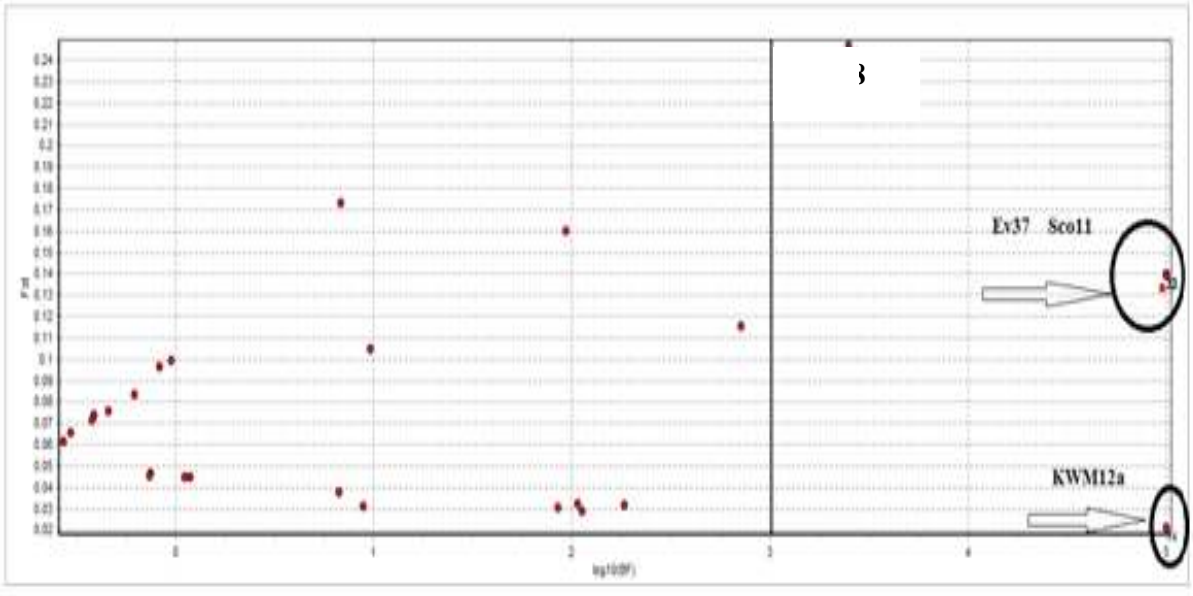


Figure 2.12. Graph of loci under natural selection (BayeScan v. 1.0). In circles the loci under positive selection (Ev37 and Sco11) and under balancing selection (KWM12a)

2.4 Discussion

In this study the bi-parental nuclear markers analysis indicates a fine population structure and genetic differentiation for contiguous populations of striped dolphin throughout the Mediterranean Sea and North Atlantic Ocean. The result was a cluster assignment of population that correspond to different habitat regions.

The North Atlantic Ocean appeared to constitute two differentiated groups. The southern part of Ireland was significant different from the northern part of Scotland (F_{ST}: 0.0238, $p<0.001$) with limited gene flow between those two regions. The analysis placed the population of Biscay Gulf into the same group with the Scotland population, and a relatively high rate of gene flow was estimated between those two regions. While a previous study also reported no genetic differentiation between Scotland and the Biscay Gulf (Bourret *et al.* 2007) the resolution of that study was very low, based on just 5 microsatellite, 41 samples from Biscay Gulf and 3 samples from Scotland. Garcia-Martinez *et al.* (1999) compared a sample of striped dolphins from the Atlantic Ocean (N=22), including Ireland (N=4) and the northeast Atlantic (N=8) at the mtDNA control region and also found no differentiation, but again the sample sizes were too small for confident inference. In this study, I use 26 microsatellite DNA loci, providing sufficient power to detect small differences, and therefore the proposed distinction between the Irish samples and those from Scotland through to Biscay is likely to be a robust result.

This genetic structure across the United Kingdom and Ireland may be is influenced by the ocean currents, in the context of predation. According to the average annual stream topography in the Atlantic Ocean there is a branching of the Gulf Stream and the hot spot of this drifting (or branching) is Ireland (Mann, 1967). One branch, also known as North Atlantic Current, curves north along the continental slope and eventually turning east. The

other branch, also known as Azores Current (Gould, 1985), flows southeast towards the Mid-Atlantic Ridge. This currents' movement is consistent with the existence of the differentiation between Scotland and Ireland. Samples from Biscay Gulf are shared alleles with both areas but mostly from Scotland. This mixing in Biscay Gulf is quite predictable as both North Atlantic Current and Azores Current are met in the Middle Eastern Atlantic (Mann, 1967).

Published studies show similar fine-scale differentiation for other marine mammals within Atlantic Ocean, but so far not for the same pattern, showing continuity from Scotland to Biscay, but differentiation between Biscay/ Scotland and Ireland. Natoli *et al.* (2005) using 9 microsatellite loci found significant genetic differentiation for bottlenose dolphin populations between Scotland (N=20) and the Biscay Gulf (N=35). Furthermore, Fontaine *et al.* (2007) found genetic differentiation between individuals of harbour porpoise from the Iberian peninsula and those further north in the Atlantic Ocean (Biscay Gulf) at 10 microsatellite loci. Roldan *et al.* (1998) found no differentiation between Ireland and Biscay, but did find differentiation suggesting a northern (Ireland and Biscay) and southern (Galacia) stock for European hake (*Merluccius merluccius*) based on allozyme loci.

Significant differentiation was observed between the North Atlantic Ocean and Mediterranean Sea. F_{ST} values between Atlantic and Mediterranean populations ranged from 0.063 to 0.038. Previous studies in striped dolphins revealed differentiation between those two areas as well. Garcia-Martinez *et al.* (1999) from a total of 63 different restriction sites that yielded 27 mtDNA haplotypes found no shared haplotypes between Atlantic Ocean (N=22) and Mediterranean Sea (N=76) striped dolphin populations. Also, Bourret *et al.* (2007) using five microsatellite loci found significant genetic differentiation

(F_{ST}=0.024, p<0.001) between Atlantic (N=45) and Mediterranean Sea (N=78) striped dolphins.

In this study the F_{ST} value between Biscay Gulf and Strait of Gibraltar was higher than the one between Valencia and Strait of Gibraltar (0.063; p<0.01 and 0.032; p>0.05 after Bonferroni correction respectively), though the Strait of Gibraltar sample was too small for robust comparisons. The five samples from the Strait of Gibraltar clustered with the Valencia population in the Structure analysis. It is possible that the represents a boundary to gene flow in this species, as has been proposed for other taxa. Garcia-Martinez *et al.* (1999) proposed that there is a very limited gene flow across the Strait of Gibraltar between the Portugues (N=5) and Balearic Sea (N=39) populations of striped dolphins, though again the sample sizes are too small for strong inference. Natoli *et al.* (2005) examined the genetic differentiation between bottlenose dolphin samples from Galicia (N=18) and Portugal (N=11) and those from Spain (N=26) and the Balearic Sea (N=5), and suggested that the Strait of Gibraltar represented a weak boundary between the Atlantic Ocean and Mediterranean Sea. Bremer *et al.* (1996) studied the genetic structure of Atlantic swordfish (*Xiphias gladius*) with nuclear genes (Idha and Calmodulin) and found that the mixing zone of Atlantic and Mediterranean Sea is restricted to small zone west of Gibraltar. Naciri *et al.* (1999) found genetic differentiation among populations of European sea bass (*Dicentrarchus labrax*) from either side of the Strait of Gibraltar based on allele-frequency variation at six microsatellite DNA loci.

Within the Mediterranean Sea my analyses showed significant differences between putative populations over relatively small geographical scales. The western Mediterranean was significantly differentiated from the eastern Mediterranean (the latter sample dominated by populations near Greece and Israel). Gaspari *et al.* (2007a) found significant differentiation between striped dolphin populations sampled from Spain (Balearic Sea)

and those from west of Italy (Ligurian sea). Furthermore, the authors found differentiation between samples from the northeastern side of Italy (Croatia, Puglia, Greece; N=22) and the western side of Italy (Ligurian Sea and Tuscany; N=112). Taken together with the results presented here, this suggests considerable fine-scale structure for this species in the eastern Mediterranean. Natoli *et al.* (2005) investigating bottlenose dolphin population structure found a strong boundary representing the western and eastern basins of Mediterranean Sea, separated by the Italian peninsula, similar to that suggested by Gaspari *et al.* (2007a) for the striped dolphin. This pattern has been demonstrated in other marine species. Tinti *et al.* (2002) studied cyt-b mtDNA sequences from sardines (*Sardina pilchardus*) in Mediterranean Sea and found differentiation between north eastern part of Italy and Balearic Sea. Moreover, Garoia *et al.* (2004) using 6 microsatellite loci found a significant differentiation of red mullet (*Mullus barbatus*) populations from either side of Italian peninsula. In those two studies, authors argue that the observed differentiation among populations within the Mediterranean Sea may be due to the complex history and different habitats of the species.

While these various studies suggest a boundary to gene flow either side of the Italian peninsula that may be relevant for a variety of marine species (as well as for striped dolphins according to Gaspari *et al.*, 2007a), the results presented here for the striped dolphin suggest differentiation between individuals from the Ionian Sea and Central Greece (Korinthiakos Gulf; F_{ST}=0.0436, $p<0.01$ after Bonferroni), and no significant differentiation between the Ionian Sea and the western Mediterranean (see Structure results). This suggests a boundary further east than the boundary across Sicily implied from the earlier studies. The Korinthiakos Gulf is semi-enclosed and consists of a unique body water due to its deep waters, the steep slopes along its coasts and the systematic occurrence of wind-driven upwelling currents (Lascaratos *et al.*, 1989). Although waters

from the Ionian Sea enter the gulf through the Rio-Antirio Strait, the Korinthiakos Gulf may provide an isolated habitat. Frantzis and Herzing (2002) in a study of Delphinidae species sightings and abundance in the Korinthiakos Gulf (Figure 2.13) suggest that striped dolphin distribution and abundance in the gulf may be due to philopatry. In addition, these authors point out the different pigmentation observed in the local population (with the pale gray flank field absent or limited, and instead a pattern similar in shape and colour to the hourglass pattern on the sides of common dolphins). While this may be a sign of introgression with common dolphins, there are no further data in support of this, and the main point is that the morphology also distinguishes this local population.

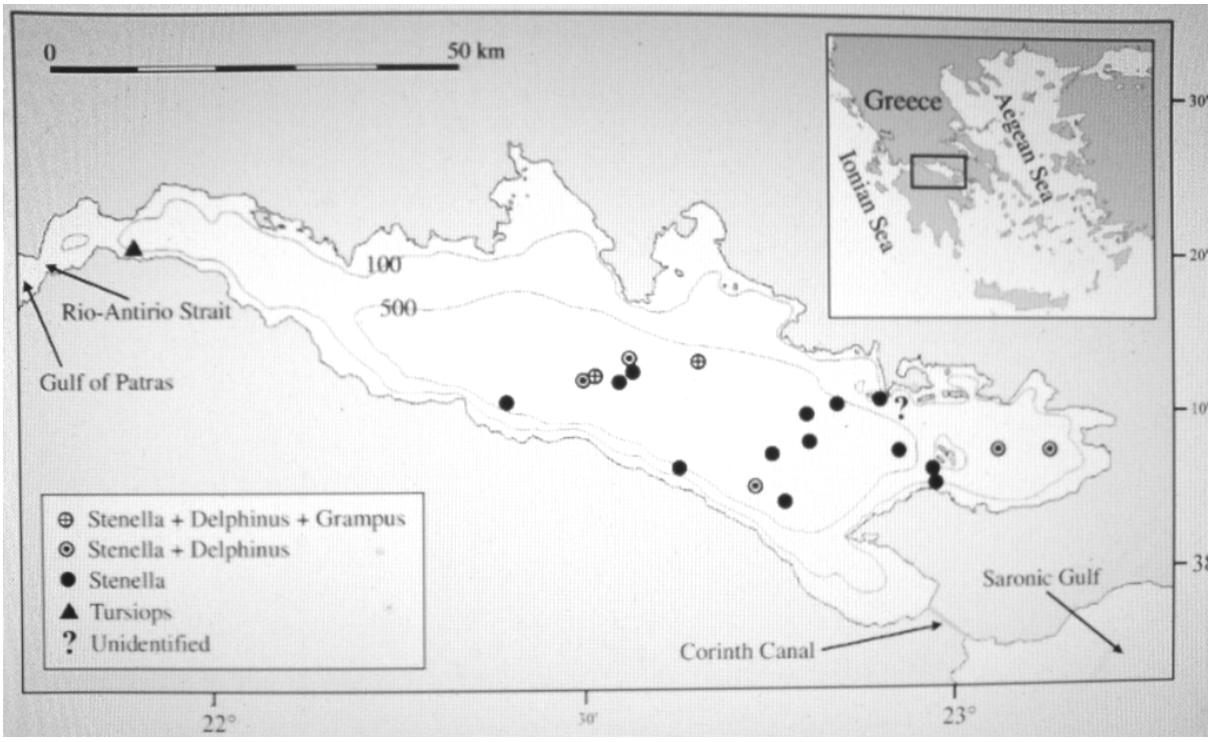


Figure 2.13. Species composition of the sightings in the Korinthiakos Gulf (Frantzis and Herzing, 2002)

In our survey while collecting samples for genetic analyses, the distribution and abundance of striped dolphin was similar to that found by Frantzis and Herzing (2002), and sightings were only in the eastern part of the Gulf (unpublished data). Personal

observations with regards the different pigmentation was also recorded. The later observation along with the F_{ST} value between those two areas may suggest that the Rio-Antirio Strait (2.4 km with a high maritime clog) seems to provide a boundary between the Ionian Sea and Korinthiakos Gulf for striped dolphins. This may be in addition to a boundary that reflects differentiation either side of Italy (Gaspari et al. 2007a). Studies of marine fishes have also suggested the differentiation of populations within the Gulf. For example, Klossa-Kilia *et al.* (2007) using control region mtDNA sequences found differences between *Atherina boyeri* populations from the eastern Korinthiakos Gulf and lagoon samples from the Rio-Antirio region suggesting two different sibling species or at least subspecies.

Genetic differentiation indices based on F_{ST} were in overall accordance with the results from STRUCTURE cluster analyses, convincingly separating the Ireland population from Scotland and the Biscay Gulf, and also distinguishing Greece from the Ionian Sea and from the western Mediterranean. However, the Ionian Sea samples group with the western Mediterranean, which could either be an effect of the small sample size from the Ionian Sea, or reflect a boundary closer to Greece than to Sicily dividing the basins of the Mediterranean for this species. The small degree of genetic differentiation between Strait of Gibraltar and Valencia may be an effect of the mixing of individuals. Similar geographical mixing of clusters may also contribute to the low differentiation between Ionian Sea samples and of those from Strait of Sicily area, though again, sample sizes are small.

The same clustering pattern was reflected in the FCA analysis, which also shows the high genetic diversity of the Atlantic Ocean populations. The isolation by distance analysis revealed significant differences only in the Atlantic Ocean. The overall average value obtained of 576 km in Atlantic is within the range of a highly mobile species such as the

striped dolphin. The lack of isolation by distance in Mediterranean Sea may reflect the importance of local boundaries to gene flow, especially in the Eastern Mediterranean. A small but significant effect of sex-biased dispersal was found suggesting greater male dispersal. This is consistent with Gaspari *et al*. (2007a) who found a significant association among adult female kin in small social groups off the western part of Italy, and found evidence that females are more philopatric than males.

This study reveals a complex pattern of genetic structure with the existence of a greater degree of genetic structure than anticipated from previous studies or from a species with such high dispersal potential. This study revealed an unexpected pattern of differentiation between Ireland and both the Biscay Gulf and Scotland. An apparent eastward shift of the boundary between the western and eastern Mediterranean Sea for this species compared to earlier studies for various taxa. These findings reveal a cryptic population structure and therefore have important implications for the effective conservation and management for this species.

CHAPTER 3

Heterozygosity Fitness Correlations in striped and common dolphins revealed by neutral markers

3.1 Introduction

Heterozygosity–fitness correlations have been studied in a variety of natural populations (Ledig *et al.*, 1983; Koehn and Gaffney, 1984; Leary *et al.*, 1984; Mitton and Grand, 1984; Zouros and Foltz, 1987; Ferguson, 1992; Bierne *et al.*, 2000;) and for many different fitness traits measures including birth weight (Coltman *et al.*, 1998), parasite load (Rijks *et al.*, 2008), longevity (Coltman *et al.*, 1999), reproductive success (Slate *et al.*, 2000), aggressiveness (Hoffman *et al.*, 2007), song complexity (Marshall *et al.*, 2003), and territory size (Seddon *et al.*, 2004). Empirical studies of Heterozygosity-fitness correlations are interpreted according to three main hypotheses (after David, 1998); the *direct effect* hypothesis (heterozygote advantage due to overdominance at the specific locus scored), the *local effect* hypothesis (heterozygote advantage detected at marker loci that are closely linked to fitness loci) and the *general effect* hypothesis (heterozygote advantage due to a high level of heterozygosity in the genome as a whole). These hypotheses assume a direct relationship between diversity and fitness.

The *direct effect* hypothesis has been proposed to account for associations between functional loci (e.g. allozyme loci and the Major Histocompatibility loci) and fitness traits, known as direct selection (David, 1998). There are various examples in the litereature, such as MHC heterozygote superiority against multiple parasites in natural population of the water vole, *Arcivola terrestris*, (Oliver *et al.*, 2009); growth effect in Glanville fritillary butterfly populations, *Melitaea cinxia*, in relation to the allelic composition of a glycolytic enzyme (Hanski and Saccheri, 2006); and the positive correlation between growth rate and variation at general non-specific proteins (*Nsp1 – Nsp2*) in a scallop (*Placopecten magellanicus*) population (Pogson and Zouros, 1994).

The *local effect* and *general effect* hypotheses have been proposed to correlate neutral markers and fitness traits. The *local effect* hypothesis associates the apparent increase of fitness to increasing heterozygosity at marker loci, when those marker loci are in linkage disequilibrium (LD) with loci affecting fitness. single locus effect show evidence of hookworm resistance on California sea lion pups (Acevedo-Whitehouse *et al.*, 2006), significant single locus Heterozygosity fitness correlations were observed in different fitness traits in a threespine stickleback population (Lieutenant-Gosselin and Bernatchez, 2006) and in a male Alpine ibex, *Capra ibex*, population (von Hardenberg *et al.*, 2007).

The *general effect* hypothesis associates the fitness cost of homozygosity at loci throughout the whole genome, that is the marker loci and loci affecting fitness are in identity disequilibrium (ID). Studies that claim general effect, suggest inbreeding depression in order to explain Heterozygosity fitness correlations, as for example Rijks *et al.* (2008) using 27 microsatellite loci found that homozygosity predicts higher hookworm burdens in young harbour seal pups (*Phoca vitulina*) due to inbreeding.

In recent years microsatellite markers have become the marker of choice in many studies due to high heterozygosity levels in most eukaryote genomes. Thus, microsatellites are the most commonly used neutral markers in studies of heterozygosity-fitness correlations. Many authors have proposed other measures of variation instead of the mean level of individual heterozygosity, all of which aim to produce a measure that correlates most strongly with the inbreeding coefficient *F*. Those estimates are the mean d^2, a measure based on microsatellite allelic distance within an individual (Coulson *et al.*, 1998), the *Standardized Heterozygosity*, a measure based on the proportion of heterozygous and mean heterozygosity at a locus (Coltman *et al.*, 1999), the *Internal Relatedness*, a measure based on influence of rare alleles (Amos *et al.*, 2001) and *HL*, a

measure based on homozygosity by locus (Aparicio *et al.*, 2006). Each one of these measurements have been proposed for different approaches and depend on the nature of the studied population, the genotyped loci, the locus diversity being scored, and the range of inbred or outbread individuals. For example, mean d^2 in isolated populations reflects founding alleles and mutations since founding, but in situations involving population admixture reflects differences due to stepwise mutation since coalescence. On the other hand, in homogeneous populations, *IR* and *HL* reflects a better measurement due to genetic relatedness between inbreeding individuals. However, most studies focus on Multilocus Heterozygosity and d^2 measurements for fitness correlations (Hedrick *et al.*, 2001; Hansson *et al.*, 2001). Slate and Pemberton (2002) in a study of red deer using a large panel of 71 loci show that Multilocus heterozygosity and not mean d^2 was associated with fitness related traits, possibly due to some loci with high mutation rates or non-stepwise mutation events. It may also be the case that the influence of marker mutation on genotype-fitness correlations is due the inbreeding history of each studied species (Tsitrone *et al.*, 2001).

Studies on marine species Heterozygosity-fitness correlations are scarce in the literature. One reason is the logistical difficulties associated with obtaining the relevant data. However, significant correlations have been reported. In a study of Amos *et al.* (2001), three marine species (gray seal – *Halichoerus grypus*, long-finned pilot whale – *Globicephala melas* and wandering albatross – *Diomedea exulans*) were tested for associations between heterozygosity and fitness and a significant correlation found between lifetime success and standardized heterozygosity. Coltman *et al.* (1998) show that homozygous individuals, based on mean d^2 measurement, of harbour seal pups (*Phoca vitulina*) had a higher mortality risk independent of birth weight. Acevedo-Whitehouse *et al.* (2006) show that homozygous individuals of California sea lion pups,

at a specific single microsatellite DNA locus based on *IR* measures, are strongly predisposed to anaemia (presumably due to linkage between that locus and a functional locus associated with that trait). Contextual to that, Rijks *et al.* (2008) found correlations between homozygosity and susceptibility to parasite infections in young harbour seal pups. Acevedo-Whitehouse *et al.* (2003) showed that heterozygous individuals of California sea lions are less likely to be infected by a range of parasites. Furthermore, Hoffman *et al.* (2010) reported a positive correlation between canine size and heterozygosity, and on this basis the authors suggest that other structures (e.g. tympano-periotic bone & otoliths) may be used to explore links between genetic variation and important life-history traits in free-ranging vertebrate populations.

The goal of this study is to investigate Heterozygosity-fitness correlations in two striped dolphin and two common dolphin populations with regards to parasite burden of lungworms and stomach digeneas.

Lungworm nematodes are quite common in the delphinidae respiratory system (Raga and Carbonell, 1985). Although the life cycle of most of the respiratory nematodes is still unknown, they can cause almost total occlusion of bronchi and bronchioles (Raga *et al.*, 1987b; Clausen and Andersen, 1988). With respect to stomach parasites, the most common for striped dolphins was the gastric digenean *Pholeter gastrophilus* which has been reported in at least 17 cetacean species worldwide (Aznar *et al.*, 1992; Raga, 1994). This species burrows into the stomach wall within the submucosal fibrotic nodules and is associated with fatal diseases (Woodart *et al.*, 1969; Migaki *et al.*, 1971; Howard *et al.*, 1983). Therefore, the level of lungworm and stomach digenean infestation is likely to be associated with fitness and appropriate for use in Heterozygosity Fitness Correlations.

The fine geographical scale of population genetic structure in the striped dolphin (Chapter 2; Bourret *et al.*, 2007; Gaspari *et al.,*2007a) and the lack of strong population genetic structuring of common dolphin in the eastern North Atlantic (Natoli *et al.*, 2006; Mirimin *et al.*, 2009) represent two different ecologies and demographic histories. The interpretation of Heterozygosity-fitness correlations from this study will be considered in this context, though just two species provides limited inference, and this is not a primary objective of the study.

Population structure affects local effective population size, and thereby affects the level of inbreeding. I test the hypothesis that local adaptation of the host may play a role with regards to heterozygosity fitness correlations. Further than that, I test the hypothesis that heterozygosity fitness correlation is due to general-effect.

Investigation of the association between heterozygosity and pathogen load may be expected to show a negative correlation. However, investigation of the single locus effect will allow the assessment of selection, assuming that the locus is in linkage disequilibrium with a gene under balancing selection. Therefore a further objective of this study is to test the hypothesis that pathogen load is associated with single locus effect.

3.2 *Materials and Methods*

3.2.1 Sample collection

115 individuals of striped dolphin were collected during the period 1990 – 2008 and 110 individuals of common dolphin were collected during the same period. The putative locations of those samples are Western Mediterranean Sea (Balearic Sea) and Ireland for striped dolphin and Ireland for common dolphin samples (Figure 3.1). All samples were obtained from adult stranded animals

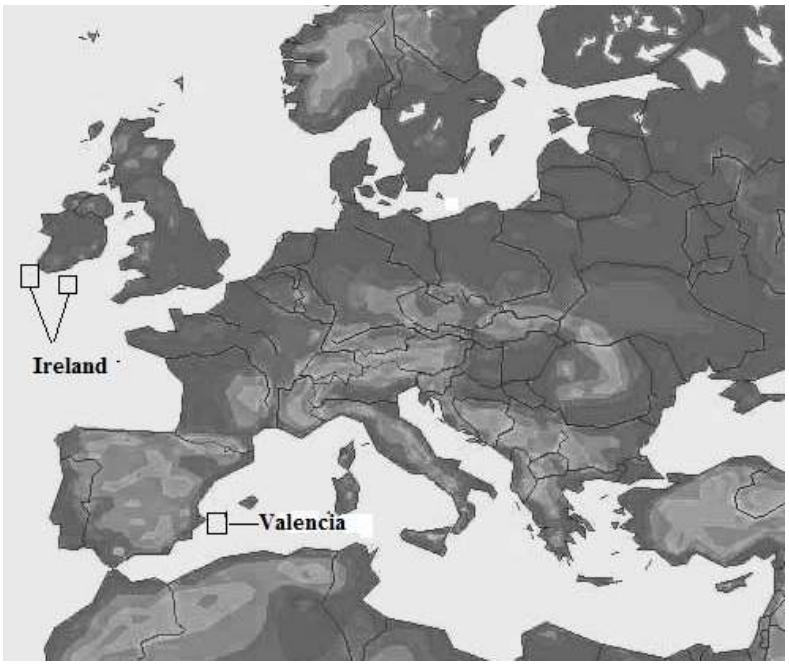


Figure 3.1. Sample sites of striped dolphin and common dolphin.

Most of the striped dolphins used in the study from the Western Mediterranean Sea stranded in association with the morbillivirus epizootic during the years 1990-2007. Individuals were transferred to the laboratory where necropsy and anatomy were carried out immediately or alternative were stored in -20^{o}C. A unique register for every individual was detailed according to the date of sight. Individual necropsies were carried

out according to Pugliares *et al.* (2007). Sex was identified visually or using genetic markers (see below). Internal organs were separated and stored in individual plastic bags in -20^{o}C. Age data wasn't available for the majority of the samples so it was excluded from the analysis.

3.2.2 Parasite extraction and counting

Parasites were extracted from the lungs and stomach of the striped dolphin and from the lungs of the common dolphin. During gross necropsy lung and stomach tissues were examined visually for the presence of parasites and associated lesions, and lesion description were registered. Parasites were cleaned in an isotonic buffer and then were stored in 70% ethanol.

i) Lung examination

Both lungs were used for parasite infestation. Each lung was weighed to the closest milligram. The lung was opened starting always from the main bronchus of the upper lobe which is connected to the trachea, and then the duct of each bronchioles and alveoli were followed through to the end of the bottom lobe (Figure 3.2).

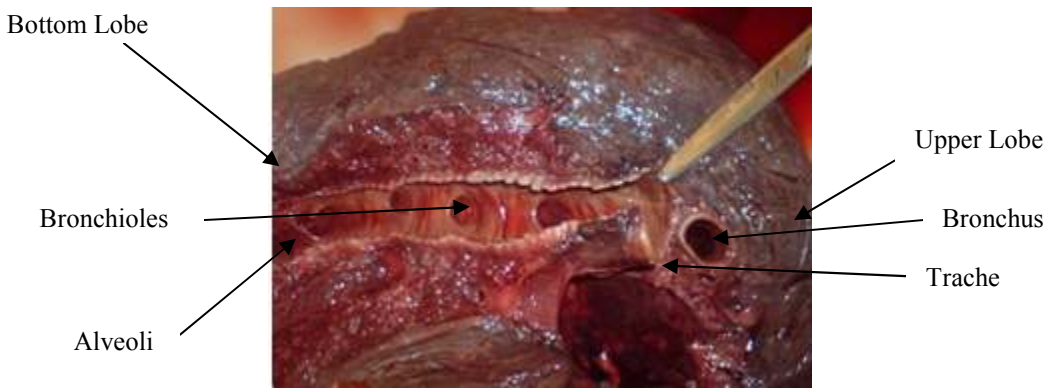


Figure 3.2.: 1 - Upper lobe, 2 - Trachea, 3- Bronchus, 4 - Bronchioles, 5 - Alveoli, 6 - Bottom lobe

Only whole parasites or the parasites' tail were collected, and then stored in saline buffer. Saline buffer is a water-based salt solution commonly used in biological research. The buffer helps to maintain a constant pH and it is an isotonic and not-toxic solution (Sambrook *et al.*, 1989). After cleaning with the isotonic buffer, parasites from each lung were preserved in 70% alcohol. After the gross examination, lungs were washed out on a 0.2 mm sifter and any parasites (whole or tails) obtained were collected. All parasites were examined in a stereoscope for species identification. Further to that, 10% of the total number of parasites were prepared and screened in a microscope to ensure the consistency of species identification. A *Petri dish* with divided areas was used for the parasite counting. Parasites of each lung were combined for the total individual lung-parasite burden. Parasites were stored in 70% ethanol for potential back up analysis.

ii) Stomach examination

All parts of the stomach were examined for parasite infestation. Each of the stomach's chambers (Fore stomach, mechanical stomach, pyloric stomach, ambula duodenum) along with the connecting channel were isolated and weighted (Figure 3.3).

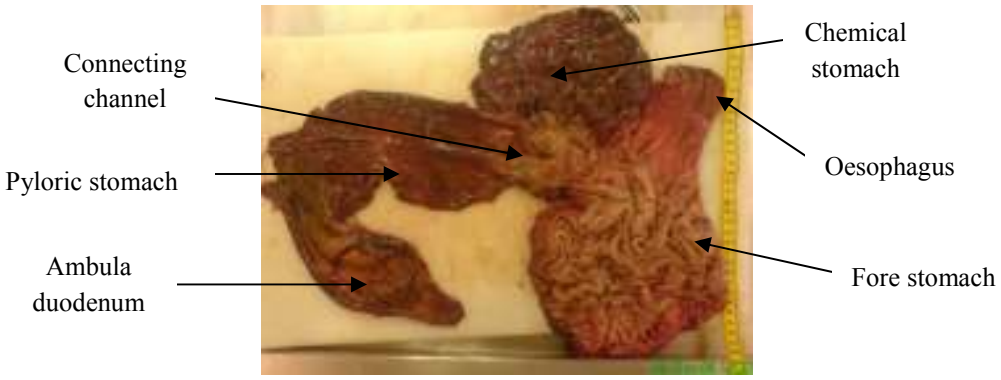


Figure 3.3.: Oesophaegus, 2: Fore stomach, 3: Chemical stomach, 4: connecting channel, 5: Pyloric stomach, 6: ambula duodenum.

The Fore Stomach or mechanical stomach was opened following the main line from the upper lobe of oesophagus to the bottom lobe. The inside of the chamber was examined manually for parasites and washed out on a 0.2 mm sifter. Parasites were collected and preserved in 70% ethanol.

The Main Stomach or chemical stomach, also known as glandular compartment, was opened following a round line from the connected point of the fore stomach to the connecting channel. Manual inspection was used to detect any lesions due to *Pholeter gastrophilus* infection. If any lesions were detected they were labelled and photographed (Figure 3.4). The chamber was then washed out on a 0.2 mm sifter and parasites were collected and preserved in 70% alcohol.

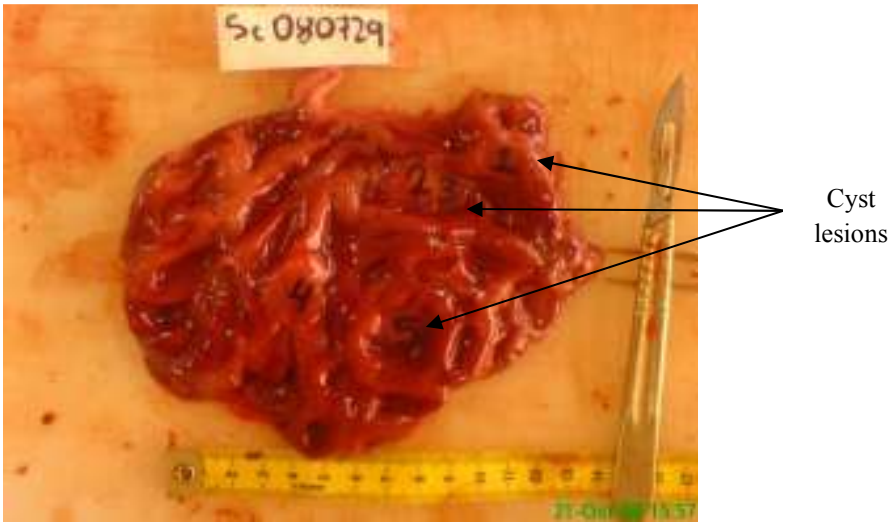


Figure 3.4. Cyst lesions on the Mechanical stomach surface

The same procedure was followed for the connecting channel, pyloric stomach and ambula duodenum. Each part was examined and washed out on a 0.2 mm shifter and parasites were collected and stored in 70% ethanol.

Parasites were screened using a stereoscope for species identification, and then 10% of the parasites were prepared and examined in microscope for species identification consistency.

3.2.3 DNA extraction and PCR amplification

Skin tissue and muscle tissue were used for DNA extraction. Tissues samples were preserved in 20% DMSO NaCl 5M or 70% ethanol. DNA was extracted following either the standard phenol/chloroform extraction protocol (Sambrook *et al.*, 1989) or a standard salt extraction protocol (see Aljanabi and Martinez, 1997).

A total of 32 universal DNA microsatellites markers were tested and optimized from which 26 polymorphic loci were used in the analysis for striped dolphins (see Chapter 2) and 18 were used for common dolphins. A multiplex PCR Kit (Qiagen) was used for the DNA amplification. The primers were divided into Primer Mix Groups (Chapter 2 for striped; Appendix, Table 6.2 for common dolphin) according to size range and florescent primer's pigment. The PCR cycling profile was: 95^{o}C for 15'; 30 cycles of 95^{o}C for 1', annealing temperature for 30'' and 72^{o}C for 30''; 72^{o}C for 15'. Amplified DNA products were screened on an ABI 3730 DNA Analyser (Applied Biosystems). Each specimen's alleles were scored by the STRand software v.2.0 (Toonen and Hughes, 2001). Sex was determined using the primers P15EZ, P23EZ for the *Zfx*/*Zfy* gene (Aasen and Medrano, 1990) and Y53-3c, Y53-3d for the *SRY* gene (Gilson *et al.*, 1998).

3.2.4 Genetic Diversity

Four alternative ways were used to calculate genetic diversity: individual *mean multilocus heterozygosity*, *mean* d^2, internal relatedness *IR* and the homosygosity by loci index *HL*. Individual *mean multilocus heterozygosity* was calculated across all scored

loci. If an individual was homozygous at a locus it was scored as "0" and if it was heterozygous was scored as "1". Then the mean across all scored loci was taken. *Mean* d^2 was calculated as the squared distance in repeat units between the two alleles of a scored locus using the following formula

$$mean\ d^2 = \sum_{i=1}^{n} \frac{(i_a - i_b)^2}{n}$$

where i_a and i_b are the lengths in repeat units of alleles a and b at locus i, and n is the total number of loci at which an individual was scored. *Internal relatedness*, a method based on Queller and Goodnight's (1989) measure of genetic relatedness between two groups or individuals, compares two alleles rather than two pairs of alleles using the formula

$$IR = {(2H - \sum f_i)}\Big/{(2N - \sum f_i)}$$

where *H* is the number of loci that are homozygous, *N* is the number of loci and f_i is the frequency of the *i* allele contained in the genotype. *Homozygosity by loci index (HL)* is a multilocus homozygosity measure that weights loci by their variability:

$$HL = {\sum E_h}\Big/{\left(\sum E_h - \sum E_j\right)}$$

where E_h and E_j are the expected heterozygosities of the loci that an individual bears in homozygosis (h) and in heterozygosis (j) respectively.

3.2.5 Statistical analysis

Parasite count intensity parameters (skewness, mean, median, exact confidence intervals) were calculated in Quantitative Parasitology v. 3.0 (Rozsa *et al.*, 2000). A Shapiro – Wilk normality test was performed to determine if parasites count distributions

were normal. All loci were tested for departures from Hardy-Weinberg equilibrium and all locus pairs were tested for linkage disequilibrium using the software Genepop v. 3.4 (Raymond and Rousset, 1995). The potential presence of null alleles was assessed using the software Micro-Checker v. 2.2.3 (van Oosterhout *et al.*, 2004). The software Bayescan v. 1.0 (Foll and Gaggioti 2008) was used to identify candidate loci under natural selection. Mean Multilocus heterozygosity and inbreeding measures were performed using the software IRmacroN v. 4.0, an EXCEL macro written in Visual Basic by W. Amos(2001) (www.zoo.cam.ac.uk/zoostaff/amos /#Computerprograms).

The relationship between heterozygosity and parasite burden was first assessed by comparing the mean levels of genetic diversity of all uninfected individuals to that of all infected ones. Linear regression was then used to investigate possible relationships between measures of genetic diversity and parasite burden using SPSS v. 15.0. The association between genetic diversity and parasite load was calculated in a generalized linear model (GLM) controlling for sex. The response variables were independently defined as a binary response in each model (female:0; male:1) and modelled using a binomial error structure.

In addition to using a direct test for the impact of marker, the method of Amos and Acevedo-Whitehouse (2009) was performed. This method is based on arranging the data to maximize the strength of association between genotype and fitness. At each locus, genotypes with above average of fitness scores are classified as “low risk” and below average fitness as “high risk”. The size of the resulting test statistic is then assessed by randomizing the genotypes repeating the process many times. The repetition was set to 10,000.

3.3 Results

3.3.1 Striped dolphin

3.3.1.1 Parasites

115 samples of striped dolphin were examined for lung parasites. Only a single species was found during the gross lung examination, the nematode *Skrjabinalius guevaraii* (Nematoda: Pseudaliidae). Forty nine individuals were uninfected and sixty six were infected. The range of infestation was 0 - 2100 worms. After the normality test of Shapiro-Wilk of the infected animals, 3 individuals were excluded from the analysis with total infestation 430, 450 and 2100 worms respectively. Individuals were divided into 4 categories according the level of infestation (Table 3.1). Those 4 categories are None, Low (1-20), Medium (21-75) and High (76 – 370). The 4 categories were created using the exact confidence limits for the median intensity (Table 3.2). Parasite count intensity parameters (skewness, mean, median, exact confidence intervals) are shown in Table 3.4. Lungworm counts fit a negative binomial distribution (skewness measure=0.159, with respect to the negative binomial, $p<0.05$).

Table 3.1. Number of females and males according to level of infestation

Level of Infestation	Females	Males	Total number of individuals
None	20	29	49
Low (1 – 20)	10	14	24
Medium (21 – 75)	9	10	19
High (76 – 370)	9	11	20
	48	64	112

Table 3.2. Striped dolphin parasite count intensity parameters

Variance/mean ratio	189.90	
Mean intensity	83.43	
Median intensity	36	99.5% exact CI:20-75
Bootstrap Confidence for mean crowding	235.86	95% CI: 159.30 – 325.56

55 samples of striped dolphin were examined for stomach parasites. During the gross stomach examination, 5 different species were found; *Pholeter gastrophilus*, *Anisakis sp*, *Tetraphyllidean plerocercoids*, *Brachycladium atlanticum*, *Tetrabothrium fosteri*. The most common parasite was the digenean *Pholeter gastrophilus* in every chamber with a higher preference in the chemical stomach (Table 3.3). 11 individuals were uninfected and 49 were infected. The range of infestation was 0 – 122. The total number of parasites counts fit a negative binomial distribution (skewness measure=0.009, with respect to the negative binomial, $p<0.05$). The parasite count intensity parameters (skewness, mean, median, exact confidence intervals) are shown in Table 3.4. 53 individuals were infected with lungworms and stomach digeneans (combined).

Table 3.3. Prevalence of stomach parasites in infected animals

Species	**Prevalence**
Pholeter gastrophilus	100%
Anisakis sp	10.2%
Tetraphyllidean plerocercoids	8.16%
Brachycladium atlanticum	14.2%
Tetrabothrium fosteri	20.4%

Table 3.4. Striped dolphin stomach parasite count intensity parameters.

Variance/mean ratio	42.74	
Mean intensity	24.80	
Median intensity	11.5	99.3% exact CI:7 – 16
Bootstrap Confidence for mean crowding	235.86	95% CI: 43.51 – 83.51

3.3.12 Genetic diversity

The number of alleles ranged from 3 (Sco11) to 26 (MK5) with expected heterozygosities 0.3936 (Sco11) to 1 (D14, D18 and D28). The mean H_{OBS} was 0.7746 and H_{EXP} was 0.7468 across all loci. Three loci showed evidence of natural selection (see Chapter 2 for details).

The range of values for mean Heterozygosity, IR, mean d^2 and HL are shown in Table 3.5. For the sample as a whole, t-test comparisons of the mean measures of genetic diversity did not vary significantly between uninfected and infected animals (Table 3.6).

Table 3.5. Mean values of *mean Heterozygosity*, *IR*, *mean* d^2 and *HL* across all loci, neutral loci and loci under positive selection.

	All loci	Neutral loci	Under positive selection
Mean Heterozygosity	0.7913	0.7860	0.7984
IR	-0.0435	-0.0416	-0.0654
Mean d^2	0.1649	0.1718	0.1132
HL	0.1703	0.1721	0.1990

Table 3.6. Mean values of *mean Heterozygosity*, *IR*, *mean* d^2 and *HL* for infected and uninfected individuals and t-test *p* values.

	All loci	t-test p	Neutral loci	t-test p	Under positive selection	t-test p
mean $H_{uninfected}$	0.800	0.181	0.793	0.304	0.836	0.190
mean $H_{infected}$	0.784		0.780		0.7702	
mean $IR_{uninfected}$	-0.043	0.978	-0.042	0.888	-0.053	0.743
mean $IR_{infected}$	-0.043		-0.040		-0.074	
mean $d^2_{uninfected}$	0.167	0.546	0.172	0.820	0.128	0.275
mean $d^2_{infected}$	0.162		0.170		0.101	
mean $HL_{uninfected}$	0.161	0.185	0.165	0.322	0.161	0.188
mean $HL_{infected}$	0.177		0.177		0.227	

3.3.1.3 Heterozygosity – parasite load associations

The linear regression analysis between lungworm, stomach and total (combined types) parasite burden, and levels of genetic diversity showed no significant correlations ($p>0.05$) for any combination of loci (all loci, neutral loci, loci under positive selection). Linear regression between 3 levels of infestation of lungworm, stomach and total parasite burden, and levels of genetic diversity did not show any correlation ($p>0.05$, after Bonferroni correction). In particular, only linear regression between the 3 different levels of lungworm infestation and the levels of genetic diversity, were found to be significant ($p<0.05$) (Table 3.7) but, not after Bonferroni correction. R^2 values were relatively low with a range between 0.13 and 0.19.

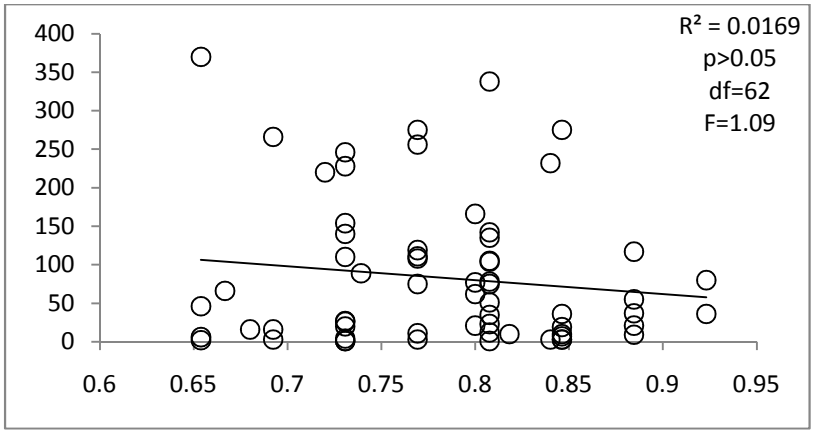


Figure 3.5. Mean heterozygosity (x axis) – Lungworm burden (y axis)

(all loci – 63 infected striped dolphins)

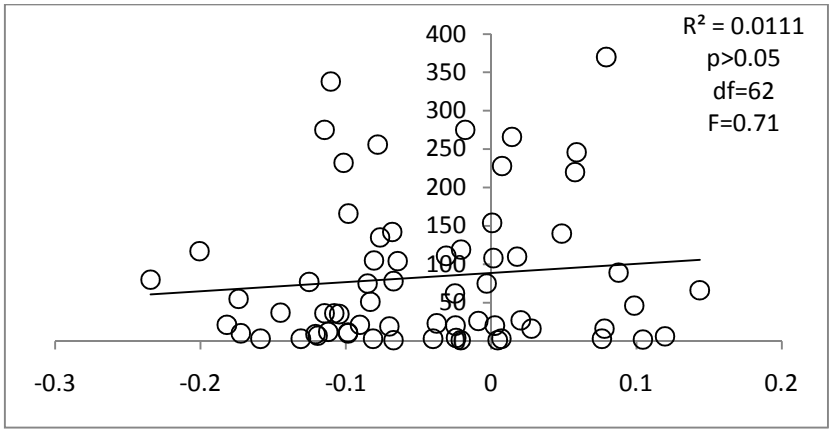


Figure 3.6. IR (x axis) – Lungworm burden (y axis)

(all loci – 63 infected striped dolphins)

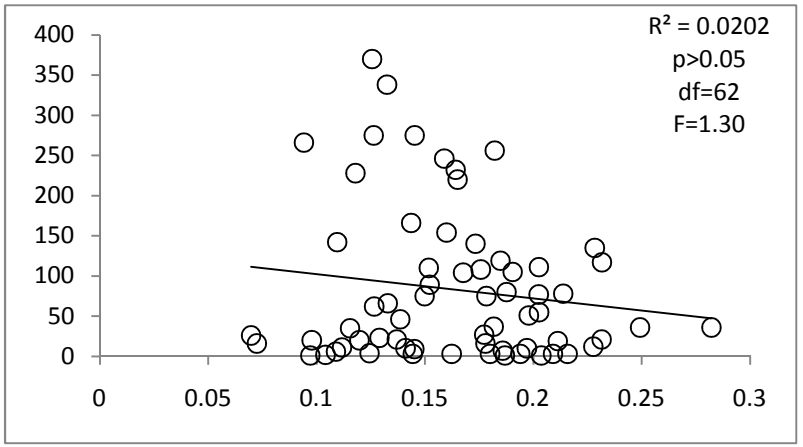


Figure 3.7. mean d^2 (x axis) – Lungworm burden (y axis)

(all loci – 63 infected striped dolphins)

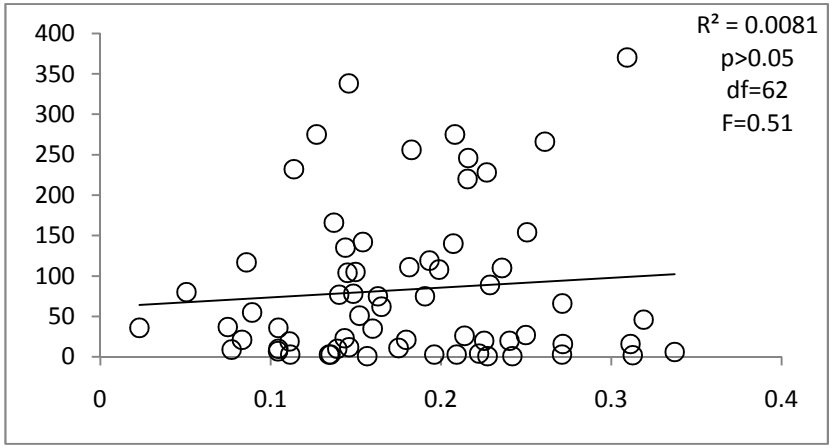


Figure 3.8. HL (x axis) – Lungworm burden (y axis)

(all loci – 63 infected striped dolphins)

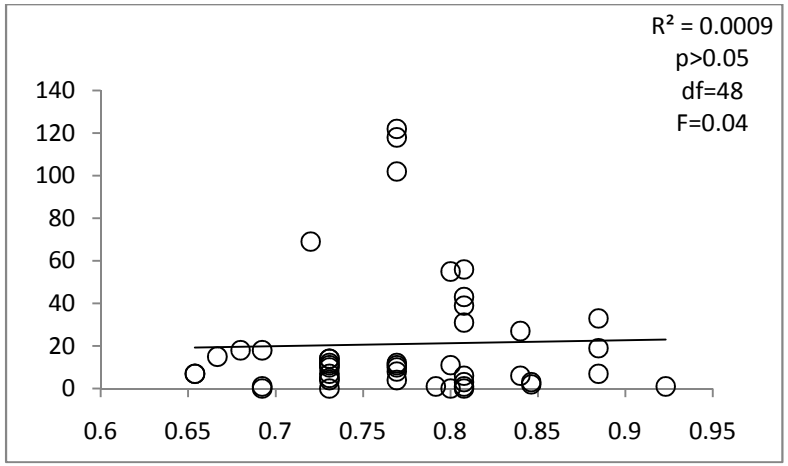


Figure 3.9. Mean heterozygosity (x axis) – stomach digeneans burden (y axis)

(all loci – 49 infected striped dolphins)

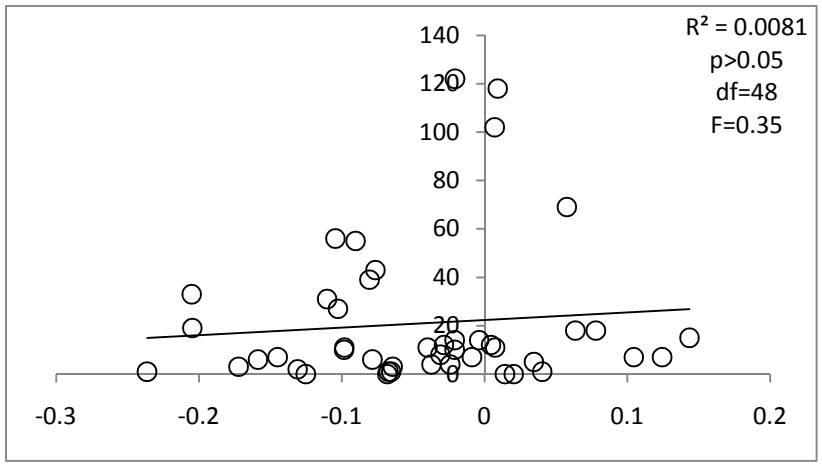


Figure 3.10. IR (x axis) – stomach digeneans burden (y axis)

(all loci – 49 infected striped dolphins)

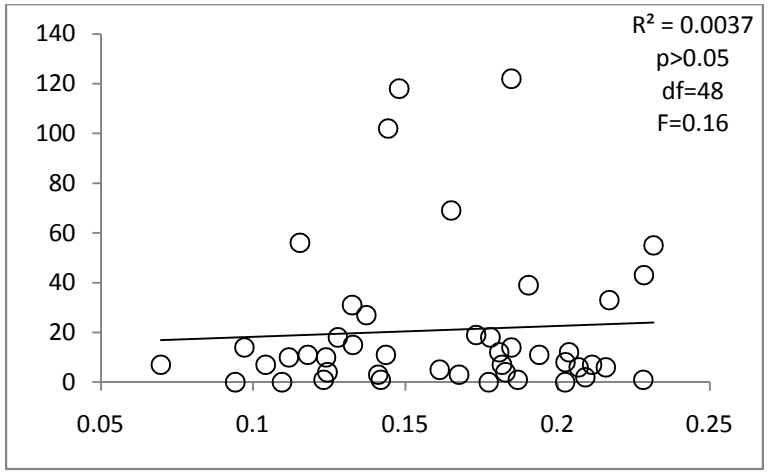


Figure 3.11. mean d^2 (x axis) – stomach digenean burden (y axis)

(all loci – 49 infected striped dolphins)

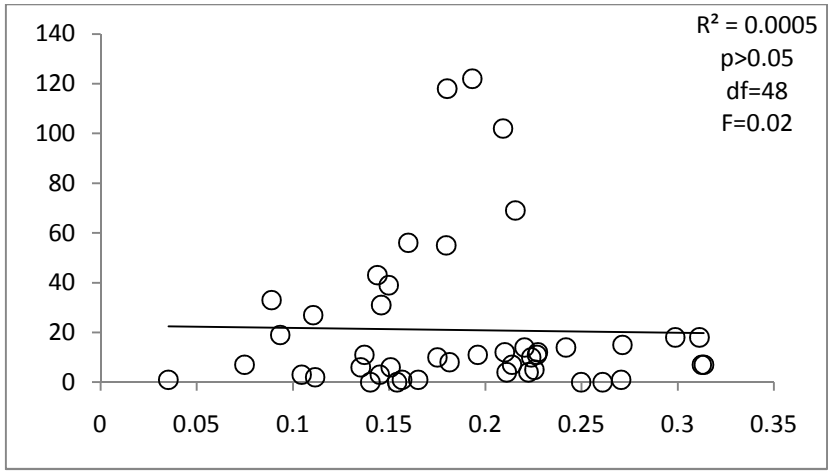


Figure 3.12. HL (x axis) – stomach digenean burden (y axis)

(all loci – 49 infected striped dolphins)

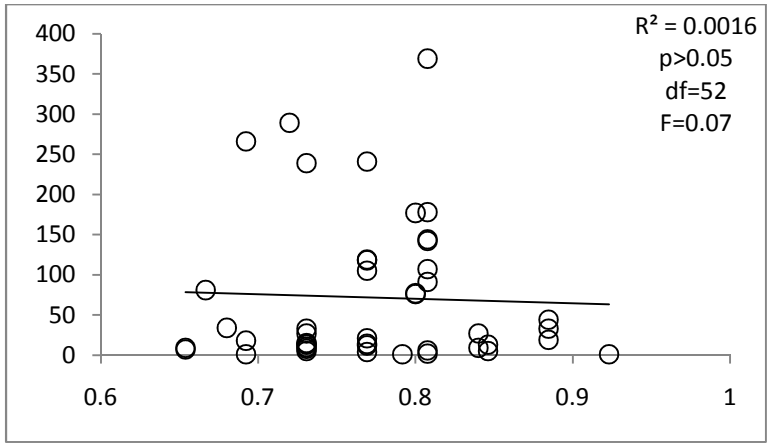


Figure 3.13. Mean heterozygosity (x axis) – Total burden (y axis)

(all loci – 53 infected striped dolphins)

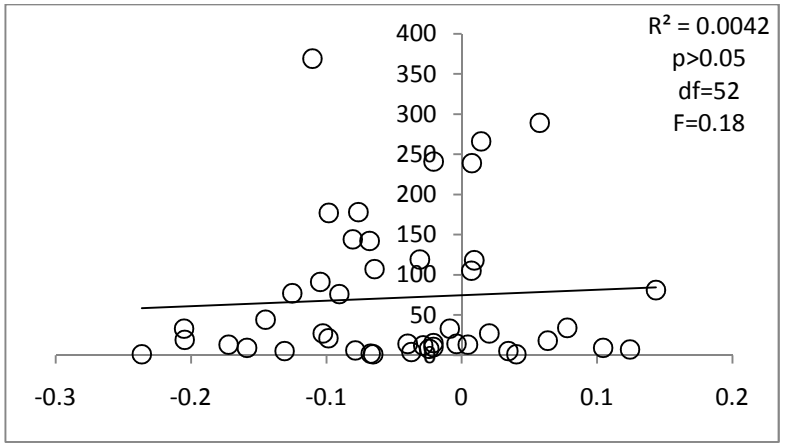


Figure 3.14. IR (x axis) –Total burden (y axis)

(all loci – 53 infected striped dolphins)

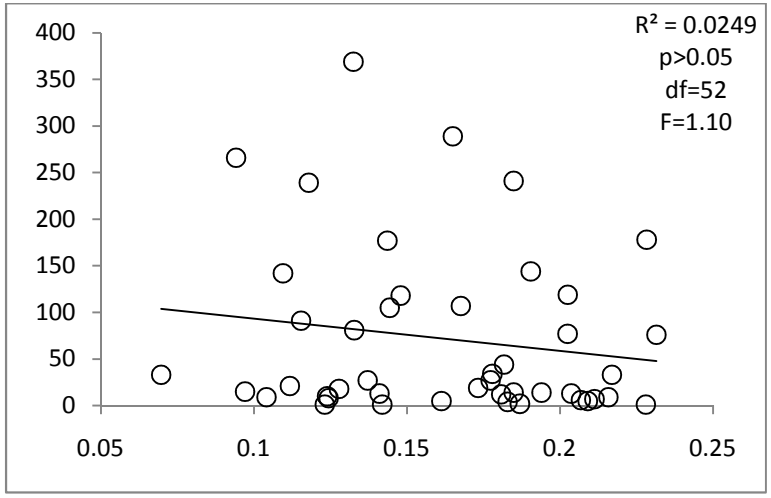


Figure 3.15. mean d^2 (x axis) – Total burden (y axis)

(all loci – 53 infected striped dolphins)

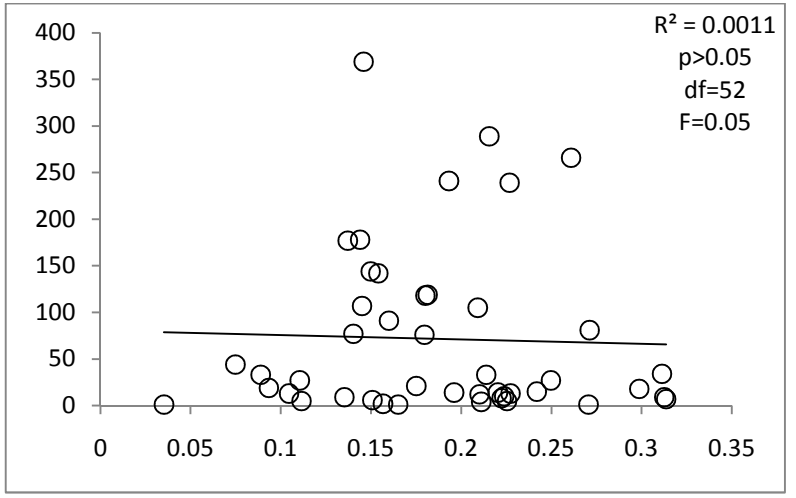


Figure 3.16. HL (x axis) – Total burden (y axis)

(all loci – 53 infected striped dolphins)

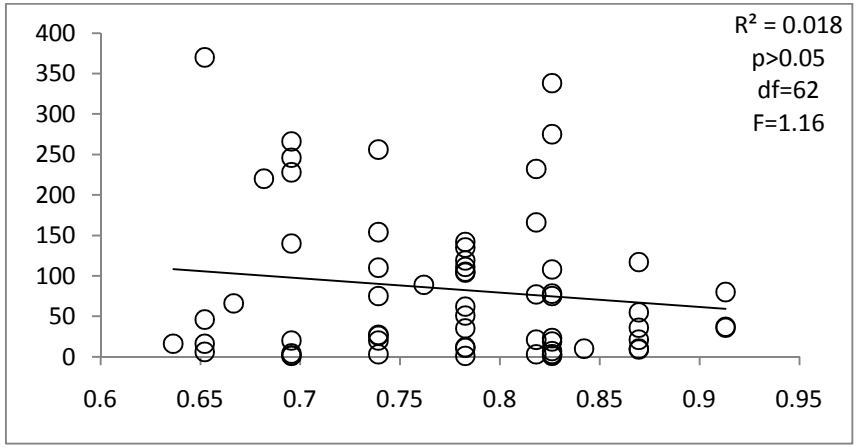


Figure 3.17. Mean heterozygosity (x axis) – Lungworm burden (y axis)

(neutral loci (23) – 63 infected striped dolphins)

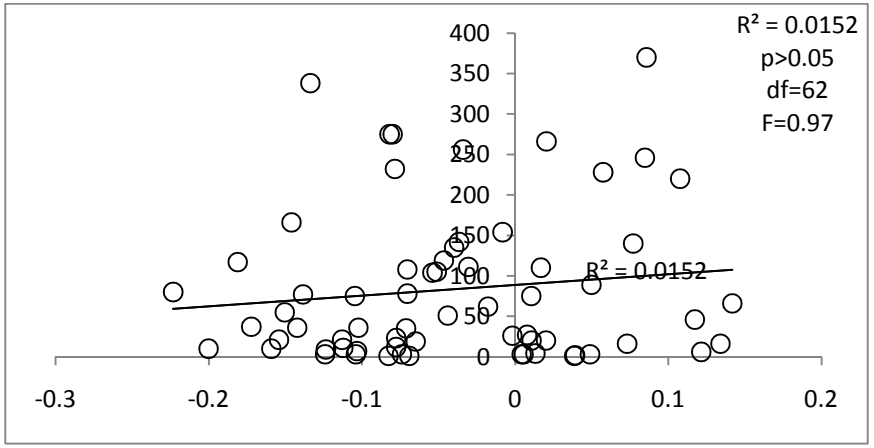


Figure 3.18. IR (x axis) – Lungworm burden (y axis)

(neutral loci (23) – 63 infected striped dolphins

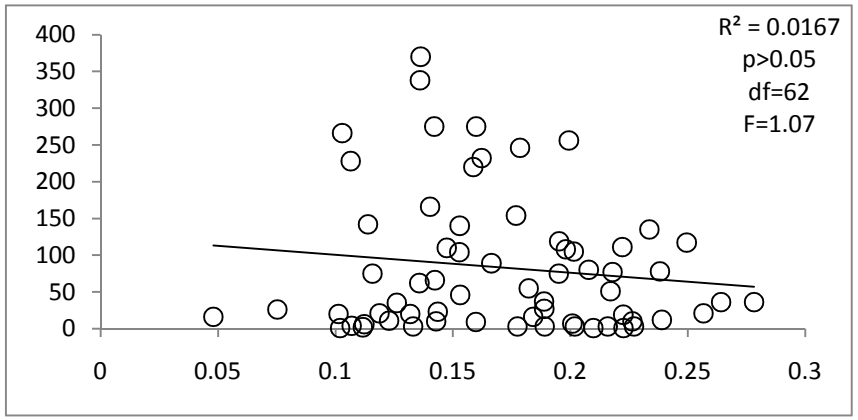


Figure 3.19. mean d^2 (x axis) – Lungworm burden (y axis)

(neutral loci (23) – 63 infected striped dolphins)

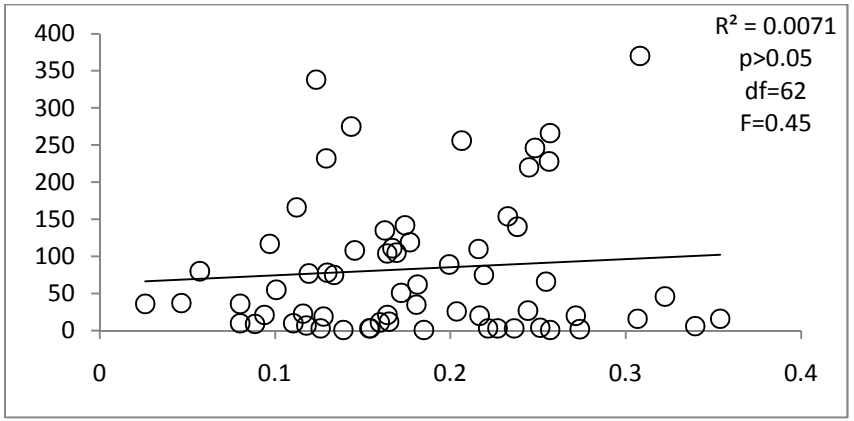


Figure 3.20. HL (x axis) – Lungworm burden (y axis)

(neutral loci (23) – 63 infected striped dolphins)

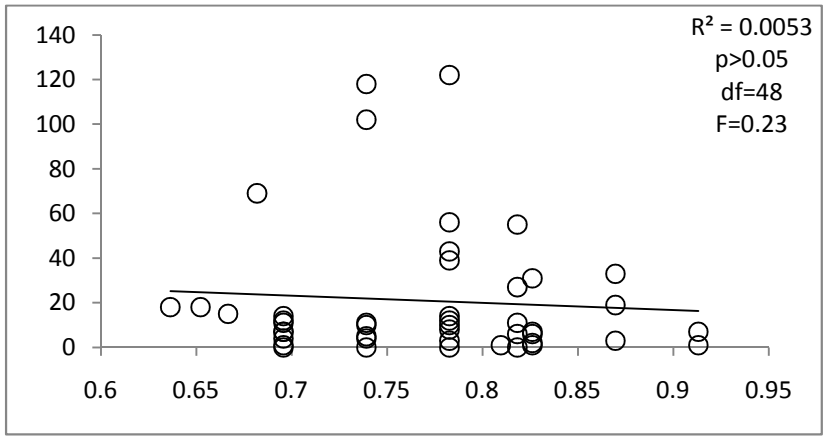


Figure 3.21 Mean heterozygosity (x axis) – stomach digeneans burden (y axis)

(neutral loci (23) – 49 infected striped dolphins)

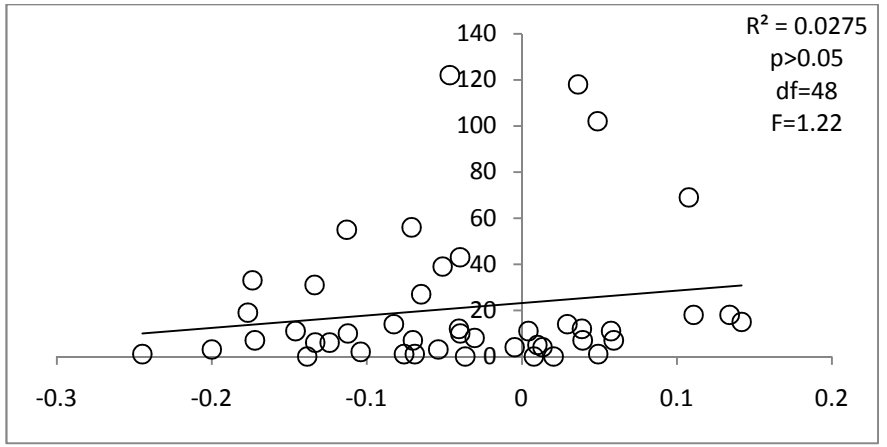


Figure 3.22. IR (x axis) – stomach digeneans burden (y axis)

(neutral loci (23) – 49 infected striped dolphins)

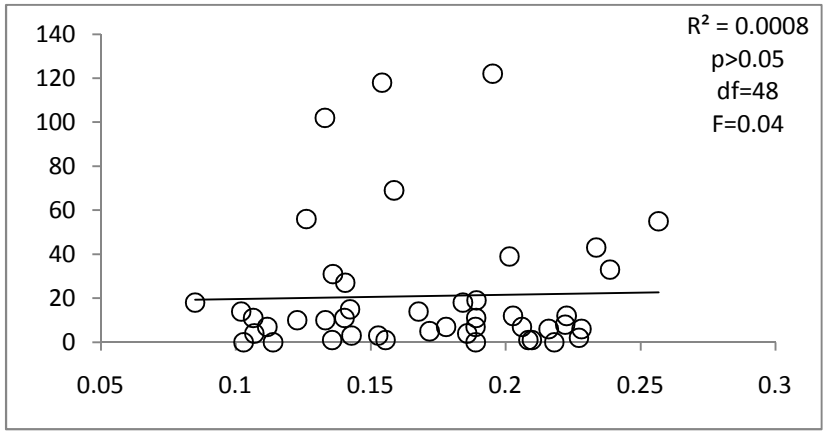


Figure 3.23. mean d^2 (x axis) – stomach digenean burden (y axis)

(neutral loci (23) – 49 infected striped dolphins)

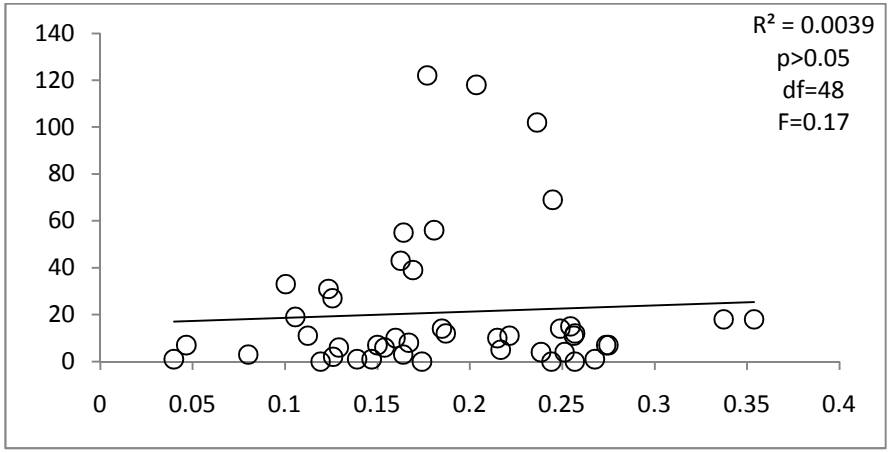


Figure 3.24. HL (x axis) – stomach digenean burden (y axis)

(neutral loci (23) – 49 infected striped dolphins)

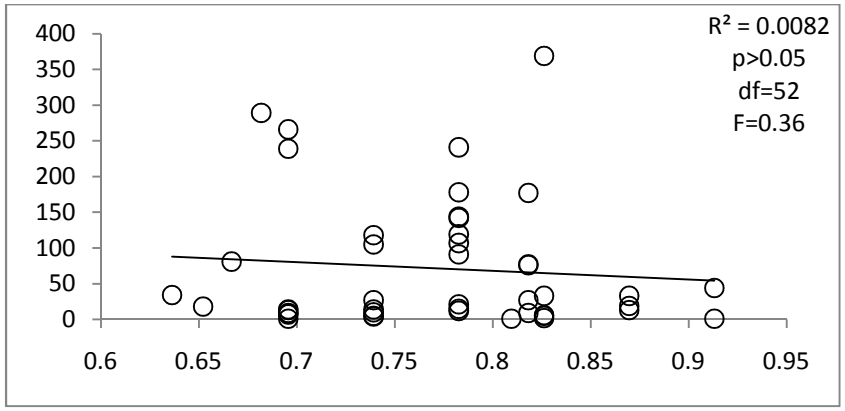


Figure 3.25. Mean heterozygosity (x axis) – Total burden (y axis)

(neutral loci (23)– 53 infected striped dolphins)

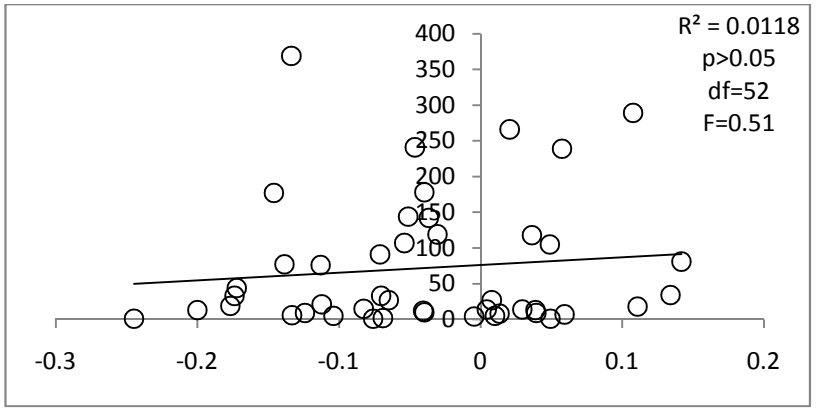


Figure 3.26. IR (x axis) –Total burden (y axis)

(neutral loci (23) – 53 infected striped dolphins)

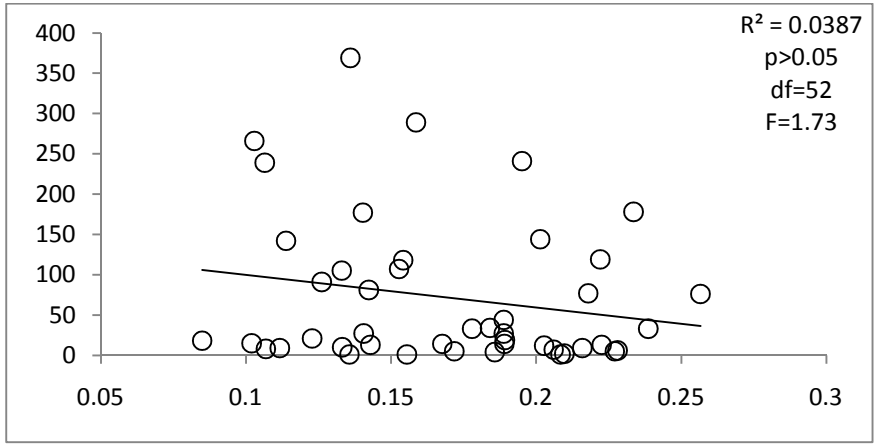


Figure 3.27. mean d^2 (x axis) – Total burden (y axis)

(neutral loci (23) – 53 infected striped dolphins)

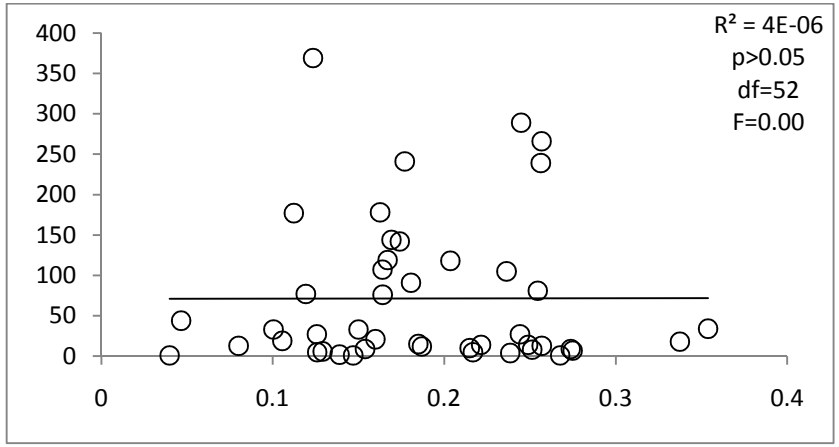


Figure 3.28. HL (x axis) – Total burden (y axis)

(neutral loci (23) – 53 infected striped dolphins)

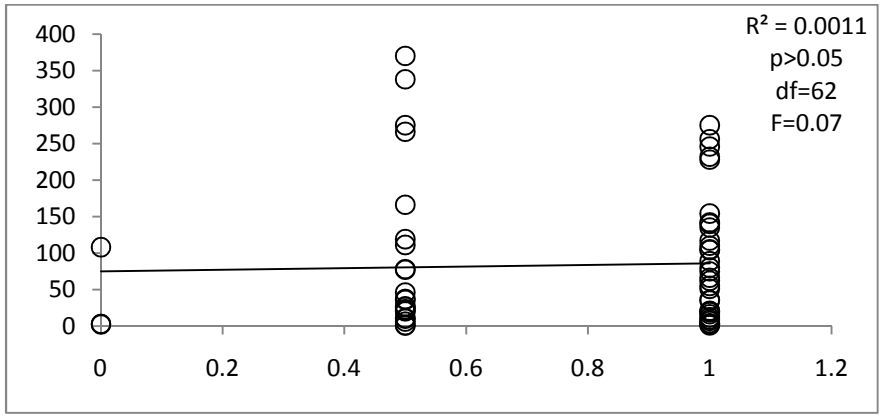


Figure 3.29. Mean heterozygosity (x axis) – Lungworm burden (y axis)

(loci under positive selection (3) – 63 infected striped dolphins)

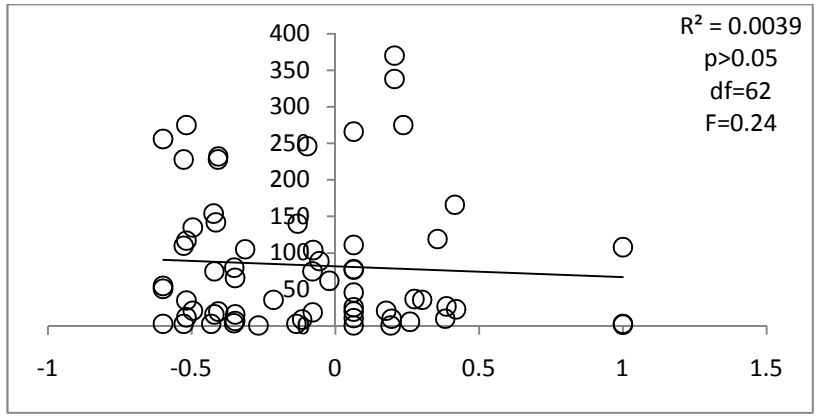


Figure 3.30. IR (x axis) – Lungworm burden (y axis)

(loci under positive selection– 63 infected striped dolphins)

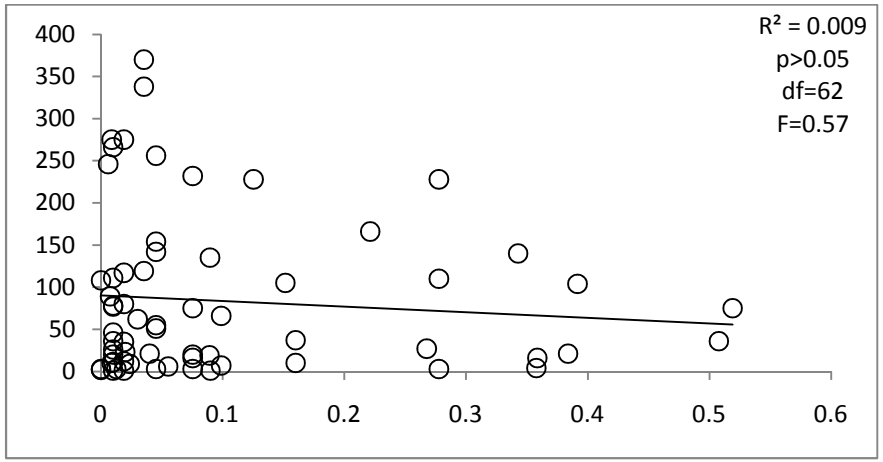


Figure 3.31. mean d^2 (x axis) – Lungworm burden (y axis)

(loci under positive selection (3) – 63 infected striped dolphins)

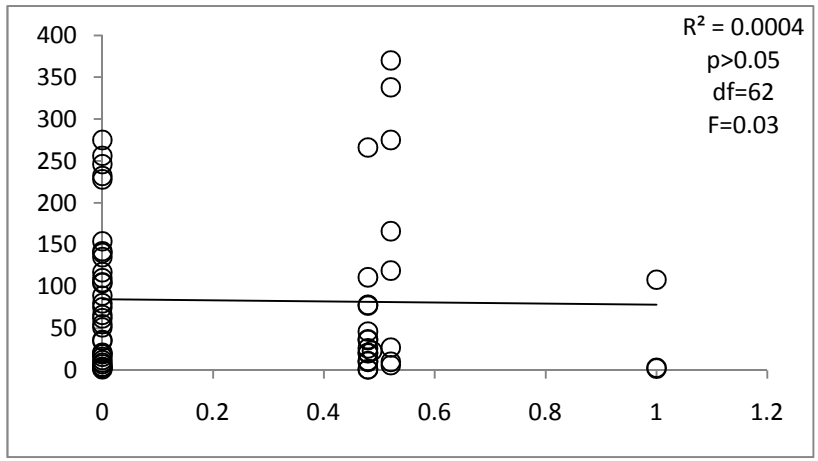


Figure 3.32. HL (x axis) – Lungworm burden (y axis)

(loci under positive selection– 63 infected striped dolphins)

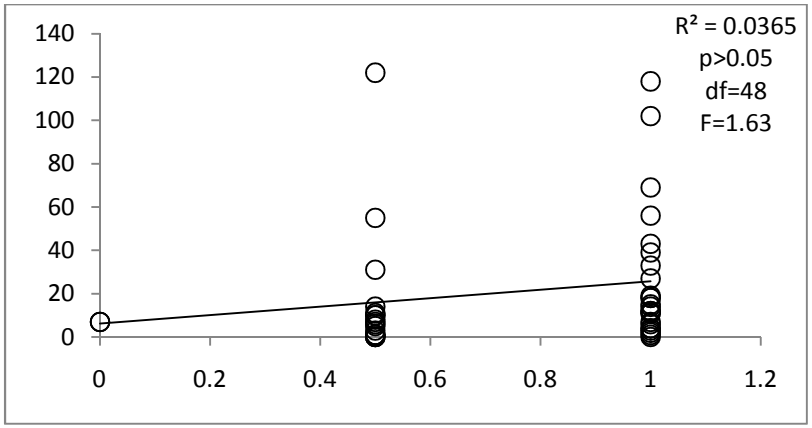


Figure 3.33. Mean heterozygosity (x axis) – stomach digeneans burden (y axis)

(loci under positive selection (3) – 49 infected striped dolphins)

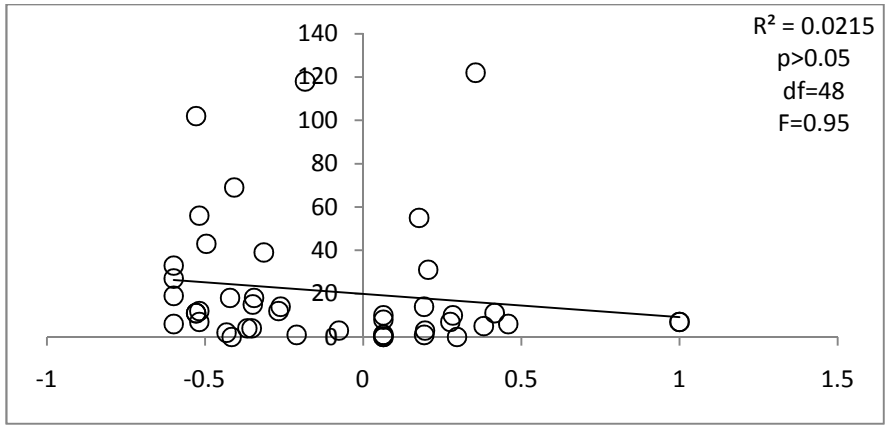


Figure 3.34. IR (x axis) – stomach digeneans burden (y axis)

(loci under positive selection– 49 infected striped dolphins)

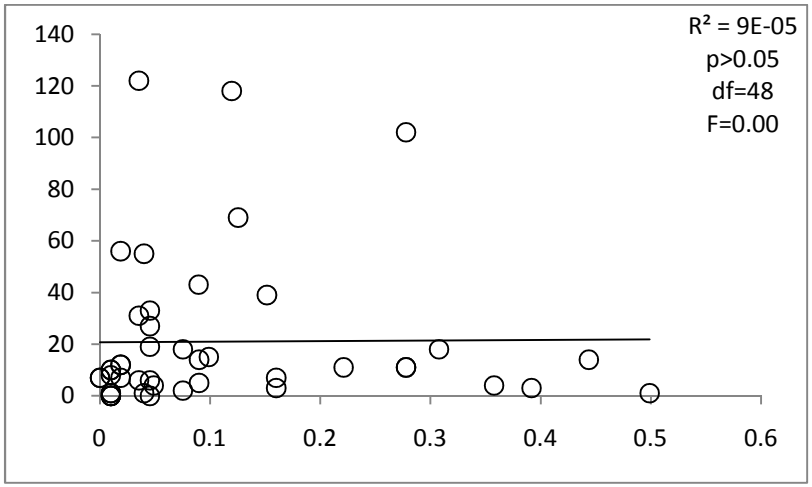


Figure 3.35. mean d^2 (x axis) – stomach digenean burden (y axis)

(loci under positive selection (3) – 49 infected striped dolphins)

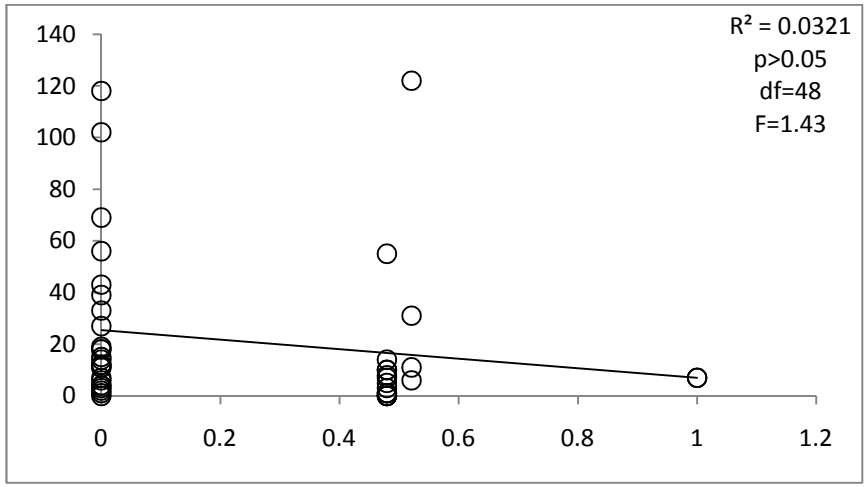


Figure 3.36. HL (x axis) – stomach digenean burden (y axis)

(loci under positive selection– 49 infected striped dolphins)

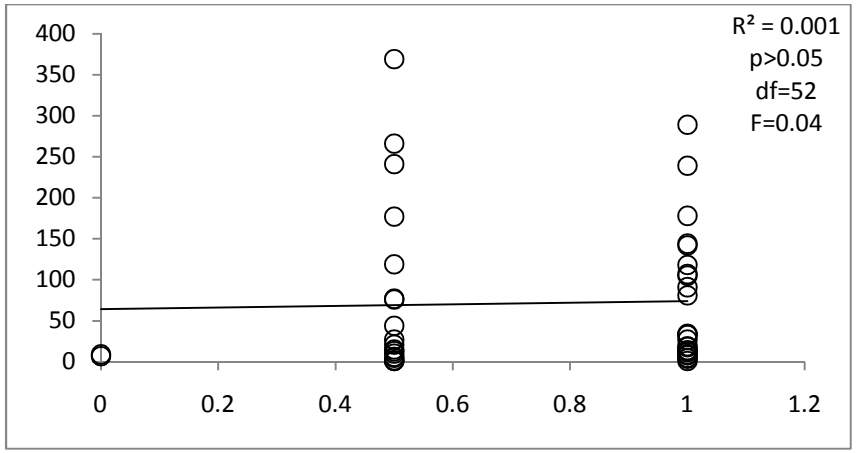


Figure 3.37. Mean heterozygosity (x axis) – Total burden (y axis)

(loci under positive selection (3) – 53 infected striped dolphins)

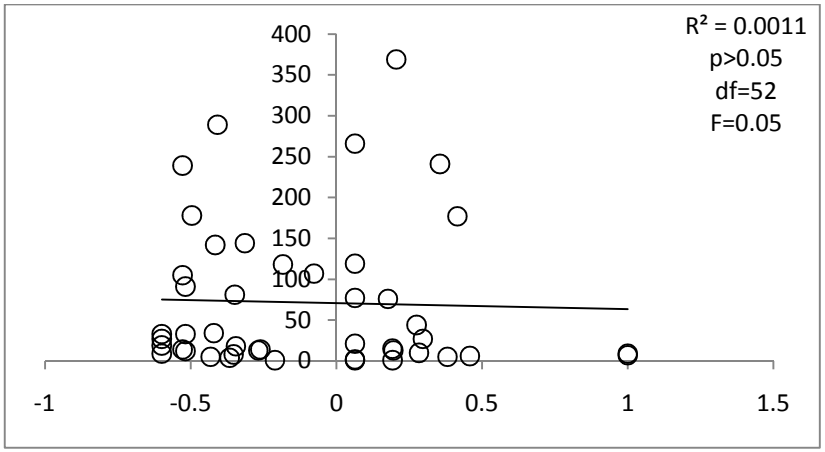


Figure 3.38. IR (x axis) –Total burden (y axis)

(loci under positive selection– 53 infected striped dolphins)

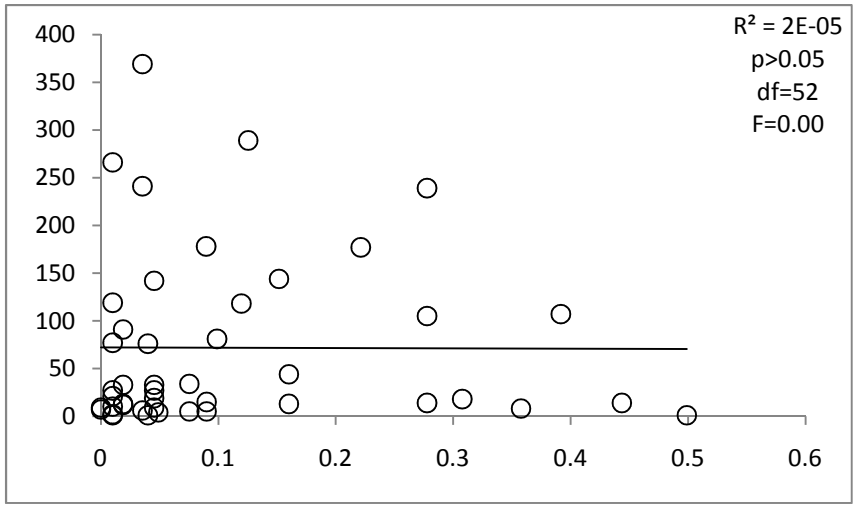


Figure 3.39. mean d^2 (x axis) – Total burden (y axis)

(loci under positive selection (3) – 53 infected striped dolphins)

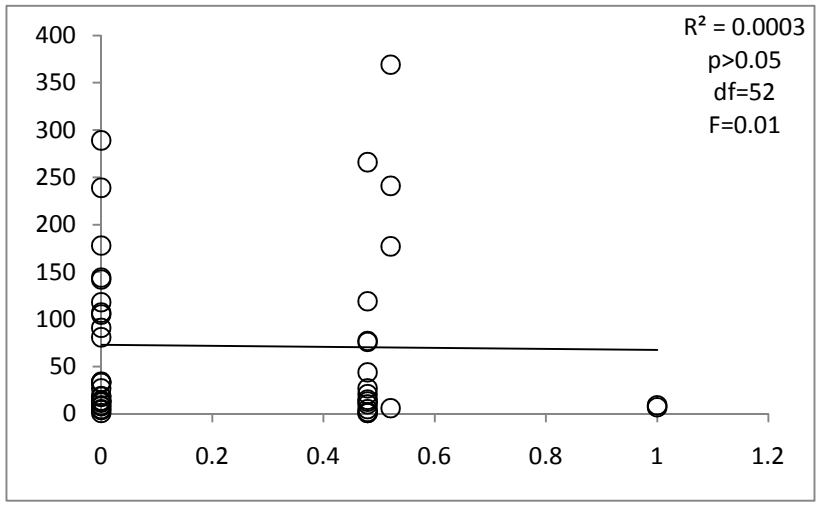


Figure 3.40. HL (x axis) – Total burden (y axis)

(loci under positive selection– 53 infected striped dolphins)

Table 3.7. *P-values* of linear regression between 3 different levels of lungworm infestation and levels of genetic diversity (*: $p<0.05$, before Bonferroni corrections)

	mean Heterozygosity			*IR*			*mean d^2*			*HL*		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
All loci	0.6	0.4	0.6	0.6	0.3	0.9	0.8	0.7	**0.04***	0.6	0.4	0.5
Neutral loci	0.9	0.3	**0.03***	0.8	0.2	0.07	0.9	0.9	0.09	0.6	0.3	**0.01***
Positive loci	0.3	**0.02***	0.12	0.4	**0.02***	0.3	0.5	0.7	0.5	0.3	**0.02***	0.12

Generalized linear models showed an association between levels of genetic diversity and lungworm parasite load when controlling for sex. Moreover, the relationship between female individuals' parasite burden and *IR* and *mean* d^2 was explained by neutral loci (df:23, F:5.96, p=0.003 for *IR* and df:23, F:5.88, p=0.004 for *mean* d^2). Furthermore, the relationship between female individuals' parasite burden and *mean Heterozygosity* was explained by the 2 loci under positive selection (df:23, F:6.62, p=0.002). *HL* did not show any association with parasite burden controlling for sex.

Gross examination showed that individuals with low infestation did not have any occlusion to the bronchioles and alveoli. Thus, a linear regression was performed for individuals with medium and high levels of infestation separately for both sexes. A strong correlation was found for female individuals (19) between parasite burden and mean Heterozygosity, $p=0.02$ (Figure 3.41), IR, $p=0.04$ (Figure 3.42) and HL, $p=0.03$ (Figure 3.44) but not when was analysed for mean d^2 (Figure 3.43). No correlation was found for male individuals ($p>0.05$) (Figures 3.45, 3.46, 3.47 and 3.48).

The new method of Amos and Acevedo-Whitehouse (2009) revealed a candidate microsatellite locus (KWM1b) under balancing selection in male individuals (p=0.002, after Bonferroni) but not in females.

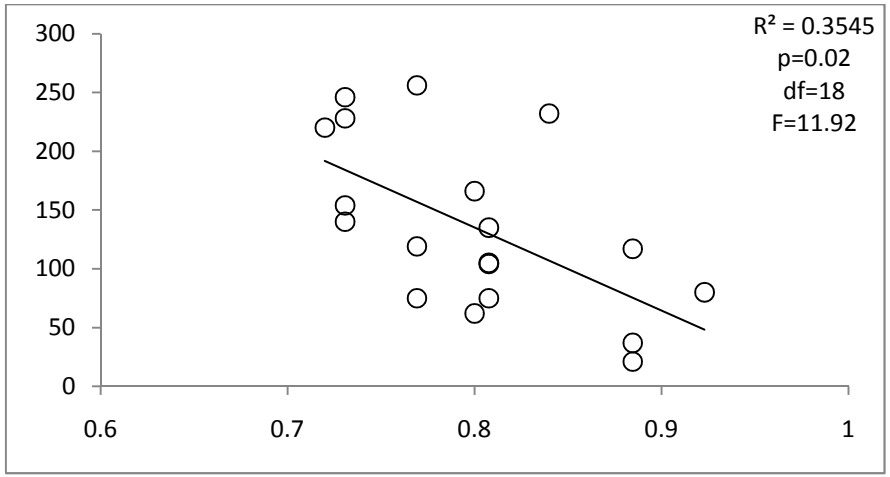


Figure 3.41. Mean Heterozygosity (x axis) – Medium/High female (N=19) infected striped dolphin individuals (y axis) – neutral loci

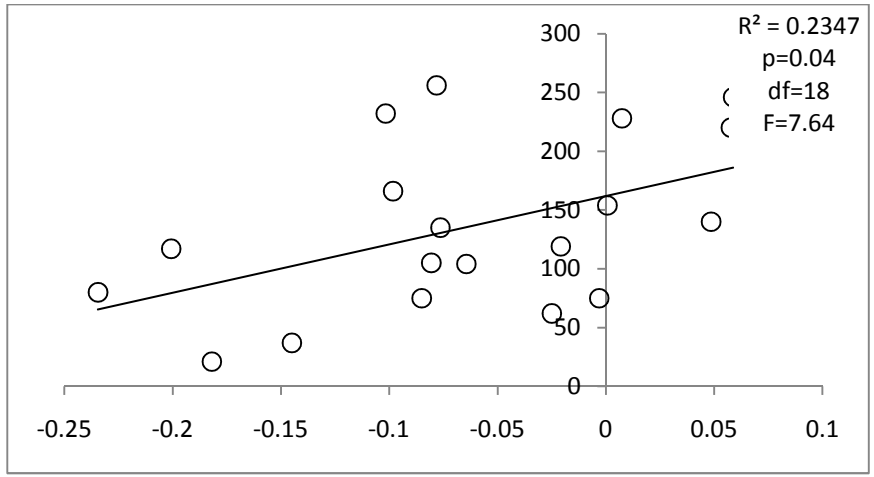


Figure 3.42. IR (x axis) - Medium/High female (N=19) infected striped dolphin individuals (y axis) – neutral loci

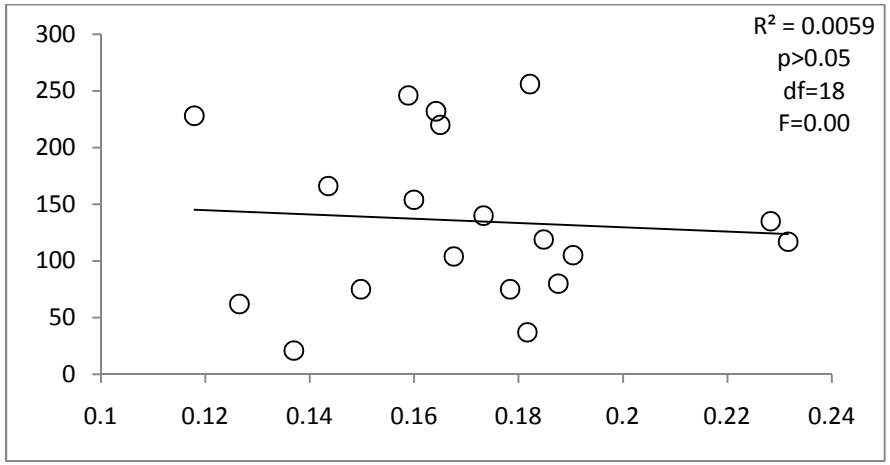


Figure 3.43. Mean d^2 (x axis) – Medium/High female (N=19) infected striped dolphin individuals (y axis) – neutral loci

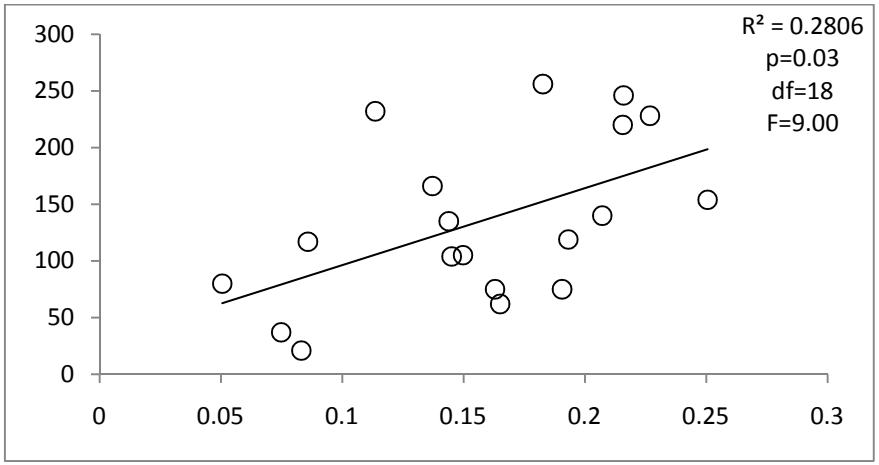


Figure 3.44. HL (x axis) - Medium/High female (N=19) infected striped dolphin individuals (y axis) – neutral loci

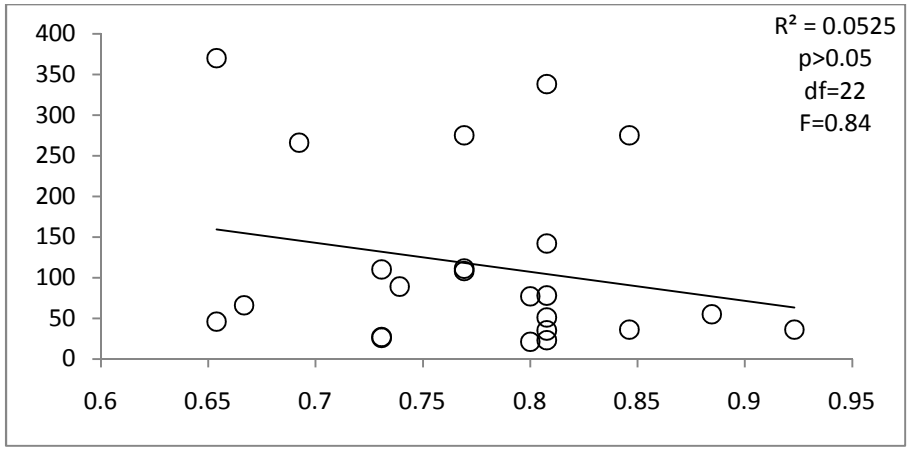


Figure 3.45. Mean Heterozygosity (x axis) – Medium/High male (N=23) infected striped dolphin individuals (y axis) – neutral loci

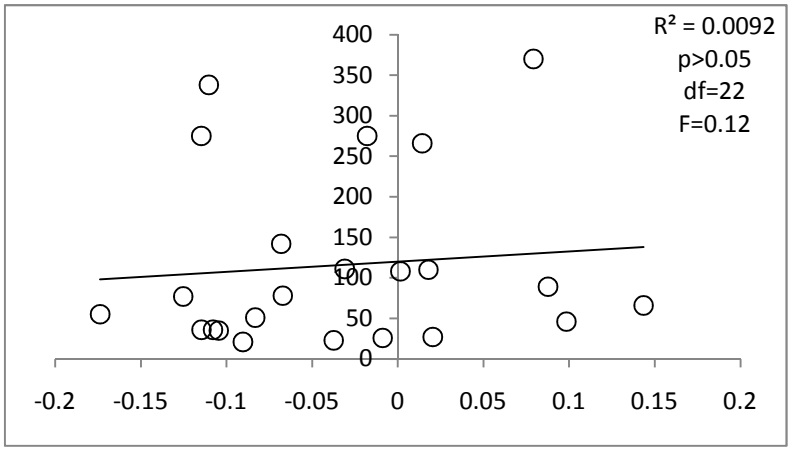


Figure 3.46. IR (x axis) - Medium/High male (N=23) infected striped dolphin individuals (y axis) – neutral loci

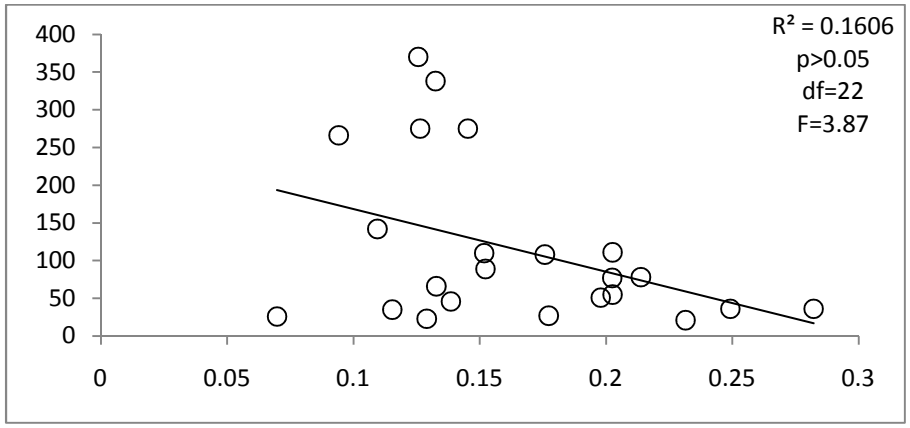


Figure 3.47. Mean d^2 (x axis) – Medium/High male (N=23) infected striped dolphin individuals (y axis) – neutral loci

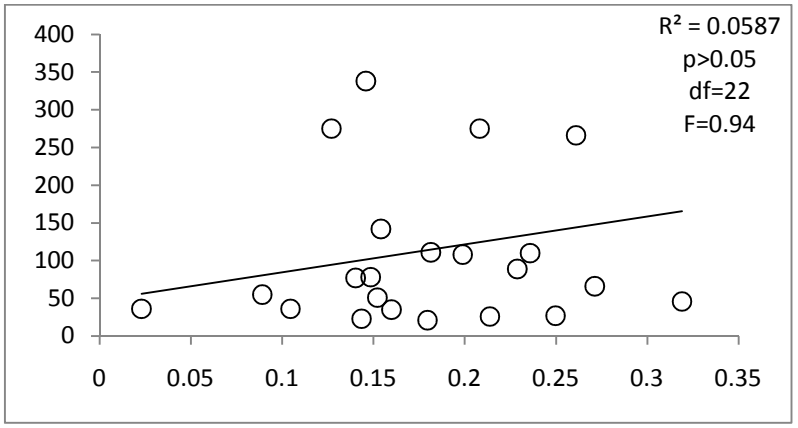


Figure 3.48. HL (x axis) - Medium/High male (N=23) infected striped dolphin individuals (y axis) – neutral loci

3.3.2 Common dolphin

3.3.2.1 Parasites

110 common dolphin samples were examined for lung parasites. Three different species were found during the gross lung examination; the nematodes *Skrjabinalius guevaraii*, *Halocerus invaginatus* and *Halocerus taurica* (Nematoda: Pseudaliidae). Fifty seven individuals were uninfected and fifty three were infected. The range of infestation was 0 - 1489 worms. After the normality test of Shapiro-Wilk assessing the distribution of infection level among animals, 2 individuals were excluded from the analysis with total infestation 1232 and 1489 worms respectively. Individuals were divided into 4 categories according the level of infestation (Table 3.8). Those 4 categories are None, Low (1-11), Medium (12-79) and High (80 – 504). The 4 categories were created using the exact confidence limits for the median intensity (Table 3.9). Parasite count intensity parameters (skewness, mean, median, exact confidence intervals) are shown on Table 3.11. Lungworm counts fitted a negative binomial distribution (skewness measure=0.099, with respect to the negative binomial, $p<0.05$).

Table 3.8. Number of females and males according to level of infestation

Level of Infestation	Females	Males	Total number of individuals
None	26	31	57
Low (1 – 11)	9	10	19
Medium (12 – 79)	8	11	19
High (80 – 504)	7	6	13
	50	58	108

Table 3.9. Common dolphin parasite count intensity parameters

Variance/mean ratio	202.48	
Mean intensity	76.19	
Median intensity	22	99.% exact CI:11 – 79
Bootstrap Confidence for mean crowding	234.83	95% CI: 159.67 – 352.04

3.3.2.2 Genetic diversity

One locus (TexVet5) showed evidence of null alleles and one locus (Ev37) showed departures from Hardy-Weinberg equilibrium. Hence 16 out of 18 loci were used for the analysis. The number of alleles ranged from 3 (TexVet9) to 20 (Ev14) with expected heterozygosities 0.10 (TexVet9) to 1 (Dde70). The mean H_{OBS} was 0.7315 and H_{EXP} was 0.7224 across all loci (Appendix, Table 6.1). Three loci showed evidence of natural selection;TexVet9 under positive selection and Dde70 and Ev14 under balancing selection (Figure 3.49).

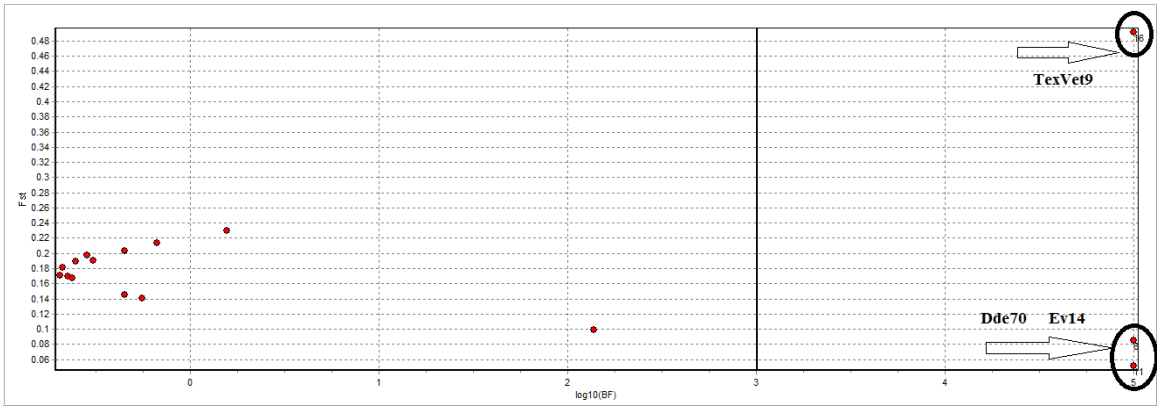


Figure 3.49. Graph of loci under natural selection (BayeScan v. 1.0). In circles the loci under positive selection (Ev37 and Sco11) and under balancing selection (KWM12a).

The ranges for mean Heterozygosity, IR, mean d^2 and HL are shown on Table 3.10. For the sample as a whole, t-tests comparing the mean measures of genetic diversity did not vary significantly between uninfected and infected animals (Table 3.11).

Table 3.10. Mean values of mean Heterozygosity, IR, mean d^2 and HL across all loci, neutral loci and locus under positive selection.

	All loci	Neutral loci	Under positive selection
Mean Heterozygosity	0.7554	0.7816	0.1081
IR	-0.030	-0.0413	0.7861
Mean d^2	0.1517	0.1586	0.054
HL	0.1766	0.1804	0.8918

Table 3.11. Mean values of mean Heterozygosity, IR, mean d^2 and HL for infected and uninfected individuals and t-test *p* values.

	All loci	t-test p	Neutral loci	t-test p	Under positive selection	t-test p
mean $H_{uninfected}$	0.754	0.87	0.785	0.72	0.132	0.44
mean $H_{infected}$	0.756		0.778		0.086	
mean $IR_{uninfected}$	-0.035	0.672	-0.050	0.44	0.829	0.43
mean $IR_{infected}$	-0.025		-0.033		0.7385	
mean $d^2_{uninfected}$	0.141	0.062	0.170	0.09	0.066	0.44
mean $d^2_{infected}$	0.163		0.148		0.043	
mean $HL_{uninfected}$	0.177	0.958	0.183	0.70	0.9137	0.44
mean $HL_{infected}$	0.176		0.176		0.867	

3.3.2.3 Heterozygosity – parasite load associations

No significant correlations were found ($p>0.05$) for linear regressions between total parasite burden and levels of genetic diversity (Figure 3.50, 3.51, 3.52 and 3.53) for all loci. Linear regression was performed also for neutral loci (Figure 3.54, 3.55, 3.56 and 3.57) and for loci under balancing selection (Figure 3.58, 3.59, 3.60 and 3.61). Generalized linear models did not show any association between levels of genetic diversity and lungworm parasite load controlling for sex.

The new method of Amos and Acevedo-Whitehouse (2009) did not reveal and microsatellite locus under balancing selection.

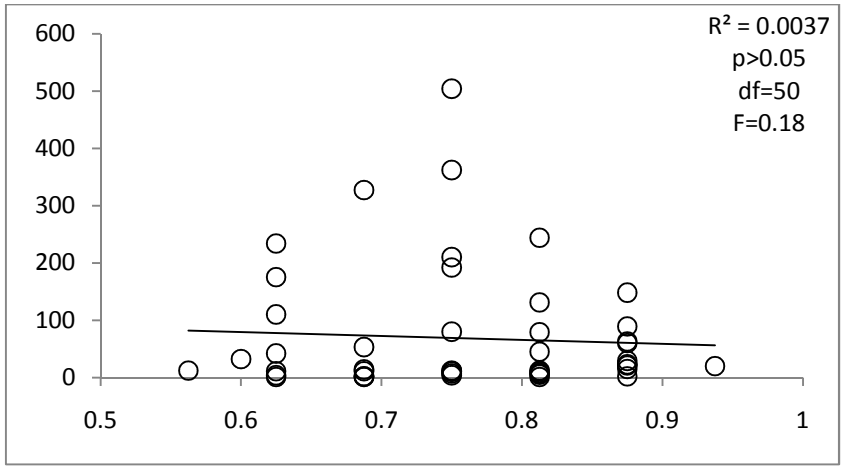


Figure 3.50. Mean Heterozygosity (x axis) – lungworm burden (x axis)

(all loci – 51 infected common dolphins)

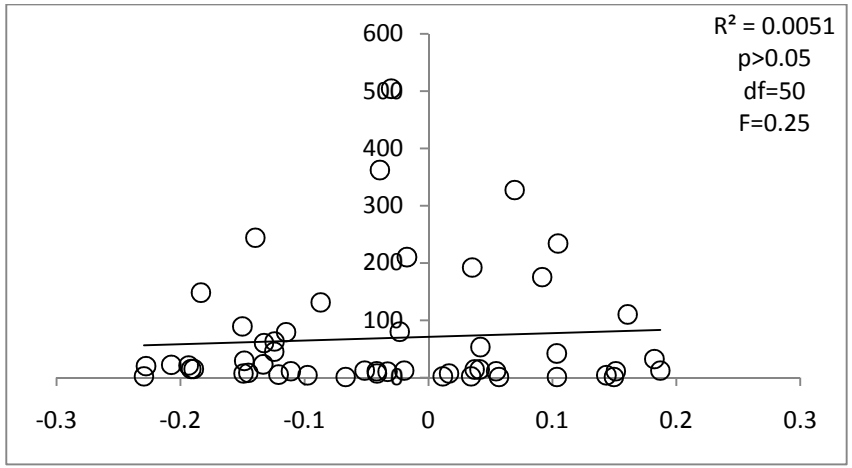


Figure 3.51. IR (x axis) – lungworm burden (y axis)

(all loci – 51 infected common dolphins)

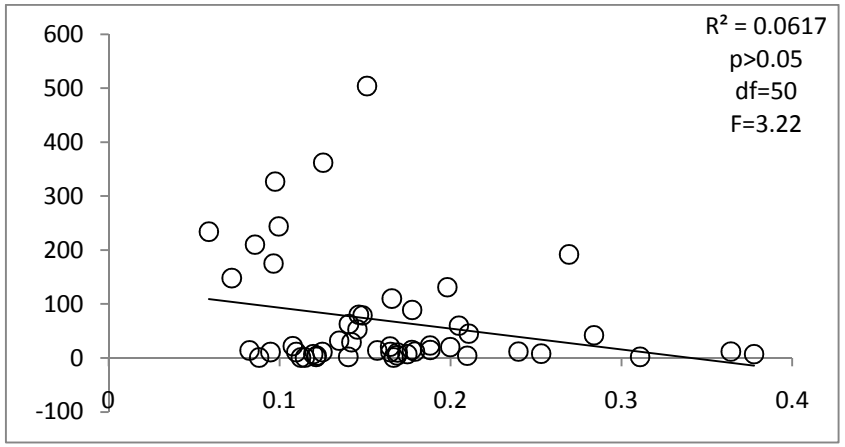


Figure 3.52. Mean d^2 (x axis) – lungworm burden (x axis)

(all loci – 51 infected common dolphins)

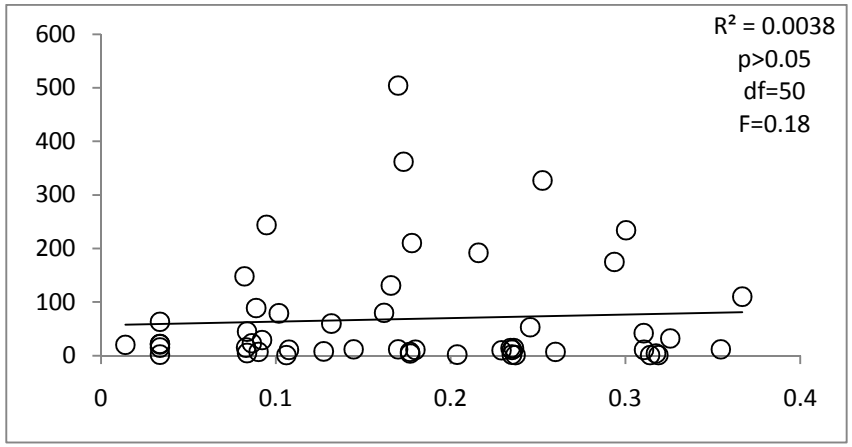


Figure 3.53. HL (x axis) – lungworm burden (y axis)

(all loci – 51 infected common dolphins)

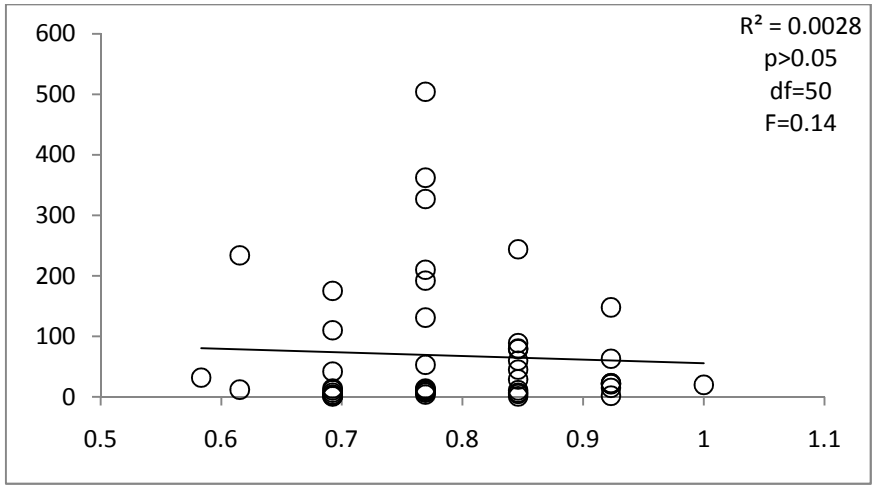


Figure 3.54. Mean Heterozygosity (x axis) – lungworm burden (x axis)

(neutral loci (13) – 51 infected common dolphins)

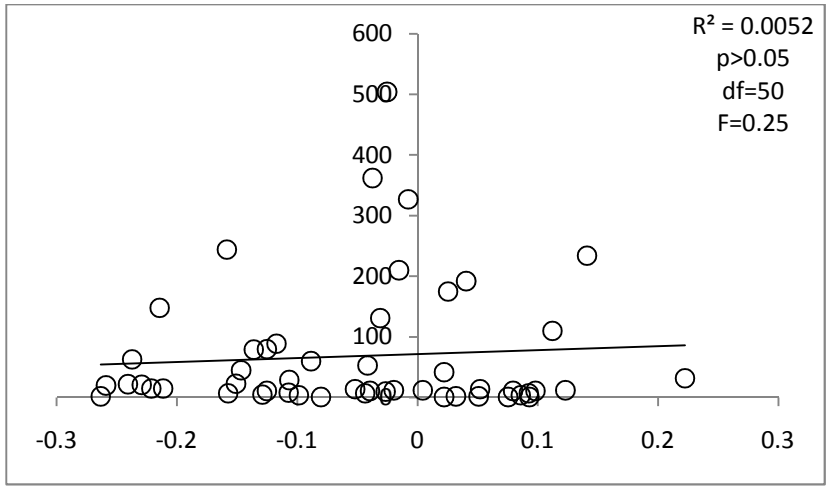


Figure 3.55. IR (x axis) – lungworm burden (y axis)

(neutral loci (13) – 51 infected common dolphins)

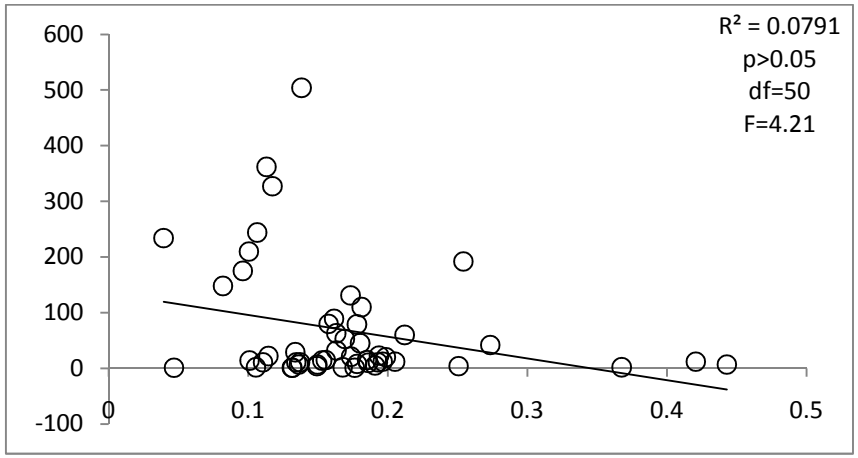


Figure 3.56. Mean d^2 (x axis) – lungworm burden (x axis)

(neutral loci (13) – 51 infected common dolphins)

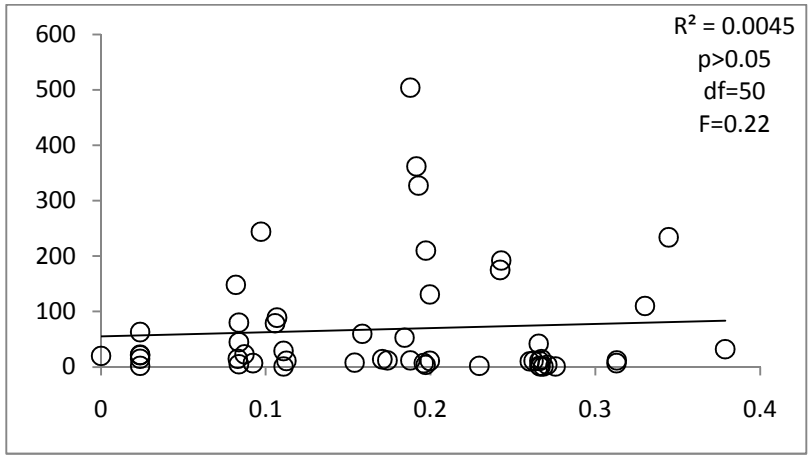


Figure 3.57. HL (x axis) – lungworm burden (y axis)

(neutral loci (13) – 51 infected common dolphins)

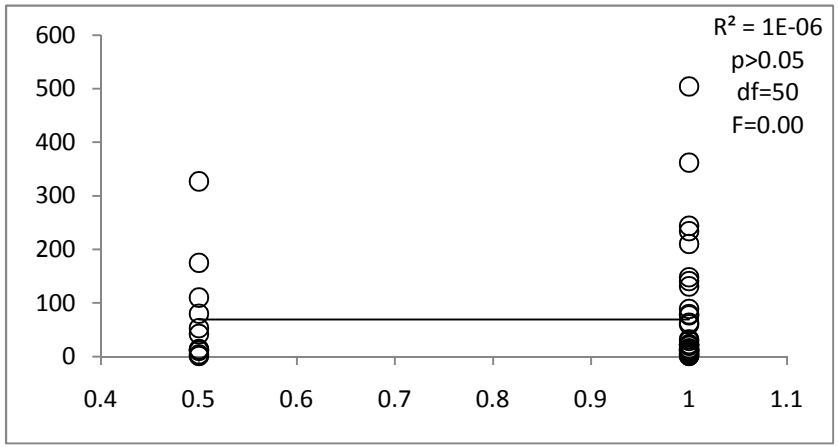


Figure 3.58. Mean Heterozygosity (x axis) – lungworm burden (x axis)

(loci under balancing selection (2) – 51 infected common dolphins)

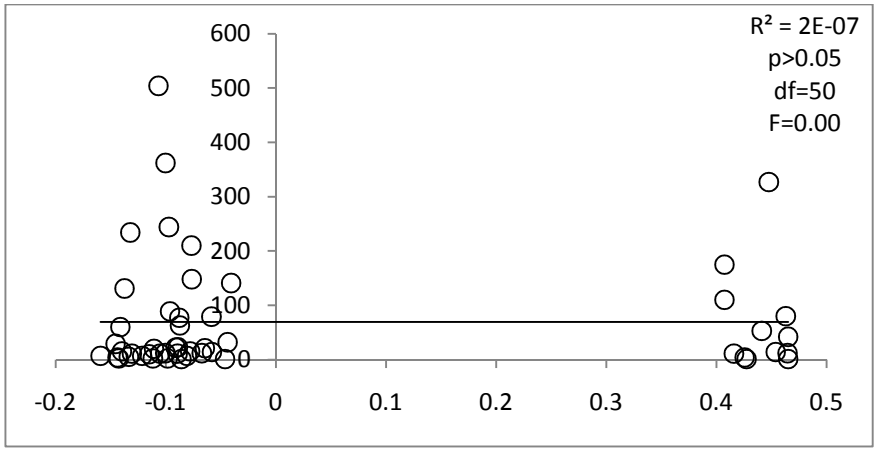


Figure 3.59. IR (x axis) – lungworm burden (y axis)

(loci under balancing selection (2) – 51 infected common dolphins)

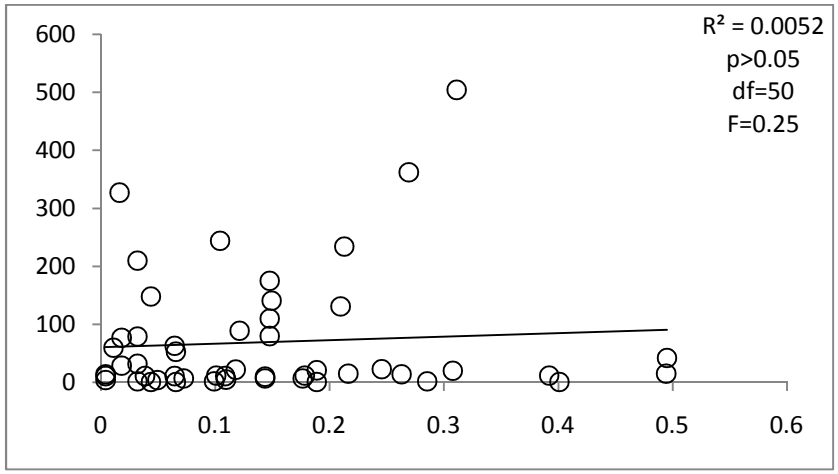


Figure 3.60. Mean d^2 (x axis) – lungworm burden (x axis)

(loci under balancing selection (2) – 51 infected common dolphins)

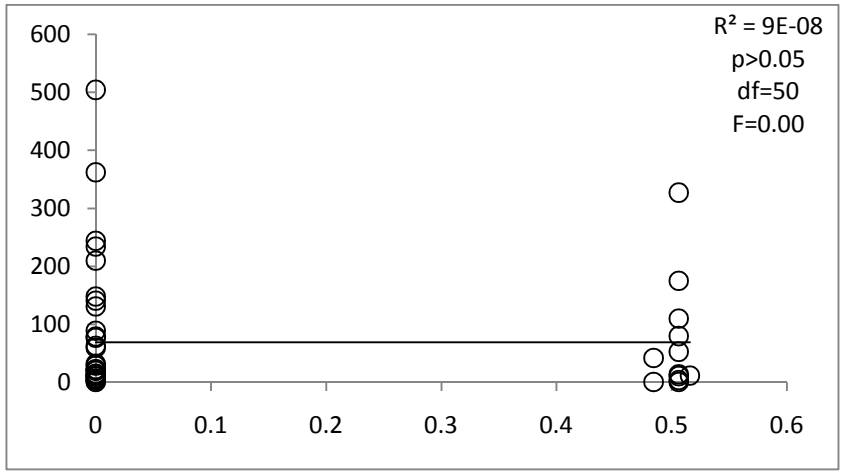


Figure 3.61. HL (x axis) – lungworm burden (y axis)

(loci under balancing selection (2) – 51 infected common dolphins)

3.4 Discussion

The relationship between infestation level from various parasites and level of genetic diversity was examined for two host species, the striped dolphin and the common dolphin. Lungworms and stomach digeneans in Delphinidae species provide useful information about the animal's health. In common dolphins, three lungworm species were found (*Skrjabinalius guevaraii*, *Halocerus invaginatus* and *Halocerus taurica*) and in striped dolphins only one (*Skrjabinalius guevaraii*). These nematodes are quite common in Delphinidae species respiratory systems (Raga and Carbonell, 1985). Although the life cycles of these nematodes are still unknown, large numbers cause total occlusion of bronchi and bronchioles due to their physical presence (Raga *et al.*, 1987b; Clausen and Andersen, 1988). Lesions associated with these lungworms include acute bronchopneumonia and other diseases such as hyperplasia and hypertrophy of the respiratory muscles (Woodart *et al.*, 1969; Migaki *et al.*, 1971; Andersen, 1974), and may cause sick infected animals to strand (Geraci, 1978). Lungworms were found only in adult animals, a fact which suggests that nematodes infect striped and common dolphins after weaning. The infection procedure is unknown, thought it is likely that nematode larvae grow in intermediate hosts (fishes and molluscs). With respect to stomach parasites, the most common for striped dolphins was the gastric digenean *Pholeter gastrophilus* which has been reported in at least 17 cetacean species worldwide (Aznar *et al.*, 1992; Raga, 1994) and molluscs and fishes may act as primary and secondary intermediate hosts, respectively (Gibson *et al.*, 1998). This species burrows into the stomach wall within the submucosal fibrotic nodules and is associated with granulomatous gastritis (Woodart *et al.*, 1969; Migaki *et al.*, 1971; Howard *et al.*, 1983) creating symptoms such as abdominal upset and indigestion.

Striped dolphin individuals from Valencia population were dominated by those found stranded during the morbillivirus epizootic between 1990 and 2007, whereas samples from Ireland were not know to be associated with the epizootic stranding. An assessment including only striped dolphins from the morbillivirus epizootic revealed no associations between the virus and different parasite species burden, suggesting that parasite loads were unaffected by virus post-mortem symptoms. Therefore, the level of lungworm and stomach digenean infestation is unlikely to be biased by this aspect of sampling.

Despite the observed high genetic diversity of the microsatellite loci, striped and common dolphin individuals (both sexes combined) did not show any significant correlation between multi-locus heterozygosity and lungworm burden for any combination of the loci (all loci, neutral loci, or just those loci under positive selection). The lack of correlation remained when using the IR, standard d^2 and HL measures. A possible explanation for the lack of a correlation would be low power from the small number of individuals examined. On the other hand, no significant differences between infected and uninfected individuals and multi-locus heterozygosy or estimates of inbreeding were found, suggesting that this pattern illustrates the fact that these microsatellite loci were variable in both species and most of the individuals were heterozygous at most loci. This study also failed to report any correlation between stomach parasite load and genetic variation in striped dolphins. A possible reason is the small sample size, and a small range for the levels of individual heterozygosity (0.7 – 0.8), each of which could cause low power. However, it is also possible that this type of infection has a relatively low impact on fitness. While the lung worms can impede respiration, stomach parasites are often born without obvious adverse effects. Howard *et al.* (1983) in a parasitological study of many cetacean taxa suggests that individuals of

Pholeter gastrophilus may leave the cyst and enter the stomach, and it is also possible that these digeneans simply die and disintegrate within the cyst.

In this study, lungworm burden levels were obtained from dead animals, and as a result, other potential factors were not taken into account such as previous health status or a possible post-mortem continuous infection. Sudden death associated with an epizootic means that some individuals will die prior to becoming infected, or never become as infected as they may have at a greater age, however this assumes a positive correlation between age and infection level, and little is known about this.

For 21.4 % of the infected animals there was a relatively low number of worms (1 – 20; for both lungs combined), and during gross examination it was apparent that this number of worms was not enough to cause any occlusion to the bronchioles and alveoli. At the same time, the occlusions caused from worms were obvious for individuals with medium or high level of infestation. This may suggest a threshold value above which an impact may begin to be seen. For this reason, an assessment including only individuals with medium and high levels of infection was undertaken and considering each sex separately. For this analysis strong significant differences were found for 18 striped dolphin female samples associated with lungworm burden and mean multi-locus heterozygosity and IR values for neutral loci. Studies in the literature that used small sample sizes also found significant heterozygosity fitness correlations. Acevedo-Whitehouse *et al.* (2006) used 27-31 California sea lion pups per each age class and found a significant negative relationship between inbreeding and hookworm burden for each class. Luikart *et al.* (2008) reported that low heterozygosity at 15 microsatellite loci was associated with significantly higher lungworm (*Protostrongylus* spp.) abundance in 17 wild bighorn sheep. However, the significant relationship remained when only seven microsatellite loci were used to compute heterozygosity. Moreover, MacDougall-

Shackleton *et al.* (2005) proposed a strong relationship between heterozygosity and risk of *Haemoproteus* infection in 12 mountain white-crowned sparrows (*Zonotrichia leucophrysorianta*).

No significant association was found for male striped dolphin individuals. The significant result for females may be due to maternal stress factors such as parturition or nursing causing females to cross a threshold such that the association with parasite resistance becomes apparent. Richardson *et al.* (2004) in a heterozygosity-fitness correlation study in the Seychelles warbler (*Acrocephalus sechellensis*) using 14 microsatellite loci proposed that the offspring of highly heterozygous females survived better than the offspring of inbred mothers, and they found no heterozygosity fitness correlation for males. Jamieson *et al.* (2003) investigating an ancestrally inbred population of the New Zealand takahe (*Porphyrio hochstetteri*) using four generations of pedigree data, showed that the mother's level of inbreeding affects offspring fitness. Moreover, in another study on pedigree data in the song sparrow (*Melospiza melodia*) Keller (1998) showed that a reduction in fitness was only seen in inbred female individuals.

Despite the lack of a multi-locus effect in males, a strong relationship was seen for the KWM1b locus associated with parasite loads. Many cases that report heterozygosity fitness correlations in wildlife studies show an effect due to a subset of loci showing heterozygote advantage. Acevedo-Whitehouse *et al.* (2009) in a study of 39 New Zealand sea lion pups and 22 microsatellite loci found no differences in the levels of heterozygosity between dead (N=25) and live (N=14) individuals and no association between overall heterozygosity and hookworm (*Uncinaria* spp.) burden, but a significant association was found between one microsatellite and the occurrence of hookworm-related anaemia. Hoffman *et al.* (2010) found evidence of three out of the nine studied

microsatellite loci being individually associated with tooth size in 84 adult male Antarctic fur seals (*Arctocephalus gazella*). Bean *et al.* (2004) studied the heterozygosity of 9 microsatellites and the survival of gray seals (*Halichoerus grypus*) and found individually significant effects of four microsatellite loci that are significantly associated with pup survival.

For common dolphins, despite the relatively high levels of heterozygosity (0.65 – 1), no heterozygosity fitness associations were identified. The lack of a general effect may be due to small sample size. It is also possible that the infecting nematodes are less virulent in common dolphins than in the striped dolphin. Two studies of New Zealand sea lions (Acevedo-Whitehouse *et al.*, 2006) and California sea lions (Acevedo-Whitehouse *et al.*, 2009) and heterozygosity fitness correlation of the same hookworm species (*Uncinaria* spp.) propose that hookworms might be less virulent in the New Zealand sea lions than in the California sea lions. In spite of the possible different pathogen effect of the lungworms in the two hosts, the post-mortem parasite developing is yet to be answered as it may influence the number of the parasite load counted. Apart from issues related to potential noise in the analyses (post-mortem infection, a smaller number of loci investigated, etc), the difference between the two species could be related to their life history. No population structure has been found in the eastern North Atlantic for the common dolphin over broad geographic areas (Natoli *et al.*, 2006; Mirimin *et al.*, 2009), only for comparisons against a local population in Greece (Natoli *et al.*, 2008). This is in contrast to the striped dolphin for which relatively fine-scale structure has been identified over the same geographic range (Gaspari *et al.*, 2007a; Chapter 2). Population structure leads to smaller local effective population size, and the possibility of a greater impact of inbreeding on fitness (c.f. Acevedo-Whitehouse *et al.*, 2009).

The heterozygosity fitness correlations of striped and common dolphins provide relatively poor support of the general effect hypothesis. This also results from the comparison between the uninfected and infected individuals where no significant differences were found. However, an interesting association was seen for female striped dolphins when only higher infection levels were considered suggesting that parasites may act as an energetic stress which is associated with maternity compounded factors (e.g. parturition). In addition, this analysis suggests a single-locus effect in male striped dolphins proposing that aspects of diseases caused by lungworm burden may be under selection (assuming linkage disequilibrium between the microsatellite DNA locus and a functional gene). Such differences between male and female striped dolphins could reflect a different pathogen pressure between sex and environmental stressors. The observed disparity with respect the heterozygosity-fitness associations in the two species in similar pathogen environments may reflect the non-identical pathogenesis of parasites and their ability to inflict damage. Studies of heterozygosity-fitness correlation can be very informative for wildlife populations regarding the population pathogen pressure which may have implications for the effective conservation of Delphinidae species.

CHAPTER 4

Exon 2- MHC Class II DQB1 locus variability and parasite load in the striped (*Stenella coeruleoalba*) and common (*Delphinus delphis*) dolphins

4.1 Introduction

In higher vertebrates the recognition of non-self is a key aspect of the immune response, and genes in the major histocompatability complex (MHC) play an important role in that process. The MHC region is ubiquitous in most vertebrates, consists of a group of closely linked genes, and plays an important role in the immune system and autoimmunity. MHC molecules display a fragment of normal proteins (self) and microbial invaders (non-self) on the cell surface and can present to a nearby immune cell, usually a T cell or natural killer cell. This cell-surface encoding that binds antigens derived from pathogens initiates the immune response. There are two general classes of MHC molecules; Class I and Class II. Class I MHC molecules bind antigens derived from viral proteins and cancer infected cells and are expressed on the surface of all cells, whereas Class II MHC molecules bind antigens derived from parasites and are expressed on the surface of macrophages and B cells (Klein and Sato, 1998). The MHC Class II is divided into clusters designated 'DQ', 'DR' and 'DP' among others, and are divided among 'alpha' and 'beta' genes. The antigen recognition site (or 'peptide binding region') is encoded in exon 2 of the DQ and DR genes (Klein, 1986). The antigen binding site is a cleft composed of two a-helices on top of a β-pleated sheet (Brown *et al.*, 1988). High genetic diversity at these elements of the MHC loci permits a broad diversity of pathogens to be recognised, and there is evidence that selection works to maintain this diversity over time (e.g. Hughes and Nei, 1988).

According to the neutrality theory, the rate of synonymous substitution (d_S; nucleotide mutations that don't alter the amino acid sequence) is predicted to be larger than non-synonymous substitution (d_N) due to purifying selection maintaining the

integrity of the functional sequence (Hughes and Nei, 1988). However, many studies on MHC variability at the antigen binding sites show a high rate of non-synonymous/synonymous substitutions ($d_N > d_S$). Although early studies of marine mammal species had suggested weak selective pressure and low diversity (e.g. Slade, 1992), later studies confirmed a similar process as seen in other mammals (e.g. Gyllensten *et al.*, 1990). For example, Murray *et al.* (1995) in a study of the beluga whale, *Delphinapterus leucas*, showed a high rate of $d_N > d_S$ substitution in the peptide binding region of exon-2 of the MHC DQB1 locus. Hoelzel *et al.* (1999) investigating variation at exon-2 of the MHC DQB1 for four pinniped species [southern elephant seal (*Mirounga leonine*, Northern elephant seal (*Mirounga angustirostris*), Antarctic fur seal (*Arctocephalus gazella*) and New Zealand fur seal (*Arctocephalus fosteri*)] and Nigenda-Morales *et al.* (2008) investigating the MHC DQB1 exon2 locus in the fin whale, *Balaenoptera physalus*, also report high d_N/d_S ratios. This high level of allelic diversity at the protein level must be due to balancing selection (either selection for the heterozygote, or frequency dependent selection; see below), as the mutation rate at these loci is not elevated (Hughes and Nei, 1998). Further evidence in support of this is provided by the persistence of this allelic diversity over extremely long time periods (Hughes and Nei, 1998). Klein (1987) referred to this as 'trans-species' evolution, whereby alleles are preserved over time and represent allelic lineages present in common ancestors. As a result, the resulting phylogenies no longer reflect the recapitulation of historical relatedness seen in trees constructed using neutral markers. For example, Hoelzel *et al.* (1999) performing a phylogenetic reconstruction of four pinniped species found a similar trans-species evolution pattern as observed in primates (Gyllensten *et al.*, 1990).

Two, primary, controversial hypotheses of balancing selection have been suggested to explain MHC allelic diversity: the *heterozygote advantage hypothesis* and the

frequency-dependent selection hypothesis. The *heterozygote advantage hypothesis* (after Hughes and Nei, 1989) presumes that heterozygous individuals are favoured because they process more different alleles than homozygous individuals do, and therefore, are able to recognize a broader spectrum of pathogens. Thursz *et al.* (1997) in a study of hepatitis B virus infection in humans and associations with HLA class-II type diversity showed an association between heterozygous individuals and viral resistance. Similar to that study, Godkin *et al.* (2005) showed an association between hepatitis B virus infection and heterozygosisty at HLA class-II loci. Carrington *et al.* (1999) in a study on the AIDS virus in humans showed that diversity in HLA class II is associated with a slower progression to AIDS after HIV-1 infection. Penn *et al.* (2002) in a laboratory experiment challenged mouse populations with *Salmonella* infection. The results showed that heterozygous individuals in MHC-congenic strains of mice (*C57BL / 10SnJH2*^{b}*, B10.D2-H2*^{d}*, B10.M-H2*^{f}*, B10.BR-H2*^{k}*, B10.Q-H2*$_{q}$) slightly enhanced the health and survival of mice, thus showing a heterozygote advantage. Ditchkoff *et al.* (2005) showed associations between groups of MHC class II DRB alleles from the same evolutionary lineage and selected pathogens among white-tailed deer, *Odocoileus virginianus*, populations suggesting that more than one allele might be associated with parasite resistance.

In contrast, the *frequency-dependent selection hypothesis* (after Takahata and Nei, 1990) assumes that MHC diversity is maintained through frequency – dependent coevolutionary processes between hosts and parasites. In the literature there are quite a few studies that show association of specific MHC alleles and parasite resistance. Paterson *et al.* (1998) studied a large unmanaged population of Soay sheep, *Ovis aries*, and associations between MHC class II DRB variation, juvenile survival and parasite resistance. The authors found that two specific MHC alleles are associated with low

survival probabilities and high levels of parasitism. In a comparative study of full-sibling families of juvenile individuals (Langerfors *et al.*, 2001) and adult individuals of the Atlantic salmon, *Salmor salar*, Lohm *et al.* (2002) showed an association between three MHC class II B alleles and a highly virulent bacteria, *Aeromonas salmonicida*. The first allele was significantly more prevalent in uninfected individuals, the second allele was significantly more prevalent to the infected and surviving individuals than in infected and dead individuals, and the third one tended to be more prevalent in infected and dead individuals. These two studies show a strong survival advantage for specific MHC alleles. Harf and Sommer (2005) show evidence of balancing selection of MHC class II DRB in the hairy-footed gerbil, *Gerbillurus paeba*, from the southern Kalahari Desert and a significant association of an allele *Gepa*-DRB*15 with infectious status of helminth egg loads. Moreover, studies on nematode parasitism in the yellow-necked mouse, *Apodemus flavicollis* (Meyer-Lucht and Sommer 2005) and parasite burden in the Malagasy mouse lemur, *Microcebus murinus* (Schad *et al.*, 2005) show evidence for pathogen-driven selection acting through specific MHC class II DRB alleles and infectious status.

HLA (MHC for humans) DR alleles have been categorised into seven groups based on their important sub-region structures and functions (Stern *et al.*, 1994). Among these sub-regions Ou *et al*. (1998) suggest a further categorization based on the sum of the charges at the Pocket 4 amino acids residues (*β70*, *β71* and *β74*) to positively charged supertype (+), negatively charged supertype (-), both positively and negatively charged or di-charged supertype (+/-) and uncharged or neutral supertype (n). These amino acid charges play an important role and influence the T-cell recognition due to selectivity for peptide binding (Ou *et al.*, 1996). A study on putative functional residues of the Pocket 4 within the MHC DQB1 exon-2 for three cetacean species (5 populations of killer whale,

Orcinus orca; 5 populations of *Tursops truncates* and one of *Trusiops aduncus*) suggests directional and balancing selection (Vassilakos *et al.*, 2009). One out of the 5 killer whale populations show evidence of directional selection based on the high average MHC Fst value (0.146) whereas the similarity of the remaining populations (MHC Fst:0.043-0.112) may suggest an overall pattern of balancing selection. For bottlenose populations Vassilakos *et al.* (2009) found that nearshore and offshore populations of *Tursiops truncates* differ significantly in Pocket 4 charge profiles with a dominance of di-charge superype (+/-) charge state in geographically distant nearshore populations.

Objectives

Klein (1986) and others have shown that the evolution of diversity in the immune system is responsible for disease resistance, and this diversity is driven by selection. Marine organisms are exposed to a diversity of pathogens, just as for terrestrial species, though we know relatively little about the pathogen environment of mammals in the sea. However, there are documented epizootic events for marine mammals that we can investigate. For example, the morbillivirus epizootic mortality in 1990 produced a massive die-off of striped dolphin (*Stenella coeruleoalba*) populations in the Mediterranean Sea (Aguilar and Raga, 1993). A second outbreak of the morbillivirus virus in 2007 produced a second large die-off of striped dolphins.

Begon *et al.* (2002) suggest that social behaviour can affect the disease transmission rate within a species. Striped dolphins, like other sympatric species (common dolphins – *Delphinus delphis*, Risso's dolphins – *Grampus griseus* and bottlenose dolphins – *Tursiops trucantus*) form fission-fusion groups, but little is known about the details of individual associations over time (Hoelzel *et al.*, 1998a; Natoli *et al.*, 2004). Gaspari *et al.* (2007a) found kinship associations between adult female striped

dolphins in small groups, but the effect dissipated in larger groups, and was not seen for males. However, each of these species shows highly gregarious behaviour that may be sufficient to permit pathogen transmission. At the same time, the degree of sociality may affect the degree of pathogen exposure (see Bowers and Turner, 1997). Striped dolphins show fine scale geographical structure within the Mediterranean Sea, eastern North Atlantic region (Garcia-Martinez *et al.*, 1999; Bourret *et al.*, 2007; Gaspari *et al.*, 2007a; Chapter 2) while the common dolphin shows little or no genetic differentiation over this range (Natoli *et al.*, 2006; Mirimin *et al.*, 2009). If philopatry is promoted by social coherence in delphinid species (see Hoelzel, 2009), then the selective pressure for pathogen resistance may be greater in a highly structured species like the striped dolphin, compared to one that shows high levels of dispersion among populations. Therefore, one objective of this study will be to test the hypothesis that evidence for selection associated with pathogen load will differ in striped dolphins compared to common dolphins.

Investigation of the association between heterozygosity at the DQB locus and pathogen load may be expected to show a negative correlation. However, investigation of functional components of the locus will allow the assessment of directional selection in the context of pathogen load. The former would be consistent with selection dominated by heterosis, while the latter would suggest frequency dependent or local directional selection (and both are possible). Therefore a further objective of this study is to test the hypothesis that pathogen load is associated with specific functional components of the DQB gene.

Finally, in chapter 2 there was an apparent sex-specific association between background individual diversity (as revealed by microsatellite DNA diversity) and pathogen load. Therefore, I will test the hypothesis that a sex-specific effect is also evident at the DQB locus.

4.2 Materials and Methods

4.2.1 Study areas and sample collection

One hundred and two adult individuals of striped dolphin from stranded dead animals were collected. Skin and muscle were obtained and stored in 20% DMSO or 70% ethanol for genetic analyses. Individuals were from two different geographical regions (Figure 4.1); Valencia Community (Western Mediterranean Sea, n=80) and Ireland (North Atlantic Ocean, n=22).

Ninety four individuals of common dolphin from stranded dead animals in Ireland were collected. Skin was obtained and stored in 20% DMSO or 70% ethanol for genetic analyses (Figure 4.1). Striped and common dolphin individuals were from the same locations in Ireland.

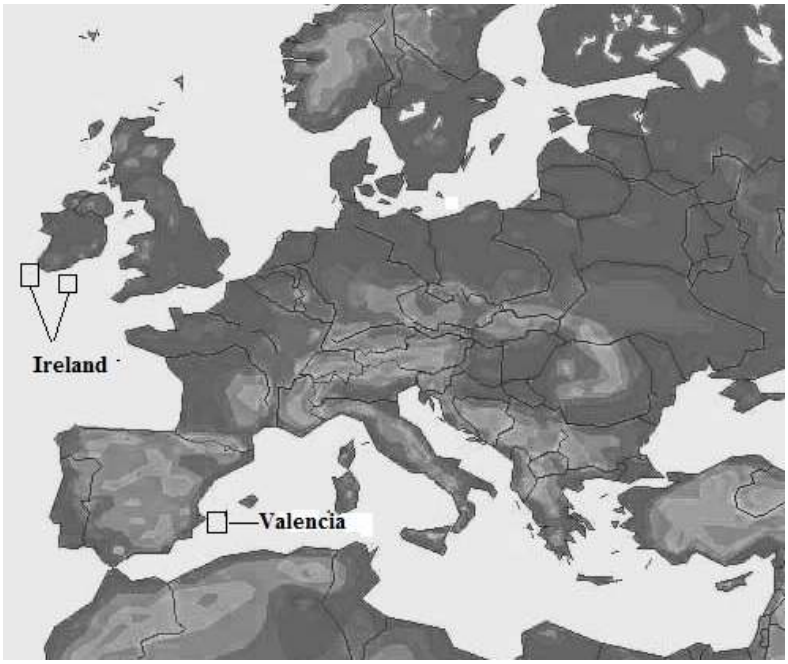


Figure 4.1. Site map locations of striped and common dolphin individuals

The majority of the striped dolphin individuals from the Mediterranean Sea died due to the high mortality of the morbillivirus in 1990 and 2007. However, further stranded samples (N=40) from the same region were collected from 1989 and 2008, and the cause of death is unknown. Similarly, striped dolphin individuals from Ireland were found stranded and the cause of death is unknown. Common dolphin individuals died between the years 1990 and 2008 and the cause of death is unknown. Necropsy for both species was carried out and internal organs were stored in -20^{o}C for parasite examination. Sex was determined either macroscopically or using genetic markers (see Chapter 3).

4.2.2 Parasite examination

Parasites were extracted from lung and stomach for the striped dolphin individuals and from lung for the common dolphin. During gross necropsy lung and stomach tissues were examined visually for the presence of parasites and associated lesions and lesion description were registered. Parasites were cleaned in an isotonic buffer and then were stored in 70% ethanol (see Chapter 3 for a detailed protocol). All individuals were screened for parasite loads and according to the level of infestation they were grouped into four categories; Uninfected, Low infected, Medium infected and High infected. Parasite count intensity parameters (skewness, mean, median, exact confidence intervals) were calculated as in Quantitative Parasitology v. 3.0 (Rozsa *et al.* 2000). A Shapiro – Wilk normality test was performed to determine if parasites count distributions were normal.

4.2.3 Molecular techniques

DNA was extracted following either the standard phenol/chloroform extraction protocol (see Chapter 2 and 3 for a detailed protocol). A highly polymorphic fragment of exon-2 MHC Class II DQB1 locus was examined. This fragment includes the functionally important antigen binding site. The exon-2 peptide binding region (PBR) was amplified using the following primers:

DQB1 F: CTGGTAGTTGTGTCTGCACAC

DBQ1 R: CATGTGCTACTTCACCTTCGG

developed by Tsuji *et al.* (1992). Reagent conditions were 10mM Tris-HCl, 50mM KCl, 2,5mM MgCl2, 0.2mM of each dNTP, 0.25μM of each primer, 2 units of high fidelity *Pfu Taq* polymerase (Promega, UK), 0.8mM DMSO 20% and 1μl of total DNA in 20μl final volume. The PCR cycling profile was an initial denaturation step at 95^{o}C for 15', following by a 30 cycles of denaturation step at 95^{o}C for 1', annealing step for 30'' and elongation at 72^{o}C for 30'' and finishing by a final elongation step at 72^{o}C for 15'. The annealing temperature for both species was 55^{o}C. PCR products were screened on an agaroze gel (1% w/v).

To identify allelic diversity all individuals were screened by Single Strand Conformation Polymorphism (SSCP) analysis. The SSCP analysis was carried out using a Bio-Rad vertical gel electrophoresis unit (Bio-Rad Labs). 2μl of PCR products were added to 2μl of denaturing loading buffer [95% (v/v) Formamide, 0.1% (w/v) Bromophenol blue, 0.1% (w/v) Xylene cyanol and 10mM NaOH; Sigma-Aldrich] and 1 μl of T.E. (10mM) and mixed thoroughly. A denaturation step of 7 minutes was carried out in a PCR thermocycler. After the denaturation samples were immediately transferred

onto ice for 3 minutes and were loaded on a non-denaturing acrylamide gel [6% (v/v) 37.5:1 acrylamide : bis-ascrylamide, 10% (v/v) glycerol, 60µl of 20% (w/v) APS, 69.2 TEMED] and 1xTBE was added to the required volume. The running time was 6 hours at 40 Watts. The gel was incubated for 30 minutes with the fluorescent GelStar™ Nucleic Acid Gel Stain (Takara, Japan). Allelic conformation was visualized by exposure to UV light. The allelic diversity for each individual was scored and genotypes were assigned (e.g. Figure 4.2).

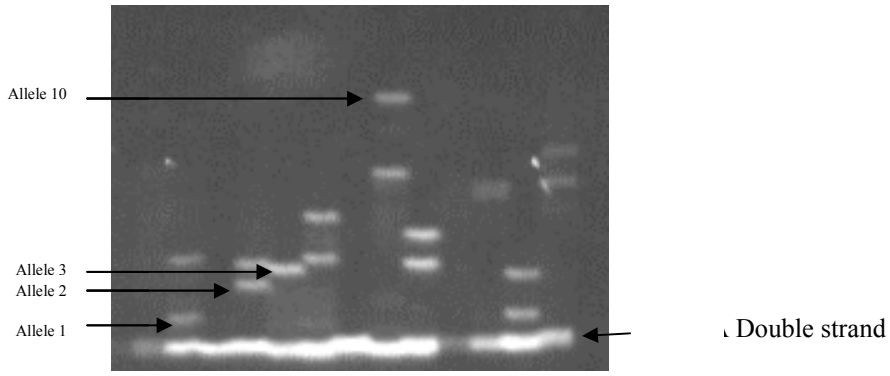


Figure 4.2. SSCP non-denaturating acrylamide gel and putative unique alleles.

After the identification of putative unique alleles, the same PCR products were loaded again onto a non-denaturing acrylamide gel (6%) and this time the bands representing unique alleles (with some replication) extracted from the gel. Gel fragments were crushed in 50µl of 10mM T.E. and incubated overnight at 37°C. One ul of the solution was then amplified by PCR (using the same concentrations and PCR profile as described before) using the high fidelity *Pfu Taq* polymerase. PCR products, prior to sequencing, were purified using a Qiagen QIAquick PCR Purification Kit™, to remove primer dimmers, unincorporated dNTPs and chemicals, according to manufacture instructions. Purified DNA was verified on 1% agaroze gel and then was sequenced on

an ABI Prism 377 automated sequencer. Each reaction was carried out using the DQB1F and DQB1R primers.

The PCR products of the putative unique alleles were cloned, using Easy T-Vector Cloning Kit (Promega) according to manufacturer instructions, in order to compare alleles and confirm that only one locus was being amplified. Up to 8 clones were screened by SSCP from different individuals.

4.2.4 Statistical analyses

A fragment of 171 bp was used in the analysis and was screened using ChromasPro v. 1.5 (Figure 4.3). Nucleotide sequences were aligned using the ClustalX v. 2.0.12 (Larkin *et al.*, 2007). BLAST v. (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi/) was used in order to confirm that DNA sequences were the result of the amplification of the exon-2 MHC Class II DQB1 locus.

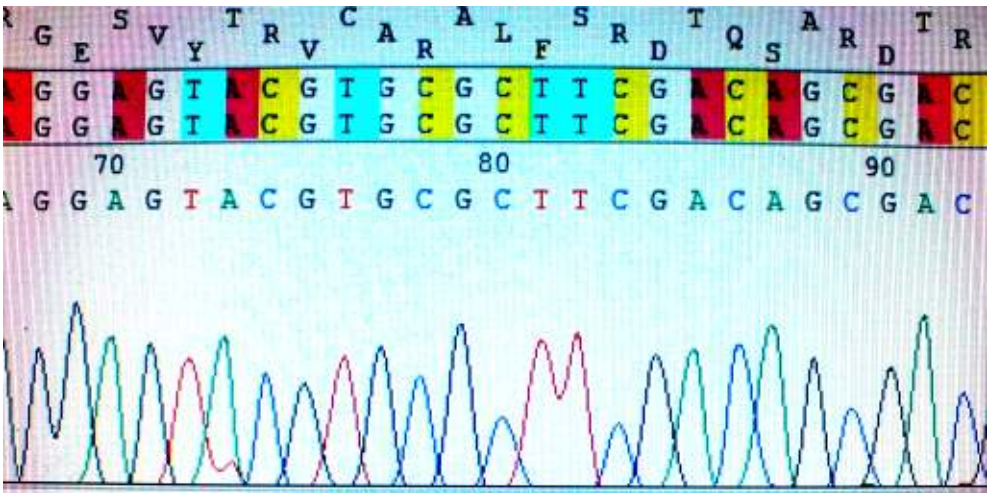


Figure 4.3. Chromatograph of MHC-DQB sequences in ChromasPro v. 1.5

Rates of non-synonymous and synonymous substitutions were calculated using the software MEGA v. 4 (Tamura *et al.*, 2007). The d_N / d_S ratio was computed according to

the Nei-Gojobori method (Nei and Gojobori, 1986). This method used the quantities of *S* (number of synonymous sites), *N* (non-synonymous sites), S_d (number of synonymous between pairs of sequences) and N_d (number of synonymous between pairs of sequences) in order to calculate the proportion if synonymous (p_s) and non-synonymous (p_n) nucleotide differences per synonymous and non-synonymous site respectively ($p_s=S_d / S$; $p_n=N_d / N$). The average estimate of number of synonymous substitutions per synonymous site and non-synonymous substitutions per non-synonymous site was calculated according to Nei-Gojobori method (1986) using the formula of Jukes – Cantor:

$$d_S = -\frac{3}{4}\ln\left(1-\frac{4}{3}ps\right), \text{ with variance } V(d_S)=\frac{ps(1-ps)}{1-(1-\frac{4}{3}ps)^2 S}$$

$$d_N = -\frac{3}{4}\ln\left(1-\frac{4}{3}pn\right), \text{with variance } V(d_N)=\frac{pn(1-pn)}{1-(1-\frac{4}{3}pn)^2 N}$$

This method have shown that these equations give an accurate estimate of d_N / d_S ration when the transition (T↔C and A↔G) and the transversion (T,C↔A,G) ratio (R) is not biased (i.e. R=1).

The charge of amino acids of P4 pocket was based on the *β70 β71 β74* residues according to physicochemical properties (Stern *et al.*, 1994). The amino acids' supertype state was determined according to the following categorization (Ou *et al.*, 1998):

(n) Neutral supertype: *F, M, W, I V, L, A, P, C, N, Q, T, Y, S, G*

(+) Positively supertype: *H, K, R*

(-) Negatively supertype: *D, E*

The total charge of each allele was the sum of each residues charge. For example if an allele was positively and negatively charge in the Pocket 4 it was classified in the di-charged supertype group.

Allele frequencies, allelic richness and F_{IS} index were estimated using the program FSTAT v. 2.9.3 (Goudet, 2001). ARLEQUIN v. 3.11 (Excoffier *et al.*, 2005) was used to estimate F_{ST}, expected (H_{EXP}) and observed (H_{OBS}) heterozygosities. The same calculations were repeated separately for female and male individuals. The Chi-square test was used to estimate statistical significance.

Contingency tables were conducted using the RxC software (www.marksgeneticsoftware.net/) running 20 batches and 2500 replicates per batch. RxC employs the metropolis algorithm to obtain an unbiased estimate of the exact p value. Contingency tables were used to compare allele profiles and charge profiles between uninfected and infected individuals. Also, calculations were performed against the different levels of infestation for the total number of individuals and against different levels of infestation and for each sex. Bonferroni corrections were calculated to deal with false discovery due to type I errors.

4.3 Results

4.3.1 Striped dolphin

4.3.1.1 Parasite load and individuals constitution

Two different parasite species were found in lungs and stomach of striped dolphin individuals; in the lungs the nematode *Skrjabinalius guevaraii* and in the stomach the gastric digenean *Pholeter gastrophilus*.

The lung-nematode is quite common in *Delphinidae* species and the only one in the striped dolphin respiratory system (Raga and Carbonell, 1985). Although the life cycle of this nematode is still unknown, it causes almost total occlusion of bronchi and bronchioles due to their physical presence to its host (Raga *et al.*, 1987b; Clausen and Andersen, 1988). Lesions associated with this lungworm include acute suppurative bronchopneumonia, acute to chronic bronchitis, endobronchitis, peribronchitis, bronchiolitis, edema, hyperplasia and hypertrophy of the mucosal epithelium and hypertrophy of peribronchiolar smooth muscle (Woodart *et al.*, 1969). All these diseases are directly linked with the death of the animal. The number of lung nematodes per individual ranged from 0 to 636.

The gastric digenean *Pholeter gastrophilus*, which has been reported in at least 17 cetacean species worldwide (Aznar *et al.*, 1992; Raga, 1994), bore into the stomach wall within the submucosal fibrotic nodules and is associated with granulomatous gastritis (Woodart *et al.*, 1969; Migaki *et al.*, 1971; Howard *et al.*, 1983). Molluscs and fishes may act as first and second intermediate hosts respectively (Gibson *et al.*, 1998). The number of gastric digeneans per individual ranged from 0 to 187.

Of the total striped dolphin sample, 43% were female and 57% were male (a sex ratio 1:1.3). The prevalence of uninfected and infected individuals carrying lungworms was 41.16% and 58.84% respectively. Out of the one hundred and two individuals, fifty three were tested for gastric digenean loads and the prevalence of uninfected and infected was 18.86% and 81.14% respectively. These fifty three individuals were also tested for lungworm and gastric digenean loads and the prevalence was 11.3% for uninfected and 88.67% for infected individuals. Table 4.1 shows the number of female and male individuals against infection and parasite species and for the combination of parasite species.

Table 4.1. Number of uninfected and infected individuals grouped by sex for each parasite species and for individuals with dual infection of both the parasite species.

	Uninfected		Infected		
	Females	Males	Females	Males	Total
Lungworms	15	27	29	31	102
Stomach gastric digenean	5	5	16	27	53
Dual infestation	4	2	17	30	53

Lungworm nematode load was further subdivided according to the level of infestation. Three groups were distinguished among the 60 infected individuals; Low infected (range: 1-20 parasites), Medium infected (range: 21-74 parasites) and High infected (range: 75-636 parasites; see Table 4.2).

Due to the small number of examined individuals for gastric digenean loads the fifty three individuals were grouped into two categories; Uninfected/Low infected (range: 0 - 20 parasites) and Medium infected/High infected (range: 75-187 parasites; see

Table 4.2) Similarly, examined individuals for lungworm and gastric digenean loads were grouped into two the same two categories (Table 4.2).

Table 4.2. Number of individuals for each of the infestation categories against parasite species and combination of the parasites.

	Uninfected	Low infected	Medium infected	High infected	Total
Lungworm load	42	19	16	25	102
gastric digenean load	33		20		53
Duel infestation load	24		29		53

4.3.1.2 MHC variability

The 102 striped dolphins showed high levels of variability in the exon-2 MHC Class II DQB1 locus. Cloning sequencing revealed no more than two sequences in each individual. Twenty three different alleles were found and among these 21 had a unique amino acid composition (Appendix, Table 6.5). Therefore 21 unique alleles were used for the analysis. Alleles were named Sc-DQB*1 to Sc-DQB*21. The comparison between each of the alleles to the complete sequence database in Genebank (MegaBlast) revealed sequence homology from 95% of up to 100% with published *Stenella coeruleoalba* MHC Class II DQB sequences (Xu *et al.*, 2009). Homologies were also revealed with other marine mammals species up to 99% such is Sc-DQB*17 and Sc-DQB*19 with Dede-a (*Delphinus delphis*; Hayashi *et al.*, 2003). The Sc-DQB*03 was 100% homologous with Stco-DQB*05 (*Stenella coeruleoalba*; Xu *et al.*, 2009), Stat-DQB*01 (*Stenella attenuata*; Xu *et al.*, 2009) and Grgr-DQB*01 (*Grampus griseus*; Xu *et al.*, 2009).

Allele frequencies ranged from 0.11 (Sc-DQB*01) to 0.02 (Sc-DQB*20) for both populations combined of striped dolphin. The allelic richness was 18.42 for Valencia and 18 for Ireland. Both populations showed a PBR d_N / d_S ratio that was significantly greater than 1, with the Ireland population showing the strongest effect (Table 3.1.3). Valencia showed a significant excess of observed heterozygotes with a negative F_{IS} value. The Ireland population showed a significant deficit of heterozygotes. The MHC F_{ST} value between the two populations was not significant (Table 4.3)

Table 4.3. Diversity and Selection Parameters of striped dolphin populations and MHC F_{ST} value between the two populations. No: number of individuals, A: number of alleles, R: allelic richness, H_{OBS}: Observed Heterozygosity, H_{EXP}: Expected Heterozygosity, dn/ds: non-synonymous synonymous ratio.

	Valencia	Ireland		MHC F_{ST} value	
No	80	22		Valencia	Ireland
A	21	18	Valencia	0	
R	18.42	18	Ireland	0.001^{NS}	0
H_{OBS}	0.98	0.90			
H_{EXP}	0.95	0.92			
F_{IS}	-0.038	0.02			
d_n / d_s	3.88^{*}	14.5^{***}			

The amino acid residues profile in Pocket 4 of the PBR showed negatively (-), positively (+) and di-charged (+/-) supertypes, but no neutral supertypes among the 21 MHC alleles (Appendix: Table 6.3). Throughout the 21 alleles 41.66% are classified as negative (-), 9.3% positive (+) and 49.04% as di-charged (+/-). The charge profiles for Valencia and Ireland striped dolphin populations are shown in Figure 4.5.

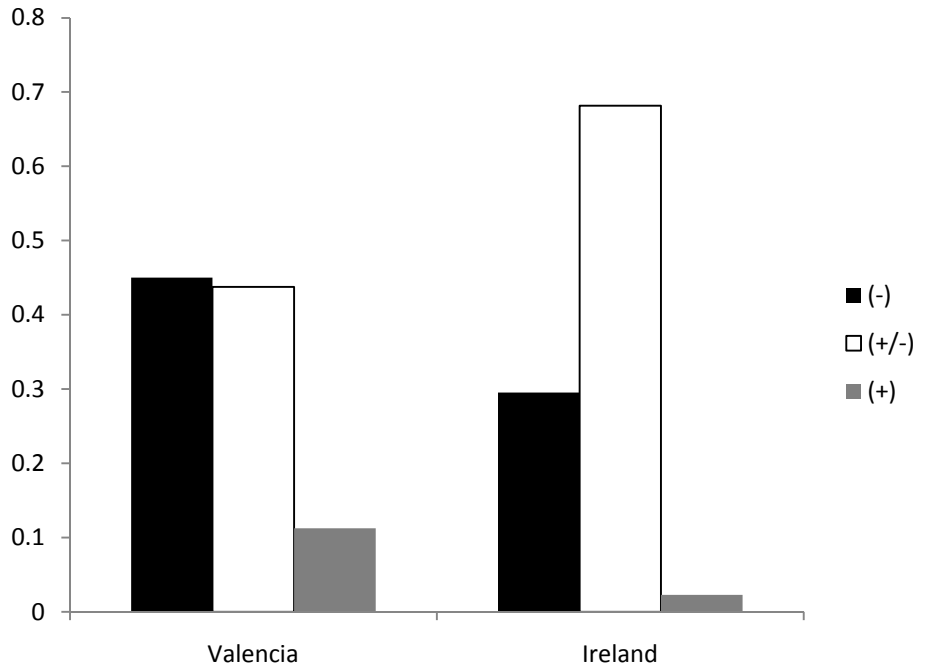


Figure 4.5. Charges of amino acid residues in Pocket 4 (β70, β71 and β74) in Valencia (N=80) and Ireland (N=22) striped dolphin populations

4.3.1.3 Parasite loads and MHC variability

i) Lungworm loads

Uninfected and infected individuals examined for lungworms shared the same number of alleles. Sc-DQB*17 and Sc-DQB*20 were absent from uninfected individuals and Sc-DQB*11 and Sc-DQB*21 were absent from infected individuals. Performing contingency tables in RxC software the association between uninfected and infected individuals with regards to total number of alleles shows a significant difference (p=0.002, S.E.=00008). Diversity analysis showed that only uninfected individuals favoured a significant excess of heterozygotes whereas infected individuals showed a significant deficit of heterozygotes. The PBR d_N / d_S ratio was significantly greater than 1, with the uninfected individuals showing the strongest effect (Table 4.4).

Table 4.4. Diversity and Selection Parameters for the different levels of infestation in the striped dolphin. (No: Number of individuals, A: number of alleles, R: allelic richness, H_{OBS}: observed heterozygosity, H_{EXP}: expected heterozygosity, F_{IS}: inbreeding coefficient, *dn/ds*: non-synonymous synonymous ratio, ***:p<0.001).

	Uninfected	Infected	Low Infected	Medium Infected	High Infected
No	42	60	19	16	25
A	19	19	14	14	17
R	12.253	13.67	12.711	14	14.328
H_{OBS}	0.94	0.82	0.78	0.81	0.88
H_{EXP}	0.92	0.94	0.94	0.94	0.96
F_{IS}	-0.02	0.13	0.176	0.143	0.084
d_n / d_s	44.33^{***}	12.79^{***}	17^{***}	11.38^{***}	10^{***}

Association of parasite loads and MHC alleles showed a significant difference in Sc-DQB*11 (χ^2=5.14, p=0.02) and Sc-DQB*21 (χ^2=5.78, p=0.01) in uninfected individuals and Sc-DQB*17 (χ^2=5.83, p=0.01) and Sc-DQB*19 (χ^2=4.41, p=0.03) in infected individuals (Figure 4.6). However, the significance did not remain after Bonferroni correction.

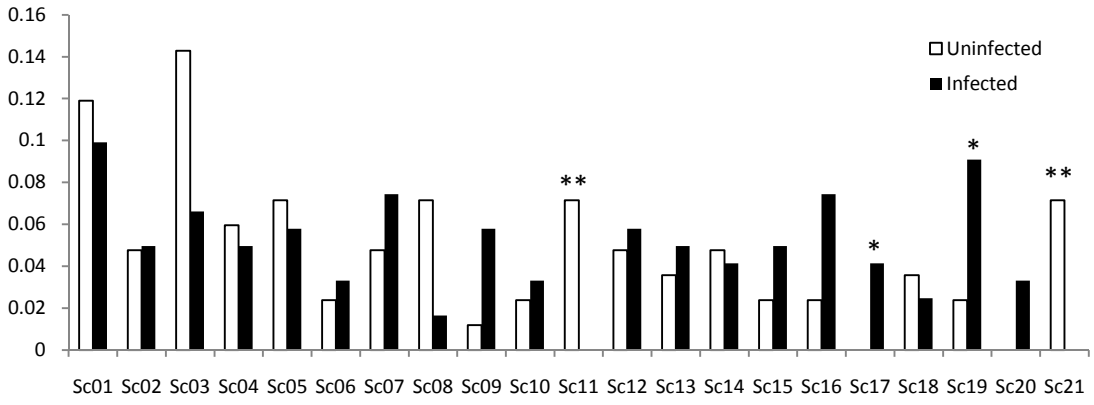


Figure 4.6. Frequency of Sc-DQB alleles in uninfected (white bars) and infected (black bars) striped dolphin individuals (N=102) examined for lungworm loads (**: p<0.01, *: p<0.05; before Bonferroni correction)

Performing contingency tables in RxC software the association between uninfected and infected individuals with regards to number of alleles and gender show a significant difference (p=0.04, S.E.=0.081). Therefore an extra analysis was performed for associations to parasite loads and MCH alleles with regard the sex (Figure 4.7 and Figure 4.8). The alleles Sc-DQB*03 (χ^2=14.19, p=0.03; after Bonferroni correction) and Sc-DQB*16 (χ^2=12.76, p=0.02; after Bonferroni correction) were significant after Bonferroni correction only in female individuals.

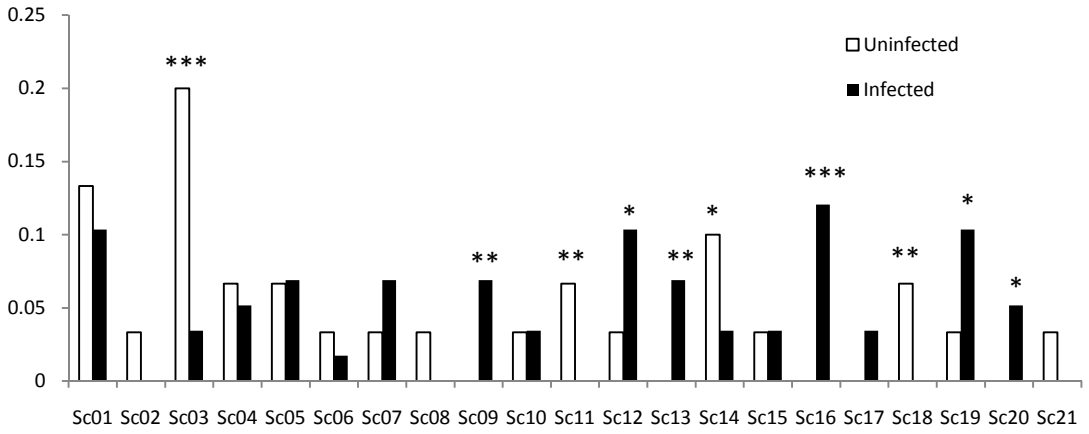


Figure 4.7. Frequency of Sc-DQB alleles in uninfected (white bars) and infected (black bars) female striped dolphin individuals (N=44) examined for lungworm loads (***: p<0.001, **: p<0.01, *: p<0.05; before Bonferroni correction)

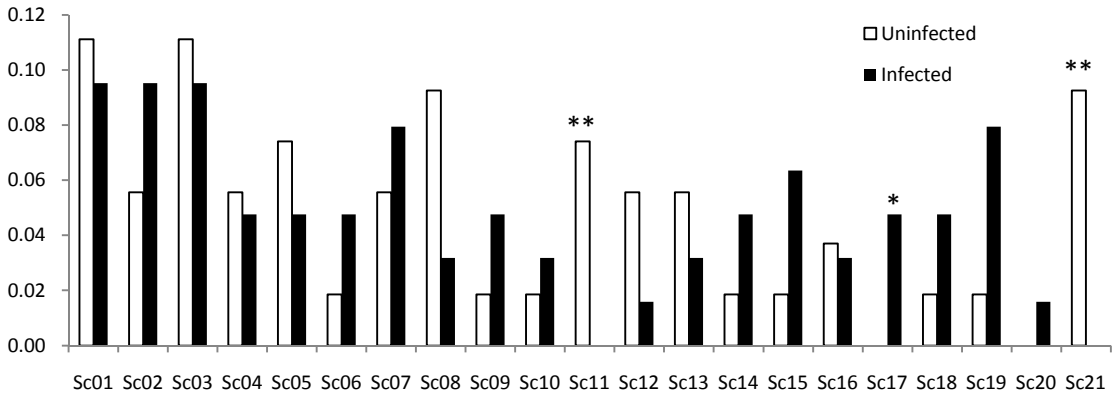


Figure 4.8. Frequency of Sc-DQB alleles in uninfected (white bars) and infected (black bars) male striped dolphin individuals (N=58) examined for lungworm loads (**: p<0.01, *: p<0.05; before Bonferroni correction)

Among the MHC alleles of uninfected individuals 38% are classified as negative (-), 7% positive (+) and 54% as di-charged (+/-) whereas among infected individuals 44% are classified as negative (-), 11% positive (+) and 45% as di-charged (+/-) (Figure 4.9). Amino acid charge profiles were calculated according to sex (Table 4.5) and charge frequencies according to sex are shown in Figures 4.10 and 4.11.

Table 4.5. Amino acid charge profile in Pocket 4 for uninfected and infected individuals grouped by sex.

	(-)	(+)	(+/-)
Uninfected Females	37%	3%	60%
Infected Females	41.3%	17.3%	41.4%
Uninfected Males	38%	9%	51%
Infected Males	46.7%	4.8%	48.3%

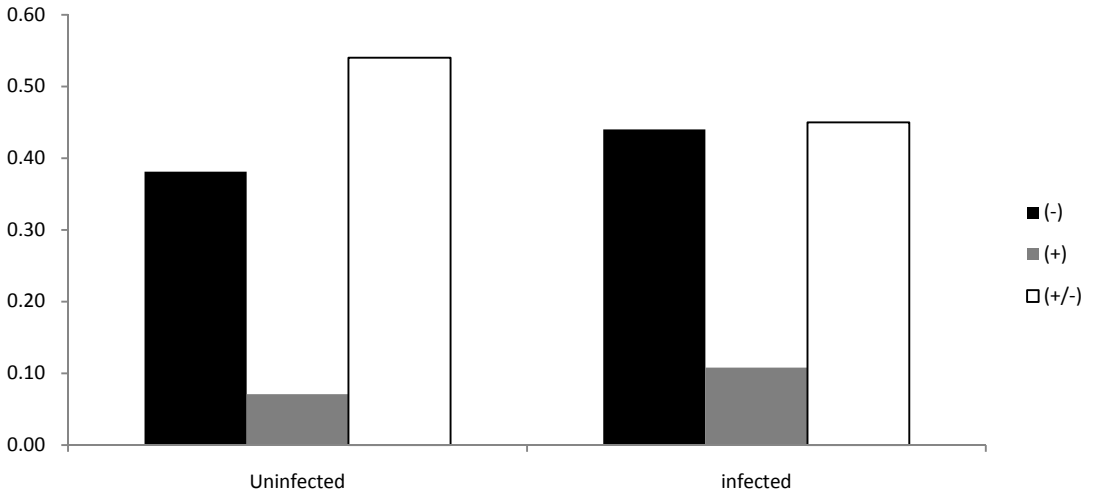


Figure 4.9. Charges of amino acid residues in Pocket 4 of uninfected and infected striped dolphin individuals (N=102) examined for lungworm loads (no significant after Bonferroni correction)

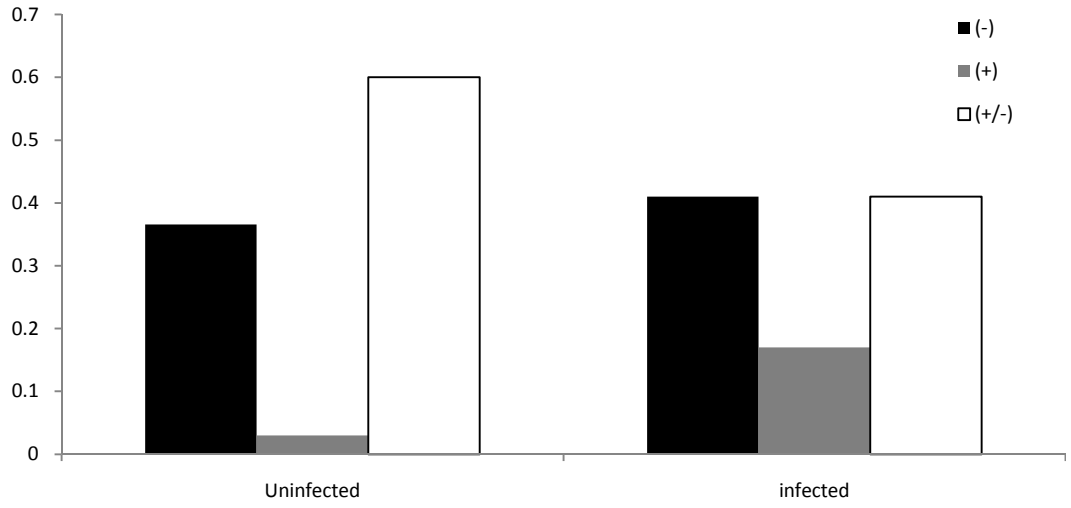


Figure 4.10. Charges of amino acid residues in Pocket 4 of uninfected and infected female striped dolphin individuals (N=44) examined for lungworm loads (no significant after Bonferroni correction)

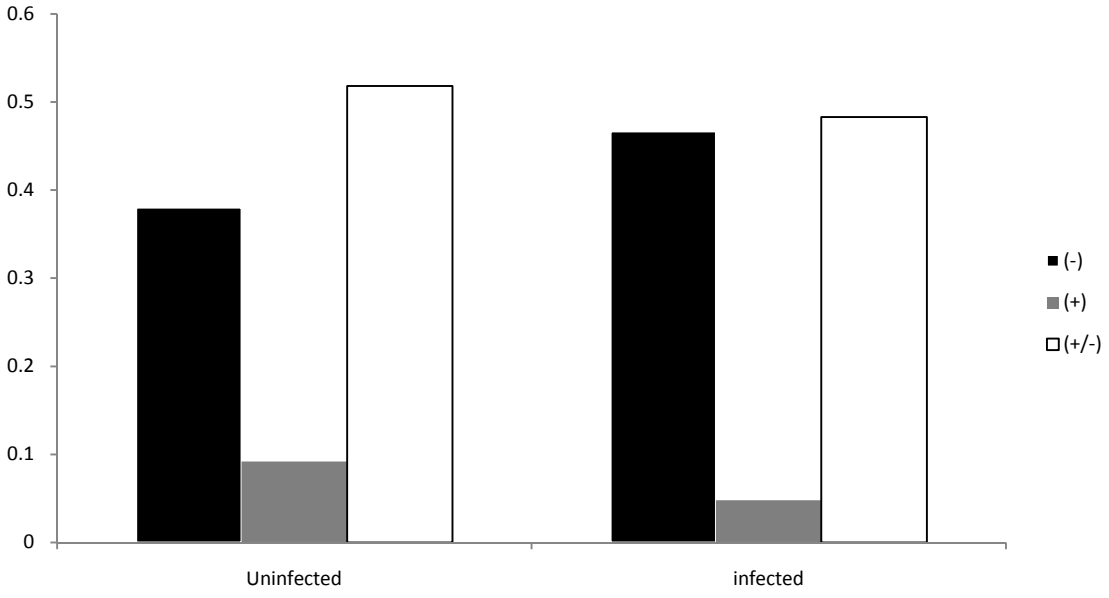


Figure 4.11. Charges of amino acid residues in Pocket 4 of uninfected and infected male striped dolphin individuals (N=58) examined for lungworm loads (no significant after Bonferroni correction)

Charge profiles for Uninfected/Low infected were classified 37.4% as negative (-), 7.9% positive (+) and 54.7% as di-charged (+/-) whereas in infected individuals 48.7% were classified as negative (-), 11.5% positive (+) and 39.8% as di-charged (+/-) (Figure

4.12). The amino acid charge profile was calculated according to sex (Table 4.6) and charge frequencies according to sex are shown in Figures 4.13 and 4.14 for Uninfected/Low infected and Medium/High infected categories.

Table 4.6. Amino acid charge profile in Pocket 4 for Uninfected/Low infected and Medium/High infected categories grouped by sex.

	(-)	(+)	(+/-)
Uninfected/Low infected Females	35.5%	8.3%	56.2%
Medium/High Infected Females	45%	17.5%	37.5%
Uninfected/Low infected Males	38.5%	7.7%	53.8%
Medium/High Infected Males	52.6%	5.2%	42.2%

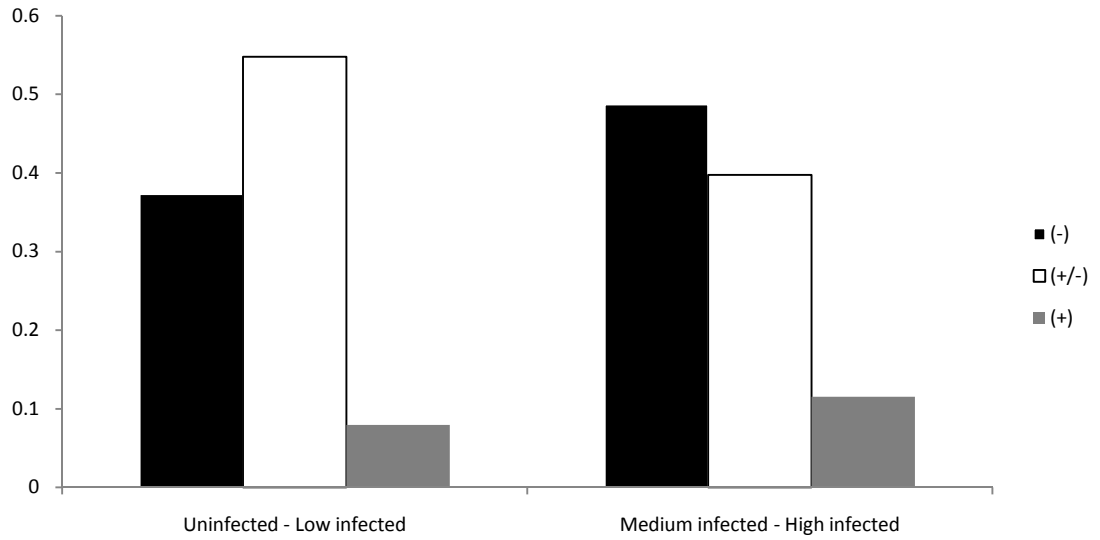


Figure 4.12. Charges of amino acid residues in Pocket 4 of uninfected/Low Infected (N=61) and Medium infected/High Infected (N=41) striped dolphin individuals examined for lungworm loads

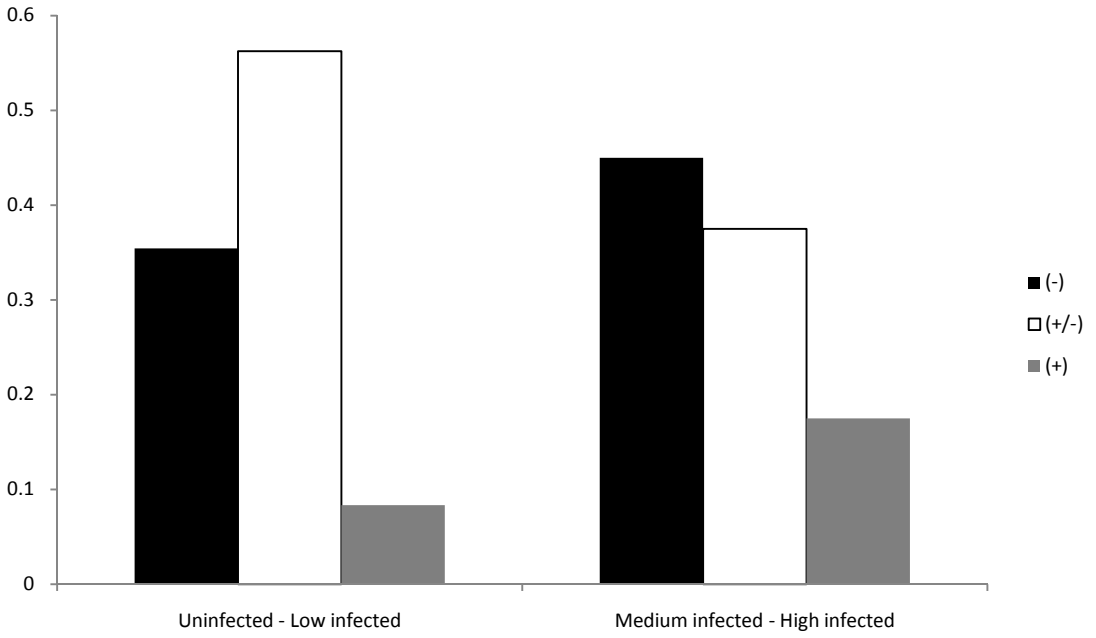


Figure 4.13. Charges of amino acid residues in Pocket 4 of uninfected/Low Infected (N=24) and Medium infected/High Infected (N=20) female striped dolphin individuals examined for lungworm loads

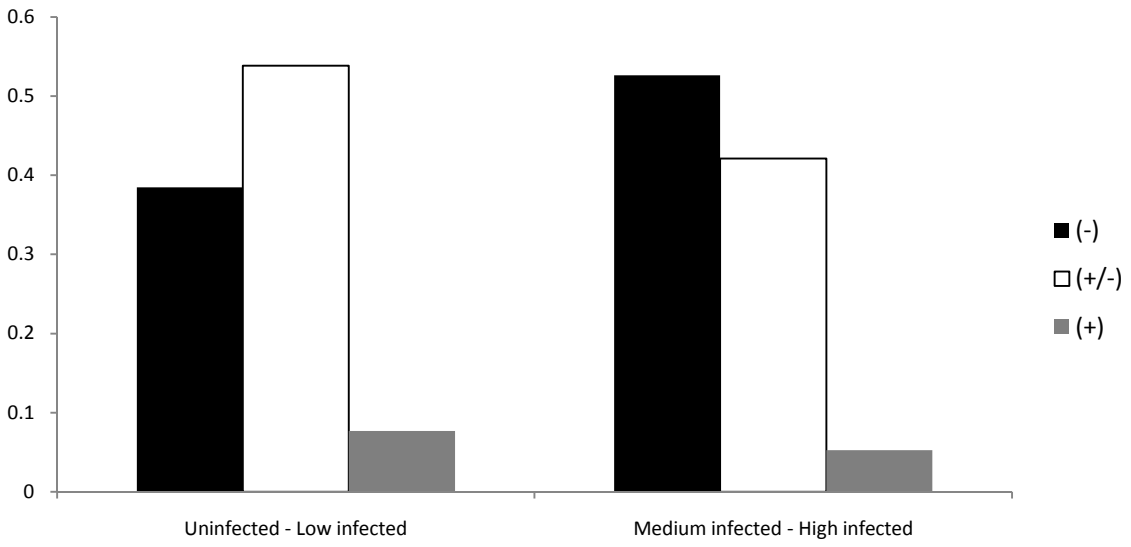


Figure 4.14. Charges of amino acid residues in Pocket 4 of uninfected/Low Infected (N=39) and Medium infected/High Infected (N=19) male striped dolphin individuals examined for lungworm loads

Performing General Linear Model the minimal adequate model used the number of alleles, the charge profile and the sex as factors with the parasite load as response variable (Table 4.7).

Table 4.7. General Linear Model; Model: Allele*Sex, Charge*Sex, Response variable: lungworm parasite load

	DF	F	P
Allele	20	3.20	0.000
Sex	1	0.50	0.481
Allele*Sex	20	1.12	0.336
Error	164		
Total	205		
S = 100.307	R-Sq = 37.07%	R-Sq(adj) = 21.33%	

	DF	F	P
Charge	2	1.14	0.322
Sex	1	0.08	0.781
Charge*Sex	2	1.26	0.285
Error	200		
Total	205		
S = 113.193	R-Sq = 2.27%	R-Sq(adj) = 0.00%	

ii) Stomach gastric digenean loads

For the 53 striped dolphin individuals that were examined for gastric digenean loads, Uninfected/Low infected and Medium/High infected individuals shared different numbers of alleles. Sc-DQB*06, Sc-DQB*09, Sc-DQB*16 and Sc-DQB*20 were absent from infected individuals. Performing contingency tables in RxC software the association between uninfected/low infected and medium/high infected individuals with regards to total number of alleles show a significant difference ($p<0.001$, S.E.=0000). Diversity analysis showed that only uninfected/Low infected individuals showed a significant excess of heterozygotes whereas Medium/High infected individuals showed a deficit of heterozygotes. The PBR d_N / d_S ratio was significantly greater than 1, with the uninfected/Low infected individuals showing the strongest effect (Table 4.8).

Table 4.8. Diversity and Selection Parameters for the different levels of gastric digenean infestation in striped dolphin. (No: Number of individuals, A: number of alleles, R: allelic richness, H_{OBS}: observed heterozygosity, H_{EXP}: expected heterozygosity, F_{IS}: inbreeding coefficient, *dn/ds*: non-synonymous synonymous ratio, ***:p<0.001).

	Uninfected – Low infected	Medium – High Infected
No	33	20
A	21	17
R	18	17.4
H_{OBS}	1	0.94
H_{EXP}	0.95	0.95
F_{IS}	-0.047	0.014
d_n / d_s	20.28^{**}	10.5^{**}

Association of parasite loads and MHC alleles show a significantly different prevalence of Sc-DQB*06 (χ^2=4.08, p=0.04), Sc-DQB*09 (χ^2=7.25, p=0.02) and Sc-DQB*16 (χ^2=6.18, p=0.01) for low parasite loads and Sc-DQB*07 with high parasite loads (Figure 4.15). However, no significance was remained after Bonferroni corrections.

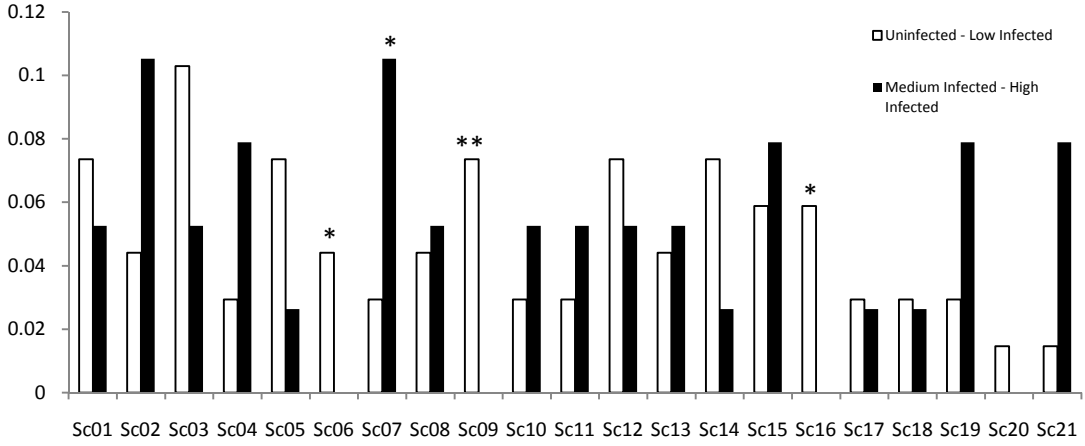


Figure 4.15. Frequency of Sc-DQB alleles of uninfected/Low Infected (white bars) (N= 34) and Medium infected/High Infected (black bars) (N=19) striped dolphin individuals examined for gastric digenean loads

Performing contingency tables in RxC software the association between uninfected and infected individuals with regards to the number of alleles and gender showed a

significant difference ($p<0.001$, S.E.=0.000). Therefore extra analyses were performed for associations to parasite loads and MHC alleles with regard to the sex (Figure 4.16 and Figure 4.17). The alleles Sc-DQB*03 ($\chi^2=10.53$, $p=0.04$; after Bonferroni correction) and Sc-DQB*19 ($\chi^2=18.57$, $p= 0.01$; after Bonferroni correction) were significant associated with parasite resistance in female striped dolphins

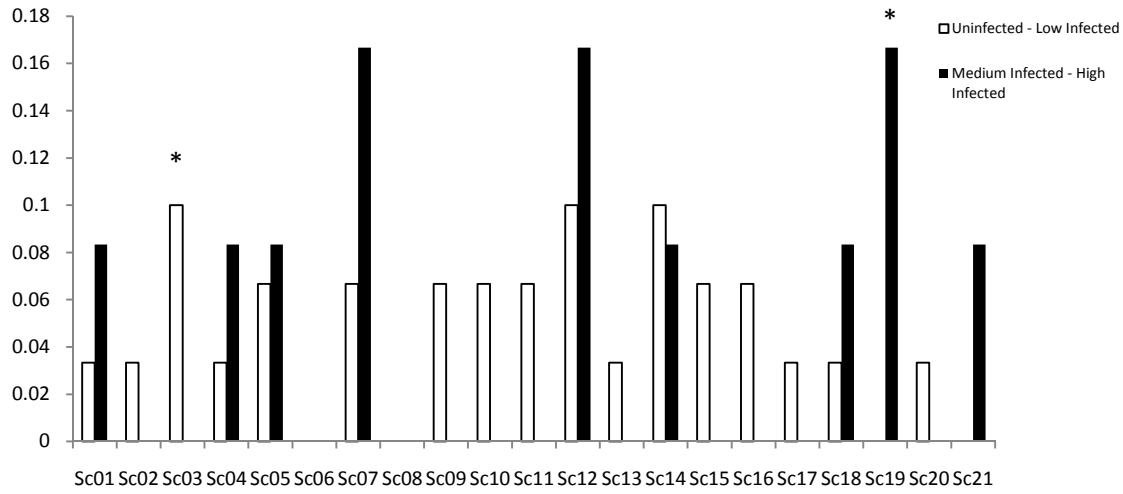


Figure 4.16. Frequency of Sc-DQB alleles of uninfected/Low Infected (white bars) (N=15) and Medium infected/High Infected (black bars) (N=6) female striped dolphin individuals examined for gastric digenean loads

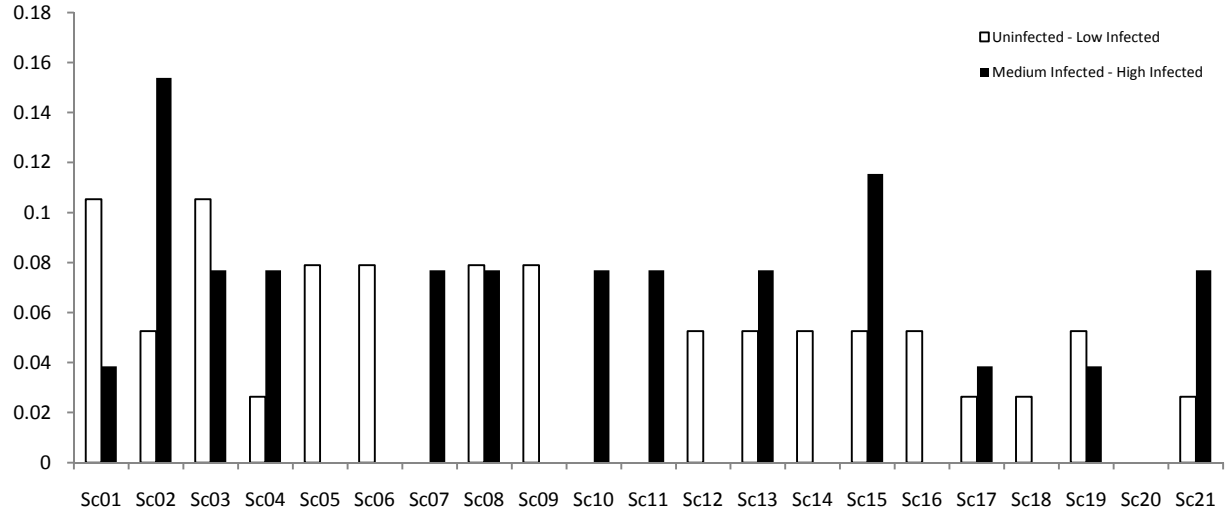


Figure 4.17. Frequency of Sc-DQB alleles of uninfected/Low Infected (white bars) (N=19) and Medium infected/High Infected (black bars) (N=13) male striped dolphin individuals examined for gastric digenean loads

Charge profiles for Uninfected/Low infected are classified 42.7% as negative (-), 11.7% positive (+) and 45.6% as di-charged (+/-) whereas for Medium/High infected

individuals 55.2% are classified as negative (-), 10.6% positive (+) and 34.2% as discharged (+/-) (Figure 4.18).

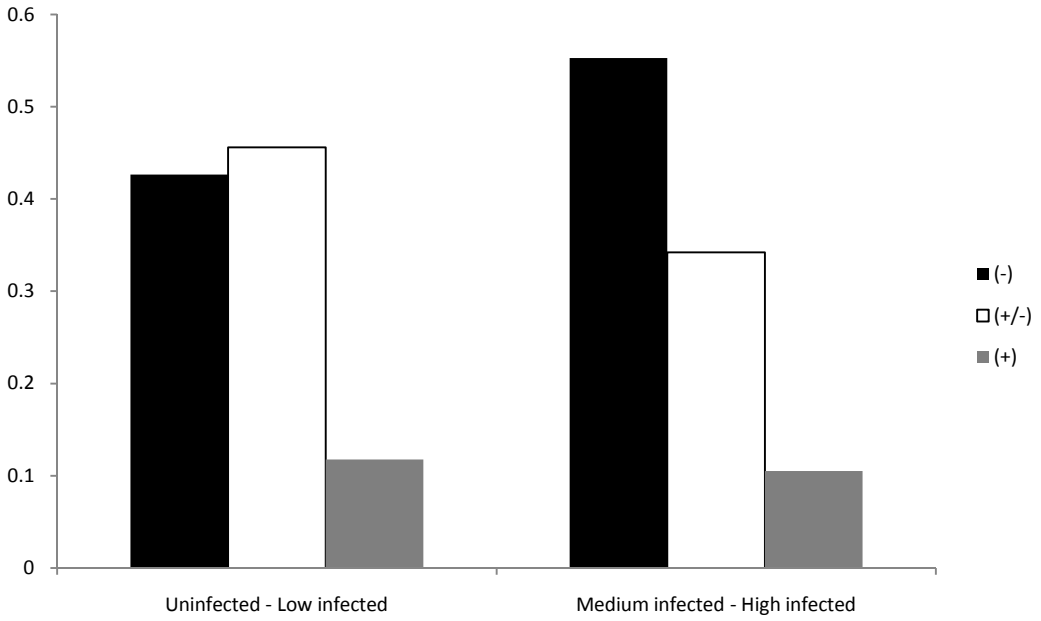


Figure 4.18. Charges of amino acid residues in Pocket 4 of uninfected/Low Infected (N=34) and Medium infected/High Infected N=19) striped dolphin individuals examined for gastric digenean loads

Amino acid charge profiles were calculated according to sex (Table 4.9) and charge frequencies according to sex are shown in Figures 4.19 and 4.20 for Uninfected/Low infected and Medium/High infected categories.

Table 4.9. Amino acid charge profile in Pocket 4 for Uninfected/Low infected and Medium/High infected categories grouped by sex.

	(-)	(+)	(+/-)
Uninfected/Low infected Females	50%	13.3%	36.7%
Medium/High Infected Females	33.3%	16.7%	50%
Uninfected/Low infected Males	36.8%	10.6%	52.6%
Medium/High Infected Males	65.4%	7.7%	26.9%

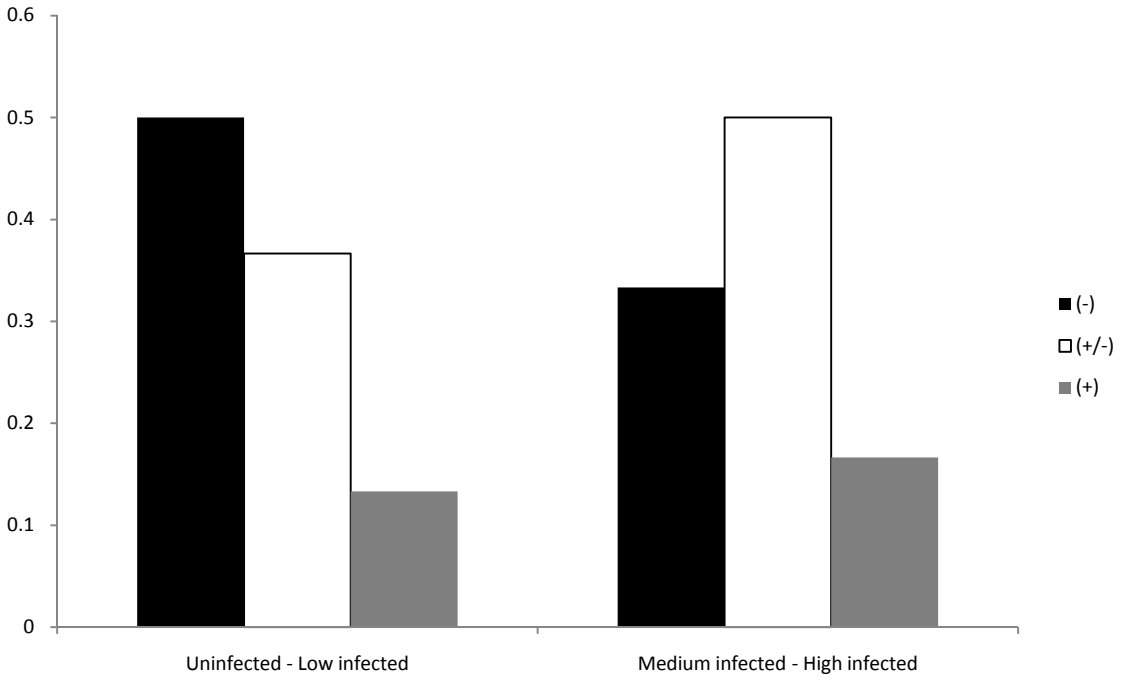


Figure 4.19. Charges of amino acid residues in Pocket 4 of uninfected/Low Infected (N=15) and Medium infected/High Infected (N=6) female striped dolphin individuals examined for gastric digenean loads

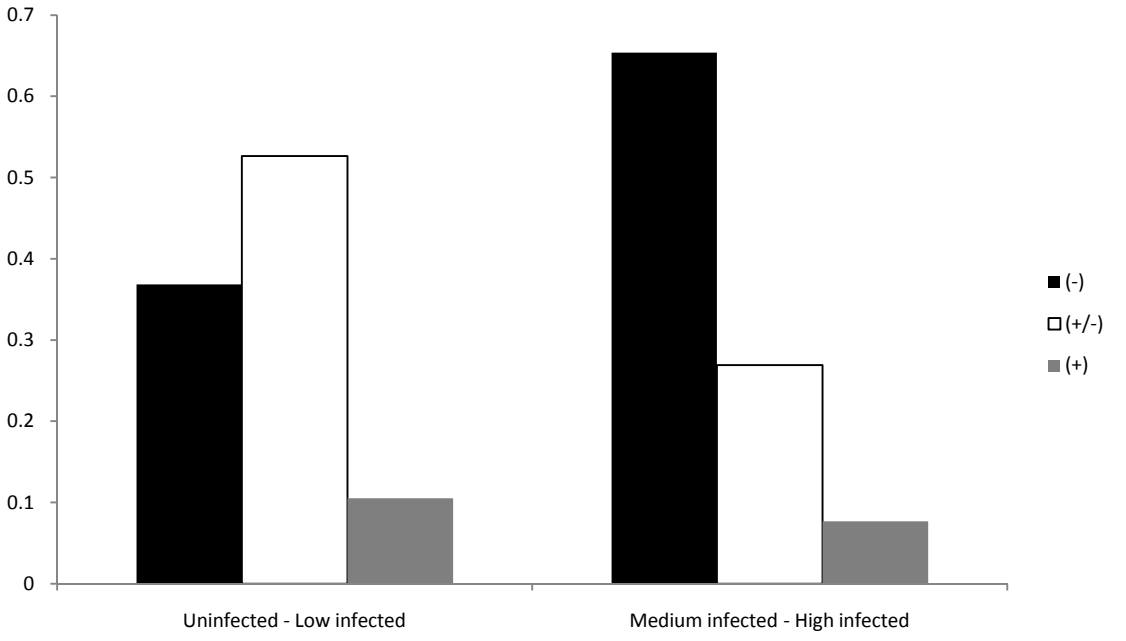


Figure 4.20. Charges of amino acid residues in Pocket 4 of uninfected/Low Infected (N=19) and Medium infected/High Infected (N=13) male striped dolphin individuals examined for gastric digenean loads

Performing General Linear Model the minimal adequate model used the charge profile and the sex as factors with the parasite load as response variable (Table 4.10).

Table 4.10. General Linear Model; Model:charge profile and sex, Response variable: gastric digenean load

Source	DF	F	P
Charge	2	0.71	0.494
Sex	1	5.86	0.017
charge*Sex	2	1.23	0.296
Error	100		
Total	105		
S = 41.5289	R-Sq = 9.77%		R-Sq(adj) = 5.26%

iii) Lungworm and gastric digenean loads

For striped dolphins examined for a combination of lungworm and gastric digenean loads Uninfected/Low infected and Medium/High infected individuals showed different number of alleles. Sc-DQB*06 and Sc-DQB*20 were absent from Medium/High infected individuals. Performing contingency tables in RxC software the association between uninfected/low infected and Medium/High infected individuals with regards to total number of alleles did not show any significant difference. Diversity analysis showed that both uninfected/Low infected individuals and Medium/High infected individuals showed a significantly deficit of heterozygotes. The PBR d_N / d_S ratio was significantly greater than 1, with the uninfected/Low infected individuals showing the strongest effect (Table 4.11).

Table 4.11. Diversity and Selection Parameters for Uninfected/Low infected and Medium/High infected individuals examined for lungworm and gastric digenean loads in striped dolphin. (No: Number of individuals, A: number of alleles, R: allelic richness, H_{OBS}: observed heterozygosity, H_{EXP}: expected heterozygosity, F_{IS}: inbreeding coefficient, *dn/ds*: non-synonymous synonymous ratio, ***:p<0.001).

	Uninfected - Low Infected	Medium-High Infected
No	24	29
A	19	20
R	13.55	13.14
H_{OBS}	0.82	0.87
H_{EXP}	0.95	0.95
F_{IS}	-0.048	-0.019
d_n / d_s	24.33	11.81

Association of parasite loads and MHC alleles showed a significant difference for prevalence of Sc-DQB*01 (χ^2=4.03, p=0.04), Sc-DQB*03 (χ^2=3.9, p=0.04) and Sc-DQB*06 (χ^2==6.18, p=0.01) with low parasite loads (Figure 4.21). After Bonferroni corrections no significant prevalence detected.

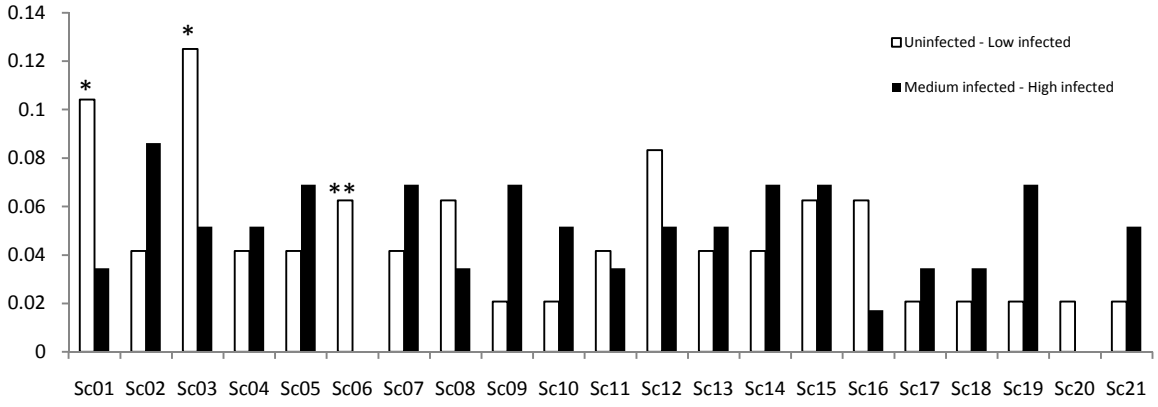


Figure 4.21. Frequency of Sc-DQB alleles in Uninfected/Low infected (white bars) (N=34) and Medium/High infected (black bars) (N=19) striped dolphin individuals examined for lungworm and stomach digenean loads

Performing contingency tables in RxC software the association between uninfected and infected individuals with regards to the number of alleles and gender showed a

significant difference ($p<0.001$, S.E.=0.000). Therefore extra analyses were performed for associations to parasite loads and MCH alleles for each sex (Figure 4.22 and Figure 4.23). In female individuals 2 alleles (Sc-DQB*09 and Sc-DQB*19; $\chi^2=10.52$, p=0.02 and $\chi^2=10.54$, p=0.02 respectively, after Bonferroni corrections) showed significant association with high parasite loads, while in male individuals 1 allele (Sc-DQB*06; $\chi^2=12.7$, p=0.02, after Bonferroni correction) showed significant association with low infection.

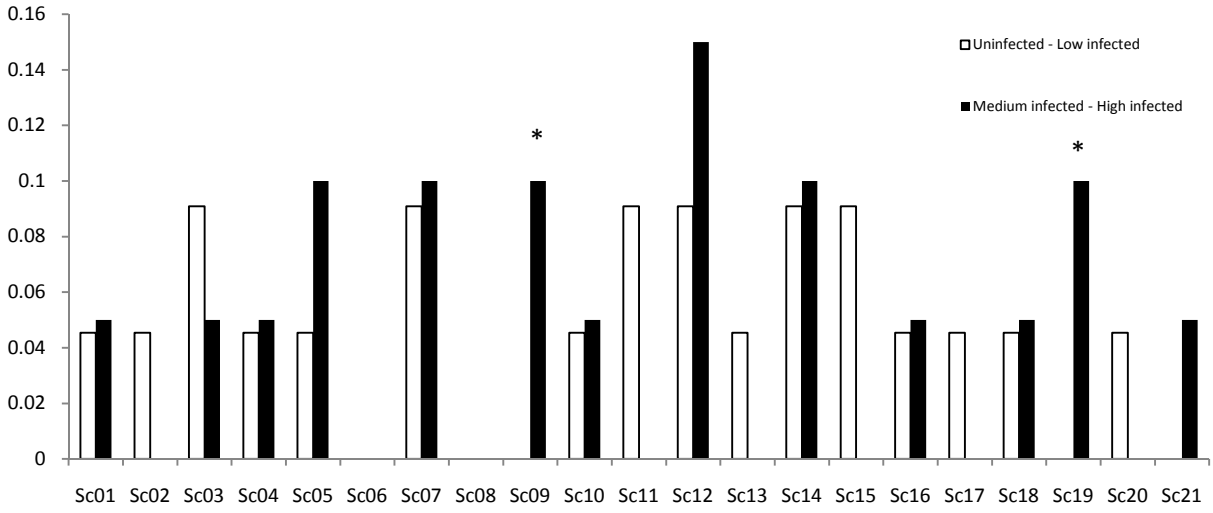


Figure 4.22. Frequency of Sc-DQB alleles in Uninfected/Low infected (white bars) (N=15) and Medium/High infected (black bars) (N=6) female striped dolphin individuals examined for lungworm and stomach digenean loads

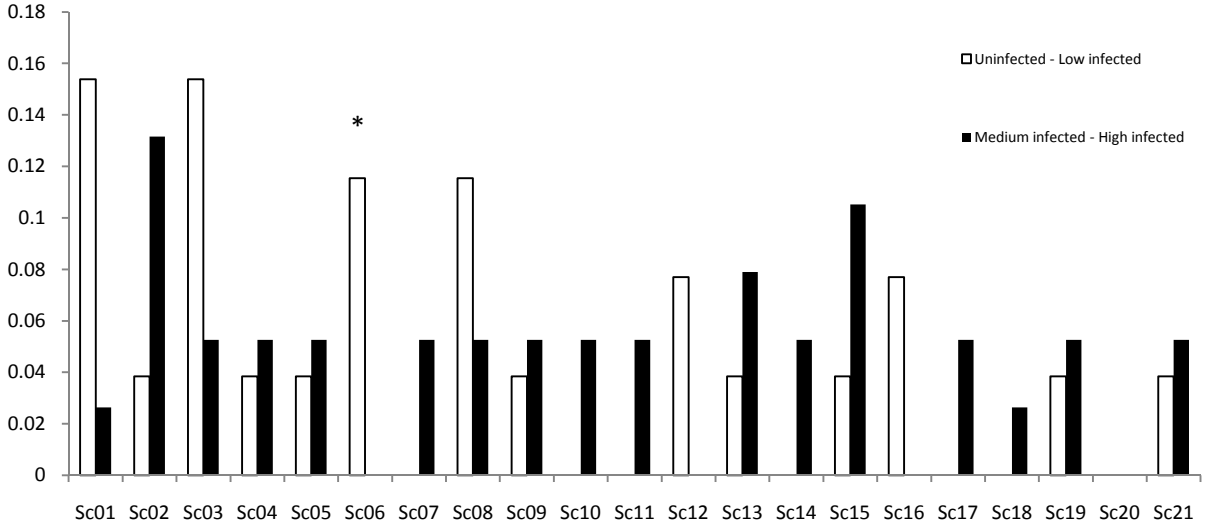


Figure 4.23. Frequency of Sc-DQB alleles in Uninfected/Low infected (white bars) (N=19) and Medium/High infected (black bars) (N=13) male striped dolphin individuals examined for lungworm and stomach digenean loads

Charge profiles for Uninfected/Low infected are classified 35.4% as negative (-), 12.5% positive (+) and 52.1% as di-charged (+/-) whereas for Medium/High infected individuals 56.8% are classified as negative (-), 10.5% positive (+) and 32.7% as di-charged (+/-) (Figure 4.24).

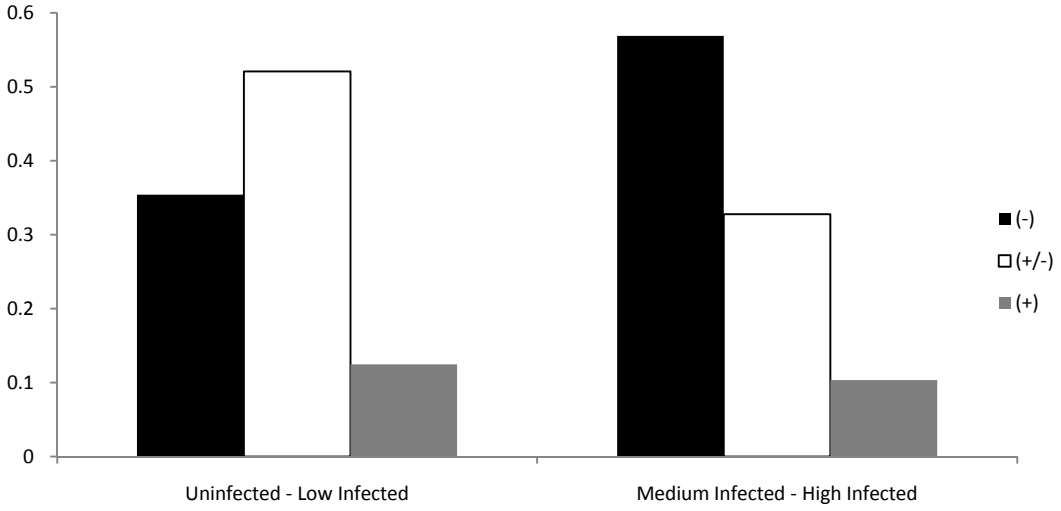


Figure 4.24. Charges of amino acid residues in Pocket 4 of uninfected/Low Infected (N=34) and Medium infected/High Infected (N=19) striped dolphin individuals examined for lungworm and stomach digenean loads.

Amino acid charge profiles were calculated according to sex (Table 4.12) and charge frequencies according to sex are shown in Figures 4.25 and 4.26 for Uninfected/Low infected and Medium/High infected categories.

Table 4.12. Amino acid charge profile in Pocket 4 for Uninfected/Low infected and Medium/High infected categories grouped by sex.

	(-)	(+)	(+/-)
Uninfected/Low infected Females	50%	13.7%	36.3%
Medium/High Infected Females	40%	15%	45%
Uninfected/Low infected Males	23%	11.5%	65.5%
Medium/High Infected Males	65.8%	7.9%	26.3%

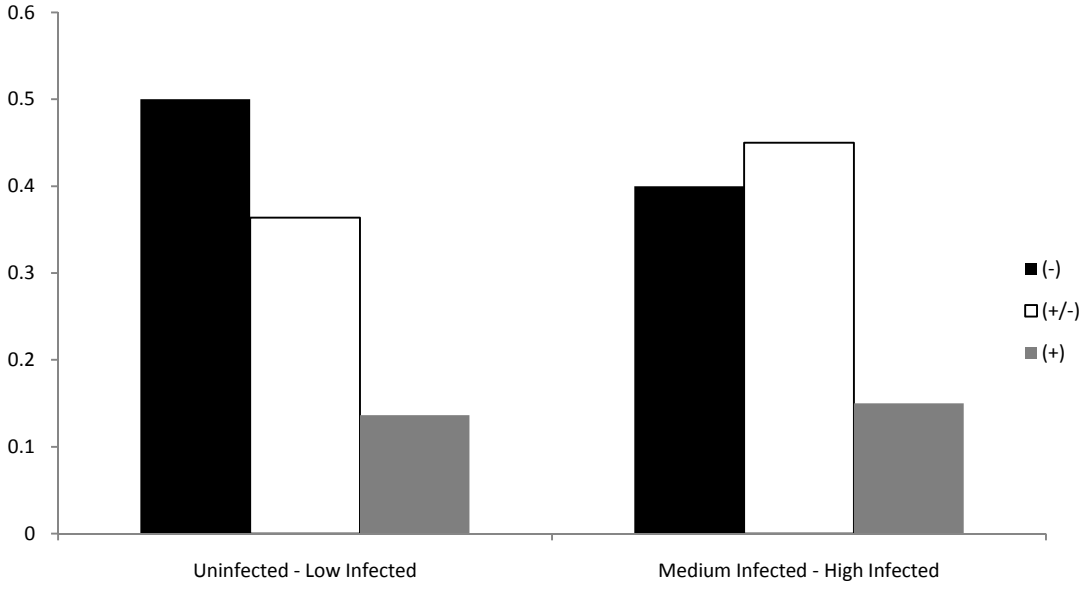


Figure 4.25. Charges of amino acid residues in Pocket 4 of uninfected/Low Infected (N=15) and Medium infected/High Infected (N=6) female striped dolphin individuals examined for lungworm and stomach digenean loads

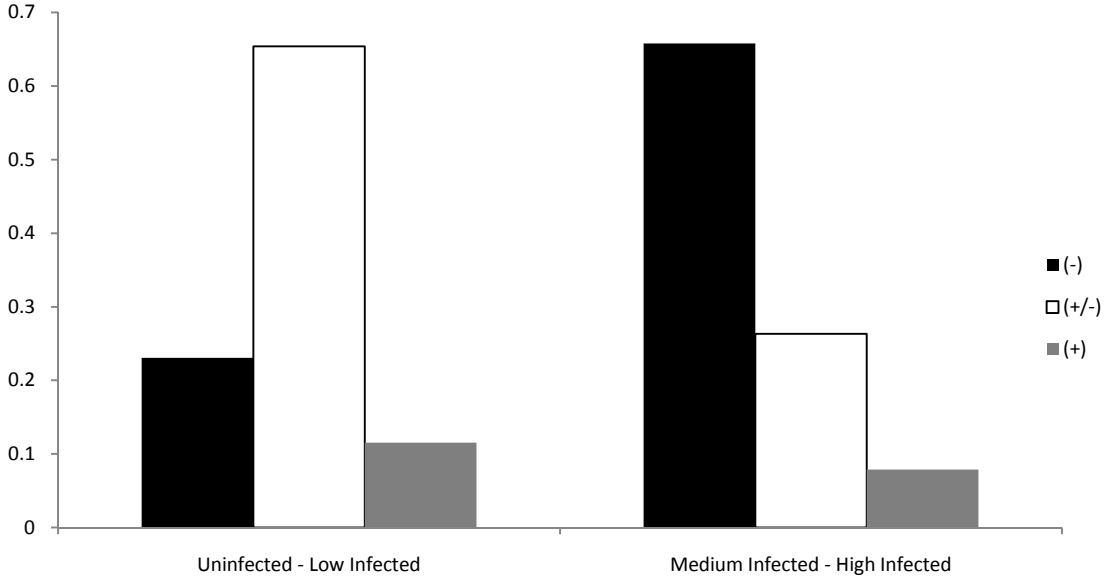


Figure 4.26. Charges of amino acid residues in Pocket 4 of uninfected/Low Infected (N=19) and Medium infected/High Infected (N=13) male striped dolphin individuals examined for lungworm and stomach digenean loads

Performing General Linear Model the minimal adequate model used the charge profile and the sex as factors with the parasite load as response variable (Table 4.13).

Table 4.13. General Linear Model; Model:charge profile and sex, Response variable: gastric digenean load

Source	DF	F	P
Charge	2	0.17	0.843
Sex	1	1.50	0.223
charge*Sex	2	0.16	0.854
Error	100		
Total	105		
S = 83.3275	R-Sq = 3.62%	R-Sq(adj) = 0.00%	

4.3.2 Common dolphin

4.3.2.1 Parasite load and individuals constitution

Three different parasite species were found in the lungs of common dolphin individuals; *Skrjabinalius guevaraii, Halocerus invaginatus* and *Halocerus taurica*. These lung-nematodes are quite common in most delphinid species respiratory systems (Raga and Carbonell, 1985). Although their life cycles are still unknown, they can cause almost total occlusion of bronchi and bronchioles due to their physical presence in the host (Raga *et al.*, 1987b; Clausen and Andersen, 1988). Diseases that are directly linked to these parasites induce the death of the animal. The number of lung nematodes per individual ranged from 0 to504. It was not possible to obtain stomach parasites from this species.

Of the total common dolphin sample 43% were female and 57% were male individuals (sex ratio 1:1.3). The prevalence of uninfected and infected individuals carrying lungworms was 50.53% and 49.47% respectively. Table 4.14 shows the number of female and male individuals compared to lungworm infection.

Table 4.14. Number of uninfected and infected females, number of infected and infected male.

	Uninfected		Infected		
	Females	Males	Females	Males	Total
Lungworms	20	27	20	26	93

Lungworm nematode load was further subdivided according to the level of infestation. Three groups were distinguished in 93 infected individuals; Low infected (range: 1-20 parasites), Medium infected (range: 21-74 parasites) and High infected (range: 75-636 parasites; Table 4.12).

4.3.2.2 MHC variability

The 93 common dolphins showed high levels of variability in the exon-2 MHC Class II DQB1 locus. Cloning sequencing revealed no more than two sequences in each individual. Eighteen different allele patterns were found and 15 alleles had a unique amino acid composition (Appendix, Table 6.6). Alleles were named Dd-DQB*01 to Dd-DQB*15. The allele Dd-DQB*7 was found only in one individual. Therefore fourteen unique alleles were used for the analysis. The comparison between each of the alleles to the complete sequence database in Genebank (MegaBlast) revealed sequence homology from 93% up to 100% with published *Delphinus delphis* MHC Class II DQB sequences (Hayashi *et al.*, 2003). However, homologies were revealed with other marine mammals species up to 99% such is Dd-DQB*05 and Turt-DQB*03 (*Tursiops truncates*; Kita *et al.*, 2007), Dd-DQB*09 and Tutr-a (*Tursiops truncatus*; Hayashi *et al.*, 2003). The Dd-DQB*15 was 100% homologous with Dede-a (*Delphinus delphis*; Xu *et al.*, 2009), Dd-DQB*06 was 100% homologous with Tutr-DQB*01 (*Tursiops truncatus*; Kita *et al.*, 2007) and Stco-DQB*04 (*Stenella coeruleoalba*; Xu *et al.*, 2009). Also, Dd-DQB*08 was 100% homologous with Tutr-DQB*03 (*Tursiops truncates*; Kita *et al.*, 2007).

4.3.2.3 Parasite loads and MHC variability

Allele frequencies ranged from 0.16 (Dd-DQB*15) to 0.01 (Dd-DQB*08). Uninfected and infected individuals examined for lungworms shared different numbers of alleles. Dd-DQB*08 and Dd-DQB*09 were absent from uninfected individuals and Dd-DQB*12 was absent from infected individuals. Performing contingency tables in RxC software the association between uninfected and infected individuals with regards to total number of alleles showed a significant difference ($p<0.001$, S.E.=0000). Diversity analysis showed that both uninfected and infected individuals showed a significant deficit of heterozygotes. The PBR d_S values were zero across uninfected and infected individuals indicating the absence of synonymous substitutions, and preventing the accurate estimation of dN/dS ratios (Table 4.15).

Table 4.15. Diversity and Selection Parameters of common dolphin populations and F_{ST} value between the two populations.

	Uninfected	Infected	Low Infected	Medium Infected	High Infected
No	47	46	20	9	17
A	13	12	8	5	6
R	12.253	13.5	12.711	14	14.328
H_{OBS}	0.73	0.7	0.8	0.55	0.77
H_{EXP}	0.90	0.82	0.86	0.79	0.81
F_{IS}	-0.02	0.14	0.176	0.143	0.084
d_n / d_s	Infinity(dn=0.201)	Infinity(dn=0.19)	Infinity(dn=0.185)	Infinity(dn=0.224)	Infinity(dn=0.163)

The association of parasite loads and MHC alleles showed a significant difference for the Dd-DQB*12 ($\chi^2 = 10.52$, p=0.04, after Bonferroni correction) allele in infected individuals (Figure 4.27).

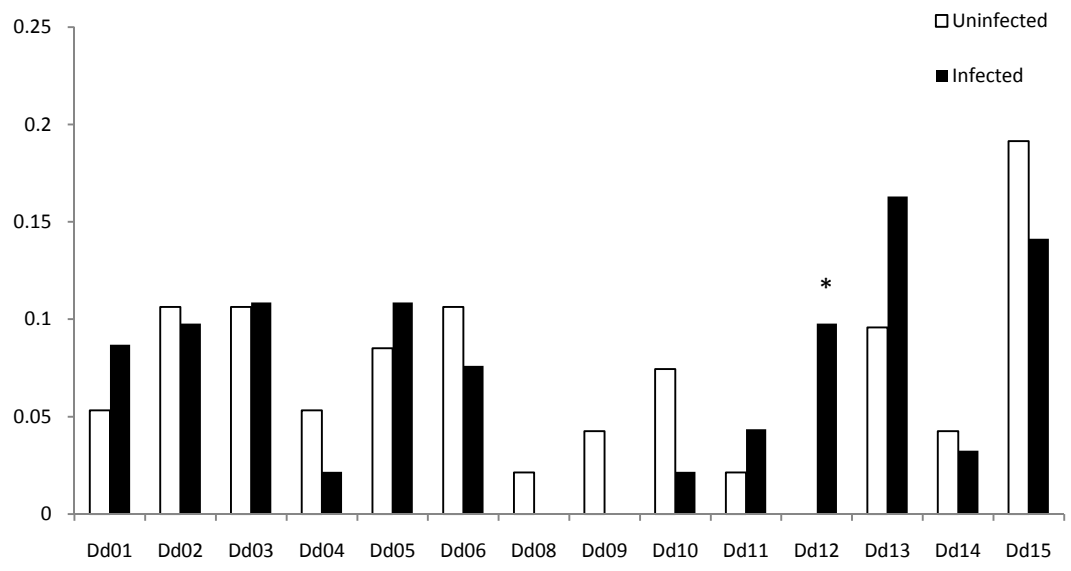


Figure 4.27. Frequency of Dd-DQB alleles of uninfected (white bars) (N=47) and infected (black bars) (N=53) common dolphin individuals

Performing contingency tables using RxC software the association between uninfected and infected individuals with regards to number of alleles and gender showed a significant difference (p=0.02, S.E.=0.07). Therefore extra analyses were performed for associations to parasite loads and MCH alleles with regard sex (Figure 4.28 and Figure 4.29). No significant associations were detected between MHC alleles and parasite loads regarding the sex.

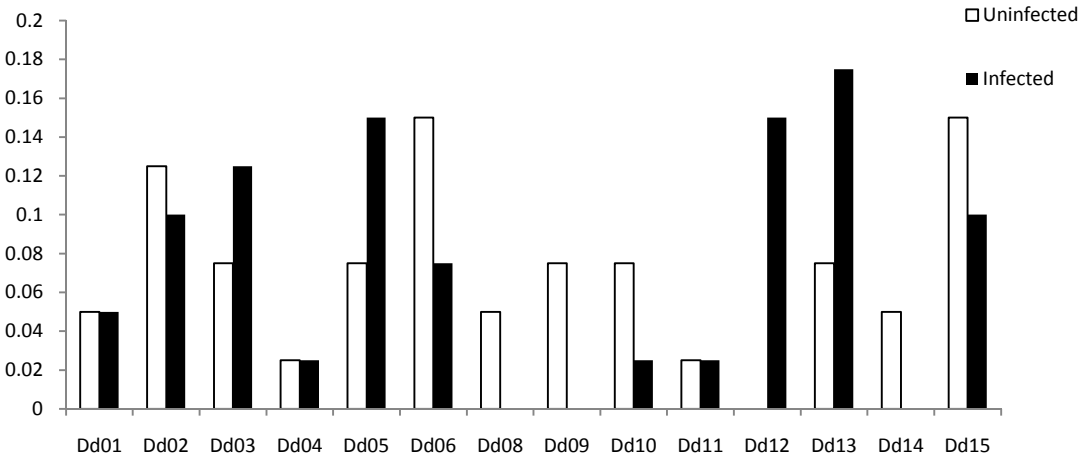


Figure 4.28. Frequency of Dd-DQB alleles of uninfected (white bars) (N=20) and infected (black bars) (N= 20) female common dolphin individuals

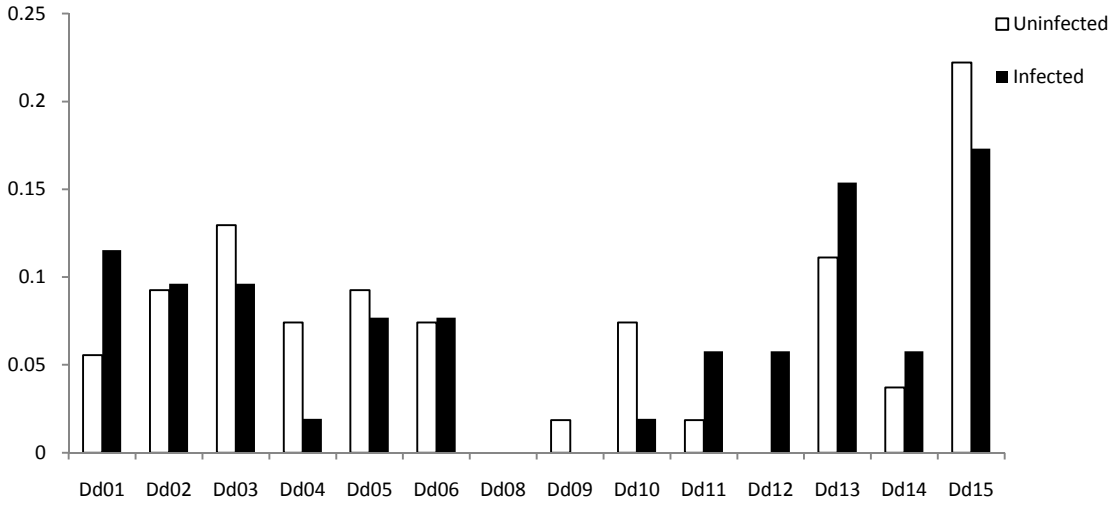


Figure 4.29. Frequency of Dd-DQB alleles in uninfected (white bars) (N= 27) and infected (black bars) (N= 26) male common dolphin individuals

The MCH alleles were classified according to Amino acid profiles based on the sum of the charges at the Pocket 4 amino acids residues (*β70*, *β71* and *β74*) (Appendix, Table 6.4).

In uninfected individuals there were 25.5% negative and 74.5% di-charges (+/-), whereas in infected individuals 21.7% are classified as negative and 78.3% as di-charged (Figure 4.30).

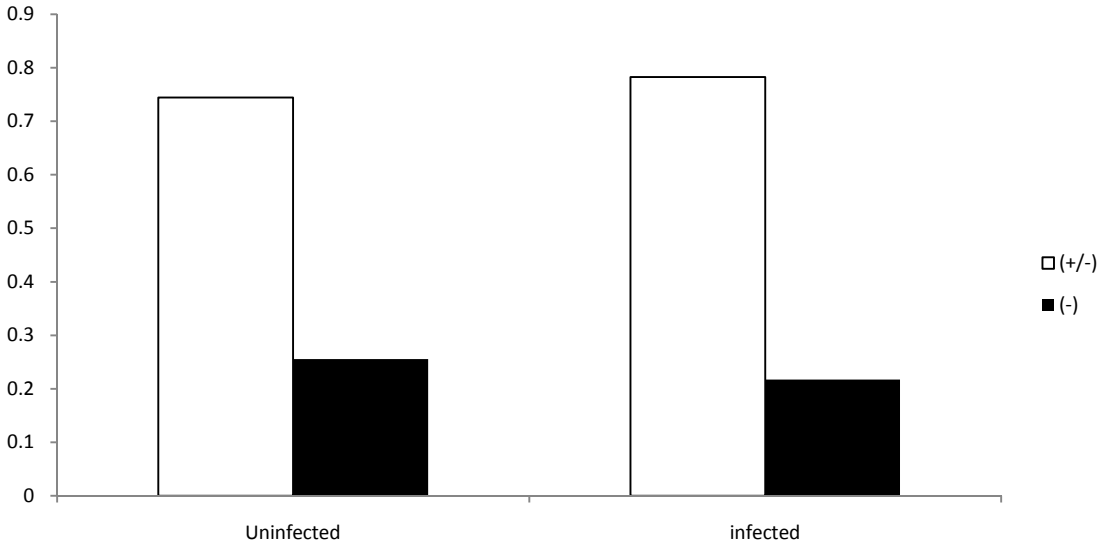


Figure 4.30. Charges of amino acid residues in Pocket 4 of uninfected (N=47) and infected (N=53) common dolphin individuals

Amino acid charge profiles were calculated according to sex (Table 4.16), shown in Figures 4.31 and 4.32.

Table 4.16. Amino acid charge profile in Pocket 4 for uninfected and infected individuals grouped by sex.

	(-)	(+/-)
Uninfected Females	23%	78%
Infected Females	13%	88%
Uninfected Males	28%	72%
Infected Males	29%	71%

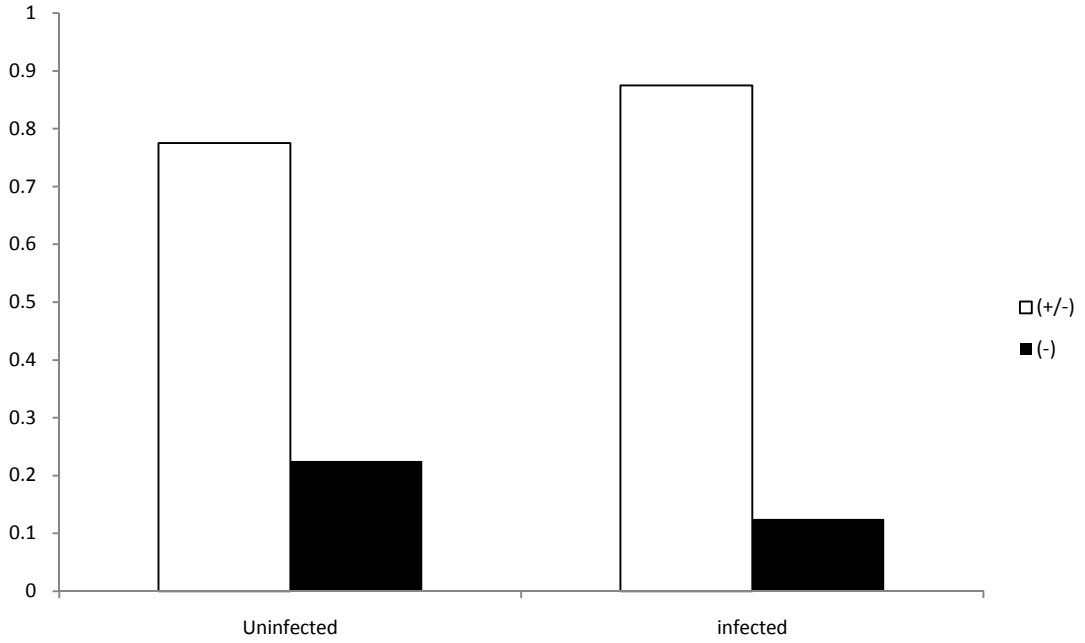


Figure 4.31. Charges of amino acid residues in Pocket 4 of uninfected (N=20) and infected (N=20) female common dolphin individuals

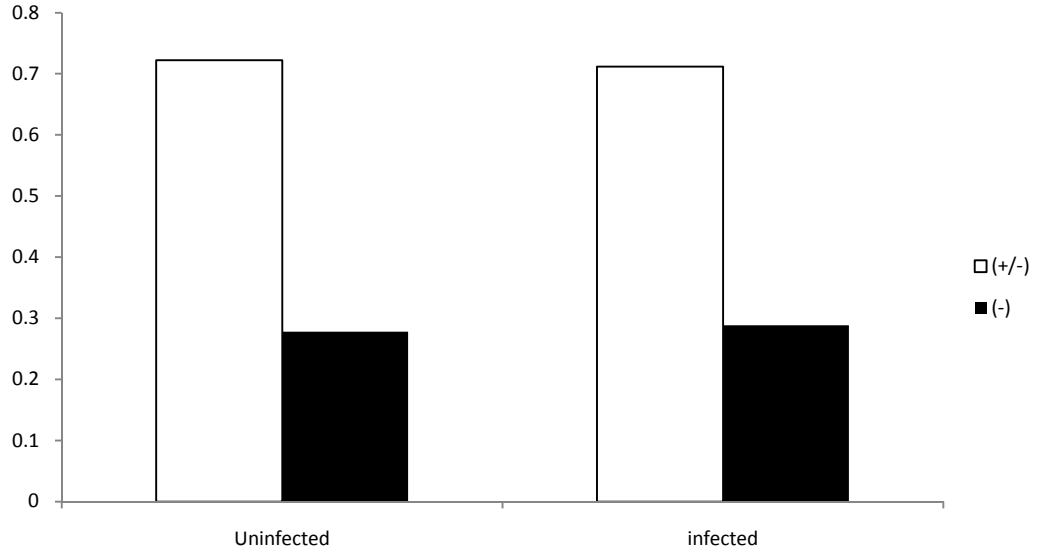


Figure 4.32. Charges of amino acid residues in Pocket 4 of uninfected (N=27) and infected (N=26) male common dolphin individuals

Charge profiles for Uninfected/Low infected were classified 23.8% as negative (-) and 76.12% as di-charged (+/-) whereas in Medium/High infected individuals 23% are classified as negative (-) and 77% as di-charged (+/-) (Figure 4.33).

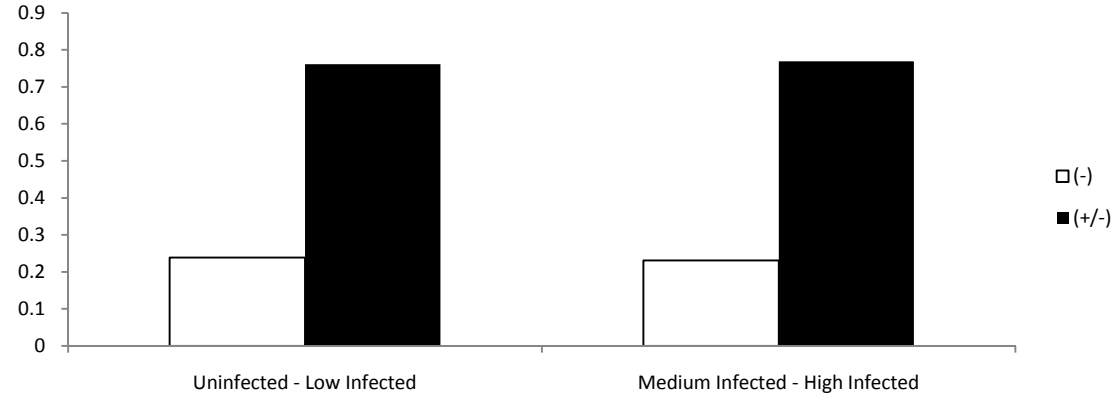


Figure 4.33. Charges of amino acid residues in Pocket 4 of uninfected/Low Infected (N=67) and Medium infected/High Infected (N=26) common dolphin individuals

Amino acid charge profile was calculated according to sex (Table 4.17) and charge frequencies according to sex are shown in Figures 4.34 and 4.35 for Uninfected/Low infected and Medium/High infected categories.

Table 4.17. Amino acid charge profile in Pocket 4 for Uninfected/Low infected and Medium/High infected categories grouped by sex.

	(-)	(+/-)
Uninfected/Low infected Females	21%	79%
Medium/High Infected Females	5.5%	94.5%
Uninfected/Low infected Males	26.4%	73.6%
Medium/High Infected Males	32.4%	67.6%

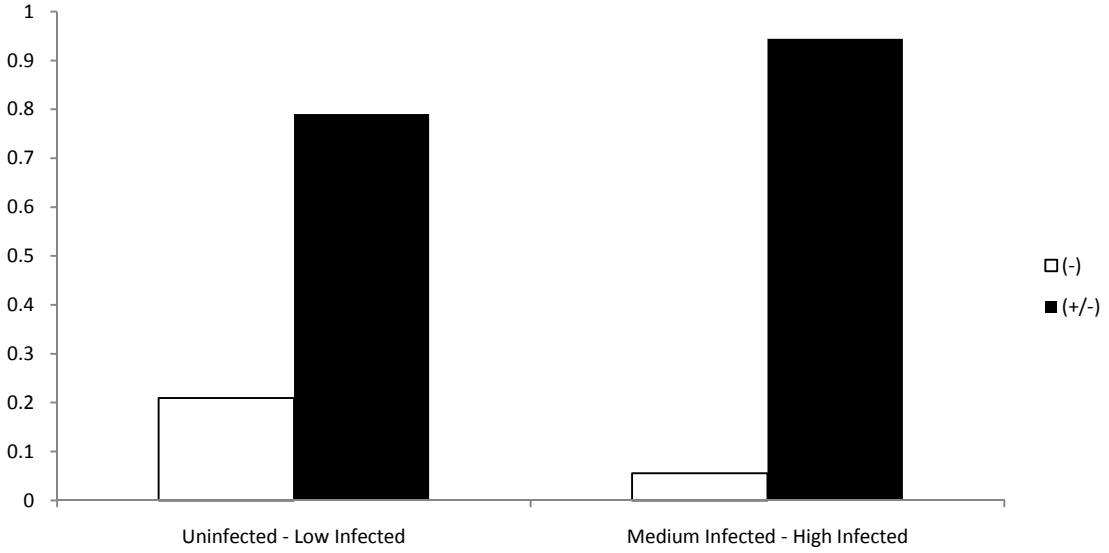


Figure 4.34. Charges of amino acid residues in Pocket 4 of uninfected/Low Infected (N=31) and Medium infected/High Infected (N=9) female common dolphin individuals

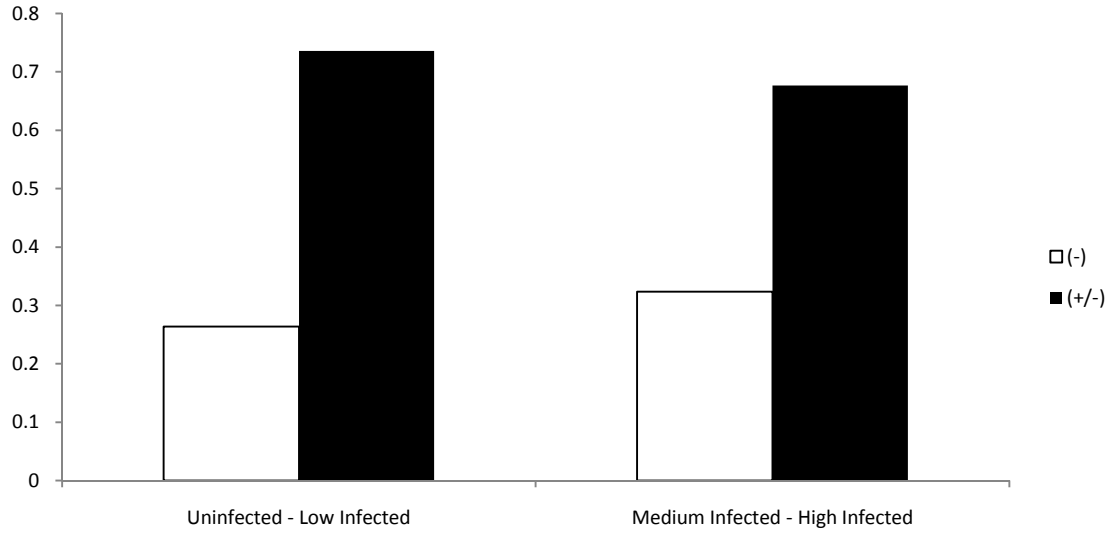


Figure 4.35. Charges of amino acid residues in Pocket 4 of uninfected/Low Infected (N=36) and Medium infected/High Infected (N=17) male common dolphin individuals

Performing General Linear Model the minimal adequate model used the charge profile and the sex as factors with the parasite load as response variable (Table 4.18).

Table 4.18. General Linear Model; Model:charge profile and sex, Response variable: gastric digenean load

Source	DF	F	P
charge_3	1	2.28	0.133
sex_3	1	0.64	0.423
charge_3*sex_3	1	0.41	0.523
Error	182		
Total	185		
S = 209.850	R-Sq = 2.71%	R-Sq(adj) = 1.11%	

4.4 Discussion

The results presented in this study indicate that alleles, of the exon-2 MHC Class II DQB1 locus, are of importance for individual fitness in Delphinidae species. The distributions of alleles were different between uninfected and infected categories, although the unique prevalence of some alleles either in infected or uninfected individuals was not statistically significant in all cases after Bonferroni correction. A significant difference in the functional Pocket 4 of the PBR between uninfected and infected categories was found to be sex-biased.

Heterozygous individuals of striped and common dolphins did not show any increased resistance to parasites and thus this study does not support heterozygous advantage hypothesis as the main mechanism for maintaining MHC variation. However, the apparent lack of association may be due to low power from small sample sizes. Other studies in the literature have also failed to detect heterozygous advantage. For example, Harf and Sommer (2005) studied 40 wild hairy-footed gerbils to test for association between MHC Class II DRB and helminths resistance and they proposed that MHC diversity had no significant effect on infection rate. Moreover, Langerfors *et al.* (2001) found that heterozygosity did not influence the infection status of 120 individuals of Atlantic salmon and MHC Class IIB with regards to resistance to furunculosis. Other studies have found a correlation (often based on analyses with greater power). For example, Penn *et al.* (2002) using 260 mice in a laboratory environment found that heterozygous individuals were associated with resistance to salmonella infection. Carrington *et al.* (1999) in a sample of 498 humans found that diversity in HLA class II is associated with a slower progression to AIDS after HIV-1 infection. However, studies that suggest heterozygote advantage may be due to dominance and not to overdominance, because the fitness of a heterozygote may frequently be greater than the

average of the two homozygotes, but not significantly greater than the most-fit homozygote.

In spite of the relatively small sample size, striped and common dolphins show an association with specific MHC alleles and parasite loads. Common dolphins individuals showed a significant association (χ^2=10.52, p=0.001, after Bonferroni p=0.04) between the Dd-DQB*12 and susceptibility to lungworms infection. This specific allele was only common in infected individuals (allele frequency: 0.1). However, this association was not significant when tested for female and male individuals separately. In male striped dolphins only a single allele was associated with the uninfected status of the co-infected individuals of lungworm and gastric digenean parasites after Bonferroni correction (Sc-DQB*06; χ^2=12.7 p=0.0001, after Bonferroni p=0.004). Interestingly, this certain allele did not show any association in female individuals and parasite resistance. This may be due to the small sample size of females that carried this allele (allele frequency in females: 0.02). On the other hand, specific MHC genotypes were significantly associated with the burden of different parasite types simultaneously in co-infected female individuals. For lungworm parasites, association with uninfected individuals was best explained by the presence of Sc-DQB*03, where female individuals that carried at least one copy of the Sc-DQB*16 allele had significantly greater association with infection than those that did not. However, female individuals with Sc-DQB*03 were associated with parasite resistance examined for the gastric digenean as well. In addition, Sc-DQB*19 allele was associated with gastric digenean infection for female individuals that carried this allele and for those that were co-infected by lungworm and stomach digeneans. Co-infected female individuals with the Sc-DQB*19 and Sc-DQB*09 were more associated with infection than those that did not favour these alleles. Other studies have also found sex-linked differences in MHC genotype, pathogen associations. For

example, Singer-Vernes *et al.* (1995) studying mice for associations between genotype at the MHC B10.A haplotype and paracoccidioidomycosis caused by *Paracoccidioided brasiliensis* fungus, found that female individuals favouring this haplotype displayed lower fungus colony-forming units. However, Prudente *et al.* (2009) studied four MHC haplotypes [B10.A(H-2a), C57BL/6(H-2b), BALB/c(H-2d), A/J (H-2a)] in individual mice and found that the genotype background affects the outcome of lagochilascariosis (*Lagochilascaris minor*) to an equal extent in both sexes. Behrens *et al.* (2010) in a study of collagen-induced arthritis in humanized mice and HLA Class II DRB found that HLA-DR4 mediates activation of autoreactive cells and autoantibodies in females while in males regulatory B cells induce protection from pathogenesis. In this study, striped dolphin MHC association with parasite loads suggests that simultaneous infection with different parasite species may induce an immunopathological cost that is higher than each parasite species effect. The fact that one allele (Sc-DQB*03) was associated with low parasite loads of the two different parasite types in either homozygote or heterozygote form suggest that this allele is under allelic dominance. The association with multiple parasite burden and specific MHC alleles may is due to linkage disequilibrium with another gene that causes the resistance. These data provide evidence that pathogen associations in common and striped dolphins are not due to heterozygous advantage or heterosis, but are instead predicted by specific alleles, suggesting frequency-dependent selection.

In common dolphins d_N substitutions in Pocket 4 of the PBR were found, but not d_S substitutions, possibly due to small sample size. No different trend was found between common dolphins uninfected (di-charge motif) and infected (di-charge motif) with lungworm parasites with respect the charge profile (according to Ou *et al.*, 1998). However, the latter supertype profile trend was opposite to uninfected/low infected and

medium/high infected groups, typically favouring negatively motif. These observations suggest that MHC DQB1 molecules may be less effective in the presentation of lungworms in common dolphins or is due to small sample size. On the other hand, the high rates of d_N/d_S in striped dolphins suggest that striped dolphins MHC polymorphism is due to balancing selection than genetic drift and mutation (Hughes and Nei, 1988). Amino acid substitution pattern of striped dolphin was found to favour diversified supertype motifs in PBR codons, according to Ou *et al* (1998) classification.

In striped dolphins, there was a trend for uninfected individuals to favour the di-charge type, and a lack of trend in infected individuals with regards the lungworm loads. When individuals were grouped according to uninfected/low infected and medium/high infected categories a consistent trend was found such that di-charged profiles were associated with low pathogen load, and negative charge with high load. This was supported both for single parasite types and when considering total infection loads. However, despite the clear and consistent trends, they did not reach the 0.05 level of significance, which may be due to low power. Published studies show this trend for other study systems. Ovsyannikova *et al*. (2007) suggest that specific supertypes of HLA Class I and Class II are strongly associated with different measles vaccine-specific antibodies in children. In particular, the supertypes B44 and B58 (according to Sette and Sidney, 1999) were associated with lower measles antibodies whereas the supertypes B7 and DR (according to Ou *et al.*, 1998) were associated with higher measles antibody responses. Bertoni *et al.* (1997) found an association of HLA Class I A2, A3 and B7 supertypes with hepatitis B-virus. Moreover, O'Hanlon *et al.* (2006) in a study of HLA-A, B, Cw, DRB1 and DQA1 and Idiopathic Inflammatory Myopathy (IIM) in African Americans found that the RSP "R" motif ($Q_{\_70}/K_{\_71}/R_{\_74}$) in the DRB1*03HVR3 domain was a significant risk factor in patients producing antisynthetase and anti-Mi-2 autoantibodies,

and the RSP "A" motif showed significant association among patients producing antisynthetase autoantibodies.

Interestingly, male and female co-infected individuals showed different trends with respect to supertype. The most common supertype, the di-charge, was associated with low co-infected male individuals and high co-infected female individuals. In the case of the negatively charged supertype the association was with high co-infected male individuals and low co-infected female individuals. These different trends of supertype motifs among infection levels of males and females may suggest that the same gene in different environments has a different effect regarding the sex. Taylor *et al.* (1998) in a study of HLA-DQA1 locus and 60 children with childhood acute lymphocytic leukemia (c-ALL) found that the DQA1Ser^{52}, DQA1Val^{57} amino acid motifs coded by DQA1*0101 and DQA1*0501 were associated with susceptibility in male but not in female patients. Song *et al.* (2009) in a study of HLA Class I genes (HLA-A, -B, -C) and Class II DRB1 locus found that the Bw4-80Ile motif, defined by HLA-B probe, is associated with an increased risk for Glioblastoma Multiforme in a extra analysis of female individuals.

Low frequencies of the alleles in the small striped and common dolphins sample size might be affected by short term neutral force that causes genetic drift (e.g. bottleneck) or long-term small effective population size (Ne). Genetic drift is actually a relatively strong force compared to selection in small populations and therefore a small Ne would make local adaptation more difficult. Although small Ne would be expected to show low genetic diversity, MHC, DQB variability in striped dolphins was high, likely due to balancing selection. Van Oosterhout *et al.* (2006) showed that in a relatively small population of Trinidadian guppies (N=100) with a long term small Ne the level of MHC variation was comparable to that of a much larger lowland population (N=2400) due to

the high balancing selection pressure being imposed by parasites and pathogens. The comparison between common and striped dolphins MHC variation showed more evidence for local adaptation in the striped dolphins. Striped dolphins live in sub-structured populations (Gaspari *et al.*, 2007a; Chapter 2) to a greater extent than common dolphins in the eastern North Atlantic (Natoli *et al.*, 2006). Therefore it is possible that local inbreeding is a greater stressor for the striped dolphin, leading to a higher selection coefficient and consequently more evident response to pathogen. The two striped dolphin populations showed high values of Heterozygosity, high dN/dS ratios, and the MHC F_{ST} value between the two populations was very low (F_{ST}=0.001), which are all consistent with the expectations of balancing selection. The microsatellite F_{ST} value between these populations (see Chapter 2) was considerably higher and significant (F_{ST}=0.053, $p<0.001$). On the other hand, the charge profile for Pocket 4 in the PBR shows significant difference in the Ireland population, typically favouring the di-charge supertype. These two observations, of F_{ST} (microsatellites and MHC) and charge profiles may provide evidence for differential directional selection, where the former suggest evidence for an underlying pattern of balancing selection and the latter suggest evidence for local selection. Vassilakos *et al.* (2009) found that nearshore and offshore populations of *Tursiops truncates* differ significantly in Pocket 4 charge profiles with a dominance of di-charge superype (+/-) charge state in the nearshore population. Although this difference between the two populations, results may be affected by the different sample size. The dominance of di-charge supertype in Ireland population may be is due to small sample size as the Sc-DQB*10, Sc-DQB*14 and Sc-DQB*15 alleles (7.4%, 11.1% and 9.8% prevalence in the Valencia population respectively) were not found in the Ireland population (negatively supertype charge profile in the Pocket 4).

In the present study, associations between the MHC Class II DQB1 locus and the load of different parasite types in striped dolphins showed sex-biased directional or frequency-dependent selection as the main mechanisms for maintaining MHC diversity. The heterozygote advantage for striped and common dolphins is poorly supported in this study, while uninfected individuals did not show any heterozygous superiority against infected ones. Certain MHC alleles were associated with parasite loads in both species and this association was stronger in striped dolphins. Further than that, associations between the MHC Class II DQB1 locus and the load of different parasite types in striped dolphins showed sex-biased directional or frequency-dependent selection as the main mechanisms for maintaining MHC diversity. A stronger association with different alleles and different parasite burden was seen for female striped dolphins, suggesting that female individual fitness is subject to environmental challenges. The functional components of the MHC gene were different for male and female striped dolphin, suggesting a different pathogen pressure between sex and environmental stressor.

CHAPTER 5

General Discussion

5. Discussion

In this study the molecular ecology and fitness of two Delphinidae species, the striped dolphin (*Stenella coeruleoalba*) and the common dolphin (*Delphinus delphis*), were investigated in the Mediterranean Sea and Atlantic Ocean. The fitness trait measure was comprised of the parasite burden of highly pathogenic lung nematodes and gastric digeneans. Genetic diversity using neutral and non-neutral markers was measured to evaluate associations with fitness. Microsatellites were used as neutral markers to test heterozygosity fitness correlations with regards to the parasite load. Alleles of the exon-2 MHC Class II DQB1 locus were used as non-neutral markers to test the association of MHC diversity and parasite loads. The population structure of the striped dolphin in the Mediterranean Sea and Atlantic Ocean was also investigated, providing context for the interpretation of the genetic diversity analyses.

The gross examination in lungs revealed one single species for the striped dolphin (*Skrjabinalius guevaraii*) and three nematode species for the common dolphin (*Skrjabinalius guevaraii*, *Halocerus invaginatus* and *Halocerus taurica*). During the gross examination in striped dolphin stomachs the most prevalent species was the digenean *Pholeter gastrophilus*. No data for stomach parasites was available for common dolphins. Lungworms and stomach digeneans are quite common in Delphinidae species (Raga and Carbonell, 1985; Raga *et al.*, 1987b). Large numbers of these parasites cause diseases to their hosts due to their physical presence (Clausen and Andersen, 1988). Lesions associated with lung nematodes can cause occlusion of bronchi and bronchioles and may lead to respiratory system diseases and could cause sick infected animals to strand (Geraci, 1978). Stomach digeneans burrows into the stomach wall and can create symptoms such abdominal upset and indigestion. Therefore, the level of lungworm and stomach digenean

is of importance for the animal's health and appropriate for use in genetic diversity fitness correlations.

Striped and common dolphins are adapted to a variety of habitats and have world-wide distributions. The most prominent distinction between the two species with respect to their known population genetics is the fine scale structure of the striped dolphin and the lack of structuring of common dolphin in the study area. Previous studies have shown no population structure for common dolphins over large geographic areas (Natoli *et al.*, 2006; Mirimin *et al.*, 2009) while the striped dolphin shows a relatively fine-scale structure (Gaspari *et al.*, 2007a). Although the study by Gaspari *et al.* (2007a) on striped dolphin in the same study area revealed structuring patterns, the methods I used were different with regards to power and the putative populations sampled, and therefore revealed population structure beyond that reported previously (Figure 5.1).

In Chapter 2, striped dolphin populations show a strong population structure within the study area. A key new finding is the structuring pattern in the Atlantic Ocean, where populations from Scotland and the Biscay Gulf were isolated from the one in Ireland. Despite the fact that previous studies on mtDNA failed to reveal any difference within the Atlantic Ocean (Garcia-Martinez *et al.*, 1999), the sample sets compared here, at high resolution, provided robust support for this pattern of differentiation. While census population sizes of striped dolphins in the Atlantic Ocean may be hundreds of individuals (Hammond *et al.*, 2008), local populations may be affected by short term neutral force that causes genetic drift.

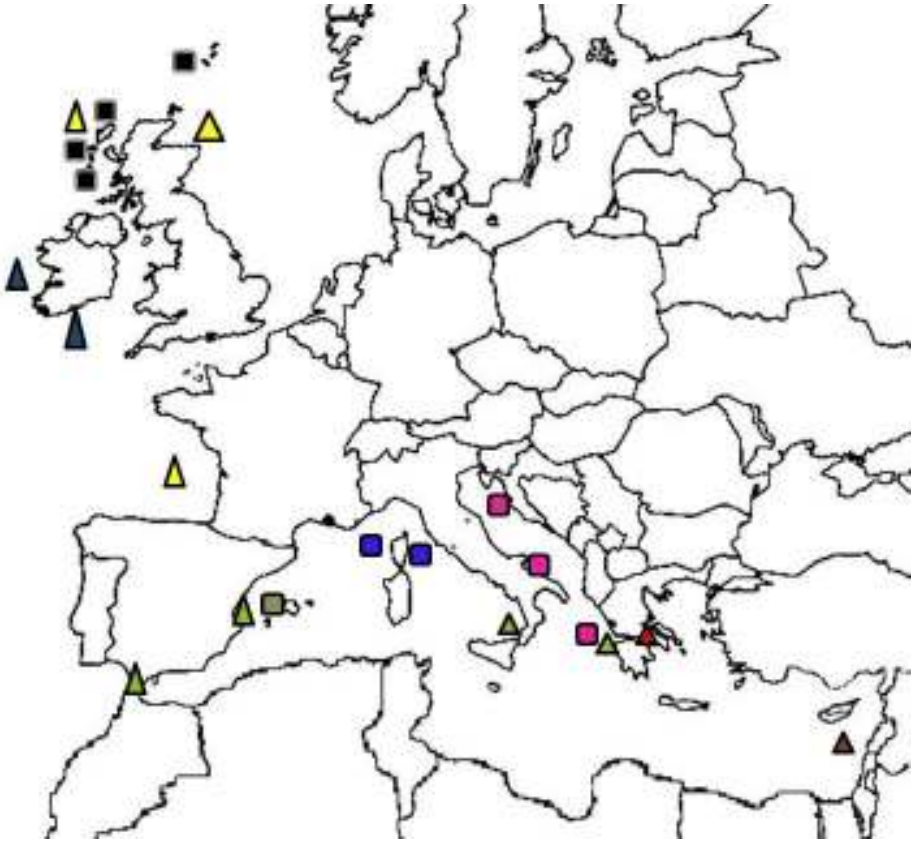


Figure 5.1. Sample sites and genetic differentiation of striped dolphin populations. (Triangle shape: this study, Square shape: Gaspari *et al.*, 2007a)

While the Atlantic Ocean was differentiated from the Mediterranean Sea as has been previously reported, in this study I found that there exists an observable boundary between these two areas located in the Gibraltar Strait. Published data on other marine mammals have shown differentiation between these two areas as well. Natoli *et al.* (2005) suggest that the Gibraltar Strait represented a weak boundary between the Atlantic Ocean and the Mediterranean Sea for bottlenose dolphin populations. Genetic differentiation has also been observed among fish populations (European sea bass - *Dicentrarchus labrax*) from either side of the Gibraltar Strait (Naciri *et al.*, 1996). Authors have suggested that this genetic differentiation reflects the differences in hydrographic characteristics that define different habitats. The significant cluster found in the western Mediterranean is dominated by those individuals that were stranded during the morbillivirus outbreak between 1990

and 2007. As has been previously reported, individuals that died due to virus outbreak were more inbred (Valsecchi *et al*; 2004) and therefore it is possible that some of the effect seen is due to biased sampling.

Another key new finding is the differentiation between the Ionian Sea and the semi-closed Korinthiakos Gulf in Greece. The previously published morphological differentiation, also observed in this study, along with known differences in hydrographic features in the Korinthiakos Gulf area are consistent with my findings on genetic differentiation. However, the Ionian Sea samples grouped with the western Mediterranean, which could either be an effect of the small sample size from the Ionian Sea, or reflect a boundary closer to Greece than to Sicily (Gaspari *et al.*, 2007a) dividing the basins of the Mediterranean for this species. The lack of detectable genetic differentiation between samples from the Ionian Sea and Sicily may also be an effect of the mixing of individuals, though sample sizes are small. Larger sample sizes could help resolve the stock structure in this region, and would contribute to the effective management of these populations.

The FCA analyses was consistent with the Structure analyses whereby in the Atlantic Ocean it revealed a cluster of Scotland and Biscay Gulf populations and a different one of Ireland population, while in the Mediterranean Sea the Korinthiakos Gulf was high distinct from the others Mediterranean populations. The isolation by distance analysis was highly significant in the Atlantic Ocean only. Chapter 2 reveals a complex pattern of striped dolphin population structure that may illustrate different habitats with respect to resources, social structure and demographic history of the species. In some respects the pattern is similar to that seen for bottlenose and Risso's dolphins (Natoli et al. 2005, Gaspari *et al.*, 2007b), though with important differences (especially regarding the

Biscay Gulf), and quite distinct from the pattern of structure seen for the common dolphin (Natoli et al. 2006, 2008).

Population structure affects local effective population size, and thereby affects the level of inbreeding. Therefore, the considerable population structure seen for the striped dolphin has the potential to inluence the impact on fitness to a greater extent than may be expected for the common dolphin, where no such structure is seen throughout most of the study area. Heterozygosity–fitness correlations have been studied in a variety of natural populations (Ledig *et al.*, 1983; Koehn and Gaffney, 1984; Leary *et al.*, 1984; Mitton and Grand, 1984; Zouros and Foltz, 1987; Ferguson, 1992; Bierne *et al.*, 2000) and for many different fitness trait measures including birth weight (Coltman *et al.*, 1998), parasite load (Rijks *et al.*, 2008), longevity (Coltman *et al.*, 1999), reproductive success (Slate *et al.*, 2000), aggressiveness (Hoffman *et al.*, 2007), song complexity (Marshall *et al.*, 2003), and territory size (Seddon *et al.*, 2004). I tested the hypothesis that low genomic diversity, based on microsatellite DNA markers, is correlated with high parasite loads. I also tested the hypothesis that local adaptation of the host may play a role with regards to heterozygosity fitness correlations.

In Chapter 3, the relationship between infestation levels from various parasites and levels of genetic diversity was examined for the two host species. For common dolphins, despite the relatively high levels of heterozygosity (0.65 – 1), no heterozygosity fitness associations were identified. This can also be seen from the comparison between the infected and uninfected individuals, where no significant differences were found. This lack of association may be due either to a small sample size or that the infecting nematode is less virulent in common dolphins. The heterozygosity fitness correlations of common dolphins provide relatively poor support of the general effect hypothesis (that overall

genomic diversity is associated with fitness, though this also assumes that the microsatellte loci investigated provide a good representation of genomic diversity). Despite the observed high genetic diversity of the microsatellite loci, only striped dolphin individuals examined for lungworms show a weak genome-wide effect possibly due to inbreeding. This study also failed to report any correlation between stomach digenean load and genetic variation in striped dolphins, possibly due to the low impact of this parasite infection on fitness. While lungworms could impede respiration (Raga *et al.*, 1987b), stomach parasites are often born without obvious adverse effects (Howard *et al.*, 1983).

In this study, lungworm burden levels were obtained from dead animals, and as a result, other potential factors were not taken into account such as previous health status or a possible post-mortem continuous infection. Sudden death associated with an epizootic outbreak means that some individuals will die prior to becoming infected, or never become as infected as they may have at a greater age. However this assumes a positive correlation between age and infection level, and very little is known about this process within the current body of literature.

During gross examination it was apparent that occlusions caused from worms were obvious in the individuals with medium or high level of infestation. This may suggest a threshold value above which an impact may begin to be seen. In this analysis, highly significant differences were found for 18 female striped dolphins associated with lungworm burden and mean multi-locus heterozygosity and IR values for neutral loci. No significant association was found for male striped dolphin individuals. Despite the lack of a multi-locus effect in males, a strong relationship was seen for the KWM1b locus associated with parasite loads. The apparent single-locus effect in male striped dolphins, suggests that aspects of diseases caused by lungworm burden may be under selection

(assuming linkage disequilibrium between the microsatellite DNA locus and a functional gene).

Despite the fact that nuclear markers are a good informative source for heterozygosity fitness correlations, in higher vertebrates the recognition of non-self is a key aspect of the immune response, and genes in the major histocompatability complex (MHC) play an important role in that process. Thus, I tested the hypothesis that pathogen load is associated with MHC diversity of the DQB gene.

In Chapter 4, the relationship between infestation levels from various parasites and levels of exon-2 MHC DQB1 locus diversity was examined for the two host species. The MHC diversity was relatively high for striped dolphins (alleles number: 21) and common dolphins (alleles number: 14). Despite the high genetic diversity heterozygous striped and common dolphin individuals did not show any increased resistance to parasites. This lack of association may be subject either to the small sample size or low frequencies of the MCH alleles. Studies in the literature, have also failed to detect heterozygous advantage. Langerfors *et al.* (2001) studied 120 Atlantic salmons and found no association between MHC Class IIB and parasites related to furunculosis. However, in some cases studies based on analyses with greater power were able to find correlations between MHC diversity and parasite burdens. For example, Penn *et al.* (2002) using 260 mice in a laboratory environment found that heterozygous individuals were associated with resistance to salmonella infection.

In spite of the relatively small sample size, striped and common dolphins show an association with specific MHC alleles and parasite loads. The distributions of alleles were different between uninfected and infected categories, although the unique prevalence of some alleles either in infected or uninfected individuals were not statistically significant in

all cases after Bonferroni correction. Published studies in the literature have also found associations with specific MHC alleles and parasite loads. Harf and Sommer (2005) found associations between one specific MHC allele and helminths resistance in wild hairy-footed gerbils. Common dolphin individuals showed a significant association between one allele and susceptibility to lungworms infection. However, this association did not remain significant when tested for female and male individuals separately. In contrast, both female and male striped dolphins showed and association between different MHC alleles and their infestation status, though the effect was stronger in females (Table 5.1).

Table 5.1. MCH alleles and infestation status of female and male striped dolphins

	Lungworms	Stomach digeneans	Duel infestation
Female striped dolphins	Sc-DQB*03 (uninfected)	Sc-DQB*03 (uninfected)	Sc-DQB*09 (infected)
	Sc-DQB*16 (infected)	Sc-DQB*19 (infected)	Sc-DQB*19 (infected)
Male striped dolphins			Sc-DQB*06 (uninfected)

In male striped dolphins only a single allele (Sc-DQB*06) was associated with the uninfected status of the co-infected individuals of lungworm and gastric digenean parasites after the Bonferroni correction. This allele did not show any association regarding parasite resistance for females. This may be due to either the small sample size of females that carried this allele or that this allele is not relevant to these particular pathogens in female striped dolphins.

The fact that one allele (Sc-DQB*03) was associated with low parasite loads (in both parasite types) in homozygote as well as in the heterozygote form, suggests that this allele is under allelic dominance. Although this allele may independently provide evidence of association to different forms of parasite type resistance, it fails to detect the fitness

correlation when the two types of parasites are combined together, and so sampling effects associated with the small sample size may also be a factor.

MHC alleles were grouped into four categories based on the sum of the amino acid charges of β70, β71 and β74 residues (positively charged group – negatively charged group – di-charged group – neutral group, see Chapter 4) of the P4 pocket in PBR according to Ou *et al.* (1998). Therefore, I tested the hypothesis that pathogen load is associated with functional components of the DQB gene.

In common dolphins no different trend was found between uninfected and infected individuals with respect the charge profile. It is possible that this locus might not be relevant to this particular pathogen in common dolphins. In striped dolphins, there was a trend for uninfected individuals to favour the di-charge type, and a lack of trend in infected individuals with regards the lungworm loads. When individuals were grouped according to uninfected/low and medium/high co-infected categories a consistent trend was found such that di-charged profiles were associated with low pathogen load, and negative charge with high load. This was supported both for single parasite types and when considering total infection loads. Interestingly, male and female co-infected individuals showed different trends with respect to supertype (Table 5.2).

Table 5.2. Amino acid supertypes of male and female striped dolphins regarding the infestation level.

	Lungworms		Stomach digeneans		Duel infestation	
	Female	Male	Female	Male	Female	Male
Uninfected Low infected	(+/-)	(+/-)	(-)	(+/-)	(-)	(+/-)
Medium infected High infected	(-)	(-)	(+/-)	(-)	(+/-)	(-)

The most common supertype, the di-charge, was associated with low co-infected male individuals and high co-infected female individuals. In the case of the negatively

charged supertype the association was with high co-infected male individuals and low co-infected female individuals. However, despite the clear and consistent trends, they did not reach the 0.05 level of significance, which may be due to low power. In the present study, I found associations between the MHC Class II DQB1 locus and the load of different parasite types in striped dolphins. Further to that, the data show evidence of a sex-biased directional or frequency-dependent selection as a mechanism for maintaining MHC diversity in striped dolphins.

In this study I found differences between the two hosts with respect their genetic diversity and parasite loads for both nuclear and functional loci. The striped dolphin show some evidence for a correlation between diversity and parasite loads in comparison with the common dolphin. This different trend between the two species may be due either to the small sample size or the smaller number of loci examined in common dolphins. Also, the post mortem infection should be taken into account for any false discovery issues.

Despite the importance of these issues related to potential noise in the analyses, the observed differences between the two hosts could be related to their life history and demographic patterns. Previous studies have shown no population structure for common dolphins in the eastern North Atlantic, over large geographic areas (Natoli *et al.*, 2006; Mirimin *et al.*, 2009) but only for comparisons against a local population in Greece (Natoli *et al.*, 2007). The striped dolphin shows a relatively fine-scale structure (Gaspari *et al.*, 2007a; Chapter 2) and local populations have been pointed out over the same geographic range.

The population structure can lead to a smaller local effective population size, as mentioned above. These local populations are more likely to be affected by short term neutral forces (genetic drift). Therefore, a small effective population size can make local

adaptation more difficult due to the stronger force of genetic drift compared to the selection force in small populations. Although, a small effective population size would be expected to show low genetic diversity, in this study high MHC DQB variability in striped dolphins (Chapter 4) combined with the single-locus effect in male striped dolphins (of one microsatellite DNA locus - Chapter 3) provides evidence that aspects of parasite loads may be under balancing selection. In spite of the fact that the genome-wide effect observed in the striped dolphin was relatively weak, there was a consistent effect seen both for neutral and the MHC marker, not seen in the common dolphin. Therefore, it is possible that local inbreeding is a greater stressor for the striped dolphin which may lead to a higher selection coefficient and the possibility of a greater impact of inbreeding in response to pathogens. While the data presented here cannot exclude the possibility that pathogen associations in striped dolphins are due in part to heterozygous advantage, they provide stronger support for frequency-dependent selection.

I also found that evidence for a fitness/ heterozygosity correlation was strongest for females, and this was true both for the correlation with genomic diversity as assessed using neutral markers, and for the functional immune system gene. This observable association of a greater effect for female striped dolphins suggests that parasites may act as an energetic stress, which could be associated with maternity compounded factors (e.g. parturition; see discussion in Chapters 3&4).

However, previous studies have often implicated mammalian males as likely being more susceptible to stress from pathogens. Based on the 'immunocompetence handicap' hypothesis (Zavahi, 1975), Folstad and Karter (1992) proposed a phenomenological model which views the cost of a secondary sexual development (e.g. body size) from an endocrinological perspective. They based this on empirical evidence of the interaction

between plasma testosterone, parasite loads and sexual reproductive success. The authors suggest that male individuals – through testosterone hormone profile – may lower their ability to resist pathogens and parasite loads. Furthermore, Schalk and Forbes (1997) using 145 testes based on mammal hosts from 38 published datasets suggest that steroids suppress immune function, inducing a different susceptibility to parasitism for the different sexes. Moreover, an empirical study by Deviche and Parris (2006) in free-ranging dark-eyed juncos (*Junco hyemalis*) on the sex-biased association between testosterone level and two hematozoan parasites (*Leucocytozoon fringillinarum* and *Trypanosoma avium*) found that testosterone leads to a higher male parasite load.

The observed differentiation with respect the sex in the striped dolphin in similar pathogen environments may reflect the non-identical pathogenesis of parasites and their ability to inflict damage through the hormone profiles. However in this case, female stress appears to be more important than male stress, possibly associated with specific aspects of life history and behaviour in this species that are not at present well understood.

This study suggested that different methods regarding to power and studied subareas show a fine-scale structure beyond that reported previously. However, important un-sampled areas such are English Channel, either side of Italy and Aegean Sea (Eastern Greece) has affected the comparison with previous studies. Also, the small sample size of some areas (e.g. the Biscay Gulf, the Ionian Sea, and the Israel) may have led to an underestimation of structuring patterns. Further sampling should be undertaken to provide a more complete understanding of population structure in support of effective conservation and management.

The smaller number of loci investigated for common dolphins may be affecting the lack of heterozygosity fitness correlations as assessed using neutral markers. Published

data in the literature showed that a small number of microsatellites are less able to detect genomic diversity correlations with parasite resistance. However, even the number applied is comparable to that used in some other studies showing a correlation, and the possibility that differences in population structure are important remains.

This study focused on the exon-2 MCH Class II DQB1 locus in order to evaluate whether MHC variability has affected the parasite loads in marine mammals. This association was greater in the striped dolphin than in the common dolphin, but I can't exclude the possibility that this specific locus might be less effective in parasite resistance in the common dolphin. Published data have reported the importance of other immune system loci regarding pathogen pressure. An extension of this study should include a broader representation of immune system genes.

Taken together the data presented here provide an advance on our understanding of the distribution and function of diversity in the focal species. They illustrate potentially important interactions between genetic drift and selection, and provide specific information that will be valuable towards the conservation and management of diversity in these species.

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6. Appendix

Table 6.1. Genetic variation at each locus of common dolphin. Gene diversity, heterozygosity observed (Ho) and heterozygosity expected (He) are reported.

AAT44		**Dde72**	
H_{EXP}	0.8108	H_{EXP}	0.8502
H_{OBS}	0.8288	H_{OBS}	0.8198
Gene diversity	0.814	Gene diversity	0.854
D08		**Dde84**	
H_{EXP}	0.6571	H_{EXP}	0.8333
H_{OBS}	0.8378	H_{OBS}	0.9182
Gene diversity	0.659	Gene diversity	0.837
Dde09		**Ev14**	
H_{EXP}	0.8001	H_{EXP}	0.9163
H_{OBS}	0.7928	H_{OBS}	0.8468
Gene diversity	0.804	Gene diversity	0.921
Dde59		**KWM12a**	
H_{EXP}	0.7897	H_{EXP}	0.8255
H_{OBS}	0.7248	H_{OBS}	0.9369
Gene diversity	0.794	Gene diversity	0.829
Dde65		**KWM1b**	
H_{EXP}	0.746	H_{EXP}	0.2242
H_{OBS}	0.6757	H_{OBS}	0.2091
Gene diversity	0.75	Gene diversity	0.225
Dde66		**KWM2a**	
H_{EXP}	0.8677	H_{EXP}	0.8967
H_{OBS}	0.8624	H_{OBS}	0.991
Gene diversity	0.872	Gene diversity	0.9
Dde69		**KWM2b**	
H_{EXP}	0.7097	H_{EXP}	0.8274
H_{OBS}	0.6972	H_{OBS}	0.8649
Gene diversity	0.713	Gene diversity	0.831
Dde70		**TexVet9**	
H_{EXP}	0.9008	H_{EXP}	0.1654
H_{OBS}	1	H_{OBS}	0.1081
Gene diversity	0.904	Gene diversity	0.166

Table 6.2. Multiplex Groups for common dolphin of the 18 microsatellites and annealing temperature.

	Primer name	Annealing Temperature	Reference		Primer name	Annealing Temperature	Reference
Group 1	AAT44	57°C	Caldwell *et al.* 2002	Group 2	D08	50°C	Rooney et al. 1999
	Dde09		Coughlan et al. 2006		KWM2a		Hoelzel *et al.* 1998
	Dde59		Coughlan et al. 2006		KWM12a		Hoelzel *et al.* 1998
	Dde65		Coughlan et al. 2006		KWM1b		Hoelzel *et al.* 1998
	Dde66		Coughlan et al. 2006		KWM2b		Hoelzel *et al.* 1998
	Dde69		Coughlan et al. 2006		TexVet5		Shinohara et al. 1997
	Dde70		Coughlan et al. 2006				
	Dde72		Coughlan et al. 2006				
	Dde84		Coughlan et al. 2006				
	Ev14		Valsecchi and Amos 1996				
	Ev37		Valsecchi and Amos 1996				
	TexVet9		Shinohara et al. 1997				

Table 6.3. Amino acid profiles of Sc-DQB* based on the sum of the charges at the Pocket 4 amino acids residues (*β70*, *β71* and *β74*)

MHC allele	Charge profile
Sc-DQB*01	(+/-)
Sc-DQB*02	(-)
Sc-DQB*03	(+/-)
Sc-DQB*04	(-)
Sc-DQB*05	(+/-)
Sc-DQB*06	(+/-)
Sc-DQB*07	(+/-)
Sc-DQB*08	(+/-)
Sc-DQB*09	(+/-)
Sc-DQB*10	(-)
Sc-DQB*11	(-)
Sc-DQB*12	(+)
Sc-DQB*13	(+)
Sc-DQB*14	(-)
Sc-DQB*15	(-)
Sc-DQB*16	(+/-)
Sc-DQB*17	(-)
Sc-DQB*18	(+/-)
Sc-DQB*19	(-)
Sc-DQB*20	(-)
Sc-DQB*21	(+/-)

Table 6.4. Amino acid profiles of Dd-DQB* based on the sum of the charges at the Pocket 4 amino acids residues (*β70*, *β71* and *β74*)

MCH Allele	Charge profile
Dd01	(+/-)
Dd02	(+/-)
Dd03	(+/-)
Dd04	(+/-)
Dd05	(+/-)
Dd06	(+/-)
Dd07	(+/-)
Dd08	(+/-)
Dd09	(+/-)
Dd10	(+/-)
Dd11	(-)
Dd12	(+/-)
Dd13	(+/-)
Dd13	(+/-)
Dd14	(-)
Dd15	(-)

Table 6.5. DQB1 exon-2 nucleotide sequence for striped dolphins

	1			10			20			30				40			50		
Sc-DQB*02	CAC	GGA	GCG	GGT	GCG	GCA	CGT	GAG	CAG	ATA	CAT	CTA	TAA	CCG	GGA	GGA	GTA	CGT	GCG
Sc-DQB*17	...	...	...	...	...	.GT	.A.	...	...	...	...	...	...	...	...	...	...	.A.	...
Sc-DQB*09	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Sc-DQB*04	...	...	...	...	...	.TT	...	..A	...	...	...	...	...	...	...	...	..T	...	...
Sc-DQB*05	...	...	...	...	...	.TT	...	.GA	...	...	...	...	...	...	...	...	..T	...	...
Sc-DQB*07	...	...	...	...	...	.T.	...	...	...	...	...	...	...	T..	...	...	..T	...	...
Sc-DQB*12	...	...	...	...	...	..T	...	...	...	...	...	...	...	T..	...	...	..T	...	...
Sc-DQB*13	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	...	..T	...	...
Sc-DQB*18	...	...	...	...	...	.?T	...	..A	...	...	...	...	...	T..	...	...	..T	...	...
Sc-DQB*14	...	...	...	...	...	.TT	...	.GA	...	...	...	...	...	...	...	...	..T	...	...
Sc-DQB*21	...	...	...	...	...	.T.	...	..A	...	...	...	...	...	T..	...	...	..T	...	...
Sc-DQB*16	...	...	...	...	...	...	...	...	...	..T	...	...	...	...	...	...	..T	...	...
Sc-DQB*03	...	...	...	...	...	.TT	...	.GA	...	...	...	...	...	...	...	...	..T	...	...
Sc-DQB*19	...	...	...	...	...	.GT	.A.	...	...	...	...	...	...	...	...	...	...	.A.	...
Sc-DQB*11	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Sc-DQB*06	...	...	...	...	...	.T.	...	..A	...	...	...	...	...	T..	...	...	..T	...	...
Sc-DQB*01	...	...	...	...	...	.TT	...	.GA	...	...	...	...	...	T..	...	...	..T	...	...
Sc-DQB*10	...	...	...	...	...	...	...	..A	...	...	...	...	...	...	...	...	..T	...	...
Sc-DQB*20	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	...	..T	...	...
Sc-DQB*15	...	...	...	...	...	..T	...	..A	...	...	...	...	...	...	...	...	..T	...	...
Sc-DQB*08	...	...	...	...	...	...	.A.	...	...	..G	...	...	...	...	...	...	..T	...	...

Table 6.5. DQB1 exon-2 nucleotide sequence for striped dolphins (continued)

	60				70			80			90			100				110	
Sc-DQB*02	CTT	CGA	CAG	CGA	CGT	GGG	CGA	GTT	CCG	GGC	GAT	GAC	CGA	GCT	GGG	CCG	GCC	GAA	CGC
Sc-DQB*17	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	.G.	...
Sc-DQB*09	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..G	..C	...
Sc-DQB*04	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	.G.	...
Sc-DQB*05	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	.G.	...
Sc-DQB*07	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	.G.	...
Sc-DQB*12	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	.G.	...
Sc-DQB*13	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	.G.	...
Sc-DQB*18	...	...	...	...	...	...	...	.C.	...	...	.G.	...	...	...	...	...	..G	..T	...
Sc-DQB*14	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	.G.	...
Sc-DQB*21	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	.G.	...
Sc-DQB*16	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	.G.	...
Sc-DQB*03	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	.G.	...
Sc-DQB*19	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	.G.	...
Sc-DQB*11	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..G	..C	...
Sc-DQB*06	...	...	...	...	...	...	...	.C.	...	...	.G.	...	...	...	...	...	...	.G.	...
Sc-DQB*01	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	.G.	...
Sc-DQB*10	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.G.	...
Sc-DQB*20	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.G.	...
Sc-DQB*15	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	.G.	...
Sc-DQB*08	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	.G.	...

Table 6.5. DQB1 exon-2 nucleotide sequence for striped dolphins (continued)

	120					130				140			150			160			170	
Sc-DQB*02	CGA	GTA	CTT	CAA	CAG	CCA	GAA	GGA	CAT	CCT	GGA	GCA	GGA	ACG	GGC	CGA	CCT	GGA	CAC	G
Sc-DQB*17	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..C	GG.	...	...	.
Sc-DQB*09	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.G.	...	...	.
Sc-DQB*04	...	...	..G	...	...	...	...	...	...	...	...	...	...	.T.	...	...	.GG	...	...	.
Sc-DQB*05	...	...	..G	G..	...	...	...	...	...	...	...	..G	...	.T.	...	...	.GG	...	...	.
Sc-DQB*07	...	...	...	G..	...	...	...	...	...	...	...	...	.A.	...	...	...	...	...	...	.
Sc-DQB*12	...	...	...	...	...	...	...	...	...	...	...	..G	.A.	...	...	.C.	...	...	...	.
Sc-DQB*13	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.G.	...	...	.
Sc-DQB*18	...	...	..G	...	...	...	...	...	...	...	...	...	.A.	...	...	...	...	...	...	.
Sc-DQB*14	...	...	..G	...	...	...	...	...	...	...	...	...	...	.T.	...	...	.GG	...	...	.
Sc-DQB*21	...	...	...	...	...	...	...	...	...	...	...	...	.A.	...	...	...	...	...	...	.
Sc-DQB*16	...	...	...	...	...	...	...	...	...	...	...	..G	...	...	...	.C.	...	...	...	.
Sc-DQB*03	...	...	..G	G..	...	...	...	...	...	...	...	..G	...	.T.	...	...	.GG	...	...	.
Sc-DQB*19	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..C	GG.	...	...	.
Sc-DQB*11	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.G.	...	..G	.
Sc-DQB*06	...	...	..G	G..	...	...	...	...	...	...	...	..G	.A.	...	...	...	...	...	...	.
Sc-DQB*01	...	...	..G	G..	...	...	...	...	...	...	...	..G	.A.	.T.	...	...	..G	...	...	.
Sc-DQB*10	...	...	..G	...	...	...	...	...	...	...	...	...	...	.T.	...	...	.G.	...	...	.
Sc-DQB*20	...	...	..G	...	...	...	...	...	...	...	...	.G.	...	...	...	...	G..	...	...	.
Sc-DQB*15	...	...	..G	...	...	...	...	...	...	...	...	...	...	.T.	...	...	.G.	...	...	.
Sc-DQB*08	...	...	...	...	...	...	...	...	...	...	...	..G	...	...	...	.C.	.G.	...	...	.

Table 6.6. DQB1 exon-2 nucleotide sequence for common dolphins

	1			10			20			30				40			50		
Dd-DQB*02	CAC	GGA	GCG	GGT	GCG	GTT	CGT	GGA	CAG	ATC	CAT	CTA	TAA	CCG	GGA	GGA	GTT	GGT	GCG
Dd-DQB*05	...	...	...	...	...	.G.	...	...	...	...	...	...	...	...	...	...	...	...	...
Dd-DQB*12	...	...	...	...	...	...	.A.	.A.	...	...	...	...	...	...	...	...	...	...	...
Dd-DQB*04	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Dd-DQB*13	...	...	...	...	...	.GG	.A.	.AG	...	..A	...	...	...	...	...	...	..A	CA.	...
Dd-DQB*06	...	...	...	...	...	.G.	...	...	...	...	...	...	...	...	...	...	...	...	...
Dd-DQB*15	...	...	...	...	...	.GG	.A.	.AG	...	..A	...	...	...	...	...	...	..A	CA.	...
Dd-DQB*07	...	...	...	...	...	...	...	.A.	...	..A	...	...	...	...	...	...	..A	C..	...
Dd-DQB*01	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Dd-DQB*03	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Dd-DQB*11	...	...	...	...	...	...	.A.	.AG	...	..A	...	...	...	...	...	...	...	...	...
Dd-DQB*10	...	...	...	...	...	...	...	...	...	..A	...	...	...	T..	...	...	...	C..	...
Dd-DQB*09	...	...	...	...	...	...	...	.A.	...	.AA	...	...	...	...	...	...	..A	C..	...
Dd-DQB*08	...	...	...	...	...	.G.	...	...	...	...	...	...	...	...	...	...	...	...	...
Dd-DQB*14	...	...	...	...	...	.GG	.A.	.AG	...	..A	...	...	...	...	...	...	..A	CA.	...

Table 6.6. DQB1 exon-2 nucleotide sequence for common dolphins (continued)

	60			70			80			90			100			110		
Dd-DQB*02	CTT	CGA	CAG	CGA	CGT	GGG	CGA	GTT	CCG	GGC	GGT	GAC	CGA	GCT	GGG	CCG	GCG	GGC	CGC
Dd-DQB*05	...	...	...	...	...	...	...	...	...	...	C..	...	...	...	...	...	.T.	.AT	...
Dd-DQB*12	...	...	...	...	...	...	...	...	...	...	C..	...	...	...	...	...	...	.AT	...
Dd-DQB*04	...	...	...	...	...	...	...	...	...	...	C..	...	...	...	...	...	...	.AT	...
Dd-DQB*13	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..C	..A	...
Dd-DQB*06	...	...	...	...	...	...	...	...	...	...	C..	...	...	...	...	...	..C	..A	...
Dd-DQB*15	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..C	..A	...
Dd-DQB*07	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.	...
Dd-DQB*01	...	...	...	...	...	...	...	...	...	...	C..	...	...	...	...	...	...	.AT	...
Dd-DQB*03	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Dd-DQB*11	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..C	..A	...
Dd-DQB*10	...	...	...	...	...	...	...	...	...	...	...	...	...	CG.	...	...	..C	..A	...
Dd-DQB*09	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.	...
Dd-DQB*08	...	...	...	...	...	...	...	...	...	...	C..	...	...	...	...	...	.T.	.AT	...
Dd-DQB*14	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..C	..A	...

Table 6.6. DQB1 exon-2 nucleotide sequence for common dolphins (continued)

	120			130			140			150			160			170			
Dd-DQB*02	CGA	GTA	CTG	GAA	CAG	CCA	GAA	GGA	CAT	CCT	GGA	GCG	GAA	ACG	GGC	CGA	GCT	GGA	CAC	G
Dd-DQB*05	...	...	...	...	...	...	...	...	.T.	...	...	...	...	...	...	...	...	...	...	.
Dd-DQB*12	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.
Dd-DQB*04	...	...	...	...	...	...	...	...	.T.	...	...	...	...	...	...	...	...	...	...	.
Dd-DQB*13	...	...	...	...	...	...	...	...	...	...	...	..A	...	...	...	...	.G.	...	...	.
Dd-DQB*06	...	...	...	...	...	...	...	...	.T.	...	...	...	...	...	...	...	...	...	...	.
Dd-DQB*15	...	...	..T	C..	...	...	...	...	...	...	...	..A	.G.	...	...	..C	.G.	...	...	.
Dd-DQB*07	...	...	...	A..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.
Dd-DQB*01	...	...	...	...	...	...	...	...	.T.	...	...	...	...	...	...	...	...	...	...	.
Dd-DQB*03	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.
Dd-DQB*11	...	...	...	...	...	...	.G.	...	...	...	...	..A	.G.	...	...	...	...	...	...	.
Dd-DQB*10	...	...	...	...	...	...	...	...	.T.	...	...	...	...	...	...	...	...	...	...	.
Dd-DQB*09	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.
Dd-DQB*08	...	...	...	...	...	...	...	...	.T.	...	...	...	...	...	...	...	...	...	...	.
Dd-DQB*14	...	...	..T	C..	...	...	...	...	...	...	...	..A	.G.	...	...	..C	.G.	...	...	.