Title

Varying disease-mediated selection at different life history stages of Atlantic salmon in fresh water.

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Selection on MH genes in wild Atlantic salmon

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[§] This paper is dedicated to the memory of René Stet, who died in September 2007, and William Jordan, who died in May 2011.

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Abstract

Laboratory studies on associations between disease resistance and susceptibility and major histocompatibility (MH) genes in Atlantic salmon *Salmo salar* have shown the importance of immunogenetics in understanding the capacity of populations to fight specific diseases. However, the occurrence and virulence of pathogens may vary spatially and temporally in the wild, making it more complicated to predict the overall effect that MH genes exert on fitness of natural populations and over several life-history stages. Here we show that MH variability is a significant determinant of salmon survival in fresh water, by comparing observed and expected genotype frequencies at MH and control microsatellite loci at parr and migrant stages in the wild. We found that additive allelic effects at immunogenetic loci were more likely to determine survival than dominance deviation, and that selection on certain MH alleles varied with life stage, possibly owing to varying pathogen prevalence and/or virulence over time. Our results highlight the importance of preserving genetic diversity (particularly at MH loci) in wild populations, so that they have the best chance of adapting to new and increased disease challenges as a result of projected climate warming and increasing aquaculture.

Keywords

MH; major histocompatibility; natural selection, Atlantic salmon, *Salmo salar*, freshwater life stages.

Introduction

Understanding the genetic basis of the immune response in fish is critical for the conservation of wild stocks which are under threat from many sources. Disease-mediated extinction of local populations is increasingly likely as a consequence of global warming, and the resulting increase in water temperatures that is likely to cause an increase in the diversity and prevalence and/or virulence of pathogens (Harvell et al. 2002; Crozier et al. 2008; Dionne et al. 2009). In addition, commercially important species such as salmonids face additional threats. The increase in salmonid aquaculture (both fish farming and stocking programmes) (ICES 2009) poses significant disease risks to wild populations; wild fish migrating past cages may be exposed to high levels of pathogens (e.g. sea lice) (Krkošek et al. 2007; Ford and Myers 2008; Costello 2009), accidental or deliberate release of farm fish may bring novel pathogens with them into the wild (Johnsen and Jensen 1994; Bergersen and Anderson 1997) and introgression between farmed escapes and wild populations may lead to changes in the variability of immunogenetic loci of wild populations (Coughlan et al. 2006). While direct genetic effects of introgression between wild and hatchery-reared salmon have been demonstrated (McGinnity et al. 2003; Araki et al. 2007), the impact of diseases originating from aquaculture (Håstein and Lindstad 1991; Johnsen and Jensen 1994; McVicar 1997) on the genetic integrity of wild fish populations has not been sufficiently addressed. A better understanding of how disease-mediated selection impacts on wild populations at all life stages is therefore crucial.

The genes of the major histocompatibility complex (MHC) encode proteins that play a crucial role in the vertebrate immune response (Klein 1986), and possibly as a result of pathogen-driven balancing selection, MHC genes are the most polymorphic coding regions

known in vertebrates (Grimholt et al. 2003; Wegner 2008). Pathogen-driven balancing selection may be the result of heterozygote advantage, negative frequency-dependent selection or varying pathogen resistance over space and time. (Hedrick et al. 1999; Hedrick et al. 2002; Kekäläinen et al. 2009; Fraser and Neff 2009). The high level of polymorphism in MH genes allows populations to mount an immune response to a wide range of pathogens, but this is only possible if populations have enough variability at MH loci and hence are 'adequately armed to face the challenge of changing environments'(Miller and Vincent 2008).

Atlantic salmon *Salmo salar* L. express single classical MH class I, class II alpha and class II beta loci (Grimholt et al. 2002; Stet et al. 2002). As class I and class II MHC genes in teleosts are unlinked and do not form a single complex they are therefore known simply as MH genes in this taxon (Stet et al. 2002).Associations between MH genes and resistance or susceptibility to several major salmonid diseases, such as amoebic gill disease (Wynne et al. 2007), furunculosis (Langefors et al. 2001; Lohm et al. 2002), sea lice (Glover et al. 2007), bacterial kidney disease (Turner et al. 2007) and infectious salmon anaemia (Grimholt et al. 2003) have been found in farmed populations, and recently, it has been shown that MH genes are linked with increased susceptibility or resistance to myxozoa in the wild (Dionne et al. 2009). There is therefore, strong evidence that MH variability can have important implications for the ability of salmon populations to fight disease, but much of this evidence comes from laboratory challenges on adults. However, associations of alleles in single challenge experiments to specific infections cannot explain how the extreme diversity of MH genes is maintained (Wegner 2008), and indeed it is highly unlikely that animals in their natural environment are only exposed to one pathogen at a time. Empirical evidence linking MH variability to survival and fitness in wild natural conditions with probable varying

pathogen assemblages is rare, but is needed in order to ascertain and predict the impact of disease-mediated selection on locally adapted wild populations of salmon.

The Atlantic salmon (*Salmo salar* L.) is an anadromous fish that spends the first part of its life in fresh water (typically one to four years) and a further one to three years feeding in the ocean, before returning to natal rivers to spawn. In the first year after hatching juvenile salmon (parr) are designated as 0+. After the spring of subsequent years parr are designated as 1+, 2+ etc until they migrate to sea as smolts. We have previously shown that natural selection on MH genes has fitness consequences for salmon in the first 6 months of their life in fresh water (de Eyto et al. 2007). As salmon generally exhibit high mortality (>90%) in the first few months of freshwater life, this life stage would be most likely to experience disease-mediated selection. However, due to their anadromous life cycle, Atlantic salmon are exposed to pathogens in both the marine and freshwater environments and as a consequence, salmon undergo a number of other potentially large mortality events both prior to smolting and in the sea, and it is likely that MH determined survival may also be important at several other life stages. The lack of 'wild immunogenetic' studies has recently been highlighted (Pedersen and Babayan 2011), particularly the need to understand how immunogenetics interact with all the other variables that affects wild animals over time, such as physiological condition, resource availability and abiotic conditions (McGinnity et al. 2009). As salmon grow from parr to smolts, they experience a wide range of these variables, e.g. two subsequent winters of potential resource limitation and cold weather, possible summer drought as 1+ fish, changing physiology as they prepare to leave freshwater as smolts and varying pathogen virulence and prevalence. In order to fully ascertain the extent of disease mediated selection, it is crucial to run an experiment for long enough to trigger the full potential of the adaptive immune response (Eizaguirre and Lenz 2010), which in the case of salmon in freshwater is at least 2 years. It may be that the immunogenetic advantage

conferred during the salmon's first life stage (de Eyto et al. 2007) ceases to be important eighteen months later, as other factors affecting survival increase. Conversely, it may be that the advantage continues to play an important role throughout the two years in freshwater. The aim of this study therefore is to assess the relationship between MH alleles and survival of salmon throughout the freshwater phase. We wanted to ascertain whether disease-mediated selection is a determinant for survival over different life stages of salmon, and if so, whether the same alleles associated with survival in parr are also important at later stages of the salmon's development in fresh water.

Material and Methods

Hatchery and field methods

Details of the experiment location and initial set up of family crosses and hatchery procedure can be found in de Eyto et al. (2007). In summary, the experiment was carried out in a contained section of the Srahrevagh river, located in the Burrishoole catchment in western Ireland. As locally adapted fish may not show any signs of disease mediated selection in their natural environment, we selected wild broodstock from the neighbouring Owenmore system in Co. Mayo (Fig. 1). Owenmore fish are not native to the experimental river and thus are probably not adapted to some of the pathogens endemic to the Burrishoole system. In addition, there has been no history of aquaculture in the Owenmore catchment or the immediate estuary, and thus fish from this system should have minimal exposure to any aquaculture-associated diseases. In order to ensure a large degree of immunogenetic diversity, we used a full reciprocal mating system, which produced 63 families with an average of 889 (±155 s.d.) eggs per family. We excluded natural spawners from the experiment river over the winter of 2001-2002, ensuring that the only salmon hatching in the river in 2002 were from the experiment. This exclusion was facilitated by the presence of a complete

upstream and downstream fish trap at the bottom of the Srahrevagh river. In early February 2002, we counted live eggs, mixed the families and 56031 eggs were planted in the upper reaches of the river in artificial redds (Donaghy and Verspoor 2000); Fig. 1). We randomly sampled approximately 1,500 0+ salmon parr from the river in the summer of 2002. A sub-sample of 746 of these parr were typed for this study. In addition, any juvenile salmon migrating through the trap at the bottom of the river were retained until after the smolt run in 2004 (the vast majority of salmon in this geographic region migrate to sea as 2+ smolts). Tail clips from each individual were preserved in 99% ethanol. Of the fish migrating through the trap, 110 pre-smolts (migrating in late 2003) and 320 smolts (migrating in Feb-April 2004) were randomly selected for genetic typing. The total number of migrants (smolts and pre-smolts combined) therefore typed for this study was 430 individuals. 0+ and 1+ densities of surviving fish in the river were calculated in August 2002 and 2003 using removal sampling (three pass electrofishing, (Zippin 1958)). The number of surviving fish was divided by the number of eggs planted in the river to calculate mortality at each life stage.

Genetic analysis

Natural selection resulting from disease should only be detectable at immunogenetic loci such as MH, while other forces such as genetic drift, migration and mutation should be detectable at control and immunogenetic loci (Garrigan and Hedrick 2003). In order to distinguish between disease-mediated selection and other forces that may impact on genetic variation, we included eight putatively neutral microsatellite loci as controls. The immunogenetic loci included in this study were: (a) *Sasa-UBA-3UTR*, a microsatellite marker embedded in the 3' untranslated region of the MH class I locus; (b) *Sasa-DAA-3UTR*, a minisatellite marker embedded in the 3' untranslated region of the MH class II alpha locus (Stet et al. 2002; Grimholt *et al.* 2003). Class II alpha loci are highly polymorphic (Stet et al.

2002) in salmonids, and class II alpha and class II beta alleles form unique haplotypes (Stet et al. 2002): each class II alpha allele is associated with a unique class II beta allele. Therefore, characterization of either the alpha allele or beta allele is sufficient to describe the polymorphism of class II genes. As previous work on the parr from this study indicated a strong signal of selection on *Sasa-DAA* alleles (de Eyto et al. 2007), we also unambiguously determined the *Sasa-DAA* genotype of all parents and progeny. As the relationship between *Sasa-DAA* alleles or genotypes and *Sasa-DAA-3UTR* markers is not one-to-one, in that some of the markers are associated with more than one allele and vice versa, the assignment of *Sasa-DAA* genotype involved the additional typing of an intron length polymorphism in the (linked) MH class II beta (*Sasa-DAB*) locus using two primers (DBIn4ctF: ATAGAACAGAATATGGGATGG; DBIn5ctR: TTCATCAGAACAGGACTCTCA). *Sasa-DAA/Sasa-DAB* haplotypes have, for the most part, a unique combination of embedded minisatellite and microsatellite markers, respectively (H. –J. Megens unpublished data). In seven out of 63 families, this additional typing did not resolve the *Sasa-DAA* allele, and so these families were excluded from this analysis. This reduced the number of eggs included in the analysis to 51931. Results from both the MH class II embedded marker (*Sasa-DAA-3UTR*), and also the actual MH class II allele (*Sasa-DAA*) are presented and discussed in the following sections, and are labelled *Sasa-DAA-3UTR* (minisatellite) and *Sasa-DAA* (allele) for clarity.

We typed 746 parr and 430 migrants for the *Sasa-UBA-3UTR* microsatellite, the *Sasa-DAA-3UTR* minisatellite, the *Sasa-DAA* allele and eight control microsatellites (*One*107 (Olsen et al. 2000); *Ssa*171, *Ssa*202, *Ssa*197 (O'Reilly et al. 1996); *Ssp*2215, *Ssa*G7SP (Paterson *et al.* 2004); SSOSL85 (Slettan *et al.* 1995); *Ssa*D144 (King *et al.* 2005)) using fluorescently labelled primers. DNA was extracted using the Wizard SVF Genomic DNA Purification System (Promega) or using a Chelex method (Estoup et al. 1996). DNA samples were amplified for all the markers in three multiplex

reactions using the Qiagen Multiplex PCR kit in a final volume of 4 µl with 30 cycles of the PCR profile recommended by manufacturers at 58 ºC annealing temperature or in ten independent PCRs (PCR profile consisted of 3min at 95ºC, followed by 30 cycles of 30s at 95ºC, 30s at 56ºC (or 50ºC for MH Class II) and 30s at 72º). Alleles were resolved in a ABI 377 automated sequencer (Applied Biosystems) and allele sizes were evaluated against a TAMRA 350/500 size standards, or on 18 or 25cm 6% polyacrylamide gels using a LiCor 4200 DNA sequencer with allele sizes evaluated against a 50-350bp size standard and a cocktail of common alleles to account for any potential differences in scoring between machines.

Statistical analysis

The null hypothesis was that under neutrality (i.e. no selection), genotype frequencies in surviving fish would be equal to those expected from parental crosses. The alternative hypotheses would therefore be that either a)frequencies of heterozygotes would be higher than expected in survivors if selection took place as a result of heterozygote advantage, or b) that frequencies of certain alleles would be higher than expected in survivors if additive allelic effects conferred fitness advantages. In theory, if disease mediated selection events had occurred in the river, we would expect no evidence of selection at the eight neutral markers and conversely evidence of selection as a result of either a) or b) (or a combination of both) at the MH loci. A generalized linear model (McCullagh and Nelder 1989) was used for the analysis of the data, which was based on the comparison of observed genotype frequencies in fish surviving after 6 months, and those surviving after 2 years, with expected genotype frequencies calculated from parental crosses. This analysis was conducted for each of the eight neutral microsatellite markers, *Sasa-UBA-3UTR* microsatellite, *Sasa-DAA-3UTR* minisatellite and the *Sasa-DAA* allele. Such models are very similar to standard regression

models but are more general in that the response variable can be non-normal (e.g. Poisson or binomially distributed). We assumed that the observed number of individuals Y_{ij} of each genotype *ij* at a particular locus (the response variable in the model) followed a Poisson distribution, with an expectation of λ_{ij}. Based on the known number of crossings made between parental genotypes, a measure of the expected number of recaptures under neutrality, x_{ij}, was calculated. In the simplest case of neutrality, the expected number of recaptures λ_{ij} should be proportional to expectations based on the crossings made, x_{ij}. Thus, under neutrality we have $\lambda_{ij} = a' x_{ij}$, or, taking logarithmic values,

$$\ln \lambda_{ij} = a + \ln x_{ij}. \qquad \text{(H00)}$$

This represents our neutral model H00, which is a generalized linear model (McCullagh and Nelder 1989) with a log link-function and a Poisson response variable. This choice of link function ensures that the response variable takes valid values (e.g. only positive values) for all values of the right hand side of equation (the linear predictor of the model). The term $\ln x_{ij}$ plays the role of an offset in the model, that is, a covariate for which the regression coefficient is not estimated but instead is known *a priori.*

Different extensions of H00 were considered by including terms representing mechanisms of selection. Firstly, terms representing additive allelic effects s_i and s_j of different alleles *i* and *j* were added to H00 to form model equation H01.

$$\ln \lambda_{ij} = a + \ln x_{ij} + s_i + s_j \qquad \text{(H01)}$$

If this model fitted the data well, it would indicate that fish with certain alleles had higher survival than fish with different alleles and that this effect was additive on the log scale. This means that the survival of a particular heterozygote, say *ij*, lies on the arithmetic mean of the

survival of the homozygotes *ii* and *jj* on the log scale. Secondly, terms representing a dominance deviation d_h where *h*=1 for *i*≠*j* (heterozygotes) and *h*=0 for *i*=*j* (homozygotes) were added to form model H10.

$$\ln \lambda_{ij} = a + \ln x_{ij} + d_h \qquad \text{(H10)}$$

With the constraint that $d_0 = 0$, the parameter d_1 can thus be interpreted as a common increase (or decrease) in survival of heterozygotes relative to the expectation at the arithmetic mean of the respective homozygotes (the expectation under the model with only additive allelic effects, model H01). The advantage of using a common parameter representing dominance deviations for all heterozygote types is increased statistical power and a more parsimonious (simpler) model. We also fitted models where all dominance deviations d_{ij} were free parameters (H20). Under this model, the survival of at least one heterozygote differs from the expectation under the additive allelic effects model (H01).

$$\ln \lambda_{ij} = a + \ln x_{ij} + d_{ij} \qquad \text{(H20)}$$

Finally, Model H11 included both allelic effects and a common dominance deviation:

$$\ln \lambda_{ij} = a + \ln x_{ij} + s_i + s_j + d_h \qquad \text{(H11)}$$

The family of fish that the individual belonged to (each family having a different combination of mother and father) was included as a random effect in each model. Theoretically, we could also have tried to model allelic effects and separate dominance deviations together, but estimating all these parameters together, along with the family effect proved to be impossible. Thus five models (H00, H01, H10, H20 and H11) were fitted to observed genotype frequencies of parr and migrant samples, and compared separately to the expected genotype frequencies calculated from parental crosses. The parr component of this analysis has been previously published in de Eyto *et al.* (2007). However owing to the different number of

298 families included, and the inclusion of 'family' as a random effect we include a reanalysis of
299 the parr data here, so that we can make direct comparison with the migrant data. .

300 The constraint that all allelic effects sum to one was introduced to avoid over-
301 parameterization of the models. The intercept and offset term was included in all models and
302 was fitted using the GLM-function of the software-package R . Models were assessed based
303 on Akaike Information Criterion (AIC)-values (Burnham and Anderson 1998) which were
304 calculated for all model alternatives. Nested model alternatives were also tested against each
305 other using standard tests based on the change in deviance (McCullagh and Nelder 1989). In
306 count data like these, the variance of the response variable is typically larger than expected
307 from the model assumptions, a phenomenon known as over-dispersion (McCullagh and
308 Nelder 1989). To assess this we also computed estimates (McCullagh and Nelder 1989) of
309 the amount of over-dispersion for each selected model, that is, the factor *c* by which the
310 variance of the response variable exceeds the theoretical variance (the variance is equal to the
311 expectation in case of the Poisson distribution). Thus, an over-dispersion value close to c=1
312 indicates that no over-dispersion is present.

313 We utilised a different statistical approach to test whether allele frequencies differed
314 significantly between parr and migrant samples, as treating the genotype counts at the parr
315 stage as true relative frequencies rather than estimates would be problematic since some of
316 the counts were zero, while they were non-zero in the migrant sample. This makes the
317 inclusion of the $\ln x_{ij}$ -offset term unfeasible and also leads to bias in the estimates of tests of
318 significance of selection between parr and migrant stage. Instead, we used a chi-square test
319 on a *n*x2 contingency table, where *n* is the number of alleles for the locus under
320 consideration. This test was also carried out on egg and parr comparisons and egg and
321 migrant comparisons to elucidate consistent patterns between the GLM models and the chi-
322 square tests.

Results

Mortality was estimated at 89% in the first 6 months (August 2002) after introduction of the eggs into the experiment river (February 2002). Based on density estimates calculated from electrofishing, 36% of fish occuring in the 0+ age class in August 2002 survived to August 2003 (1+ age class), i.e. 64% mortality was recorded between 0+ and 1+ age classes. An estimated 41% of 1+ fish subsequently migrated through the Srahrevagh river trap, either in late summer of 2003, or as presmolts or 2+smolts. We presume that the 59% of 1+ fish which did not migrate through the trap died, although there may have been a very small proportion surviving to migrate as 3+smolts, or staying in the river for a third winter as sexually mature male parr. This number, however, is likely to be very small in the Burrishoole catchment. Cumulative estimated mortality from egg to smolt for the study population was 98.7%. It should be noted that survival to migrate through the Srahrevagh river trap does not give a total estimate of freshwater mortality, as the base of the Srahrevagh river is 10km upstream of the top of the tide in the Burrishoole catchment, so additional mortality may occur during the migration between the trap and the ocean.

AIC values of the five model alternatives indicated that the observed parr genotypes of five control microsatellites (*One*107, *Ssa*171, *Ssa*202, *Ssa*D144b, *Ssp*2215) and the *Sasa-UBA-3UTR* marker were close to expectations based on neutrality and the genotypes of parental crosses. Thus, for these loci, the neutral model (H00) was most appropriate as indicated by lower AIC values than for the alternative models (Table 1). AIC values indicated that observed genotypes of two control microsatellites, *Ssa*197 and *Ssa*G7SP, were better fitted by models H10 (common heterozygote advantage) and H01 (additive allelic effects), respectively. However, an explicit test of H10 versus H00 for *Ssa*197 was not significant

348 (p=0.13), while an explicit test of H01 versus H00 for *Ssa*G7SP test was marginally
349 significant (p=0.016), making it unlikely that selection had acted at these loci. For SSOSL85,
350 the H10 model had a lower AIC value than the neutral model (H00), and the difference was
351 highly significant (H10 vs H00, p=0.007). Observed genotypes of the *Sasa-DAA-3UTR*
352 marker and *Sasa-DAA* allele also deviated significantly from neutral expectation and the
353 model with the lowest AIC values was H01 which included additive allelic effects. In
354 summary, the results from the model selection indicate that at parr stage, the observed
355 genotypes of 7 out of 8 control microsatellites conformed to neutral expectations and that
356 selection occurred at one control microsatellite (heterozygotes at this locus had higher
357 survival) and at both the Sasa_DAA-3UTR marker and allele (fish with certain alleles had
358 higher or lower survival than expected).

359 Observed genotypes of migrant fish showed a very similar signal. Observed
360 genotypes of six control microsatellites (*Ssa*171, *Ssa*202, *Ssa*197, *Ssa*D144b, SSOSL85 and
361 *SSp*2215) and the *Sasa-UBA-3UTR* marker were not significantly different from expectations
362 based on neutrality (H00) (Table 1, lower part). The *Sasa-DAA-3UTR* marker and *Sasa-DAA*
363 allele, as well as two control microsatellites (*One*107 and *Ssa*G7SP) displayed a signal of
364 selection as the best model for these loci included additive allelic effects (H01) (Table 1). In
365 these four cases, explicit tests of H01 vs H00 were significant ($p<0.010$).

366 Changes in allele frequencies between egg, parr and migrant were generally very
367 small for most of the loci, with a maximum in the order of 3%. The change in frequency at
368 the *Sasa-DAA* locus was much more substantial, with the maximum change in allele
369 frequency being 7.6% at *DAA*0302* between egg and migrants (Fig. 2). This is further
370 illustrated in Fig. 3, where the pattern and magnitude change in allele frequency is similar for
371 the egg-parr and egg-migrant comparison, with the control microsatellites having a smaller
372 range in frequency change than the two *Sasa-DAA* loci. The change in allele frequency

between parr and migrant is fairly equal across all loci (Fig 3). However, Chi squared tests for the changes in allele frequencies indicated that significant changes were observed between parr and migrant for two control microsatellites (*One*107 and *Ssa*G7SP) and the two *Sasa-DAA* loci (Table 2), which is consistent with the patterns identified by the GLM analysis.

Estimates of the allelic effects (parameters s_i and s_j) were calculated for each *Sasa-DAA* allele by fitting observed and expected genotype frequencies to model H01. Selection coefficients for each *Sasa-DAA* allele were then calculated from the estimated allelic effect. For example, an estimated allelic effect of 0.25 means that an individual carrying one copy of this particular allele on average experiences an increase in survival by a factor of $e^{(0.25)}$ = 1.28; that is, a 28% increase in survival or a selection coefficient of 0.28, relative to the first allele in the dataset. Selections coefficients for the *DAA* alleles ranged from -0.48 to 0.81 for parr and -0.39 and 1.03 for migrants, indicating that within this population, the *DAA* specific allele could affect survival negatively by up to 48% or positively by up to 103% (Fig. 4). The *DAA* alleles **0301* and **0302* were associated with increased survival in both parr and migrant fish while *DAA*0304*, **1202* and **0601* were associated with decreased survival in both parr and migrant. In six out of 12 alleles, selection was either positive or negative depending on the life stage in question – i.e. the direction of selection varied between life stages. For example *DAA*0901* had a selection coefficient of 0.24 in parr, but -0.39 in migrants (Fig. 4).

In order to assess whether large allelic effects (and hence selection coefficients) and sign changes were important, confidence intervals based on standard errors of the allelic estimates (estimate +/- $z_{0.95}$ * s.e.) were calculated (Fig. 5). For three alleles which displayed sign changes in their selection coefficients (*DAA*0901*, *DAA*1001* and *DAA*0201*), the confidence intervals indicate minimal overlap between confidence intervals

of egg-parr and egg-migrant estimates, indicating that the sign change in selection coefficient was important. For the three other alleles which displayed sign changes, (*DAA*0501*, *DAA*0101* and *DAA*0401*), the confidence intervals overlapped to a large extent between life stages, and were not that different from zero. The two alleles which showed the highest degree of positive selection were *DAA*0301* and *DAA*0302*, and the confidence intervals of these alleles did not include zero. Similarly, the confidence intervals of *DAA*0304*, which was associated with negative selection coefficients at both life stages were, on the whole, negative. In summary the confidence intervals around the allelic estimates support the view that three of the alleles displayed a strong selection signal consistent between life stages (positive: **0301*, **0302*, negative: **0304*) and three displayed a selection signal that varied between life stage (**0901, *1001, *0201*). For the other five alleles *(*0501, *0101, *0401 *1202, *0602*), the confidence intervals for allelic estimates were generally centred around zero, which indicates that in this experiment these alleles were probably not associated with survival to any large degree.

Discussion

The results presented here indicate the importance of immune genes in determining survival of salmon throughout their life stages in fresh water. At both parr and migrant stages, allele frequencies for the *Sasa-DAA* allele and marker deviated significantly from neutrality, while allele frequencies for the majority of control microsatellites and the *Sasa-UBA-3UTR* marker did not. As natural selection resulting from disease should only be detectable at immunogenetic loci, while other forces such as genetic drift, migration and mutation should be detectable at both control and immunogenetic loci, we conclude that disease-mediated selection during the two years of freshwater life was the cause of the deviation of the *Sasa-*

423 *DAA* locus from neutrality. Although two of the control microsatellites, *One*107 and
424 *Ssa*G7SP, are putatively neutral, our results indicate that they might not necessarily be so,
425 and may in fact be linked to, or are in linkage disequilibrium with another locus or loci which
426 were under selection. The additive allelic effects in migrant fish (as indicated by the selection
427 of model H01) were much stronger for the Sasa-*DAA* immunogenetic locus than for *One*107
428 and *Ssa*G7SP, as indicated by the difference between observed and expected allele
429 frequencies, which were substantially larger for the *DAA* locus than for the control
430 microsatellites (Fig. 3). This indicates that even though there was some evidence of selection
431 on these two control microsatellites, it was not as strong as the selection acting on the DAA
432 loci.

433 There is still much debate over the mechanism by which MH polymorphism is
434 maintained, and how fitness differences are conferred by MH genes. Heterozygote advantage
435 has been shown to be an important evolutionary mechanism in Arctic charr populations
436 (Kekäläinen et al. 2009), water voles (Oliver et al. 2009) mice (Penn et al. 2002) and salmon
437 (Turner et al. 2007), but has been discounted in other animals (Tollenaere et al. 2008). There
438 is also evidence supporting the view that specific alleles are more important for disease
439 resistance than heterozygosity (Paterson et al. 1998; Schad et al. 2005; de Eyto et al. 2007).
440 The results presented here support the latter view, as additive allelic effects were more
441 important than dominance deviations in determining the survival of an individual in response
442 to disease-mediated selection. While we cannot rule out heterozygote advantage or
443 superiority as playing a role in determining survival in this experiment, we must conclude
444 that it is less important than the role of specific alleles, as demonstrated by the best fit of the
445 H01 (additive allelic effects) to the data over model H10 (common dominance deviations) or
446 H20 (separate dominance deviations). It is worth noting that the roles of heterozygote
447 advantage and frequency dependant selection (or specific allele advantage) are probably not

448 mutually exclusive (Eizaguirre and Lenz 2010), and as the number of alleles in a population
449 increases, the difficulty in separating out the effects becomes increasingly challenging (Oliver
450 et al. 2009). This is especially true in our case, where we had 56 families and 12 *Sasa*-DAA
451 alleles in total. A similar experiment with a smaller number of alleles may allow the fitting of
452 a model including additive allelic effects and separate dominance deviations without
453 overparameterization (Oliver et al. 2009), which was the problem that we encountered in this
454 analysis. However, it is worth noting that the allelic variation we encountered in our
455 experimental population (12 Sasa-DAA alleles) is what we would expect to find in any
456 locally adapted population in this part of Ireland (14 *Sasa*-DAA alleles were sampled from
457 four river close to, and including the Burrishoole catchment (Consuegra et al. 2005)), and
458 indeed is what we were aiming for when we designed the experiment. This is in line with the
459 suggestion that to test the extent of disease mediated selection as a result of local
460 immunogenetic adaptation, the allele diversity in the translocated population should reflect
461 the natural diversity present in the wild (Eizaguirre and Lenz 2010). As a result of this trade
462 off between statistical power and a true representation of the likely adaptive response in a
463 wild population, it is difficult to be definitive about the mechanism accounting for differential
464 survival.

465 Additive allelic effects are consistent with the theory of frequency-dependant
466 selection, or variable selection in time and space (Hedrick 2002). This study indicates that the
467 second theory may be more applicable to Atlantic salmon, as several *Sasa-DAA* alleles were
468 significantly associated with positive selection coefficients between egg and parr stage, but
469 with negative selection between egg and migrant stage, or *vice versa*. This is in agreement
470 with the view that temporal variation in selection means that selective mortality in one time
471 period is a poor predictor of selection coefficients during other time periods (Kekäläinen et
472 al. 2009). Temporal variation in selection may be due to different life stages being susceptible

473 to different pathogens, or alternatively, that the prevalence and/or virulence of pathogens may
474 vary seasonally and annually. Spatial variation in pathogenicity is also very likely in the case
475 of the salmon life cycles, as 0+, 1+ and 2+ salmon use different habitats (Bardonnet and
476 Baglinière 2000), which may harbour differing pathogens. As either spatial or temporal
477 variation in selection (or a combination of the two) are a likely cause of the sign changes in
478 selection coefficients between salmon life stages, our results support the theory that the high
479 polymorphism observed at immunogentic loci may be maintained by a combination of
480 spatial and temporal variation in selection pressures.

481 This study highlights the fact that experiments carried out at particular life stages (e.g.
482 parr) may be insufficient to gain a full understanding of the action of disease-mediated
483 selection. For example, the two *DAA* alleles (*0501* and *0101*) associated with susceptibility to
484 furunculosis in laboratory conditions (Grimholt et al. 2003) were not strongly correlated with
485 survival at parr or migrant stage, even though furunculosis is known to occur in the
486 Burrishoole system. This confirms that resistance or susceptibility in the laboratory does not
487 translate easily to wild conditions, possibly because laboratory studies are generally confined
488 to one age class of fish, most commonly adults (Dionne et al. 2009).

489 The results presented here show that disease mediated selection, either carried
490 forward as a selective advantage from the parr mortality event, or as a result of subsequent
491 disease episodes, continues to be an important, but variable determinant of survival until
492 salmon migrate to sea as smolts. Both of these scenarios are supported by our data. For
493 example, *DAA *0301* and *DAA*0302* were both strongly associated with positive selection at
494 parr stage, and this large effect was carried forward to the migrant stage, when these two
495 alleles still displayed high levels of positive selection. It is also possible that the same
496 pathogen which these two alleles were conferring some kind of resistance to, continued to be
497 a factor for 1+ and 2+ fish. In contrast, allele *DAA*0901* displayed positive selection at parr

498 stage, but displayed negative selection by the migrant stage. This indicates that fish with this
499 allele, which had survived the initially large mortality event as parr, were disadvantaged
500 during the subsequent 18 months, perhaps as a result of a different pathogen affecting the
501 population. It is interesting to note that the signal of disease mediated selection (as indicated
502 by the difference in the scale of allele frequency changes between neutral and *Sasa-DAA* loci)
503 appears to be strongest at the 0+ life stage of salmon (Fig. 3). This may be simply a reflection
504 of the sheer size of the mortality event which occurs in the first couple of months after the fry
505 emerge from the redds, and the susceptibility of emerged fish to a new habitat. Nevertheless,
506 the fact that three DAA alleles (*DAA*0901*, *DAA*1001* and *DAA*0201*) displayed a sign
507 change in selection coefficient between parr and migrant stages indicates that disease
508 mediated selection continues to be an important determinant of survival throughout the
509 freshwater stage of the salmon lifecycle, and that the selection event at parr stage is not
510 simply carried forward through the fresh water stages.
511
512 In the time period of this study, egg to smolt survival (to the experiment stream
513 downstream trap) was 1.3%. In other words, for every 1000 eggs spawned, 13 fish survived
514 to migrate towards the sea. However, freshwater mortality is highly variable, as indicated by
515 the egg to smolt survival recorded for the entire Burrishoole catchment, which varied up to
516 400% between 1969 and 2006 (minimum 0.3%, maximum 1.2%, McGinnity et al. 2009). It
517 has been shown that warmer winters reduce the survival of juvenile salmon in their first two
518 years in fresh water in the Burrishoole catchment (McGinnity et al. 2009), and it has been
519 hypothesised that this may be the result of a mismatch between photoperiod-determined
520 emergence of fry and temperature determined energetics of hibernating salmon. It is also
521 possible that it is the result of disease-mediated selection which may be greater in warmer
522 winters, as pathogen virulence and diversity has been shown to increase with temperature

523 (Hari et al. 2006; Okamura et al. in press). The results presented here indicate that *Sasa*-DAA
524 variability within a population can play a significant role in determining which fish survive
525 the freshwater stage of the salmon life cycle, and hence how many will migrate to sea.
526 Disease-mediated selection, therefore, may be an important factor determining the annual
527 variation in freshwater survival in salmon. It is highly likely that disease-mediated selection
528 on salmonids in fresh water as reported in this paper also occurs during the marine phase over
529 very short time periods, particularly as some of the pathogens associated with MH variability
530 occur at sea, such as sea lice (Glover et al. 2007) and infectious salmon anaemia (Grimholt et
531 al. 2003). The importance of MH genes in determining marine survival maybe a fruitful
532 avenue of exploration in trying to understand the continuing decline in marine survival of
533 Atlantic salmon (ICES 2010).
534
535 The polymorphic nature of immunogenetic loci such as *Sasa-DAA* and other MH loci
536 indicate that these loci are capable of adapting to new pathogen challenges and it has been
537 shown that increasing temperature may be one of the drivers of immunogenetic diversity in
538 populations (Tonteri et al. 2010). Dionne et al. (2007) showed that large scale genetic
539 variability at a major histocompatibility (MH) class IIβ gene in Atlantic salmon increased
540 with increasing temperature and bacterial diversity in rivers contrary to patterns with neutral
541 microsatellite markers. It is possible therefore, that climate change may increase selection on
542 MH genes, and hence increase polymorphism within wild populations in the long term, but
543 only if they are not demographically compromised in the short term by a pathogen which
544 they do not have the ability to fight at a population level. Locally-adapted wild populations
545 may be most at risk of extinction in this case. The increasing production of captive bred fish
546 for aquaculture and stocking, and the related disease risks, further increase the risk of disease-
547 mediated extinction. The combination of warmer climates and potential increases in novel

pathogens and their virulence will undoubtedly put huge pressure on wild populations (Jonsson and Jonsson 2009; McGinnity et al. 2009). While it might be possible to breed disease resistance into cultured strains using information gathered about susceptibility and resistance conferred by specific MH alleles, it would be impractical to attempt this with wild populations. A more practical application of our results would be to avoid interactions between wild and farmed fish by not locating farms where projected changes in temperature are expected to be the most extreme i.e. northern latitudes (McGinnity et al, 2009) In the face of the unpredictable nature of climate change, our results highlight the importance of conserving genetic diversity (in this case immunogenetic diversity), so that wild populations have the greatest chance to adapt to emerging pathogen challenges.

Data for this study are available in the supplementary data, Table S1.

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735

Table 1. Akaike Information Criterion values and of five model alternatives (see text for more detail) for each locus typed in Atlantic salmon surviving in the wild in a section of the Burrishoole river system six months (parr) and two years (migrants) after introduction. The lowest AIC values (in bold) are considered to be the best fit for that locus. Values for *c* are the overdispersion estimates for the best fit model, and *n* is the number of fish successfully genotyped at that locus.

	H00 Parr	H01	H10	H20	H11	n	*c*	H00 Migrants	H01	H10	H20	H11	n	*c*
*One*107	**225.7**	236.0	227.1	349.4	237.7	746	1.83	272.0	**271.5**	272.3	371.3	273.1	428	1.31
*Ssa*171	**223.5**	234.1	225.1	271.6	235.5	746	1.85	**258.8**	259.3	260.5	280.5	261.0	430	1.49
*Ssa*202	**178.1**	184.4	180.1	237.1	186.3	746	2.00	**209.8**	217.0	211.7	264.1	218.9	430	1.46
*Ssa*197	293.1	298.4	**292.9**	337.5	299.3	729	1.99	**268.2**	270.7	270.2	310.1	272.0	367	1.28
*Ssa*D144b	**198.1**	208.9	199.3	293.1	210.8	727	1.77	**266.0**	277.7	268.0	331.9	279.3	369	1.37
*Ssa*G7SP	234.2	**231.0**	234.0	247.6	231.9	720	1.62	320.2	**313.3**	317.9	335.5	315.2	363	1.29
SSOSL85	227.9	237.6	**222.7**	261.4	233.8	730	1.92	**240.0**	247.3	241.7	271.8	249.2	365	1.29
*Ssp*2215	**198.7**	204.2	200.6	247.4	206.1	746	1.88	**211.5**	222.0	213.4	270.9	224.0	430	1.39
Sasa-DAA allele	452.5	**361.8**	454.4	414.9	363.7	746	2.02	374.7	**316.5**	376.7	380.0	318.4	430	1.53
Sasa-DAA-3UTR	438.5	**375.1**	440.3	404.6	376.1	746	2.35	367.7	**338.0**	365.9	367.2	339.2	430	1.74
Sasa-UBA-3UTR	**247.4**	258.7	**247.4**	309.6	259.0	746	1.83	**306.3**	310.0	311.1	364.5	311.8	430	1.39

Table 2. Per marker *p*-values of chi-square tests on nx2 contingency tables comparing allele frequencies between two life stages indicated above the column; number of alleles n = df+1. Significant values ($p<0.01$) are in bold.

Locus	d.f.	Egg – parr	Egg – migrant	Parr – migrant
One_107	18	0.132	**0.005**	**0.000**
Ssa171	9	0.638	0.014	0.072
Ssa197	13	0.107	0.035	0.069
Ssa202	10	0.257	0.095	0.068
SsaD144b	14	0.270	0.172	0.196
SsaG7SP	7	0.021	**0.004**	**0.009**
SSOSL85	9	0.533	0.280	0.292
Ssp2215	11	0.129	0.206	0.210
Sasa-DAA-3UTR	6	**0.000**	**0.000**	**0.003**
Sasa-DAA allele	11	**0.000**	**0.000**	**0.000**
Sasa-UBA-3UTR	12	0.419	0.030	0.178

Figure 1.Location of the Burrishoole and Owenmore catchments in Ireland (top left), the experiment river in the Burrishoole catchment (bottom left) and the position of the fish trap and artificial redds in the experiment river (right).

Figure 2. Changes in allele frequencies between egg and parr (white circles), egg and migrant (black circles) and parr and migrant (grey circles). Expected egg allele frequencies were calculated from parental crosses and observed allele frequencies in salmon parr and salmon migrants stage were observed after 6 months and 2 years respectively in a wild environment. Alleles are ordered left to right with increasing frequency in eggs.

Figure 3. Changes in allele frequencies between egg and parr (top) and egg and migrant (middle) and parr-migrant (bottom) for eight control microsatellites and three immunogenetic loci. Whiskers indicate minimum and maximum changes in allele frequencies. Allele frequencies at egg stage were calculated from parental crosses and allele frequencies in salmon parr and salmon migrants stage were observed after 6 months (n=746) and 2 years (n=430) respectively in a wild environment.

Figure 4. Selection coefficients for *Sasa*-DAA alleles in salmon surviving at 6 months in their natural environment as parr (grey bars) and at 2 years as migrants (black bars). The selection coefficients are derived from allelic point estimates calculated by fitting observed and expected genotype frequencies to model H01 (see text for details). Alleles are ordered left to right with increasing frequency in eggs. DAA*0601 is not included in the graph as it was used as the baseline in the model, against which all other selection coefficients were measured.

Figure 5. Allelic effects calculated for *Sasa*-DAA alleles in salmon surviving at 6 months in their natural environment as parr (left bars) and at 2 years as migrants (right bars). Allelic effects are calculated by fitting observed and expected genotype frequencies to model H01 (see text for details). Alleles are ordered left to right with increasing frequency in eggs. Whiskers represent the 95% confidence interval of the estimates. DAA*0601 is not included in the graph as it was used as the baseline in the model, against which all other allelic effects were measured.

Fig. 1

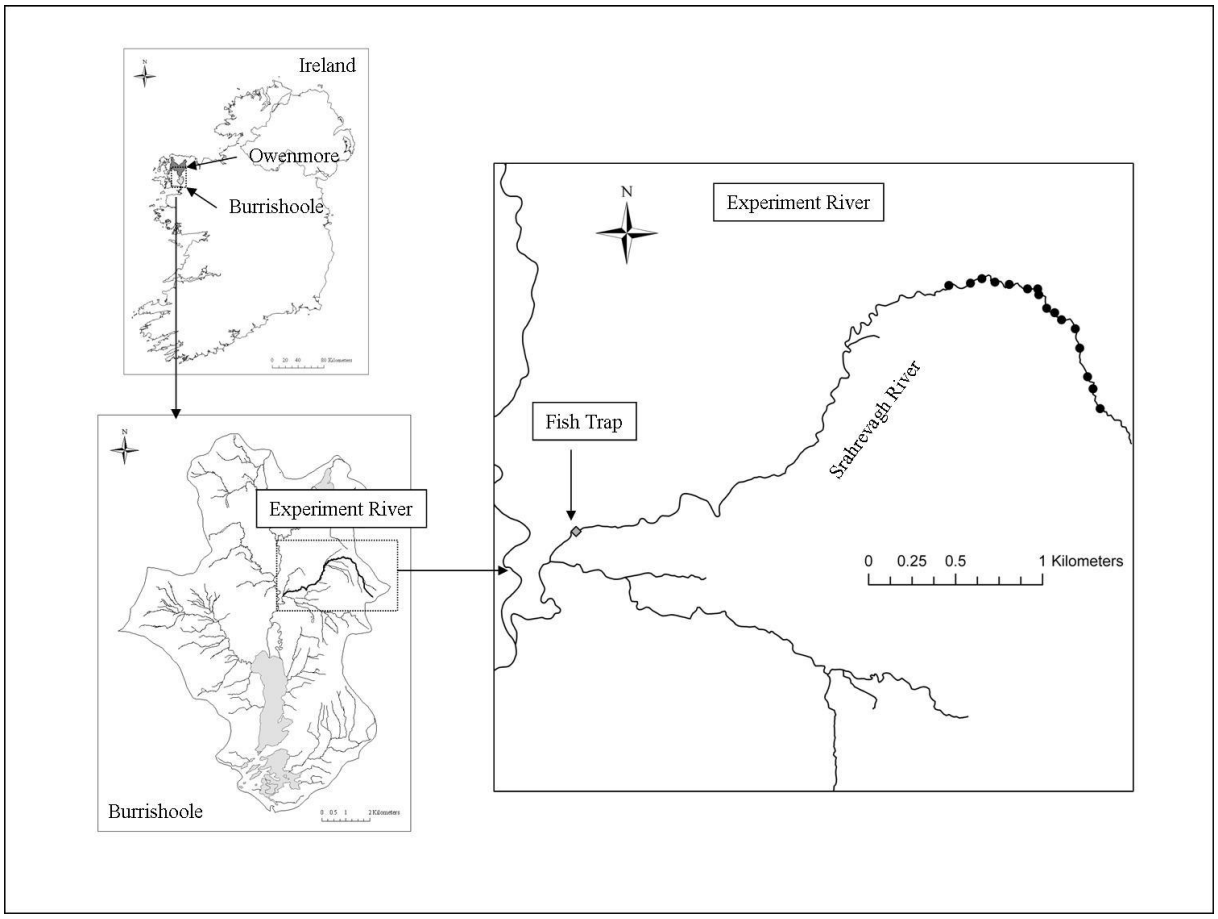

Ireland
Owenmore
Burrishoole
Experiment River
Burrishoole
N
Experiment River
Fish Trap
Srahrevagh River
0 0.25 0.5 1 Kilometers

Fig. 2

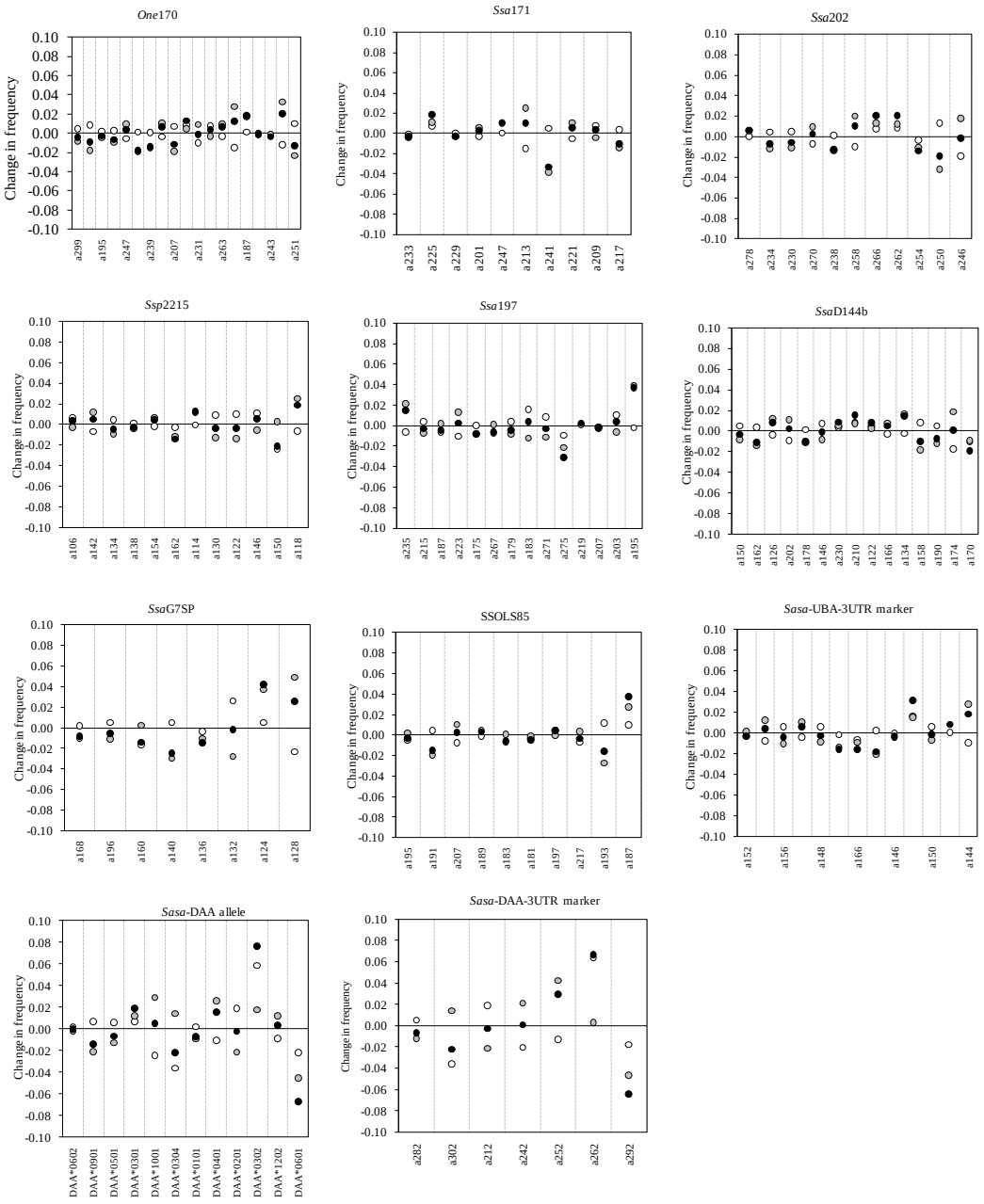

*One*170
*Ssa*171
*Ssa*202
*Ssp*2215
*Ssa*197
*Ssa*D144b
*Ssa*G7SP
SSOLS85
Sasa-UBA-3UTR marker
Sasa-DAA allele
Sasa-DAA-3UTR marker
Change in frequency

Fig. 3

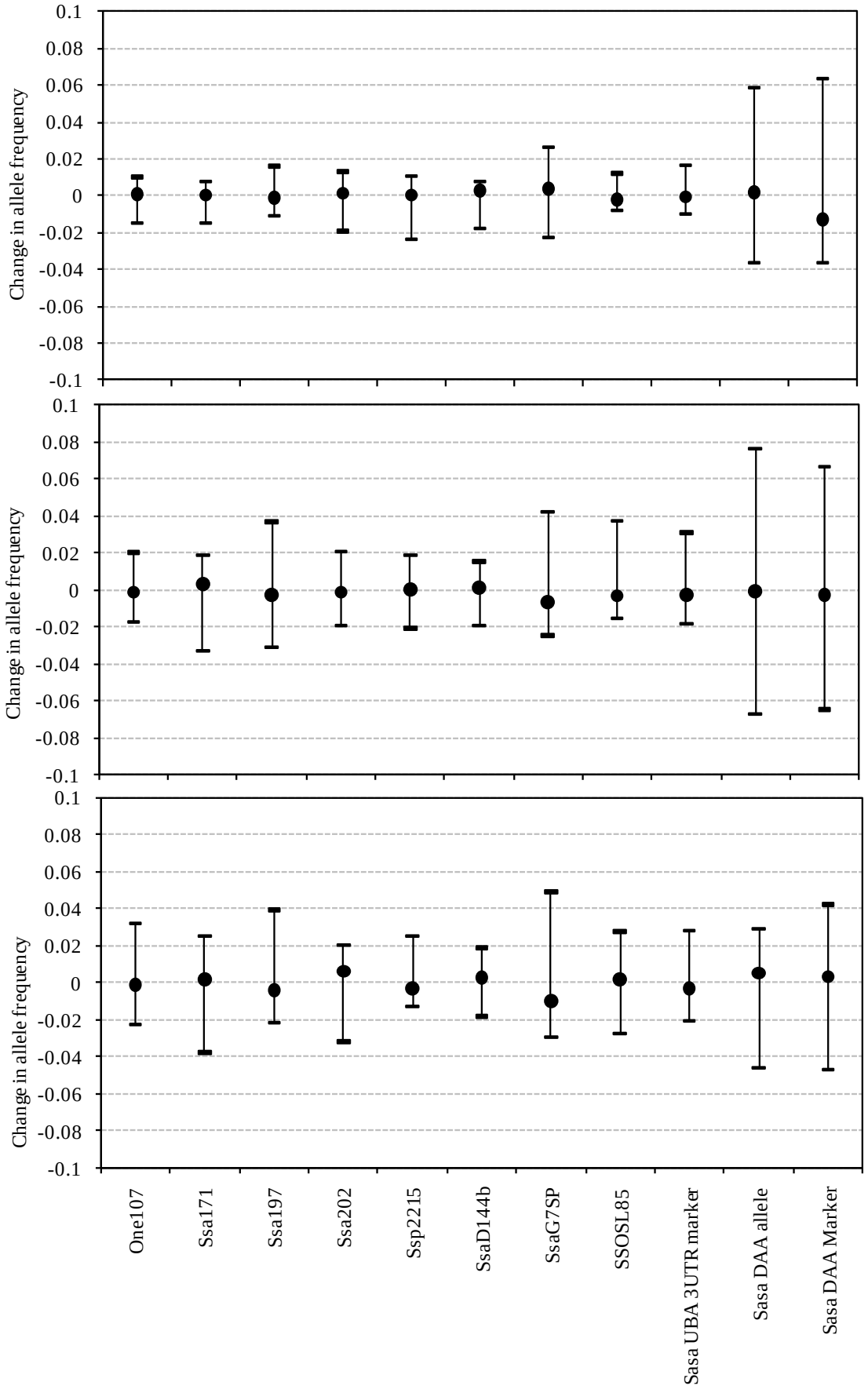

0.1
0.08
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
-0.08
-0.1
Change in allele frequency
One107
Ssa171
Ssa197
Ssa202
Ssp2215
SsaD144b
SsaG7SP
SSOSL85
Sasa UBA 3UTR marker
Sasa DAA allele
Sasa DAA Marker

Fig. 4

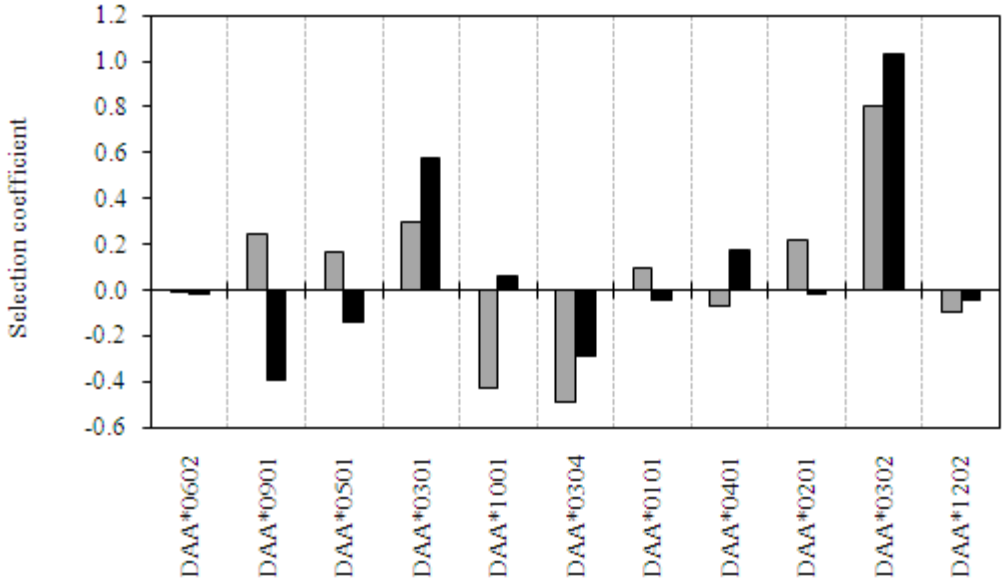
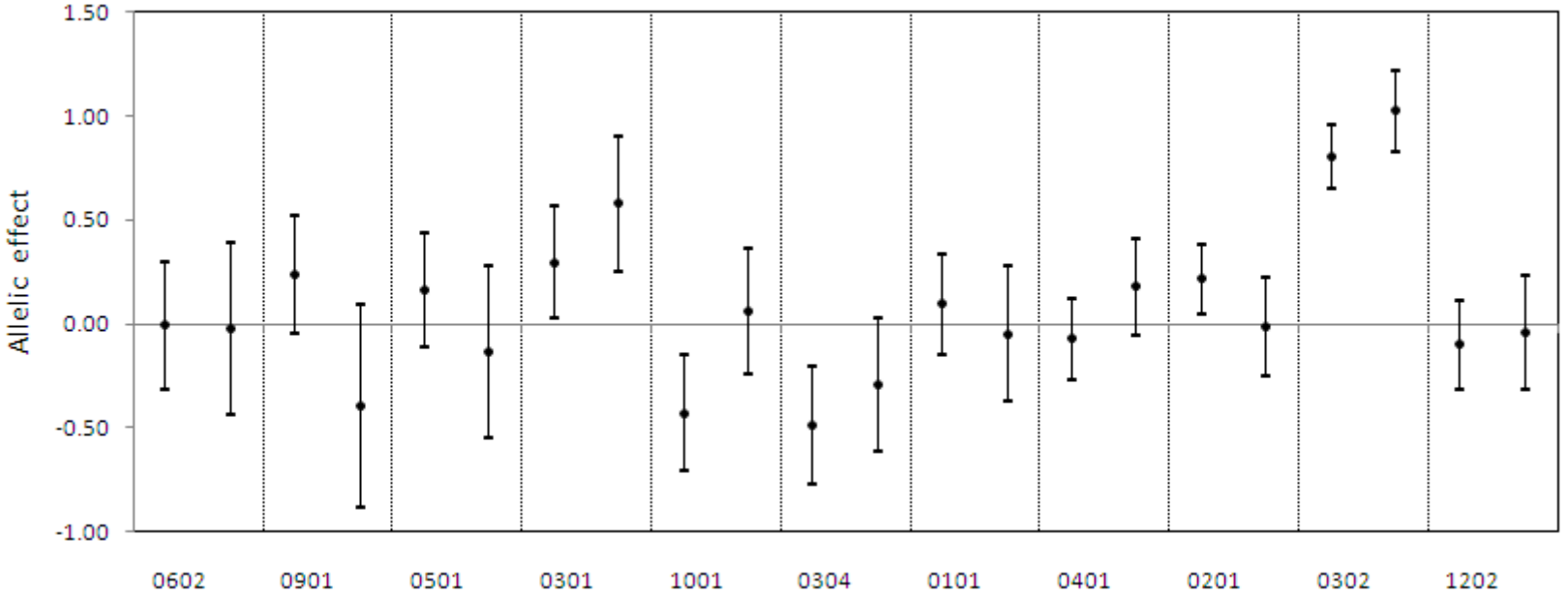

Selection coefficient
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
DAA*0602
DAA*0901
DAA*0501
DAA*0301
DAA*1001
DAA*0304
DAA*0101
DAA*0401
DAA*0201
DAA*0302
DAA*1202

Fig. 5


1.50
1.00
0.50
0.00
-0.50
-1.00
Allelic effect
0602
0901
0501
0301
1001
0304
0101
0401
0201
0302
1202