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1 **Balancing selection on MHC class I in wild brown trout (*Salmo trutta*)**

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36 **Running title:** Balancing selection on MHC in trout

37

38 **Abstract**

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41 Evidence is reported for balancing selection acting on variation at Major Histocompatibility
42 Complex (MHC) in wild populations of brown trout (*Salmo trutta*). First, variation at an
43 MHC class I–linked microsatellite locus is retained in small trout populations isolated
44 above waterfalls although variation is lost at neutral microsatellite markers. Second,
45 populations across several catchments are less differentiated at the MHC-linked locus
46 than at neutral markers, as predicted by theory. The population structure of these trout was
47 also elucidated.

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50 **Keywords**

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53 Major Histocompatibility Complex, variation, fish, genetic diversity, isolated populations

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Introduction

Genes which are under selection and which, in turn, may be involved in local adaptation are of clear interest to evolutionary biology (Nielsen, 2005). Many studies have attempted to identify genes under selection by contrasting patterns and levels of variation at these genes with genes which are presumed to conform to neutral expectations (Kimura, 1983; Karl & Avise, 1992; Pogson *et al.*, 1995; Jordan *et al.*, 1997; Vitalis & Couvet, 2001; Ford, 2002; Dufresne *et al.*, 2002; Goldringer & Bataillon, 2004; Dhuyvetter *et al.*, 2004; Beaumont & Balding, 2004; Vasemagi *et al.*, 2005; Consuegra *et al.*, 2011).

A particularly interesting phenomenon is the maintenance of extensive allelic polymorphism at a locus by selection. This is usually termed balancing polymorphism, where some form of rare allele advantage is necessarily implicated in the prevention of a particular allelic lineage predominating (Takahata & Nei, 1990). The Major Histocompatibility Complex (MHC) gene family, which is critical for determining self from non-self in immune system responses in vertebrates, is commonly held to be under this type of balancing selection (Hedrick, 1994; Apanius *et al.*, 1997). Classical MHC Class Ia molecules are found on the surface of all the nucleated cells of the body. These are composed of a heavy and light chain encoded by polymorphic MHC Class Ia genes and the invariant β2-microglobulin gene. MHC Class II genes, in contrast, are only expressed in a reduced set of cells, e.g. the antigen-presenting cells such as dendritic cells, B cells and macrophages. MHC Class I and II function in the presentation of self and non-self peptides derived from endogenously (i.e. mutated, misfolded or viral) and exogenously (e.g. bacterial or macroparasitic) derived proteins, to cytotoxic T lymphocytes (CTL) or

helper T cells (Th), respectively. Variation in residues within the peptide binding region of different MHC alleles allows binding of different antigenic peptides.

There are three primary non-mutually exclusive theories on how pathogen-driven balancing selection occurs. These are generalised overdominance (Doherty & Zinkernagel, 1975; Hughes & Nei, 1988), whereby heterozygotes have improved immune surveillance over homozygotes; negative frequency dependence (Clarke & Kirby, 1966; Slade & McCallum, 1992), whereby rare alleles may incur an advantage through, for example, pathogen adaptation to more common MHC alleles; and fluctuating selection (Spurgin & Richardson, 2010) among time and place, which may lead to localised patterns of MHC polymorphism and adaptation (Hill, 1991; Hedrick, 2002; Bernatchez & Landry, 2003; Loiseau *et al.*, 2011). Selection for heterozygosity implies selective equivalence of different alleles and, by extension, that different alleles are maintained at a similar frequency (1/k, where k is the number of alleles) in a given population (Richman, 2000). However, this does not appear to be the case with significantly uneven distribution of alleles being the norm even where many MHC alleles are maintained at an appreciable frequency (Salamon *et al.*, 1999). Additionally, it has been proposed that there may be a sexual selection component to MHC evolution, arising from mate selection improving the inclusive fitness of offspring through assortative or disassortative mating (Trivers, 1972; Hamilton & Zuk, 1982). Sexual selection on MHC has clearly been demonstrated in salmonids (Landry *et al.*, 2001; Bernatchez & Landry, 2003; Pitcher & Neff, 2006; Neff *et al.*, 2008; Consuegra & Garcia de Leaniz, 2008). Recently, a role for kin association in maintaining MHC polymorphism in salmonids has been posited (O'Farrell *et al.*, 2012).

108 MHC diversity is considered crucial for the ability of populations to resist disease
109 challenges (O'Brien & Evermann, 1988; Muirhead, 2001; Bernatchez & Landry, 2003;
110 Kurtz *et al.*, 2004). In keeping with this hypothesis, MHC variation has been shown to be
111 vulnerable to genetic erosion arising from bottleneck events in New Zealand robins
112 (Petroicidae) (Miller & Lambert, 2004) and in northern elephant seals (*Mirounga*
113 *angustirostris*) (Weber *et al.*, 2004). Populations which have lost MHC variation may not be
114 viable in the medium to long-term, being less capable of fending off novel disease
115 challenges (O'Brien & Evermann, 1988).

116

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118 However, the situation appears to be complicated by balancing selection acting to
119 maintain genetic variation at MHC despite the loss of genetic variation at neutral genetic
120 markers (Hedrick *et al.*, 2000a; Hedrick *et al.*, 2000b; Hedrick, 2003; Richardson &
121 Westerdahl, 2003; Richman *et al.*, 2003; Aguilar *et al.*, 2004; Jarvi *et al.*, 2004; van
122 Oosterhout *et al.*, 2006). The suggestion is that intense balancing selection serves to
123 maintain MHC variation in the face of these demographic factors, acting through
124 pathogenic pressures (Klein & O'Huigin, 1994; Jeffery & Bangham, 2000; Prugnolle *et al.*,
125 2005). Despite theoretical predictions, a meta analysis of published empirical data has
126 shown a moderate but significantly greater loss of variation at MHC than neutral loci
127 (Sutton *et al.*, 2011). This study mainly included data from MHC class II (94%) due to an
128 apparent publication bias.

129

130

131 Selective pressure on MHC may lead to differential maintenance of variation at
132 MHC and at neutral loci. In isolated populations, variation at MHC may be maintained
133 where variation is lost at neutral markers, despite genetic drift and lack of inward gene flow

134 (Bernatchez & Landry, 2003; Aguilar & Garza, 2006; van Oosterhout *et al.*, 2006; Oliver *et*
135 *al.*, 2009); and populations should be less differentiated at MHC than at neutral markers,
136 due to higher effective gene flow and more even allele frequency distributions (Muirhead,
137 2001). Reasonably, population differentiation at a MHC-linked microsatellite should also be
138 less than that at neutral microsatellites. However, higher population differentiation than
139 neutral expectations is normally observed empirically at MHC loci (Bernatchez & Landry,
140 2003; Sutton *et al.*, 2011). This has been attributed to fluctuating selection (Spurgin &
141 Richardson, 2010) on MHC alleles arising from differential pathogen pressures (Muirhead,
142 2001). These predictions of balancing selection at MHC class I in wild brown trout (*Salmo*
143 *trutta* L.*)* populations are tested. Previous work on MHC in non-model vertebrates has
144 tended to focus on MHC class II (Bernatchez & Landry, 2003; Sutton *et al.*, 2011). MHC
145 class I (*UBA*) and class II (*DAA/DAB*) have only one expressed locus each in salmonids
146 and these loci are not linked (Shum *et al.*, 2001; Grimholt *et al.*, 2002; Stet *et al.*, 2002;
147 Aoyagi *et al.*, 2002). In this respect, these genes are not a “complex”, as in other
148 vertebrates, and are referred to as “Major Histocompatibility” (MH) genes in salmonids.
149 However, the common acronym “MHC” is used to refer to them in this paper. The unlinked
150 nature of the class I and class II loci in salmonids allows independent detection of
151 selection on each locus, something which is confounded in similar studies in most other
152 vertebrates.

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154

155 **Materials and Methods**

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157

158 Samples were taken from eight Irish River catchments; (from North to South, see
159 Figure 1) the Owenmore, Owenduff, Burrishoole (Goulaun and Srahrevagh tributaries),
160 Newport (Skerdagh tributary), Owenwee, Carrowniskey, Erriff and Mulkear) (Table I) which
161 were 25-370km apart. In the case of the Burrishoole (Srahrevagh tributary) and Mulkear
162 Rivers (300km apart), these have populations isolated above waterfalls for over 12,200
163 years. The ice cleared from the Mulkear waterfall 16,500±300 years ago and isostatic
164 uplifting would have created this waterfall 3,000-4,000 years after that (McCabe, 2007). In
165 the case of the Srahrevagh waterfall, ice would have cleared 16,950±50 years ago and
166 isostatic uplifting would, again, have created the waterfall 3,000-4,000 years later
167 (Ballantyne *et al.*, 2008). These two long-term isolated *S. trutta* populations were
168 compared with their downstream counterparts and offer a unique opportunity to study the
169 maintenance of genetic diversity. The data from the broader geographical area across all
170 eight catchments allowed us to test the relative population differentiation of *S. trutta*
171 populations at neutral loci and MHC.

172

173

174 A total of 964 individuals were screened at eight selectively neutral microsatellite
175 loci: *Str73* (Estoup *et al.*, 1993), *Ssa85* and *Ssa197* (O'Reilly *et al.*, 1996), *Ssa2216*
176 (Paterson *et al.*, 2004), *SsOsl417* and *SsOsl85* (Slettan *et al.*, 1995), *F43* (Sanchez *et al.*,
177 1996) and *Str543* (Presa & Guyomard, 1996) and a microsatellite locus embedded in the
178 untranslated 3' end of the MHC class I locus (*Satr-UBA*). This dinucleotide microsatellite
179 marker has been demonstrated to be tightly linked to the class I locus in Atlantic salmon

180 (*Salmo salar* L.) (Grimholt *et al.*, 2002). It has been successfully employed previously in
181 studies in *S. salar* (de Eyto *et al.*, 2007; Consuegra *et al.*, 2011) and *S. trutta* (Coughlan *et*
182 *al.*, 2006; Hansen *et al.*, 2007; O'Farrell *et al.*, 2012). The pattern of linkage has been
183 discussed in some detail in these publications. Briefly, the marker locus is less variable
184 than *Satr-UBA*, with one marker allele often being linked to more than one *Satr-UBA* allele
185 (Coughlan *et al.*, 2006; O'Farrell *et al.*, 2012). It should be noted that marker alleles do not
186 necessarily reflect functional characteristics of linked *UBA* alleles and the proteins they
187 encode. No similar marker was available for MHC class II in these *S. trutta*.
188
189
190 DNA extractions were conducted by dissecting small pieces of tissue (1-5µg) from
191 the samples and added to 0.5ml tubes containing 300µl of 10% (weight/volume) Chelex™
192 solution. The mixture was heated at 99ºC for 1 hour. Samples were centrifuged at 3000
193 rpm for 3 min and then stored at -20ºC.
194
195
196 PCR amplifications were carried out in a 10µl reaction volume under the following
197 conditions: 95℃ 3min; (95℃ for 30s, 56℃ for 30s, and 72℃ for 30s) X 30 cycles. Alleles
198 were resolved on 18cm or 25 cm 6% polyacrylamide gels, using a Li-Cor 4200 DNA
199 sequencer. Allele sizes were determined by reference to a 50-350bp size ladder and locus-
200 specific allele size standards. These allele size standards were constructed in the
201 laboratory using the full complement of allele sizes observed in pilot studies, to enable
202 consistent scoring amongst batches of individuals screened for each locus. When initial
203 genotyping was unclear due to gel electrophoresis problems or weak amplification (~4% of
204 genotypes), *S. trutta* fry samples were re-extracted and re-screened. Large allele dropout
205 was identified as an occasional problem but large alleles could usually be reliably scored
206 after re-screening. Following re-screening, the final estimated error rate was ≤0.5% of

207 composite genotypes per individual (Coughlan *et al.*, 2006).

208

209

210 The MICROCHECKER application (Van Oosterhout *et al.*, 2004) was used to help
211 identify general problems such as mistyping, typographical and scoring errors together
212 with a number of null allele tests prior to further analysis of these *S. trutta* populations. This
213 helped establish whether particular loci might best be removed from further analysis due to
214 unsatisfactory error rates. MICROCHECKER was run with a maximum expected allele size
215 of 300bp. Unusual observations were checked and a randomisation procedure (1,000
216 randomisations) with Bonferroni correction was used for all tests. Missing or suspect data
217 were omitted from the analysis. MICROCHECKER analysis uncovered no evidence of loci
218 presenting problems.

219

220

221 Only the two known samples from *S. trutta* isolated above waterfalls in the Mulkear
222 and Srahrevagh were assumed *a priori* to be populations. Instead, an analysis was
223 conducted on all *S. trutta* samples using the software STRUCTURE (Pritchard *et al.*, 2000;
224 Falush *et al.*, 2003) with input files created using CONVERT (Glaubitz, 2004). The range of
225 values for the number of clusters (K) was narrowed using three short runs (10,000 burn-in
226 and 10,000 MCMC iterations thereafter) of STRUCTURE (Pritchard *et al.*, 2000; Falush *et*
227 *al.*, 2003; Evanno *et al.*, 2005), with an admixture model with correlated allele frequencies,
228 in the range K=2 to K=17. Having narrowed the range suitably and plotted the log
229 likelihood values for these runs, an admixture model with correlated allele frequencies was
230 again used and the appropriate number of clusters (K) identified using three runs each at
231 each value of K between 9 and 14 (Burn-in 100,000 with subsequent 1,000,000 MCMC
232 iterations). The appropriate number of clusters (K) was identified with reference to plots of
233 Ln P(D) values while setting a cut off for K at that level wherein additional clusters had no

234 obvious explanation in geography and where few, if any, individuals strongly assigned to
235 the additional cluster (Falush *et al.*, 2003). The program DISTRUCT was used to generate
236 high quality graphical outputs of the STRUCTURE results (Rosenberg, 2004).
237
238
239 F_{ST} statistics (Φ) (Weir & Cockerham, 1984) between all populations were
240 calculated for the neutral microsatellite loci and the MHC-linked locus, separately, in
241 GENETIX v4.04 (Belkhir *et al*., 2004). These Φ values have been tabulated using Python
242 software developed in this study, CREATEMATRIX. A jacknifing approach was used to
243 estimate neutral Φ statistics. Φ statistics were re-calculated with the removal of one of the
244 eight loci each time. This allowed us to calculate the 99.9% confidence limits in neutral Φ
245 statistics for each population pair. It was then assessed whether the Φ statistic for the
246 MHC-linked microsatellite was greater or less than the respective neutral Φ statistic and
247 whether they fell inside or outside the 99.9% confidence limits for the neutral Φ statistic.
248 Hardy-Weinberg exact tests were implemented in GENEPOP (Raymond & Rousset,
249 1995).
250
251
252 Unless specified, subsequent statistical analyses were implemented in Python
253 scripts using standard approaches. Individual heterozygosity was calculated across the
254 eight neutral loci and, separately, at the MHC class I linked marker. For each pairwise
255 comparison, unpaired t-tests on the binomial data for heterozygosity were conducted for
256 each locus. Paired t-tests were also conducted in SPSS on the proportion of
257 heterozygotes over the eight neutral loci.
258
259

Allelic richness (AR) for each population was estimated by a bootstrap procedure which corrected for sample size differences. The smallest sample was the Mulkear BW sample, with data for 27 diploid individuals for one locus (Table I). At each iteration of a bootstrap procedure, allelic richness was estimated for each population by taking a random sample of 54 gene copies (27 X2) (*g*) from the frequency distribution at each locus and counting the number of alleles observed for that locus. Allelic richness for each of the eight neutral loci and MHC was calculated as the average over 100,000 bootstraps. A summary statistic of neutral allelic richness for each population was further calculated as the average AR across the eight neutral microsatellite loci for each bootstrap iteration and then the average of those values across the 100,000 bootstraps.

A bootstrap method (Coughlan *et al.*, 2006) was adapted for significance tests on allelic richness at neutral loci and MHC in each case study. In each pairwise population comparison of variability, and under the null model that the two samples do not differ in variability, an allele frequency distribution for each locus was estimated from pooled genotypic data from the two samples. As per the calculation of allelic richness, two samples of 54 gene copies were drawn, with replacement, from this distribution to create a pair of simulated samples. Neutral and MHC allelic richness statistics were calculated (as above). The absolute difference in neutral allelic richness between the two simulated samples was used as the test statistic. This was repeated over 100,000 bootstraps to provide a null distribution for the test statistic. A null distribution for MHC allelic richness was constructed similarly. The proportion of simulated test statistic values which exceeded the test statistic value for the real samples provided a test for significant differences in variability above and below the waterfalls in the Srahrevagh and Mulkear. Additionally, paired sample t-tests were conducted in SPSS on AR statistics for the eight neutral loci.

Results

STRUCTURE analysis found that $K=11$ was the most appropriate number of clusters across the eight river catchments sampled. Values of $K \geq 12$ were not well supported by Ln P(D) values and additional clusters had no individuals strongly assigning to them nor any explicable geographical basis. The eleven clusters identified tended to have broad agreement with river catchments. For instance, good assignment of most individuals to a particular cluster was found in the Owenwee (Cluster 8; 56.3%), Owenduff (Cluster 11; 70.5%), the Goulaun (Cluster 2; 56.0%), Owenmore (Cluster 9; 45.5%) and Erriff (Cluster 6; 44.0%) (see Table I, Fig. 2).

The two populations isolated above waterfalls in the Srahrevagh and Mulkear demonstrated the least admixture amongst clusters. Virtually all individuals strongly assigned to cluster 4 in the Srahrevagh AW sample and the overall sample assignment was 86.1%. However, individuals could be identified in the Srahrevagh below the waterfall (BW) which strongly assigned to the same cluster, 4, at $Q \geq 0.5$ (number of individuals, $n=8$). These were considered likely to be downstream migrants. This was not unexpected as it is possible that fry may pass down over the waterfall but fish are not able to get up the waterfall. This does suggest the possibility for some unidirectional gene flow from above the waterfall to below and there were a further small number ($n=12$) of fry in the Srahrevagh (BW) sample which may be admixed, showing intermediate assignment ($0.1 < Q \leq 0.5$) to the “above waterfall cluster”. However, there was strong differentiation

between the above and below waterfall populations in the Srahrevagh ($\Phi = 0.077$, $p<0.001$). This was not the case in the Mulkear ($\Phi = 0.007$, ns) where the same cluster was found in both samples (n=5) although there was more evidence of admixture below the waterfall (Mulkear AW 90.5%, Mulkear BW 78.9%).

There were some important exceptions to the general trend of agreement between river systems and clustering patterns. The Carrowniskey (Cluster 6 44.7%) and Lough Alisheen (Cluster 1 77.0%, see Fig 2) samples were initially considered to be taken from the same population but preliminary tests, including the use of STRUCTURE, identified strong population differentiation ($\Phi = 0.074$, $p<0.001$). The two were considered separately in subsequent analyses. Cluster 6 was the most common cluster in both the Carrowniskey (44.7%) and Erriff (44.0%). The Carrowniskey and the Erriff have a much lower Φ (0.0194) than do the Carrowniskey and the next nearest neighbour, the Owenwee ($\Phi = 0.055$). There is no obvious reason for this similarity between the Carrowniskey and Erriff, although the mouths of the rivers are reasonably close. Cryptic population structure was identified in the Skerdagh S1 and S4 samples. Both are largely composed of cluster 7 (77.2% and 33.3%, respectively, Fig. 2) but the Skerdagh S4 sample demonstrates far more admixture with cluster 8 (23.5%) (which is mainly found in the Owenwee) and is significantly differentiated from Skerdagh S1 ($\Phi = 0.055$, $p<0.001$). The Skerdagh S4 sample is less differentiated from the Owenwee ($\Phi = 0.038$) than the Skerdagh S1 sample is from Owenwee ($\Phi = 0.079$). The Goulaun (56.0%) and Srahrevagh (BW) (17.5%) samples are both in the Burrishoole system, cluster 2 was common in both but the Srahrevagh (BW) demonstrated admixture with two other clusters, 3 (29.2%) and 10 (29.5%), which were much less common in the Goulaun (4.4% and 11.3%, respectively).

338

339 STRUCTURE identified clear incidences of cryptic population structure which
340 could be resolved, *post hoc*, to discrete sub-samples of small tributaries or different
341 reaches of the same tributary, as in the Carrowniskey and Skerdagh systems, and where
342 Φ estimates of population differentiation were significant. These were considered as the
343 Skerdagh (S1), Skerdagh (S4), Carrowniskey and Lough Alisheen populations in
344 subsequent analyses. The Mulkear above and below samples did not show significant
345 population differentiation. Consequently, the Mulkear (BW) and Mulkear (AW) samples
346 were combined for the analysis comparing population differentiation at neutral loci and
347 MHC.

348

349

350 Hardy-Weinberg exact tests found no deviations from expectations across loci in
351 any of the populations. It was concluded from STRUCTURE and Φ estimates of population
352 differentiation (Table II) that the overall population structure was best defined by 12
353 populations (data are presented for the Mulkear (BW) and Mulkear (AW) samples
354 separately in Table I).

355

356

357 Variation at the neutral markers was significantly lower in populations isolated
358 above waterfalls in both rivers, as measured by individual heterozygosity and allelic
359 richness (Table I, Fig. 3, 4). Individual heterozygosity at neutral markers in the Srahrevagh
360 had a median value of 0.750 below the waterfall and 0.625 above (Mann-Whitney test, Z=-
361 6.587, p=0.001). A paired t-test on locus by locus proportions of heterozygotes at neutral
362 loci also showed significantly lower variation above the waterfall (t=-4.812, df=7, p=0.002).
363 In the Mulkear, median individual heterozygosity at neutral markers was 0.571 below the

364 waterfall and 0.470 above (Mann-Whitney test, Z=-2.976, p=0.003) while the paired t-test
365 on locus by locus proportions of heterozygotes at the neutral loci was also significant (t=-
366 3.185, df=7, p=0.015).
367
368
369 Neutral allelic richness was significantly greater below the waterfall in the
370 Srahrevagh (7.59, CI95%±0.0019) than above the waterfall (5.21, CI95%±0.0012)
371 (Bootstrap test, p=0.000001; Paired sample t-test, t=-3.722, df=7, p=0.007). Neutral allelic
372 richness was also significantly greater below the waterfall in the Mulkear (4.70,
373 CI95%±0.0007) than above the waterfall (3.47, CI95%±0.0013) (Bootstrap test, p=0.046;
374 Paired sample t-test t=-3.087, df=7, p=0.018).
375
376
377 Variation at the MHC class I-linked locus was not significantly reduced for either
378 individual heterozygosity or allelic richness in the populations isolated above waterfalls
379 (Table I, Fig. 3, 4). The proportion of heterozygotes in the Srahrevagh above the waterfall
380 was 0.852 while the proportion below was 0.885 (t-test, df=193, t=0.511, p=0.610). In the
381 Mulkear, the proportion of heterozygotes was not significantly different above (0.778) and
382 below (0.795) the waterfall (t-test, df=100, t=0.188, p=0.851). Allelic richness was not
383 significantly different above (8.58, CI95%±0.0040) and below (7.84, CI95%±0.0062) the
384 waterfall in the Srahrevagh (bootstrap test, p=0.636). However, allelic richness was
385 actually, marginally, significantly higher above the waterfall in the Mulkear (6.54,
386 CI95%±0.0037) than below (5.00, CI95%±0.0000) (bootstrap test, p=0.047).
387
388
389 Population differentiation (Φ) (Table II) was significantly less at the MHC class I-

390 linked locus (mean 0.078±0.0050) than at neutral loci (mean 0.104±0.0074, Wilcoxon
391 Signed Rank Test, Z=-2.701, p<0.001) amongst the twelve distinct populations identified
392 (the Mulkear was considered one population for this analysis due to the lack of a
393 significant Φ between the two Mulkear samples), with significantly lower MHC class I Φ
394 seen in 43 of 66 population pairs (Jacknife test, CI 99.9% on neutral expectations, Fig 5).
395
396
397 **Discussion**
398
399
400 This study tested two predictions of balancing selection on MHC class I in wild *S.*
401 *trutta* and found strong evidence for both. First, variation was significantly lower at neutral
402 genetic markers in isolated populations but was maintained at the MHC class I-linked
403 locus. Balancing selection on MHC is thought to be largely driven by exposure to a high
404 diversity of pathogens (Klein & O'Huigin, 1994; Jeffery & Bangham, 2000; Prugnolle *et al.*,
405 2005) but it should be noted that the waterfalls pose barriers to many pathogens and novel
406 disease vectoring. One study of *S. salar* gut microfauna found it to be remarkably
407 depauperate and dominated by *Mycoplasma* spp., ordinarily obligate intracellular parasites
408 (Holben *et al.*, 2002). This may not be surprising given these salmonids are found in
409 upland systems which amount to freshwater flow-through systems. There are reasonable
410 grounds to conclude that pathogenic pressures above waterfalls differ markedly from those
411 below the waterfall and are likely to be reduced. Sexual selection may also influence the
412 maintenance of genetic variation at MHC in these salmonids (Landry *et al.*, 2001;
413 Bernatchez & Landry, 2003; Pitcher & Neff, 2006; Neff *et al.*, 2008; Consuegra & Garcia de
414 Leaniz, 2008). O' Farrell *et al.* (2012) found evidence for kin association in *S. trutta* below
415 the waterfall in the Srahrevagh tributary based on the sharing of MHC alleles. They went

416 on to argue how this phenomenon could lead to a form of kin recognition-driven rare allele
417 advantage (Grafen, 1990) leading to balancing selection on MHC in these *S. trutta*. This
418 could also explain the maintenance of MHC variation above the waterfall in the
419 Srahrevagh.
420
421
422 It is not known whether the suite of MHC alleles maintained by alternate
423 mechanisms above waterfalls provide downstream migrants with good resistance to
424 pathogens encountered below the waterfall. Within the isolated populations, this type of
425 behaviourally-mediated balancing selection is decoupled from broader disease pressures.
426 Behaviourally-mediated balancing selection relies on the finite ability of individuals to
427 identify alleles which are different from their own. MHC alleles which have a large number
428 of amino acid pairwise difference to other alleles, such as recombinant alleles, may tend to
429 be favoured. Interestingly, preliminary MHC class I (*Satr-UBA*) sequence data from a
430 sample of adult *S. trutta* in 2004 (unpublished data) found that fish assigned to the above
431 waterfall population had a significantly more divergent suite of MHC alleles when mean
432 amino acid pairwise distances (0.40±0.024) were looked at, than those found in fish
433 assigned to the below waterfall population (0.32±0.022), Mann-Whitney U=624.0, Z=-
434 3.684, $P<0.001$.
435
436
437 A suite of MHC alleles with a large mean amino acid sequence distance does not
438 imply a similarly diverse immuno-surveillance capacity. In practice, MHC alleles fall into a
439 smaller number of “supertypes” based on their antigen binding capacity (Sette *et al.*,
440 2003). Behaviourally-mediated selection on MHC alleles, when divorced from pathogen-
441 driven balancing selection over any considerable length of time, may lead to a form of

442 runaway selection, wherein alleles with rare or even maladaptive antigen binding capacity
443 are favoured. Consequently, some MHC alleles in isolated populations may prove
444 maladaptive in downstream migrants placing them at a selective disadvantage if they are
445 poorly able to deal with more varied pathogenic pressures downstream. Conversely, some
446 of these exotic MHC alleles may provide migrants with unique capacity to defend against
447 epidemics of novel pathogens in the downstream population.
448
449
450 Second, population differentiation (Φ) was significantly less at MHC class I-linked
451 locus across the study as a whole. This is as predicted for a gene under balancing
452 selection or one closely linked to such a locus (Muirhead, 2001). The opposite has been
453 observed for most studies of MHC (Muirhead, 2001; Landry & Bernatchez, 2001;
454 Bernatchez & Landry, 2003; Aguilar & Garza, 2006; Sutton *et al.*, 2011). However, these
455 have usually compared MHC sequence data-derived F_{ST} values with those from neutral
456 microsatellites. Neutral data in the Sutton *et al.* (2011) meta-analysis paper was largely
457 derived from neutral microsatellites (74%). The approach here, comparing F_{ST} (Φ) at
458 neutral and a MHC-linked microsatellite, avoids a potential bias in comparing F_{ST} derived
459 from different types of genetic marker. For example, if these previous studies had
460 compared their MHC sequence data with neutral SNP data (neutral SNP F_{ST} is nearly
461 three times that at neutral microsatellites in salmonids (Narum *et al.*, 2008)) it is possible,
462 even likely, that they would have found lower population differentiation at MHC. Another
463 possibility may be that there are more issues with homology at the MHC-linked
464 microsatellite locus than the neutral loci, which would depress Φ estimates at the former,
465 although there are no data to support this.
466
467

468 Directional selection can also cause lower differentiation at MHC through exposure
469 to the same pathogen (Teacher *et al.*, 2009; Fraser & Neff, 2010). This occurs because a
470 specific pathogen will select for and against the same MHC alleles in separate
471 populations, causing their allele frequencies to become more similar. No agent of
472 homogenising, directional selection could explain the lower differentiation across the *S.*
473 *trutta* populations that have been monitored for several decades. However, this issue was
474 not examined directly and this may be an interesting avenue for future research.
475
476
477 However, the significantly higher Φ values for MHC seen in 12 of 66 population
478 comparisons may be explained by directional selection, as each of these comparisons
479 involved the Burrishoole, Erriff and Skerdagh rivers (Muirhead, 2001). *S. trutta* in these
480 rivers have a history of disease exposure associated with *S. salar* aquaculture and
481 perturbations associated with fisheries management, not experienced by the other
482 populations sampled. Localised bursts of directional selection (selective sweeps) may
483 have occurred in the Burrishoole, Erriff and Skerdagh rivers and it is clear that an interplay
484 of directional and balancing selection may occur. In the case of the Skerdagh, where
485 cryptic population structure was observed, the neutral Φ between Skerdagh S1 and S4
486 was 0.058 while the Φ value at the MHC-linked locus was 0.139. Disease might help
487 explain the cryptic population structure in the Skerdagh. The overall contrast in pattern for
488 disturbed/disease-affected populations versus pristine populations in this study may be
489 noteworthy given the growing interest in using selected markers like MHC for identifying
490 stocks in conservation genetics. This is an interesting anecdotal finding. The aquaculture
491 practices in the Mayo region involve only *S. salar*. As such, there is potential for disease
492 exposure to both native *S. salar* and *S. trutta* but only the potential for gene flow from
493 aquaculture escapes in one species. One possible follow-on study would be to examine

494 gene flow at neutral and at selected loci such as MHC in native *S. salar* and *S. trutta*
495 populations within the study region.
496
497
498 It is concluded that balancing selection at MHC best explains the overall
499 observations of lower than expected differentiation at MHC, and the maintenance of
500 significantly higher variation at MHC than expected in the isolated populations. This study
501 has presented clear evidence of balancing selection on MHC class I in the wild.
502
503
504 **Acknowledgments**
505
506
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511
512
513 **Conflict of Interest**
514
515
516 The authors declare no conflict of interest.
517

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519

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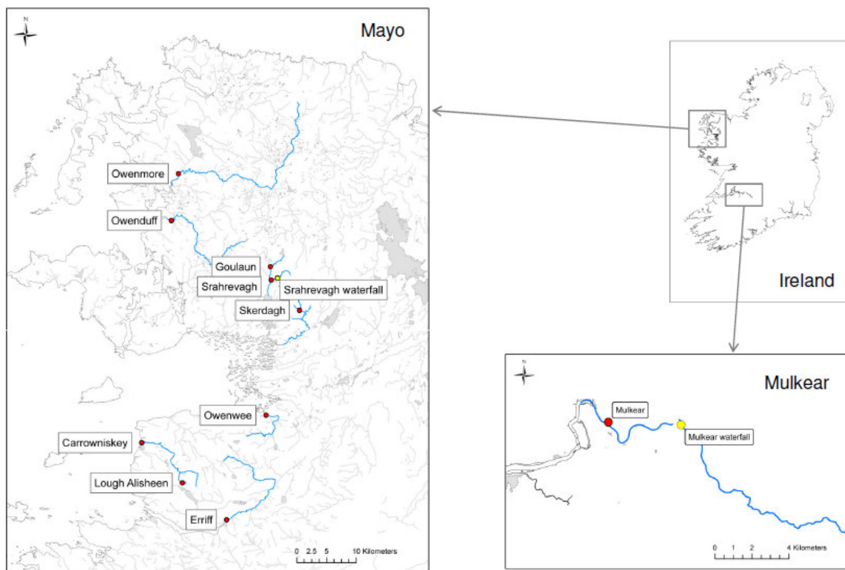
755



756

Figure 1

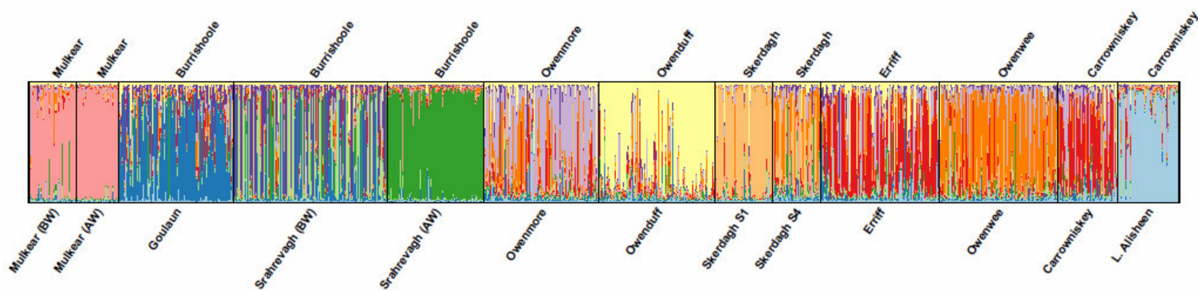


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Figure 2

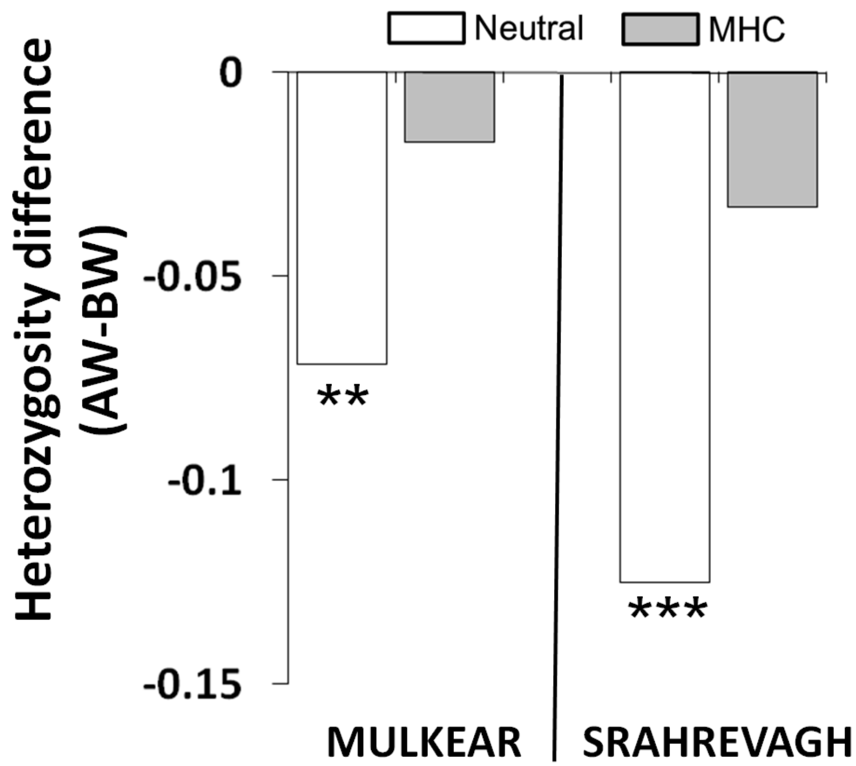

Figure 3

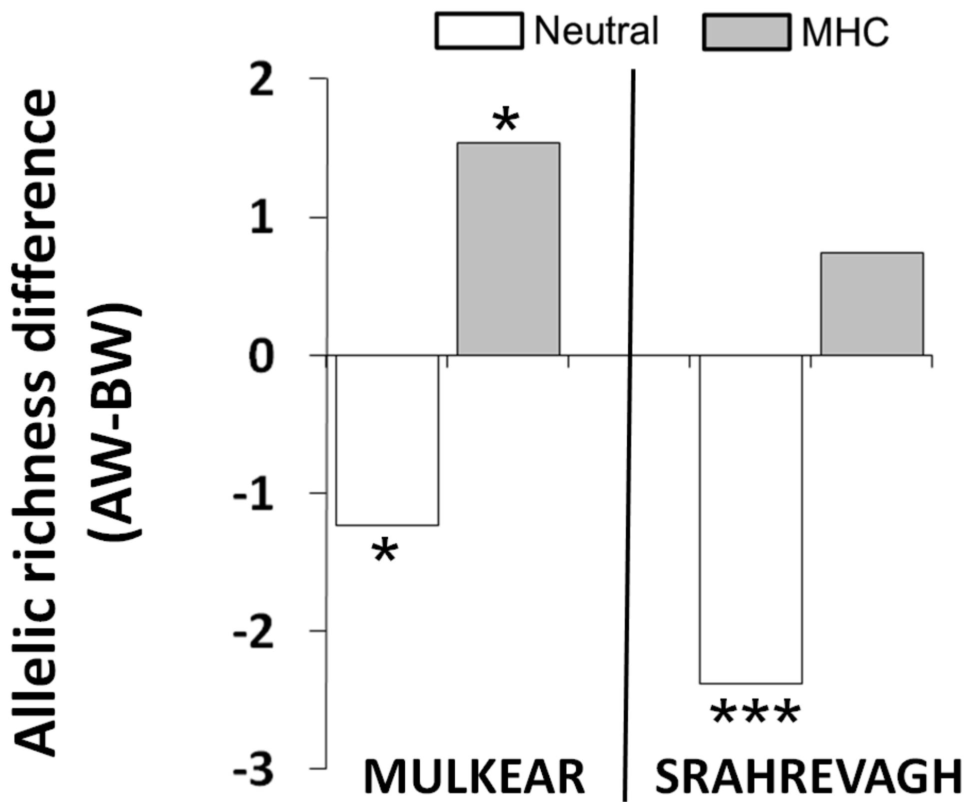

Figure 4

758

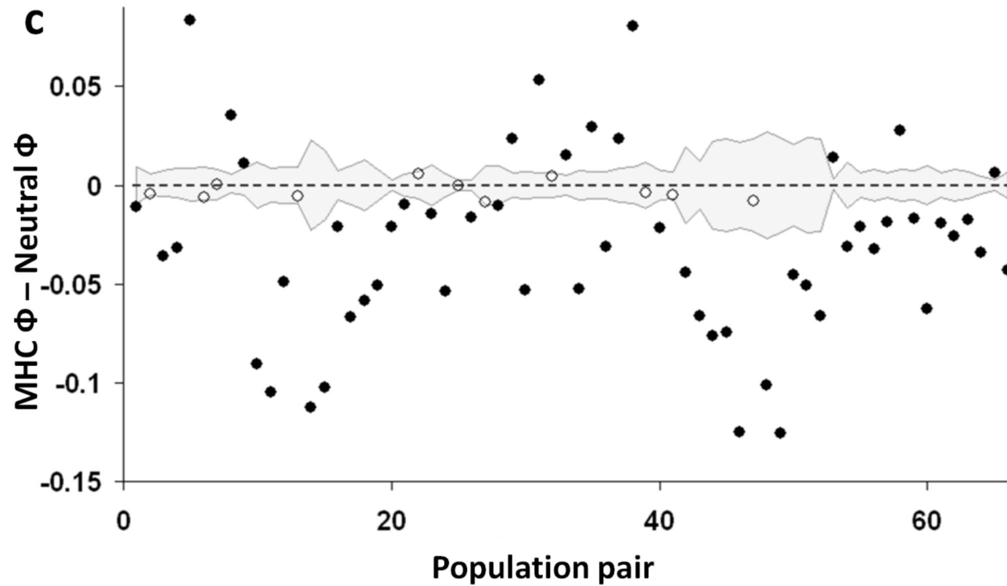

Figure 5

759 **Captions**

760

761

762 **Figure 1**

763 Map of Ireland showing the areas containing the eight river systems from which brown
764 trout samples in this paper were taken. The Burrishoole system contains the Srahrevagh
765 and Goulaun tributaries shown while the Newport system contains the Skerdagh tributary
766 shown.

767

768

769 **Figure 2**

770 STRUCTURE analysis (K = 11) of brown trout sampled. The number of clusters was
771 arrived at following the approach of Evanno *et al.* (2005) but then followed by longer runs
772 (Burn-in 100,000 with subsequent 1,000,000 MCMC iterations) over the range K = 9 to K =
773 14. Trout population names are included at the bottom of the figure with the river

774 catchment from which they are from on the top of the figure. The Mulkear BW and Mulkear
775 AW are presented separately despite lack of significant population differentiation. The
776 populations isolated above waterfalls (Mulkear AW and Srahrevagh AW) demonstrate
777 good assignment of all individuals to a particular cluster. The cryptic population in the
778 Carrowniskey system, now referred to as the Lough Alisheen population, has strong
779 assignment to a unique cluster. This is interesting given there are no physical barriers
780 between this cluster and the downstream population which we term Carrowniskey. Indeed,
781 there is more evidence of admixture between the Carrowniskey and the Erriff population to
782 the South (see Figure 1) than between the Carrowniskey and Lough Alisheen.
783
784
785 **Figure 3** The difference in heterozygosity above waterfalls (AW) and below waterfalls
786 (BW) is presented for neutral loci (median values across individuals) and the MHC-linked
787 locus (proportion of heterozygotes at the locus). Significance levels ($P < 0.001$,***; $P <$
788 0.01,**; $P < 0.05$,*) are also indicated. Heterozygosity is significantly lower at neutral loci
789 above waterfalls than below waterfalls (BW) but not at MHC in both the Srahrevagh and
790 the Mulkear.
791
792 **Figure 4** The difference in allelic richness above waterfalls (AW) and below waterfalls
793 (BW) is presented for neutral loci and the MHC-linked locus. Significance levels ($P <$
794 0.001,***; $P < 0.01$,**; $P < 0.05$,*) are also indicated. Allelic richness above waterfalls (AW)
795 is significantly lower at neutral loci than below waterfalls (BW) but not at MHC in the
796 Srahrevagh and the Mulkear. Allelic richness is actually somewhat higher above waterfalls
797 in both case studies and significantly so in the case of the Mulkear.
798 **Figure 5** Difference between MHC and neutral loci for all population pairs (•=significant,
799 o=not-significant): those which show a significantly lower level of differentiation (φ) at MHC

800 than at neutral loci are those below the zero line and outside the 99.9% confidence
801 intervals for the neutral loci (grey), those showing higher levels of differentiation at MHC
802 are above the line.
803

804 **Table I** Sample sizes & descriptive data. Note the Mulkear (BW) and Mulkear (AW) are
805 presented separately as the values are relevant to the waterfall case studies and the
806 above waterfall population is implicitly reproductively isolated. It's interesting to note that
807 although neutral allelic richness is lower in the Srahrevagh (AW) sample than all open
808 populations bar the Mulkear (BW) sample, MHC allelic richness is higher than that found in
809 both open populations in the Burrishoole system. The Skerdagh S1 population also shows
810 some signs of reduced allelic richness at both neutral and MHC loci whereas, curiously,
811 the Skerdagh S4 has reduced neutral allelic richness but much higher allelic richness at
812 MHC.

Population	Mulkear BW	Mulkear AW	Goulaun	Srahrevagh BW	Srahrevagh AW	Owenmore	Owenduff	Skerdagh S1	Skerdagh S4	Erriff	Owenwee	Carrowniskey	Lough Alisheen
Sample size	39	45	97	130	74	99	98	48	41	100	100	51	51
River System	Mulkear	Mulkear	Burrishoole	Burrishoole	Burrishoole	Owenmore	Owenduff	Newport	Newport	Erriff	Owenwee	Carrowniskey	Carrowniskey
STRUCTURE Assignment (K=11)	5 (78.9%)	5 (90.5%)	2 (56.0%)	3/10 (29.2%/29.5%)	4 (86.1%)	9 (45.5%)	11 (70.5%)	7 (77.2%)	7 (33.3%)	6 (44.0%)	8 (56.3%)	6 (44.7%)	1 (77.0%)
Allelic Richness													
Neutral allelic	4.699	3.471	7.513	7.589	5.207	7.746	6.822	5.118	6.073	7.138	6.801	8.0606	6.656

richness (bootstrap)													
MHC allelic richness (bootstrap)	5.000	6.538	7.867	7.841	8.584	11.551	11.259	5.906	8.799	8.076	9.302	8.988	8.32
Neutral heterozygosity													
Neutral individual hete	0.571	0.500	0.750	0.750	0.625	0.750	0.750	0.625	0.625	0.750	0.625	0.75	0.625

rozygosity (median) MHC (proportion of heterozygotes)	0.795	0.778	0.794	0.885	0.852	0.707	0.776	0.625	0.707	0.750	0.910	0.725	0.902

Table I Sample sizes & descriptive data. Note the Mulkear (BW) and Mulkear (AW) are presented separately as the values are relevant to the waterfall case studies and the above waterfall population is implicitly reproductively isolated. It’s interesting to note that although neutral allelic richness is lower in the Srahrevagh (AW) sample than all open populations bar the Mulkear (BW) sample, MHC allelic richness is higher than that found in both open populations in the Burrishoole system. The Skerdagh S1 population also shows some signs of reduced allelic richness at both neutral and MHC loci whereas, curiously, the Skerdagh S4 has reduced neutral allelic richness but much higher allelic richness at MHC.

Population	**Mulkear BW**	**Mulkear AW**	**Goulaun**	**Srahrevagh BW**	**Srahrevagh AW**	**Owenmore**	**Owenduff**	**Skerdagh S1**	**Skerdagh S4**	**Erriff**	**Owenwee**	**Carrowniskey**	**Lough Alisheen**
Sample size	39	45	97	130	74	99	98	48	41	100	100	51	51
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