Allegato 2a_6

Heterozygosity-Fitness Correlation despite low variation at Major Histocompatibility Complex in Alpine ibex (*Capra ibex*), evidence for selection or signal of inbreeding?

Alice Brambilla[1,2], Iris Biebach [3]

[1] DSTA - Department of Earth and Environmental Science, University of Pavia, Via A. Ferrata 9, 27100 Pavia (PV), Italy.

[2] Alpine Wildlife Research Centre, Gran Paradiso National Park, Degioz 11, Valsavarenche (AO).

[3] Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland.

Corresponding author: Alice Brambilla

E-mail address: alicebrambilla1@gmail.com

Abstract

The Major Histocompatibility Complex (MHC) is a large gene complex responsible for the immune response in vertebrates. MHC is known to be one of the most variable regions of the entire genomes. The mechanisms involved in the maintenance of such a diversity are still debated but it is well accepted that the high genetic variability at MHC plays an important role in the recognition of a large number of pathogen agents and should therefore be maintained through some form of selection. Bottlenecks could strongly reduce genome-wide diversity; balancing selection, on the other side, should maintain higher levels of diversity at MHC regions. We tested this hypothesis in Alpine ibex (*Capra ibex*) of Gran Paradiso National Park. We tested if there are differences in heterozygosity between MHC linked and neutral microsatellites; we also tested for Heterozygosity-Fitness Correlations (HFCs) between MHC linked microsatellites and some fitness related traits (body mass, horn length and parasitic infection) and compared them with HFCs at neutral markers. As expected due to the recent bottleneck events experienced by Gran Paradiso population, we found very low genetic diversity at MHC linked microsatellites. Heterozygosity at the MHC linked and neutral markers was not correlated. We found some MHC linked microsatellites deviating from

Hardy-Weinberg Equilibrium thus suggesting selection at these loci even if there are no strong evidences. We found HFCs and local effects on weight and horn growth for MHC linked microsatellites while we did not find an effect of heterozygosity on parasite infection. It is till not clear if MHC markers are themselves responsible for the HFCs, reinforcing the idea of selection at these markers, or if it is just a signal of inbreeding like that at neutral markers.

Introduction

Genetic variation is the raw material necessary for evolution. In absence of genetic variation, populations could not evolve in response to environmental changes, changes in the ecosystems, new diseases or parasites (Frankham 1996). Moreover, genetic variation, and particularly heterozygosity, is known to be correlated to fitness (Reed and Frankham 2003; Chapman 2009). Low levels of heterozygosity can arise after bottlenecks due to the reduction in population size and thus in the genetic pool. Bottlenecks can also cause inbreeding which can result in inbreeding depression (Szulkin et al. 2010) and in a decrease of fitness (Crnokrak and Roff 1999; Slate et al. 2000; Keller and Waller 2002). Loss of genetic variation moreover is predicted to reduce the adaptive potential of populations (Frankham et al. 2002; Willi et al. 2006).

A well known consequence of the loss of genetic diversity is the decreased resistance of species to disease (Coltman et al. 1999; Frankham et al. 2002; Spielman et al. 2004). Immunitary response in vertebrates is mainly driven by the Major Histocompatibility Complex (MHC), MHC is a large gene complex that encodes proteins involved in the innate immunity; it is divided in three subregions (MHC I, MHC II and MHC III). Most of the identified loci of MHC are expressed and encodes for MHC molecules. The most important function of these molecules is to bind immunogenetic peptides inside cells and to present them to lymphocytes for recognition and activation of the immunitary response (Janeway et al. 2001). The major histocompatibility complex, particularly MHC I and MHC II, are known to be one of the most variable region of the entire vertebrate genomes (Janeway et al. 2001). MHC I and MHC II are polymorphic and polygenic greatly increasing the number of pathogens recognized by the immune system. In large outbred populations, polymorphism at each locus can potentially double the number of different MHC molecules expressed by an individual, as most individuals will be heterozygotes. Moreover, due to polymorphism, individuals in a population will differ in the combinations of MHC molecules they express, making it unlikely that all individuals in a population will be equally susceptible to a given pathogen which spread will therefore be limited.

Many studies indicate that high levels of genetic variation at MHC are maintained through some form of selection (Garrigan and Hedrick 2003; Aguilar et al. 2004). Other lines of evidences are

reviewed in Paterson et al. 1998 and can be summarized as follow: in some studies, in which MHC genealogies were reconstructed, the divergence of MHC lineages predates speciation events originating separate taxa (Klein et al. 1993; Yuhki and O'Brien 1997). Another support to the hypothesis of balancing selection as a cause of maintenance of high allelic diversity at MHC is that, at the antigen presenting site, the rate of non synonymous exceed the rate of synonymous substitution favoring variation at MHC (Hughes and Nei 1988; 1989; Hughes et al. 1994). Finally, allele frequency distribution at MHC markers are often more even than expected under neutrality indicating some form of selection going on (Hedrick and Tomson 1993). Despite evidences of selective advantage of MHC polymorphism the mechanisms behind its maintenance are still debated (Spurgin and Richardson 2010). The three mechanism proposed to explain the advantage of MHC variability are: a) overdominance or heterozygote advantage (Heterozygosity at MHC loci should increase the range of parasites recognized by the immune system leading heterozygous individuals advantaged compared to homozygous in immunitary response); b) rare allele advantage (negative frequency-dependence selection driven by coevolution between host and pathogens (Borghans et al. 2004) and c) fluctuating selection. Theory predicts that that any of the three mechanisms, or any combination of them, could maintain MHC diversity (Spurgin and Richardson 2010). Despite it is often not possible to detect which is the exact mechanism by which higher variation at MHC are selected (Spurgin and Richardson 2010), the consequences of the loss of genetic variation are well known: it can reduce resistance to pathogens and, more generally, decrease fitness of individuals. Heterozygosity-Fitness Correlation (HFC) are thus expected to arise also at MHC level even if the mechanism underlying it are still debated (Da Silva et al. 2008).

An important cause of the reduction of genetic variation are bottlenecks. Alpine ibex (*Capra ibex*) is a mountain ungulates once well spread in the entire Alpine arc which underwent severe bottlenecks. The species almost went extinct in the 18th century with less than 100 individuals left in the Gran Paradiso area (Grodinsky and Stuwe 1987; Apollonio et al. 2003). During world war second, the Gran Paradiso population suffered another strong numerical reduction with no more than 600 animals left (unpublished data). Nowadays Alpine ibex is again spread in all the Alps; all the existing populations however derived from reintroduction programs and originated from few founders individuals. The species is considered as “Least concern” in IUCN Red List of Threatened Species (Aulagnier et al, 2008), however, even the original Gran Paradiso population has a very low genetic variability at neutral markers. Alpine ibex thus represent a good model species to test the effect of genetic variability at MHC on fitness of individuals and possible evidences of selection at these loci.

We investigated whether there is signal of selection at MHC level testing if heterozygosity at MHC

is different from heterozygosity at neutral microsatellites (Piertney and Oliver 2006). We then calculated HFC at MHC loci. Particularly, we tested if MHC linked microsatellites are correlated with fitness related traits and we compared the magnitude effect of HFC at neutral and MHC linked microsatellites. We then tried to disentangle whether HFC at MHC is a signal of inbreeding depression reflecting genome-wide heterozygosity, as expected from the so called "general effect" hypothesis (Chapman et al. 2009), or if MHC genes themselves are responsible for HFC indicating a "direct effect" on fitness highlighted by the local effect at MHC closely linked microsatellites.

Materials and methods

Study site, population and life history data collection:

This study has been carried out in the framework of a long term monitoring project on ecology and life-history of Alpine ibex going on in Gran Paradiso National Park (GPNP, North-Western Italian Alps; 45° 25' N, 07° 34' W) since 1999 (Bassano et al. 2003; Grignolio et al. 2004; von Hardenberg et al. 2004; 2007; Bergeron et al. 2010). The intensive study site of GPNP is situated in the Levionaz basin (Valsavarenche, AO) where most of the animals are marked and individually followed life-long collecting behavioural and life history data. During captures, skin or blood samples were collected for molecular analysis. Cohort of marked animals ranged between 1985 and 2009 providing 25 years of molecular data. For more details on the marking protocol see Brambilla et al. 2013.

Life history data (body mass and annual horn growth) are collected every year on the marked individuals in Levionaz. Male ibex were repeatedly weighed during summer with an electronic platform scale baited with salt; to allow comparison between individuals, body mass is then adjusted at the 1st of August. More details on data collection and adjustment can be found in Bassano et al. (2003) and von Hardenberg (2005).

Annual horn growth and total horn length of males ibex were measured during captures. In the years after captures, annual horn growth of marked individuals were measured from remote pictures following Bergeron (2007) and Brambilla and Canedoli (2013).

Parasite load data are also collected every year during summer on marked individuals: faecal samples were collected monthly (from May to September) and individual Faecal Eggs Count (FEC) was determined following a modified McMaster technique (Ministry of Agriculture, Fisheries and Food 1971). FEC were expressed as the number of eggs per gram of fresh faeces (EPG). We then calculated the average EPG for each animal during the season. (Brambilla et al. 2013). FEC can be considered as an index of resistance and resilience of Alpine ibex to nematode infection (Abomasal trichostrongyle: *Marshallagia marshalli*, *Teladorsagia circumcincta* and *Ostertagia occidentalis*),

since host resistance and resilience may influence parasite fecundity (Coltman et al. 1999).

Genetic analysis:

We performed microsatellite analysis on N=248 samples of individual male Alpine ibex. Tissue samples were stored in 95% ethanol solution after collection while blood samples were stored in EDTA vacutainer tubes at a temperature of -35°C or applied on absorbent paper cards for blood DNA (Whatman FTA ® cards) and stored at room temperature before analysis.

DNA extraction and genotyping: for tissue samples we used QIAmp DNA Mini Standard protocol; for blood samples on FTA ® cards we used QIAmp DNA Mini Dried Blood Spot Protocol; for frozen entire blood samples we modified Biosprint 96 DNA protocol. We genotyped all samples at two sets of loci for a total of 51 polymorphic microsatellites. The first set was composed of 37 loci known to be neutral (Biebach and Keller 2009) while the second set was composed of other 9 microsatellites that are known to be linked to MHC in Alpine ibex (See Table 1 for details).

Table1: name, position (chromosome and gene region) and references of the MHC-linked microsatellites used in the present study.

Microsatellites	Location	References
DYMS1	23, MHC I	Buitkamp et al. 1996
OLADRB1	23, MHC II	Paterson 1998
OLADRB2	23, MHC II	Blattman and Beh 1992
TCRVb6		Buitkamp et al. 1993
OMHC1	23, MHC I	Groth and Wetherall 1994
BM1818	23, flanking MHC	Bishop et al. 1994
Bf94.1		Groth and Wetherall 1995
SPS113		Movahedin et al. 2010
BM1258	23, flanking MHC	Bishop et al. 1994

The remaining 5 microsatellites were found to be not in HWE in many population or were supposed to be not neutral (SR-CRSP07; BM4208; ETH10; OarHH62; OarkP6) and were thus not used for this study. The microsatellite we used have been originally isolated in cattle, sheep or domestic goat (von Hardenberg et al. 2007; Biebach and Keller 2009). PCR conditions and Fragment Analysis are described in Appendix 1. We did up to 3 PCR repetitions for non-amplified or unreliable samples and then we manually built consensus genotypes. We did 2 PCR repetitions for all the FTA ® cards samples. We calculated locus specific dropout and false allele rates with the software Gimlet

(Valiere 2002). The error rates are reported in Appendix 1.

Data analysis:

Hardy-Weinberg Equilibrium (HWE) with sequential Bonferroni correction was calculated for the 9 MHC linked microsatellites using the Excel ® plugin GeneAlEx 6.5 (Peakall and Smouse 2012). HWE of the neutral microsatellites were already calculated in a previous study (Brambilla et al. under review). Linkage Disequilibrium (LD) between all pair of loci (MHC linked and neutral) was obtained using the software GenePop (Rousset 2008) and sequential Bonferroni correction was again calculated to avoid problems due to multiple testing.

Identity Disequilibrium (ID) between MHC linked microsatellites was assessed using the software RMES calculating g2 (David et al. 2007).

Statistical analysis were performed using R 3.0.0 (R Development Core 2013). For an explorative analysis we calculated average multilocus heterozygosity (MLH) at MHC linked and at neutral microsatellites in the population, we then calculated multilocus standardized heterozygosity (Hst) for each individual (N=237) for the two sets of markers: standardization avoid errors due to possible differences in loci typed between individuals and was calculated as the heterozygosity of the individual divided by the mean heterozygosity of the loci at which the individual was typed (Coltman et al. 1999). Hst at the two sets of markers was then compared through a paired t-test and moreover a correlation between Hst at neutral and at MHC linked microsatellites was calculated.

HFCs were calculated correlating Hst at MHC linked microsatellites and three fitness-related traits: body mass, horn growth and parasite infection (EPG). We standardized all the variables to allow comparison of the effect of Hst on the different traits. HFCs were tested using linear mixed effects models (package lme4, Bates et al. 2011). We included in the models for body size and horn growth Age and Age squared of individuals and the interaction between Hst and Age and in the model for parasite infection the interaction between Hst and Age. We also added in the models individual Identity and Year as random effect since we had repeated measures for individuals taken in different years. For the three fitness traits analyzed we fitted a set of models with all the combinations of the variables of the full model. Using the dredge and model.avg functions of the R package MuMIn (Barton, 2013) we tested the importance of the variables. Model selection was done using Akaike's Information Criterion, AICc (Burnham and Anderson 2002), comparing the corrected values of AIC and choosing a threshold of ΔAICc = 6 to select the best models. HFCs at neutral microsatellites were calculated in a previous work done on the same species (Brambilla et al. under review)

Local effects of single MHC linked microsatellites on parasite load were tested using binary variable (0-1) for Heterozygosity at the different MHC linked loci and FEC as response variable.

Age and interaction between Age and Hst were added in the models and Year and individual Identity were added as random effects.

Results

Three out of nine MHC linked microsatellites were not in Hardy-Weinberg Equilibrium after Bonferroni sequential correction: DYMS1, OLA1, OLADRB2. All these three microsatellites showed a slight excess of homozygotes.

Four pair of markers were in Linkage Disequilibrium after Bonferroni sequential correction: Bf94.1-OLA1; OLA1-OLADRB2; Bf94.1-OMHC1; OLA1-OMHC1.

G2 for the MHC markers was different from zero (g2 = 0.036, S.D. = 0.018, p = 0.027) indicating the presence of Identity Disequilibrium within this set of microsatellites.

Heterozygosity at neutral microsatellites was: MLH ± S.E. = 0.449 ± 0.084 while heterozygosity at MHC linked microsatellites was: MLH ± S.E. = 0.363 ± 0.188.

The paired t-test for differences in standardized heterozygosity between neutral and MHC linked microsatellites was not significant (t = -0.1946, df = 246, p = 0.4229) indicating no directional differences in Hst between the two sets of markers. There was instead no correlation between Hst at neutral and MHC linked microsatellites (Multiple R-squared = 0.0054, p-value = 0.249).

A summary of the models of Heterozygosity-Fitness Correlations at MHC linked microsatellites are reported in Tables 2 (Body Mass) 3 (Horn Growth) and 4 (EPG).

Table 2. HFC at MHC linked microsatellites: model average of mixed effects model for testing correlation between Body Mass and Hst at MHC linked microsatellites. We reported only the first 4 models of the set.

Components of the model (Body Mass)	df	log likelihood	AICc	Δ AICc	Weight
Age + Age^2 + Hst	7	-223,20	460,70	0,00	0,87
Age + Age^2 + Hst + Age*Hst	8	-225,39	467,17	6,47	0,13
Age + Age^2	6	-238,27	488,75	28,05	0,00
Age + Hst	6	-348,22	708,67	247,97	0,00

Table 3. HFC at MHC linked microsatellites: model average of mixed effects model for testing correlation between Horn Growth and Hst at MHC linked microsatellites. We reported only the first 4 models of the set.

Components of the model (Horn Growth)	df	log likelihood	AICc	Δ AICc	Weight
Age + Age^2 + Hst	6	-998,92	2009,44	0,00	0,90
Age + Age^2 + Hst + Age*Hst	7	-1000,11	2014,36	4,42	0,10
Age + Hst	5	-1013,85	2043,77	33,83	0,00
Age + Hst + Age*Hst	6	-1017,57	2047,24	37,30	0,00

Table 4. HFC at MHC linked microsatellites: model average of mixed effects model for testing correlation between EPG and Hst at MHC linked microsatellites. We reported only the first 4 models of the set.

Components of the model (EPG)	df	log likelihood	AICc	Δ AICc	Weight
Age + Hst	6	-588,22	1188,62	0,00	0,96
Age + Hst + Age*Hst	7	-590,29	1194,82	6,20	0,04
Hst	5	-607,16	1224,45	35,83	0,00
Age	5	-643,50	1297,20	108,50	0,00

We reported here the model averaged coefficients of the models for HFCs MHC linked microsatellites. In brackets we also reported the corresponding coefficients of Hst in HFCs models done with neutral microsatellites found in Brambilla et al. (under review). Hst at MHC linked microsatellites had an effect on all the three traits analyzed very similar to the effect of Hst at neutral markers: particularly it had a weak positive effect on horn growth (model averaged coefficient $\beta \pm$ S.E.: MHC = 0.056 ± 0.039, neutral = 0.080 ± 0.040), a stronger positive effect on body mass (model averaged coefficient $\beta \pm$ S.E.: MHC = 0.154 ± 0.052, neutral = 0.043 ± 0.058) and a weak positive effect on parasite infection (model averaged coefficient $\beta \pm$ S.E.: MHC = 0.066 ± 0.053, neutral = -0.087 ± 0.053). The interaction between Hst and age was retained only in the model for horn growth and had a very weak negative effect (model averaged coefficient $\beta \pm$ S.E.: MHC = -0.053 ± 0.033).

Local effects of heterozygosity at single loci on fitness-related traits were found for only for horn growth and body mass while no effect was found for parasite infection (Table 5).

Table 5: Per-locus analysis: model averaged coefficients β ± S.E. of models testing the local effects of heterozygosity at single loci on fitness-related traits. We reported only the coefficients for the traits for which H was retained in the best models (NR= not retained in the set of the best models). Models for DYMS1 were not reported since models did not run because of problems of colinearity.

Locus	**Horn Growth ± S.E.**	**Body Mass ± S.E.**	**EPG ± S.E.**
Bf94.1	0,0360 ± 0,091	0,2563 ± 0,127	NR
BM1258	0,1275 ± 0,091	0,1851 ± 0,141	-0,0023 ± 0,022
BM1818	0,0336 ± 0,094	0,1636 ± 0,126	0,0004 ± 0,023
OLADRB1	0,0273 ± 0,087	0,2494 ± 0,124	NR
OLADRB2	-0,1505 ± 0,038	0,0765 ± 0,314	NR
OMHC1	0,0384 ± 0,081	-0,0712 ± 0,117	NR
SPS113	0,0706 ± 0,081	0,2868 ± 0,116	NR
TCRVb6	0,0301 ± 0,088	0,1487 ± 0,121	NR

Discussion

The level of heterozygosity at neutral marker that we found in the Alpine ibex population of Gran Paradiso National Park was very low as reported also in previous studies (Biebach and Keller 2012). Despite multilocus heterozygosity at neutral microsatellites is thought to be representative of genome-wide heterozygosity (Hansson and Westerberg 2002), H at MHC markers is expected to be higher since this region of the genome is harshly selected for being highly variable (Hedrick 1994; Aguilar et al. 2004). Nevertheless we found heterozygosity at MHC linked microsatellites of Alpine ibex population of Gran Paradiso National Park being equally low as microsatellites derived heterozygosity. The recent bottlenecks trough which ibex passed may represent a possible explanation of this finding: after the first bottleneck in the 18th century (Grodinsky and Stuwe 1987) indeed, the population underwent another severe numerical reduction during world war second (unpublished data). These two extreme events may have decreased variability at genome-wide level including also MHC region. The same MHC microsatellites that we used in this study, analyzed in other species, showed more allele richness and heterozygosity (Buitkamp et al. 1996; Paterson et al. 1998; Paterson 1998; Roy et al. 1999). Theory (Bernatchez and Landry 2003) and empirical evidences (Aguilar et al. 2004) suggested that high variability at MHC level are maintained through some form of balancing selection. Selection, however, needs some level of variability to act on. If bottlenecks have deleted most of the existing variability at MHC level the years past till then may have been not enough to bring back variability at pre-bottlenecks levels (Eldridge et al. 1999; Withehouse and Harley 2001). In absence of variability, indeed, selection can not act and it is thus

necessary that new variability is added to the population through mutations or introduction of new genotypes of immigrant individuals; mutation processes, however, are extremely slow and need several generations to have a significant effect and immigration is unlikely to contribute to restore diversity since all individuals of other populations that can eventually immigrate derived from few founders taken from the Gran Paradiso population itself (Biebach and Keller 2010). We can thus state that genetic variability at MHC region in Alpine ibex is very low and this may represent a limitation to the adaptive potential of the species.

Three MHC linked microsatellites (DYMS1, OLA1 and OLADRB2) were not in Hardy-Weinberg Equilibrium. As expected due to the physical proximity of the MHC markers, we found some of them in Linkage Disequilibrium. The direction of the deviation from HWE however was unexpected: we found indeed a slight excess of homozygotes while, since MHC region should be selected for variability, we were expecting an excess of heterozygotes. Previous studies carried out on four Swiss ibex populations found most of the same markers being in HWE in all the populations and an excess of homozygotes only in one marker (OMHC1) in one population (unpublished data). Since the direction of the deviation from HWE that we found is toward an excess of homozygotes we can not argue that this is a signal of selection, it may rather be an evidence of an unknown sub-group structure of Gran Paradiso population. As argued by Biebach and Keller (2009), LD may instead be the consequence of of genetic drift driven by the reduced population size due to the bottleneck. ID, as well, may only be an effect of the recent bottlenecks and does not provide any evidence of selection.

The absence of a clear signal of selection in this study however is not a conclusive results, it would be indeed important to analyze several populations and to compare their heterozygosity levels.

Despite the average level of heterozygosity at MHC linked microsatellites of Gran Paradiso population is similar to that at neutral microsatellites, the two values for each individual were not correlated. For this reason we further investigated HFCs at MHC level considering it as independent from HFCs at neutral microsatellites. HFC at neutral microsatellites is a signal of inbreeding depression and it can be considered as an evidence of the "general effect" hypothesis following which the heterozygosity at neutral microsatellites is representative of genome-wide heterozygosity which is advantageous for individuals (Hansson and Westerberg 2002). MHC linked microsatellites instead are closely linked to genes under selection (Paterson 1998; Paterson et al. 1998) and a correlation between heterozygosity and fitness can be an evidence of the "direct effect" that is the advantage of heterozygosity at loci directly affecting fitness (Hansson and Westerberg 2002) that in our case is highlighted by heterozygosity at loci closely linked to expressed genes.

Heterozygosity of MHC markers had a positive effect on body mass and horn growth; for both the

traits the interaction between Hst and age was negative indicating that the effect of Hst on body mass and horn growth decreases with age. As expected (Szulkin et al. 2010), the effect size of Hst on horn growth was weak. The effect size was moreover very similar to that of Hst at neutral microsatellites. On the other hand, the effect of MHC heterozygosity on body mass was stronger both comparing it with the effect on horn growth and with the effect of Hst at neutral microsatellites on body mass itself. The single-locus models moreover indicate a positive influence of heterozygosity of all the analyzed loci on horn growth (except for OLADRB2) and also a positive influence on body mass of heterozygosity at all analyzed loci except for OMHC1; however, the high standard errors of the model averaged coefficient lead to cautiously interpret the results. Anyway, the same effect of different single loci on fitness traits would be expected in loci that are in LD and are linked to the same genes under expression (as our microsatellites are).

The heterozygote advantage observed at these loci might be explained by direct selection on MHC genes closely linked to microsatellites analyzed as suggested by Da Silva et al. (2008). The linkage between microsatellites itself however can partially explain the fact that the positive correlation was found for all the markers analyzed.

In regard to parasite infection, instead, we found a weak positive of Hst at MHC on EPG but in the opposite direction than expected. Also the results of the single-locus models did not evidence an effect of heterozygosity on EPG. This result is in contrast with what we found for neutral microsatellites which, on the contrary, showed a negative effect of Hst on parasitic load. Other researches, instead, found an effect of variation within MHC on resistance to intestinal nematodes (Paterson et al. 1998); this study, however, highlighted the effect of single alleles instead that the effect of heterozygosity itself. Unfortunately, our data did not allow us to compute per-allele models since we had problems of co-linearity due to the low genetic variability of Gran Paradiso population and the very low frequencies of some alleles. Body mass and horn growth are honest signals of individual quality in Alpine ibex (von Hardenberg et al. 2004; 2007; Bergeron et al. 2010) and may represent good proxies for fitness. However, despite a stronger effect size may be expected for these traits than for a physiological traits such as parasite infection (Chapman et al. 2009), the lack of correlation between Hst at MHC marker and EPG was surprisingly especially if we consider that the correlation was found with Hst at neutral microsatellites.

The linkage of MHC microsatellites and the presence of ID suggested that heterozygosity of MHC markers is likely to reflect heterozygosity at expressed MHC genes and thus that HFC at MHC microsatellites is driven by local effect, the advantage for the individuals indeed would be to be heterozygous at MHC genes. HFC at neutral microsatellites, on the other hand, suggested a general positive effect of genome-wide heterozygosity on fitness (Hansson and Westerberg 2002). Also Da

Silva et al. (2008) found a correlation of fitness traits both with neutral microsatellites and candidate gene markers. As stated by Szulkin et al. (2010), however, the general and local effect hypothesis are not necessarily in contrast and can be partial explanation of the same phenomenon.

Our results showed that high rates of heterozygosity at MHC linked microsatellites are favorable for horn growth and body mass of Alpine ibex while they seemed not to have effect on parasitic load. Even if we did not find molecular evidences of selection acting at MHC level and MHC markers did not show an excess of heterozygotes, we found that there is an advantage for individuals with higher heterozygosity at MHC linked microsatellites. This result suggests that overdominance is a mechanism acting also at MHC region in Gran Paradiso population.

Gran Paradiso Alpine ibex population showed a very low heterozygosity at MHC region, this results *per se* would worth special attention as MHC is involved in immunitary response and low levels of heterozygosity in this region of the genome could decrease the adaptive potential of the species. MHC heterozygosity, moreover, was found to have a direct effect on body mass and horn growth of male ibex confirming the importance of this genome region. The analysis of more than one population is needed to see whether, despite the low levels of heterozygosity, there is differentiation between populations and if there is selection acting differently at these loci.

References

Aguilar, A., Roemer, G., Debenham, S., Binns, M., Garcelon, D., and R.K. Waine. 2004. High MHC diversity maintained by balancing selection in an otherwise genetically monomorphic mammal. PNAS 101:3490-3494.

Apollonio, M., Bassano, B., and A. Mustoni. 2003. Behavioral aspects of conservation and management of european mammals. pp. 157-170. *In:* Festa-Bianchet M. and Apollonio M. eds, Animal Behavior and Wildlife Conservation.

Aulagnier, S., Kranz, A., Lovari, S., Jdeidi, T., Masseti, M., Nader, I., de Smet, K. and F. Cuzin. 2008. *Capra ibex*. *In:* IUCN 2013. IUCN Red List of Threatened Species. Version 2013.1.

Bassano, B., von Hardenberg, A., Pelletier, F., and G. Gobbi. 2003. A method to weigh free-ranging ungulates without handling. Wildlife Soc. B. 31:1205-1209.

Barton, K. 2013. MuMIn: Multi-model inference. R package version 1.9.0. http://CRAN.R-project.org/package=MuMIn

Bates, D., Maechler, M., and B. Bolker. 2011. lme4: Linear mixed-effects models using S4 classes. R package version 0.999375-42/r1414. http://R-Forge.R-project.org/projects/lme4/

Bergeron, P. 2007. Parallel lasers for remote measurements of morphological traits. J. Wildl. Manage. 71:289-292.

Bergeron, P., Grignolio, S., Apollonio, M., Shipley, B., and M. Festa-Bianchet. 2010. Secondary sexual characters signal fighting ability and determine social rank in Alpine ibex (Capra ibex). Behav. Ecol. Sociobiol. 64:1299-1307.

Bernatchez, L., and C. Landry. 2003. MHC studies in nonmodel vertebrates: what have we learned about natural selection in 15 years? Journal of Evolutionary Biology 16: 363-377.

Biebach, I., and L. F. Keller. 2009. A strong genetic footprint of the re-introduction history of Alpine ibex (*Capra ibex ibex*). Mol. Ecol. 18:5046-5058.

Biebach, I., and L. F. Keller. 2010. Inbreeding in reintroduced populations: the effects of early reintroduction history and contemporary processes. Conserv. Genet. 11:527-538.

Biebach, I., and L. F. Keller. 2012. Genetic variation depends more on admixture than number of founders in reintroduced Alpine ibex populations. Biol. Conserv. 147:197-203.

Bishop, M. D., Kappes, S. M., Keele, J. W., Stone, R. T., Sunden, S. L. F., Hawkins, G. A., Toldo, S. S., Fries, R., Grosz, M. D., Yoo, J. Y. and C.W. Beattie. 1994. A Genetic Linkage Map for Cattle. Genetics 136: 619-639.

Blattman, A. N., and K. J. Beh. 1992. Dinucleotide repeat polymorphism within the ovine major histocompatibility complex. Animal Genetics 23:392-392.

Borghans, J.A.M., Beltman, J.B., and R.J. De Boer. 2004. MHC polymorphism under host-pathogen coevolution. Immunogenetics 55:732-739.

Brambilla, A., and C. Canedoli. 2013. How to continue measuring horn growth after capture in long term studies. J Mount Ecol (in press).

Brambilla, A., von Hardenberg, A., Kristo, O., Bassano, B., and G. Bogliani. 2013. Don't spit in the soup: faecal avoidance in foraging wild Alpine ibex (*Capra ibex*). Anim. Beh. 86:153-158.

Buitkamp, J., Schwaiger, F.W., and J.T. Epplen. 1993. Vb6 T-cell receptor elements in artiodactyls: conservation and germline polymorphisms. Mammalian Genome. 4:504-510.

Buitkamp, J., Filmether, P., Stear, M.J., and J.T. Epplen. 1996. Class I and class II major histocompatibility complex alleles are associated with faecal egg counts following natural, predominantly *Ostertagia circumcincta* infection. Parasitology Research. 82: 693-696.

Burnham, K.P., and D. R. Anderson. 2002. Model selection and multimodel inference: a practical information-theoretic approach. 2nd ed. New York, Springer-Verlag.

Chapman, J. R., Nakagawa, S., Coltman D. W., Slate J., and B. C. Sheldon., 2009. A quantitative review of heterozygosity-fitness correlations in animal populations. Molecular Ecology, 18:2746-2765.

Coltman, D. W., Pilkington, J. G., Smith, J. A., and J. M. Pemberton. 1999. Parasite-mediated selection against inbred Soay sheep in a free-living, island population. Evolution 53:1259-1267.

Crnokrak, P., and D. A. Roff. 1999. Inbreeding depression in the wild. Heredity 83: 260-270.

Da Silva, A., Gaillard, J.M., Yoccoz, N.G., Hewison, A.J.M., Galan, M., Coulson, T., Allainé, D., Vial, L., Delorme, D., Van Laere, G., Klein, F., and G. Luikart. 2008. Heterozygosity-fitness correlations revealed by neutal and candidate gene markers in roe deer from a long-term study. Evolution 63: 403-417.

David, P., Pujol, B., Viard, F., Castella, V., and J. Goudet. 2007. Reliable selfing rate estimates from imperfect population genetic data. Mol. Ecol. 16:2474–2487.

Eldridge, M.D.B., King, J.M., Loupis, A.K., Spencer, P.B.S., Taylor, A.C., Pope, L.C., and G.P. Hall. 1999. Unprecedent low levels of genetic variation and inbreeding depression in an island population of the black-footed rock-wallaby. Conservation Biology 13: 531-541.

Frankham, R., 1996. Relationship of genetic variation to population size in wildlife. Conservation Biology, 10:1500-1508.

Frankham, R., Ballou, J.D. and D.A. Briscoe, 2002. Introduction to Conservation Genetics. Cambridge University Press, Cambridge, UK.

Garrigan D., and P.W. Hedrick. 2003. Detecting adaptive molecular polymorphism: lessons from the MHC. Evolution 57:1707-1722.

Grignolio, S., Rossi, I., Bassano, B., Parrini, F., and M. Apollonio. 2004. Seasonal variations of spatial behaviour in female Alpine ibex (*Capra ibex ibex*) in relation to climatic conditions and age. Ethol. Ecol. Evol. 16:255-264.

Grodinsky, C, and M. Stuwe. 1987. The reintroduction of the Alpine Ibex to the Swiss Alps. Smithsonian 18:68–77.

Groth, D. M., and J. D. Wetherall. 1994. Dinucleotide repeat polymorphism within the ovine major histocompatibility complex class-I region. Animal Genetics 25:61-61.

Groth, D.M., and J.D. Wetherall. 1995. Dinucleotide repeat polymorphism adjacent to sheep complement factor B. Animal Genetics, 26:282-283.

Hannson, B., and L. Westerberg. 2002. On the correlation between heterozygosity and fitness in natural populations. Mol. Ecol. 11: 2467-2474.

Hedrick, P.W., and Thomson, G. 1983. Evidence for balancing selection at HLA. Genetics 104:449-456.

Hedrick, P.W. 1994. Evolutionary Genetics of the Major Histocompatibility Complex. The American Naturalist. 143: 945-964.

Huges, A.L., Huges, M.K., Howell, C.Y., and M. Nei. 1994. Natural selection at the class II major histocompatibility complex loci of mammals. Phil Trans R Soc Lond B 345:359-367.

Huges, A.L., and Nei, M. 1998. Pattern of nucleotide substititution at major histocompatibility complex class-I loci reveals overdominant selection. Nature 335:167-170.

Janeway, C.A., Travers, P., Walport, M., and M.J. Shlomchik. 2001. The major histocompatibility complex and its functions *in* The Immune System in Health and Disease, Immunobiology 5th ed. Garland Science, New York.

Keller, L. and P. Waller. 2002. Inbreeding effects in wild populations. TRENDS Ecol. Evol. 17:230-241.

Klein, J., Satta, Y., Ohuigin, C., and N. Takahata. 1993. The molecular descent of the major hystocompatibility complex. Annu Rev Immunol 11:269:295.

Ministry of Agriculture, Fisheries and Food. 1971. *In*: Manual of veterinary parasitological laboratory techniques, London (UK): Her Majesty's Stationery Office.

Movahedin, M.R., Amirinia, C., Noshary, A., and S.A. Mirhadi. 2010. Detection of genetic variation in sample of Iranian proofed Holstein cattle by using microsatellite marker. African Journal of Biotechnology, 9:9042-9045.

Paterson, S. 1998. Evidence for balancing selection at the major histocompatibility complex in a free living ruminant. The American Genetic Association. 89:289-294.

Paterson, S. Wilson, K., and J.M. Pemberton. 1998. Major histocompatibility complex variation associated with juvenile survival and parasite resistance in a large unmanaged ungulate population (*Ovis aries L.*). Proc. Natl. Acad. Sci. 95: 3714-3719.

Peakall, R., and P. E. Smouse. 2012. GenAlEx 6.5, J. Bioinf. 28:2537-2539.

Piertney, S.B., and M.K. Oliver. 2006. The evolutionary ecology of the major histocompatibility complex. Heredity 96:7–21.

R Development Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, http://www.R-project.org/

Reed, D.H., and R. Frankham. 2003. Correlation between fitness and genetic diversity. Conservation Biology 17:230-237.

Rousset, F. 2008. GENEPOP ’007: a complete re-implementation of the GENEPOP software for Windows and Linux. Mol Ecol Res 8:103-106.

Roy, F., Maurel, M.C., Combes, B., Vaiman, D., Cribiu, E.P., Lantier, I., Pobel, T., Deletang, F., Combarnous, Y., and F. Guillou. 1999. The negative effect of repeated equine chorionic gonadotropin treatment on subsequent fertility in Alpine goats is due to a humoral immune response involving the major histocompatibility complex. Biology of Reproduction 60:805-813.

Slate, J., Kruuk, L. E. B., Marshall, T. C., Pemberton, J. M., and T. H. Clutton-Brock. 2000. Inbreeding depression influences lifetime breeding success in a wild population of red deer (*Cervus elaphus*). Proc. R. Soc. Lond. 267: 1657-1662.

Spielman, D., Brook, B.W., Briscoe, D.A., and R. Frankham. 2004. Does inbreeding and loss of genetic diversity decrease disease resistance? Conservation Genetics 5: 439-448, 2004.

Spurgin, L.G., and D.S. Richardson. 2010. How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. Proc. R. Soc. B 277:979–988.

Szulkin, M., Bierne, N., and P. David. 2010. Heterozygosity-Fitness Correlations: a time for reappraisal. Evolution 64:1202-1217.

Valière, N. 2002. GIMLET : a computer program for analysing genetic individual identification data. Mol. Ecol. Notes 2:377–379.

von Hardenberg, A., Bassano, B., Zummel Arranz, M.d.P., and G. Bogliani. 2004. Horn growth but not asymmetry heralds the onset of senescence in male Alpine ibex (*Capra ibex*). J. Zool. 263:425-432.

von Hardenberg, A. 2005. Sénescence, sélection sexuelle et dynamique de population du bouquetin des Alpes (*Capra ibex*). Ph.D. thesis, Université de Sherbrooke.

von Hardenberg, A., Bassano, B., Festa-Bianchet, M., Luikart, G., Lanfranchi, P., and D. Coltman. 2007. Age-dependent genetic effects on a secundary sexual trait in male Alpine ibex, *Capra ibex*. Mol. Ecol. 16:1969-1980.

Whitehouse, A.M., and E.H. Harley. 2001. Post-bottleneck genetic diversity of elephant populations in South Africa, revealed using microsatellite analysis. 10: 2139–214.

Willi, Y., van Buskirk, J., and A.A. Hoffmann. 2006. Limits to the adaptive potential of small populations. Annual Review of Ecology, Evolution, and Systematics 37:433-458.

Yuhki, N, and S.J. O'Brien, 1997. Nature and origin of polymorphism in feline MHC class II DRA and DRB genes. J Immunol 158:2822-2833.