CHAPTER VII:

Growth rate correlates to individual heterozygosity in European eel, *Anguilla anguilla* L.

Pujolar J.M., Maes G.E., Vancoillie C. and Volckaert F.A.M.

SUMMARY

Heterozygosity-fitness correlations (HFCs) have been reported in populations of many species. We provide evidence for a positive correlation between genetic variability and growth rate at 12 allozyme loci in a catadromous marine fish species, the European eel (*Anguilla anguilla* L.). More heterozygous individuals show a significantly higher length and weight increase and an above average condition index in comparison with more homozygous individuals. To a lesser extent, 6 microsatellite loci show a similar pattern, with positive but not significant correlations between heterozygosity and growth rate. The HFCs observed could be explained either by an effect of direct allozyme overdominance or associative overdominance. Selection affecting some of the allozyme loci would explain the greater strength of the HFCs found at allozymes in comparison with microsatellites, and the lack of correlation between MLH at allozymes and MLH at microsatellites. Associative overdominance (where allozyme loci are merely acting as neutral markers of closely linked fitness loci) might provide an explanation for the HFCs if we consider that allozyme loci have a higher chance than microsatellites to be in linkage disequilibrium with fitness loci.

Keywords: allozymes, associative overdominance, direct overdominance, heterozygosity, growth rate, microsatellites, selection

This chapter is published in: Evolution 59(1): 189-199.

INTRODUCTION

Heterozygosity-fitness correlations (HFCs), the correlation between heterozygosity observed at marker loci and fitness-related traits such as growth, survival, fecundity or developmental stability, have been under study for decades in populations of many species. Positive HFCs have been reported in organisms as diverse as plants (Schaal & Levin, 1976; Ledig *et al.*, 1983), marine bivalves (Zouros *et al.*, 1980; Koehn & Gaffney, 1984), crustaceans (Bierne *et al.* 2000), amphibians (Pierce & Mitton, 1982), salmonids (Leary *et al.*, 1984; Danzmann *et al.*, 1987; Thelen & Allendorf 2001) and mammals (Slate & Pemberton 2002; Hildner *et al.* 2003). Null results are likely to be under-represented in literature (Hansson & Westerberg 2002). The correlation between genetic variability and fitness components as reflected by molecular marker heterozygosity in natural populations usually accounts for a small percentage (1-5%) of the observed phenotypic variance (David, 1998).

The first HFCs were observed in studies using allozyme markers, which led to the hypothesis of direct overdominance, where the correlation is due to a direct heterozygous genotype advantage at allozyme loci compared to the corresponding homozygous genotypes. It has been proposed that heterozygotes at allozyme loci might have an intrinsically higher fitness than homozygotes, which would be related to lower energy consumption and greater metabolic efficiency (Mitton, 1993).

The observation of positive HFCs with putative neutral DNA markers proves that at least some correlations are not due to the direct effect of the marker genes but to the genetic association between the neutral markers and fitness genes, resulting in associative overdominance (David, 1998). When marker loci are not directly responsible, the observed correlation can be either due to the effect of linkage disequilibrium restricted to a narrow chromosomal section affecting closely-linked fitness loci ("local effect") or due to partial inbreeding caused by the non-random association of diploid genotypes in zygotes ("general effect") (David *et al.*, 1995).

Few studies correlating heterozygosity and growth have been carried out in fish, mainly due to the difficulty in rearing experiments, especially for marine species. In salmonids, Danzmann *et al.* (1987) found a positive correlation between allozyme heterozygosity and growth (length or weight up to 6 months) in cultured rainbow trout (*Oncorhynchus mykiss*), suggesting that heterozygotes have enhanced growth rates compared to more homozygous individuals. Nevertheless, salmonids seem to produce different HFCs at different life stages, and negative correlations were found between (1) allozyme variation and length at one year,

and (2) length and weight at maturation (Ferguson, 1990, 1992). Pogson & Fevolden (1998) examined the relationships between growth and the degree of individual heterozygosity at ten nuclear RFLP loci in two natural populations of Atlantic cod (*Gadus morhua*), using a rough measure of growth (size at age). A significant positive correlation was found in one population, supporting the hypothesis that neutral DNA markers can detect HFCs.

The species of interest in this study is the European eel (*Anguilla anguilla* L.; Anguillidae; Teleostei), a catadromous fish species with a particularly complex life cycle, which moves between marine (spawning, larval phase and maturation) and freshwater (feeding, growth and maturation) environments. After spawning in the Sargasso Sea, larvae (leptocephali) migrate with the Gulf Stream and North Atlantic Drift Current to the shores of Europe and North Africa, where they metamorphose into glass eels upon reaching the continental shelf. Glass eels move into freshwater systems (rivers, lagoons or lakes), where they feed for up to 25 years (on average, 7-8 years for males and 11 years for females) before metamorphosing into silver eels and migrating back to the Sargasso Sea, where they spawn once and die (Tesch 2003).

Recruitment abundance of European eel has declined dramatically in recent decades, jeopardizing the future of the species (Dekker 2003). A better understanding of crucial aspects of its biology, including genetic diversity, may promote effective measures to protect the species. In the case of European eel, no previous studies have been carried out to correlate individual growth and genetic variability. Despite progress in artificial propagation, completion of the European eel lifecycle in captivity is not possible yet (Tesch 2003). Alternatively, fattening farms dedicated to the growth of stocked glass eel (varying from postlarvae having completed the leptocephalus metamorphosis to the full pigmentation stage), are readily available in many coastal regions of southern and western Europe. There is a long history of stocking and extensive rearing of eels in ponds, offering an adequate setting for growth experiments (Tesch 2003). When growth is tested under artificial conditions, it must be taken into account that environmental stress might enhance HFCs (Danzmann *et al.*, 1988) and that HFCs seem to decrease with age (David, 1998).

The aim of this study is to identify the existence of HFCs in European eel in order to evaluate genetic variability and selection in cultured eel populations and to assess the contribution of genetic and environmental factors in the growth of the species. At the same time, such experiments provide a model for natural populations of European eel and for other fish species. The association between heterozygosity and two phenotypic measures of growth (length and weight increase) was tested in two groups of farmed European eel individuals

using thirteen allozyme and six microsatellite markers. In case of direct overdominance, HFCs should not be expressed at putative neutral markers (microsatellites) but only at markers affected by natural selection (allozymes). Under the hypothesis of associative overdominance, an apparent advantage observed at heterozygous individuals would be independent of the genetic marker used. Since linkage is not limited to allozymes, a similar HFC would be expected using either allozyme or microsatellite markers. Both hypotheses are not mutually exclusive since natural selection might not be acting equally across all allozyme loci. Besides, despite the usual interpretation that microsatellites evolve neutrally, recent studies have documented the functional role of microsatellites in gene regulation, chromatin organization, cell cycle and DNA metabolic processes (Li *et al.*, 2002).

MATERIAL AND METHODS

Glass eel individuals collected in the mouth of the river Adour (South-western France) were transferred and raised in a closed recirculation system at the eel farm of Royaal BV (The Netherlands). Two batches of glass eels, collected in December 2001 (Batch 1; ROY101) and February 2002 (Batch 2; ROY 201), respectively, were monitored for one year. A sub-sample of 100 individuals was collected from each batch at the start of the experiment and after one year in the tanks (ROY102 and ROY202) for genetic analyses (Table 1). Once measured, individuals were split in two, with the tail kept in ethanol for microsatellite analysis and the rest of the body kept frozen at –80°C for allozyme analysis.

Table 1. *Anguilla anguilla*. Summary of genetic samples of farmed glass eels including sampling date, number of individuals analysed (N), mean length (L) and mean weight (W). Standard deviation in parentheses.

Sample	Batch	Sampling date	N	Mean L (mm) ± S.D.	Mean W (g) ± S.D.
ROY101	1	December 2001	100	71.69 (3.80)	0.41 (0.07)
ROY102	1	December 2002	100	233.04 (69.80)	29.16 (31.95)
ROY201	2	February 2002	100	70.36 (4.47)	0.29 (0.07)
ROY202	2	February 2003	100	241.34 (87.74)	43.37 (44.80)
ROY2F	2	February 2003	50	612.9 (41.1)	602.2 (117.5)

Additionally, genetic analysis also included a sub-sample of 50 individuals from batch 2 (ROY2F), which were regarded as "fast-growth individuals" due to their exceptional growth rate after one year in the tanks in the same conditions as the rest of the individuals (mean length: 612.9 ± 41.1 mm/ mean weight: 602.2 ± 117.5 g).

Allozyme electrophoresis – A total of 450 individuals (Table 1) were analysed for protein variation using Cellulose Acetate Gel Electrophoresis (CAGE, Harris and Hopkinson, 1976; Richardson *et al.*, 1986). Tissue extraction, electrophoresis and procedures for visualising proteins, and buffer systems used (Tris Glycine (TG) and Tris Malate (TM)) are described in Maes and Volckaert (2002). Nine enzymatic systems were examined: aspartate aminotransferase (*AAT-1**, *AAT-2**, *AAT-3**, EC 2.6.1.1, TM), alcohol dehydrogenase (*ADH**, EC 1.1.1.1, TG), glucose-6-phosphate isomerase (*GPI-1**, *GPI-2**, EC 5.3.1.9, TG), L-Iditol dehydrogenase (*IDDH**, EC 1.1.1.14, TG), isocitrate dehydrogenase (*IDHP**, EC 1.1.1.42, TM), malate dehydrogenase (*MDH-2**, EC 1.1.1.37, TM), mannose-6-phosphate isomerase (*MPI**, EC 5.3.1.8, TG), phosphogluconate dehydrogenase (*PGDH**, EC 1.1.1.44, TM) and phosphoglucomutase (*PGM**, EC 5.4.2.2, TG). Genetic nomenclature followed the suggestions of Shaklee *et al.* (1990). Allele assignment was carried out comparing the relative distance with the most common allele (**100*).

Microsatellite analysis – DNA was extracted in a sub-sample of 60 out of the 100 individuals from each batch at the start of the experiment and after one year in the tanks, and 50 fast-growth individuals. Minute sections of tissue from ethanol preserved glass eels were digested in a lysis buffer containing 100 µl TE Buffer, 7 µl 1M DTT (Dithiothreitol) solution pH 5.2 (diluted in 0.08M NaAc) and 5 µl Proteinase K solution (10 $mg.ml^{-1}$) for at least 4 h at 56°C. After incubation at 96°C for 10 min, samples were centrifuged at 13,000 rpm for 11 min; the supernatant was stored at –20°C for further analysis.

Genotypes were examined at 6 dinucleotide repeat microsatellite loci: AAN 01, AAN 03, AAN 05 (Daemen et al., 2001); ARO 063, ARO 095 and ANG 151 (Wirth & Bernatchez 2001). Loci were amplified in two separate multiplexes. Multiplex PCR reactions consisted of 1 X PCR buffer (supplied with polymerase), 1.5 mM $MgCl_2$, 80 µM dNTP, 0.4 µM (on average) fluorochrome labelled forward and non-labelled reverse primer, 0.5 U Goldstar *Taq* polymerase (Eurogentec) and 1 µl DNA template. DdH_2O was added up to 25 µl. PCR cycling conditions were as follows: 5 min at 94°C, 24 cycles of 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C, and final elongation for 8 min at 72°C. PCR products were visualized on an automated sequencer (LICOR 4200), using a molecular ladder (Westburg) in order to quantify allele sizes. Fragment data were analysed using Gene ImagIR ver 4.03 (Scanalytics Inc).

Data analysis – All individuals were measured for standard length (L) and body weight (W). Length and weight increase are calculated as the difference between individual length and

weight after one year in the tanks and mean length and weight after arriving in the facilities. Ricker's (1975) condition factor ($CI= 1000(W/L^b)$) was calculated for each individual, where L is standard length in mm, W is body weight in mg and b is the slope from the log length-log weight regression for all samples. The condition factor is based on the analysis of length-weight data, assuming that heavier individuals at a given length are in a better condition. Additionally, the relative condition factor (K) has been calculated as described in Le Cren (1951). $K= W/\hat{W}$ compares the observed (W) and expected ($\hat{W}$) weight of each individual, where expected weight is obtained using the length-weight regression ($W=aL^b$) of each individual. Differences in morphometric measures among groups were tested by an analysis of variance (ANOVA).

Within sample genetic variation was assessed by observed heterozygosity per locus (H_o), level of polymorphism using the 95% criterion ($P_{0.05}$) and mean and total number of alleles using GENETIX version 4.02 (Belkhir *et al.*, 1999). Values among groups were compared by an analysis of variance (ANOVA). Deviations from Hardy-Weinberg equilibrium, genotypic associations for all possible pairs of loci in each sample, and differences in allele frequencies among samples were tested using GENEPOP version 3.1d (Raymond & Rousset, 1995). Significance levels for multiple simultaneous comparisons were adjusted using the sequential Bonferroni technique (Rice, 1989). Patterns of variation among samples were assessed by hierarchical gene diversity analysis as implemented in GENETIX version 4.02 (Belkhir *et al.*, 1999).

Individual multi-locus heterozygosity (MLH) was calculated as the proportion of loci that are heterozygous (corrected for non-scored loci). MLH among groups was compared using an ANOVA. In the case of microsatellite loci, the squared difference in repeat units between two alleles in an individual at a given locus (d^2) averaged over all loci (mean d^2) was calculated following Coulson *et al.* (1998). Mean d^2 is an alternative method to MLH in order to infer fitness differences between individuals; in addition to taking into account whether individuals are homozygotes or heterozygotes, it also considers the differences in allele size in heterozygous individuals. Coltman *et al.* (1998) suggested that mean d^2 provides a better measure of individual genetic variability than MLH for microsatellite data. Outbreeding mean d^2 (calculated in the same way as mean d^2 but excluding homozygous loci in each individual) and standardized mean $d^2_{var\ (d2)}$ (the average d^2 at each locus scaled by the variance of d^2 at that locus) were calculated for each individual as described by Hedrick *et al.* (2001).

All data were checked for normality and a logarithmic transformation was conducted if necessary before parametric statistical analysis. Regression analysis (Pearson's correlation)

was performed between individual heterozygosity (MLH and all calculations of d^2) values and growth estimators (length increase, weight increase, condition index and relative condition index) in order to test for possible HFCs. When fast-growth individuals were included in the analysis, Spearman's correlation was used since variables did not approximate a normal distribution following logarithmic transformation. All analyses were performed in STATISTICA version 6.0 (Statsoft). Significance for all statistical tests was taken as 0.05.

RESULTS

A comparison between the newly arrived batches 1 and 2 showed no significant differences in length and weight (Table 1). After one year at the farm, a substantial range of sizes was observed in both batches. In batch 1, average length was 233.04 ± 69.80 mm, with a maximum length of 444 mm and a minimum length of 135 mm. Differences were not significant in comparison with batch 2, with an average length of 241.34 ± 87.74 mm ranging from 110 - 433 mm. Larger differences were observed in the weight distribution, with individuals from batch 2 (mean weight: 43.37 ± 44.80 g; maximum weight: 183.2 g) being heavier than individuals from batch 1 (mean weight: 29.16 ± 31.95 g; maximum weight: 136.2 g), although differences were not significant. Individuals from batch 1 showed an average increase of 167.58 ± 66.83 mm and 29.29 ± 31.48 g, while individuals from batch 2 showed an average increase of 176.18 ± 84.97 mm and 44.45 ± 44.62 g. Increases of both batches were not significantly different.

In order to correlate genetic variability with growth, the samples from batch 1 and 2 which had spent one year at the farm (ROY102 and ROY202) were split for the analysis into small (< 40 g, N = 136) and large individuals (40-200 g, N = 64), and compared with the fast-growth individuals (500-800 g, N = 50). Differences in length and weight among groups were statistically significant.

Allozymes - The 9 enzymatic systems examined resulted in 12 polymorphic loci. Overall tests for Hardy-Weinberg proportions with all polymorphic loci, and for linkage disequilibrium among all loci showed no significant departures from expected values. When observed heterozygosities were compared between growth groups, the group of small individuals presented lower heterozygosities (H_o= 0.192 ± 0.217) than the group of large individuals (H_o= 0.217 ± 0.200), while the highest heterozygosities were observed in the fast-growth individuals (H_o= 0.235 ± 0.225) (Table 2). Differences in observed heterozygosities between

groups were statistically significant ($p< 0.05$). The group of fast-growth individuals presented higher values of observed heterozygosity for most of the loci in comparison with small and large individuals, including *AAT-1**, *GPI-1**, *GPI-2**, *IDHP**, *MPI**, *PGDH** and *PGM** (Table 2). Additionally, polymorphism was also higher in fast-growth individuals (0.692) than in large (0.615) and small (0.539) individuals.

Table 2. *Anguilla anguilla*. Values of observed heterozygosity (H_o), level of polymorphism (P_{95}) and mean number of alleles (MNA) at all allozyme and microsatellite loci for small (<40 g), large (40 - 200 g) and fast-growth (500 - 800 g) individuals. Standard error in parentheses. $*p < 0.05$

Locus	Small	Large	Fast growth	Locus	Slow	Large	Fast growth
Allozymes				Microsatellites			
*AAT-1**	0.051	0.047	0.061	AAN 01	0.761	0.762	0.750
*AAT-2**	0.029	0.000	0.041	AAN 03	0.209	0.227	0.178
*AAT-3**	0.103	0.188	0.102	AAN 05	0.728	1.000	0.778
*ADH**	0.504	0.468	0.490	ARO 063	0.951	0.949	0.905
*GPI-1**	0.267	0.375	0.440	ARO 095	0.899	0.950	0.846
*GPI-2**	0.052	0.094	0.100	ANG 151	0.858	0.818	0.900
*IDDH**	0.431	0.547	0.500				
*IDHP**	0.037	0.094	0.102				
*MDH-2**	0.193	0.328	0.245				
*MPI**	0.140	0.171	0.260				
*PGDH**	0.675	0.492	0.688				
*PGM**	0.007	0.016	0.020				
Mean H_o	0.192*	0.217*	0.235*	Mean H_o	0.711	0.753	0.726
P_{95}	0.539	0.615	0.692	P_{95}	1.000	1.000	1.000
MNA	3.308	3.000	2.769	MNA	18.167	11.333	13.667

In order to correlate genetic variability and growth at the individual level, multi-locus heterozygosity (MLH) was calculated for each individual (Figure 1). When pooling all individuals (combining batches 1 and 2), a highly significant positive association was observed between MLH - length increase ($r= 0.177$; $p= 0.005$) and between MLH - weight increase ($r= 0.164$; $p= 0.009$). Similarly, when the fast-growth individuals were not included in the analysis, a significant correlation was found between MLH - length increase ($r= 0.153$; $p= 0.030$) and MLH - weight increase ($r= 0.143$; $p= 0.044$). In batch 1, the correlations between MLH - length increase ($r= 0.065$; $p= 0.518$) and between MLH - weight increase ($r= 0.066$; $p= 0.517$) were positive but not statistically significant. By contrast, a significant correlation was found in batch 2 between MLH - length increase ($r= 0.240$; $p= 0.016$) and MLH - weight increase ($r= 0.230$; $p= 0.021$).

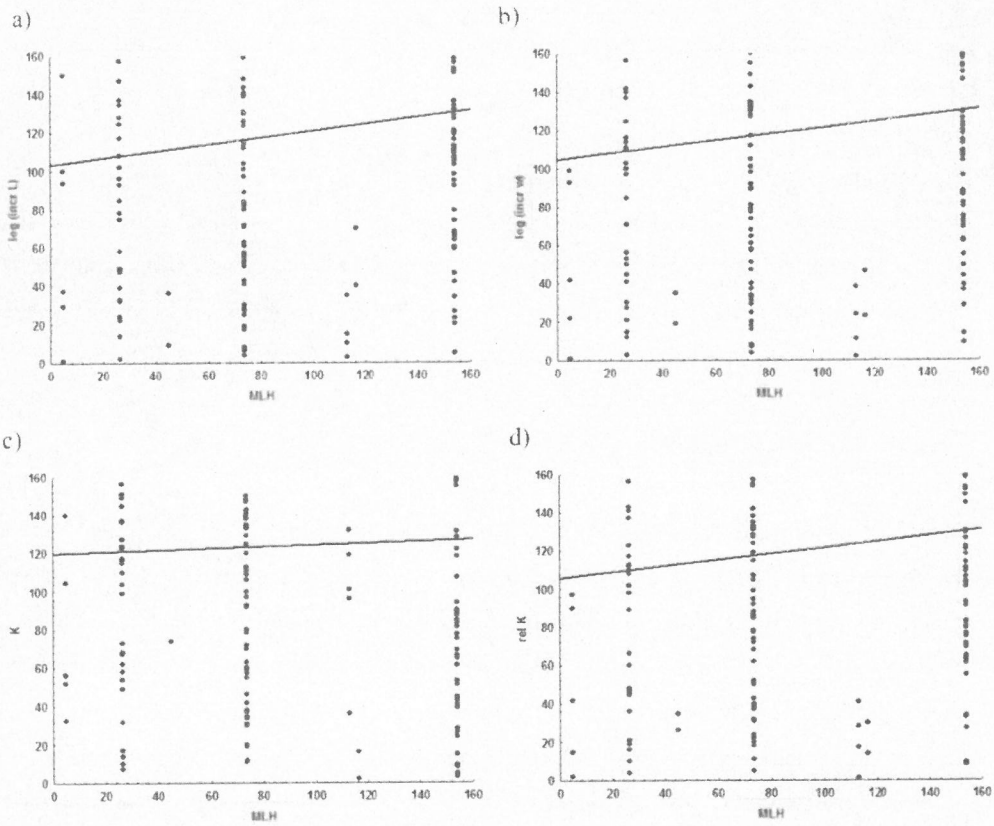

Figure 1. *Anguilla anguilla*. Spearman rank correlation of multi-locus heterozygosity (MLH) versus (a) length (cm) increase (r= 0.177; p= 0.005*), (b) weight (g) increase (r= 0.164; p= 0.009*), (c) condition index (r= 0.044; p= 0.484), and (d) relative condition index at 12 allozyme loci in all individuals (r= 0.157; p= 0.013*).

Multi-locus heterozygosity at allozymes explained 3.1% of the variation in length increase and 3.3% of the variation in weight increase. When mean MLH was calculated for each growth group, slow individuals presented the lowest MLH (0.188 ± 0.087) in comparison with large individuals (0.216 ± 0.102), while the greatest MLH was found in fast-growth individuals (0.229 ± 0.108). When HFCs were tested within growth groups, no significant associations were observed between MLH and growth rate for either small, large or fast growth individuals.

Ricker's condition index (CI) was calculated as $1000(W/L^{3.348})$ for each individual, using the length-weight regression for all samples in order to correct for population effects. The correlation found between MLH - condition factor was positive but not significant when pooling all samples (r= 0.045; p= 0.484), but negative when considering the batches

separately or when excluding fast growth individuals in the analysis (Figure 1). When using the relative condition index (K), significant correlations were found for all pooled individuals ($r= 0.157$; $p= 0.013$) and for batch 2 ($r= 0.215$; $p= 0.032$), and a positive but not significant correlation for batch 1 ($r= 0.063$; $p= 0.537$).

Examination of individual loci suggests that the effect of allozyme heterozygosity on growth is attributed to most of the loci examined. Out of 10 loci (in which all genotypes were sufficiently represented), heterozygotes presented a greater length than homozygotes at 10 loci and a greater weight and relative condition at 9 loci (Table 3). Differences between homozygotes and heterozygotes were maximal at *GPI-1** and *MPI**, with a difference of 5.5 cm in mean length and >70 g in mean weight (70.5 g at *GPI-1**and 80.2 g at *MPI**). These were the only statistically different comparisons ($p< 0.05$).

Microsatellites - Tests for Hardy-Weinberg proportions with all polymorphic loci, and for genotypic disequilibrium among all loci showed no significant departures from expected values. In contrast to allozyme data, the highest heterozygosities were found in large individuals ($H_o= 0.753 \pm 0.276$) in comparison with small ($H_o= 0.711 \pm 0.253$) and fast-growth individuals ($H_o= 0.726 \pm 0.275$); differences were not statistically significant (Table 2). Fast growth individuals only presented a higher (but not significant) heterozygosity at one of the six microsatellite loci examined (ANG 151) in comparison with the other growth groups. Small individuals also presented a higher mean number of alleles (18.167) in comparison with large (11.333) and fast-growth (13.667) individuals.

A small positive effect of microsatellite heterozygosity on growth was observed in most loci examined, although no comparisons were statistically significant. Heterozygotes presented a greater length and weight than homozygotes at 4 out of 6 loci examined and a higher relative condition at 5 loci (Table 3). Differences between heterozygotes and homozygotes were maximal at ARO 095 (4.14 cm in length; 58.6 g in weight) and ANG 151 (3.15 cm in length; 54.1 g in weight). Individual multi-locus heterozygosity (MLH) showed a positive correlation between growth rate (measured as length and weight increase) and heterozygosity at 6 microsatellite loci, although this association was not as strong as found for the allozyme markers (Figure 2).

Table 3. *Anguilla anguilla*. Difference in mean length (L), mean weight (W) and relative condition index (K) values for homozygotes and heterozygotes at ten allozyme and six microsatellite loci. Standard deviation in parentheses. D indicates the sign of difference in all three factors between heterozygotes and homozygotes.

Locus	L (mm)	D	W (g)	D	K	D
Allozymes						
*ADH**						
Homozygotes	315.15		143.02		0.390	
Heterozygotes	320.10	+	158.35	+	0.392	+
*AAT-1**						
Homozygotes	313.32		148.40		0.390	
Heterozygotes	331.07	+	135.48	-	0.396	+
*AAT-3**						
Homozygotes	316.07		148.64		0.391	
Heterozygotes	333.23	+	178.56	+	0.394	+
*GPI-1**						
Homozygotes	300.28		128.39		0.387	
Heterozygotes	354.92	+*	198.92	+*	0.400	+
*GPI-2**						
Homozygotes	315.12		147.69		0.391	
Heterozygotes	349.78	+	190.22	+	0.398	+
*IDDH**						
Homozygotes	310.05		144.29		0.390	
Heterozygotes	327.08	+	156.61	+	0.394	+
*IDHP**						
Homozygotes	312.53		144.66		0.390	
Heterozygotes	364.19	+	203.05	+	0.404	+
*MDH-2**						
Homozygotes	310.24		142.86		0.390	
Heterozygotes	336.67	+	169.03	+	0.394	+
*MPI**						
Homozygotes	307.76		136.71		0.389	
Heterozygotes	362.57	+*	216.95	+*	0.399	+
*PGDH**						
Homozygotes	320.69		141.99		0.394	
Heterozygotes	320.94	+	158.94	+	0.391	-
Microsatellites						
AAN 01						
Homozygotes	346.22		222.45		0.392	
Heterozygotes	340.59	-	198.99	-	0.394	+
AAN 03						
Homozygotes	328.38		186.17		0.390	
Heterozygotes	311.83	-	164.82	-	0.388	-
AAN 05						
Homozygotes	315.64		190.37		0.386	
Heterozygotes	335.13	+	192.93	+	0.393	+
ARO 063						
Homozygotes	341.50		206.22		0.391	
Heterozygotes	350.57	+	215.61	+	0.395	+
ARO 095						
Homozygotes	290.14		132.33		0.384	
Heterozygotes	331.49	+	190.91	+	0.391	+
ANG 151						
Homozygotes	294.19		130.77		0.387	
Heterozygotes	325.67	+	184.85	+	0.390	+

(*) = $p < 0.05$

Positive but not significant correlations were observed between MLH - length increase ($r= 0.142$; $p= 0.070$) and MLH - weight increase ($r= 0.145$; $p= 0.063$) and when fast-growth individuals were not included in the analysis (MLH - length increase: $r= 0.163$; $p= 0.081$; MLH - weight increase: $r=0.166$; $p= 0.076$). In batch 1, correlations were positive but not

significant between MLH - length increase (r= 0.237; p= 0.076) and significant between MLH - weight increase (r= 0.264; p= 0.047). In batch 2 correlations were positive but not significant (MLH - length increase: r= 0.037; p= 0.786; MLH - weight increase: r=0.037; p= 0.782). Multi-locus heterozygosity at microsatellite loci explained 1.0% of the variation in length increase and 1.3% of the variation in weight increase. When mean MLH was calculated for each growth group using microsatellite loci, large individuals presented the largest MLH (0.751 ± 0.146) in comparison with fast-growth (0.733 ± 0.166) and slow (0.703 ± 0.158) individuals. No HFCs were observed within growth groups.

Negative associations were observed between MLH and Ricker's condition index in all possible comparisons. Using the relative condition index, positive but not significant correlations were observed for batch 1 (r= 0.117; p= 0.137), batch 2 (r= 0.032; p= 0.815) and when pooling all individuals (r= 0.152; p= 0.052).

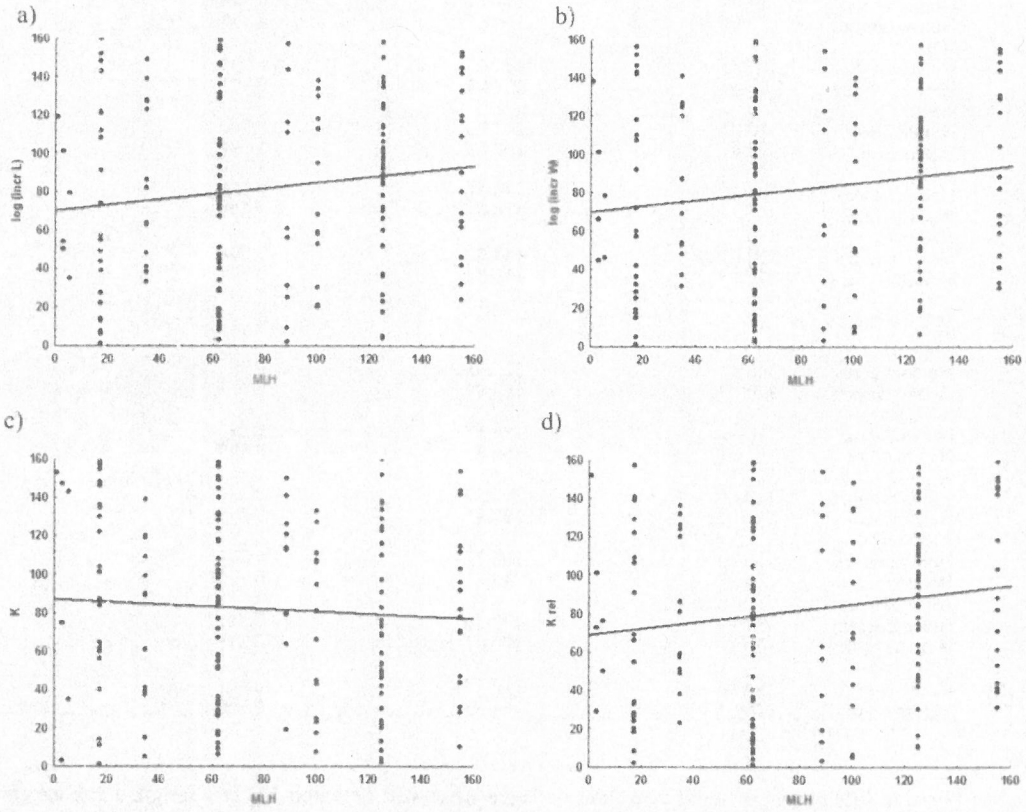

Figure 2. *Anguilla anguilla*. Spearman rank correlation of multi-locus heterozygosity (MLH) versus (a) length (cm) increase (r= 0.142; p= 0.070), (b) weight (g) increase (r= 0.145; p= 0.063), (c) condition index (r= -0.061; p= 0.433), and (d) relative condition index at 6 microsatellite loci in all individuals (r= 0.152; p= 0.052).

The logarithmic transformation of mean d^2 was negatively correlated to growth rate (Figure 3). Negative associations were found between mean d^2 and length increase (r= -0.045; p= 0.557), weight increase (r= -0.050; p= 0.537), condition index (r= -0.058; p= 0.478) and relative condition index (r= -0.055; p= 0.494), respectively. Similar negative correlations were obtained when homozygous loci in each individual were excluded (outbreeding mean d^2) or when mean d^2 was standardized at each locus by the variance of d^2 (mean $d^2_{var\ (d2)}$).

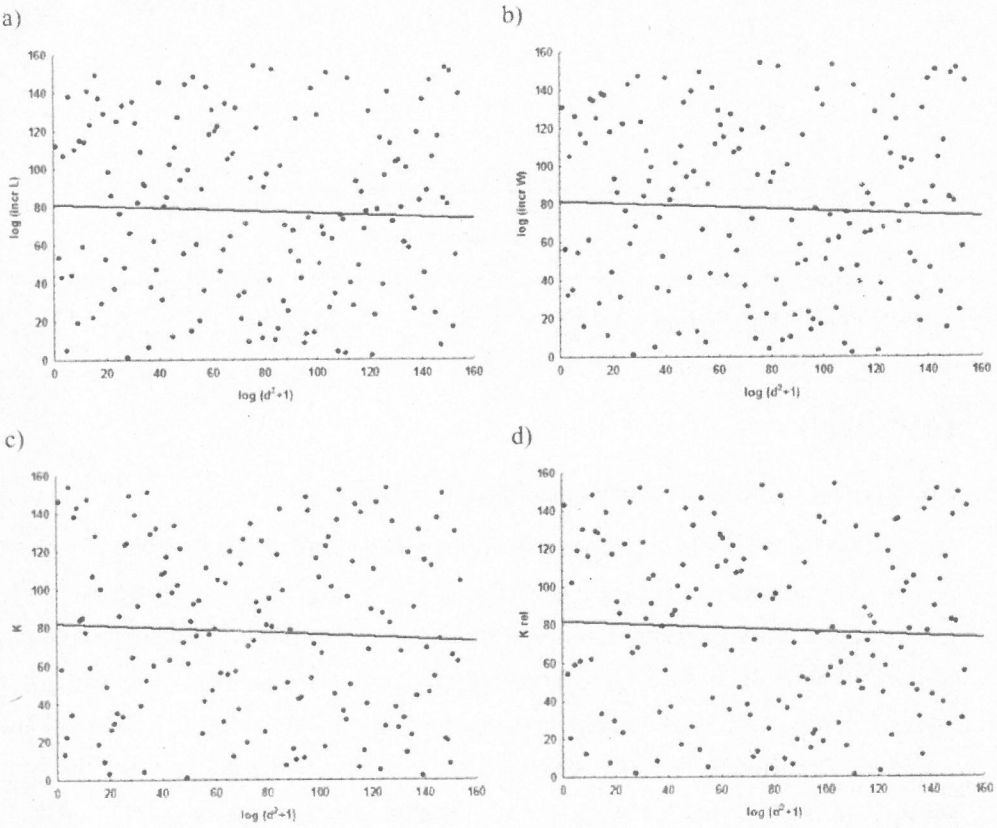

Figure 3. *Anguilla anguilla*. Regression of mean d^2 versus (a) length (cm) increase (r= -0.045; p= 0.557), (b) weight (g) increase (r= -0.050; p= 0.537), (c) condition index (r= -0.058; p= 0.478), and (d) relative condition index at 6 microsatellite loci in all individuals (r= -0.055; p= 0.494).

When testing the association between genetic variation at allozymes and microsatellite markers, a non significant negative correlation was found between individual MLH at allozyme loci and individual MLH at microsatellite loci (Figure 4).

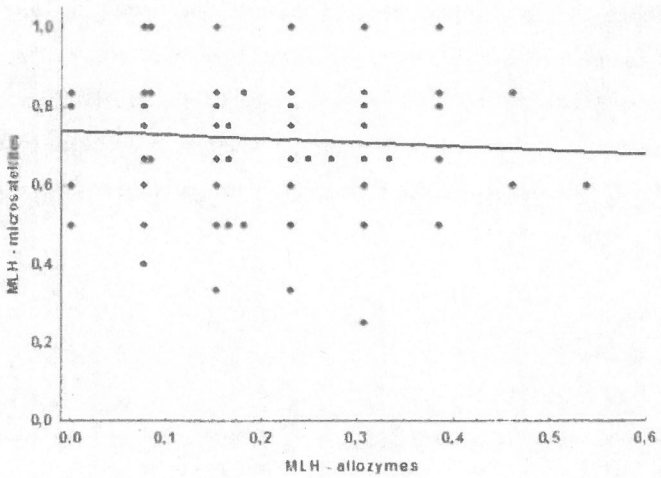

Figure 4. *Anguilla anguilla.* Regression of multi-locus (MLH) at 12 allozyme loci versus MLH at 6 microsatellite loci in all individuals ($r = 0.062$; $p = 0.431$).

DISCUSSION

Evidence for heterozygosity-fitness correlation in European eel

We clearly show a positive correlation between heterozygosity at allozyme loci and growth rate; more heterozygous individuals presented a higher length and weight increase and a greater relative condition index in two separate batches of farmed European eel. The stronger correlations observed in batch 2 in comparison with batch 1 might be related to their greater variation in length and weight; differences in growth rate should be easier to detect in such conditions. At microsatellite loci, a general pattern was observed that individuals with a larger growth rate mostly presented higher heterozygosities. Correlations were positive but not significant, and HFCs at microsatellites cannot be considered to be as strong as HFCs at allozymes. Multi-locus heterozygosity at allozymes also explained more variation in length and weight increase (3%) than MLH at microsatellites (1%) in multiple regressions. As heterozygosity in European eel explains less than 5% of the individual variation in growth rate, the large fraction of unexplained variation in growth rate is partitioned between environmental effects, genetic factors other than the loci studied and epistatic interactions. Similar correlation values have been reported in previous HFC studies, usually accounting for a small proportion of the observed phenotypic variance (3 - 6%) (Britten, 1996). A meta-

analysis of correlations between phenotypic variation and genetic variation revealed that the strength of the associations are generally weak (mean $r < 0.10$), with a mean r of 0.0274 for MLH and 0.0156 for mean d^2, respectively (Coltman & Slate 2003). Examining HFCs in fish, multi-locus allozyme heterozygosity explained more than 10% of the total variance of the condition factor in rainbow trout (*Oncorhynchus mykiss*) (Thelen & Allendorf 2001). In the positive correlations between genetic variation (both microsatellite heterozygosity and d^2) and four reproductive fitness-related traits in chinook salmon (*Oncorhynchus tshawytscha*), regressions explained between 6 - 8% of the variance (Heath *et al.* 2002).

Our results show that heterozygosity at allozyme and microsatellite loci, albeit scored for an unequal number of loci, has a different effect on the individual phenotype and supports the view that natural selection does not affect allozymes and microsatellites similarly. A negative correlation was observed between individual MLH at allozymes and individual MLH at microsatellites (Figure 4), implicating that a high level of genetic variability at allozyme loci does not necessarily correspond with a high genetic variation at microsatellite loci. In fish, Thelen & Allendorf (2001) examined the relationship between MLH and condition factor at 10 allozyme and 10 microsatellite loci in 217 farmed rainbow trout (*O. mykiss*). While more heterozygous individuals at allozyme loci showed a significantly higher condition factor, increased heterozygosity at microsatellite loci was not associated with a higher condition. More recently, Borrell *et al.* (2004) reported a positive correlation between heterozygosity and fluctuating asymmetry, length and weight in two samples of Atlantic salmon (*Salmo salar*) with different timings of first active feeding at six allozyme loci but not at eight microsatellite loci.

Positive correlations were observed in our study between MLH and all growth measures except condition. Weight is highly variable among eel populations; optimal feeding conditions may cause an eel's weight to be twice that of another eel of equal length (Tesch 2003). This is especially valid in aquaculture and might explain the lack of correlation between MLH and condition observed in our study. On the other hand, relative condition mostly reflects changes in weight regardless of changes in length. Hence the positive association between MLH and relative condition is a direct consequence of the positive correlation observed between MLH and weight.

Regarding the performance of MLH and mean d^2, our results are concordant with the meta-analysis of published and unpublished HFCs carried out by Coltman and Slate (2003), where correlations reported for mean d^2 were smaller than those reported for MLH. Contrasting with the positive correlation between MLH and growth in our study, the negative

association between mean d^2 and growth in European eel (Figure 3) suggests that mean d^2 might not be an adequate estimator of genetic variability. Positive associations between mean d^2 and fitness traits have been reported in species with high inbreeding coefficients (Coltman *et al.*, 1998; Coulson *et al.*, 1998), where mean d^2 refers to the relatedness of individuals and not genetic variation. In the absence of inbreeding, which is typical for species with large population sizes, high migration rates and lack of population substructuring, mean d^2 does not necessarily reflect genetic variation. This explains the negative relation in our study between mean d^2 and growth rate.

Support for the direct overdominance hypothesis

A prediction of the direct overdominance hypothesis is that only heterozygosity scored at selection-sensitive markers should correlate with fitness-traits, while no relationship should be expected at neutral markers. This contrasts with the associative overdominance hypothesis where HFCs are not specific to the type of genetic marker used. In our study, both markers showed positive correlations between the degree of heterozygosity and growth rate, but only significantly so at allozymes. Similarly, fast-growth individuals presented significantly higher heterozygosities than the rest of individuals at allozymes but not at microsatellites. Allozymes produced a more significant and consistent association than microsatellites, thus providing evidence for the direct overdominance hypothesis as an explanation for the HFCs observed. Nevertheless, it must be taken into account that although microsatellites are usually considered as evolutionarily neutral DNA markers (Schlötterer & Wiehe, 1999), recent evidence indicates otherwise and at least some microsatellites are functionally important and may not be neutral (Li *et al.*, 2002). Pogson & Zouros (1994) proposed that under the associative overdominance hypothesis re-calculation of MLH combining both allozymes and microsatellites loci would result in stronger HFCs than each set of markers independently. In our study, correlations with the combined set of 18 loci were identical to the ones obtained with allozyme data, with an apparent lack of effect of microsatellite loci.

Selection affecting some of the allozyme loci would explain the stronger HFCs at allozymes in comparison with microsatellites, and the lack of correlation between MLH at allozymes and MLH at microsatellites. At allozymes, more heterozygous individuals present an intrinsically higher fitness due to increased biochemical efficiency in comparison with more homozygous individuals (Mitton, 1993, 1997). Model organisms like *Drosophila melanogaster* and *Fundulus heteroclitus* have provided clear evidence for selection acting on

enzyme polymorphisms in metabolic genes (Eanes, 1999). Koehn *et al.* (1988) reported significant effects of heterozygosity on growth at enzymes involved in metabolic functions such as protein catabolism or glycolysis, while genes without significant effect on growth rate code for enzymes with other miscellaneous functions including the pentose shunt, redox balance or digestion. Metabolic responses to selection are essentially multi-locus in nature (reviewed in Eanes, 1999). The effects of allozyme heterozygosity on growth are the result of many loci, although not all contribute equally (Pogson & Zouros, 1994; Thelen & Allendorf 2001). In the scallop *Placopecten magellanicus*, the mean shell length in heterozygotes was larger than in homozygotes at 8 loci, but only significant at 2 loci (Pogson & Zouros, 1994). In our study, positive differences in growth between homozygotes and heterozygotes were observed at almost all loci. Statistically significant values were only obtained when comparing differences in length and weight increase at *GPI** and *MPI**. Both loci are involved in important metabolic functions: mannose-6-phosphate isomerase (MPI) is a pre-glycolytic enzyme, which supplies carbon skeletons to glycolysis. Glucose phosphate isomerase (GPI) is a main-line glycolytic enzyme that catalyses the reversible interconversion of d-fructose-6-phosphate and d-glucose-6-phosphate. By contrast, the smallest differences between homozygotes and heterozygotes in growth rate were observed at phosphogluconate dehydrogenase (PGDH), which codes for an enzyme with a secondary metabolic function (pentose shunt). Since PGDH is not involved in protein catabolism or glycolysis, it would play a smaller role in growth.

Alternative hypothesis: associative overdominance

Associative overdominance could provide an explanation for the HFCs in this study if we consider that allozyme loci have a higher chance than microsatellites to be in linkage disequilibrium with fitness loci. A greater mutation rate of microsatellites would cause homozygous genotypes to present identical alleles by state (homoplasy) and not by descent, and therefore are less likely to reflect homozygosity at linked fitness loci. Alternatively, allozyme and microsatellite loci might not have the same distribution in the genome. If allozyme loci are located in richer gene regions and microsatellite loci in poorer gene regions, allozymes would be more likely to be in linkage disequilibrium with fitness loci (Thelen & Allendorf 2001). Little information is available on the distribution of genes in the European eel, although linkage maps in for example salmonids (pink salmon, Lindner *et al.* 2000; brown trout, Sakamoto *et al.* 2000) suggest that allozyme and microsatellite loci are found at

similar genomic locations. Tóth *et al.* (2000) reported that despite microsatellites being less abundant in exons than in noncoding regions, microsatellite distribution in introns and intergenic regions is similar and differs only in the abundance of certain triplets.

The main mechanisms explaining HFCs detected by neutral markers relate to inbreeding due to low effective population sizes or linkage disequilibrium caused by bottleneck/founder events followed by rapid population expansion (Hansson & Westerberg 2002). Glass eel recruitment is estimated at about 2 x 10^9 individuals per year, although mortality is close to 100% in some areas (Dekker 2000). As in many marine fish species, anthropogenic impacts (fisheries overexploitation, pollution, habitat loss and migration barriers) cause populations to decline. Since the 1980's, a steadily downward trend has been observed in recruitment of glass eel arriving to the European continent, which is affecting or will shortly affect the continental (yellow and silver eel) stock (ICES 2003). Using a Bayesian approach to infer demographic parameters from microsatellite data, Wirth & Bernatchez (2003) suggested a contemporary effective population size of about 5 x 10^3 – 10^4 eels. Despite the population of European eel being possibly at an historical minimum, it would remain large enough not to be affected by inbreeding.

Conclusions and future directions

Our results provide sound evidence for a heterozygosity-fitness correlation in farmed European eel individuals. Multi-locus heterozygosity is positively correlated with growth, so that more heterozygous individuals at allozyme loci (and to a lesser extent microsatellite loci) show a significantly higher length and weight increase and a higher condition index in comparison with more homozygous individuals. The HFCs observed could be explained by an effect of either direct allozyme overdominance or associative overdominance.

HFCs are expected to decrease or disappear with age since growth and survival differences are maximal early in life (David, 1998). It has been proposed that early and late growth may be controlled by different sets of genes (Vaugh *et al.*, 1999). In our study, individuals will be kept in the farm for another year, which will allow us to check for time consistency of the observed HFCs. We intend to estimate genotype-specific survival and possible heterozygosity-viability correlations by analyzing individuals that died after handling and parasite infection, and the influence of grading (early splitting of individuals in size classes) in the association MLH-growth.

ACKNOWLEDGMENTS

We thank G. Pogson and three anonymous reviewers for their useful comments on the manuscript. We also thank V. van Ginneken and all the personnel at Royaal BV who assisted us with sampling. Research was funded by the EU contract EELREP (Q5RS-2001-01836). G.E. Maes received a PhD fellowship from the I.W.T. (Institute for the Promotion of Innovation by Science and Technology in Flanders). J.M. Pujolar received a postdoctoral fellowship from the Ministerio de Educación, Cultura y Deporte (Spain). Both authors were supported by the EU contract EELREP (Q5RS-2001-01836).