MOLECULAR ECOLOGY

Molecular Ecology (2009) **18**, 4604–4616 doi: 10.1111/j.1365-294X.2009.04390.x

Genetic variability is unrelated to growth and parasite infestation in natural populations of the European eel (*Anguilla anguilla*)

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Abstract

Positive correlations between individual genetic heterozygosity and fitness-related traits (HFCs) have been observed in organisms as diverse as plants, marine bivalves, fish or mammals. HFCs are not universal and the strength and stability of HFCs seem to be variable across species, populations and ages. We analysed the relationship between individual genetic variability and two different estimators of fitness in natural samples of European eel, growth rate (using back-calculated length-at-age 1, 2 and 3) and parasite infestation by the swimbladder nematode *Anguillicola crassus*. Despite using a large data set of 22 expressed sequence tags-derived microsatellite loci and a large sample size of 346 individuals, no heterozygote advantage was observed in terms of growth rate or parasite load. The lack of association was evidenced by (i) nonsignificant global HFCs, (ii) a Multivariate General Linear Model showing no effect of heterozygosity on fitness components, (iii) single-locus analysis showing a lower number of significant tests than the expected false discovery rate, (iv) sign tests showing only a significant departure from expectations at one component, and, (v) a random distribution of significant single-locus HFCs that was not consistent across fitness components or sampling sites. This contrasts with the positive association observed in farmed eels in a previous study using allozymes, which can be explained by the nature of the markers used, with the allozyme study including many loci involved in metabolic energy pathways, while the expressed sequence tags-linked microsatellites might be located in genes or in the proximity of genes uncoupled with metabolism/growth.

Keywords: European eel, growth, heterozygosity, microsatellites, parasites

Received 27 July 2009; revision received 11 September 2009; accepted 15 September 2009

Introduction

Heterozygosity–fitness correlations (HFCs), the correlation between the degree of individual genetic heterozygosity and fitness-related traits such as growth rate, survival, fecundity or development stability, have been studied for more than two decades in populations of many species. Significant positive correlations have been observed in organisms as diverse as plants (Ledig *et al.* 1983), marine bivalves (Koehn & Gaffney 1984), crustaceans (Bierne *et al.* 2000), fish (Leary *et al.* 1984; Ferguson 1992; Thelen & Allendorf 2001) and mammals (Coltman *et al.* 1998, 1999; Coulson *et al.* 1998). Despite being widely accepted, HFCs are not universal and seem to be variable among taxa, species and populations (David 1998). A recent meta-analysis suggested that HFCs may be a general phenomenon but of small effect (less than 1% of the observed phenotypic variance) (Chapman *et al.* 2009). The generality of HFCs is difficult to assess owing to the publication bias in favour of positive correlations, making null results likely to be under-represented in literature (Hansson & Westerberg 2002; Chapman *et al.* 2009).

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The first HFCs were observed in studies using allozyme markers, which led to the hypothesis of a direct heterozygous genotype advantage resulting in higher fitness compared with the corresponding homozygous genotype (direct overdominance). The observation of positive HFCs with putative neutral DNA markers proves that at least some correlations are not because of the direct effect of the marker genes but owing to the genetic association between the neutral markers and fitness (associative overdominance; David 1998). When marker loci are not directly responsible, the observed correlation can be because of the effect of linkage disequilibrium affecting closely linked fitness loci located in a narrow chromosomal section (local effect) or the effect of homozygosity at genome-wide distributed fitness loci generated by partial inbreeding (general effect; David *et al.* 1995; Hansson & Westerberg 2002; Pemberton 2004). Slate *et al.* (2004) suggested that the relationship between heterozygosity and inbreeding is only strong in the case of unusually high variance in individual inbreeding coefficient. Similarly, the study of Balloux *et al.* (2004) using stochastic individual-based simulations showed that heterozygosity and inbreeding are only correlated in situations involving strong population structure or highly skewed mating systems (selfing, polygyny) that led to both frequent and severe inbreeding events.

Many studies have documented positive correlations between heterozygosity and growth rate, although the strength and stability of the HFCs can clearly differ across species, temporal and geographic samples and sexes (Liskauskas & Ferguson 1991; David 1998). One source contributing to such variation is the prediction that HFCs might decrease with age as differences in growth are maximal early in life (energy is mostly directed to growth at early but not at later stages; David 1998), and because unfit genotypes are selectively eliminated in ageing cohorts (Koehn & Gaffney 1984). For instance, Bierne *et al.* (1998) observed significant microsatellite heterozygosity–growth correlations in larvae and post-larval-stage individuals in the European oyster *Ostrea edulis*, which were not observed in 1-year-old cohorts using allozymes (Marsic-Lucic & David 2003). In fish, positive correlations were observed between allozyme heterozygosity and growth up to 6 months in cultured rainbow trout *Oncorrhynchus mykiss*, while negative correlations were found with length at 1 year and length at maturity (Ferguson 1992). When examining the relationship between heterozygosity at 10 nuclear restriction fragment length polymorphism loci and size-at-age in two populations of Atlantic cod *Gadus morhua*, Pogson & Fevolden (1998) found significant correlations in only one of the two populations, supporting the hypothesis that different samples may have different genetic backgrounds and consequently different HFC outputs.

Over the past decade, a growing body of evidence has emerged showing a direct relation between genetic variability and parasitism in wild mammal animals, with the less heterozygous individuals being more susceptible to parasite infection and less likely to survive, including studies on Soay sheep *Ovis aries* (Coltman *et al.* 1999), Cuvier's gazelle *Gazella cuvieri* (Cassinello *et al.* 2001) and bighorn sheep *Ovis canadensis* (Luikart *et al.* 2008). Similarly, in the case of marine mammals, heterozygous California sea lions were less likely to be infected by a range of pathogens (Acevedo-Whitehouse *et al.* 2003), whereas heterozygosity predicted lower lungworm burden in young harbour seals (Rijks *et al.* 2008). In fish, higher heterozygosity had also been associated with greater disease resistance and survival in rainbow trout challenged with bacterial gill disease (Drahushchack 1990). Several lines of evidence indicate that survival after pathogen infection is connected to major histocompatibility complex (MHC) loci (Bernatchez & Landry 2003).

The species of interest in our study is the European eel *Anguilla anguilla* (Anguillidae; Teleostei), a catadromous highly migratory fish species that moves between marine and continental environments. After spawning in the Sargasso Sea, larvae (leptocephali) cross the Atlantic Ocean and metamorphose into glass eels that complete the migration into continental growth habitats. Glass eels metamorphose into yellow eels and after an average feeding period of 7–15 years metamorphose into silver eels that migrate back to the Sargasso Sea, where they reproduce once and die (Van den Thillart *et al.* 2009). Recruitment has declined beyond safety limits in recent years (ICES 2008), prompting for a better understanding of the genetic factors influencing the survival of the species. The maintenance of a high level of genetic diversity is expected to be crucial to safeguard its evolutionary potential given the broad number of anthropogenic (overfishing, migration barriers, habitat destruction, pollution, introduced diseases and parasites) and environmental (climate and current ocean change) factors influencing its decline (Knights 2003; Friedland *et al.* 2007; Bonhommeau *et al.* 2008).

Pujolar *et al.* (2005) reported for the first time a correlation between genetic variability and growth rate in farmed European eel individuals. More heterozygous individuals at 10 allozyme loci showed a significantly greater length and weight increase and an above-average condition index in comparison with more homozygous individuals after 12 months. When testing the temporal stability of the HFCs found, 22-month-old individuals showed no evidence that genetic variation was correlated with growth rate (Pujolar *et al.* 2006).

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The decrease of HFCs in time was attributed to the relaxation of environmental conditions related to population-density effects, which fits the prediction that environmental stress enhances HFCs (Lesbarreres *et al.* 2005). Pujolar *et al.* (2006) also compared genetic variability in surviving and dead individuals under handling stress and parasite (*Pseudogyrodactylus*) infection, two conditions that cause high mortalities in eel aquaculture. Highly heterozygous individuals did not exhibit an above-average survival under either of the two stressors, thus the authors suggested that the markers used might have an influence on growth (related to a metabolic advantage) but not survival.

The aim of the study is to test the association between genetic variability and two measures of fitness, namely growth rate and parasite infestation, in natural populations of the European eel. Genetic variation was estimated using microsatellite markers developed from expressed sequence tags (EST), which are single-read sequences produced from messenger RNA (mRNA). EST-derived microsatellites have the advantage of being putative type I markers (non-neutral), as it is possible to connect the function of the transcript of the genes from an EST sequence with the presence of a microsatellite. EST-derived microsatellites show a potential applicability in HFC studies as they are located in coding regions (either inside genes or adjacent to genes) so they have a higher chance to detect a direct or associated effect of the marker genes than traditional genomic microsatellites obtained from enriched libraries, which are generally type II markers (neutral) located in noncoding regions, and therefore not linked to genes of known function. Regarding number of markers, we used a large panel of 22 EST-derived microsatellite loci, given the lack of evidence that small (<10) marker sets have any power to detect HFCs (Hansson & Westerberg 2002; Balloux *et al.* 2004; Slate *et al.* 2004). In terms of HFCs, first we examined the association between genetic variation and growth by testing the null hypothesis that more heterozygous individuals show an above-average growth rate in comparison with more homozygous individuals. Aquaculture experiments have shown that a faster growth has a clear direct fitness benefit at the individual level during competitive struggles for food, in which the smallest individuals in the hierarchy usually die either after withering owing to their inability to obtain food or after predation by larger individuals (Pujolar *et al.* 2005). As HFCs may be environmentally dependent (Chapman *et al.* 2009), we tested whether HFCs previously found in farmed individuals (obtained from the wild but grown in aquaculture facilities; Pujolar *et al.* 2005) could be detected in natural populations, and whether HFCs were consistent across different natural sampling sites. Growth was estimated at ages 1, 2 and 3 using otolith readings and back-calculation methods to test the stability of HFCs over time and the general prediction that HFCs tend to decrease with age (David 1998; Pujolar *et al.* 2006 in eels). Second, we tested a possible correlation between genetic variability and parasite infestation by the swimbladder nematode *Anguillicola crassus*. The parasite is native to the Far East and was introduced into Europe in the early 1980s when Japanese eel *Anguilla japonica* was imported for aquaculture, which resulted in infection of wild populations of European eel (De Charleroy *et al.* 1990). While the Japanese eel maintains low numbers of parasites (<10) in the swimblader without obvious pathological effects, the European eel can be infected with as many as 50 mature worms, and although the parasite is usually not lethal, it causes significant tissue damage in the swimbladder. As the swimbladder contributes to the ability of a fish to control its buoyancy, a severe parasite infestation could hamper its potential to swim back to the spawning grounds in the Sargasso Sea (Sioberg *et al.* 2009). We tested the general prediction that individuals with low genetic variation may be less able to cope with parasite infection in comparison with more heterozygous individuals. Given the severe decline of the European eel stocks, understanding the interaction among genetic diversity loss, survival to parasite infection and fitness in natural populations is of key importance.

Materials and methods

Sampling collection

A total of 1234 European eel *Anguilla anguilla* individuals were caught using fyke nets in three separate geographic sites in the Mediterranean Sea: (i) the low course of the Tiber river (41°48′N; 12°25′E) in the Tyrrhenian Sea. Samples were collected during May–November 2007 ($N = 422$); (ii) Laguna di Lesina (41°53′N; 15°51′E), a brackish lagoon connected to the Adriatic Sea, with an area of 51.4 km^2 and characterized by variable salinity (11–33%) and high productivity (8–15% total organic matter). Samples were collected during October 2007–March 2008 ($N = 407$); (iii) Laguna di Caprolace (41°21′N; 12°59′E) in the National Park of Circeo, a small lagoon connected to the Tyrrhenian Sea, with an area of 2.26 km^2 and high average salinity (40%). Samples were collected during October 2007–March 2008 ($N = 405$). The parasitic nematode *Anguillicola crassus* has been recorded in both Tiber and Lesina, whereas Caprolace is parasite-free, which might be related to its high salinity, as parasite development and transmission is lower in brackish and marine waters than in fresh waters (Kirk *et al.* 2000).

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All individuals were measured for total length (L) and weight (W). Each individual was dissected and the swimbladder was removed, maintained damp in distilled water and dissected in longitudinal sense to identify and single count *A. crassus* nematodes using a binocular stereomicroscope. Otoliths were extracted from all individuals, embedded in epoxyn resin and stained with 3% toluidine blue following grinding of the convex side. Individual age was determined from reading annual otolith rings (annuli) starting from the first ring after the marine nucleus edge using a light stereomicroscope. Ages ranged from 2 to 13 in Tiber, 2 to 11 in Lesina and 3 to 15 in Caprolace.

A subsample of 346 individuals from the three sampling sites (Tiber, $N = 109$; Lesina, $N = 134$; Caprolace, $N = 103$) was selected for the genetic analysis on the basis of good quality of otolith reading (Table 1). This allowed for three age classes in Tiber (3+, 4+, 5+), four in Lesina (3+, 4+, 5+, 6+) and three in Caprolace (6+, 7+, 8+). Growth rate was estimated for all individuals included in the genetic analysis by means of back-calculated lengths-at-age.

Back-calculation procedures

The body length–otolith radius relation was used for back-calculation of length-at-age 1 (BL1), age 2 (BL2) and age 3 (BL3), based on the proportionality between otolith and somatic growth (Jessop *et al.* 2004). Length-at-age i (BL_i) was calculated as $BL_i = L_o + (r_i/R)^{\beta} (L_c - L_o)$, where L_o is the average length at recruitment (63 mm at each of the three sites), L_c is length at capture, r_i is the radius length corresponding to the ith ring of the otolith, R is the total radius length of the otolith (distance from the marine nucleus edge) and β is the slope of the total length–total radius length of the otolith regression for all samples (Tiber, $\beta = 1.06$; Lesina, $\beta = 0.86$; Caprolace, $\beta = 0.68$). All BLs were highly correlated with both otolith radius (BL1: $r = 0.562$, $P < 0.001$; BL2: $r = 0.677$, $P < 0.001$; BL3: $r = 0.641$; $P < 0.001$), and length at capture ($L_c - BL1$: $r = 0.430$; $P < 0.001$; $L_c - BL2$: $r = 0.742$; $P < 0.001$; $L_c - BL3$: $r = 0.745$; $P < 0.001$).

Microsatellite analysis

Minute sections of tissue from ethanol-preserved eel finclips were digested in a lysis buffer containing 100 μL TE Buffer, 7 μL of 1 M DTT (dithiothreitol) solution, pH 5.2 (diluted in 0.08 M NaAC) and 2 μL of proteinase K solution (20 mg/mL) for at least 8 h at 56 °C. After incubation at 96 °C for 10 min, samples were centrifuged at 13 000 rpm for 11 min, and the supernatant was stored at −20 °C.

Genotypes were examined at a total of 22 EST-linked microsatellite loci (Pujolar *et al.* 2008), after excluding locus 41A22 owing to an homozygote excess linked to the presence of null alleles. Microsatellites were grouped into two separate multiplexes to reduce polymerase chain reaction (PCR) and genotyping costs (Pujolar *et al.* 2009). PCR products were obtained in a GeneAmp PCR System 2700 Thermocycler (Applied Biosystems) using the Qiagen Multiplex PCR Kit. PCR reactions consisted of 2 μL template DNA, 5 μL Qiagen Multiplex PCR Master Mix, 0.2 μL of 10 μM forward and reverse primers and water up to 10 μL. PCR conditions were as follows: 15 min at 95 °C, 35 cycles of 30 s at 94 °C, 90 s at 57 °C and 1 min at 72 °C, and final elongation for 30 min at 60 °C. PCR products were visualized in 1.8% agarose gels and screened for

Table 1 Sampling details for genetic analysis of *Anguilla anguilla* including age class groups per sampling site, number of individuals, mean total length (L; cm), mean total weight (W; g) and % of parasited individuals (prevalence). Summary of genetic variability includes expected (H_E) and observed (H_O) heterozygosities and allelic richness (AR). Standard deviation (SD) is given in parentheses

Sampling site/age class	N	L (SD)	W (SD)	Prevalence	H_E (SD)	H_O (SD)	AR
Tiber (109)							
Age 3+	34	23.49 (2.97)	21.94 (10.03)	77.42	0.59 (0.24)	0.54 (0.23)	6.54 (3.89)
Age 4+	42	28.62 (4.55)	45.20 (28.05)	69.44	0.55 (0.27)	0.52 (0.27)	6.41 (4.02)
Age 5+	33	31.93 (7.21)	65.71 (61.81)	59.38	0.56 (0.26)	0.54 (0.25)	6.16 (3.91)
Lesina (134)							
Age 3+	27	39.12 (6.19)	96.68 (43.98)	39.46	0.55 (0.27)	0.51 (0.27)	6.40 (4.34)
Age 4+	46	41.43 (5.93)	116.86 (55.82)	29.27	0.56 (0.26)	0.50 (0.21)	6.25 (3.94)
Age 5+	31	47.34 (8.41)	199.17 (115.27)	26.13	0.56 (0.26)	0.52 (0.24)	6.02 (3.46)
Age 6+	30	53.58 (7.63)	289.26 (139.16)	25.00	0.57 (0.24)	0.55 (0.24)	6.19 (3.41)
Caprolace (103)							
Age 6+	29	34.57 (4.72)	62.56 (33.35)	—	0.57 (0.23)	0.54 (0.23)	6.10 (3.40)
Age 7+	41	37.36 (4.18)	79.83 (37.35)	—	0.55 (0.25)	0.50 (0.24)	6.12 (3.56)
Age 8+	33	40.53 (6.37)	100.33 (35.53)	—	0.57 (0.26)	0.50 (0.24)	6.33 (4.23)

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microsatellite polymorphism using an ABI 3130 AVANT automatic capillary sequencer (Applied Biosystems). Alleles were sized according to a Liz500 (50–500 bp) marker.

Data analysis

Within sample genetic diversity statistics were assessed by observed (H_O) and expected (H_E) heterozygosities per locus using GENETIX version 4.05 (Belkhir *et al.* 2005) and allelic richness (AR) using FSTAT (Goudet 2002), and compared statistically using one-way ANOVA. Deviations from Hardy–Weinberg equilibrium, linkage disequilibrium and differences in allele and genotype frequencies among samples were tested using Genepop version 3.4 (Raymond & Rousset 1995). Significance levels for multiple comparisons were adjusted using the sequential Bonferroni technique (Rice 1989). Presence of null alleles was tested using the program MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al.* 2004).

Individual genetic variability was estimated using multilocus heterozygosity (MLH), calculated as the proportion of loci that were heterozygously corrected for nonscored loci. A mean of 21.48 ± 1.06 loci were scored per individual. Individuals genotyped at fewer than 17 loci were excluded from the analysis. MLH was chosen over alternative metrics such as standardized mean d^2 (Coulson *et al.* 1998), internal relatedness (IR; Amos *et al.* 2001) or homozygosity by loci (HL; Aparicio *et al.* 2006) as recommended by the recent review by Chapman *et al.* (2009), which argued that newly proposed measures of variation were no more powerful in detecting HFCs than MLH, and advocated the use of the simplest metric, MLH, in HFC studies. The review also showed most genetic metrics to be highly correlated and nonindependent, prompting researchers to report only one of these genetic metrics to avoid pseudoreplication. In our data, all metrics were highly correlated to MLH, with a positive relationship between MLH and mean d^2 ($r = 0.34$; $P < 0.001$) and a negative association between MLH-HL ($r = -0.93$; $P < 0.001$) and MLH-IR ($r = -0.96$; $P < 0.001$). While MLH and mean d^2 measure heterozygosity, both HL and IR measure homozygosity, which explains the negative correlation observed. In accordance with the review of Chapman *et al.* (2009), previous reviews concluded that if HFCs exist, they will be better detected by individual heterozygosity than by d^2 values (Tsitrone *et al.* 2001; Slate & Pemberton 2002; Coltman & Slate 2003). MLH has also been the metric used in most HFC studies on fish (Drahushchack 1990; Ferguson 1992; Pogson & Fevolden 1998; Thelen & Allendorf 2001; Borrell *et al.* 2004; Lieutenant-Gosselin & Bernatchez 2006), including the only study to date reporting positive HFCs on European eel (Pujolar *et al.* 2005), in which the positive associations found at MLH contrasted with the lack of association using d^2 measurements. Previous studies in which d^2 outperformed MLH seems to be restricted to species with particular inbreeding patterns (harbour seal, Coltman *et al.* 1998; red deer, Coulson *et al.* 1998) that do not apply to the outbred European eel.

The existence of possible HFCs was tested by multiple linear regression analysis. All data were checked for normality prior to parametrical statistical analysis and a logarithmic transformation was conducted if necessary. Correlations were performed between MLH and the following fitness estimators: (i) growth rate using BL1, BL2 and BL3 for all locations, and (ii) parasite infestation measured as absence/presence of parasites (prevalence) and number of parasites (MNP).

In addition, the association between MLH and fitness components was assessed using a Multivariate General Linear Model (MGLM), which allows controlling for differences between localities. A model was fit containing sampling location as fixed factor with two levels (Caprolace was omitted because it is parasite-free), back-calculated lengths (BL1, BL2, BL3) and MNP as dependent variables and MLH as covariate. All variables were checked for normality prior to the analysis and a logarithmic transformation was conducted if necessary.

At both parasite infestation and growth rate, individual loci effects were examined by comparing the performance of homozygotes and heterozygotes at all loci. The significance of positive or negative effects was investigated by two-tailed *t*-tests (BL1, BL2, BL3, MNP) and chi-squared tests (prevalence). Correction for multiple tests was applied by comparing the observed number of significant associations with the expected number of false-positive associations (14.3 in total, 13 groups × 22 loci at $\alpha = 0.05$) using a chi-squared test.

The predominance of positive vs. negative single-locus HFCs independent of the absolute level of significance was investigated by (i) a cumulative binomial exact probability test on the direction of the associations assuming equal probabilities for positive or negative results (50%), and (ii) a Wilcoxon signed-ranks test. We also tested whether the combination of positive or negative single-locus HFCs had a significant impact on each fitness component using the Fisher's method combining probabilities of independent tests (Sokal & Rohlf 1995). Finally, we determined whether the distribution of significant single-locus HFCs was random among loci by comparing the distribution of the observed associations with the expected uniform distribution using a chi-squared test. All statistical analyses were performed in STATISTICA version 6.0 (Statsoft Inc.) and SPSS version 16.0 (SPSS Inc.).

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Results

All samples showed similar levels of genetic variation at 22 microsatellite loci, with no significant differences in H_E ($F = 0.05$; d.f. = 9; $P = 1.000$), H_O ($F = 0.15$; d.f. = 9; $P = 0.998$) or AR ($F = 0.04$; d,f. = 9; $P = 1.000$; Table 1). Comparable values were found across sampling sites regarding heterozygosities (Tiber: H_E = 0.55–0.59; Lesina: H_E = 0.55–0.57; Caprolace: H_E = 0.55–0.57) and AR (Tiber: AR = 6.2–6.5; Lesina: AR = 6.0–6.4; Caprolace: AR = 6.1–6.3). No differences were found in mean MLH calculated over 22 loci across sites (Tiber: 0.524; Lesina: 0.511; Caprolace: 0.509) using a one-way ANOVA ($F = 0.75$; d.f. = 2; $P = 0.474$). All loci were in Hardy–Weinberg equilibrium.

Figure 1 shows BLs at the three sampling locations (Tiber, Lesina, Caprolace) in our study. All comparisons of growth rate across locations were highly significant ($P < 0.001$). At age 1, individuals in Caprolace showed a faster growth (mean BL1: 10.45 ± 1.19) than Lesina (mean BL1: 9.40 ± 1.11) and Tiber (mean BL1: 8.08 ± 1.45). At ages 2 and 3, the fastest growth was observed in Lesina (mean BL2: 20.53 ± 3.79; mean BL3: 28.28 ± 5.07), with Caprolace showing an intermediate growth rate (mean BL2: 17.28 ± 2.67; mean BL3: 22.38 ± 3.15), and Tiber showing the slowest growth (mean BL2: 13.16 ± 2.34; mean BL3: 19.10 ± 3.48). Out of the two sites at which parasites were present (Caprolace is parasite-free), Tiber showed a higher infestation with 70.90% of the individuals parasited, a mean load of 3.871 ± 3.635 parasites per individual and a maximum load of 19 parasites. Infestation was less severe in Lesina, with 37.07% of the individuals parasited, a mean load of 2.912 ± 2.234 parasites per individual and a maximum load of 10 parasites. A preliminary analysis showed that age-at-capture was not correlated with MLH at any of the sites (Tiber: $r = 0.07$; $F = 0.47$; d.f. = 1.1; $P = 0.494$; Lesina: $r = 0.15$; $F = 3.02$; d.f. = 1.1; $P = 0.084$; Caprolace: $r = 0.15$; $F = 2.26$; d.f. = 2.0; $P = 0.136$). Similarly, age-at-capture was not associated with parasite load at both Tiber ($F = 1.19$; d.f. = 2; $P = 0.308$) and Lesina ($F = 1.22$; d.f. = 3; $P = 0.304$) when prevalence was compared using one-way ANOVA.

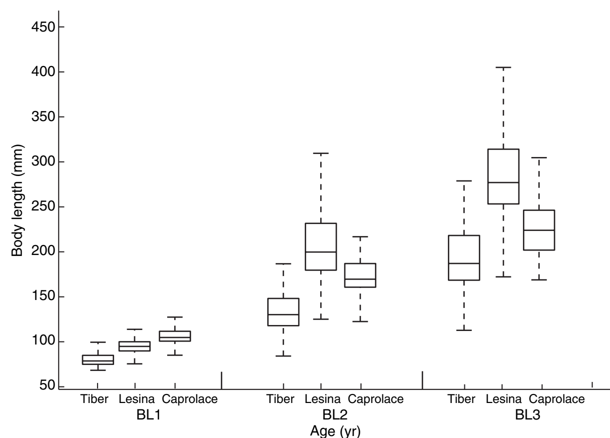

Fig. 1 Box plots of back-calculated lengths-at-age 1 (BL1), age 2 (BL2) and age 3 (BL3) at all sampling sites (Tiber, Lesina, Caprolace). Lines in the boxes represent lower quartile, median and upper quartile values. Whiskers extend from quartile values to the most extreme data value within 1.5 times interquartile range.

Global HFCs

No significant relationship was observed between growth and MLH calculated over 22 loci (Table 2 and Fig. 2). All regressions of BL1, BL2 and BL3 on MLH were not significant at the three sampling sites. Regarding parasite infestation, infected and noninfected individuals showed no difference in mean MLH at either Tiber (infected: 0.522 ± 0.096/noninfected: 0528 ± 0.095; $P = 0.743$) or Lesina (infected: 0.511 ± 0.099/noninfected: 0.511 ± 0.107; $P = 0.992$). No association was detected between MLH and the number of parasites at Tiber ($r = -0.014$; $F = 0.02$; d.f. = 1.97; $P = 0.891$) or Lesina ($r = -0.002$; $F = 0.01$; d.f. = 1.13; $P = 0.975$; Table 2).

Table 3 details results from the MGLM. Prior to the analysis, all values were checked for normality and a logarithmic transformation was conducted for all BLs (BL1, BL2 and BL3) and for number of parasites. The model suggested no effect of MLH on growth (BL1: $F = 1.00$, $P = 0.319$; BL2: $F = 0.88$, $P = 0.351$; BL3: $F = 0.34$, $P = 0.563$) and no effect of MLH on parasites ($F = 0.02$, $P = 0.887$). Nevertheless, an effect of location was observed on all fitness traits ($P < 0.001$ in all cases), which is consistent with the statistically significant differences found in growth rate and parasite load across

Table 2 Multiple linear regression between multilocus heterozygosity (MLH) and fitness components (all log-transformed) including back-calculated lengths-at-age 1 (BL1), age 2 (BL2) and age 3 (BL3) and mean number of parasites (MNP) at all sampling sites (Tiber, Lesina, Caprolace)

	Tiber (N = 109)		Lesina (N = 134)		Caprolace (N = 103)	
Association	r	P	r	P	r	P
MLH–BL1	−0.158	0.105	−0.060	0.492	0.086	0.397
MLH–BL2	−0.100	0.301	−0.017	0.843	−0.025	0.807
MLH–BL3	−0.018	0.854	−0.069	0.428	0.028	0.778
MLH–MNP	−0.014	0.891	−0.002	0.975	—	—

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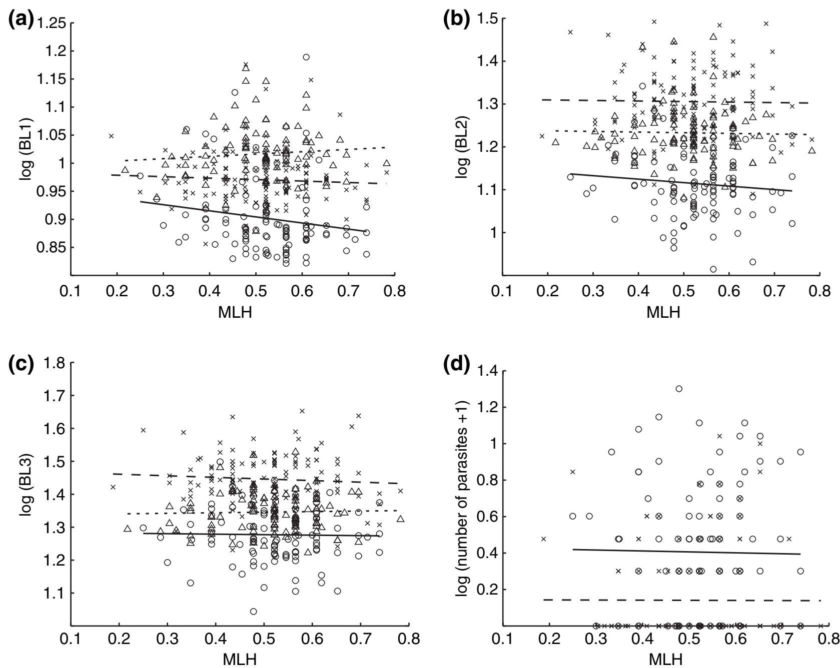

Fig. 2 Plots of multilocus heterozygosity vs. (a) length-at-age 1 (BL1), (b) age 2 (BL2), (c) age 3 (BL3) and (d) number of parasites for all sampling sites. Tiber, circles/solid lines; Lesina, crosses/dashed lines; Caprolace, triangles/dotted lines.

Table 3 Multivariate General Linear Model (MGLM) using back-calculated lengths-at-age 1 (BL1), age 2 (BL2) and age 3 (BL3) and mean number of parasites (MNP) (all log-transformed) as dependent variables, sampling location as fixed factor with two levels (Caprolace was omitted because none of the fish sampled there were parasited) and multilocus heterozygosity (MLH) as covariate

Effect	Dependent variable	*F*	d.f.	*P*
Intercept	BL1	2343.29	1.226	<0.001*
	BL2	2090.68	1.226	<0.001*
	BL3	2564.95	1.226	<0.001*
	MNP	7.30	1.226	<0.001*
MLH	BL1	1.00	1.226	0.319
	BL2	0.88	1.226	0.351
	BL3	0.34	1.226	0.563
	MNP	0.02	1.226	0.887
Location	BL1	80.58	1.226	<0.001*
	BL2	341.96	1.226	<0.001*
	BL3	255.09	1.226	<0.001*
	MNP	41.73	1.226	<0.001*

*$P < 0.05$.

sampling locations. No outliers or points with high leverage were observed (Fig. 2).

Single-locus HFCs

Occurrence of positive and negative single-locus HFCs was calculated for BL1, BL2, BL3, presence of parasites (prevalence) and MNP at Tiber (Table S1) and Lesina (Table S2), and, for BL1, BL2 and BL3 at Caprolace (Table S3). A total of 13 out of 286 single-locus HFCs were statistically significant, which was not significantly different from the expected number of false positives (14.3; $\chi^2 = 0.125$, d.f. = 1, $P = 0.724$). Only 4 out of the 13 significant HFCs were positive, and 9 were negative. Similarly, the number of significant single-locus HFCs when taking into account one back-calculated measure at a time was not significantly different from the expected false discovery rate (7.7): BL1 ($\chi^2 = 0.375$, d.f. = 1, $P = 0.540$), BL2 ($\chi^2 = 0.375$, d.f. = 1, $P = 0.540$) and BL3 ($\chi^2 = 0.063$, d.f. = 1, $P = 0.802$). Considering all single-locus HFCs independently of their significance, positive relationships represented 138 out of THE 286 associations (48.25%), whereas negative relationships represented 51.75% of all associations (148/286). The proportion of positive and negative relationships was not significantly different from random expectation (cumulate binomial probability test, $P = 0.616$; Wilcoxon signed-ranks test, $P = 0.703$; Table 4). When considering fitness components and sample locations separately, a highly significant deviation was only found at Tiber for BL1, after observing 18 out of 22 negative associations (cumulate binomial probability test, $P = 0.026$; Wilcoxon signed-ranks test, $P = 0.012$). The overall significance of multiple tests was assessed using Fisher's method separately for loci showing either positive or negative effects (Table 5). No significant effect was observed between any fitness component and loci with either a positive or negative association. Accordingly, the distribution of

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Table 4 Comparison of positive vs. negative single-locus heterozygosity–fitness correlations (HFCs) for all associations – back-calculated lengths-at-age 1 (BL1), age 2 (BL2) and age 3 (BL3), parasite prevalence (PREV) and mean number of parasites (MNP) – at three sampling sites (Tiber, Lesina and Caprolace) using (i) a cumulative binomial exact probability test and (ii) a Wilcoxon signed-ranks test

Association	*N*	Positive	Negative	*P* (1)	*P* (2)
BL1/Tiber	22	4	18	0.026*	0.012*
BL2/Tiber	22	9	13	0.545	0.363
BL3/Tiber	22	10	12	0.763	0.758
PREV/Tiber	22	14	8	0.361	0.277
MNP/Tiber	22	11	11	1.000	0.783
BL1/Lesina	22	9	13	0.545	0.200
BL2/Lesina	22	12	10	0.793	0.974
BL3/Lesina	22	11	11	1.000	0.426
PREV/Lesina	22	14	8	0.361	0.140
MNP/Lesina	22	9	13	0.545	0.795
BL1/Caprolace	22	14	8	0.361	0.211
BL2/Caprolace	22	9	13	0.545	0.650
BL3/Caprolace	22	12	10	0.793	0.495
BL1	66	27	39	0.294	0.135
BL2	66	30	36	0.601	0.451
BL3	66	33	33	1.000	0.474
PREV	44	28	16	0.197	0.067
MNP	44	20	24	0.670	0.912
Tiber	110	48	62	0.344	0.888
Lesina	110	55	55	1.000	0.374
Caprolace	66	35	31	0.728	0.488
All	286	138	148	0.616	0.703

*$P < 0.05$.

Table 5 Fisher's method for combining probabilities for the positive and negative single-locus heterozygosity–fitness correlations (HFCs) for all associations – back-calculated lengths-at-age 1 (BL1), age 2 (BL2) and age 3 (BL3), parasite prevalence (PREV) and mean number of parasites (MNP) – at three sampling sites (Tiber, Lesina and Caprolace)

	Positive associations			Negative associations		
Association	χ^2	d.f.	*P*	χ^2	d.f.	*P*
BL1/Tiber	5.77	8	0.674	28.13	36	0.823
BL2/Tiber	13.45	18	0.764	27.34	26	0.392
BL3/Tiber	24.33	20	0.228	23.26	24	0.504
PREV/Tiber	21.59	28	0.800	14.39	16	0.570
MNP/Tiber	22.69	22	0.419	17.07	22	0.759
BL1/Lesina	10.85	18	0.908	27.01	26	0.409
BL2/Lesina	15.84	24	0.894	24.42	20	0.225
BL3/Lesina	18.62	22	0.669	31.56	22	0.085
PREV/Lesina	27.82	28	0.474	13.86	16	0.609
MNP/Lesina	11.79	18	0.858	17.68	26	0.887
BL1/Caprolace	24.63	28	0.648	21.72	16	0.152
BL2/Caprolace	13.50	18	0.761	15.37	26	0.950
BL3/Caprolace	17.92	24	0.807	11.00	20	0.341
BL1	41.24	54	0.899	76.86	78	0.515
BL2	42.80	60	0.954	67.13	72	0.640
BL3	60.86	66	0.656	65.81	66	0.658
PREV	49.41	56	0.721	28.24	32	0.657
MNP	34.48	40	0.712	34.37	48	0.931
Tiber	87.82	96	0.711	110.20	124	0.807
Lesina	84.92	108	0.951	114.50	108	0.316
Caprolace	56.60	70	0.876	48.10	62	0.902
All	228.79	274	0.978	272.80	294	0.807

the significant single-locus HFCs among loci for the different fitness components was random ($\chi^2 = 15.00$, d.f. = 21, $P = 0.853$). Only two loci were associated with more than one fitness component: locus CT812 in Caprolace was positively associated with BL2 and BL3, whereas locus 33D15 was negatively associated with BL2 and BL3 in Lesina. The remaining nine loci were associated with a single component, and none involved at the same time a growth and a parasite-related component. All significant single-locus HFCs were location-specific, meaning that they were limited to one location but not found in the others.

Discussion

We analysed the relationship between individual genetic variability and two different estimators of fitness in natural samples of European eel. Despite using a large data set of 22 EST-derived microsatellite loci and a large sample size of 346 individuals, no significant correlations were observed between heterozygosity and growth rate or parasite infestation by the swimbladder nematode *Anguillicola crassus*. The lack of association was evidenced by (i) nonsignificant global HFCs, (ii) an MGLM showing no effect of heterozygosity on fitness components, (iii) single-locus analysis showing a lower number of significant tests than the expected false-discovery rate, (iv) sign tests showing only a significant departure from expectations at one component (BL1 in Tiber), and, (v) the distribution of significant single-locus HFCs was random and not consistent across fitness components or sampling sites. The power of the test (the probability of correctly rejecting a false null hypothesis) was >0.9 (or $B < 0.1$) for all fitness comparisons. Conventionally, a test with a power greater than 0.8 (or $B < 0.2$) is considered to be statistically powerful (Mazen *et al.* 1987).

Null heterozygote advantage at growth rate

Highly heterozygous individuals did not exhibit an above-average growth rate in comparison with more homozygous individuals, suggesting that at least for the markers surveyed in this study faster growth was not coupled with genetic variability. Most HFCs in fish

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have been described in salmonids, including positive associations between heterozygosity and development stability (Leary *et al.* 1984), condition (Thelen & Allendorf 2001), fluctuating asymmetry (Borrell *et al.* 2004) and growth (Ferguson 1992). The strength and stability of correlations with growth in fish can vary across conspecific populations as it was shown in the case of Atlantic cod (*Gadus morhua*) where only one of the two populations studied showed a positive association between size-at-age and genetic diversity at 10 nuclear restriction fragment length polymorphism loci (Pogson & Fevolden 1998). In this sense, our sampling included three separate sites with particular environmental conditions so that the fastest growth was found at Lesina, whereas Tiber and Caprolace showed a similar growth, which might be related to Lesina presenting a higher primary production (Rossi & Villani 1980). A clear heterozygote advantage in growth was not found at any of the three locations, which suggests that the failure to detect a correlation was not location-related. Only one single component (BL1 in Tiber) showed a statistically significant difference in the proportion of single-locus HFCs, but showed a homozygote advantage instead. It should be noted that back-calculations in Caprolace were obtained from older eels (ages 6–8) than in Tiber (ages 3–5) or Lesina (ages 3–6), which might be a potential drawback for comparisons. Nevertheless, no differences were found in MLH/genetic variability across sampling sites despite the differences in age at capture.

HFCs can also produce different patterns at different life stages as observed in rainbow trout *Oncorrhynchus mykiss*, with a positive correlation between allozyme heterozygosity and growth up to 6 months but negative at 1 year and at maturity (Ferguson 1992). A drop of HFCs over time is expected under the prediction that energy is mostly directed to growth early in life but not during late life stages (David 1998) and because unfit genotypes are selectively eliminated in ageing cohorts (Koehn & Gaffney 1984). Nevertheless, our data show the same output across age classes, with no evidence of ontogenetic variation in gene activity. Several studies have argued that early and late growth may be controlled by a different set of genes. Using quantitative trait loci (QTL) data, Vaughn *et al.* (1999) showed that QTLs controlling early growth in mice mapped to separate chromosomal locations from those of late growth QTLs. McElroy & Diehl (2005) showed an inconsistent ontogenetic pattern among loci in HFCs in the earthworm *Eisinia andrei*, suggesting different roles of allozymes at different life stages of the species. The lack of ontogenetic variance found in the present study does not rule out that this might have resulted from the activity of different gene sets.

The null heterozygote advantage found at growth can also be explained by selection influence, that is, sampling a group of individuals after differential selective mortality has occurred and eliminated the most unfit individuals. An individual with an overall low level of genetic diversity may show a general disadvantage at many levels, including metabolism, as homozygotes might have a lower intrinsic fitness than heterozygotes related to a higher energy consumption and lower metabolic efficiency (Mitton 1993). Individuals with a slower growth rate may have a clear direct disadvantage in competitive struggles with fast-growing individuals, and the smallest ones might die either after failing to obtain food or after being predated by larger individuals. Behavioural studies have shown that yellow eels severely compete for food intake, and that larger eels tend to attack more readily, be dominant in threat encounters, consume more food and be strongly cannibalistic (Knights 1987). Differential selective mortality of smaller and more homozygous individuals could mechanically decrease the MLH–fitness correlation and explain the lack of an HFC pattern in our study. Nevertheless, no departures were observed from Hardy–Weinberg expectations at any of the 22 loci included in the MLH calculation, and particularly, no homozygote deficit was found at any locus, which could have been an indication of differential selection mortality affecting homozygotes/slow-growing eels.

The null heterozygote advantage at growth rate in natural samples of European eel using data from EST-linked loci (this study) contrasts with the positive association observed in farmed individuals using 12 allozyme loci (Pujolar *et al.* 2005). After 1 year in the aquaculture facilities, more heterozygous individuals showed a significantly faster growth and attained a larger length and weight than the more homozygous individuals (length increase: $r = 0.177$; $P = 0.005$; weight increase: $r = 0.164$; $P = 0.009$). The authors advocated for a direct overdominance of allozyme loci on the basis of a significant heterozygote advantage in growth at two enzymes involved in key metabolic functions (GPI: protein catabolism; MPI: glycolysis), whereas the smallest differences in growth between homozygotes and heterozygotes were observed at an enzyme with a secondary metabolic function (PGDH: pentose shunt); thus, it would play a smaller role in growth. The lack of correspondence between the positive HFCs at allozymes and the null heterozygote advantage at growth using EST-linked microsatellites might be related to the nature of the markers used, with the allozyme study including many loci involved in metabolic energy pathways (i.e. GPI, IDHP, MDH, MPI or PGM), whereas the EST-linked markers might be located in genes or in the proximity of genes uncoupled with metabolism/growth.

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In this sense, the study of Pujolar *et al.* (2005) found no correlation between growth and genetic variation at six neutral microsatellite loci using the same inidividuals, which was attributed to their location in nonfunctional regions of the genome and their uncoupling with coding genes. In the European eel, a genomic (genome-wide) HFC can be ruled out as this requires identity disequilibria which are mainly generated by partial inbreeding. Despite the population of European eel being in severe decline for the last 30 years and possibly at an historical low, Wirth & Bernatchez (2003) suggested a contemporary effective population size of about 5×10^3–10^4 eels; thus, it would remain large enough not to be affected by inbreeding.

Alternatively, the discrepancies between studies might be related to the allozyme study being conducted in farmed individuals reared in aquaculture facilities under controlled conditions, whereas the present study corresponds to natural samples. It has been suggested that HFCs are highly dependent on environmental conditions and that environmental stress enhances HFCs (David 1998; Lesbarreres *et al.* 2005), although the effects are generally poorly understood. If environmental conditions are too relaxed, a heterozygote advantage might not be detected if the differences among individuals are subtle, but might become more apparent under extreme duress. In the case of farmed eels, a positive correlation was observed in 12-month-old individuals raised in high-population densities that generate an extreme competition for food. After eels were graded (sorted by size) to lower competitive conditions, a drop in HFCs was observed and no HFCs were found in 22-month-old individuals, which was attributed to a relaxation of environmental stress (Pujolar *et al.* 2006). We hypothesize that wild populations might be in a similar situation to that of farmed eels after grading, resulting in only very subtle fitness differences among individuals of equal or similar size.

Parasite-associated genetic variability

Prevalence and severity of the nematode *A. crassus* did not correlate with genetic variation at EST-derived microsatellite loci in two wild eel populations showing a different intensity of parasite infestation. The general prediction that heterozygotes may be selected in systems where parasites impose a fitness cost on their host because they are more resistant to parasite infection than homozygotes was not corroborated in our study, in which unparasited individuals did not show a higher genetic diversity than parasited ones. The null heterozygote advantage at parasite load was independent of environmental effect as (i) Tiber (high infestation) and Lesina (mild infestation) showed comparable heterozygosities, suggesting no apparent effect of parasite intensity, and (ii) the noninfested Caprolace sample did not differ from the diversity levels observed in the rest of the samples, which suggests a null effect of the prevalence of parasites.

Similarly, Coté *et al.* (2005) reported no significant relationship between resistance to gastrointestinal nematodes and individual genetic variability at nine microsatellite loci in Svalbard reindeer *Rangifer tarandus*. In birds, prevalence of avian malaria was not correlated with genetic diversity at microsatellites in great reed warblers *Acrocephalus arundinaceus* (Westerdahl *et al.* 2005) or in a migratory kestrel (Ortego *et al.* 2007). In contrast, MacDougall-Shackleton *et al.* (2005) suggested that haematozoan parasite load was associated with genetic variability at eight microsatellite loci in a sample of 48 mountain white-crowned sparrows (*Zonotrichia leucophrys oriantha*), which was the first study to show a positive HFC with parasite load in an outbred population. Previous studies showing the existence of parasite-mediated selection favouring genetic variability had mostly been limited to island populations where positive associations between mean d^2 and fitness traits have been reported in species with inbreeding being the main cause of homozygosity (Coltman *et al.* 1999; Cassinello *et al.* 2001; Luikart *et al.* 2008).

The null heterozygosity advantage at parasite infestation is concordant with the study of Pujolar *et al.* (2006), where heterozygous individuals did not show a higher survival rate than more homozygous individuals at 12 allozyme and 8 microsatellite loci following a severe infection by the monogenean trematode *Pseudodactylogyrus*. Previous studies reporting a positive correlation between genetic variability and survival in fish include a superior heterozygote viability in *Fundulus heteroclitus* (Koehn 1975) and a greater survival to bacterial gill disease in rainbow trout *O. mykiss* (Drahushchack 1990). More recent studies have shown that the MHC in vertebrates is the main example for resistance genes with extensive genetic polymorphism attributed to balancing selection (Bernatchez & Landry 2003). Infection seems to be modulated by specific alleles in functional genes directly involved in immune defence rather than by genome-wide heterozygosity, as it has been shown in Atlantic salmon where resistance to the highly pathogenic bacterium *Aeromonas salmonicida* was associated with particular MHC class IIB alleles significantly more prevalent in high-resistance families (Langefors *et al.* 2001).

Prevalence (presence of parasites) and severity (mean number of parasites) of the infestation showed a different behaviour in many single-locus HFCs, although not statistically significant. This was mostly apparent at Tiber, with seven HFCs showing a different sign of the

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difference between heterozygotes and homozygotes. In this sense, MacDougall-Shackleton *et al.* (2005) found that different genetic measures predicted distinct aspects of parasitaemia (risk of infection vs. severity of infection) in mountain white-crowned sparrows, suggesting that prevalence might be linked to the likelihood of presenting a parasite-specific immune factor, whereas parasite load might be associated with general, highly polygenic measures of health.

We conclude that the EST-derived microsatellite markers used in our study might be putatively selected but uncoupled with genes with a direct effect on either growth rate or parasite infestation. More heterozygous individuals did not show a significant fitness advantage in comparison with more homozygous individuals under the two estimators of fitness investigated. At growth, heterozygotes did not exhibit a faster growth rate than homozygotes and attained a similar length at all ages. At parasite infestation, heterozygotes did not outperform homozygotes, as infected and uninfected individuals presented comparable levels of genetic diversity. A candidate-gene approach might be more adequate for further research on HFCs in the European eel and other species if any genetic variation in fitness traits is associated with specific alleles rather than genomic heterozygosity, including genes related to important metabolic functions in the case of growth or specific genes in the MHC for parasite resistance.

Acknowledgements

This work was funded by an Italian Research Program Grant to L. Zane (PRIN 2006, national coordinator G. De Leo) and by the University of Padova grant CPDA 085158/08 to L. Zane (Ateneo Project 2008). The authors thank the CNR Lesina, the Parco Nazionale del Circeo and the Tiber fishers for support in eel samplings. They also thank A. Pilastro for support on the Multivariate General Linear Model analysis.

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JMP works on population genetic structure, natural selection and the relationship between genetic diversity and fitness. DB is interested in the use of mathematics to analyse population dynamics of marine and freshwater species and to explore pro and cons of different alternatives for natural resource management. The research interests of FC cover the disciplines of fish ecology and biology and is currently working on his PhD, focused on eel (*Anguilla anguilla*) growth and ecology in the Mediterranean area. The research interest of EC focuses on fish ecology and biology, with particular reference to conservation and sustainable management of exploited fish resources in transitional habitats (estuaries, Mediterranean coastal lagoons). GADL's primary research is in the use of ecological theory, particularly life history-based models, in conservation of threatened species and management of renewable resources. LZ is primarily interested in evolutionary processes and research in his laboratory is currently focused on population genetics of marine organisms and conservation genetics of endangered species.

Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 Difference in back-calculated lengths-at-age 1 (BL1), age 2 (BL2) and age 3 (BL3; all in cm), parasite prevalence (PREV) and mean number of parasites (MNP) between homozygotes (HOMO) and heterozygotes (HET) at Tiber. Standard deviation (SD) in parentheses. *D* indicates the sign of the difference between heterozygotes and homozygotes; $^*P < 0.05$

Table S2 Difference in back-calculated lengths-at-age 1 (BL1), age 2 (BL2) and age 3 (BL3; all in cm), parasite prevalence (PREV) and mean number of parasites (MNP) between homozygotes (HOMO) and heterozygotes (HET) at Lesina. Standard deviation (SD) in parentheses. *D* indicates the sign of the difference between heterozygotes and homozygotes; $^*\ P < 0.05$

Table S3 Difference in back-calculated lengths-at-age 1 (BL1), age 2 (BL2) and age 3 (BL3; all in cm) between homozygotes (HOMO) and heterozygotes (HET) at Caprolace. Standard deviation (SD) in parentheses. *D* indicates the sign of the difference between heterozygotes and homozygotes; $^*P < 0.05$

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