

# Immunocompetence and heterozygosity in the mussel *Mytilus edulis*

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Multiple locus allozyme heterozygosity has been shown to be weakly, but significantly correlated with certain fitness parameters in bivalves. Immune function of individual *Mytilus edulis* from the Menai Strait, Wales, UK was assessed monthly over one year (1997–1998) by measuring total blood cell (haemocyte) counts, differential haemocyte counts (% basophils and eosinophils), phagocytic capability (by zymozan uptake) and intracellular superoxide generation. Sampled mussels were also scored at 9 allozyme loci. Mussels were then grouped into multiple locus heterozygosity (MLH) classes and plotted separately against the different measures of immunocompetence. No significant association was present between MLH and total haemocyte count, phagocytic capability or intracellular superoxide generation. However, there was a significant association between MLH and the character (basophilic or eosinophilic) of circulating haemocytes ( $r^2=0.057$ ,  $P=0.002$ ). Highly heterozygous individuals tended to have significantly more eosinophilic haemocytes circulating in the blood than highly homozygous individuals. Eosinophilic haemocytes are known to have a high capacity for phagocytosis of invading organisms and these results may therefore be interpreted as increased health (=fitness) in more highly heterozygous individuals.

## INTRODUCTION

The immune system of mussels relies on non-specific adaptive reactions including both an enhanced production of different humoral components and an increase in the number of circulating blood cells (haemocytes). Haemocytes, the first line of defence against micro-invaders, are involved in inflammation, wound repair, encapsulation, and elimination of pathogens (Renwrautz, 1990). Bivalve haemocytes can be granulocytes (with granules) or hyalinocytes (without granules) (Cheng, 1981) and in mussels two types of granulocytes can be identified, those with small granules which are basophilic and those with larger granules which are eosinophilic. Eosinophils are significantly more phagocytic than basophils (Pipe et al., 1997) therefore individuals with a higher proportion of eosinophils in their blood are more able to eliminate potential pathogens by phagocytosis.

Immune parameters in bivalves have been measured in a number of ways (e.g. haemocyte numbers and type, level of phagocytosis, locomotion, and respiratory burst activity) (Pipe et al., 1995) and results demonstrate that immune function is significantly affected by environmental factors such as season, temperature, salinity, disease, pollutants and physical disturbance (Coles et al., 1995; Pipe & Coles, 1995; Pipe et al., 1999; Fournier et al., 2002; Lacoste et al., 2002). Nevertheless, the greatest contributor to individual variance in immune response is likely to be genetic variation.

Weak, but often significant correlations between multiple locus heterozygosity at allozyme loci and fitness-related characters (heterozygosity/fitness correlations, HFC) have been detected in mussels and a number of

other organisms (Zouros & Pogson, 1994; David, 1998). The HFC can be summarized as meaning that individuals which are more heterozygous, on average, grow faster, have a lower standard respiration rate, or a greater efficiency for protein synthesis and a higher scope for growth (Koehn & Shumway, 1982; Koehn & Gaffney, 1984; Hawkins et al., 1989) than individuals which are more homozygous. Most estimates of HFC give values of  $r^2$  from 0.01–0.05 which border on significance. According to David (1998), for really convincing confirmation of correlation, ideally sample sizes in the thousands, rather than the hundreds that are usually used, are required. Although the use of different terminology by different authors has caused some confusion, HFC is thought to be a result of either (a) direct overdominance at the scored loci and/or (b) the association between homozygosity at the scored loci and homozygosity for deleterious genes at loci in linkage disequilibrium with the scored loci (David, 1998).

Irrespective of the causes of HFC, one critical component of an individual's fitness is the strength of its immune system and its ability to resist challenge from disease organisms. Here we consider the potential association between the multiple-locus heterozygosity of an individual *Mytilus edulis* L. and its immunocompetence. We have measured immunocompetence as density of haemocytes, their phagocytosis capability, the proportions of basophils and eosinophils, and quantification of intracellular attack by the release of superoxide anions (nitroblue tetrazolium test). *Mytilus edulis* were collected from a single site regularly during the year, assessed for immunocompetence and then scored at 9 allozyme loci allowing us to test for multiple-locus heterozygosity.

## MATERIALS AND METHODS

Specimens of *Mytilus edulis* L. of a size range 40–60 mm shell length were collected from below Bangor Pier, North Wales UK, each month, from November 1997 to October 1998 and were held in a laboratory tank with a constant flow of 10 µm-filtered seawater for at least one week before analysis.

### *Immunocompetence assays*

Two methods were used for extraction of mussel haemolymph depending on the assay to be carried out (modified from Pipe et al., 1995). Each mussel was prised partially open without cutting the adductor muscle and 500 µl of haemolymph was extracted from the posterior adductor muscle using a 2.5 ml syringe (21 gauge needle). Depending on the assay required, the syringe had been previously charged with different solutions. For the phagocytosis and nitroblue tetrazolium (NBT) assays, which were to be carried out within 2–3 hours, tris buffered saline (TBS), 0.05M, pH 7.6 plus 2% sodium chloride (NaCl) was used to maintain the blood cells alive. For later analysis of total and differential blood cell counts the syringe was charged with 500 µl of Baker's formol calcium with 2% NaCl added to give a final dilution of 1:1. These samples were stored in 1.5 ml microtubes at 4°C until analysed.

### *Total and differential blood cell counts*

Fixed samples were mixed by shaking and the total numbers of blood cells ml<sup>-1</sup> of haemolymph were estimated using a haemocytometer. Two sub-populations of blood cells, basophils and eosinophils, were then differentiated using Wright's staining method. Samples of fixed haemolymph (250 µl) were spun onto glass microscope slides using a cytocentrifuge at 1000 rpm for 5 min and post-fixed with methanol for 2 min. After the methanol was drained off, the haemocytes were stained with a 1:4 dilution of Wright's stain in phosphate buffered saline, pH 6.8, for 1.5 min. Excess stain was rinsed off with tap water, the slides were allowed to air-dry and the samples were mounted in Canada balsam. Percentages of pink staining eosinophilic blood cells and blue staining basophilic blood cells were estimated by counting 200 blood cells from each sample using an electronic differential cell counter.

### *Microtitre plate reader assays*

A microtitre plate assay was used to determine levels of phagocytosis of stained zymozan (Pipe et al., 1995). In

addition, release of superoxide dismutase inhibitable intracellular superoxide radicals was determined, without membrane stimulation (NBT test). Blood cell protein determinations were also carried out so that the results could be expressed per unit of haemocyte protein.

### *Allozymes*

Following haemolymph removal, small pieces of adductor muscle and digestive gland were dissected out, placed in replicate microtubes and held frozen at -70°C until electrophoresis. Fifteen mussels which were analysed for both total and differential blood cell counts were sampled for allozyme analysis. A different 12 mussels which were tested for phagocytosis, and a further 12 mussels which were assayed for NBT were also sampled giving a total of 39 mussels scored each month in the allozyme study.

Allozyme electrophoresis was carried out on standard horizontal 12% starch gels (Beaumont, 1991) using three different buffers according to the enzymes being stained. The enzymes diaphorase (*Dia*: EC No. 1.6.2.2), esterase D (*Es-D*: EC No. 3.1.1.1), glutathione reductase (*Gsr*: EC No. 1.6.4.2), and leucine aminopeptidase (*Lap*: EC No. 3.4.11.-) were run on a 0.1 M tris/EDTA/maleic acid buffer at pH 7.4. Octopine dehydrogenase (*Odh*: EC No. 1.5.1.11) and phosphoglucomutase (*Pgm*: EC No. 2.7.5.1) were stained on a 0.1 M tris/EDTA/maleic acid buffer at pH 6.0. A 0.15 M tris/citrate pH 8.0 buffer was used to run gels which were stained for glucose phosphate isomerase (*Gpi*: EC No. 5.3.1.9), mannose phosphate isomerase (*Mpi*: EC No. 5.3.1.8) and 6-phosphogluconic dehydrogenase (*Pgd*: EC No. 1.1.1.44). Alleles at a locus were designated by a superscript according to their mobility relative to the most common allele (=100) at that locus.

## RESULTS

The immunological and genetic results gathered monthly were pooled together for analysis (Tables 1 & 2). From Table 1, we can note that for all immunocompetence parameters, the range of variation was extensive (sometimes ten fold seen in the percentage of basophils or even 1000 fold seen in the NBT results). Allozyme variation (Table 2) was similar to that already found for this species (Beaumont, 1991). The number of alleles at the loci ranged from 3 to 8 and all 9 loci presented significant deficiencies of heterozygotes against the Hardy-Weinberg model after Bonferroni adjustment.

Firstly, in order to visualize any potential association, scatter diagrams (Figures 1–4) were drawn of immune

**Table 1.** Mean values over one year for immunoassay tests in *Mytilus edulis*: total blood cell counts, percentage of circulating basophils, phagocytosis and nitroblue tetrazolium (NBT) reduction (OD, optical densities).

	Total cell counts (cells. 10 <sup>-5</sup> ml <sup>-1</sup> )	% Basophils	Phagocytosis (zymozan. µg <sup>-1</sup> .ml <sup>-1</sup> )	NBT reduction (OD. µg <sup>-1</sup> .ml <sup>-1</sup> )
Mean	21.5	42.4	17775	3.27.10 <sup>-4</sup>
SE	2.8	2.4	1752	4.61.10 <sup>-5</sup>
Maximum	130.5	85.0	99300	2.52.10 <sup>-3</sup>
Minimum	1.5	8.0	1047	3.39.10 <sup>-6</sup>

**Table 2.** Allele frequency data for 9 allozyme loci in a population of *Mytilus edulis* tested monthly for immunocompetence through a year. Monthly data are pooled. *p*-HW, probability of agreement with the Hardy–Weinberg model (all values remain significant following Bonferroni adjustment).

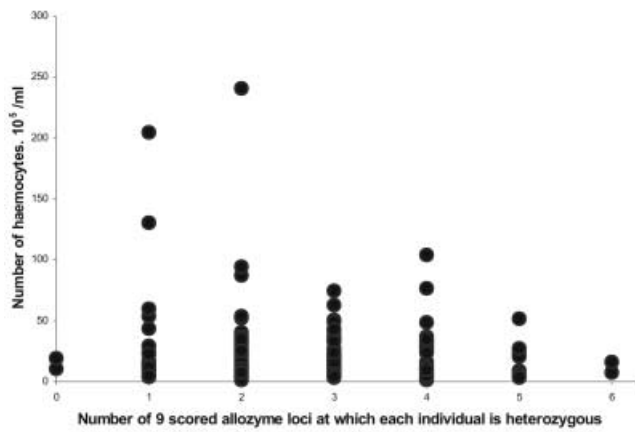
Locus	Relative mobility of alleles	Allele frequencies	Observed heterozygosity	Expected heterozygosity	p-HW
<i>Dia</i>	115	0.0732	0.1634	0.2595	<0.0001
	105	0.0012			
	100	0.8561			
	97	0.0427			
	90	0.0268			
<i>Es-D</i>	133	0.0122	0.1098	0.2026	<0.0001
	117	0.0146			
	100	0.8890			
	85	0.0829			
	75	0.0012			
<i>Gpi</i>	112	0.0537	0.5610	0.6371	<0.0001
	104	0.0512			
	100	0.5744			
	93	0.1146			
	90	0.0622			
	83	0.0841			
	77	0.0585			
	70	0.0012			
<i>Gsr</i>	110	0.0146	0.1707	0.1900	<0.0001
	100	0.8963			
	88	0.0085			
	75	0.0805			
<i>Lap</i>	117	0.0780	0.4902	0.5767	<0.0001
	109	0.1463			
	100	0.6195			
	94	0.0939			
	90	0.0622			
<i>Mpi</i>	106	0.0098	0.1634	0.1879	<0.0001
	100	0.8963			
	72	0.9390			
<i>Odh</i>	130	0.0110	0.1512	0.2282	<0.0001
	114	0.0122			
	100	0.8756			
	90	0.0524			
	82	0.0488			
<i>Pgd</i>	160	0.0098	0.1171	0.1941	<0.0001
	130	0.0366			
	100	0.8951			
	70	0.0585			
<i>Pgm</i>	115	0.1280	0.5732	0.6814	<0.0001
	110	0.1268			
	100	0.5195			
	96	0.0817			
	89	0.0744			
	85	0.0695			

response character against the number of scored allozyme loci at which individual *Mytilus edulis* were heterozygous from 0 (heterozygous at no loci) to 7 (heterozygous at 7 loci [none were heterozygous at 8 or 9 loci]).

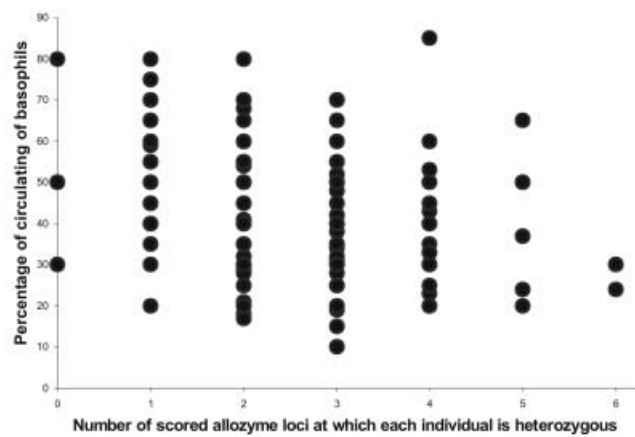
The coefficient of determination ( $r^2$ ) and associated statistical probability from the regression of heterozygosity on the specific immunocompetence assays were obtained (Table 3). Because both total and differential blood cell counts were carried out on the same individual mussels, the number of circulating basophils  $\text{ml}^{-1}$  of haemolymph could be estimated and also compared with heterozygosity.

There was no significant relationship between heterozygosity and total haemolymph cell count, zymosan phagocytosis or NBT reduction (Table 3). However a significant relationship was evident between heterozygosity and the percentage of basophils in the haemolymph ( $r^2=0.057$ ,  $P=0.002$ ). Figure 2 shows that the greater the number of loci at which an individual is heterozygous, the lower is the percentage of basophils (and the higher the percentage of eosinophils) in the haemolymph.

Because there were very few mussels amongst the extreme classes of heterozygosity, the data set was re-tested following pooling of the highly homozygous classes



**Figure 1.** Relationship between the total haemocyte counts and the number of allozyme loci at which individual *Mytilus edulis* are heterozygous.



**Figure 2.** Relationship between the percentage of circulating haemocytes that are basophilic and the number of loci at which individual *Mytilus edulis* are heterozygous.

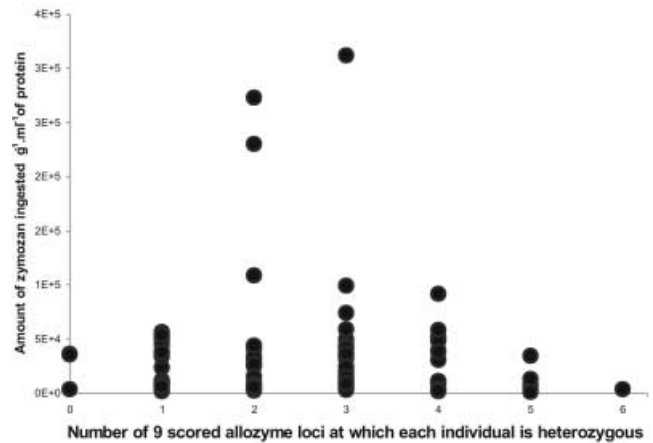
0 and 1 into one group, and pooling the highly heterozygous classes 5, 6, and 7 together (Table 3—pooled data set). This test confirms the significance of the relationship between multiple locus heterozygosity (MLH) and the percentage of basophils in the haemolymph.

Because there was clear seasonal pattern to the percentage of basophils in the haemolymph (Carissan-Lloyd,

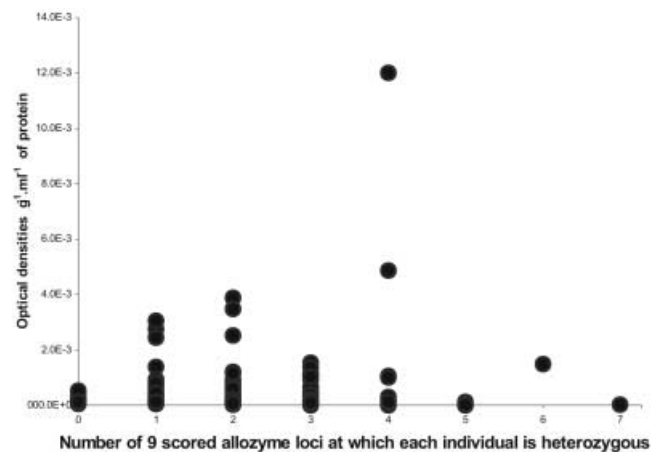
**Table 3.** Coefficient of determination ( $r^2$ ) and probability ( $P$ ) from the regression of heterozygosity at 9 allozyme loci on immunological assays in *Mytilus edulis*. Pooled data set, extreme classes of heterozygosity and homozygosity pooled—see text for details.

Immunoassay	Full data set		Pooled data set	
	$r^2$	$P$	$r^2$	$P$
Total haemolymph cell count	0.012	0.156	0.013	0.139
Percentage of basophils	0.057	0.002	0.062	0.001
Total basophil count	0.009	0.208	0.008	0.237
Zymosan phagocytosis	0.000	0.839	0.002	0.652
NBT reduction	0.007	0.380	0.011	0.265

NBT, nitroblue tetrazolium.



**Figure 3.** Relationship between phagocytosis by haemocytes and the number of allozyme loci at which individual *Mytilus edulis* are heterozygous.



**Figure 4.** Relationship between the intra-cellular release of reactive oxygen species (NBT-reduction) by the haemocytes and the number of allozyme loci at which individual *Mytilus edulis* are heterozygous.

2000)—a high percentage in summer (May to October) and a low percentage in winter (November to April)—we examined the relationship between percentage basophils and MLH in summer and winter. The correlation remains significant for the winter months ( $r^2=0.067$ ,  $P=0.022$ ) but is not significant for the summer months ( $r^2=0.011$ ,  $P=0.332$ ).

To investigate the effect at individual loci, mean percentage basophil values were calculated for heterozygotes and homozygotes at each locus. The mean percentage of basophils were significantly lower in heterozygotes (compared with homozygotes) at the *Dia*, *Gsr*, *Mpi* and *Pgm* loci, but significantly higher in heterozygotes at the *Es-D*, *Gpi*, *Odh* and *Pgd* loci. Therefore there is no trend across all loci for heterozygotes to exhibit lower percentages of basophils.

## DISCUSSION

It is well known that mussels exhibit inter-individual variation in immune function (Pipe et al., 1995; Pipe et al., 1999). Our data include not only this variation, but also seasonal variation in the data (Carissan-Lloyd, 2000)

and this explains the very extensive variation in the measurements of immune characters shown in Table 1. Additional variation can be caused by collection effects, but to reduce this we acclimatized mussels in the laboratory for a week prior to assay (Pipe et al., 1995).

Significant deficiencies of heterozygotes against Hardy–Weinberg expectations (Table 2) are common in other studies of bivalves (Zouros & Foltz, 1984). Potential causes include the presence of null alleles (Gaffney, 1994), selection against heterozygotes (Beaumont, 1991) or the Wahlund effect (sampling mixtures of populations with different allele frequencies, Zouros & Foltz, 1984). Unexpectedly, allele frequencies did vary between monthly samples (Carissan-Lloyd, 2000), so some of the heterozygote deficiency could be due to the Wahlund effect.

The results indicate that there is a weak, but significant, negative correlation between the percentage of basophils in the haemolymph and the number of loci at which the mussels were heterozygous. Highly heterozygous individuals, on average, had lower percentages of circulating basophilic haemocytes than highly homozygous individuals. This heterozygosity/fitness correlation (HFC) was significant in the winter months (November to April) when there was generally a low percentage of circulating basophils in the blood, but not in the summer when the percentage of basophils was generally higher. Mussels possess an open circulatory system allowing haemocytes to migrate through tissues. It is known that the proportion of basophils and eosinophils in bivalve blood can be influenced by environmental and physiological demands (Rudell & Rains, 1975; Cheng, 1988; Pipe et al., 1999). Such variations may be partly explained by (a) differential blood cell mortality or (b) simply migration of a certain type of blood cells into or out of tissues. When there are increased numbers of basophilic cells circulating in the blood, increased numbers of eosinophils occur in the tissues where they are believed to perform the function of wound repair and aid in digestion (Pipe et al., 1999). Our results, showing that eosinophils were in higher numbers in the blood of highly heterozygous mussels, could be a sign that the circulating blood in such mussels has a greater capacity for phagocytosis of invading organisms—an important fitness character. Similarly, the reduced numbers of eosinophils in the tissues could be interpreted as an indication of good tissue health.

Although many studies on natural populations have shown positive HFCs, others have not (David, 1998) and our failure to detect HFCs for immune characters other than percentage of basophils could be due to a number of causes. The sample sizes in this study are low, in the hundreds rather than the thousands, which can influence the likelihood of detecting HFCs (David, 1998). Also HFC is sometimes more likely to be detected if the organisms are under some kind of environmental stress (Gentili & Beaumont, 1988). However, mussels used in this study were from a natural mussel bed in an area with a high tidal throughput of unpolluted coastal water so were unlikely to have been nutritionally stressed or challenged by pollution.

The strength of HFC can vary over time within a population (Gaffney, 1990). We have identified summer/winter variation in the strength of the relationship with percentage basophils and HFCs with other immune

parameters may have been confounded by seasonal variation within our samples.

Finally, due to practical limitations (volume of blood in an individual mussel) we were unable to provide a single index of immunocompetence for each individual based on all parameters and this may partly account for differing results between immune parameters.

If the HFC is principally due to overdominance at allozyme loci which are directly involved, or linked to the fitness trait in question, then the choice of loci is important to the study. Initial explorations of staining systems for a number of enzymes (e.g. *N*-acetyl-hexosaminidase, arylsulphatase, catalase,  $\beta$ -glucuronidase, lysozyme, phenoloxidase and superoxidase dismutase) which are known to be directly or indirectly involved in the biochemical pathways critical to the mussel immune system, were unsuccessful. As it turned out, heterozygotes at the *Dia*, *Gsr*, *Mpi* and *Pgm* loci had a significantly lower average percentage of basophils than homozygotes at these loci. These are therefore the loci which have contributed most to the HFC. Mannose phosphate isomerase and phosphoglucosyltransferase are both enzymes associated with the metabolism of sugars, diaphorase catalyses the oxidation of NADH or NADPH and glutathione reductase reduces the tripeptide, glutathione. None of these enzymes seem closely related to immune function. It is possible that these enzyme loci are simply acting as markers for other linked loci which do have a role in immune function.

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