Differential immunity as a factor influencing mussel hybrid zone structure

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Running title: Role of immunity in hybrid zones

Keywords: hybridisation, immunocompetence, invertebrate immunity, metabolic rate, pathogen, species integrity, sympatry

Abstract

Interspecific hybridisation can alter fitness-related traits, including the response to pathogens, yet immunity is rarely investigated as a potential driver of hybrid zone dynamics, particularly in invertebrates. We investigated the immune response of mussels from a sympatric population at Croyde Bay, within the hybrid zone of *Mytilus edulis* and *M. galloprovincialis* in Southwest England. The site is characterised by size-dependent variation in genotype frequencies, with a higher frequency of *Mytilus galloprovincialis* alleles in large mussels, largely attributed to selective mortality in favour of the *M. galloprovincialis* genotype. To determine if differences in immune response may

contribute to this size-dependent variation in genotype frequencies, we assessed the two pure species and their hybrids in their phagocytic abilities when subject to immune challenge as a measure of immunocompetence and measured the metabolic cost of mounting an antigen-stimulated immune response. Mussels identified as *M. galloprovincialis* had a greater immunocompetence response at a lower metabolic cost compared to mussels identified as *M. edulis.* Mussels identified as hybrids had intermediate values for both parameters, providing no evidence for heterosis but suggesting that increased susceptibility compared to *M. galloprovincialis* may be attributed to the *M. edulis* genotype. The results indicate phenotypic differences in the face of pathogenic infection, which may be a contributing factor to the differential mortality in favour of *M. galloprovincialis*, and the size-dependent variation in genotype frequencies associated with this contact zone. We propose that immunity may contribute to European mussel hybrid zone dynamics.

Introduction

A hybrid zone is a location in which there is a genetic cline between two closely related but genetically distinct lineages and hybrid individuals of the two parental forms persist. These are commonly due to cases of secondary contact between recently diverged species, whereby previously allopatric lineages come into contact, allowing interbreeding. Hybrid zones can provide excellent opportunities for the study of various stages of speciation and to understand mechanisms by which gene flow is impeded (Barton and Hewitt 1985; Jiggins and Mallet 2000). Genetic incompatibilities between two divergent taxa can cause their hybrids to be unviable or at a fitness disadvantage, thus creating a barrier to gene flow. Incomplete reproductive isolation maintains hybrid zones, wherein species interbreed without compromising their genetic integrity (Barton and Hewitt 1985). Isolating mechanisms can be either prezygotic, in which hybrid zygotes are never formed, or postzygotic, in which hybrid offspring have a fitness disadvantage. Reduced fitness of hybrids (hybrid depression) is reported in various taxa including molluscs (Wiwegweaw et al. 2009), fish (Goldberg et al. 2005), amphibians (Parris 2004), birds (Prager and Wilson 1975) and plants (Alcázar et al. 2010).

Postzygotic barriers to gene flow may also arise from immunological traits. The immune system plays a role in many evolutionary processes (e.g. Hamilton 1980; Lawniczak et al. 2007). Resistance to pathogens is important for survival, and infection by parasites can drive differentiation among invertebrate populations (Sanford and Kelly 2011). Plant models demonstrate that incompatibilities and incomplete isolation can arise from immune gene differentiation (Bomblies and Weigel 2007), provoking studies of immunity as a mechanism of postzygotic isolation. For example, hybrids of

certain *Arabidopsis thaliana* accessions are incompatible dwarfs due to an overactive immune response which demands considerable metabolic activity at the cost of growth (Alcázar et al. 2010). Hybrid depression may also result from increased co-infection of pathogens associated with both parental species (Zabal-Aguirre et al. 2009). However, hybrid traits can also demonstrate increased fitness compared to their parental species, termed hybrid vigour or heterosis. Contrary to cases of hybrid depression (Goldberg et al. 2005; Zabal-Aguirre et al. 2009; Alcázar et al. 2010), the potential resistance against pathogens conferred from new allele combinations is proposed as a mechanism of hybrid vigour (Day and Day 1974; Maxwell and Jennings 1980), of which there are some known cases (e.g. Wendling and Wegner 2015). Despite the role of immunity in evolutionary processes, its role in forming the structure of hybrid zones has not been thoroughly investigated in animals besides vertebrates, namely mice (de Bellocq et al. 2012; Baird et al. 2012) and cyprinid fish (Brun et al. 1992; Krasnovyd et al. 2017). As vertebrates have adaptive immune systems, little work has drawn comparisons to invertebrate species with innate immune systems (e.g. Piertney and Oliver 2006).

The mussels *Mytilus edulis* and *Mytilus galloprovincialis* occur sympatrically on European Atlantic coasts, where they hybridise and introgress (Skibinski et al. 1983; Bierne et al. 2003). We use the secondary contact mosaic *Mytilus* hybrid zone on the British coast as a model, where *M. edulis* (Linnaeus)*, M. galloprovincialis* (Lamarck) and hybrid individuals locally coexist (Gardner and Skibinski 1988). Several pre- and postzygotic mechanisms have been demonstrated to contribute to their reproductive isolation including gamete incompatibility (Miranda et al. 2010), spawning asynchrony (Gardner and Skibinski 1990), assortative fertilization (Bierne et al. 2006), habitat specialization (Gosling and McGrath 1990), and hybrid fitness depression (Beaumont et al. 1993; Bierne et al. 2002). Previous research has provided evidence for differential susceptibility to parasitic infection of genotypes in the *Mytilus* hybrid zone (Coustau et al. 1991; Fuentes et al. 2002). Parasitism by the trematode *Prosorhynchus squamatus* occurring in individuals with a predominantly *M .edulis* genome, either 'pure' or introgressed (Coustau et al. 1991). There is also higher prevalence of the copepod *Mytilicola intestinalis* in hybrid than in *M. galloprovincialis* crosses (Fuentes et al. 2002). This evidence suggests that *Mytilus* interspecies gene flow may be associated with differences in immune capability and possibly hybrid depression.

The aim of this study was to investigate whether immunity may be a factor in the maintenance of species boundaries in invertebrate hybrid zones. To address our aim, we used the secondary contact mosaic *Mytilus* hybrid zone on the British coast as a model and examined the immunocompetence and metabolic cost of immune challenge in mussels identified as *M. edulis*, *M. galloprovincialis*, or hybrids. The selected site, Croyde, is characterised by size dependent variation in genotype frequencies, with a higher frequency of alleles characterising *M. gallopronvincialis* in larger mussels as a result of differential mortality between the two species (Gardner and Skibinski, 1988). Croyde is typical of and representative of the larger hybrid zone in Southwest England. The association between genotype and immunity was tested by subjecting the different genotypes to an immune challenge and assessing 1) the extent of their immune response based on the number of phagocytosing haemocytes and 2) the associated cost of mounting an immune response using metabolic rate upon infection as a proxy for the energetic demand of the immune challenge. Previous studies have shown hybrids of these species to be intermediate between the two parental genotypes across several traits (Gosling and McGrath 1990; Willis and Skibinski 1992; Gardner et al. 1993), thus we predicted that hybrids would be intermediate in their immune capabilities and metabolic demands when presented with an immune challenge. Given the evidence for selective

mortality in favour of the *M. galloprovincialis* phenotype with size observed in this hybrid zone (Gardner and Skibinski 1988; Skibinski and Roderick 1991), we also predicted that *M. galloprovincialis* would present a stronger immune response compared to *M. edulis*.

Materials and Methods

Study Organisms

Mussels were collected from a population containing *M. edulis, M. galloprovincialis* and their hybrids at the low shore of Croyde Bay in North Devon, UK (51.1346° N, 4.2342° W). The population at this site exhibits low rates of introgression (Skibinski et al. 1983; Gardner and Skibinski 1993). Roughly equal numbers of *M. edulis* (Linnaeus), *M. galloprovincialis* (Lamarck), and putative hybrids were selected based on initial morphological identification within a size range of 28-34 mm external shell length. Within this size range, genotype frequencies for marker allozyme loci are roughly equal between the parent species at the collection site (Gardner and Skibinski 1988). The sample of mussels identified as hybrids are expected to contain individuals of various types of mixed ancestry. Mussels were returned to the laboratory where they were randomly allocated to five 20 L aquaria containing aerated seawater at pre-exposure conditions (temperature: 15 °C, salinity: 36.1 ± 0.4, PO_2: 7.2 mL L^{-1}, light cycle: 12:12 h light:dark) for four weeks and fed Liquifry Marine (Interpet Ltd., Surrey, UK) daily by adding 5 mL directly to each aquarium. Subsequently, mussels were used in either immunocompetence assays (n=23) or respirometry (n=47). Upon completion of the assays, mussels were dissected out of their shells and a sample (<1 mg) of mantle tissue was taken, fast frozen in liquid nitrogen, and stored at -20°C to be used in genetic identification.

Prior to assessment of immunocompetency and metabolic rate, mussels were putatively identified as *M. edulis*, *M. galloprovincialis* or hybrid, based on morphological characteristics of the shell, with genetic identification performed after the assays. Accordingly, final sample sizes were reduced in some treatments.

Immunocompetence Assay

The immunocompetence of mussels was assessed to compare genotypes in their ability to mount an immune response upon exposure to simulated infection. Bacterial incubation methods were used in accordance with Roth et al. (2010). Briefly, mussels were removed from the aquarium and 5 µL of haemolymph was withdrawn from the anterior adductor muscle using a Hamilton syringe. Immediately after, a 5 µL solution of heat-killed *Bacillus thuringensis* bacteria (approximately 10^8 cells mL^{-1}) suspended in mussel physiological saline and labelled with FITC dye was injected in to the same area (Kurtz 2002; Wood et al. 2014). Mussels were placed in aquaria for a 2 h *in vivo* incubation period. Then for each mussel, 15 µL of haemolymph was withdrawn and mixed with 250 µL mussel physiological saline in a chamber of a LabTek multi-well chamber slide, which was placed on ice for 15 min and subsequently placed in a wet chamber for 30 min. Trypan Blue was added to the chamber for 15 min to quench free (non-phagocytosed) bacteria, after which all liquid was pipetted off and the slide washed with mussel physiological saline. DAPI mountant was added to fluorescently stain haemocytes. After 24 h, the total number of haemocytes and the number of those phagocytosing bacteria (fluorescing once engulfed) were counted using a Nikon eclipse 80i under an epifluorescent light in three fields of vision per well selected at random (one individual per well). Total haemocyte count was elicited by exciting the DAPI stained haemocytes which present

blue under UV light (458 nm), while phagocytosing haemocytes were identified by the encapsulated FITC labelled bacteria which show as green (488 nm). The number of phagocytosing haemocytes was divided by the total number counted to give a ratio as a measure of immunocompetence for each mussel. As this method relies on detection of fluorescently labelled bacteria within haemocytes to determine the occurrence of phagocytosis, saline-injected controls could not be examined.

Respirometry

Mussels (n=22 and n=24 for control and immune challenged respectively) were starved for two weeks before oxygen consumption rate was measured (Bayne 1973). Immune-challenged mussels were exposed to the same injection procedure as in the immunocompetence assay twice (48 h and 24 h prior to measurement), to elicit a sustained metabolic response. Control mussels were injected with an equal volume of physiological saline. Oxygen consumption rate was measured using closed, gas-tight, glass incubation chambers (150 mL), fitted with a Presens oxygen sensor spot (Precision Sensing GmbH, Regensburg, Germany) and supplied with filtered (22 µm), autoclaved, diluted sea water and a magnetic flea. Individual mussels were left to settle in their unsealed chambers for 30 min, after which the containers were sealed while submerged and placed onto a multi-channel magnetic stirrer to ensure mixing of water and to prevent stratification of oxygen within the respirometer. Once sealed, oxygen levels in the chambers were measured every 10 min using a calibrated optical oxygen sensor (Fibox4, PreSens, Regensburg, Germany) until O_2 saturation reached 80 % of the initial measurement (~2 h on average). Mussels were continually observed, and any individual seen to have closed its valves during the measurement period was excluded from analysis, as these could be relying on anaerobic metabolism. A blank, containing no animal, was run simultaneously to control for microbial respiration. The experiment was terminated by removing individuals from the chamber, dissecting them out of their shells, gently blotting them dry, and weighing them. Mantle tissue samples for genetic identification were taken after weighing. The difference between oxygen tension levels in water in the chamber at the beginning and at the end of the experiment was used to calculate rate of O_2 uptake, expressed as µg O_2 g wet mass^{-1} h^{-1} salinity-temperature-pressure, and used as a proxy for resting metabolic rate.

Genotype Identification

All mussels were genotyped using the species diagnostic marker *Glu-5*, amplified using the primers *Me 15* (5'-CCAGTATACAAACCTGTGAAGA-3') and *Me16* (5'-TGTTGTCTTA ATAGGTTTGTAAGA-3') (Inoue et al. 1995). Alleles at this locus are represented by fragments of different lengths for *M. edulis* (180 bp) and *M. galloprovincialis* (126 bp). DNA was extracted from <1 mg of foot tissue using the HotSHOT protocol. Briefly, tissue was digested in 100 µL alkaline lysis reagent (25 mM NAOH and 0.2 mM disodium EDTA) at 95 °C for 30 min and cooled on ice for 5 min, after which 100 µL neutralising agent (40 mM Tris-HCl added) was added. PCR reactions were carried out in a 12.5 µL volume containing 30-50 ng DNA, 6.25 µL 2x MyTaq Mix (Bioline) and 0.25 µL each primer, under the following cycling conditions: 94°C for 5 min, 30 cycles of 94°C for 30 s, 56°C for 30 s, 70°C for 1 min 30 s, and 72°C for 5 min.

The *Glu-5* marker has been used extensive for the identification of species within the Mytilus complex (REFS needed). While it is possible for backcrosses to appear homozygous at this locus, the population used in the present study has been found to have limited introgression (Gardner et al.

1993), giving us reasonable confidence in the marker's ability to detect pure and hybrid individuals, or individuals with highly constrasting ancestry.

Data Analyses

Statistical analyses were conducted using RStudio v.3.3.2 and SPSS. Assumptions of normality and homogeneity of variance were met following Shapiro-Wilk and Levene's tests respectively unless stated otherwise. Tukey's HSD post hoc test was used to detect significant differences between individual groups.

Results

Immunocompetence Assay

Immunocompetency assays were performed in *M. galloprovincialis* (G/G, n=7), *M. edulis* (E/E, n=7), and their hybrids (E/G, n=9). Sample sizes were in line with those used in Wood et al.(2014). Immunocompetency, assessed as the ratio of phagocytosing to non-phagocytosing haemocytes, differed between the genotypes (Fig. 1a) with *M. galloprovincialis* (G/G) higher than *M. edulis* (E/E) and hybrids (E/G) intermediate. The distributions of the EE did not overlap, however EG overlapped with both EE and GG. The variation between genotypes was significant (ANOVA, $F(2,20) = 13.091$, $P < 0.001$). According to the Tukey HSD post hoc test, phagocytosis of G/G is significantly greater than E/E ($P = 0.000$) and E/G ($P = 0.011$).Consistent with Fig. 1a, the mean values for the three genotypes fell close to a straight line (Fig. 1b) with G/G having the highest ratio value. E/G was not significantly different from the midpoint between EE and G/G (ANOVA, $F(1,20) = 0.435$, $P = 0.517$). Thus, there was no statistical evidence for heterosis or hybrid depression, when this is defined as a deviation from the midpoint value rather than the more extreme situation where E/G might lie outside the range separating E/E and G/G.

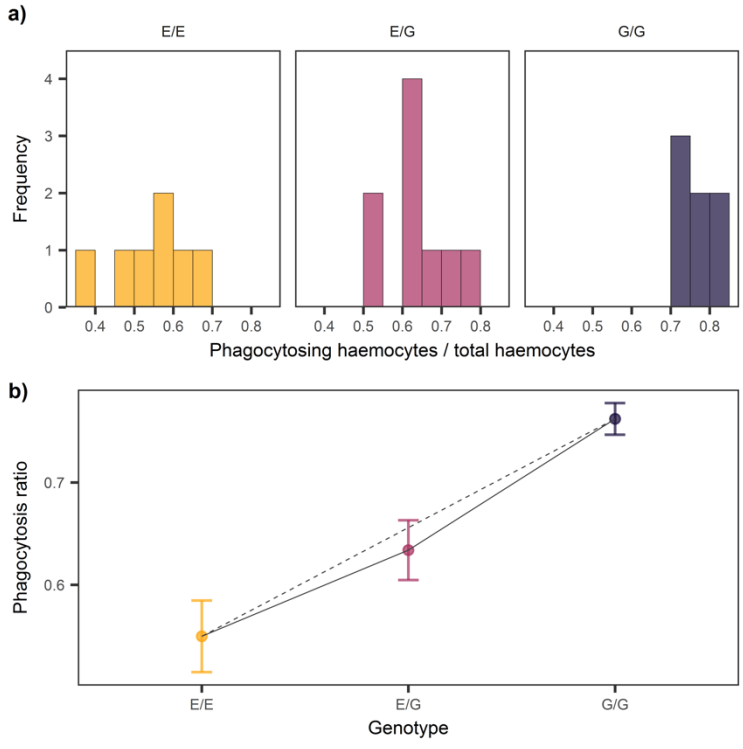

Fig.1 Immunocompentency in mussels presented as a) histograms of the distributions of the ratio of phagocytising dividing by total haemocytes and b) mean (±SE) number of phagocytosing haemocytes divided by total number of haemocytes (phagocytosis ratio) in the haemolymph of immune-challenged mussels identified as *M. edulis* (E/E, n=7, yellow), hybrid (E/G, n=9, pink) or *M. galloprovincialis* (G/G, n=7, purple).

Respirometry

Respirometry assays were performed in *M. galloprovincialis* (G/G, n=10 and 12 for control and immune challenged respectively) *M. edulis* (E/E, n= 5 and 4 for control and immune challenged respectively), and their hybrids (E/G, n=6 and 9 for control and immune challenged respectively). Histograms of the distributions of O_2 uptake are shown for the six different treatment and genotype combinations in Fig. 2 panelled in two different ways. The difference between control and immune challenged groups was greatest for E/E, less marked for E/G and showing no difference for G/G (Fig. 2a). The difference between genotypes was marked for the immune challenged mussels but showing no difference for the controls (Fig. 2b). In line with the histograms, one-way ANOVA showed no significant differences between genotypes for the control (ANOVA, $F(2,18) = 0.210$, $P = 0.794$). There was, however, a significant result for the immune challenged group ($F(2,22) = 4.821$, $P = 0.020$). According to the Tukey HSD post hoc test, G/G was significantly different from E/E ($P = 0.040$) and at the borderline of significance in the comparison with E/G ($P = 0.060$). The presence of significant differences for the immune challenged but not the control group is also consistent with a significant treatment-genotype interaction (ANOVA, $F(2,40) = 3.949$, $P = 0.027$). For the immune challenged group, the genotype means fall close to a straight line (Fig. 3), with a decline in O_2 uptake as the number of G alleles increases. E/G was not significantly different from the midpoint between E/E and G/G ($P = 0.113$). Thus, there is no statistical evidence for heterosis or hybrid depression. For the control group, the genotype means, though not significantly different, trend in the opposite direction and the difference in slope will contribute to the significant interaction in the two-way ANOVA.

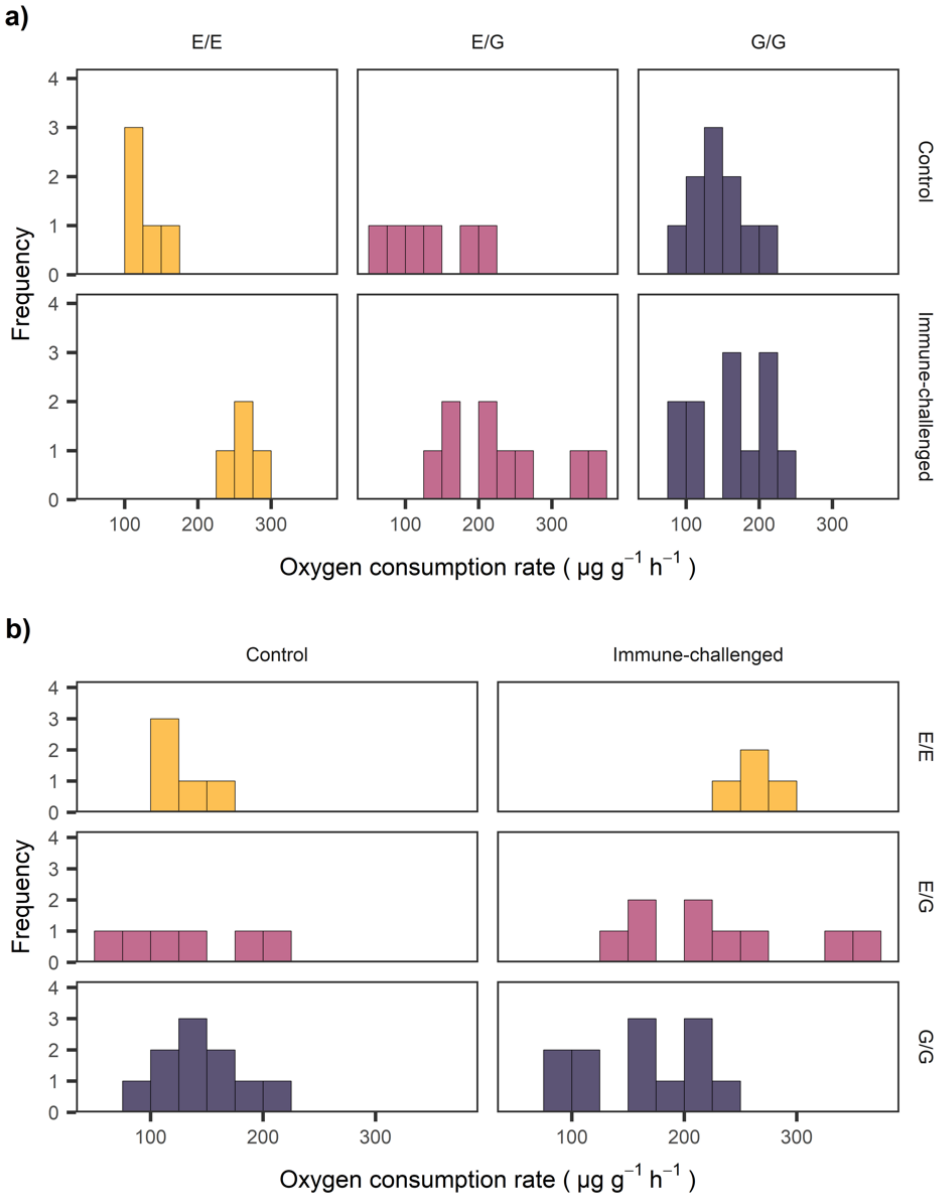

Fig.2 Mass specific rates of oxygen uptake in mussels presented as a-b) histograms of the distributions of oxygen uptake (µg g^{-1} h^{-1}) for the six different treatment and genotype combinations panelled in two different ways (axes inverted) to facilitate visualisation. Mussels have been identified as *M. edulis* (E/E: control n=5, immune-challenged n=4, yellow), hybrid (E/G: control n=6, immune-challenged n=9, pink), or *M. galloprovincialis* (G/G: control n=10, immune-challenged n=12, purple)

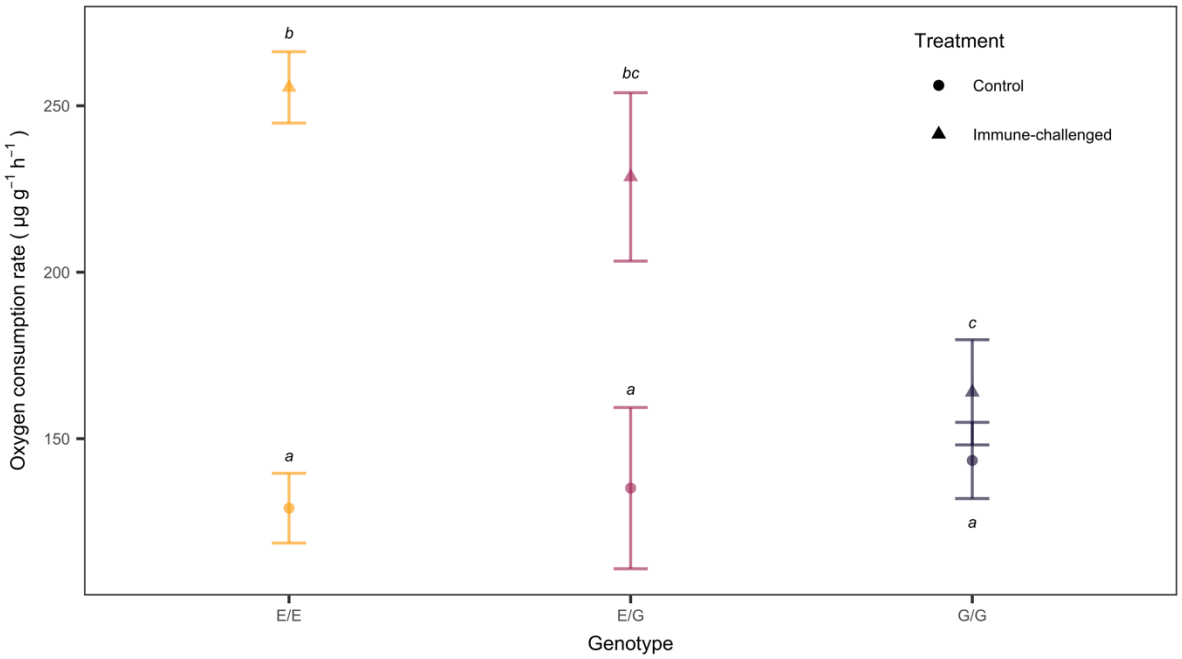

Fig.3 Mean (±SE) mass specific rates of O_2 uptake (µg g^{-1} h^{-1}) of *M. edulis* (E/E: control n=5, immune-challenged n=4, yellow), hybrids (E/G: control n=6, immune-challenged n=9, pink) and *M. galloprovincialis* (G/G: control n=10, immune-challenged n=12, purple), presented by treatment: control mussels (circles) and those immune-challenged by bacterial injection (triangles). Letters by error bars represent significant differences between genotypes, within each treatment group.

Discussion

The mussel hybrid zone in Southwest England is characterised by a size-dependent genotypic variation, suggesting differences in viability among *M. edulis*, *M. galloprovincialis* and hybrids in sympatric populations. Here, we aimed to determine whether differential immunity may be a factor influencing such differences in viability. As predicted, *M. galloprovincialis* was able to mount a stronger immunocompetence response at a lower metabolic cost compared to *M. edulis* when subjected to a novel immune challenge, with hybrids presenting intermediate values for both parameters. The decreased ability to mount an immune response to pathogens in hybrids compared to *M. galloprovincialis* could be attributed to introgression of the less resistant *M. edulis* genome. The observed differential immunity may account for some of the differential mortality observed in favour of *M. galloprovincialis* at Croyde (Gardner and Skibinski 1988) and could be a contributing factor in European *Mytilus* hybrid zone dynamics.

The stronger immune response to bacterial infection measured in mussels identified as *M. galloprovincialis* compared to *M. edulis* add to a large body of studies that found differentiation in fitness-related traits apparently in favour of *M. galloprovincialis* genotypes over *M. edulis* ones (Skibinski et al. 1983; Coustau et al. 1991; Gardner 1994; Hilbish et al. 1994; Bierne et al. 2006), and provides new evidence of this for a previously underappreciated trait. As we were not able to measure phagocytic ability under control conditions, it is not possible to determine whether *M. galloprovincialis* has as constitutively higher phagocytosis rate, or whether it is able to mount a response faster than *M. edulis*. Nonetheless, our results concur with others recording a strong immune response in *M. galloprovincialis* from transcriptomic (Moreira et al. 2018) and parasite load

(Coustau et al. 1991) approaches. The intermediate immune response observed in hybrids when compared to the parental genotype suggests no heterosis or hybrid depression for immunity. This agrees with previous studies, describing hybrids as intermediate for several traits, such as length-at-age values (Gardner et al. 1993), habitat specialisation (Gosling and McGrath 1990), and attachment strength (Willis and Skibinski 1992). In contrast, hybrid depression has been observed in larval viability (Beaumont et al. 1993; Bierne et al. 2002).

Our results complement those of Coustau et al. (1991), who discovered a pattern of susceptibility to the trematode *P. squamatus*, which causes total castration and mortality, in which the *M. galloprovincialis* genotype was least parasitised in the hybrid zone. The authors could not determine whether this could be ascribed to immune mechanisms of the host or specific mesologic requirements of the parasite. *Bacillus thuringiensis*, used in the present study, is not a pathogen that is encountered by mussels in nature. It is however useful for many invertebrate immunological studies, inducing a phagocytic response independent of exposure history. Specific host-pathogen interactions may present different patterns, such as the apparent hybrid susceptibility to disseminated haemic neoplasia (Fuentes et al. 2002).The enhanced immune capabilities associated with the *M. galloprovincialis* genotype support the hypothesis that intense selection may favour the spread of *M. galloprovincialis* genes. Fuentes et al. (2002) found greater mortality in hatchery-produced hybrid crosses, compared to *M. galloprovincialis* crosses, when reared in aquaculture conditions. Increased mortality in hybrids was associated with higher parasitisation by the protist *Marteilia refringens* in hybrid crosses when compared to *M. galloprovincialis* crosses. They provide further evidence for strong pathogen resistance in *M. galloprovincialis,* observed at a range of sites around Europe under natural and aquaculture conditions. In conjunction with our results, this suggests immunity may be a contributing factor outside of Croyde Bay, in *Mytilus* hybrid zone dynamics throughout Europe.

Most knowledge about immunity in hybrids is regarding plant-pathogen or plant-herbivore interactions. In a review of hybrid resistance to pathogens and herbivores, Fritz et al (1994) hypothesised that resistance to pathogens may be a more common feature in animal hybrids than in plant hybrids. More recent data (Derothe et al. 2001; Parris 2004; Wolinska et al. 2004), including those presented here, so far suggests that animal hybrids are not different to plants in their patterns of susceptibility. The present study contributes to the currently limited invertebrate hybrid literature, finding intermediate hybrid immunocompetency as in other invertebrates such as mosquitoes (Mancini et al. 2015) and vertebrates (which possess adaptive immune systems) such as birds (Wiley et al. 2009)

Size-dependent variation in genotype frequency at the low shore of Croyde Bay (Gardner and Skibinski 1988) informed the size range of mussels collected for this study. Below the chosen size range of 28-34 mm, Gardner and Skibinski (1988) found the *M. edulis* genotype to be most prevalent, accounting for almost 80% of individuals sampled. Above this size range, they observed a drastic switch to *M. galloprovincialis* as the most prevalent genotype, accounting for up to 60% abundance. Hybrid genotype frequency remained stable with size, increasing in frequency only within the size range selected in this study. Though this pattern has been partly explained by attachment strength (Willis and Skibinski 1992), the distribution of genotypes predicted by this is dependent on wave action and does not adequately match the observed distributions in Southwestern England (Hilbish et al. 2002). Differential immunity between the genotypes across distinct sizes could therefore be a causative factor. The greater immunocompetence of *M. galloprovincialis* compared to *M. edulis* observed in the size range used in this study may represent a

threshold at which a combination of differential immunity, attachment strength, and possibly other factors, cause the preferential survival and geographic extension of *M. galloprovincialis* with increasing shell length.

Our results suggest that there is a fitness advantage conferred by the more powerful immunocompetence of the *M. galloprovincialis* genome implied by our phenotypic results in addition to those of genetic (Boon et al. 2009), transcriptomic (Moreira et al. 2018), and parasite load (Coustau et al. 1991) studies. Hybrids are intermediate suggesting additivity in immunocompetence. We can thus infer directional selection in favour of *M. galloprovincialis*-like immune genotypes, in consensus with the complete genome (Edwards and Skibinski 1987; Wilhelm and Hilbish 1998), which is balanced by immigration of *M. edulis* (Gilg and Hilbish 2003). What maintains these pure populations of *M. edulis* remains unclear, and future work should explore this important factor. It might also be fruitful to investigate the effects of immunity alongside environmental gradients, as *M. galloprovincialis* is limited by other environmental factors. Further studies might also examine whether the proportion of parent genotype directly correlates with immune capability as in an additive model of hybrid pathogen resistance.

Acknowledgements

The authors thank Dr Jo Triner for her technical assistance and Dr Luke Holmes for providing graphical outputs. Work was funded by the School of Biological and Marine Sciences, University of Plymouth.

Compliance with Ethical Standards

All applicable international, national and/or institutional guidelines for sampling, care and experimental use of organisms for the study have been followed. The authors declare no conflict of interest. The datasets during and/or analysed during the current study are available from the corresponding author on reasonable request.

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498

499