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2019-12

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Tolman, D

http://hdl.handle.net/10026.1/15223

10.1007/s00227-019-3604-3
Marine Biology
Springer Science and Business Media LLC

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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

29 **Abstract**

30 Interspecific hybridisation can alter fitness-related traits, including the response to
31 pathogens, yet immunity is rarely investigated as a potential driver of hybrid zone
32 dynamics, particularly in invertebrates. We investigated the immune response of
33 mussels from a sympatric population at Croyde Bay, within the hybrid zone of *Mytilus*
34 *edulis* and *M. galloprovincialis* in Southwest England. The site is characterised by
35 size-dependent variation in genotype frequencies, with a higher frequency of *Mytilus*
36 *galloprovincialis* alleles in large mussels, largely attributed to selective mortality in
37 favour of the *M. galloprovincialis* genotype. To determine if differences in immune
38 response may contribute to this size-dependent variation in genotype frequencies,
39 we assessed the two pure species and their hybrids in their phagocytic abilities when
40 subject to immune challenge as a measure of immunocompetence and measured
41 the metabolic cost of mounting an antigen-stimulated immune response. Mussels
42 identified as *M. galloprovincialis* had a greater immunocompetence response at a
43 lower metabolic cost compared to mussels identified as *M. edulis.* Mussels identified
44 as hybrids had intermediate values for both parameters, providing no evidence for
45 heterosis but suggesting that increased susceptibility compared to *M.*
46 *galloprovincialis* may be attributed to the *M. edulis* genotype. The results indicate
47 phenotypic differences in the face of pathogenic infection, which may be a
48 contributing factor to the differential mortality in favour of *M. galloprovincialis*, and the
49 size-dependent variation in genotype frequencies associated with this contact zone.
50 We propose that immunity may contribute to European mussel hybrid zone
51 dynamics.

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60 ## Introduction

61 A hybrid zone is a location in which there is a genetic cline between two closely
62 related but genetically distinct lineages and hybrid individuals of the two parental
63 forms persist. These are commonly due to cases of secondary contact between
64 recently diverged species, whereby previously allopatric lineages come into contact,
65 allowing interbreeding. Hybrid zones can provide excellent opportunities for the study
66 of various stages of speciation and to understand mechanisms by which gene flow is
67 impeded (Barton and Hewitt 1985; Jiggins and Mallet 2000). Genetic
68 incompatibilities between two divergent taxa can cause their hybrids to be unviable
69 or at a fitness disadvantage, thus creating a barrier to gene flow. Incomplete
70 reproductive isolation maintains hybrid zones, wherein species interbreed without
71 compromising their genetic integrity (Barton and Hewitt 1985). Isolating mechanisms
72 can be either prezygotic, in which hybrid zygotes are never formed, or postzygotic, in
73 which hybrid offspring have a fitness disadvantage. Reduced fitness of hybrids
74 (hybrid depression) is reported in various taxa including molluscs (Wiwegweaw et al.
75 2009), fish (Goldberg et al. 2005), amphibians (Parris 2004), birds (Prager and
76 Wilson 1975) and plants (Alcázar et al. 2010).

77

78 Postzygotic barriers to gene flow may also arise from immunological traits. The
79 immune system plays a role in many evolutionary processes (e.g. Hamilton 1980;
80 Lawniczak et al. 2007). Resistance to pathogens is important for survival, and
81 infection by parasites can drive differentiation among invertebrate populations
82 (Sanford and Kelly 2011). Plant models demonstrate that incompatibilities and
83 incomplete isolation can arise from immune gene differentiation (Bomblies and
84 Weigel 2007), provoking studies of immunity as a mechanism of postzygotic
85 isolation. For example, hybrids of certain *Arabidopsis thaliana* accessions are
86 incompatible dwarfs due to an overactive immune response which demands
87 considerable metabolic activity at the cost of growth (Alcázar et al. 2010). Hybrid
88 depression may also result from increased co-infection of pathogens associated with
89 both parental species (Zabal-Aguirre et al. 2009). However, hybrid traits can also
90 demonstrate increased fitness compared to their parental species, termed hybrid

91 vigour or heterosis. Contrary to cases of hybrid depression (Goldberg et al. 2005;
92 Zabal-Aguirre et al. 2009; Alcázar et al. 2010), the potential resistance against
93 pathogens conferred from new allele combinations is proposed as a mechanism of
94 hybrid vigour (Day and Day 1974; Maxwell and Jennings 1980). Despite the role of
95 immunity in evolutionary processes, its role in forming the structure of hybrid zones
96 has not been investigated extensively in animals besides vertebrates (but see e.g.
97 (Wendling and Wegner 2015), namely mice (de Bellocq et al. 2012; Baird et al.
98 2012) and cyprinid fish (Brun et al. 1992; Krasnovyd et al. 2017). As vertebrates
99 have adaptive immune systems, little work has drawn comparisons to invertebrate
100 species with innate immune systems (e.g. Piertney and Oliver 2006).

101

102 The mussels *Mytilus edulis* and *Mytilus galloprovincialis* occur sympatrically on
103 European Atlantic coasts, where they hybridise and introgress (Skibinski et al. 1983;
104 Bierne et al. 2003). We use the secondary contact mosaic *Mytilus* hybrid zone on the
105 British coast as a model, where *M. edulis* (Linnaeus)*, M. galloprovincialis* (Lamarck)
106 and hybrid individuals locally coexist (Gardner and Skibinski 1988). Several pre- and
107 postzygotic mechanisms have been demonstrated to contribute to their reproductive
108 isolation including gamete incompatibility (Miranda et al. 2010), spawning
109 asynchrony (Gardner and Skibinski 1990), assortative fertilization (Bierne et al.
110 2006), habitat specialization (Gosling and McGrath 1990), and hybrid fitness
111 depression (Beaumont et al. 1993; Bierne et al. 2002). Previous research has
112 provided evidence for differential susceptibility to parasitic infection of genotypes in
113 the *Mytilus* hybrid zone (Coustau et al. 1991; Fuentes et al. 2002). Parasitism by the
114 trematode *Prosorhynchus squamatus* occurring in individuals with a predominantly *M*
115 *.edulis* genome, either 'pure' or introgressed (Coustau et al. 1991). There is also
116 higher prevalence of the copepod *Mytilicola intestinalis* in hybrid than in *M.*
117 *galloprovincialis* crosses (Fuentes et al. 2002). This evidence suggests that *Mytilus*
118 interspecies gene flow may be associated with differences in immune capability and
119 possibly hybrid depression.

120

121 The aim of this study was to investigate whether immunity may be a factor in the
122 maintenance of species boundaries in invertebrate hybrid zones. To address our

123 aim, we used the secondary contact mosaic *Mytilus* hybrid zone on the British coast
124 as a model and examined the immunocompetence and metabolic cost of immune
125 challenge in mussels identified as *M. edulis*, *M. galloprovincialis*, or hybrids. The
126 selected site, Croyde, is characterised by size dependent variation in genotype
127 frequencies, with a higher frequency of alleles characterising *M. gallopronvincialis* in
128 larger mussels as a result of differential mortality between the two species (Gardner
129 and Skibinski, 1988). Croyde is typical of and representative of the larger hybrid
130 zone in Southwest England. The association between genotype and immunity was
131 tested by subjecting the different genotypes to an immune challenge and assessing
132 1) the extent of their immune response based on the number of phagocytosing
133 haemocytes and 2) the associated cost of mounting an immune response using
134 metabolic rate upon infection as a proxy for the energetic demand of the immune
135 challenge. Previous studies have shown hybrids of these species to be intermediate
136 between the two parental genotypes across several traits (Gosling and McGrath
137 1990; Willis and Skibinski 1992; Gardner et al. 1993), thus we predicted that hybrids
138 would be intermediate in their immune capabilities and metabolic demands when
139 presented with an immune challenge. Given the evidence for selective mortality in
140 favour of the *M. galloprovincialis* phenotype with size observed in this hybrid zone
141 (Gardner and Skibinski 1988; Skibinski and Roderick 1991), we also predicted that
142 *M. galloprovincialis* would present a stronger immune response compared to *M.*
143 *edulis*.

144

145 **<u>Materials and Methods</u>**

146 <u>Study Organisms</u>

147 Mussels were collected from a population containing *M. edulis, M. galloprovincialis*
148 and their hybrids at the low shore of Croyde Bay in North Devon, UK (51.1346° N,
149 4.2342° W). The population at this site exhibits low rates of introgression (Skibinski
150 et al. 1983; Gardner and Skibinski 1993). Roughly equal numbers of *M. edulis*
151 (Linnaeus), *M. galloprovincialis* (Lamarck), and putative hybrids were selected based
152 on initial morphological identification within a size range of 28-34 mm external shell
153 length. Within this size range, genotype frequencies for marker allozyme loci are
154 roughly equal between the parent species at the collection site (Gardner and

155 Skibinski 1988). The sample of mussels identified as hybrids are expected to contain
156 individuals of various types of mixed ancestry. Mussels were returned to the
157 laboratory where they were randomly allocated to five 20 L aquaria containing
158 aerated seawater at pre-exposure conditions (temperature: 15 °C, salinity: 36.1 ±
159 0.4, PO_2: 7.2 mL L^{-1}, light cycle: 12:12 h light:dark) for four weeks and fed Liquifry
160 Marine (Interpet Ltd., Surrey, UK) daily by adding 5 mL directly to each aquarium.
161 Subsequently, mussels were used in either immunocompetence assays (n=23) or
162 respirometry (n=47). Upon completion of the assays, mussels were dissected out of
163 their shells and a sample (<1 mg) of mantle tissue was taken, fast frozen in liquid
164 nitrogen, and stored at -20°C to be used in genetic identification.

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

169

170 <u>Immunocompetence Assay</u>

171 The immunocompetence of mussels was assessed to compare genotypes in their
172 ability to mount an immune response upon exposure to simulated infection. Bacterial
173 incubation methods were used in accordance with Roth et al. (2010). Briefly,
174 mussels were removed from the aquarium and 5 µL of haemolymph was withdrawn
175 from the anterior adductor muscle using a Hamilton syringe. Immediately after, a 5
176 µL solution of heat-killed *Bacillus thuringensis* bacteria (approximately 10^8 cells mL^{-1})
177 suspended in mussel physiological saline and labelled with FITC dye was injected in
178 to the same area (Kurtz 2002; Wood et al. 2014). Mussels were placed in aquaria for
179 a 2 h *in vivo* incubation period. Then for each mussel, 15 µL of haemolymph was
180 withdrawn and mixed with 250 µL mussel physiological saline in a chamber of a
181 LabTek multi-well chamber slide, which was placed on ice for 15 min and
182 subsequently placed in a wet chamber for 30 min. Trypan Blue was added to the
183 chamber for 15 min to quench free (non-phagocytosed) bacteria, after which all liquid
184 was pipetted off and the slide washed with mussel physiological saline. DAPI
185 mountant was added to fluorescently stain haemocytes. After 24 h, the total number
186 of haemocytes and the number of those phagocytosing bacteria (fluorescing once

187 engulfed) were counted using a Nikon eclipse 80i under an epifluorescent light in
188 three fields of vision per well selected at random (one individual per well). Total
189 haemocyte count was elicited by exciting the DAPI stained haemocytes which
190 present blue under UV light (458 nm), while phagocytosing haemocytes were
191 identified by the encapsulated FITC labelled bacteria which show as green (488 nm).
192 The number of phagocytosing haemocytes was divided by the total number counted
193 to give a ratio as a measure of immunocompetence for each mussel. As this method
194 relies on detection of fluorescently labelled bacteria within haemocytes to determine
195 the occurrence of phagocytosis, saline-injected controls could not be examined.

196

197 Respirometry

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

219 after weighing. The difference between oxygen tension levels in water in the
220 chamber at the beginning and at the end of the experiment was used to calculate
221 rate of O_2 uptake, expressed as µg O_2 g wet mass^{-1} h^{-1} salinity-temperature-
222 pressure, and used as a proxy for resting metabolic rate.

223

224 Genotype Identification

225 All mussels were genotyped using the species diagnostic marker *Glu-5*, amplified
226 using the primers *Me 15* (5'-CCAGTATACAAACCTGTGAAGA-3') and *Me16* (5'-
227 TGTTGTCTTA ATAGGTTTGTAAGA-3') (Inoue et al. 1995). Alleles at this locus are
228 represented by fragments of different lengths for *M. edulis* (180 bp) and *M.*
229 *galloprovincialis* (126 bp). DNA was extracted from <1 mg of foot tissue using the
230 HotSHOT protocol. Briefly, tissue was digested in 100 µL alkaline lysis reagent (25
231 mM NAOH and 0.2 mM disodium EDTA) at 95 °C for 30 min and cooled on ice for 5
232 min, after which 100 µL neutralising agent (40 mM Tris-HCl added) was added. PCR
233 reactions were carried out in a 12.5 µL volume containing 30-50 ng DNA, 6.25 µL 2x
234 MyTaq Mix (Bioline) and 0.25 µL each primer, under the following cycling conditions:
235 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
236 72°C for 5 min.

237 The *Glu-5* marker has been used extensive for the identification of species within the
238 Mytilus complex (REFS needed). While it is possible for backcrosses to appear
239 homozygous at this locus, the population used in the present study has been found
240 to have limited introgression (Gardner et al. 1993), giving us reasonable confidence
241 in the marker's ability to detect pure and hybrid individuals, or individuals with highly
242 constrasting ancestry.

243

244 Data Analyses

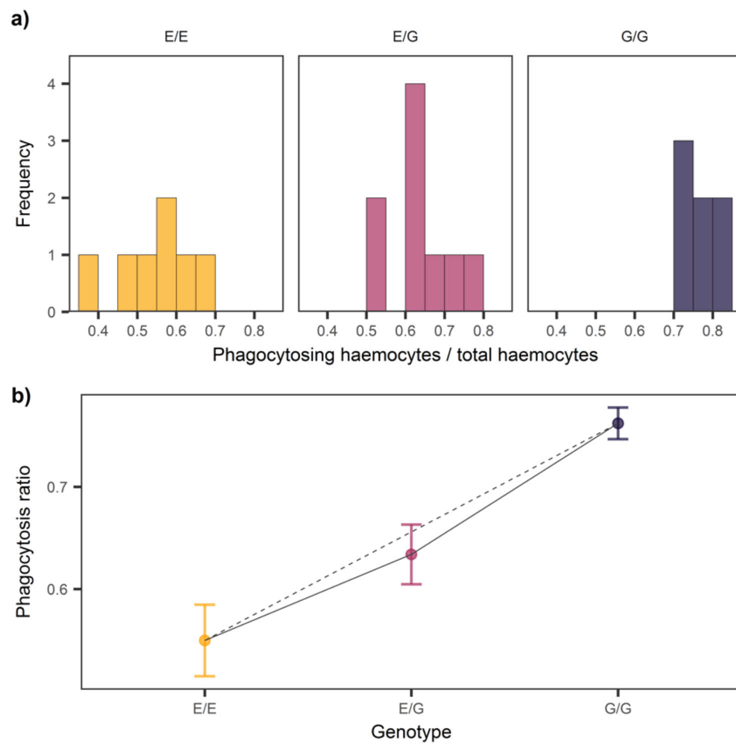
245 Statistical analyses were conducted using RStudio v.3.3.2 and SPSS. Assumptions
246 of normality and homogeneity of variance were met following Shapiro-Wilk and
247 Levene's tests respectively unless stated otherwise. Tukey's HSD post hoc test was
248 used to detect significant differences between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

249

250 **Results**

251 Immunocompetence Assay

252 Immunocompetency assays were performed in *M. galloprovincialis* (G/G, n=7), *M.*
253 *edulis* (E/E, n=7), and their hybrids (E/G, n=9). Sample sizes were in line with those
254 used in Wood et al.(2014). Immunocompetency, assessed as the ratio of
255 phagocytosing to non-phagocytosing haemocytes, differed between the genotypes
256 (Fig. 1a) with *M. galloprovincialis* (G/G) higher than *M. edulis* (E/E) and hybrids (E/G)
257 intermediate. The distributions of the EE did not overlap, however EG overlapped
258 with both EE and GG. The variation between genotypes was significant (ANOVA,
259 $F(2,20) = 13.091$, $P< 0.001$). According to the Tukey HSD post hoc test,
260 phagocytosis of G/G is significantly greater than E/E ($P = 0.000$) and E/G ($P =$
261 0.011).Consistent with Fig. 1a, the mean values for the three genotypes fell close to
262 a straight line (Fig. 1b) with G/G having the highest ratio value. E/G was not
263 significantly different from the midpoint between EE and G/G (ANOVA, $F(1,20) =$
264 0.435, $P= 0.517$). Thus, there was no statistical evidence for heterosis or hybrid
265 depression, when this is defined as a deviation from the midpoint value rather than
266 the more extreme situation where E/G might lie outside the range separating E/E
267 and G/G.



268

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

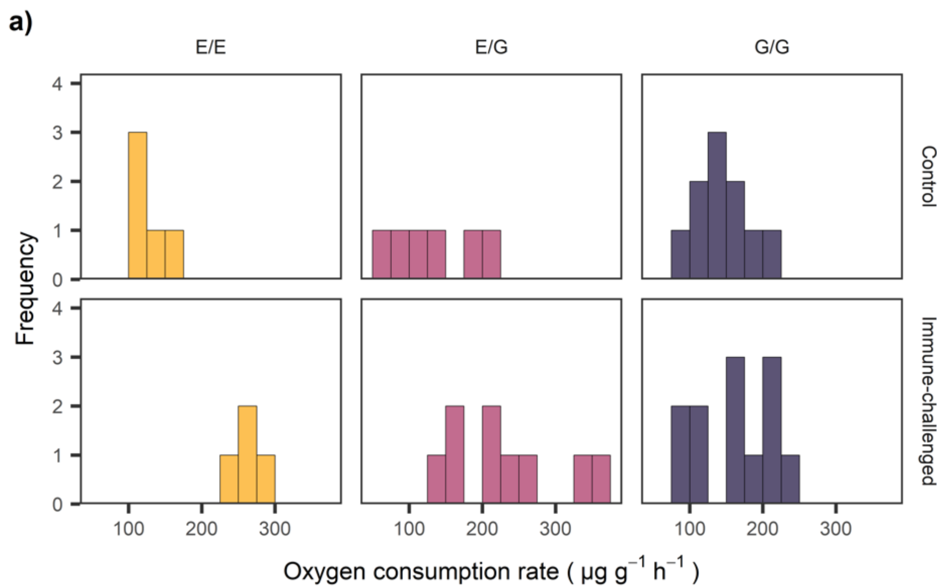
275

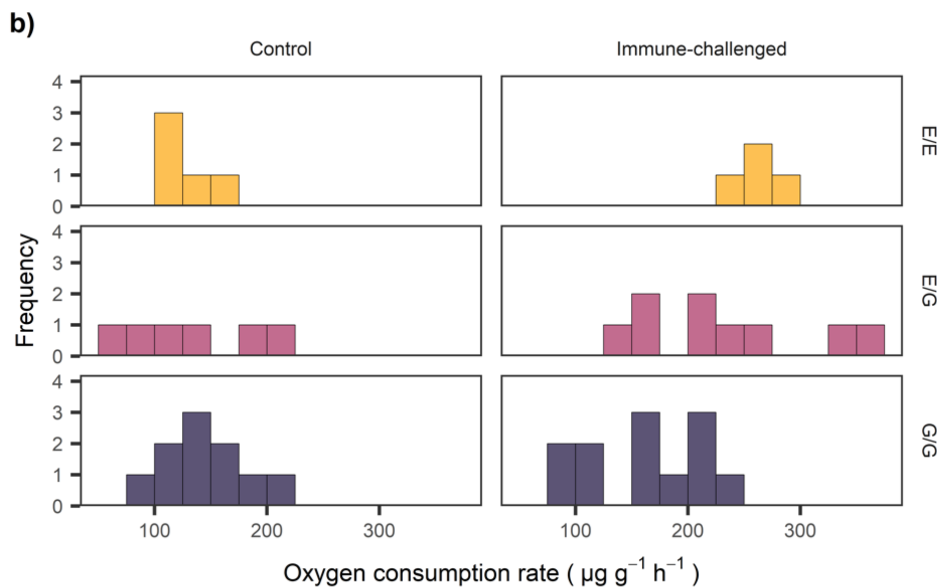
276 <u>Respirometry</u>

277 Respirometry assays were performed in *M. galloprovincialis* (G/G, n=10 and 12 for
278 control and immune challenged respectively) *M. edulis* (E/E, n= 5 and 4 for control
279 and immune challenged respectively), and their hybrids (E/G, n=6 and 9 for control
280 and immune challenged respectively). Histograms of the distributions of O_2 uptake
281 are shown for the six different treatment and genotype combinations in Fig. 2
282 panelled in two different ways. The difference between control and immune
283 challenged groups was greatest for E/E, less marked for E/G and showing no
284 difference for G/G (Fig. 2a). The difference between genotypes was marked for the
285 immune challenged mussels but showing no difference for the controls (Fig. 2b). In
286 line with the histograms, one-way ANOVA showed no significant differences

287 between genotypes for the control (ANOVA, $F(2,18) = 0.210$, $P = 0.794$). There was,
288 however, a significant result for the immune challenged group ($F(2,22) = 4.821$, $P =$
289 0.020). According to the Tukey HSD post hoc test, G/G was significantly different
290 from E/E ($P = 0.040$) and at the borderline of significance in the comparison with E/G
291 ($P = 0.060$). The presence of significant differences for the immune challenged but
292 not the control group is also consistent with a significant treatment-genotype
293 interaction (ANOVA, $F(2,40) = 3.949$, $P = 0.027$). For the immune challenged group,
294 the genotype means fall close to a straight line (Fig. 3), with a decline in O_2 uptake
295 as the number of G alleles increases. E/G was not significantly different from the
296 midpoint between E/E and G/G ($P = 0.113$). Thus, there is no statistical evidence for
297 heterosis or hybrid depression. For the control group, the genotype means, though
298 not significantly different, trend in the opposite direction and the difference in slope
299 will contribute to the significant interaction in the two-way ANOVA.

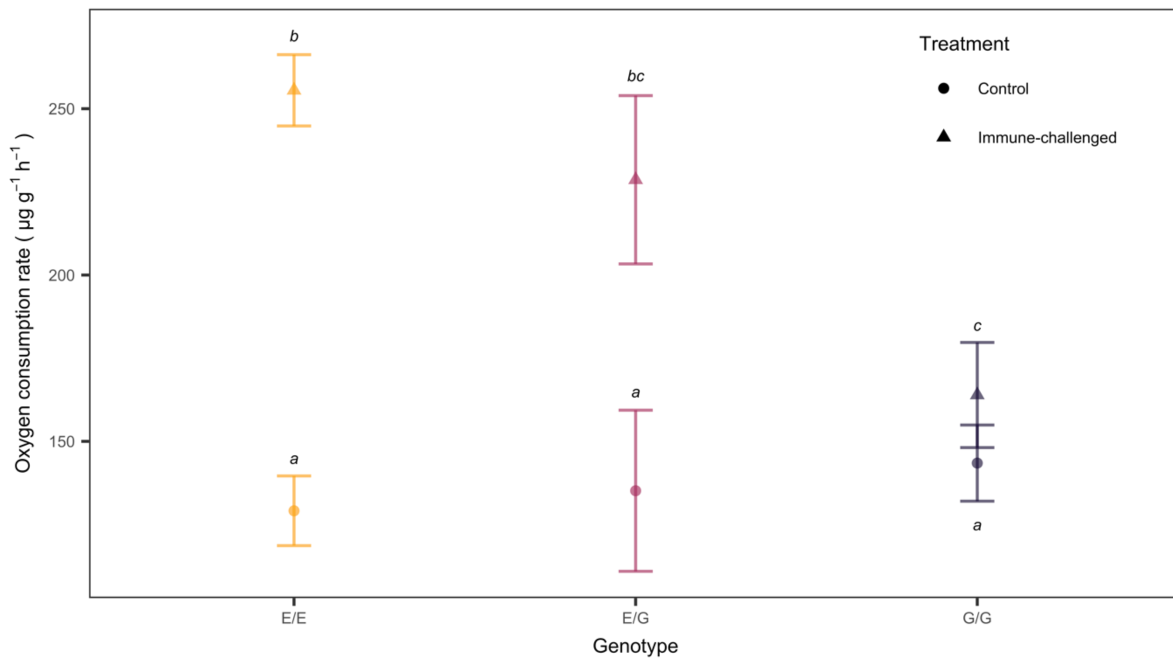
300





301

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



308

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

315

316 **Discussion**

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

328 (Gardner and Skibinski 1988) and could be a contributing factor in European *Mytilus*
329 hybrid zone dynamics.

330 The stronger immune response to bacterial infection measured in mussels identified
331 as *M. galloprovincialis* compared to *M. edulis* add to a large body of studies that
332 found differentiation in fitness-related traits apparently in favour of *M.*
333 *galloprovincialis* genotypes over *M. edulis* ones (Skibinski et al. 1983; Coustau et al.
334 1991; Gardner 1994; Hilbish et al. 1994; Bierne et al. 2006), and provides new
335 evidence of this for a previously underappreciated trait. As we were not able to
336 measure phagocytic ability under control conditions, it is not possible to determine
337 whether *M. galloprovincialis* has as constitutively higher phagocytosis rate, or
338 whether it is able to mount a response faster than *M. edulis*. Nonetheless, our results
339 concur with others recording a strong immune response in *M. galloprovincialis* from
340 transcriptomic (Moreira et al. 2018) and parasite load (Coustau et al. 1991)
341 approaches. The intermediate immune response observed in hybrids when
342 compared to the parental genotype suggests no heterosis or hybrid depression for
343 immunity. This agrees with previous studies, describing hybrids as intermediate for
344 several traits, such as length-at-age values (Gardner et al. 1993), habitat
345 specialisation (Gosling and McGrath 1990), and attachment strength (Willis and
346 Skibinski 1992). In contrast, hybrid depression has been observed in larval viability
347 (Beaumont et al. 1993; Bierne et al. 2002).

348 Our results complement those of Coustau et al. (1991), who discovered a pattern of
349 susceptibility to the trematode *P. squamatus*, which causes total castration and
350 mortality, in which the *M. galloprovincialis* genotype was least parasitised in the
351 hybrid zone. The authors could not determine whether this could be ascribed to
352 immune mechanisms of the host or specific mesologic requirements of the parasite.
353 *Bacillus thuringiensis*, used in the present study, is not a pathogen that is
354 encountered by mussels in nature. It is however useful for many invertebrate
355 immunological studies, inducing a phagocytic response independent of exposure
356 history. Specific host-pathogen interactions may present different patterns, such as
357 the apparent hybrid susceptibility to disseminated haemic neoplasia (Fuentes et al.
358 2002).The enhanced immune capabilities associated with the *M. galloprovincialis*
359 genotype support the hypothesis that intense selection may favour the spread of *M.*
360 *galloprovincialis* genes. Fuentes et al. (2002) found greater mortality in hatchery-

361 produced hybrid crosses, compared to *M. galloprovincialis* crosses, when reared in
362 aquaculture conditions. Increased mortality in hybrids was associated with higher
363 parasitisation by the protist *Marteilia refringens* in hybrid crosses when compared to
364 *M. galloprovincialis* crosses. They provide further evidence for strong pathogen
365 resistance in *M. galloprovincialis,* observed at a range of sites around Europe under
366 natural and aquaculture conditions. In conjunction with our results, this suggests
367 immunity may be a contributing factor outside of Croyde Bay, in *Mytilus* hybrid zone
368 dynamics throughout Europe.

369 Most knowledge about immunity in hybrids is regarding plant-pathogen or plant-
370 herbivore interactions. In a review of hybrid resistance to pathogens and herbivores,
371 Fritz et al (1994) hypothesised that resistance to pathogens may be a more common
372 feature in animal hybrids than in plant hybrids. More recent data (Derothe et al.
373 2001; Parris 2004; Wolinska et al. 2004), including those presented here, so far
374 suggests that animal hybrids are not different to plants in their patterns of
375 susceptibility. The present study contributes to the currently limited invertebrate
376 hybrid literature, finding intermediate hybrid immunocompetency as in other
377 invertebrates such as mosquitoes (Mancini et al. 2015) and vertebrates (which
378 possess adaptive immune systems) such as birds (Wiley et al. 2009)

379

380 Size-dependent variation in genotype frequency at the low shore of Croyde Bay
381 (Gardner and Skibinski 1988) informed the size range of mussels collected for this
382 study. Below the chosen size range of 28-34 mm, Gardner and Skibinski (1988)
383 found the *M. edulis* genotype to be most prevalent, accounting for almost 80% of
384 individuals sampled. Above this size range, they observed a drastic switch to *M.*
385 *galloprovincialis* as the most prevalent genotype, accounting for up to 60%
386 abundance. Hybrid genotype frequency remained stable with size, increasing in
387 frequency only within the size range selected in this study. Though this pattern has
388 been partly explained by attachment strength (Willis and Skibinski 1992), the
389 distribution of genotypes predicted by this is dependent on wave action and does not
390 adequately match the observed distributions in Southwestern England (Hilbish et al.
391 2002). Differential immunity between the genotypes across distinct sizes could
392 therefore be a causative factor. The greater immunocompetence of *M.*

393 *galloprovincialis* compared to *M. edulis* observed in the size range used in this study
394 may represent a threshold at which a combination of differential immunity,
395 attachment strength, and possibly other factors, cause the preferential survival and
396 geographic extension of *M. galloprovincialis* with increasing shell length.

397

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

412

413 **Acknowledgements**

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

417

418 **Compliance with Ethical Standards**

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

423

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