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Thermal selection of PGM allozymes in newly founded populations of the thermotolerant vent polychaete *Alvinella pompejana*

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Alvinella pompejana lives on the top of chimneys at deep-sea hydrothermal vents of the East Pacific Rise. It is thought to be one of the most thermotolerant and eurythermal metazoans. Our experimental approach combines methods of population genetics and biochemistry, considering temperature as a potential selective factor. Phosphoglucomutase (*Pgm-1* locus) is one of the most polymorphic loci of *A. pompejana* and exhibits four alleles, from which alleles 90 and 100 dominate with frequencies of approximately 0.5 in populations. Results from previous studies suggested that allele 90 might be more thermostable than allele 100. Significant genetic differentiation was found by comparing contrasted microhabitats, especially the young, still hot, versus older and colder chimneys, with allele 90 being at highest frequency on young chimneys. Moreover the frequency of allele 90 was positively correlated with mean temperature at the opening of *Alvinella* tubes. In parallel, thermostability and thermal optimum experiments demonstrated that allele 90 is more thermostable and more active at higher temperatures than allele 100. This dataset supports an additive model of diversifying selection in which allele 90 is favoured on young hot chimneys but counterbalanced over the whole metapopulation by the dynamics of the vent ecosystem.

Keywords: phosphoglucomutase; population genetics; enzyme kinetics; thermostability; hydrothermal vents; Alvinella pompejana

1. INTRODUCTION

Sewall Wright (1932) proposed that a species might adapt to a heterogeneous environment by means of a 'shifting balance' between natural selection, migration and genetic drift. Natural selection tends to increase the average fitness of individuals, and thus populations evolve towards the nearest 'adaptive peak' in the 'adaptive landscape' by local adaptation. Illustrating such a theory requires the study of fragmented populations small enough to undergo marked genetic drift and subjected to a heterogeneous environment in which a given factor greatly affects the individual fitness (Hedrick 1986). Among many environmental parameters, temperature has been recognized as one of the most important factors that affect species' distribution through their thermal sensitivities, optima and tolerances (Somero 1995). Ultimately, temperature constrains every biological process (macromolecular structures and interactions, reaction flux: Hochachka & Somero (1984)) and has been identified as a strong selective factor (Eanes 1999; Watt & Dean 2000). However, previous studies generally dealt with large-scale latitudinal or altitudinal clines, where historical processes or migration patterns can hide selective effects. Very few studies have addressed the impact of heterogeneous environments on population structure over small spatial scales (Johannesson & Tatarenkov 1997; Schmidt & Rand 1999). Among eurythermal marine eukaryotes, invertebrates associated with deep-sea hydrothermal vents are one of the most exposed organisms to highly fluctuating thermal regimes over small spatial scales (Chevaldonné *et al.* 1991). The hydrothermal vent habitat is therefore a very good model for the study of selective forces: it is characterized by highly contrasted habitats in close proximity, with high temporal and spatial short-scale heterogeneity of the physico-chemical parameters.

The observation of vent sites and their associated fauna at 13° N / East Pacific Rise (EPR) every 2 or 3 years since 1982 allowed us to carefully describe the evolution of both the vent fluid displacements and the assemblages (Desbruyères 1995; Jollivet 1993). Both tectonism and the intrinsic heat flow convection act in displacing vents along the axial *graben* of the rift and thus generate the appearance of new vent sites in close proximity to old sites that become extinct. The number of new active vent sites is low and represents only a small fraction of the total number of active sites (*ca.* 5–10%). These new vent sites correspond to 0.5–1 m high porous anhydrite chimneys through which hot translucent (100–250 °C) hydrothermal fluid diffuses. Diffusers cool down rapidly (a few months) and grow both laterally and vertically to become snowballs and white smokers (if the inner fluid pipe still remains porous) or black smokers (if the inner conduit is impermeable), which evolve more slowly afterwards, from years to tens of years, with temperatures varying between 5 °C and 20 °C at the entrance of alvinellid tubes until the chimney becomes extinct by clogging of the conduits.

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Alvinella pompejana, the 'Pompeii worm', is a tubicolous polychaete that lives on the walls of active chimneys at deep-sea hydrothermal vents on the EPR, a patchy, harsh and unstable environment (Desbruyères *et al.* 1998). It is among the first metazoans to colonize young hot chimneys (Chevaldonné 1996). Because *A. pompejana* grazes on chemolithoautotrophic bacteria, the worm must live within the turbulent mixing zone that chaotically switches from hydrothermal fluid- to seawater-dominated conditions and thus is exposed to periodic alternation of oxic and anoxic conditions (Desbruyères *et al.* 1998). This terebellomorph worm is known to be one of the most thermophilic marine eukaryotes on Earth (Cary *et al.* 1998) although great controversy exists about the thermal limits encountered by this peculiar organism (Chevaldonné *et al.* 2000). To live in this harsh environment, the worm displays specific biochemical adaptations to relatively high temperatures, such as enzyme optima, mitochondrial respiration or collagen melting temperature, which are unique from those of other marine invertebrates (Dahlhoff *et al.* 1991; Dahlhoff & Somero 1991; Gaill *et al.* 1995). It forms highly fragmented populations subjected to local extinctions. *Alvinella* is gonochoric, uses internal fertilization to reproduce and displays a peculiar mating behaviour in which males and females pseudocopulate (Desbruyères *et al.* 1998). It is also suspected to have benthic development with embryo/erpochaete larvae that need a very narrow thermal window to develop (Desbruyères *et al.* 1998; Pradillon *et al.* 2001).

Unlike other key enzyme loci from the glycolytic and Krebs pathways investigated so far in *A. pompejana* (Jollivet *et al.* 1995*a*) (which could be subject to temperature selection), such as glucose phosphate isomerase (Gpi), the *Pgm-1* locus (coding for phosphoglucomutase (PGM)) is polymorphic and consists of two major allozymes, *Pgm90* and *Pgm100*, and two rare ones, *Pgm78* and *Pgm112*. A previous study (Jollivet *et al.* 1995*b*) suggested that allozyme 90 might be more thermostable than allozyme 100. PGM is a relay enzyme between glycogen metabolism and glycolysis, the pathway that provides the most energy in many organisms, especially in those living in hypoxic to anoxic environments such as *A. pompejana*. As a branch point enzyme, PGM is expected to undergo strong selective pressures (Verrelli & Eanes 2001*b*). In fact, PGM is a highly polymorphic enzyme in many invertebrate organisms (Dawson & Jaeger 1970) and functional studies in the sea anemone *Metridium senile* (Hoffmann 1985), the oyster *Crassostrea gigas* (Pogson 1989, 1991), *Colias* butterflies (Carter & Watt 1988; Watt *et al.* 1985) and *Drosophila melanogaster* (Verrelli & Eanes 2000, 2001*a*,*b*) showed strong evidence for selection at this locus, most cases implying temperature (Nevo *et al.* 1977; Wilkins *et al.* 1978) as a selective factor.

Because *A. pompejana* experiences contrasting thermal regimes, temperature could be an important environmental factor governing *Pgm1* allele frequencies in populations. Using the *Pgm-1* locus, we studied the adaptive strategy of *A. pompejana* to such an unstable and heterogeneous environment. In particular, we tried to understand if the *Pgm-1* locus is under stabilizing or diversifying selection and if temperature affects the population structure at this locus and ultimately the microevolution of populations. Because one needs an integrative approach to know more about microevolutionary forces acting at one single locus, this study combines population genetics, ecology and biochemistry in an original approach that could be applied to many single genes.

2. MATERIAL AND METHODS

(a) *Study site and population sampling*

A total of 652 worms from 18 populations of *A. pompejana* were sampled during the oceanographic cruises *Hydronaut* (1987), *Mmvt* (1990), *Hot* (1996) and *Hope* (1999) on young and old chimney structures at a depth of *ca.* 2500 m within the 13° N hydrothermal vent field on the EPR (figure 1). Chimneys were classified into three main recognized types: young diffusers, white smokers and black smokers. Individuals were collected by using the robot arm of the manned submersibles *Nautile* and *Alvin* and brought back to the surface in an insulated basket. All individuals were preserved in liquid nitrogen until further analysis.

(b) *Temperature measurements*

Temperature was measured using either the submersible temperature probe (series of discrete measurements over a few minutes), autonomous probes (time series over days with measurements every 2 min) or the autonomous temperature probe of the *in situ* analyser ALCHIMIST (coupled to the inlet which was used to measure hydrogen sulphide concentration at different locations in the environment continuously over periods of a few hours; table 1). Because of sharp thermal gradients occurring through the chimney's walls, we retained only temperature measurements taken at the opening of *Alvinella* tubes (measurements were taken into consideration only when the probe had touched the opening of the tube but still remained totally visible on video surveys). Mean temperature values and standard errors were calculated for submersible probe measurements and time series (autonomous and ALCHIMIST probes).

(c) *Genetic structure at the* Pgm-1 *locus*

Proteins were extracted in a grinding buffer (0.01 M Tris–HCl pH 6.8, 2 mM EDTA, 0.5 mM NADP, 0.05% β-mercaptoethanol and 0.1 mM PMSF) using the procedure described in Jollivet (1993). Individuals were genotyped at the *Pgm-1* locus by using starch-gel electrophoresis (Tris–citrate pH 8.0 buffer system, 100 V, 80 mA, 4 h; PGM staining method 2 following Pasteur *et al.* (1987)). For each population, significant deviations from Hardy–Weinberg equilibrium were tested using an exact test as implemented in GENEPOP software (Raymond & Rousset 1995). Populations sampled during *Hope* 1999 (nine populations; 333 individuals) clustered according to the kind of chimney on which they were collected (diffusers, white and black smokers), and a hierarchical analysis was performed with TFPGA (Miller 1997) to test for the habitat effect. A correlation between geographical distances and genetic differentiation was also tested according to the one-dimensional isolation-by-distance model using a Mantel procedure from GENEPOP software. All the populations (1987, 1990, 1996 and 1999) were then considered and exact tests between habitats and between populations from each habitat were performed to test for an inter- or intra-habitat differentiation. A correlation between temperature at the opening of *Alvinella* tubes and *Pgm90* allele frequency (12 samples for which a temperature dataset was available) was tested by a Spearman rank correlation test (STATISTICA software).

(d) *Thermal stability of allozymes*

As pressure variations do not seem to affect enzyme kinetics for deep-sea organisms including vent species (Dahlhoff & Somero

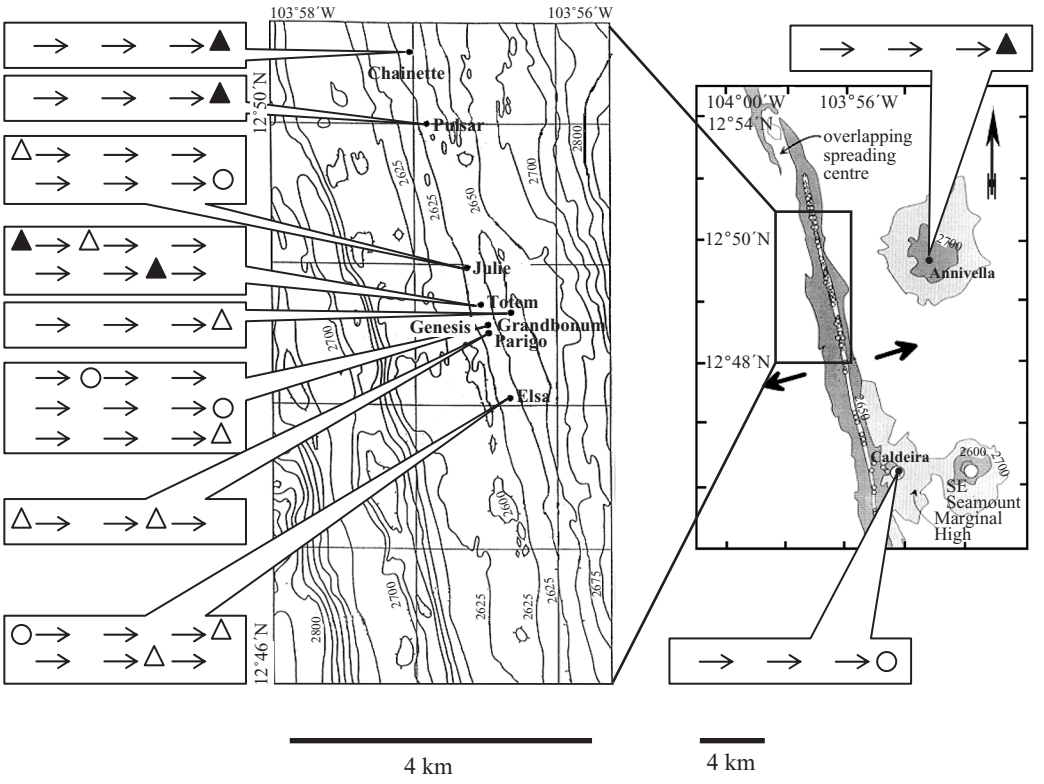

Figure 1. Study site: 13° N on the East Pacific Rise. Populations were sampled along the axial *graben* (except Caldeira and Annivella). For each sampling site, one (Chainette, Pulsar, Grandbonum, Parigo, Annivella, Caldeira), two (Julie, Totem, Elsa) or three (Genesis) different chimneys were sampled (maximum distance: 10 m). Moreover, half of the sites were sampled repeatedly (in 1987, 1990, 1996 or 1999), as described on the figure by arrows symbolizing time. Open circles: diffuser; open triangles: white smoker; filled triangles: black smoker. For example, Elsa was sampled in 1987 (diffuser) and 1999 (white smoker; same chimney), and in 1996 (white smoker; another chimney separated from the first one by less than 10 m).

1991), biochemical experiments were all realized at one atmospheric pressure.

A first thermostability experiment was conducted on PGM allozymes of homozygous individuals (crude extracts from five different individuals for both genotypes *Pgm* 90/90 and *Pgm* 100/100). Aliquots of each crude protein extract were incubated respectively 30 and 60 min at either 35 °C or 45 °C before the enzyme assay. A control aliquot was kept on ice during incubation. Assays were conducted for 10 min in a reaction solution adapted from Jollivet *et al.* (1995*b*). The initial enzyme velocity was then estimated for each aliquot according to a linear regression and the percentage of residual activity was calculated according to the control aliquot. A second and more resolute experiment was conducted using non-denaturing polyacrylamide gel electrophoresis for heterozygous individuals. The use of heterozygous individuals allows for a better comparison of thermostabilities between alleles 90 and 100 by avoiding inter-individual effects. Ten crude extracts of individuals 90/100 containing the same amount of proteins (50 or 100 μg; standard Bradford assays) were incubated for 0, 60 or 90 min at 35 °C and loaded onto a polyacrylamide gel. PGM activity was then detected on gel following method 1 of Pasteur *et al.* (1987) and the mean product density per spot (allozyme 90 or 100) was quantified using a numerical camera (Kodak DCS 420) and the Scion Image 4.0.2 software (www.scioncorp.com).

A third thermal stability experiment based on the incubation of partly purified PGM extracts on starch gel slides was finally conducted. Such an approach restricts most macromolecular interactions during the incubation (enzyme refolding by chaperones or specific enzyme inhibitions), which may affect the enzyme thermostability in the crude protein extract. Extracts were loaded on a starch gel and run at 80 mA for 5 h in the buffer system described above. After migration, four slides of the gel (replicates) containing crude protein extracts of five individuals' 90/100 were incubated for 30 min at 4, 37, 48 and 60 °C in their gel buffer to avoid desiccation, assayed for PGM activity (Pasteur *et al.* 1987; method 2) and the mean product density per spot was quantified on gel following the method described in the previous paragraph.

(e) *Enzyme kinetics and temperature*

The PGM initial velocities were measured at 20, 27, 37, 45, 50 and 55 °C using the same method as previously described in spectrophotometric assays for *Pgm-1* 90/90 and 100/100 genotypes. Additional initial velocities were also estimated over a wider range of temperatures (20–65 °C) with an alternative method adapted from Manchenko (1994) and Aminot & Chaussepied (1983) to avoid the use of *G6Pdh*, which could be denatured at high temperatures. Assays were conducted with two distinct pools of five individuals of each homozygous genotype. In this method, the assay solution corresponds to 0.2 M Tris–HCl pH 8 containing 20 mM $MgCl_2$ and 1 mM glucose-1-phosphate to which 50 μl of crude protein extract of known protein content (Bradford method) were added. Every 30 s, 50 μl of the 1 ml assay solution were dropped into 330 μl of 2 M H_2SO_4, incubated for 1 h at 45 °C (selective acid hydrolysis of glucose-1-phosphate to glucose and inorganic phosphate) and centrifuged for 5 min at 10 000 r.p.m. to discard protein precipitates. Acid hydrolysis was then neutralized by adding 420 μl of 2.5 M NaOH and 200 μl of a staining solution containing 2.5 M H_2SO_4, 0.6% ammonium molybdate, 2.16% ascorbic acid, 0.0136% potassium and antimony (III) oxytartrate

Table 1. Populations for which temperature was recorded at the tube openings of the *Alvinella pompejana*.
(Mean temperature (degrees Celcius), minimum and maximum recorded temperatures (min/max; degrees Celcius), standard deviation (degrees Celcius) and recording time (minutes) are given. When more than one temperature-measurement device was used, recording time is indicated for each of them. *Submersible probe measurements; †water sampling device measurements; ‡temporal measurements, autonomous probes; §temporal measurements, *in situ* analyser ALCHIMIST. The end of autonomous probes was not always totally visible on videos and might slightly overestimate our temperature estimates in time series.)

site	habitat	sampling device	recording time	mean temperature	standard deviation[a]	min/max
Genesis99	diffuser	† ‡ §	8410/20/37	60.75	12.2	12.1/93.3
Julie99	diffuser	*	10	65.00		50.0/80.0
Genesis90	diffuser	*	10	37.50		35.0/40.0
Genesis99	white smoker	‡	10170	6.40	1.02	2.0/11.3
Elsa99	white smoker	‡ §	3426/17	18.73	2.25	13.2/26.0
GrandBonum99	white smoker	*	10	10.50		6.0/15.0
Parigo96	white smoker	*	10	12.50		5.0/20.0
Parigo87	white smoker	*	10	12.50		5.0/20.0
Totem96	black smoker	‡	25344	12.20		3.0/26.3
Totem87	black smoker	‡	2820	6.09	2.01	2.3/11.5

[a] Standard deviation was calculated only for time-series measures.

to quantify the inorganic phosphate contents. The phosphate complex quantities were measured at 885 nm and 25 °C using a spectrophotometer (Uvikon 930, Kontron).

3. RESULTS AND DISCUSSION

(a) *Allele frequencies at the* Pgm-1 *locus*

Previous studies already showed that the polychaete *A. pompejana* possesses four alleles at the *Pgm-1* locus of which two are found to be nearly equifrequent in populations (Jollivet *et al.* 1995*a*,*b*). Mean allele frequencies estimated over the individuals collected confirmed this allelic distribution (table 2). However, these frequencies are not homogeneous between populations. A UPGMA tree based on the PGM allele frequencies and Rogers' genetic distance revealed the occurrence of three distinct groupings (figure 2). With the exception of Totem90 for which no rare alleles (78 and 112) are found and allele 100 dominates, most of the genetic divergence occurs between group A, in which the allele 90 frequency exceeds 0.453 in all populations, and groups B and C, in which the allele 90 frequency falls below 0.429. These two major clusters correspond to individuals recently settled on new chimneys, i.e. diffusers (group A) and to individuals living on either white or black smokers (groups B and C), which represent older and cooler edifices. A hierarchical analysis on populations sampled in 1999 (the first exhaustive and equilibrated sampling of *A. pompejana* according to different kinds of chimneys) was performed to avoid any temporal effects. Significant positive values of F (0.0821; Weir and Cockerham's estimator of *Fit*) and f (0.0792; Weir and Cockerham's estimator of *Fis*) were observed, indicating that most of the overall genetic differentiation is explained by a heterozygote deficiency (exact test, $p < 0.01$). The inter-group differentiation θ_p (0.0152) is significantly different from zero (exact test; $p < 0.05$) and five times greater than the intra-group differentiation estimate θ_s (0.0032), which is not different from zero. There is therefore significant differentiation between groups of populations from different habitats (diffusers, white or black smokers). When the whole dataset is considered (1987, 1990, 1996 and 1999), the genetic differentiation between groups was also significant (table 3) and not affected if Totem90 is excluded. Like the 1999 dataset, intra-group differentiation was not significantly different from zero. This pattern of differentiation does not correspond to a peculiar spatial organization of the vent habitat within the segment 13° N / EPR as no isolation by distance has been detected from a Mantel test (GENEPOP test, $p = 0.85$). These observed differences in allele frequencies between populations therefore appear to reflect only the temporal state of the vent chimneys, which evolve from 'hot' porous anhydrite diffusers to large cooler chimneys, with allele 90 being more frequent in recently settled populations (table 2).

The survey of the vent ecosystem at 13° N / EPR every 2–4 years from 1982 to 1999 (Desbruyères 1995; Fustec *et al.* 1987; Jollivet 1993) provided useful information about the temporal evolution of vent chimneys. Interestingly, during the past decade, chimneys sampled twice (figure 1) model exactly the pattern of frequencies previously highlighted (figure 2): evolution from a diffuser to a white smoker led to a decrease of allele 90 frequency (Elsa 87/99), whereas no change of habitat led to no genetic changes (Elsa96/99; Parigo87/96; Totem87/96). Similar changes in allele frequencies at both the *Pgm* and *Aat* loci have been previously reported by Tunnicliffe *et al.* (1993) for the northeastern Pacific vent polychaete *Paralvinella palmiformis* after an artefactual increase of the ambient temperature of a vent chimney owing to sampling. Our findings therefore demonstrate the role of selective processes in shaping population structure on small spatial scales and corroborate previous studies that attempted to demystify the effects of geographical and habitat variations (Johannesson & Tatarenkov 1997; Schmidt & Rand 1999).

The chaotic distribution of PGM alleles in populations of *A. pompejana* greatly contrasts with classical latitudinal clines of allele frequencies found at broader spatial scales (Place & Powers 1979; McDonald & Kreitman 1991; Dahlhoff & Rank 2000) as it mimics vent dynamics and especially changes in thermal regimes.

Table 2. Mean temperature recorded (T °C ± s.d.) at the opening of the tubes and allele frequencies (f) at the *Pgm-1* locus for each habitat (populations pooled are those listed in table 2, excluding Totem).
(N, number of genotyped individuals.)

habitat	diffuser	white smoker	black smoker	all
T °C (± s.d.)	54.4 (±14.8)	12.1 (±4.5)	9.1 (±4.3)	
N	156	346	132	634
f(78)	0.058	0.026	0.051	0.043
f(90)	0.471	0.396	0.355	0.399
f(100)	0.465	0.562	0.577	0.540
f(112)	0.006	0.016	0.017	0.018

(b) *Is temperature a selective factor governing frequencies at* Pgm-1 *locus?*

The thermal habitat of *Alvinella* worms was recently documented (Chevaldonné *et al.* 1991; Desbruyères *et al.* 1998; Sarradin *et al.* 1998) and time-series analyses showed that temperature displays tremendous variations both spatially and temporally from 7 °C to 91 °C with an amplitude of more than 25 °C over short periods of time (Chevaldonné 1996; Sarradin *et al.* 1998). These vent conditions are much warmer than those encountered by other species living in diffuse venting around the chimney (Sarradin *et al.* 1998). Moreover, thermal regime differences can occur between edifices according to their temporal state with temperature means (time series over few days) varying at the opening of the tubes from 3.8 °C to 12.2 °C (Chevaldonné 1996; Chevaldonné *et al.* 1991) on white and black smokers to 54.4 °C on diffusers (this study; table 2). Our observations in the field indicated that the worms settled on diffusers (young chimneys) sustained on average higher temperatures than those living on older chimneys such as white or black smokers, based on temperatures taken at the opening of *Alvinella* tubes (table 1). The average temperature at the opening of the tubes correlated positively with the frequency of allele 90 and was found to be highly significant (Spearman rank correlation test, $R = 0.85$, $p = 0.0005$; figure 3). This finding indicated that there was either a direct link between temperature and the *Pgm-1* allele frequencies by a temperature-driven selective effect on the *Pgm-1* locus (or a linked locus) or an indirect link by additive selective effects implying other physico-chemical parameters (H_2S, O_2 or CO_2 concentrations). Indeed, we could not rule out the influence of other environmental factors such as anoxia or H_2S simply because temperature could be viewed as a proxy for the chemical environment in the hydrothermal vent habitat. Temperature is a semi-conservative factor that reflects the level of mixing between hydrothermal fluid and ambient seawater and thus is positively correlated with H_2S, CO_2 and Fe^{2+} and negatively correlated with O_2 concentrations (Johnson *et al.* 1986; Le Bris *et al.* 2003).

Although enzyme catalytic efficiencies could be slightly affected by hydrostatic pressures, we investigated the thermal properties of allozymes 90 and 100 by simple biochemical methods on either crude protein extracts of homozygotes 90/90 and 100/100 and heterozygotes 90/100 or gel-purified extracts of heterozygotes 90/100. The incubation times and temperatures were selected to fall within the range of temperatures encountered by *A. pompejana* on diffusers (Chevaldonné *et al.* 2000; Desbruyères *et al.* 1998; Jollivet 1993). In the crude protein extract spectrophotometric assays, the average residual activity of PGM-1 was higher for the five homozygotes 90/90 than for the five homozygotes 100/100 at each incubation temperature and time (figure 4) and it became significant (Student's t-test; $p < 0.001$) for the longest incubation time at the higher temperature (60 min at 45 °C). However, because PGM activity could be affected by other molecules in crude extracts such as chaperones, ions and proteases, additional biochemical experiments were performed on gel-separated extracts. Inter-individual biases were also avoided by using heterozygotes. For the five heterozygotes 90/100, the average density of the spot (proportional to the level of the PGM-1 activity) was estimated at four distinct temperatures and was found to be significantly higher for allozyme 90 compared with allozyme 100 (same individuals) at the highest incubation temperatures (30 min at 48 °C or 60 °C; $p < 0.05$; figure 5). The other experiment using acrylamide-purified extracts of heterozygotes 90/100 (figure 6) also showed positive differences between residual activities of allozyme 90 and allozyme 100 all significantly different from zero (Student's t-tests; $p < 0.01$). These findings suggest that allele 90 is more thermostable than allele 100 and fits well with previous studies, indicating that allelic selection for key enzymes of the glycolysis could be either due to a better thermal stability of allozymes (Dahlhoff & Rank 2000), the functional impact of variants on energy allocation (Verrelli & Eanes 2001*b*) or to variations in the level of heat shock protein expression (Koban *et al.* 1991; Dahlhoff & Rank 2000).

Better thermostability of an enzyme does not always imply that this enzyme is more active and efficient at higher temperatures. Differences between the optimal kinetics of isoforms should also be tested. Initial velocities of crude protein extracts of homozygotes 90/90 and 100/100 over a wide range of temperatures also showed that the thermal optimum is 3 °C higher (55 °C versus 52 °C) for homozygotes 90/90 than for homozygotes 100/100 (figure 7), and that the activity was always higher for homozygotes 90/90, at all of the tested temperatures. This strongly suggests that allozyme 90 is either more expressed or more active in gill tissues than allozyme 100 when ambient temperature exceeds 20 °C. These findings fully support previous work of Verrelli & Eanes (2001*b*) indicating that the most thermostable PGM variants of *Drosophila melanogaster* are also those exhibiting the highest level of activity and the highest glycogen content in tissues. These latter authors indeed showed that PGM is a quantitative trait locus for glycogen content. As glycogen content is essential for vent organisms that are often subjected to hypoxic conditions (especially populations experiencing high temperatures), one can

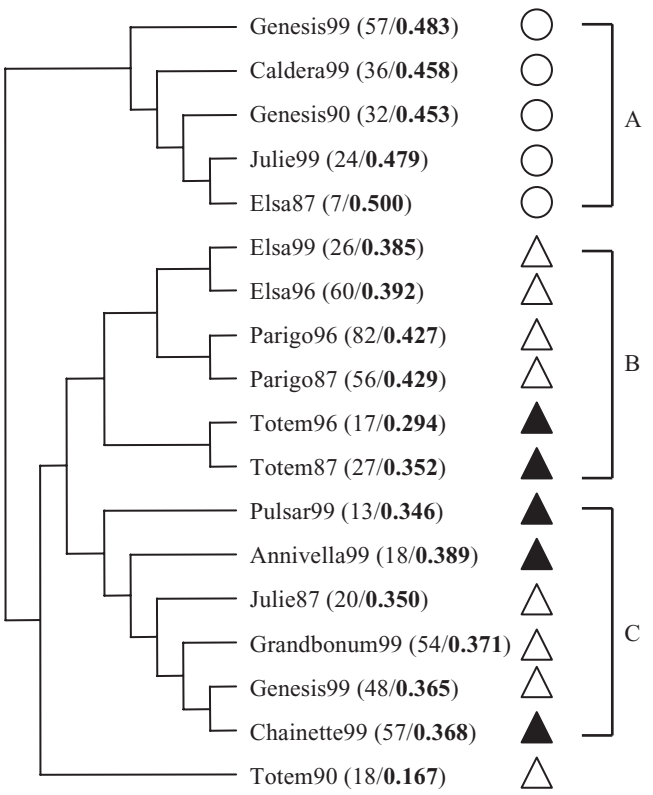

Figure 2. UPGMA tree based on PGM-1 allele frequencies using Rogers' genetic distance (1972). Habitat type according to the classification given in figure 1. Number of individuals per allele 90 frequency is given in parentheses for each population sampled.

Table 3. Exact tests on the whole dataset for inter-habitat differentiation.
(p-values above the diagonal are for the whole dataset whereas below the diagonal only the Totem90 population is excluded. $^{*}p < 0.05$; $^{**}p < 0.01$.)

	diffuser	white smoker	black smoker
diffuser		0.001^{**}	0.018^{*}
white smoker	0.003^{**}		0.247
black smoker	0.023^{*}	0.259	

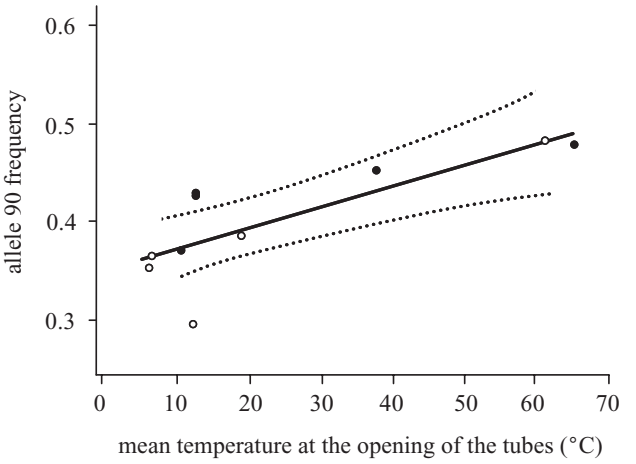

Figure 3. Correlation between the mean temperature at the opening of the tube of *A. pompejana* and allele 90 frequency for each population for which temperature data are available (see table 1). Open circles: submersible probe measurements; filled circles: temporal measurements. Regression line: $T = 0.35161 + 0.00215f$; T, temperature (degrees Celcius); f, frequency of allele 90. Confidence interval 0.95 (dotted line). Significant correlation is detected (Spearman rank correlation: $R = 0.875$; $p = 0.0005$).

easily understand whether 'hot' environments may change the regulation of specific metabolic fluxes and thus lead to the co-occurrence of allozymes that are constrained by a trade-off between molecular stability and catalysis (Somero 1995).

Our results therefore tend to demonstrate that allele 90 is more adapted to endure temperatures higher than 20 °C and is selected differentially between newly active chimneys and older chimneys (white or black smokers) according to the thermal regime.

(c) *Which microevolution model can maintain polymorphism at* Pgm-1 *locus?*

Daily wide fluctuations of the vent chemistry and especially the temperature at hydrothermal vents (Chevaldonné 1996) were proposed as a first explanation for the maintenance of polymorphism in *A. pompejana* (Jollivet *et al.* 1995*a*,*b*). Indeed, the maintenance of two equifrequent alleles (90 and 100) at the *Pgm-1* locus in most populations of *A. pompejana* along the northern EPR encourages the hypothesis of balancing selection with an overdominance of the heterozygote *Pgm1* 90/100, as already proposed in other marine organisms (Karl & Avise 1992). Such a selective regime should display heterozygote excesses in populations if no other evolutionary forces act. In our study, all but one of the 18 sampled populations were in Hardy–Weinberg equilibrium (no significant heterozygote excess) and a mean heterozygote deficiency was even observed. Although not demonstrated here, the balancing selection hypothesis could not be ruled out as other evolutionary forces could counterbalance a heterozygote excess. To confirm whether balancing or directional selection occurs at the *Pgm-1* locus, survival and reproductive success of individuals have to be explored by surveying the mortality of individuals' *Pgm1* 100/90, *Pgm1* 90/90 and *Pgm1* 100/100 under 'low' and 'high' thermal regimes through *in vivo* experiments and by estimating their fecundity according to habitat.

Because the spatial heterogeneity of the vent conditions seems more relevant in shaping frequencies at the *Pgm-1* locus, our results favour a model of diversifying selection, with allele 90 being more adapted to the 'warm' environment (diffusers) than allele 100. The most parsimonious explanation is that allele 90 is more efficient at high temperatures and increases the fitness of individuals' *Pgm1* 90/90 and *Pgm1* 90/100 during the colonization phase. A similar hypothesis was proposed for the intertidal snail *Littorina saxatilis* to explain the occurrence of an allozyme microcline at the aspartate aminotransferase locus associated with the position of individuals on the shore (Johannesson *et al.* 1995). However, in this particular case, the microcline was more pronounced and might also reflect disruptions in gene flow as proposed by Engel & Destombe (2002) to explain local adaptation to desiccation of the alga *Gracilaria gracilis*. A more complete population

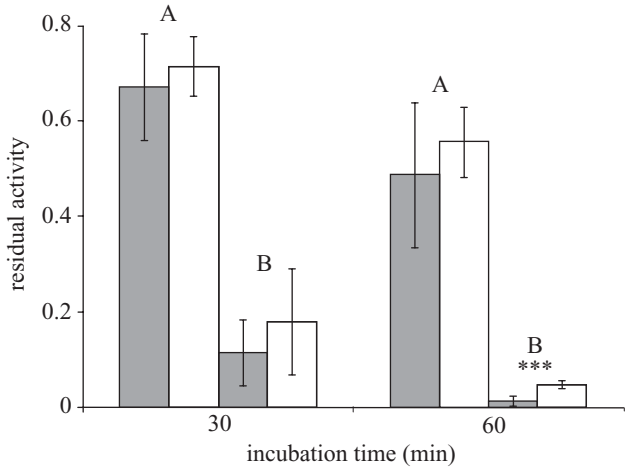

Figure 4. PGM residual activity (initial velocity $_{\text{after incubation}}$ / initial velocity $_{\text{before incubation}}$) after 30 or 60 min incubation at 35 °C (A) or 45 °C (B). Five homozygotes 90/90 (open bars) and five homozygotes 100/100 (grey bars) were assayed for each incubation temperature. For the longest incubation at the highest temperature, differences between the two groups are significant (Student's *t*-test; $^{***}p < 0.001$).

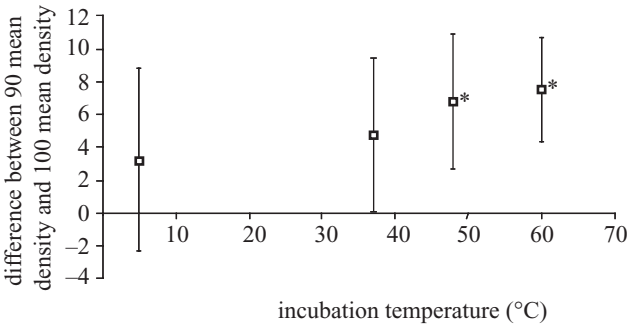

Figure 5. Mean density of allele 90 minus mean density of allele 100 calculated by SCION IMAGE software after starch gel electrophoresis of four heterozygotes 90/100 and incubation of slides (replicates) at four temperatures (5, 37, 48 and 60 °C). Mean value and standard deviation (four heterozygotes) are given for each temperature (Student's *t*-test; $^{*}p < 0.05$).

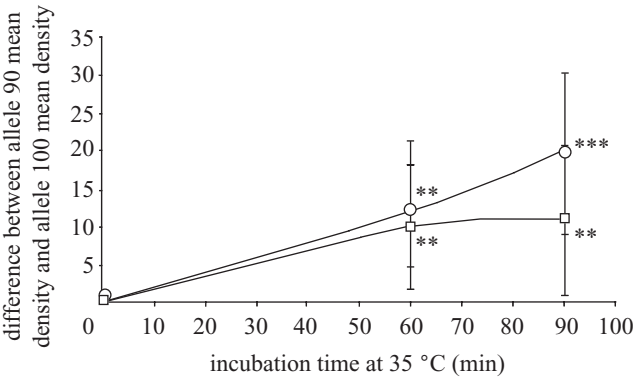

Figure 6. Mean density of allele 90 minus mean density of allele 100 calculated by Scion Image software after incubation of crude protein extracts for 0 (kept at 4 °C), 60 and 90 min at 35 °C and polyacrylamide native gel electrophoresis of 10 heterozygotes 90/100. Total protein quantity loaded on the gel: 50 μg (squares) or 100 μg (circles). Mean value and standard deviation (10 heterozygotes) are given for each incubation time and total protein quantity (Student's *t*-test; $^{**}p < 0.01$; $^{***}p < 0.001$).

genetics study performed for the mannose phosphate isomerase of the barnacle *Semibalanus balanoides* (Schmidt & Rand 1999), using other allozyme loci as neutral markers, also proposed an additive model of selection to explain why diversifying selection occurs at such a locus in this barnacle. Although differences in allele frequency are weak between habitats, our results suggest in a similar fashion that diversifying selection driven by temperature acts on *A. pompejana* populations. Because our study was performed on a small spatial scale (less than 1 km), one can propose that selection is probably strong enough to counterbalance high gene flow. However, if allele 90 is favoured in colonists, why does not it spread throughout the whole population?

To counteract the spread of allele 90 throughout the whole population, allele 90 must be counterselected or allele 100 must be favoured in the 'cold' environment (white or black smokers). However, asymmetrical migration rates between habitats could also be invoked to maintain an unstable polymorphism for more than 15 000 generations (P. Piccino, GeneWright's simulations (data not shown)). From our dataset, there is no argument allowing us to propose that allele 100 could be advantageous in the 'cold' environment. If so, our data could fit the model of Levene (1953), the first up-to-date theoretical model of selection to examine the effects of spatial variation of fitness in a heterogeneous environment characterized by a patchwork of two distinct habitats where migration occurs at random. In our case, the hydrothermal vent environment could be simplified as a mosaic of two distinct habitats (young versus old chimneys) in which the migrant pool is proportional to the number of individuals living in each niche (Hedrick 1986). Previous temporal surveys of 13° N / EPR sites indicate that young diffusers correspond to a small proportion of the vent habitat (less than 8%; D. Jollivet, personal observation). In this simple selection-by-habitat model, an additive model of fitness of the three main genotypes led to a significant increase of the allele 90 frequency in the 'hot' habitat and the maintenance of polymorphism. In addition, temporal heterogeneity would enhance the maintenance of polymorphism by reducing values of the selection coefficient (Hedrick 2000). If physical or chemical parameters are cycling with the tidal regime as shown by Chevaldonné *et al.* (1991), differential selective pressure could favour either allele 90 or allele 100 in such a way that a polymorphism is maintained. However, Levene's model does not take into account frequencies driven by habitat changes. Thus, these two models could act together to widen the conditions for a stable polymorphism. Habitat selection could also act in shaping frequencies at the *Pgm-1* locus if there is a selective choice of habitat, with individuals carrying the *Pgm1* 90/90, and/or *Pgm1* 90/100 alleles being colonists, and individuals carrying the *Pgm1* 100/100 allele true migrants (Olivieri & Gouyon 1997).

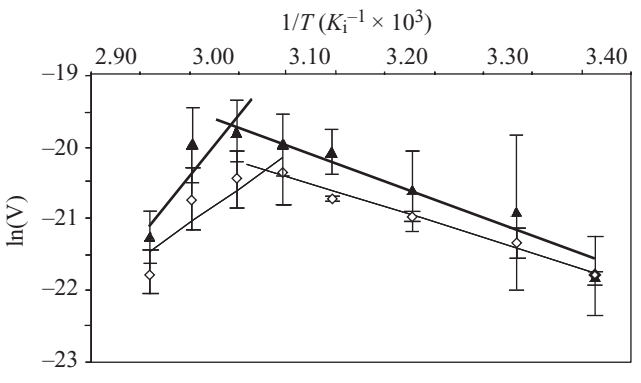

Figure 7. Arrhenius break graph constructed as a function of initial velocities ln(V) for each homozygote and $1/T$ (expressed in kelvin). Mean values and standard deviations for two pools of five crude protein extracts are plotted for each genotype (filled triangle: homozygote 90/90; open diamond: homozygote 100/100). After conversion, the thermal optimum is 52 °C for homozygotes 100/100 and 55 °C for homozygotes 90/90 (15 min assays).

4. CONCLUSION

(a) *Is the* Pgm-1 *locus under selection?*

Our biochemical experiments and population genetics analyses strongly suggest that PGM alleles are under strong diversifying selection that is probably minimized by demographic effects due to vent instability and the way in which *A. pompejana* colonizes new vents. However, despite sharp thermostability and activity differences between *Pgm1* alleles, it is still possible that selective forces act on other loci in strong linkage disequilibrium with the *Pgm-1* locus (no other polymorphic enzymes besides PGM have been genotyped here). Other physico-chemical parameters could also induce the selective effects observed here. More work is required to fully understand the impact of selective forces at the molecular level and how PGM polymorphism is maintained in such a heterogeneous habitat (asymmetric migration from one habitat to another or a cost on allele 90).

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