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RESEARCH ARTICLE

Understanding climate change response in the age of genomics

Gene expression plasticity, genetic variation and fatty acid remodelling in divergent populations of a tropical bivalve species

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Abstract

1. Ocean warming challenges marine organisms' resilience, especially for species experiencing temperatures close to their upper thermal limits. A potential increase in thermal tolerance might significantly reduce the risk of population decline, which is intrinsically linked to variability in local habitat temperatures.
2. Our goal was to assess the plastic and genetic potential of response to elevated temperatures in a tropical bivalve model, *Pinctada margaritifera*. We benefit from two ecotypes for which local environmental conditions are characterized by either large diurnal variations in the tide pools (Marquesas archipelago) or low mean temperature with stable to moderate seasonal variations (Gambier archipelago).
3. We explored the physiological basis of individual responses to elevated temperature, genetic divergence as well as plasticity and acclimation by combining lipidomic and transcriptomic approaches.
4. We show that *P. margaritifera* has certain capacities to adjust to long-term elevated temperatures that was thus far largely underestimated. Genetic variation across populations overlaps with gene expression and involves the mitochondrial respiration machinery, a central physiological process that contributes to species thermal sensitivity and their distribution ranges.
5. Our results present evidence for acclimation potential in *P. margaritifera* and urge for longer term studies to assess populations resilience in the face of climate change.

KEYWORDS
acclimation, fatty acids remodelling, genetic divergence, physiology, RNAseq, thermal plasticity

1 | INTRODUCTION

Temperature is a major determinant of marine species distribution (Hochachka & Somero, 2002). The expected increase in temperature caused by global warming is particularly challenging for stenothermal species and for those that already experience temperatures close to their upper thermal limit (Dahlke et al., 2020; Tewksbury et al., 2008). In the tropical black-lip pearl oyster *Pinctada margaritifera*, the physiological thermal performance peaks at *c*. 28–29°C, while at 22 and 34°C, growth and reproduction are suspended (Le Moullac et al., 2016). The seawater temperature in French Polynesian archipelagoes is usually below 31°C, with limited daily and annual variation (Van Wynsberge et al., 2017). However, temperatures recorded across the region reveal that in some lagoons *P. margaritifera* experiences temperatures warmer than their thermal optimum for ~121 days per year. These populations therefore live close to their upper physiological limits (Le Moullac et al., 2016), a threat that is now being amplified by ongoing climate change. The contrasting thermal regimes that exist across French Polynesian lagoons offer an opportunity to explore mechanisms of thermal adaptation. Particularly interesting is the divergent intertidal population of the Marquesas archipelago, which experiences temperatures that can vary between 27 and >34°C within a daily tide cycle (Reisser et al., 2019). The temperatures experienced by the Gambier population, in contrast, are considerably lower and governed by a seasonal rather than a daily tidal cycle (sea surface temperatures: mean 25.04°C ± 1.80 *SD*, min.: 21.38°C, max.: 29.35°C, recorded between 2015 and 2017). How these organisms perform under heat stress and which potential physiological capacities allow the intertidal population to cope with high and fluctuating temperatures remain unknown. We know however that Marquesas populations are genetically and phenotypically distinct from *P. margaritifera* populations present in other archipelagos, reflecting possible local adaptation and/or acclimation potential (i.e. phenotypic plasticity) (Reisser et al., 2019).

Phenotypic plasticity allows individuals to survive to stochastic and/or variable environmental conditions when response time is shorter than the speed and the intensity of the change (Fox et al., 2019; Seebacher et al., 2015). Chronic exposure to sublethal elevated temperature should request specific and reversible plastic processes to allow individuals acclimate to these conditions. Among the biological processes involved in the thermal response, membrane remodelling is considered a pivotal mechanism for preventing membrane dysfunction. The remodelling of membrane lipids in poikilotherms, via changes in phospholipid head groups, fatty acid composition and cholesterol content, allows maintaining membranes fluidity and functions in elevated temperature conditions, a process known as homeoviscous adaptation (HVA) (Hazel, 1995). In temperate bivalve species (e.g. the scallop *Placopecten magellanicus*, the oysters *Crassostrea gigas* and *C. virginica* and the blue mussel *Mytilus edulis*), the long-chain polyunsaturated fatty acids increase with decreasing acclimation/acclimatization temperature (Delisle et al., 2020; Hall et al., 2002; Pernet et al., 2007). Data remain scarce for tropical species, yet the giant clam *Tridacna maxima* shows membrane lipid remodelling in response to temperature consistent with HVA (Dubousquet et al., 2016). Additionally, genome-wide transcriptomic approaches have also uncovered additional details on the repertoire of molecular and cellular mechanisms responsible for thermal plasticity (Evans, 2015; Lee et al., 2016; Yeaman, 2015). For example, transcriptomics and proteomics have revealed the complex interplay between chaperone regulation, redox and metabolic shifts exhibited by thermally stressed bivalves (Chen et al., 2019; Tomanek, 2010).

Environmental pressure also confers genetic-level adaptation. For instance, natural selection has contributed to the higher thermal tolerance and increased invasive potential of *M. galloprovencialis* compared to other *Mytilus* species (Popovic & Riginos, 2020; Saarman et al., 2017). In *C. gigas*, thermal performances shaped the fine-scale genetic structure of populations living in a highly connected and temperature-contrasted area (Li et al., 2018). The genetic divergence observed among these populations of *C. gigas* also correlates with phenotypic divergence in response to thermal stress and suggests that plasticity might be favourably selected for populations subject to more variable environments (Li et al., 2018). Efforts to quantify the contributions of genetics and plastic responses to temperature exhibited by organisms are challenging but critical for implementing sound conservation policies and ensuring the persistence of natural and managed populations under climate change (Chevin et al., 2010; Duarte et al., 2020). In the Pacific basin, local adaptation associated with heterogenic environmental conditions was suggested to explain the weak albeit observed structure of the *P. margaritifera* population in five main clusters (Lal et al., 2017). In French Polynesia, the *P. margaritifera* populations from Marquesas and Gambier archipelagoes live under contrasting environments and exhibit strong genetic differentiation (Reisser et al., 2019); however, no study has explored the possible adaptive potential across heterogenous habitats.

Using two ecologically divergent populations (Gambier population: stable—older environment and Marquesas tidepool population: highly variable—hotter environment), our goal was to (a) quantify the plastic (transcriptomic and lipidomic) response to temperatures over different time-scales (from days to several weeks) and (b) to assess genetic divergence possibly involved in the response to elevated temperatures. We expected that Marquesas population would present specific genetic or plastic variations to resist in highly fluctuating environment.

2 | MATERIALS AND METHODS

Marquesan breeders were collected from tide pools (Ua Pou; French Polynesia; 9°25′18.7″S, 140°03′07.5″W) and air-shipped to Ifremer facilities (Taravao, Tahiti, French Polynesia; 17°48′31.7″S, 149°17′41.4″W) in October 2015. Five females were crossed with seven males following a full-factorial breeding scheme according to a standard procedure developed in the laboratory (Ky et al., 2018). Concurrently, in a local private hatchery at Gambier (CA Regahiga Pearls Farm & Hatchery; 23°07′05.2″S, 134°58′56.8″W), eight

females were crossed with 10 males (full-factorial breeding scheme). Spat of the F1 progenies were placed in their respective location (Tahiti or Gambier) for a 2-year period. During this time, no extreme temperature values were recorded during this time (max. temperature of 30.1 and 29.3°C in Tahiti and Gambier respectively), suggesting no additional thermal stress forced by transfer. Detailed information on temperatures experienced by F1 progenies is provided in Figure S1. Progenies from the Marquesas and Gambier crosses were then transferred to a lagoon nearby Ifremer (Tahiti) and maintained for 1 month in common garden before the acclimation period in the laboratory (September 2017). At the start of the experiment, 16 individuals per population were sampled randomly to examine the initial physiological condition. Populations were not significantly different in mean wet weight (g) of 30.80 ± 6.96 and 35.04 ± 8.74 (*SE*) for Gambier and Marquesas, respectively (*t* test; $t = -1.53$; $p = 0.14$), and mean shell width (cm) of 6.60 ± 0.60 and 6.70 ± 0.58 (*SE*) for Gambier and Marquesas respectively (*t* test; $t = -0.51$; $p = 0.61$). Individuals from Marquesas were longer than those from Gambier, with a mean length (cm) of 6.39 ± 0.49 and 6.82 ± 0.46 (*SE*) for Gambier and Marquesas respectively (*t* test; $t = -2.56$; $p = 0.02$). Individuals sampled at the start of the experiment were all males. This male bias is expected as *P. margaritifera* is a protandrous hermaphrodite meaning they all start being males to turn females after around 3 years. The choice of using only 2-year-old F1 individuals was motivated to (a) reduce the possible gene expression variability driven by differences in age classes or sex even if no sex-specific difference related to biomineralization or to stress response has been reported to our knowledge and to (b) use individuals of a reasonable size to increase the number of replicates per tank. Specimens used in this study were transferred and held under a special permit (no specific permit number provided) delivered from the French Polynesian government. No further ethical approval was required.

2.1 | Experimental design, physiological measurements and tissue sampling

After the 1-month acclimatization in the lagoon (September 2017; temperature), animals were further acclimated for 3 weeks to the laboratory conditions at 27°C (i.e. ambient). A total of 192 male individuals (96 individual per population) were placed in 20-L tanks nested in flow-through raceways. Each tank contained 12 individuals, six per population (Figure S2). Animals were supplied continuously (ad libitum) with a mix of *Isochrysis galbana* and *Chaetoceros minus* microalgae, at a daily ratio equal to 7%–8% dry-weight-algae/dry-weight-oyster, for limiting pseudo-faeces production (Gardon et al., 2018).

At the end of the acclimation period (6 October), animals were subjected to a gradual decrease/increase in temperature, reaching either 23°C (cooling), 32 or 34°C (warming) within 2 days. Another group was kept at 27°C. These temperatures reflect normal/reference (27°C), extreme-cold (23°C), warm (32°C) and extreme-hot (34°C) conditions for oysters in French Polynesia. Note that the temperature treatments were not replicated due to logistical constraint, thus precluding our ability to unambiguously separate the treatment effect from among-tank differences (Hurlbert, 1984). Temperatures were recorded hourly using iBWetLand biochip (Alpha Mach inc., Canada) throughout the experiment. Seawater was heated by an electric heater or cooled with a heat exchanger (calorie exchange with cold freshwater), both operated by a temperature controller.

Animals were maintained at their respective temperatures for 48 days. Individuals were sampled on days 2 and 48 for molecular (transcriptomics) analyses, on days 48 for lipids and on days 0, 2, 28 and 48 for biometry (wet weight, length and sex determination). Each sampling consisted of four oysters per combination of population × temperature × time. Sex was determined by microscopic examination of the gametes. Two pieces of mantle tissue from the same area (*c.* 1 cm^2) were stored in RNA-Later at −80°C or in chloroform/methanol (2:1, v/v) at −20°C for molecular and lipid analyses respectively. The sampling was done at the same time of the day across all sampling dates to prevent confounding circadian rhythm effects on gene expression (Doherty & Kay, 2010).

2.2 | Lipids

Sample handling, storage and analysis followed the recommendations for best practices for lipid research in aquatic sciences (Couturier et al., 2020). The detailed protocol of fatty acid quantification is available in Supplementary Material. Fatty acid compositions were expressed as the mass percentage of the total fatty acid content. Here we particularly focused on polyunsaturated fatty acids (PUFAs) and unsaturation index, that is the average number of double bonds per acyl chain, because they generally vary with temperature in a way consistent with HVA in other bivalves (Hazel, 1995; Pernet et al., 2007).

2.3 | Statistics for biometry and fatty acids

Statistics were done using R (R Core Team, 2012), and differences were considered significant when $p < 0.05$, unless otherwise specified.

2.3.1 | Biometry

Variation in weight data was assessed for each time point using ANOVA with population (two levels), temperature (four levels) and time (three levels) as explanatory variables. Normality and homoscedasticity were assessed visually and statistically (Shapiro and Levene tests respectively). When significant effects were detected, Tukey's HSD post hoc tests were used to assess variation for factors with more than two levels. Variation in the proportion of sex categories was assessed using the Chi-squared test. When significant effects were detected, we computed the contribution (%) of each sex category to the difference observed (calculated as follows: contribution (%) = r^2/χ^2, with r the Pearson residual for each cell (standardized residual) and χ^2 the Chi-square statistic).

2.3.2 | Fatty acids

We used a subset of the most common fatty acid type (at least 1% of the total FA content) to assess the variation explained by both temperature and population on the FA content on day 48. We first computed a Euclidean distance matrix on the FA content across individuals and performed a principal coordinate analysis (PCoA) on this Euclidean distance matrix. Only PCo factors showing a relative eigenvalue higher than 2% ($n = 6$) were retained for the analysis, accounting for 97.3% of the total variance. The Euclidean distance and the PCoA were computed, respectively, using the functions '*daisy*' and '*pcoa*' available in the R package APE (Paradis et al., 2004). We first produced a stepwise model selection on variables temperature and population using the function '*ordistep*' in the VEGAN R package (Oksanen et al., 2012). A distance-based redundancy analysis (db-RDA) was then computed using the retained PCo factors as a response matrix and the variables temperature and population as the explanatory factors. Partial db-RDAs were produced to test for the effect of rearing temperature or population alone, while controlling for the other variable.

We used two-way ANOVA with interaction to assess the effects of population and temperature on specific FA on day 48. Pearson's correlations were used to visualize pairwise similarities across FA classes; associations were considered significant when $p < 0.01$.

2.4 | RNA extraction and sequencing

Total RNA was extracted from *P. margaritifera* by lacerating the mantle tissues with a scalpel and rinsing with 1X PBS. Cellular lysis was induced with 1.5 ml of TRIzol (Invitrogen, USA) according to the manufacturer's recommendations. The supernatant was transferred into a 2-ml tube and incubated for 10 min in ice. The phase separation was achieved by adding 300 µl of chloroform followed by centrifugation at 12,000×*g* for 12 min at 4°C. Total RNA from each individual was subjected to a DNase treatment using Qiagen's RNA cleanup kit (USA). Total RNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo-Fisher, USA), and quality was further evaluated using a Bioanalyzer 2100 (Agilent, USA). High-quality RNA was sent to McGill University's 'Genome Quebec Innovation Center' (Montréal, QC, Canada) for Nextera XT (Illumina, USA) stranded library preparation and sequencing on an Illumina HiSeq4000 100 bp paired-end platform, randomly multiplexing 24 samples per lane.

2.5 | Mapping and coding region genotyping

Raw reads provided by RNA sequencing were filtered for quality, length and putative contaminant and mapped against *P. margaritifera* reference genome (Le Luyer et al., 2019). Detailed pipeline of the RNAseq analysis is provided in Supplementary Material and codes are available online (https://github.com/jleluyer/gamma).

For variants discovery (single nucleotide polymorphisms; SNPs), mappings were processed with GATKv-4.0.3.0 suite (Auwera et al., 2013) following recommendations for RNAseq data. SNP discovery and filtration information are provided in Supplementary Material.

2.6 | Genetic structuring, differentiation and candidate SNPs

The VCFR package (Knaus & Grünwald, 2016) was used to integrate the SNP dataset into R. We did two exploratory analyses: first, we performed a principal component analysis (PCA) using the ADEGENET package (Jombart & Ahmed, 2011) and the '*glPca*' function, with three PC axes kept, explaining 25.47%, 4.92% and 4.04% of the variance, for PC1, PC2 and PC3 respectively. To search for putative candidate SNPs that significantly contribute to the differentiation of genetic clusters, we used the R package PCADAPT using optimal K of 2. A variant was considered as putative outlier if $q < 0.05$. We further explored the effect of the SNPs on the protein sequences (synonymous vs. non-synonymous) using SnpEff (Cingolani et al., 2012).

Gene ontology (GO) enrichment for transcripts carrying candidate SNPs was conducted using Fisher's test in the GOATOOLS python library (Klopfenstein et al., 2018) implemented in '*go_enrichment*' GitHub repository (https://github.com/enormandeau/go_enrichment). We retained enriched GO if $p < 0.001$. Finally, population genetic differentiation was estimated by computing the pairwise Fst and associated *p*-values using 1,000 permutations across populations with the R package STAMPP (Pembleton et al., 2013). Finally, per site nucleotide diversity (π) using VCFtools v0.1.16 (Danecek et al., 2011) and kinship estimates (KING indexes) were evaluated with the '*sngdsIBDKING*' function implemented in the SNPRELATE R package with prior linkage disequilibrium (LED) pruning filtering (*ld.treshold* = 0.1).

2.7 | Variance partitioning analysis of gene expression

For gene expression quantification, raw counts were computed using HTSeq v0.9.1 software (Anders et al., 2015) based on the gene feature information in the reference genome (Le Luyer et al., 2019) using htseq-count tools in '*mode:union*'. Genes showing residual expression (<1 count per million; CPM; in at least four individuals) were removed for downstream analysis using DESEQ2 v1.22.1 R package (Love et al., 2014). Counts were prior transformed using the '*vst*' function implemented in DESEQ2 R package. To assess the relative contribution of population, time and/or temperature on the gene expression of *P. margaritifera*, we followed the same procedure as for the lipid analysis using the combination of RDAs. For gene expression, the selection of the optimal number of PCoA axes was done using the broken stick approach (Legendre & Gallagher, 2001; Legendre & Legendre, 2012), which resulted in a total of 17 axes explaining 62.89% of the total variance. Three partial db-RDAs and analysis of variance (ANOVA, 1,000 permutations) were used to validate that effect of each factor alone,

while controlling for the other factor. The effect of a given factor was considered significant when *p*-values were <0.05.

2.8 | Gene network analysis

Signed co-expression network was built based on the variance stabilized read counts (*vst* transformed) using the R package WGCNA following the protocol developed by Langfelder and Horvath (2008) (for more details, see Supplementary Material). Only modules with significant correlation with one of the condition factors (population, time or temperature; $p < 0.001$) were conserved for downstream functional analysis. GO enrichment analyses were conducted for each module separately using the same procedure as described above (*Genetic structuring*, *differentiation* and *candidate SNPs*).

2.9 | Identification of differentially expressed genes affected by extreme elevated temperature

We explored variation in gene expression using pairwise group comparisons (per time × population × temperature) using contrasts and Wald tests. For contrasts, genes were considered differentially expressed if false discovery rate (FDR) < 0.05 and absolute fold-change (|FC|) was >2. GO enrichment analyses were conducted following same procedure than described above (*Genetic structuring*, *differentiation* and *candidate SNPs*) and enrichment was considered significant for Bonferroni adj. $p < 0.01$.

We finally explored the genotype-by-environment interaction (GEI) effect in gene expression across temperatures, populations for each sampling time separately using a series of GLMs and likelihood-ratio tests (LRT) implemented in the DESeq2 v1.22.1 R package (Love et al., 2014). We focus on GEI effect between control (27°C) and extreme elevated temperature (34°C). The full model containing the interaction term was retained if false discovery rate (FDR) < 0.05.

3 | RESULTS

3.1 | Individual-level performance

Mortality was negligible throughout the experiment (<2%) and independent of the acclimation temperature or origin. Gametes were detected in oysters on all sampling dates and temperatures, but 75% of individuals had no gametes after 28 days at 34°C (Figure 1). The absence of gametes at 34°C on day 28 was the main contributor (40.40%) explaining the effect of temperature (Pearson's $\chi^2 = 19.8$; $p = 0.02$). The absence of gametes is considered as a regression of gonads that is commonly observed in mature individuals subjected to stress affecting the energy budget (Gardon et al., 2018; Teaniniuraitemoana et al., 2016). The wet weight of animals was unaffected by temperature or population, but varied across time. Individuals sampled on day 48 were heavier than those sampled earlier (Tukey's HSD; $p = 0.02$ and 0.04 for the day 48–day 2 and day 48–day 28 contrasts respectively; Figure S3).

3.2 | Genetic structuring, differentiation and candidate SNPs

We removed six individuals from the analysis that showed more than 15% missing genotypes and identified 55,546 SNPs genotyped in 66 individuals (30 individuals from Gambier and 36 individuals from the Marquesas). The PCA shows the segregation of the two populations across PC1 with high Fst value of 0.21, despite some substructure observed in the Marquesas population (Figure 2a). Nucleotide diversity was comparable across populations (0.32 ± 0.16 and 0.32 ± 0.16 for Gambier and Marquesas respectively) and kinship matrix suggests higher relatedness in Marquesas than in Gambier (Figure S4). In total, 887 SNPs were identified as outliers with *PCAdapt* (FDR < 0.05) belonging to 128 genes, with a mean *F*st of 0.59 [CI: 0.57–0.61]. These SNPs show enrichment ($p < 0.001$) for positive regulation of ion transmembrane transporter activity (biological process; BP), response to stimulus (BP), generation of precursor metabolites and energy (BP), regulation of mitochondrial membrane potential (BP), regulation of reactive oxygen species metabolic process (BP), mitochondrial inner membrane (cellular component; CC), RNA binding (molecular function; MF), antioxidant and oxidoreductase activity (MF). Among the genes containing outlier SNPs, we identify specific genes involved in mitochondrial functioning and regulation of oxidative damages in molluscs such as the heat shock protein 90-alpha 1 (HS90A), the NADH–ubiquinone oxidoreductase chain 5 (NU5M), the serine/threonine-protein kinase PINK1, mitochondrial precursor, the glutathione S-transferase 1 (GST1)-coding genes or the superoxide dismutase C (SODC). For the latter, the mutation is non-synonymous (Val74Ala, missense variant) and occurred in the protein β-chain.

3.3 | Fatty acids

After 48 days of exposure, the FA profile of oysters is strongly affected by temperature (RDA; ANOVA; $p < 0.01$; 33.49% of the total variance explained, Figure 3a), as indicated by the reduction of the unsaturation index (UI) with increasing temperature (Pearson's correlation; $R = -0.87$; $p < 0.001$; Figure 3b). Reduction of UI is mainly explained by a decrease in polyunsaturated fatty acids (PUFAs), including docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3, ANOVA; DHA, $F = 31.95$, $p < 0.001$; EPA, $F = 20.17$, $p < 0.001$), detected at higher temperatures. In contrast to these two PUFAs, arachidonic acid (ARA, 20:4n-6) increased with temperature (ANOVA; $F = 7.77$, $p < 0.001$). The remodelling of FA content was similar between populations (partial db-RDA; ANOVA; $F = 2.31$; $p = 0.07$) and no significant interaction (temperature × population) was detected for these FAs (ANOVA, $p > 0.05$). A detailed account of FA level contents is provided in the Table S1).

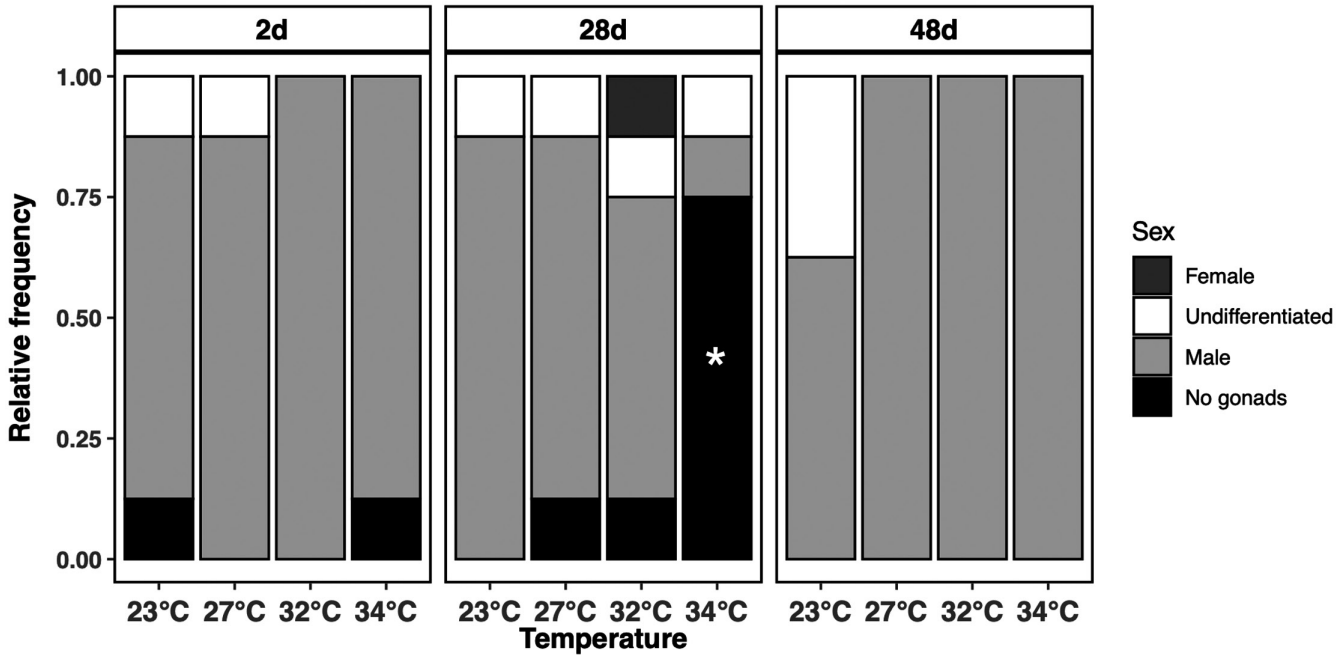


FIGURE 1 Categories of sex determination across sampling dates. Gametes were evaluated by gonad biopsy and microscopic examination. The asterisk represents the category contributing most to the difference across temperature treatments on day 28

FIGURE 2 Genetic variation across populations. (a) Clustering of populations through principal component analysis (PCA2021-12-30 5:14:00 PM) based on SNP variation (n = 55,546 SNPs). (b) RDA of gene expression level (n = 31,518 genes) measured when the set temperature was reached (day 2) and at the end of the experiment (day 48)

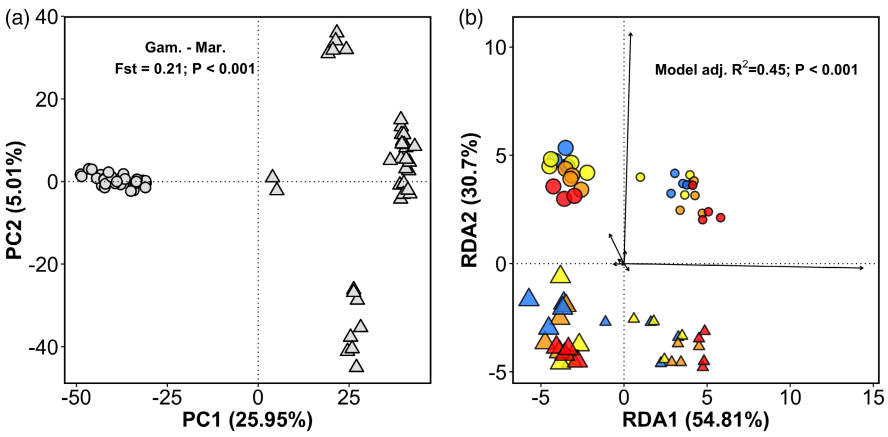
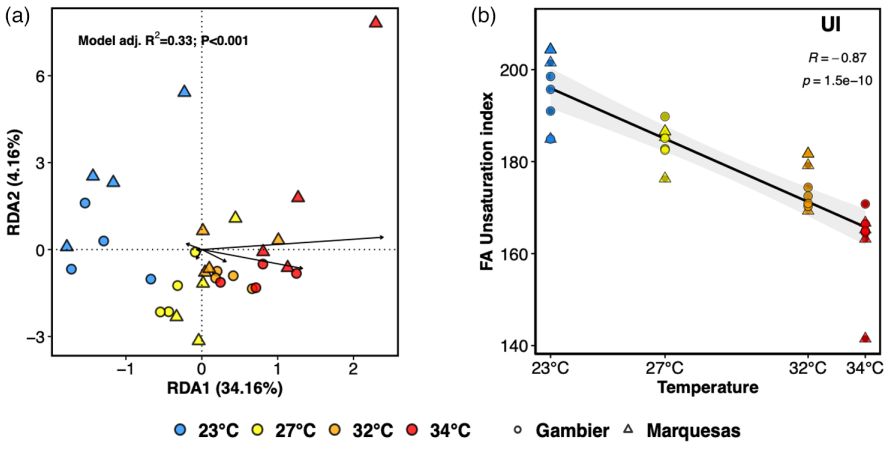


FIGURE 3 Fatty acid composition across temperature conditions and populations on day 48. (a) Redundant discriminant analysis (RDA) of fatty acid profile (% FA) across population and temperature treatment. (b) Pearson's correlation of unsaturated index



3.4 | Gene expression

Overall, we found 8.95 ± 1.67 M (*M* ± SD) uniquely mapped paired-end reads per individual (details of sequencing statistics is provided in Table S2). The RDA model that included time, temperature and population explained 43.6% of the total variance in gene expression (RDA; ANOVA; $p < 0.001$). Time alone accounted for 26.5% of the variance explained by the model (ANOVA; partial db-RDA; $F = 25.24$; $p < 0.001$). Gene expression was also affected by population (ANOVA; partial db-RDA; $F = 8.67$; $p < 0.001$) and temperature (ANOVA; partial db-RDA; $F = 7.19$; $p < 0.001$), which explained 10.2% and 6.9% of the variance explained by the model respectively.

3.5 | Functional gene expression variation across population, time and temperature

To identify modules of associated genes, we used a co-expression network analysis. We then examined the relationship between temperature, population and time on the expression of these modules. We identified 15 different modules encompassing a total of 31,481 genes.

3.5.1 | Temperature

We observed seven modules that are correlated significantly with temperature factors—three positively and four negatively ($p < 0.001$; Figure 4). One module (dark grey) is highly positively correlated with temperature variation (Pearson's correlation $R = 0.87$; $N = 85$ genes). This module shows enrichment for mRNA processing and splicing (BP), apoptotic process (BP) as well as binding (MF) and heat shock protein binding (MF). Among the top hub genes (defined by the highest module membership and the highest gene significance), we also identify several heat shock-related proteins including HSP70A1 and HSP74, HS90A and the DNnaJ homologue subfamily B4 (DNAJB4), which show elevated expression at ≥32°C. The antioxidant response seems negatively affected at higher temperatures (32 and 34°C) as suggested by the reduced expression of key actors of the defences against oxidative stress including GST1, microsomal GST1 as well as several thioredoxins (TRXR2, TXD17 and TXNRDR). Inversely, the black module ($R = -0.83$; $N = 1{,}083$) shows enrichment for chromatin binding (MF), mRNA binding (MF), oxidoreductase activity (MF), catalytic activity (MF), mitochondrion (CC), response to stimulus (BP) and regulation of DNA replication (BP). A complete list of genes with correlation with temperature and GO enrichment is provided in Table S3.

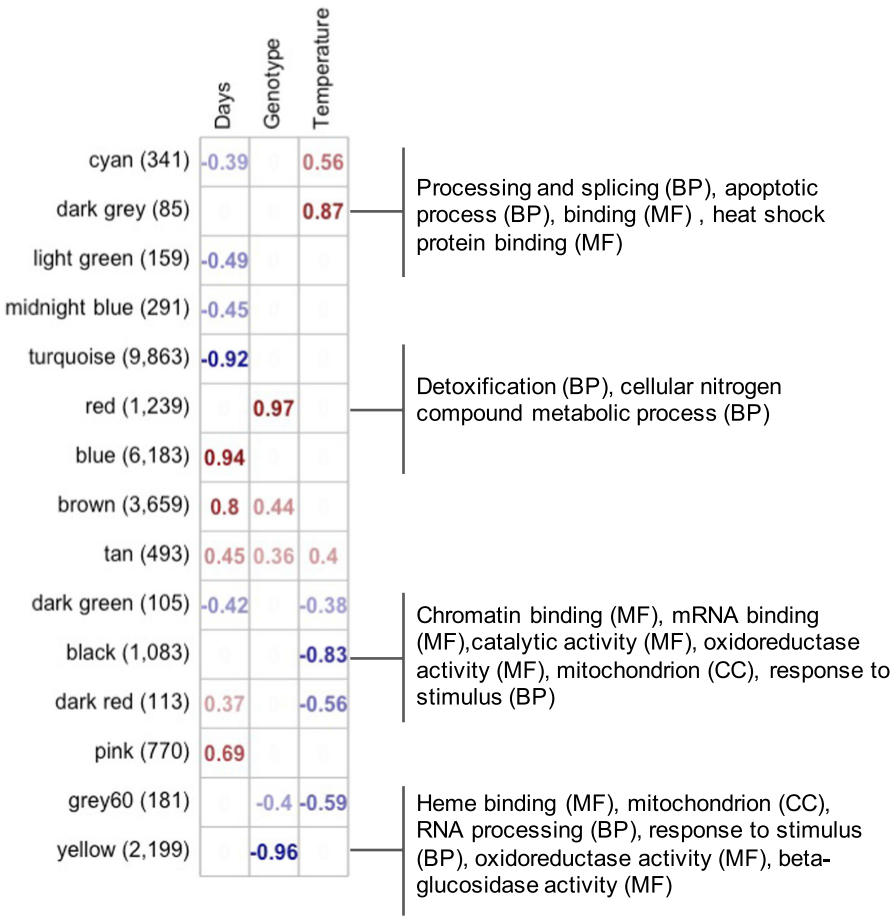


FIGURE 4 Gene expression analysis. Correlation matrix obtained for signed co-expression network of gene expression ($n = 31{,}481$ genes). Values in the cells represent significant ($p < 0.001$) Pearson's correlation of module eigenvalue to physiological parameters (top panel). Names (left panel) are arbitrary colour-coded for each module; values in parenthesis represent the number of genes per module. Empty cells indicate non-significant correlations ($p \geq 0.001$). Expression values were *vst* transformed prior to the analysis. Days were coded as numerical values (2 and 48); populations were coded as numerical (1 = Gambier; 2 = Marquesas); temperatures were coded as numerical (22, 27, 32 and 34). Main GO terms enriched associated with the major modules correlate with temperature or population

Other modules show correlation with temperature but also days and/or population. These modules are particularly relevant to explore the interaction effects on gene expression. For instance, the cyan module shows negative correlation with time ($R = -0.39$; $N = 341$) and positive correlation with temperature ($R = 0.56$). This module shows enrichment for immune system process (BP), response to stimulus (BP), carbohydrate metabolic process (BP), glucose homeostasis (BP), nitrogen compound metabolic process (BP), regulation of gene expression and epigenetic (BP), autophagy of mitochondrion (BP), protein binding (MF), ubiquitin-protein transferase activity (MF) or regulation of apoptotic process (BP). These results primarily confirm that apoptotic process is solicited in elevated temperature but that this process is reduced after 48-d exposure compared to day 2 at elevated temperature. Finally, the tan module shows correlation with all the factor with $R = 0.45$, $R = 0.36$ and $R = 0.4$ for times, population and temperature respectively ($N = 493$). This module shows enrichment for ribonucleoprotein complex subunit organization (BP), methylation (BP), RNA modification (BP), catalytic activity (MF), acetyltransferase activity (MF), RNA binding (MF), respirasome (CC), mitochondrion (CC) and mitochondrial matrix (CC).

We computed the magnitude of the shift (27–34°C) on day 2 based either on the whole dataset or the temperature-responsive genes based on the genes correlated with temperature ($p < 0.001$) as a proxy of the plastic potential. The magnitude of transcriptional shift is higher in the Gambier populations when using the whole dataset ($n = 31{,}481$ genes; $p_{mcmc} = 0.01$) or only temperature-responsive genes ($n = 1{,}879$ genes; $p_{mcmc} < 0.001$) suggesting higher plasticity potential for this ecotype (Figure 5).

3.5.2 | Population

Population factor is significantly associated with five modules, notably the red ($R = 0.97$; $N = 1{,}239$) and yellow ($R = -0.96$; $N = 2{,}199$) modules. The red module (correlating with higher expression in Marquesas) shows enrichment for detoxification (BP) and cellular nitrogen compound metabolic process (BP), while the yellow module shows enrichment for heme binding (MF), mitochondrion (CC), RNA processing (BP), response to stimulus (BP), oxidoreductase activity acting on paired donors with incorporation or reduction of molecular oxygen (MF) or beta-glucosidase activity (MF). A complete list of genes with correlation with population and GO enrichment is provided in Table S3.

3.6 | Molecular mechanism of short and long-term responses to elevated temperature

On day 2, contrasts (27 vs. 34°C) showed a total of 1,331 and 492 genes differentially expressed, for Gambier et Marquesas populations respectively. Common GO enrichments across populations include for enzyme-regulation activity (MF), regulation of cell death (BP), regulation of nitrogen compounds and protein metabolic processes (BP), regulation of proteolysis (BP) and regulation of response to stress (BP). The response to stress included common HSP, namely: HSP70A1 and HSP70B2. The HS cognate 71 kDa protein-coding gene was upregulated in the Gambier population only (Table S4). The HS90A, also identified in the outlier SNPs, was differentially expressed in Marquesas population only. We also found that oxidoreductase activity (MF) was enriched for the Marquesas population only. A complete list of GO enrichments and differential expression visualizations are provided in the Table S4 and Figure S5 respectively.

On day 48, fewer genes ($n = 182$ and 186, for Gambier and Marquesas respectively) were differentially expressed between the conditions 27 and 34°C. Compared to the short-term response, the expression of most inducible HSPs did not differ between 27 and 34°C, except for the Gambier population where the expression of HSP70A1 and HSP70B2-coding genes was higher at the warmer

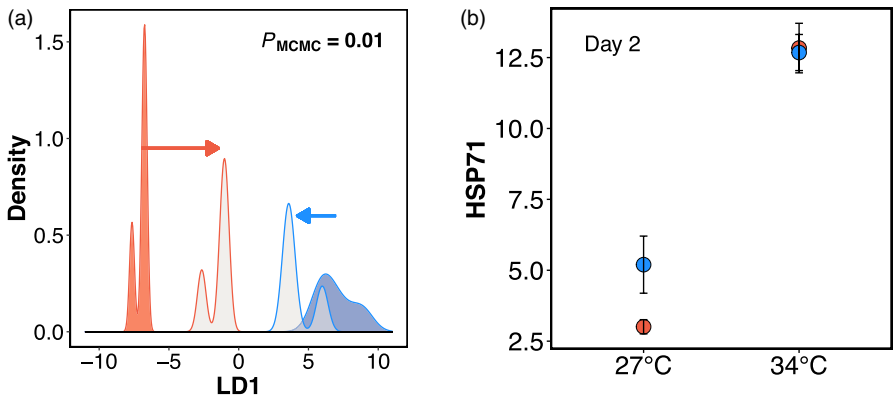


FIGURE 5 Gene plasticity variation across population. (a) Population-level variation gene expression plasticity at day 2 ($n = 31{,}481$ genes). Blue = Marquesas and red = Gambier. Solid fills indicate the control (27°C) temperature, whereas transparent fills indicate thermal stress (34°C). Arrows indicate the mean changes in the gene expression profiles as a result to exposure to thermal stress; (b) Representation of the *hsp71* expression across populations and temperatures at day 2. Blue = Marquesas and red = Gambier. Values are represented as log2 (counts + 1) counts per million (log2CPM)

treatment. Common genes differentially expressed for Gambier and Marquesas population show enrichment for binding (BP). Gambier population specifically show enrichment for protein-containing complex (CC) and extracellular region (CC), while Marquesas show enrichment for intracellular part (CC) and membrane-bounded organelle (CC).

The differential gene expression at 34°C varied over time. The chronic exposure (34°C-day 2 vs. 34°C-day 48) is associated with a reduced expression of genes involved in responses to stimulus (BP) functions, regulation of lipid metabolism processes (BP), carbohydrate derivative binding (MF), positive regulation of apoptotic process (BP), immune responses (BP), hydrolase activity (MF) and response to steroid hormones (MF). In contrast, genes with increased expression at the end of the experiment were involved in functions associated with mitochondrial respiratory chain complex I (CC), peptide biosynthetic process (BP) and chemical homeostasis (BP). We also observe that at 34°C on day 48, genes involved in the regulation of reactive oxygen species (ROS) are upregulated in Marquesas, but not in Gambier, individuals.

Finally, relatively few genes (50 and 109 on days 2 and 48 respectively) showed a significant pattern of GEI at 27 versus 34°C. To explore GEI on the plastic response to elevated temperature, we used 27°C (i.e. control temperature, closer to the optimal) and 34°C (i.e. extreme high temperature). For each of the time point, mean of absolute log2FC 27°C versus 34°C was higher for Gambier population compared to Marquesas (Wilcoxon, $p < 0.01$), further supporting the observation of larger plastic variation across populations. This observation is also true for HSP71A-coding gene (Figure 5b). At day 2, enrichment includes only the protein K48-linked deubiquitination and no significant enrichment was detected at day 48 (Bonferroni adj. $p < 0.05$).

4 | DISCUSSION

Ocean warming is a particularly challenging threat for tropical marine bivalve species because most of them live already near their upper thermal limits (Huey et al., 2009; Stuart-Smith et al., 2017). Here, we show that tropical sedentary organisms might be able to cope with abnormally elevated temperature over an extended period (several weeks), and that divergent populations that naturally experience contrasting habitat temperatures exhibit similar physiological mechanisms to tolerate and acclimate to thermal stress. Nevertheless, differences in genetic and transcriptomic profiles between populations suggest some possible plastic and adaptative divergence specifically in managing oxidative damages and mitochondrial functioning.

4.1 | Acclimation potential to long-term exposure to elevated temperatures in *Pinctada*

The acute detrimental effect of elevated temperature was partially compensated after chronic exposure, revealing an underestimated acclimation potential in *P. margaritifera*. We show that exposure to elevated temperature induces a rapid activation of molecular indicators of systemic stress (e.g. HSP) and is accompanied by a significant reduction in gamete production (75% of the individuals showing no gametes) at 28 days, all together strongly supporting the notion that 34°C lays beyond the species' thermal range (Kooijman, 2000; Sokolova, 2013; Sokolova et al., 2012). These results support previous observations that estimated the optimal temperature range of *P. margaritifera* to fall between 23 and 29°C, depending on the source population, and negative scope-for-growth after 7 days of exposure to 34°C (Le Moullac et al., 2016; Yukihira et al., 2000). However, after a longer exposure (48 days) to 34°C, we show that gonads were differentiating again, indicating that sufficient energy was available for basal somatic maintenance and to supply part of the reproduction needs (Sokolova, 2013). The discrepancy between the short- and long-term observations reveals an overall physiological capacity of *P. margaritifera* to somehow adjust to stressful thermal conditions. Similarly, the fatty acid unsaturation index shows a steady decrease, as predicted by the HVA theory (Sinensky, 1974), with no signs of plateau suggesting that at least the membrane is well-acclimated at 34°C after 48 days of exposure. These observations remain true for the time and conditions of the experiment (steady temperature control, feeding ad libitum, continuous oxygen supply and quality-controlled water) and might not imply the resilience of *Pinctada* populations when facing a combination of multiple stressors or that fitness-related traits (e.g. reproductive effort and fertilization rate) are not impacted. Furthermore, as thermal tolerance can increase from embryos to adults in marine ectotherms (Dahlke et al., 2020), studies based only on the response of adults might underestimate the effect of global warming on the species' resilience. Nevertheless, the thermal acclimation capacity has been largely underestimated, especially with the over-representation of short-term experiments (Dahlke et al., 2020), hence hampering the identification of the physiological and molecular mechanisms of acclimation. If both populations are apparently able to cope with extreme temperatures, further studies looking at the differences between populations' upper thermal limits should be informative on assessing population functional divergence.

4.2 | Mechanisms of acclimation to long-term extreme temperature exposure

The heat shock response (HSR) was triggered quickly after exposure to elevated temperature (32 and 34°C). Protein turnover chaperoned by HSPs is energetically demanding, representing up to 10% of the total protein synthesis costs (Semsar-kazerouni & Verberk, 2018). Therefore, covering both maintenance and reproduction costs under prolonged exposure to extreme temperatures, as we show here, would necessarily require other physiological adjustments, different from the highly energy demanding HSR. The transition (between 2 and 48 days) is characterized by the solicitation of genes involved in the mitochondrial machinery, notably via the respiratory

chain complex I. Besides its role on regulating apoptosis and ROS, the complex I is tightly correlated with thermotolerance capacity because it catalyses the entry of electrons into the mitochondria which represents a bottleneck of energy in ectotherms (Sokolova, 2021).

In parallel, we showed a strong remodelling of membrane fatty acids that is consistent with the HVA theory (Sokolova, 2021). Maintenance of membrane fluidity is an essential component of the acclimation process and also mediate respiration machinery (Sinensky, 1974). Our observations support previous data on marine temperate mollusc species exposed to elevated temperatures, which showed decreases in 22:6n-3 and 20:5n-3 with increasing temperatures (Budin et al., 2018; Hazel, 1995; Pernet et al., 2007). Therefore, lipid remodelling, specially PUFAs, is also a common mechanism of thermal acclimation in marine ectotherms including stenothermal tropical species. In previous studies, we found differences in remodelling of membrane lipids among populations of bivalves. For example, the pattern of lipid remodelling in response to temperature in oysters *C. virginica* and clams *Mercenaria mercenaria* shows strong intraspecific variation together with differences in metabolic and growth rates despite the fact that animals were maintained in the same environment (Parent et al., 2008; Pernet et al., 2006, 2008). Similarity in FA profiles suggests that HVA is common in *P. margaritifera* across its latitudinal range, hence, similarities in FA profiles indicate here similar physiological state across temperatures.

4.3 | Evidence for plastic and adaptive divergence between populations

Divergence between populations was observed at the gene expression levels and was pervasive after almost 3 months exposure to common environment (acclimatization in the wild and the acclimation in the laboratory). Overall, we observed that the magnitude of the response (shift) to elevated temperature is higher in Gambier population compared to Marquesas, implying a higher plastic potential for the former. Our observation supports the theory predicting that environmental conditions fluctuating over longer periods, typically seasons, might primarily trigger higher plasticity (Kenkel & Matz, 2017) and this plasticity capacity might have an underlying adaptive genetic variation (Li et al., 2018). On the other hand, in environments where conditions changes are too quick and of high intensity compared to the response time, adaptive genetic variation not associated with higher plasticity might be favoured (Kenkel & Matz, 2017). Among the genes under GEI, the expression of HSP71-coding gene suggest that Marquesas have baseline higher HSP transcript levels at 27°C, yet levels of expression reach similar maximum in both populations at 34°C. Furthermore, several of the genes showing different expression levels across populations overlap with differentiated genes such as genes involved in the respiration chain complex I gene or in the detoxification such as SODC-coding gene, also recognized as explaining both the thermotolerance potential and biogeographic distribution of ectotherm species (Schlichting & Pigliucci, 1998). Our design (F1 families) does not distinguish real genotype effects from putative confounded carry-over effects or transgenerational plasticity and further studies are thus required to define onset temperatures of different candidate genes (Barua & Heckathorn, 2004; Dong & Somero, 2009; Liao et al., 2019).

Nevertheless, the genetic and phenotypic (shell morphology and coloration) differences between Gambier and Marquesas populations (Kellermann et al., 2017) are maintained in the F1 progeny under laboratory conditions. Genetic variation segregating populations affects the oxidoreductase activity, mainly through NADH dehydrogenase activity, a complex involved in the mitochondrial oxidative phosphorylation system (OXPHOS). Mutation on the mitogenome, particularly the OXPHOS genes complex, can have a profound effect on respiration and overall energy budget in animals, hence playing a key role in their adaptation to climate change (Reisser et al., 2019). For example, variation in complex I genes (including NADH:ubiquinone oxidoreductase) differentiated ecologically divergent populations of temperate fishes (and F1 progenies) (Narum & Campbell, 2015). The physiological condition at the beginning of the experiment did not differ among populations, strongly suggesting that their responses were most likely genetically coded. A careful examination of the effect of maintaining F1 Marquesas population in different conditions than their F0 needs to be assessed. Yet, we interpret the data as a putative mark of local adaptation although further studies will be needed to differentiate putative adaptation from isolation by distance marks.

5 | CONCLUSIONS

Understanding and quantifying the response of organisms and populations under ongoing global changes is critical for environmental-resource managers and policymakers. We showed that *P. margaritifera* has acclimation capacities to elevated temperatures largely underestimated to date. Individual- and molecular-level responses showed major differences across conditions and time. The time component is too often ignored when interpretations are based only on short-term experiments, which can mislead predictions of long-term dynamics. We now urge for comprehensive studies integrating long-term monitoring (several developmental stages and/or several generation) that would integrate population diversity to draw significant patterns and accurate predictions. Finally, while the molecular response to temperature was largely shared between populations, there was a marked divergence for loci involved in the respiration machinery. Further studies will be needed to validate difference in maximum critical temperature across ecotypes and possible selective mortality that might shape local adaptation.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

F.P. and J.L.L. conceived the experiment; J.L.L. and C.S. conducted the experiment; C.-L.K. provided the biological material; C.B. carried out the laboratory benchwork for the transcriptomic data; F.P. conducted lipid analyses; H.R. provided SST calculations; C.J.M., C.R., F.P., G.L.M., J.L.L. and L.M. analysed the data; C.J.M., C.R., J.L.L. and F.P. wrote the manuscript. All co-authors contributed substantially to reviewed drafts and gave final approval for publication of the manuscript.

DATA AVAILABILITY STATEMENT

Raw sequencing RNAseq data have been made publicly available on NCBI's Sequence Read Archive (SRA) under bioproject accession PRJNA672139 and is available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA672139. Lipid data are deposited on the Dryad Digital Repository: https://doi.org/10.5061/dryad.8cz8w9gt2 (Le Luyer, 2022a). Codes are deposited on Zenodo https://doi.org/10.5281/zenodo.6459807 (Le Luyer, 2022b)

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