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Abstract

The use of marine mussels as biological models encompasses a broad range of research fields, in which the application of RNA analyses disclosed novel biomarkers of environmental stress and investigated biochemical mechanisms of action. Quantitative real-time PCR (qPCR) is the gold standard for these studies, and despite its wide use and available protocols, it may be affected by technical flaws requiring reference gene data normalization. In this study, stability of housekeeping genes commonly employed as reference genes in qPCR analyses with *Mytilus galloprovincialis* was explored under field conditions. Mussels were collected from farms in the Northwestern Adriatic Sea. The sampling strategy considered latitudinal gradients of environmental parameters (proxied by location), gender, and their interactions with seasonality. Analyses of gene stability were performed using different algorithms. BestKeeper and geNorm agreed that combination of the ribosomal genes 18S ribosomal RNA (18S) and 28S ribosomal RNA (*28S)* was the best normalization strategy in the conditions tested, which agrees with available evidence. NormFinder provided different normalization strategies, involving combinations of tubulin (*TUB)/28S* (Gender/Season effect) or *TUB/*helicase *(HEL)* (Location/Season effect). Since NormFinder considers data grouping and computes both intra- and inter-group stability variations, it should work better with complex experimental designs and dataset structuring. Under the selected normalization strategies, expressions of the variable housekeeping genes actin (*ACT)* and elongation factor-1*α* (*EF1)* correlated with seasonal and latitudinal changes of abiotic environmental factors and mussel physiological status. Results point to consider *ACT*

and *EF1* expressions as molecular biomarkers of mussel general physiological status in field studies.

Keywords: real-time PCR; mussel; gene transcription; qPCR data normalization; field studies; actin; tubulin; ribosomal genes

Highlights

- Stability of 6 commonly used housekeeping reference genes in qPCR analyses with *Mytilus galloprovincialis* was explored
- Gradients of environmental parameters with locations, gender, and interactions with season were considered.
- BestKeeper and geNorm selected *18S/28S* rRNA genes combination as best normalization strategy
- NormFinder selected *TUB/28S* (Gender/Season effect) or *TUB/HEL* (Location/Season effect) combinations.
- The selected normalization strategies were assessed on *ACT* and *EF1*.
- *ACT* and *EF1* expression correlated with season and location related abiotic and mussel biotic parameters
- *ACT* and *EF1* profiling may be a valuable molecular biomarker of mussel physiological status in field studies.

1. INTRODUCTION

The use of marine mussels (Mytilus spp) as biological models encompasses a broad range of research fields, from evolutionary biology (Ghiselli et al., 2021, 2018), environmental monitoring and ecotoxicology (Capolupo et al., 2017; Li et al., 2019; Świacka et al., 2019), comparative physiology (Franzellitti et al., 2010; Zhao et al., 2024), to translational medicine (Gan et al., 2022; Taghizadeh et al., 2022). In these fields, technologies based on RNA analysis, with their capacity for in-detail evaluation of key gene features, are employed to explore novel genes, disclose novel biomarkers of environmental stress response, and to investigate biochemical mechanisms of action (Counihan et al., 2019). Quantitative real-time PCR (qPCR) using intercalating dyes, such as SYBR Green®, is the gold standard for transcriptional studies, given its simplicity, rapidity, high specificity, and inexpensiveness, which yields reproducible results with convenient experimental effort (Bustin et al., 2009). Despite its wide use and available protocols, this technique may be affected by variability sources related to the intrinsic diversity of RNA populations and amounts among samples, and to the presence of technical and experimental biases (Bustin et al., 2013, 2009) that may cause data misinterpretation (Huggett et al., 2005; Sanders et al., 2018; Svec et al., 2015). To account for these technical flaws in the qPCR workflow, generated data are usually normalized with one or more internal control, defined as reference gene (RG). Ideal RGs should be expressed at stable levels irrespective of tissue type, treatment, metabolism, or sampling conditions (Wong and Medrano, 2005). Evidence show that using single or inappropriate RGs for normalization may dramatically bias quantification

of mRNA levels (Franzellitti et al., 2015; Volland et al., 2017). Therefore, RG selection and validation is required for robust qPCR datasets (Bustin et al., 2009).

Housekeeping genes are considered suitable RGs, since they are supposed to fulfill the primary task of stable expression, and further they belong to cellular maintenance pathways, they are evolutionarily conserved, and they are expressed at high levels (Joshi et al., 2022). However, comprehensive investigations on multiple species and experimental conditions reported that the influence of environmental conditions of the organism on housekeeping gene expression stabilities is largely underrated (Joshi et al., 2022), while there is evidence that environmental pressures do affect animal global transcriptional profiles (Gracey et al., 2008; Lockwood et al., 2015; Milan et al., 2023; Wathsala et al., 2021).

In this study, the variability of housekeeping genes commonly employed as RGs in qPCR investigations with the Mediterranean mussel (*Mytilus galloprovincialis*) was explored under field conditions. Analyses of gene stability were performed on mussels collected over different seasons, sampling locations, and considering gender. Once the most viable qPCR normalization strategy being established, transcriptional profiles of variable housekeeping gene transcripts were investigated to evaluate putative influences of abiotic factors employing mussels farmed in the study area of the Northwestern (NW) Adriatic Sea, which is characterized by extensive gradients of temperature, salinity, and nutrients (Cozzi and Giani, 2011; Grilli et al., 2020; Kraus et al., 2019). The goal of this study is to provide evidence for accurate qPCR data normalization under real exposure scenarios, and to explore the putative use of variable

housekeeping gene products as molecular biomarkers of mussel health status in field biomonitoring and environmental risk assessment.

2. METHODS

2.1 Study area and experimental design

Mussel samplings were performed by professional fishermen in farms of the NW Adriatic Sea (Fig 1). The experimental setup was implemented to account for gender and location effects, and their interaction with season:

- *Experiment I - Gender/Season effect*. Five sampling campaigns were performed in 2018 from a commercial mussel farm located off Cesenatico (N 44.2316863°, E 12.5048955°; Fig 1A). Mussels were collected once a month, from March to July. Sex and gonad development was determined in individual mussels in a previous study (Wathsala et al., 2021).
- *Experiment II - Location/Season effect*. Mussels were collected from three farms located at Goro (N 44.737075°, E 12.3011157°), Cattolica (N 43.9991147°, E 12.7348203°), and Senigallia (N 43.7486°, E 13.232767°) along a North-South gradient of environmental parameters (in particular salinity and chlorophyll-a), hence of physiological conditions of farmed mussels (Fig 1A,B). Samplings were performed across winter, spring and summer seasons between 2019 and 2020.

In both experiments, data for sea surface temperature (SST), salinity, and chlorophyll-a were retrieved from the web portal of the Regional Agencies of Environmental Protection of Emilia Romagna (https://www.arpae.it) and Marche

(https://www.arpa.marche.it) (Fig 1B). Upon collection mussels were immediately stored in coolers (+4°C) and transferred to the laboratory. Biometric parameters, including shell length, total tissue weight, soft tissue weight, and shell weight were as reported previously (Palladino et al., 2023; Wathsala et al., 2021). The meat yield (soft tissue weight/total weight) and the condition index (dried soft tissue weight/dried shell weight) were calculated according to Kurtay and Lök (2023) to be employed for multivariate analyses of gene transcription data. Values are reported in Table S1. The digestive gland was selected as the target tissue for transcriptional analyses because of its role in food digestion, detoxification processes and immune responses, and because of its use in biomonitoring studies with biomarkers and mussels as sentinel organisms (Faggio et al., 2018; Viarengo et al., 2007). Tissues were snap-frozen in liquid nitrogen and stored at −80°C until analyzed.

2.2 Selection of housekeeping candidate reference genes

Housekeeping gene products assessed as candidate reference genes and their functions are listed in Table 1. Primer sequences were from previous studies (Table 1), and their specificities were checked both *in silico* and empirically by Basic Local Alignment Search Tool (BLAST) analysis and using melting profiles. BLAST analyses against *M. galloprovincialis* and *M. edulis* nucleotide sequences in the GenBank indicated all primers were specific (data not shown), which was confirmed by melting profiles, electrophoresis and sequencing of PCR products.

2.3 Mussel RNA extraction, cDNA preparation, and qPCR analyses

In both experiments, 7 mussels for each condition were used. For each animal, 200 mg of digestive gland were homogenized in a suitable volume of the TRI Reagent (Sigma Aldrich, Milan, Italy) and total RNA was extracted using the DirectZol kit (Zymo Research, Freiburg, Germany) following the manufacturer's instructions. RNA concentration and quality were confirmed using the Qubit system (Thermo Fisher, Milan, Italy) and electrophoresis with a 1.2% agarose gel under denaturing conditions. First strand cDNA for each sample was synthesized from 1 µg of total RNA using the iScript supermix (BioRad Laboratories, Milan, Italy) following the manufacturer's instructions.

Expression profiles of selected transcripts were assessed by real time qPCR using primer pairs reported in Table 1. qPCR reactions were performed in duplicate, in a final volume of 10 µL containing 5 µL iTaq Universal Master Mix with ROX (BioRad Laboratories, Milan, Italy), 2 µL diluted cDNA, and 0.2 µM specific primers. A control lacking cDNA template was included in the qPCR analysis to determine the specificity of target cDNA amplifications. Amplifications were performed in a StepOne real time PCR system apparatus (Life Technologies, Milan, Italy) using a standard “fast mode” thermal protocol. For each target mRNA, analysis of melting curves and gel pictures verified amplicon specificity and the absence of artifacts. The amplification efficiency of each primer pair was checked using a cDNA dilution series, being consistent with results of previous studies (Balbi et al., 2016; Franzellitti and Fabbri, 2013; Wathsala et al., 2021).

2.4 Data analysis and statistics

All data visualizations and graphics were obtained with the *ggplot2* package in R (R Development Core Team, 2018).

To assess transcript stability and identify the best performing RGs, the algorithms BestKeeper and geNorm were employed using the package *ctrlGene* in R (R Development Core Team, 2018), and NormFinder using an Excel-based Macro (Andersen et al., 2004). BestKeeper calculates a geometric average for the entire dataset of threshold cycle (C_T), generating a hypothetical global normalization factor, the 'bestkeeper'. By making pairwise comparisons between expression data from individual transcripts and this 'bestkeeper', the algorithm ranks RGs from high (low standard deviation, SD) to low (high SD) stability. geNorm utilizes pairwise comparisons to rank candidate RGs by summed individual variation. Transcript stability is expressed by a stability measure (M value) calculated on the geometric mean of all studied gene products and the pairwise variation. The lower is the geNorm M value the more stable is the gene. NormFinder calculates individual variations for each gene relative to the dataset average, computing intergroup (i.e. different conditions in the two Experiments) and intragroup (here comparing mussel individuals within each condition in each Experiment) variations to provide a gene-specific stability value in a given set of conditions. The application of this group-specific analysis identifies transcripts with the greatest stability across the conditions considered. The lower is the NormFinder stability index the more stable is the gene.

Relative mRNA expression values of variable housekeeping gene transcripts were calculated by the comparative C_T method (Schmittgen and Livak, 2008) using the

StepOne and DataAssist softwares (Thermo Fisher, Milan, Italy). Data were reported as relative expression (log_2-transformed fold change). The transcriptional datasets were further analyzed by a 2-way permutation multivariate analysis of variance (PERMANOVA) and PERMANOVA pairwise comparisons using PRIMER v6 (Anderson et al., 2008) to test for variations of transcriptional profiles among tested factors. Log-transformed variations of the transcripts were used to calculate similarity matrices based on the Euclidean distance (999 permutations).

A DISTLM analysis (Distance-based redundancy linear modeling) with a test of marginality in PRIMER was further performed to account for the contribution of environmental parameters in explaining the total observed variance in the transcriptional profiles. DISTLM used the BEST selection procedure and adjusted R^2 selection criteria. Statistical differences were achieved with $P < 0.05$. Correlation analyses were performed using the Spearman's test. In any case, statistical differences were accepted when $P < 0.05$.

3. RESULTS

3.1 Amplifications

A set of 6 housekeeping gene transcripts has been assessed as putative candidate mussel RGs (Table 1). In both experiments, there were no samples with missing C_T or with inconsistency between replicates (C_T SD < 0.5). A single amplification product for each primer pair was confirmed by a single peak in the melting curve analysis and by electrophoresis analysis of PCR products. After averaging replicates, boxplots were

obtained for each candidate RG showing C_T variations across conditions in the two experiments (Fig S1). In the dataset of either Experiment I or Experiment II, helicase (*HEL*) showed the lowest expression (i.e. highest average C_T) and 18S ribosomal RNA (*18S)* the highest expression (i.e. lowest average C_T) (Table 1). When considering intergroup variability in the datasets, certain transcripts showed consistent indications of being regulated across the different conditions in the Experiments (Fig S1).

3.2 Analyses of candidate RG stability

Three algorithms were used (BestKeeper, NormFinder and geNorm) to analyze the expression stability of the mussel housekeeping genes (Fig 2). The results of geNorm agreed with those of BestKeeper, indicating that *18S* was consistently the top-ranked or part of the top-ranked pair of transcripts (in combination with 28S ribosomal RNA, *28S*, according to geNorm) in both experiments, whereas elongation factor-1*α* (*EF1*) and actin (*ACT*) were the least stable (Fig 2A,B). Therefore, the combination *18S/28S* was selected as the best normalization strategy according to BestKeeper and geNorm. The output of NormFinder indicated a different normalization strategy from the previous algorithms and between the datasets of the two experiments. Indeed, tubulin (*TUB*)*/28S* was the top-ranked pair of transcripts in the dataset of Experiment I (Fig 2A), while *TUB/ HEL* was the top-ranked pair of transcripts in the dataset of Experiment II (Fig 2B).

3.3. Expression patterns of housekeeping gene transcripts

Variations of transcriptional profiles of *ACT* and *EF1*, assessed as the most variable housekeeping gene transcripts by the stability analyses, are reported in Fig 3. Results from PERMANOVA analyses demonstrated that in Experiment I only the single factor “Season” had a significant effect on *ACT* and *EF1* gene transcriptions and under both selected normalization strategies (Table 2). In Experiment II, both factors “Location” and “Season” had a significant effect on *ACT* and *EF1* transcription and under both selected normalization strategies (Table 2); furthermore, a significant interaction between the factors was observed (Table 2). In both Experiments, *ACT* levels showed a general trend to significantly increase from winter to summer with the *18S/28S* normalization strategy (Fig 3A,C), while normalizing over *28S/TUB* (Experiment I) or *TUB/HEL* (Experiment II) produced more complex expression profiles (Fig 3B,D). In Experiment II, both normalization strategies revealed location-related increase (*ACT*) or decrease (*EF1*) (Fig 3C,D). The season-based comparisons between genders or locations are reported in Table S2.

The tests of marginality from the DISTLM analyses performed on separate *ACT* and *EF1* results under the different normalization strategies and by considering environmental parameters, gonad maturation level (for Experiment I, assessed through *VCL/VERL* expression profiling reported by Wathsala et al. (2021)), and meat yield as well as condition index are reported in Fig 4. In Experiment I (Gender/Season Effect), all environmental parameters, meat yield and condition index, and gonad maturation significantly contribute to explaining the total variance observed either for *ACT* or *EF1* under the *18S/28S* normalization strategy ($P< 0.01$) (Fig 4A). Under the *28S/TUB* normalization strategy, salinity, chlorophyll-a, explained most of the variation observed

for *ACT* transcription, meat yield and condition index, gonad maturation, as well as all environmental parameters except SST explained most of the variation of the observed for *EF1* transcription (Fig 4A). In Experiment II (Location/Season effect), meat yield and condition index, and all environmental parameters except SST explained most of the variation of the observed for *ACT* under the 18S/28S normalization strategy ($P < 0.05$) (Fig 4B), whereas no significant contribution by any environmental parameter or meat yield and condition index was observed for *EF1* (Fig 4B). Under the *TUB/HEL* normalization strategy, all environmental parameters, meat yield, and condition index significantly contribute to explaining the total variance observed for *ACT*, with SST, salinity and chlorophyll-a having a bigger contribution with respect to the physiological indices (Fig 4B). Salinity, chlorophyll-a explained most of the variation observed for *EF1* transcription (Fig 4B).

Spearman's correlation analyses showed that in Experiment I both *ACT* and *EF1* transcriptional profiles significantly correlated with meat yield and condition index, as w withell as gonad maturation trends under the *18S/28S* normalization strategy (Fig 5A), whereas under the *28S/TUB* normalization strategy only *EF1* positively correlated with meat yield and condition index ($P< 0.05$), and negatively correlated with gonad maturation ($P < 0.05$) (Fig 5A). In Experiment II, only *ACT* showed significant (negative) correlation with both meat yield and condition index under both qPCR normalization strategy employed ($P < 0.01$) (Fig 5B). The BEST/BioEnV analysis reported in Table S3, further showed the most significant correlations of *ACT* and *EF1* expressions with levels of contaminants (pesticides and pharmaceuticals) previously assessed in soft tissues of

mussels collected at the same sampling locations and season of Experiment II (Palladino et al., 2023).

4. DISCUSSION

This study is part of our ongoing investigations on basal expression of transcripts involved in physiological processes of adult mussels under environmental changes of physical and chemical parameters (Capolupo et al., 2017; Musella et al., 2020; Palladino et al., 2023; Wathsala et al., 2021). The Mediterranean mussel (*M. galloprovincialis*) is one of the most intensively cultured bivalve species in the NW Adriatic Sea (Minarelli et al., 2018; Tamburini et al., 2020), and it is also the most employed sentinel organism in biomonitoring plans that assessed habitat quality and environmental threats in the area (Capolupo et al., 2017; Franzellitti et al., 2010; Gomiero et al., 2015; Mezzelani et al., 2020; Palladino et al., 2023). In this study, transcript levels of six common housekeeping genes were investigated in digestive glands of mussels collected from four farms, and their expression stability and the putative influence of environmental parameters on their expression patterns were evaluated. The sampling strategy considered the influence of gradients of environmental parameters (here proxied by location), gender, and their interactions with season, which were shown to affect several molecular biomarkers (Palladino et al., 2023; Wathsala et al., 2021), and to influence mussel sensitivity to pollutants (Blanco-Rayón et al., 2020; Lacroix et al., 2017).

The housekeeping genes selected in this study are commonly used for qPCR data normalization with mussels, showing heterogeneity in stability orders depending on

the different ranking strategies and experimental constraints employed (Balbi et al., 2016; Cubero-Leon et al., 2012; Lacroix et al., 2014; Martínez-Escauriaza et al., 2018; Salatiello et al., 2022). BestKeeper and geNorm agreed that combination of *18S* and *28S* were the best suited RGs in both experiments. In general, ribosomal RNAs (rRNAs) are advocated for use as RGs in many experimental systems because their levels are thought to vary less under conditions that affect mRNA processing (Filby and Tyler, 2007; Goidin et al., 2001). Nevertheless, other studies reported these transcripts being regulated under specific conditions, for example in developing embryonal stages of *M. galloprovincialis* (Balbi et al., 2016), in herpesvirus infected ark clams *Scapharca broughtonii* (Xin et al., 2018), in haemocytes from copper exposed clams *Ruditapes philippinarum* (Volland et al., 2017), or in gills of mangrove oysters *Crassostrea brasiliana* exposed to different combinations of abiotic and chemical stressors (Müller et al., 2017). NormFinder provided different normalization strategies for the two experiments, which involve the combination of *TUB* and *28S* (Experiment I - Gender/Season effect), or *TUB* and *HEL* (Experiment II - Location/Season effect). *TUB* and *HEL* carry out essential functions in cell physiology, as composition and maintenance of cytoskeletal structures (*TUB*) (Janke and Magiera, 2020), or shaping the form of RNA molecules during transcription, splicing, and translation (*HEL*) (Abdelhaleem, 2010), whose maintenance is suggested as a general mechanism for increased tolerance to environmental stress (Lockwood et al., 2010; Negri et al., 2013). *TUB* differential expression at the mRNA or protein level has been reported in bivalves exposed to seasonal diarrhetic shellfish toxin contamination (*Cerastoderma edule*) (Domínguez-Pérez et al., 2021), n-TiO_2 (*M. galloprovincialis*) (Banni et al., 2016),

copper (Châtel et al., 2018; Hall et al., 2020), or to hypo-salinity (*M. galloprovincialis* and *M. trossulus*) (Tomanek et al., 2012). *HEL*, which was not selected by NormfFinder as RG in the Gender/Season Experiment, is known to be affected by the stage of mussel gametogenesis (Cubero-Leon et al., 2012); thus, it appears not suited as RG in studies considering gender or gametogenic status of the animals. It is worth noting that the NormFinder algorithm considers data grouping and computes both intra- and inter-group variations of candidate RGs to calculate expression stabilities and relative rankings. It may be postulated that the output of this algorithm may disentangle the relative importance of factors contributing to the whole dataset variance, hence it should work better to select the proper normalization strategy with complex experimental designs and dataset structuring.

ACT and *EF1* resulted the most variable housekeeping gene products under the conditions tested in this study, which is in line with previous investigations on bivalves (Feng et al., 2013; Moreira et al., 2015; Rossi et al., 2016). Therefore, *ACT* and *EF1* expression profiles were investigated applying the normalization strategies selected by geNorm/Bestkeeper and NormFinder to address putative influences of environmental factors that characterize the study area. Indeed, coastal ecosystems of the NW Adriatic Sea are the most strongly dependent on atmosphere–(in)land–sea interactions in the Mediterranean (Cozzi and Giani, 2011). The general circulation pattern, dominated by the North Adriatic Current originating from density gradients off the Po Delta, determines strong latitudinal gradients particularly of salinity, chlorophyll-a, and nutrients (Cozzi and Giani, 2011; Grilli et al., 2020; Kraus et al., 2019). These features sustain the high mussel productivity of these coastal zones (Brigolin et al., 2017;

Minarelli et al., 2018), but they are also responsible for the environmental variability and natural stress pressures on marine fauna, as well as its exposure to pollutants (Hala and Bakiu, 2024). The area is also well-known for its widespread occurrence of contaminants, including metals, polycyclic aromatic hydrocarbons (PAHs), pesticides, microplastics and pharmaceuticals (Combi et al., 2016; Loos et al., 2013; Nödler et al., 2014, 2013; Vianello et al., 2018). In particular, spatial trends of legacy (PAHs, polychlorinated biphenyls, PCBs, and chlorinated pesticides) and emerging (fragances, UV-filters, endocrine disruptors) contaminants assessed in sediments from the NW Adriatic coastal areas revealed decreasing concentrations from the Po River prodelta southward (Combi et al., 2016). Accordingly, north-to-south trends of contaminants accumulated in mussel tissues are reported (Gomiero et al., 2019; Interino et al., 2023; Palladino et al., 2023), likely reflecting the relevant riverine inputs from the Italian border and coastal anthropogenic activities (aquaculture farms, tourisms, etc.), as well as the hydrological features of the Adriatic Sea (Combi et al., 2016; Gomiero et al., 2019). Colletively, these evidence led to appoint mussel farms located along such gradients of environmental conditions as field laboratories to explore the connection between animal health and environmental changes (Bocchetti and Regoli, 2006; Buratti et al., 2013; Ferrara et al., 2024; Mezzelani et al., 2020; Palladino et al., 2023; Wathsala et al., 2021).

According to both qPCR normalization strategies, in the Gender/Season Experiment, seasonality is the main factor affecting transcriptional patterns, whereas in the Location/Season Experiment, *ACT* and *EF1* profiles are significantly influenced by both Season and Location factors. *ACT* is involved in the structure and function of the

cytoskeleton and is a component of cell microfilaments (Heissler and Chinthalapudi, 2024), while *EF1* is engaged in control of translation (Sasikumar et al., 2012). Comprehensively, expression profiles of these transcripts have been related to the variability of protein synthesis rate that may change according to season progression (Banni et al., 2011; Pytharopoulou et al., 2006; Rossi et al., 2016), and to cell oxidative and pro-apoptotic stress levels, likely resulting from animal exposure to pollutants (Vincenzetti et al., 2017). Furthermore, increased growth of actin filaments to expand cell membrane and cell volume was observed in mussels as a homeostatic response to hyposalinity stress (Tomanek et al., 2012). In this study, *ACT/EF1* profiles correlated with variations of meat yield and condition index, that are known indicators of eco-physiological status and growth of mussels, and they are widely used to assess the general health status (fitness) and the nutritive status of the animals (Andral et al., 2004; Kurtay and Lök, 2023; Okumuş and Stirling, 1998). Changes of these parameters may result from the complex interaction of a variety of environmental and endogenous factors, but food supply is considered the most important factor after gametogenic cycle (Okumuş and Stirling, 199\8). Indeed, the DISTLM analyses showed that chlorophyll-a (proxying phytoplankton, i.e. food availability), together with salinity, is the environmental factor that explains most of the variance of either *ACT* or *EF1* transcriptional variations in both experiments. These factors may have a significant influence on the seasonal metabolic makeup of mussels and growth, and sensitivity of biochemical and physiological biomarkers (Bal et al., 2021; Nahrgang et al., 2013; Wathsala et al., 2021). Furthermore, *ACT* showed remarkably high Spearman correlations with gonad maturation endpoints. Indeed, *ACT* expression may be affected

by the stage of gametogenesis, and be related to tissue regeneration (Cubero-Leon et al., 2012; Jarque et al., 2014) or reflect variations in protein content related to changes in the utilization of energy storage compounds, where the highest values are achieved when carbohydrates and lipids are utilized (Mladineo et al., 2007). Nevertheless, no statistically significant differences in *ACT* or *EF1* expression between females and males was observed in this study, leading to consider gender bias not a relevant factor in housekeeping gene transcription. Anyway, our previous study on mussels from Experiment I pointed out that male and females displayed a different season-based regulation of metabolic and cytoprotective gene transcripts which is likely related to reproductive cycle progression (Wathsala et al., 2021). It must be noted that both metabolic and cytoprotective mechanisms peak in the digestive gland because of its role in food digestion, pollutant bioaccumulation and detoxification (Faggio et al., 2018). Accordingly, previous whole transcriptomic analyses of males *vs* females digestive glands did not highlight processes or molecular functions related to pathways specific to gonadal development, but rather indicate differences for catabolic and biosynthetic processes (Banni et al., 2011). Therefore, a wider dynamic of transcriptional regulation for gene underlying those functions can be reasonably expected compared to that of the assessed housekeeping genes. Finally, effects of pollutants already detected in mussels collected in the same sampling area cannot be ruled out. Taking advantage from a datatset of pollutants detected in soft tissues of mussels from the sampling locations and seasons of Experiment II (Palladino et al., 2023), a BEST/BioEnV analysis revealed that *ACT* expression may be signifcanlty correlated with combination of antibiotics (sulfomethoxazole, erythromycin) and pesticides (atrazine and metolachlor)

accumulated in mussels. Despite their diverse modes of action, these pollutants have been shown to impair protein synthesis through alternative pathways, i.e. by disrupting aminoacids biosynthesis (sulfomethoxazole), accelerating protein metabolism (erythromycin), altering biosynthesis of ribosomes (atrazine), or by impairing cell cycle progression (metolachlor) (Hartnett et al., 2013; Liang et al., 2020; Serra-Compte et al., 2019; Zuo et al., 2024). More generally, as the requirement to conserve energy by modulating the protein synthesis machinery is an important feature of all stress response strategies (Gasch et al., 2000; Somero, 2012), the differences observed in animals from the different locations of Experiment II could be related to their aquired plasticity to cope with the local threats, that included the cumulative/synergistic effects of physical and chemical stress factors (Gracey et al., 2008; Lockwood et al., 2015; Núñez-Acuña et al., 2012; Serra-Compte et al., 2019; Yévenes et al., 2021).

5. CONCLUSION

This study explored the transcriptional variability of housekeeping genes commonly employed as RGs in qPCR investigations with *M. galloprovincialis*. Transcripts encoding the ribosomal proteins *18S* and *28S* are suggested as the most stable among the housekeeping gene transcripts assessed in this study, which is generally in line with available evidence on mussels (Cubero-Leon et al., 2012; Lacroix et al., 2014). Furthermore, present results showed that different algorithms performing stability analyses may differentially estimate the influence of data structuring giving different outputs. This finding is particularly relevant in field investigations, where multiple environmental and endogenous factors may influence mussel physiology and

underlying gene expression, and complex experimental designs are frequently employed. Therefore, in these conditions, comparing different RG stability analysis methods seems particularly advisable to select the proper normalization strategy. In this regard, the second stage of this study showed the influence of the different selected normalization strategies on expression analysis of the variable housekeeping genes *ACT* and *EF1*, revealing that seasonal and latitudinal changes of abiotic environmental factors (likely including the occurrence of pollutants affecting protein synthesis) and mussel physiological status may heavily affect *ACT/EF1* transcription. Overall, reported results point to consider *ACT* and *EF1* mRNA profilings as valuable molecular biomarkers to be related to the mussel general physiological status in field studies.

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FIGURE LEGENDS

FIG 1. Study area, sampling setup, mussel condition factors, variations of environmental parameters. (A) Location of the mussel farms assessed in this study in the Northwetsern Adriatic Sea (Italy). Size of sampling points in the map show average meat yield (i.e. the ratio of the soft tissue weight to dry shell weight). Map was generated using the *ggplot2* and *ggmaps* packages in R. (B) Violin plots reporting temporal trends of seawater parameters at the sampling locations. Data are retrieved from the web portal of the Regional Agencies of Environmental Protection of Emilia Romagna (https://www.arpae.it) and Marche (https://www.arpa.marche.it). Biometric parameters employed for the calculations are as reported previously (Palladino et al., 2023; Wathsala et al., 2021). ***Colored figure is intended only for the online and PDF version***

FIG 2. Stability rankings of the Mediterranean mussel candidate reference gene products obtained using different computational methods. In each graph, candidate reference gene products are ordered from the least stable (high stability index) to the most stable (low stability index). (A) Experiment I (Gender/Season) or (B) Experiment II (Location/Season). ***Colored figure is intended only for the online and PDF version.***

FIG 3. Transcriptional profiles of the variable housekeeping gene products *ACT* and *EF1*. For each target transcript, box plots (median, upper and lower quartiles; N = 7) show variations across condition tested within each experiment. Different letters indicate statistical differences between sampling seasons within each gender/location ($P < 0.05$, pairwise PERMANOVA comparisons). Season-based comparisons between genders or locations are reported in Table S2. ***Colored figure is intended only for the online and PDF version.***

FIG 4. DISTLM analysis to explore trends of biological parameters with environmental variables. Results from the test of marginality related to the DISTLM analysis showing contribution of each environmental variable to the total variance observed in season/location and season/gender datasets of housekeeping gene transcription profiles. *VERL/VCL* expression levels (which are proxies of gonad maturation in females/males) reported by Wathsala et al. (2021) have been included as a predictor variable. DISTLM used the BEST selection procedure and adjusted R^2 selection criteria. ***Colored figure is intended only for the online and PDF version.***

FIG 5. Correlations between *ACT* or *EF1* mRNA levels, gonad maturation, and physiological indices in Mediterranean mussels under the condition tested. Bubble graphs report Spearman's Rho values and their levels of significance (**$p \leq 0.01$, *$p \leq 0.05$) to show positive or negative correlations between *ACT/EF1* levels and physiological indices as well as gonad maturation in Experiment I (A), and physiological indices in Experiment II (B) under the different selected qPCR normalization strategies. ***Colored figure is intended only for the online and PDF version.***

Table 1. Mediterranean mussel housekeeping genes candidate as reference gene products, their functions, primers and parameters derived from qPCR analyses.

Transcript name (Acronym)	Function	Primers (5'-3')	Accession number (Reference)	Descriptive statistics of C_T (mean ± SD, CV, N = 7)
18S rRNA (18S)	Protein synthesis	TCGATGGTACGTGATATGCC CGTTTCTCATGCTCCCTCTC	L33451 (Dondero et al., 2005)	Experiment I: 10.75 ± 1.31, 12.18 % Experiment II: 11.00 ± 2.14, 19.48 %
28S rRNA (28S)	mRNA translation	AGCCACTGCTTGCAGTTCTC ACTCGCGCACATGTTAGACTC	DQ158078 (Ciocan et al., 2011)	Experiment I: 18.19 ± 1.59, 10.92 % Experiment II: 18.90 ± 2.71, 14.34 %
Actin (ACT)	Structure and function of the cytoskeleton, and component of cell microfilaments	GTGTGATGTCATATCCGTAAGGA GCTTGGAGCAAGTGCTGTGA	AF157491 (Banni et al., 2011)	Experiment I: 23.67 ± 2.32, 9.79 % Experiment II: 23.45 ± 2.37, 10.10 %
Tubulin (TUB)	Composition and maintenance of cytoskeletal structures	TTGCAACCATCAAGACCAAG TGCAGACGGCTCTCTGT	HM537081 (Cubero-Leon et al., 2012)	Experiment I: 17.65 ± 1.63, 9.22 % Experiment II: 18.52 ± 2.10, 11.36 %
Elongation factor-1α (EF1)	Control of translation	CGTTTTGCTGTCCGAGACATG CCACGCCTCACATCATTTCTTG	AB162021 (Ciocan et al., 2011)	Experiment I: 17.26 ± 1.89, 10.92 % Experiment II: 17.93 ± 3.09, 17.25 %
Helicase (HEL)	Maintaining RNA structures during transcription, splicing, and translation	GCACTCATCAGAAGAAGGTGGC GCTCTCACTTGTGAAGGGTGAC	DQ158075 (Cubero-Leon et al., 2012)	Experiment I: 26.98 ± 1.85, 6.84 % Experiment II: 27.19 ± 2.95, 10.84 %

N: total number of samples; SD: standard deviations of the C_T values; CV: coefficients of variation of the C_T values.

Table 2. Two-way PERMANOVA results on *ACT* and *EF1* transcriptional profiles (998 permutations). Significant P values are in bold.

	df	*ACT*				*EF1*			
Experiment I: Gender/Season effect									
qPCR normalization strategy		*18S/28S*		*TUB/28S*		*18S/28S*		*TUB/28S*	
		F	P	F	P	F	P	F	P
Gender	1	0.66	0.43	0.48	0.52	1.20	0.27	0.06	0.81
Season	2	20.56	**0.001**	7.65	**0.004**	7.57	**0.005**	7.12	**0.004**
Gender x Season	2	0.72	0.50	0.04	0.96	0.13	0.87	0.31	0.71
Experiment II: Location/Season effect									
qPCR normalization strategy		*18S/28S*		*TUB/HEL*		*18S/28S*		*TUB/HEL*	
		F	P	F	P	F	P	F	P
Location	2	18.84	**0.001**	17.05	**0.001**	3.51	**0.04**	12.33	**0.001**
Season	2	5.08	**0.015**	41.14	**0.001**	7.96	**0.001**	5.81	**0.008**
Location x Season	4	5.69	**0.001**	11.65	**0.001**	6.20	**0.003**	4.68	**0.005**

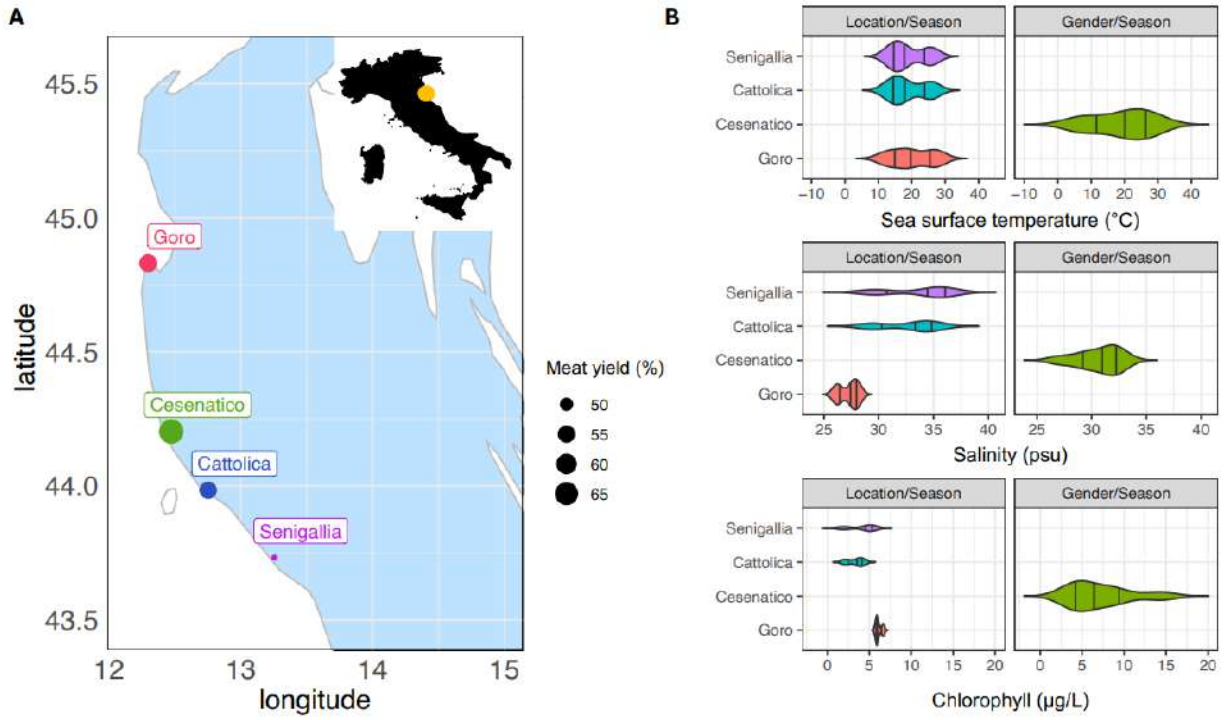
df: degree of freedom; Pseudo-F: F value by permutation (Anderson et al., 2008); P(perm): probability of

pseudo-F.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Figure 1


A
B
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45.0
44.5
44.0
43.5
latitude
12
13
14
15
longitude
Goro
Cesenatico
Cattolica
Senigallia
Meat yield (%)
50
55
60
65
Location/Season
Gender/Season
Senigallia
Cattolica
Cesenatico
Goro
Sea surface temperature (°C)
Salinity (psu)
Chlorophyll (µg/L)

Figure 2

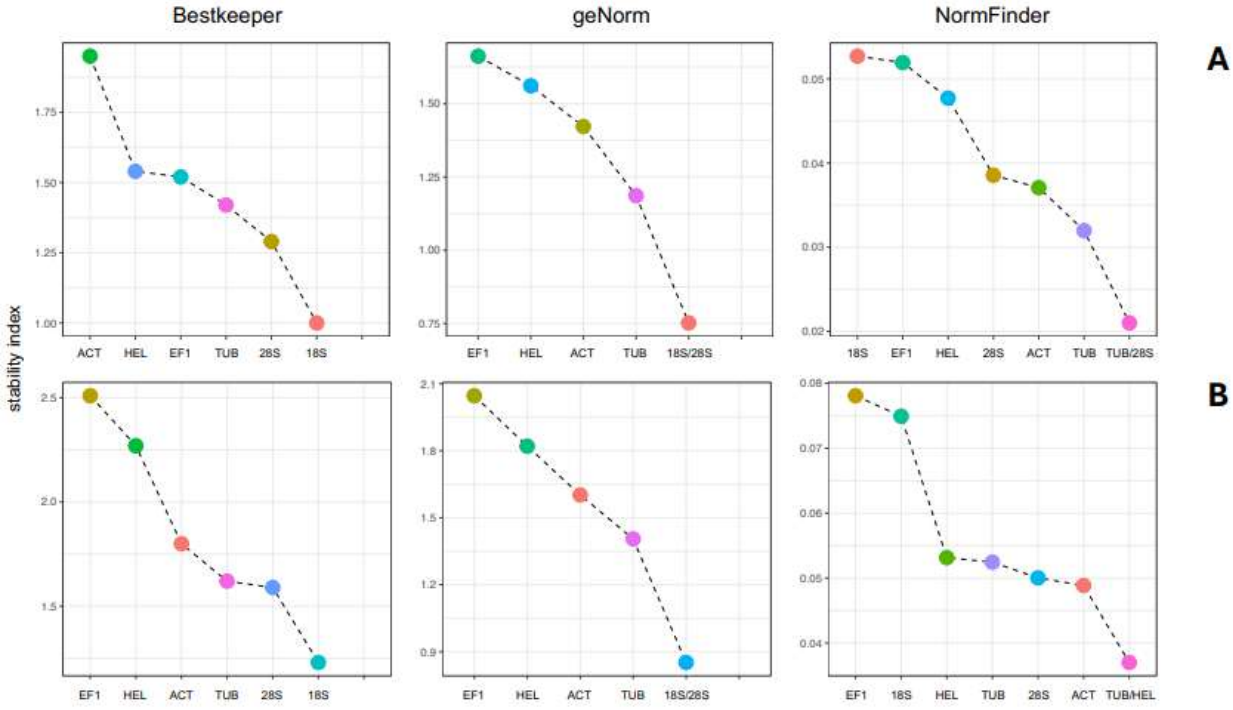

Bestkeeper
geNorm
NormFinder
A
B
stability index
ACT HEL EF1 TUB 28S 18S
EF1 HEL ACT TUB 18S/28S
18S EF1 HEL 28S ACT TUB TUB/28S
EF1 HEL ACT TUB 28S 18S
EF1 HEL ACT TUB 18S/28S
EF1 18S HEL TUB 28S ACT TUB/HEL

Figure 3

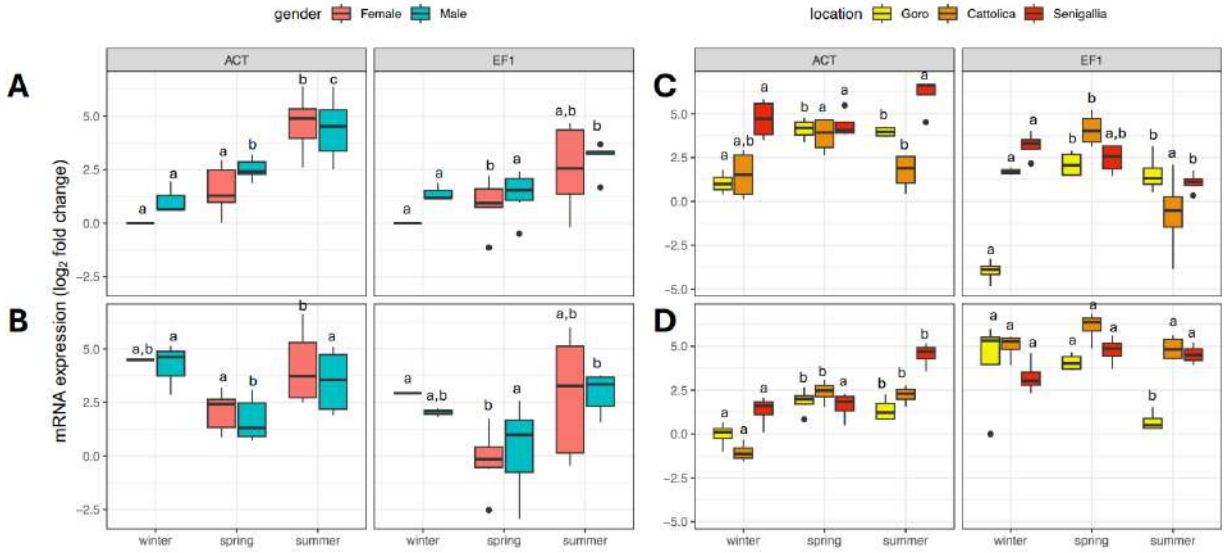
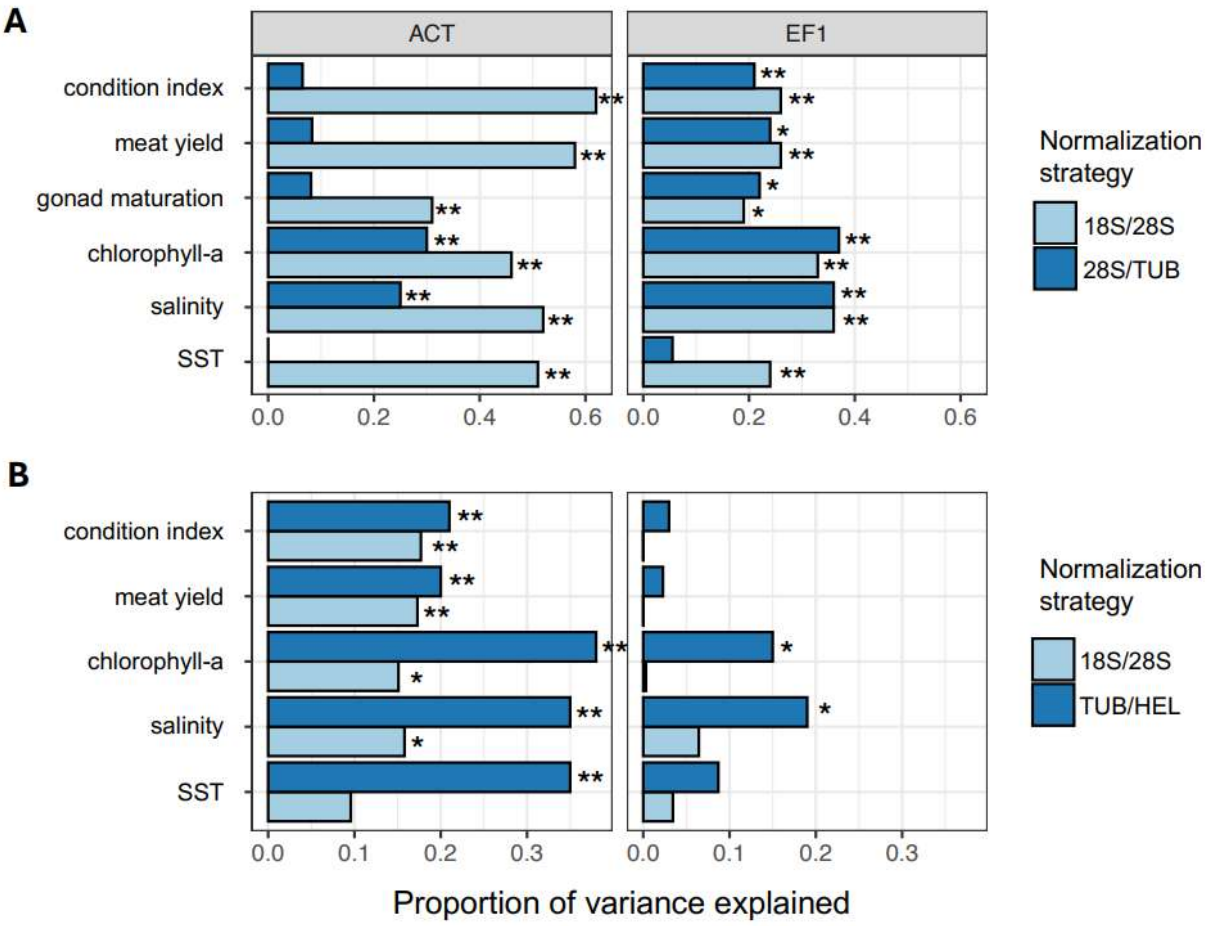
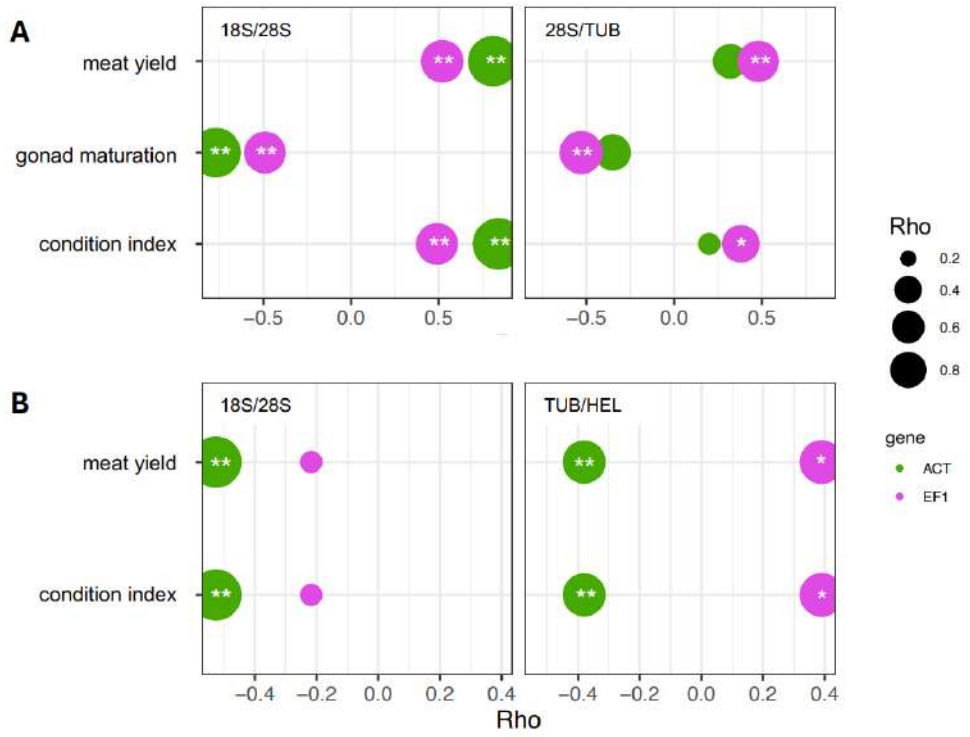
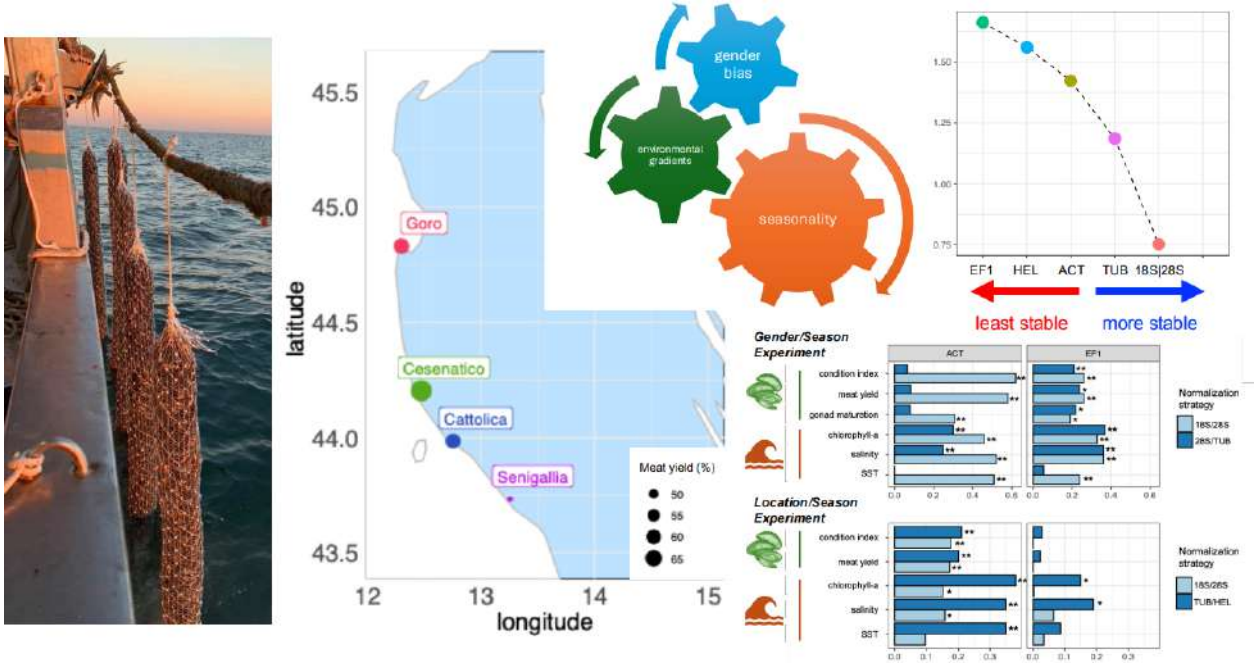


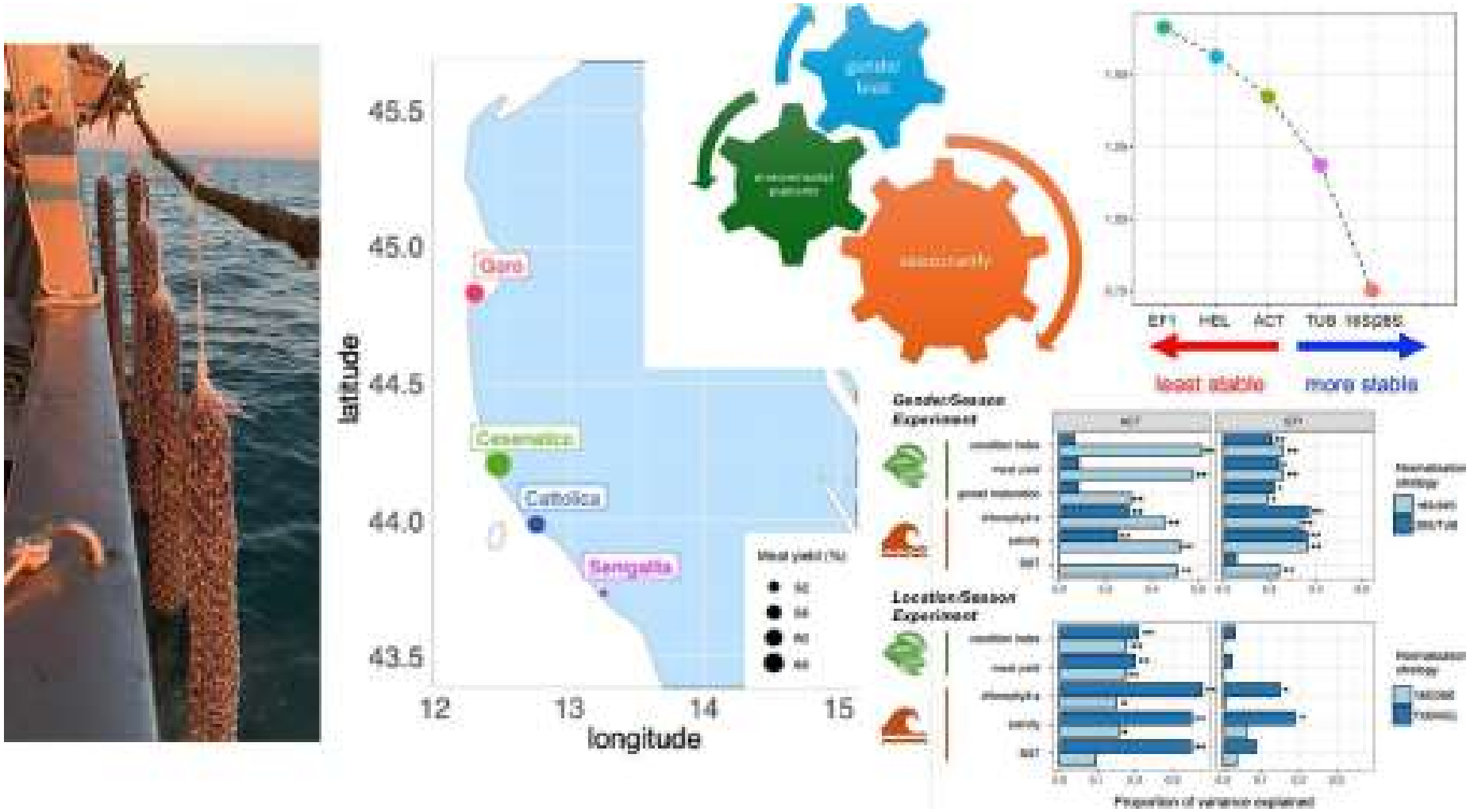
Figure 4


A
ACT
EF1
condition index
meat yield
gonad maturation
chlorophyll-a
salinity
SST
0.0
0.2
0.4
0.6
Normalization strategy
18S/28S
28S/TUB
B
condition index
meat yield
chlorophyll-a
salinity
SST
0.0
0.1
0.2
0.3
Normalization strategy
18S/28S
TUB/HEL
Proportion of variance explained

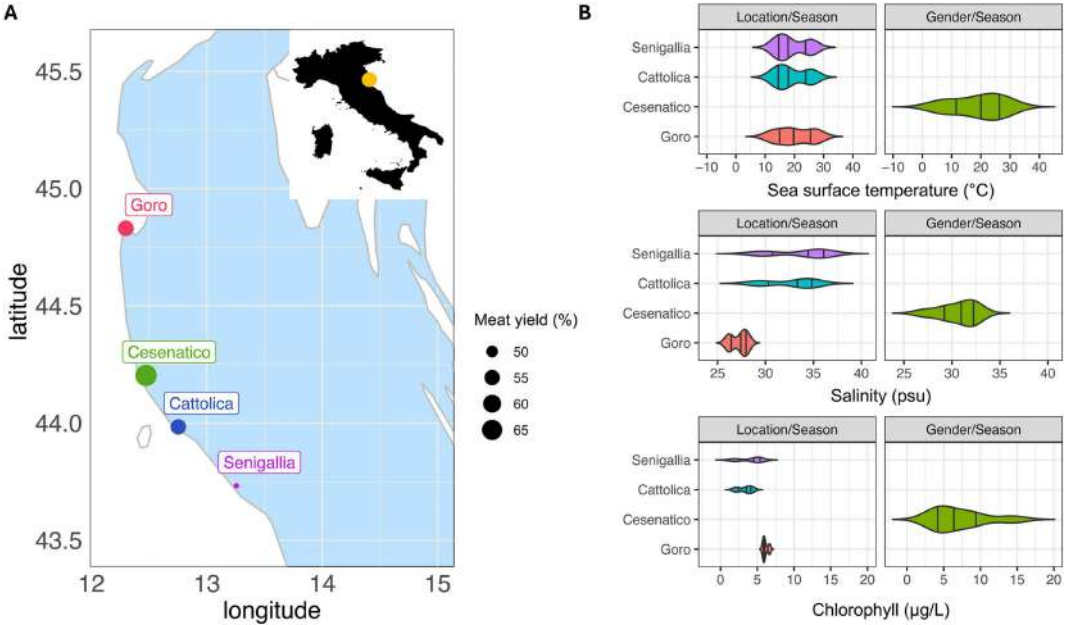


Graphical abstract





Graphics Abstract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45.5
45.0
44.5
44.0
43.5
latitude
12
13
14
15
longitude
Goro
Cesenatico
Cattolica
Senigallia
Meat yield (%)
50
55
60
65
B
Location/Season
Gender/Season
Senigallia
Cattolica
Cesenatico
Goro
−10 0 10 20 30 40
Sea surface temperature (°C)
25 30 35 40
Salinity (psu)
0 5 10 15 20
Chlorophyll (µg/L)

Figure 1

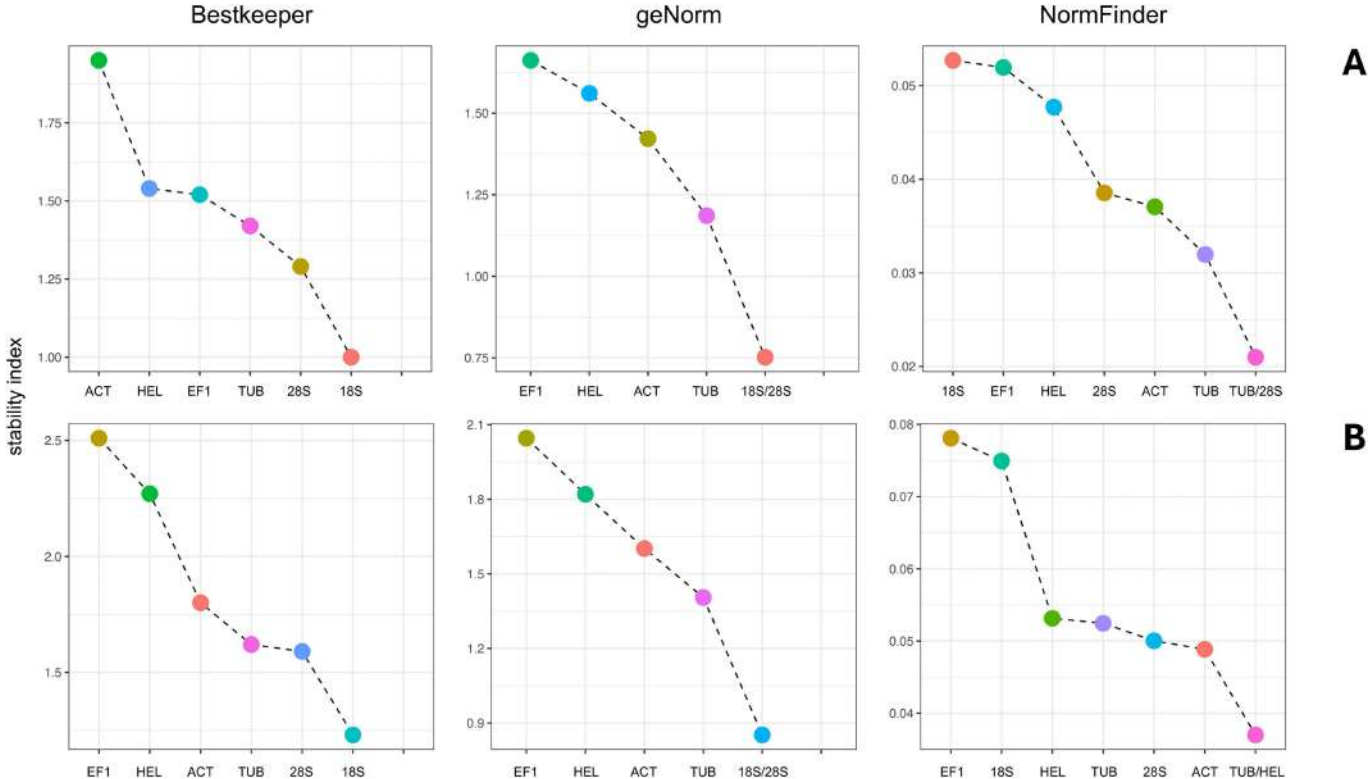
Bestkeeper
geNorm
NormFinder
A
B
stability index
ACT HEL EF1 TUB 28S 18S
EF1 HEL ACT TUB 18S/28S
18S EF1 HEL 28S ACT TUB TUB/28S
EF1 HEL ACT TUB 28S 18S
EF1 HEL ACT TUB 18S/28S
EF1 18S HEL TUB 28S ACT TUB/HEL

Figure 2

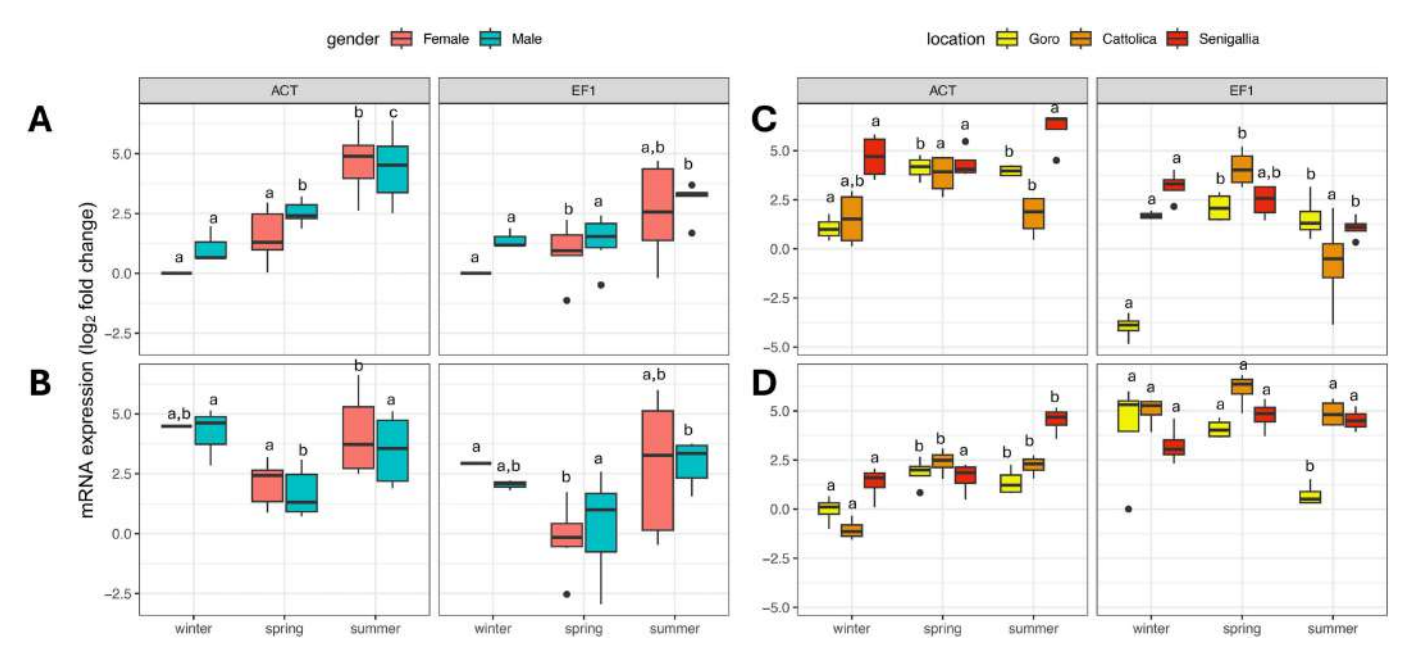
gender
Female
Male
location
Goro
Cattolica
Senigallia
A
B
C
D
ACT
EF1
mRNA expression (log2 fold change)
winter
spring
summer

Figure 3

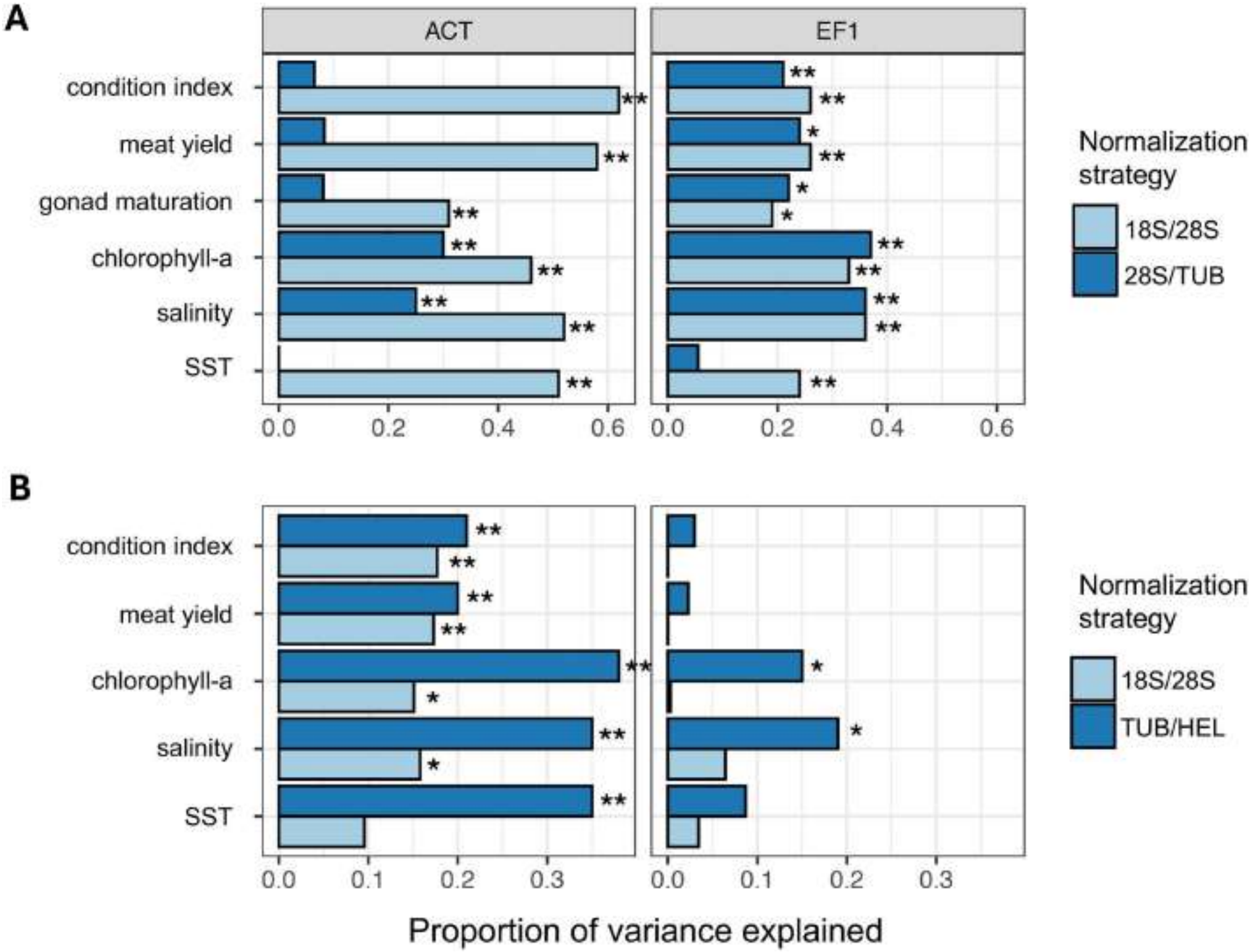
A
ACT
EF1
condition index
meat yield
gonad maturation
chlorophyll-a
salinity
SST
0.0
0.2
0.4
0.6
Normalization strategy
18S/28S
28S/TUB
B
condition index
meat yield
chlorophyll-a
salinity
SST
0.0
0.1
0.2
0.3
Normalization strategy
18S/28S
TUB/HEL
Proportion of variance explained

Figure 4

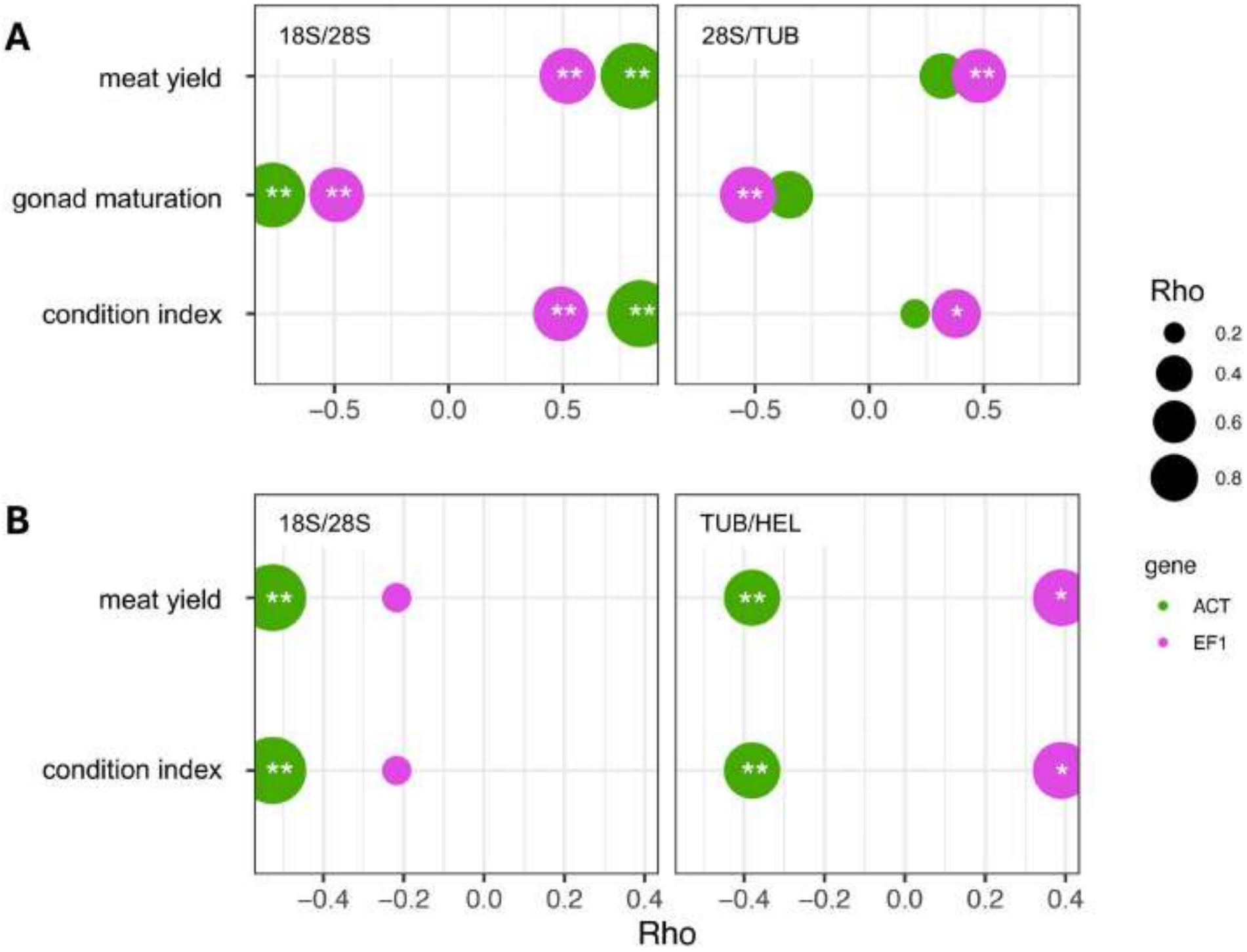
A
18S/28S
28S/TUB
meat yield
gonad maturation
condition index
**
*
−0.5
0.0
0.5
B
18S/28S
TUB/HEL
meat yield
condition index
−0.4
−0.2
0.0
0.2
0.4
Rho
Rho
0.2
0.4
0.6
0.8
gene
ACT
EF1

Figure 5