Identification and selection of optimal reference genes for qPCR-based gene expression analysis in *Fucus distichus* under various abiotic stresses

Marina Linardić[1,2*] and Siobhan A Braybrook[1,2,3,*]

[1] Department of Molecular, Cell and Developmental Biology, University of California Los Angeles, USA

[2] Department of Energy Institute of Genomics and Proteomics, University of California Los Angeles, Los Angeles, USA

[3] Molecular Biology Institute, University of Los Angeles, Los Angeles, USA

* Corresponding Author

E-mail: marina.linardic@cantab.net (UCLA) siobhanb@ucla.edu (UCLA)

Abstract

Quantitative gene expression analysis is an important tool in the scientist's belt. The identification of evenly expressed reference genes is necessary for accurate quantitative gene expression analysis, whether by traditional RT-PCR (reverse-transcription polymerase chain reaction) or by qRT-PCR (quantitative real-time PCR; qPCR). In the Stramenopiles (the major line of eukaryotes that includes brown algae) there is a noted lack of known reference genes for such studies, largely due to the absence of available molecular tools. Here we present a set of nine reference genes (*Elongation Factor 1 alpha (EF1A), Elongation Factor 2 alpha (EF2A), Elongation Factor 1 beta (EF1B), 14-3-3 Protein, Ubiquitin Conjugating Enzyme (UBCE2), Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH), Actin Related Protein Complex (ARP2/3), Ribosomal Protein (40s; S23), and Actin*) for the brown alga *Fucus distichus*. These reference genes were tested on adult sporophytes across six abiotic stress conditions (desiccation, light and temperature modification, hormone addition, pollutant exposure, nutrient addition, and wounding). Suitability of these genes as reference genes was quantitatively evaluated across conditions using standard methods and the majority of the tested genes were evaluated favorably. However, we show that normalization genes should be chosen on a condition-by-condition basis. We provide a recommendation that at least two reference genes be used per experiment, a list of recommended pairs for the conditions tested here, and a procedure for identifying a suitable set for an experimenter's unique design. With the recent expansion of interest in brown algal biology and accompanied molecular tools development, the variety of experimental conditions tested here makes this study a valuable resource for future work in basic biology and understanding stress responses in the brown algal lineage.

Introduction

Brown algae represent one of the five major lineages that have developed a multicellular body organization independently from other species (with red algae, plants/green algae, fungi, and metazoans as the other four) (1,2). Among all of the algal groups, the brown algae have the largest diversity in size and morphology, from filamentous to 'complex' thalli (bodies) (1). In addition to their complex morphology, brown algal life cycles are similarly diverse, exhibiting a broad range of variation between the gametophyte and sporophyte generations (3). As the main inhabitants of the intertidal zone, brown algae have to deal with extreme conditions; tidal cycles expose them to desiccation and osmotic shock, evaporation, and heat shock daily. Furthermore, anthropogenic impact (e.g. pollution) serves as an additional source of abiotic stress in these already challenging environments. How brown algae survive, and thrive, in these environments remains unknown. Taking into account their divergent evolutionary history (4), importance as an ecological and economical resource (5,6), and their astonishing ecology (7), the brown algae represent a unique and important group to explore. However, they have been extremely understudied on a molecular level. It is only within the past decades that scientists have begun to explore their rich molecular biology.

Gene expression analyses are necessary to understand how brown algal genetic networks dynamically change upon stimulus to regulate their responses to stressful life conditions. In recent years, microarrays and next-generation sequencing (NGS) technologies have arisen as the most used methods for quantification of gene expression on a global level. In spite of these advancements, qPCR remains one of the simplest and accessible methods for studies of small gene numbers. It also serves as a confirmational tool for NGS-derived results. qPCR is most widely used for fast and reliable quantification of mRNA steady-state levels because of its high sensitivity, accuracy, specificity, reproducibility, and low cost (8,9). However, the accuracy and

reliability of qPCR experiments are highly affected by several factors such as RNA integrity, reverse transcription efficiency, and primer efficiency (10). It is therefore of utmost importance to combat methodologically introduced variation by normalizing to stable reference genes as internal controls. An ideal reference gene should have a similar expression in all tissues and experimental conditions. Choosing reference genes with unstable expression could result in misleading results and inappropriate conclusions regarding target gene expression. However, the expression of commonly used reference genes may vary depending on the life stage of the organism, experimental conditions, as well as tissue source (10). Therefore, it is unlikely that a single reference gene would exist for all experiments and it is necessary to evaluate the best reference genes for each experiment. To quantitatively evaluate the efficiency and stability of reference genes for qPCR experiments, several statistical algorithms have been developed, such as geNorm (8), NormFinder (11), and BestKeeper (12). These methods have been used to determine the best reference genes in red, green, and brown algae (13–16), plants (17–22), and metazoans (23–27).

In some brown algae such as *Ectocarpus siliculosus* and *Undaria pinnatifida*, efforts have been made to identify reference genes, with results indicating variable stability of candidate reference genes depending on experimental treatments (13,14). The brown alga *Fucus* has been an important cell biology model. Experiments in *Fucus* led to several crucial discoveries in cell polarization and asymmetric cell division (28–32). Recent studies in *Fucus* further show its scientific importance as a cell biology model (33–39), but also as a tool to investigate ecological and physiological effects of abiotic stresses in natural habitats (40–46). As new molecular methods become available, the potential for *Fucus* to serve as a modern model system for exploring biology in extreme environments becomes tractable. qPCR serves as a basis to address questions of gene expression; it is, therefore, necessary to develop a set of normalization genes in *Fucus* to enable reproducible quantitative gene analysis by qPCR. In *Fucus*, *Elongation Factor*

alpha, Beta-Actin, Tubulin, and/or a *14-3-3* protein have all been used as reference genes for qPCR (41,42,44,45). However, there has been no systematic study of the stability and suitability of such reference genes over a wide array of conditions.

In this study, we identified and tested a set of potential normalization genes for *Fucus distichus*. Nine 'housekeeping' genes were selected as candidate reference genes: *Elongation Factor 1 alpha (EF1A), Elongation Factor 2 alpha (EF2A), Elongation Factor 1 beta (EF1B),* a *14-3-3* gene*, Ubiquitin Conjugating Enzyme (UBCE2), Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH), Actin Related Protein Complex (ARP2/3), Ribosomal protein (40s; S23), and Actin (ACT).* Their expression was analyzed by qPCR in samples submitted to various stress conditions; salinity, desiccation, pollution, nutrient deprivation, wounding as well as temperature, light, and phytohormone treatment. Three algorithms were used to evaluate the expression stability of the reference genes: geNorm, NormFinder, BestKeeper; a rank aggregation algorithm was employed to reach a consensus between the three. The number of reference genes to use for qPCR-based normalization was also explored. We provide a recommendation of paired reference genes for the conditions tested, alongside a recommended method for suitability assessment in an individual's experiments. To validate our recommendations, the differential expression of *Hsp70* and *Hsp90* genes, encoding stress-responsive heat shock proteins, were examined under salinity stress.

Results

Choosing candidate reference genes

To identify the 'housekeeping' gene sequences for potential normalization genes, two approaches were taken. First, housekeeping gene sequences previously reported for *Ectocarpus siliculosus* (13) were aligned against the related species *Fucus serratus* embryo development

transcriptome (36) to identify putative homologs. Furthermore, additional sequences were identified directly from the *F. serratus* transcriptome dataset, by identifying highly and constantly expressed genes across the four embryo developmental stages in the study. As such, candidate gene sequences were chosen based on three criteria: 1) a high percentage of alignment to *E. siliqulosus* (for those with *Ectocarpus* sequence matches), 2) high expression values in the *Fucus* embryo transcriptome (normalized expression metric 'transcripts per million transcripts'; 'TPM') and 3) a relatively constant expression across samples in the *Fucus* embryo transcriptome. Nine housekeeping genes were selected that satisfied these criteria: *elongation factor 1 alpha (EF1A), elongation factor 2 alpha (EF2A), elongation factor 1 beta (EF1B),* a *14-3-3 gene, ubiquitin conjugating enzyme (UBCE2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin related protein complex (ARP2/3), ribosomal protein (40s; S23),* and *actin (ACT)* (Table 1, sequences in S1 Fig). Primer sets were designed for all nine genes and their efficiencies and melting temperatures determined by qPCR (Table 1, S1 Fig; See Materials and Methods). The efficiency of the primer sets varied from 98.5% (*GAPDH*) to 106.9% (*EF1A*), and correlation coefficients ranged between 0.987 and 0.999 (Table 1). All efficiencies were considered acceptable for use. We strongly recommend the calculation of primer efficiencies for use qPCR-based differential gene expression analyses.

138 **Table 1. Description statistics of housekeeping gene candidates.** T_m, melting temperature;
139 P*E*, PCR efficiency; r^2, correlation coefficient.

Gene name	Gene symbol	primer F	primer R	Fragment length	Tm [ºC]	PE[%]	r2
Elongation factor 1 alpha	*EF1A*	ATGAGGTGGCCATCTACCTG	CCCTTGTACCAAGGCATGTT	124	59.85	106.90	0.999
14-3-3	*14-3-3*	CGAGACAGAGTTGACGGACA	CGCAAGATACCGGTGGTAGT	133	60.02	99.76	0.999
Actin	*ACT*	GACCTTTACGGCAACATCGT	GGTGCCACAACCTTGATCTT	122	59.97	106.86	0.989
Ubiquitin conjugating enzyme 2	*UBCE2*	AAGCTCAACATGGGCTGTGT	GCCACCAGTACCTGCTCAAT	110	60.14	103.52	0.995
Ribosomal subunit 40S	*40s*	ACGGCTGTCTGAACTTCACC	ACCTTCACCACCTTGAAACG	112	60.01	105.70	0.989
Elongation factor 1 beta	*EF1B*	TTCGGAGTGAAGAAGCTCGT	CAGAGGCGGTTCATCGTAGT	134	60.28	104.70	0.997
Elongation factor 2 alpha	*EF2A*	TGGACCACGGAAAGTCTACC	GGTGATACATCGGTCCTGCT	125	59.96	106.34	0.996
Actin related protein complex	*ARP2/3*	GGAAGCCTCTGGCTATTGGT	GTGGTCTTGGCTTGGAACAT	123	59.97	105.05	0.998
Glyceraldehyde -3-phosphate dehydrogenase	*GAPDH*	TCTTGGGTTACACCGAGGAC	GTACCACGACACGAGCTTGA	125	59.9	98.43	0.987

140

141 **Expression of reference genes across samples**

142 To determine reference gene expression across a wide set of conditions, the steady-state
143 levels of all nine genes were analyzed in cDNA samples from fifteen different treatments (in
144 biological triplicate) with two time-points each (3 hours and 3 days post-treatment): auxin,
145 gibberellic acid, EtOH control, imidacloprid, $CuSO_4$, hypersaline 2x artificial seawater (ASW),
146 hyposaline 0.5X ASW, desiccation, wounding, high light, low light, high temperature, low
147 temperature, ASW + Provasoli nutrients, and ASW with no additional nutrients as the control

(detailed in Materials and Methods; S3 Table). The quantification cycle (Cq) was identified for each gene in the triplicate samples by qPCR. The Cq values of all tested genes lay between 18.63 and 28.21 (except a single replicate outlier in *EF2A* at Cq = 32.7) (S4 Fig). The most highly expressed gene was *EF1A* (Cq_{mean} = 20.6), followed by *40S*. The lowest expression level was observed for *GAPDH* (Cq_{mean} = 26.9) (Fig 1). For each gene, the variation across samples did not exceed 3 cycles, which suggests a relatively constant expression in all conditions. All nine candidate genes were determined as suitably 'consistent' (qualitatively) across conditions to move forward in our analyses.

Figure 1. Expression level of tested housekeeping genes. The distribution of Cq (quantification cycle) values of 9 reference genes pooled across 30 samples (15 experimental conditions x 2 time-points) obtained using qPCR. The boxplot marks the median (line) and 25th (lower) and 75th (upper) percentile; x marks the mean; the underlying violin plots show the data distribution for each housekeeping gene. Outliers are depicted as black dots. Data for individual experimental conditions may be found in S4 Fig.

Reference gene expression stability in all samples

The stability of expression for a given reference gene is a quantitative measure by which the suitability of the reference gene may be assessed. Stability refers to how invariant the expression pattern of a given gene is in an experimental setup. The stability of our nine candidate reference genes was evaluated across all 15 conditions and 2 time-points (Fig. 2). Stability was analyzed using three algorithms: geNorm (8), NormFinder (11), and BestKeeper (12). These algorithms rank the reference genes according to the calculated gene expression stability values and acceptability threshold (geNorm (M<1.5); NormFinder (SV<1.0)) or standard deviation and

172 threshold (SD<1.0, BestKeeper) (detailed in Materials and Methods). Figure 2 shows the
173 calculated stability metric for each gene in all samples combined, by method, ranked from best to
174 worst (left to right).

175 The rankings generated by geNorm and NormFinder were similar; *EF1B* and *GAPDH*
176 were identified as the most stable genes, followed by *ACT*, *14-3-3*, *EF1A*, and *40S* (Fig. 2AB).
177 BestKeeper, on the other hand, ranked *40S* and *14-3-3* as the most stable (Fig. 2C). The three
178 least stable genes identified by all three algorithms were *UBCE2*, *EF2A*, and *ARP2*/3 (Fig. 2ABC).
179 To create a consensus of the best-to-worst gene ranking across all three programs, a rank
180 aggregation method was performed using a Cross Entropy Monte Carlo algorithm (R-package
181 RankAggreg; See Materials and Methods). This analysis indicated that *GAPDH* and *EF1B* were
182 the two most stable genes and *EF2A* and *ARP2/3* the most unstable (Fig. 2D). Based on these
183 analyses, *GAPDH* and *EF1B* are recommended as general normalization genes for qPCR gene
184 expression studied in *Fucus*.

185

186 **Figure 2. Individual ranking of housekeeping genes by stability**. Stability values for nine
187 candidate reference genes generated by the following algorithms: A) geNorm, B) NormFinder, C)
188 BestKeeper, and D) a consensus (by rank aggregation). The maxiumum scale for A-C represent
189 the maximum acceptability value for each stability metric.

190

191 **Reference gene expression stability in sample groups (conditions)**

192 Numerous reports acknowledge the importance of using an appropriate reference gene
193 for a given experiment since individual reference genes may not maintain normalized expression
194 in all conditions (10,47,48). To test which reference genes would be most suitable for each of our

195 stress-condition groups, expression stability was analyzed per group using geNorm, NormFider,
196 and BestKeeper. The samples were grouped based on the nature of their stressor as follows:
197 *Fucus* is an intertidal alga that is challenged by a very harsh natural environment where it
198 undergoes daily desiccation and osmotic shock (group 1 – physiological stress); furthermore,
199 these environments are under the constant pressure of pollutants, such as copper and
200 herbicides/pesticides (group 2 - pollution); also, brown algae serve as food for marine organisms
201 such as mollusks, so the effect of grazing (proxied by mechanical wounding) was examined as
202 well (group 3 – wounding); in Stramenopiles, phytohormones have been found to influence
203 developmental processes (49–51) and as such we have tested the effect of auxin and gibberellic
204 acid (group 4 - hormones); *Fucus* was also grown with and without nutrients to identify the best
205 housekeeping genes for nutritional studies (group 5 - nutrients); lastly, we cultured *Fucus* under
206 different light and temperature regimes (group 6 – temperature-light). The ranking of reference
207 genes in each treatment group, based on stability, is shown in Fig 3. The Cq values for each
208 reference gene, by treatment group, can be found in S4 Fig. The stability values for each gene
209 by treatment group, per algorithm, can be found in S5 Fig.

210 GAPDH was identified as one of the top three most stable genes across multiple
211 conditions (4 out of 6) and ARP2/3 as one of three least stable genes (4 out of 6), whereas the
212 stability of other candidate reference genes varied depending on the condition examined (Fig 3;
213 S5 Fig). As mentioned earlier, the three algorithms rank reference genes according to their
214 calculated gene expression stability values and acceptability thresholds (geNorm: M<1.5;
215 NormFinder: SV<1.0) or standard deviation (BestKeeper: SD<1.0). Based on these thresholds, in
216 the hormone and the temperature-light groups, *ARP2/3* and *UBCE2* were rejected as suitable
217 reference genes (S4 S5 Fig).

218 geNorm, BestKeeper, and NormFinder showed differences in the ranking of candidate
219 reference genes within treatment groups, to some extent (Fig 3). To achieve a consensus ranking
220 across all three methods that could be used for recommendation of normalization genes by

condition, we again performed a rank aggregation analysis (Fig 3, red line and left to right order). For pollution, hormone, nutrient and temperature-light treatments there was less variation in ranking by the three algorithms and the consensus appears to be a reasonable recommendation. However, for physiological stress and wounding, the results of the three algorithms differed more, and the consensus seemed qualitatively less reliable. As such, for these two conditions, we recommend using the ranking produced by geNorm or NormFinder; our recommendation here is based on geNorm (See Discussion).

Figure 3. Optimal stability ranking of candidate reference genes using geNorm, NormFinder, BestKeeper, and rank aggregation method. Gene ranks from the three stability algorithms are shown by treatment group. Line colors represent: purple (geNorm), green (NormFinder), yellow (BestKeeper), black (mean rank), and red (consensus rank).

Identifying the optimal number of reference genes for normalization

Our previous analyses allowed us to provide recommendations for normalization genes to be used in general and specific experimental designs. However, using a single reference gene during qPCR experiments can cause bias and the use of multiple reference genes is suggested as standard practice (8). To determine the optimal number of reference genes to be used in *Fucus* qPCR experiments, pairwise variation $V_{n/n+1}$ was calculated for the proposed reference genes using the geNorm algorithm (8). In this method, the reference gene variances were combined additively and successively according to geNorm rank. After each addition the pairwise variance was calculated between that sum (V_{n+1}) and the variance of the prior sum (V_n); for example, $V_{2/3}$ was the pairwise variance calculated when using the top two genes was compared with using the top three. In general, the more reference genes that are added, the lower the successive pairwise

variance becomes. When the pairwise variance was below 0.15, *n* number of normalization genes was considered sufficient.

When all conditions were pooled, to simulate a general experiment, $V_{2/3}$ was below the 0.15 threshold, suggesting that using the top two stable reference genes was sufficient (Fig 4; $V_{2/3}$=0.145; optimal reference gene set *GAPDH+EF1B*). When the analysis was performed by condition group, $V_{2/3}$ was below 0.15 for all conditions except pollution (physiological stress=0.130, hormones=0.096, nutrients=0.103, temperature-light=0.120, wounding=0.105, pollution = 0.173; Fig. 4). For pollution stress, $V_{3/4}$ was less than the threshold ($V_{3/4}$= 0.145) suggesting the need for three reference genes for these experiments (Fig 4). Taken together, two stable reference genes are sufficient for most conditions tested here. By combining the recommended number of reference genes from this analysis with the consensus rank achieved for stability, we generated a table of most recommended and least recommended reference genes (Table 2).

Figure 4. Calculation of optimal number of housekeeping genes. Pairwise variation ($V_{n/n+1}$) was calculated in all tested samples; all (all conditions together), hor (hormones), nutr (nutrients), phy (physiological stress), pol (pollution), t-l (temperature-light), wou (wounding). The $V_{n/n+1}$ values below the 0.15 threshold (dotted line) indicate that *n* normalization genes are sufficient.

Table 2. List of recommended reference genes for each of the tested conditions. The recommendation is based on the result of a rank aggregation consensus gene stability analysis (pollution, wounding, hormones, nutrients, temperature-light) and geNorm stability analysis (physiological stress).

	Reference gene pair with highest stability	Reference gene pair with lowest stability
All samples	*GAPDH*	*EF2A*
	EF1B	*ARP2/3*
Physiological stress	*GAPDH*	*EF2A*
	EF1A	*ARP2/3*
Pollution	*GAPDH*	*UBCE2*
	EF1B	*ARP2/3*
	14-3-3	
Wounding	*EF1B*	*14-3-3*
	ARP2/3	*ACT*
Hormones	*GAPDH*	*40S*
	14-3-3	*EF2A*
Nutrients	*ARP2/3*	*40S*
	UBCE2	*EF2A*
Temperature - light	*EF1B*	*UBCE2*
	ACT	*ARP2/3*

Validation of reference genes in conditions of physiological stress

To experimentally validate our reference gene recommendations, we performed qPCR for two heat shock proteins, *Hsp70* and *Hsp90*, in samples from *Fucus distichus* under physiological stress (salinity and desiccation). *Hsp70* and *Hsp90* were chosen as potential target genes as they had exhibited differential expression in *Fucus* under various stress conditions (40,42,52). *Hsp70* and *Hsp90* sequences were identified in the *F. serratus* embryo transcriptome (36) and primers were designed and tested (S6 Fig). We then examined gene expression using the ΔΔCq method with 1) normalization to the two most stable reference genes (*GAPDH* and *EF1A*) or 2) with

normalization to the least stable genes (*EF2A* and *ARP2/3*) for physiological stress (Table 2). Gene expression analysis with the most stable pair (*EF1A* + *GAPDH)* showed a significant increase of *Hsp70* cDNA levels with 0.5x ASW treatment and a significant decrease in levels with the 2x ASW stress and desiccation treatments (Fig 5A). Conversely, normalizing to the *ARP2/3/EF2A* (least stable) reference gene set resulted in a loss of significant differential expression in the 0.5X ASW treatment (Fig 5B). *Hsp90* cDNA levels did not show significant change with treatment using either normalization set (Fig 5). These data indicate that using the most stable pair of normalization genes allowed for the capture of more information on differential gene expression for *Hsp70*; however, even the lowest-ranked normalization pair did result in some differential expression detection. Using a suboptimal normalization pair would likely be most detrimental when high Cq variance within technical replicates or between biological replicates is present. As such, using the most recommended set of normalization genes, in general or by condition, is likely to increase experimental sensitivity and reduce false negatives.

Figure 5. Detection of significant change in gene expression depending on the reference gene choice. Expression of two heat shock protein genes (*Hsp70* and *Hsp90*) normalized by the two most stable (A: *EF1A* and *GAPDH*) and two least stable (B: *ARP2/3* and *EF2A*) reference gene pairs. Statistically different gene expression from ASWP is indicated by * (Student's t-test; normal distribution, unequal variance; $p<0.05$).

Discussion

The use of stable and suitable reference genes when analyzing qPCR data is of utmost importance to correctly assess, present, and interpret gene expression in any experimental

304 design. In this study, we analyzed the suitability of nine candidate genes for normalization of gene
305 expression data obtained from qPCR in a brown alga *Fucus distichus*. This paper reports
306 recommendations for reference gene pairs to be used in *Fucus* experimental qPCR studies (Table
307 2). The recommendation is a result of two analyses: stability assessment and calculation of an
308 optimal number of reference genes to be used. Below we discuss the outcomes and comparative
309 merits of these analyses.

310

311 **Assessing stability**

312 The expression stability of the candidate genes was tested using the geNorm,
313 NormFinder, and BestKeeper algorithms. Our analysis with the three algorithms showed that the
314 stability of most candidate genes strongly depended on the experimental condition used, with
315 *GAPDH* being the most stable gene in most conditions and *ARP2/3* being the least stable in most
316 conditions. We did observe some discrepancies in ranking candidate gene stability within
317 conditions when using these three different algorithms.

318 The differences in stability values observed between different algorithms were not entirely
319 unexpected, taking into account that there are differences inherent in the three approaches
320 (8,11,12). geNorm is based on the principle that the most stable housekeeping genes should have
321 a nearly identical variation in expression ratio, or co-expression pattern. This approach, however,
322 may identify co-differentially expressed genes as highly stable in the chosen experimental system
323 (8,11). Conversely, NormFinder is not affected by the co-differential expression problem and as
324 such should be more robust; it estimates both inter- and intra-group variation and then combines
325 them into a stability value (11). BestKeeper, on the other hand, uses raw Cp values as input to
326 identify the best among the investigated candidate genes. It uses a Pearson correlation analysis,
327 a parametric method, which is valid for normally distributed data with a homogeneous variance;

328 if the data do not match these dependencies it may lead to a false interpretation of the obtained
329 results.

330 When the stability of genes in all the treatments was tested together, some discrepancies
331 were detected in the ranking of the most stable candidate reference genes using the three
332 algorithms, but there was an agreement between ranking the least stable genes. *GAPDH* and
333 *EF1B* were ranked as the most stable housekeeping genes tested by geNorm and NormFinder,
334 however, BestKeeper only placed them 3rd and 5th, respectively. The least stable genes were
335 identified as *ARP2/3*, *EF2alpha*, and *UBCE*. This is contradictory to results in *Ectocarpus*
336 *siliculosus*, where both *ARP2/3* and *UBCE* were the most stable genes and *GAPDH* was one of
337 the most unstable genes (13). It may be that patterns of gene expression vary between different
338 brown algal species, which further emphasizes the need to precisely define best reference genes
339 for specific studies.

340 According to our results, in four out of seven tested conditions *GAPDH* and *EF1B* were
341 the most stable reference gene candidates. It is valuable to note that *GAPDH* and *EF1B* had
342 different Cq_{mean} values (26.9 and 20.6, respectively) providing candidate normalization genes at
343 different expression level tiers. These genes have been previously identified as stably expressed
344 in other systems such as green algae, brown algae, plants, and animals (14,15,17,18,53,54).
345 Some studies in *Fucus* directly selected common reference genes such as *EFAs* to normalize
346 their target genes (41,42,45). *EFA* genes have been one of the most widely used in normalizing
347 gene expression of algal and plant species under stress conditions (13,17,22,54,55). However,
348 *EFAs* are not necessarily optimal for all stress conditions in *Fucus*, as shown here.

349 Some of the candidate genes were mostly present in the middle of the stability range, such
350 as *14-3-3*, *40S*, and *actin*. *Actin* and ribosomal subunit or RNA genes have previously been
351 reported as variably expressed in algae (13,16) and other organisms (17,22,56,57). The most
352 unstable genes from our study were *ARP2/3*, *UBCE2*, and *EF2A*. Even though their stability

353 ranking position varied depending on the treatment, for all of the conditions except wounding, at
354 least one of the aforementioned genes was ranked last. *ARP2/3* or *UBCE* have previously been
355 used as normalization genes in the brown alga *Ectocarpus siliculosus* and were stably expressed
356 in that system (13), consistent with our analysis. In line with our results, some previous reports in
357 algae and plants identified *UBCE* as a variable and non-suitable reference gene (16,54).

358 What is perhaps most pertinent to note is that even though ranking differences were
359 observed between the different algorithms, the majority of the calculated stability values were still
360 suitable as the fell under the rejection threshold (geNorm >1.5, BestKeeper>1, NormFinder>1;
361 Fig. S2). This suggests that, generally, all nine of the proposed reference genes we started with
362 should be suitable for normalization. We note that there are specific stress conditions where a
363 handful of candidate genes were rejected for use (hormone and temperature-light; Fig. S2) and
364 so care in specific stress conditions should be taken when selecting the exact pair of normalization
365 genes.

366

367 **The optimal number of reference genes**

368 It has been shown that the inclusion of more than one reference gene is required to detect
369 subtle changes in gene transcript levels by qPCR analysis (8,11,58). Vandesompele et al. (8)
370 provide evidence that normalization using a single gene can lead to an inaccurate normalization
371 of up to 25% of cases (with up to 6.4-fold difference). In our experiments and analyses, we could
372 conclude that in all treatment groups, except pollution, using the two most stable genes should
373 be sufficient for normalization. For the pollution treatment, our experiment would require three
374 normalization genes according to analysis. We strongly recommend that experimental designs
375 for *Fucus* RT-PCR and qPCR include at least two reference normalization genes.

376

377 **Evaluating normalization pairs in a desiccation and salinity stress study**

378 Overall, our recommendation of a 'best' normalization pair for physiological stress
379 condition (*GAPDH* and *EF1A*) proved to be more sound when compared to the 'least' suitable
380 pair when evaluating the differential gene expression of *Hsp70* during salinity stress and
381 desiccation. The statistically significant difference in the expression of *Hsp70* in low salinity failed
382 to be detected when normalizing to the two least stable genes. This has previously been shown
383 in other reports as well, where the choice of unstable genes led to misinterpretation of expression
384 data (47,59,60). It should be noted that we have assumed that the differential expression of *Hsp70*
385 in low salinity is true and not a false positive; we believe this to be the most likely case given the
386 higher stability of the 'best' pair.

387

388 **Conclusion**

389 The regulation of genes involved in algal biology including stress responses and
390 development is complex, and exploring the molecular mechanisms behind them requires high
391 precision and understanding. The selection of suitable reference genes to test qPCR based gene
392 expression is a necessary first step in understanding key molecular networks in the brown algal
393 lineage. Here we recommend the usage of specific reference gene sets in specific conditions
394 when performing comparable experiments in the brown alga *Fucus distichus*. In general, at least
395 two reference genes are recommended, and the best 'general' pair to use are *GAPDH* and *EF1B*.
396 When designing new experiments, we recommend checking the top five general recommended
397 genes from this study (Table 2) to test their stability in one's experimental system, as conducted
398 here, before deciding on the best normalization gene set for your study.

Materials and methods

Culture conditions and experimental design

Fucus distichus (Fucales, Phaeophyceae) individuals were collected from their natural rockpool habitat at the University of California Kenneth S. Norris Rancho Marino Reserve (Cambria, CA). Multiple apical segments were cut from adult individuals and cultivated in 2L glass flasks in a Percival incubator (Percival Scientific, USA) at 16ºC in filter-sterilized artificial seawater (ASW; 450mM NaCl, 10mM KCl, 9mM $CaCl_2$, 30mM $MgCL_2 \cdot 6H_2O$, 16mM $MgSO_4 \cdot 7H_2O$) enriched with Provasoli medium (PES; Anderson, 2005) for acclimation. Samples were cultured under a white fluorescent light at 60 µmol m^{-2} s^{-1} with a 12h:12h light: dark cycle. After 7 days, individual apical segments were transferred into Petri dishes with 15 different treatments, one segment per condition (Fig. ST1). Three biological replicates were obtained for each treatment at two time-points: 3 hours and 3 days after treatment start, resulting in 90 samples in total. A summary of the treatments may be found in Table S1.

The chemical treatments were: control ASW with nutrients (PES), nutrient-deficient ASW, 0.2µg/L imidacloprid (Marathon 1%, OHP, Inc., USA), 50µM indole-3-acetic acid (IAA; Cat#102037, MP Biochemicals, Irvine, CA.), 50µM gibberellic acid (GA; Cat#G7645, Sigma). An equal volume of absolute ethanol was used as a control for the GA and IAA treatment. In addition, a saline shock was performed using a hypersaline solution (2x ASW) and a hyposaline solution (0.5x ASW). The desiccation treatment was affected by placing the algal segments onto dry Petri dishes, after blotting gently, under the same environmental conditions as in other treatments. A mechanical wounding treatment, to simulate the effect of grazing, was performed by damaging the algal segments with a razor blade in several places along the thallus. Alteration of light and temperature was achieved as follows: a portion of samples was cultured under modified light

422 conditions (120 µmol m^{-2} s^{-1} and complete darkness) or two non-standard temperatures (8ºC and
423 22ºC).

424 **RNA extraction and cDNA synthesis**

425 Tissue was flash frozen in liquid nitrogen after treatment (3 hours and 3 days) and
426 immediately ground in liquid nitrogen with a pestle and a mortar. In our hands, extraction of RNA
427 from *Fucus* tissue is impaired by storage of the intact and/or ground tissue at -80 for longer periods
428 of time. To further refine the tissue homogenate samples were ground in 3mL Duall glass grinders
429 (Cat# K885451/0021, Smith Scientific, UK) grinder in 1ml of CTAB buffer (2% CTAB, 100 mM
430 Tris-HCl, 1.5 M NaCl, 50 mM EDTA, 50 mM DTT). RNA extraction was adapted from Apt et al.
431 (62) as follows briefly. Samples were shaken on a tilt shaker at room temperature for 20 minutes
432 after which 1V of chloroform was added to each. Solutions were mixed by inverting the tubes
433 several times and additionally incubating for 20 minutes while gently shaking. Samples were then
434 centrifuged at 10,000g for 20 minutes at 4ºC, after which 0.3V of 100% EtOH was added to
435 remove the polysaccharides. Polysaccharides were further extracted with 1V of chloroform and
436 centrifuged for 20 minutes at 10,000g at 4ºC. The upper phase was transferred to a new tube and
437 RNA was precipitated overnight at -20ºC by adding 0.25V LiCl and 1% (v/v) beta-
438 mercaptoethanol. Samples were centrifuged for 30 minutes 13,000g (4ºC), after which the pellet
439 was re-suspended in 50 µl of DEPC-treated MiliQ water. To remove residual DNA, a DNase
440 treatment was performed (TURBO DNase, ThermoFisher) according to manufacturer's
441 instructions. The final volume was adjusted to 500 µl with RNAse free water and extraction was
442 performed by adding 1V of phenol: chloroform: isoamylic alcohol (25:24:1 V/V). The samples were
443 centrifuged at 10,000g for 20 minutes (4ºC), after which another 1V chloroform extraction was
444 carried out and centrifuged again. The upper phase was transferred to a clean RNAse free tube
445 and RNA was precipitated by addition of 0.1V NaOAc (pH5.5) and 2.5V of 100% EtOH overnight
446 at -20ºC. After centrifugation (30 minutes at full speed, 4ºC), the supernatant was carefully

aspirated with a pipette and the pellet was washed with 75% ethanol. The tubes were centrifuged for 10 minutes at full speed (4ºC). The supernatant was carefully removed and the tubes were left to air dry for 5-10 minutes. The pellet was then re-suspended in RNAse free water.

The purity of RNA was assessed by measuring the ratio OD_{260}/OD_{280} and OD_{230}/OD_{260} using a NanoDrop 2000 (Thermo Fisher Scientific, USA). RNA integrity was measured using the High Sensitvity RNA ScreenTape Assay in an Agilent TapeStation (Agilent Technologies, Inc.; Fig. S1). The RNA sample (40ng) was reverse-transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit with oligo (dT)20 primers (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. cDNA samples were diluted to 10ng/ul for qPCR.

Quantitative Real-Time PCR (qPCR)

For each candidate gene, a pair of oligonucleotide primers was designed using Primer3 Input (v. 4.1.0) online tool (Table 1). qPCR was performed using a CFX384 Real Time PCR System (Bio-rad Laboratories Inc., USA). For each test, 3ul of cDNA (in technical duplicate wells) template was amplified using the SsoAdvanced™ SYBR® Green Supermix (Bio-rad Laboratories Inc., USA) in a final volume of 15 µl to test housekeeping gene expression levels. The cycling was performed as follows: 95ºC for 5 min followed by 41 cycles of 30s at 95ºC, 30s at 60ºC and 30s at 72ºC and a final step of 95ºC for 1 min. Each run was finished with heating up the samples from 65ºC to 95ºC to obtain a melting curve to test the specificity of amplification. All of the amplicons tested here had single melting peaks indicating a unique amplification product (Fig. S2).

Primer efficiencies were calculated as follows: a pooled cDNA sample of all samples was mixed and a dilution series generated to yield 1x, 0.1x, 0.01x, and 0.001x dilutions. Each primer set was used to amplify from the dilution series using the conditions above. The primer amplification efficiency (PE) and the correlation coefficient (R2) of each primer pair were

471 calculated (Table 1). All of the primer sets reported here fell within accepted boundaries of PE
472 and R2.

473 **Assessing the stability of candidate gene expression**

474 Data stability analysis was performed on all samples (15 conditions x 2-time points x 3
475 biological replicates) for all of the 9 genes identified, as well as separate groups with respect to
476 the nature of the treatment (physiological stress, hormone addition, pollution stress, temperature-
477 light modification, nutrient modification, and wounding/grazing (Fig. ST1). Quantification cycle
478 (Cq) values were analyzed with geNorm, NormFinder, and BestKeeper.

479 GeNorm calculates an average expression stability value (M) for each reference gene.
480 The analysis allows ranking of the genes according to their expression stability based on an
481 iterative stepwise exclusion of the genes with the highest M value (lowest stability). Additionally,
482 it calculates the optimal number of reference genes to be used for normalization, through a
483 pairwise variation (V) test (8). NormFinder calculates the expression stability value for each gene
484 using ANOVA-based mathematical analysis, taking into account intra- and inter-group variations
485 of the samples. A low SV-value indicates the high expression stability of this gene (11).
486 BestKeeper is an Excel-based tool that uses the standard deviation (SD) and the coefficient of
487 variance (CV) as evaluation criteria for stably expressed reference genes. Stability values for
488 geNorm were analyzed using R package NormqPCR (63) (NormFinder stability values were
489 calculated using the NormFinder R script (Andersen et al., 2004), and BestKeeper Excel-based
490 analysis was performed and standard deviation (SD) was used as a stability measure (12). In
491 addition, a rank aggregation method based on a Monte Carlo cross-entropy algorithm (R-
492 package; RankAggreg 0.6.5; https://CRAN.R-project.org/package=RankAggreg) was used to
493 combine the gene ranks of the three above algorithms and create a consensus housekeeping
494 gene ranking.

Validation of chosen housekeeping genes

Apical segments were placed the following physiological stress conditions: 2x salinity, 0.5x salinity, and desiccation condition for 6 hours. Untreated samples were transferred to 1X ASW. After 6 hours, RNA was extracted as detailed above and gene expression analyzed. To test the normalization efficiency of reference genes in a specific condition (ΔΔCq method), two of the most stable and two of the most unstable candidates for this group (physiological stress) were used to test the expression level of two heat-shock proteins (Hsp). Hsp gene sequences were identified through a local BLAST of the *Ectocarpus* Hsp70 and Hsp90 to the *Fucus serratus* transcriptome (36) and primers were designed using Primer3 Input (v. 4.1.0) online tool. ΔΔCq was calculated by normalization of the Hsp genes with the two housekeeping genes and to the targeted gene expression detected in a separate control sample.

Acknowledgements

The Braybrook group at UCLA is funded by The Department of Cell, Molecular and Developmental Biology and The College of Life Sciences (S.A.B); this work was majorly supported by the U.S. Department of Energy Office of Science, Office of Biological and Environmental Research program under Award Number DE-FC02-02ER63421 and the US Department of Energy (Biological and Environmental Research (BER), the Biological Systems Science Division (BSSD); M.L, S.A.B). We thank the developers of geNorm, NormFinder, and BestKeeper for their tools. We also thank Dr. Lauren Dedow for critical reading of the manuscript.

References

1. Charrier B, Le Bail A, De Reviers B. Plant Proteus: Brown algal morphological plasticity and underlying developmental mechanisms. Trends Plant Sci. 2012;17(8):468–77.

2. Silberfeld T, Leigh JW, Verbruggen H, Cruaud C, de Reviers B, Rousseau F. A multi-locus

time-calibrated phylogeny of the brown algae (Heterokonta, Ochrophyta, Phaeophyceae): Investigating the evolutionary nature of the “brown algal crown radiation.” Mol Phylogenet Evol. 2010;56(2):659–74.

3. Cock JM, Godfroy O, Macaisne N, Peters AF, Coelho SM. Evolution and regulation of complex life cycles: a brown algal perspective. Curr Opin Plant Biol. 2014;17:1–6.

4. Baldauf SL. The deep roots of eukaryotes. Science. 2003;300:1703–6.

5. Barbier M, Araújo R, Rebours C, Jacquemin B, Holdt SL, Charrier B. Development and objectives of the PHYCOMORPH European Guidelines for the Sustainable Aquaculture of Seaweeds (PEGASUS). Bot Mar. 2020;63(1):5–16.

6. FAO. The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Rome; 2018. 227 p.

7. Hurd CL, Harrison P, Bischof K, Lobban CS. Seaweed Ecology and Physiology. Cambridge University Press; 2014. 551 p.

8. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3(7):RESEARCH0034.

9. Phillips MA, D’Auria JC, Luck K, Gershenzon J. Evaluation of candidate reference genes for real-time quantitative PCR of plant samples using purified cDNA as template. Plant Mol Biol Report. 2009;27(3):407–16.

10. Kozera B, Rapacz M. Reference genes in real-time PCR. J Appl Genet. 2013;54(4):391–406.

11. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes

suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 2004;64(15):5245–50.

12. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol Lett. 2004;26(6):509–15.

13. Bail A Le, Dittami SM, Franco P-O de, Rousvoal S, Cock MJ, Tonon T, et al. Normalisation genes for expression analyses in the brown alga model *Ectocarpus siliculosus*. BMC Mol Biol. 2008;9:75.

14. Li J, Huang H, Shan T, Pang S. Selection of reference genes for real-time RT-PCR normalization in brown alga *Undaria pinnatifida*. J Appl Phycol. 2019;31(1):787–93.

15. Liu C, Wu G, Huang X, Liu S, Cong B. Validation of housekeeping genes for gene expression studies in an ice alga *Chlamydomonas* during freezing acclimation. Extremophiles. 2012;16(3):419–25.

16. Kowalczyk N, Rousvoal S, Hervé C, Boyen C, Collén J. RT-qPCR Normalization Genes in the Red Alga *Chondrus crispus*. PLoS One. 2014;9(2):e86574.

17. Hong S-Y, Seo P, Yang M-S, Xiang F, Park C-M. Exploring valid reference genes for gene expression studies in *Brachypodium distachyon* by real-time PCR. BMC Plant Biol. 2008;8(1):112.

18. Iskandar HM, Simpson RS, Casu RE, Bonnett GD, Maclean DJ, Manners JM. Comparison of reference genes for quantitative real-time polymerase chain reaction analysis of gene expression in sugarcane. Plant Mol Biol Report. 2004;22(4):325–37.

19. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible W-R. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant

564 Physiol. 2005;139(1):5–17.

565 20. Dekkers BJW, Willems L, Bassel GW, van Bolderen-Veldkamp RP (Marieke), Ligterink W,
566 Hilhorst HWM, et al. Identification of Reference Genes for RT–qPCR Expression Analysis
567 in Arabidopsis and Tomato Seeds. Plant Cell Physiol. 2012;53(1):28–37.

568 21. Souček P, Pavlů J, Medveďová Z, Reinöhl V, Brzobohatý B. Stability of housekeeping gene
569 expression in *Arabidopsis thaliana* seedlings under differing macronutrient and hormonal
570 conditions. J Plant Biochem Biotechnol. 2017;26(4):415–24.

571 22. Nicot N, Hausman J-F, Hoffmann L, Evers D. Housekeeping gene selection for real-time
572 RT-PCR normalization in potato during biotic and abiotic stress. J Exp Bot.
573 2005;56(421):2907–14.

574 23. Gong H, Sun L, Chen B, Han Y, Pang J, Wu W, et al. Evaluation of candidate reference
575 genes for RT-qPCR studies in three metabolism related tissues of mice after caloric
576 restriction. Sci Rep. 2016;6(1):38513.

577 24. Holmgren G, Ghosheh N, Zeng X, Bogestål Y, Sartipy P, Synnergren J. Identification of
578 stable reference genes in differentiating human pluripotent stem cells. Physiol Genomics.
579 2015;47(6):232–9.

580 25. Zhang Y, Chen D, Smith MA, Zhang B, Pan X. Selection of Reliable Reference Genes in
581 Caenorhabditis elegans for Analysis of Nanotoxicity. PLoS One. 2012;7(3):e31849.

582 26. Pombo-Suarez M, Calaza M, Gomez-Reino JJ, Gonzalez A. Reference genes for
583 normalization of gene expression studies in human osteoarthritic articular cartilage. BMC
584 Mol Biol. 2008 9(1):17.

585 27. McNeill RE, Miller N, Kerin MJ. Evaluation and validation of candidate endogenous control
586 genes for real-time quantitative PCR studies of breast cancer. BMC Mol Biol.

2007;8(1):107.

28. Bothwell JHF, Kisielewska J, Genner MJ, McAinsh MR, Brownlee C. Ca2+ signals coordinate zygotic polarization and cell cycle progression in the brown alga *Fucus serratus*. Development. 2008;135(12):2173–81.

29. Bouget FY, Berger F, Brownlee C. Position dependent control of cell fate in the *Fucus* embryo: role of intercellular communication. Development. 1998;125(11):1999–2008. 5

30. Goodner B, Quatrano RS. *Fucus* embryogenesis - a model to study the establishment of polarity. Plant Cell. 1993;5(10):1471–81.

31. Brownlee C, Bouget FY. Polarity determination in *Fucus*: from zygote to multicellular embryo. Semin Cell Dev Biol. 1998;9:179–85.

32. Kropf DL, Kloareg B, Quatrano RS. Cell wall is required for fixation of the embryonic axis in *Fucus* zygotes. Science. 1988;239(4836):187–90.

33. Torode TA, Marcus SE, Jam M, Tonon T, Blackburn RS, Herve C, et al. Monoclonal antibodies directed to fucoidan preparations from brown algae. PLoS One. 2015;10(2):e0118366.

34. Torode TA, Siméon A, Marcus SE, Jam M, Le Moigne MA, Duffieux D, et al. Dynamics of cell wall assembly during early embryogenesis in the brown alga *Fucus*. J Exp Bot. 2016;67(21):6089–100.

35. Hervé C, Siméon A, Jam M, Cassin A, Johnson KL, Salmeán AA, et al. Arabinogalactan proteins have deep roots in eukaryotes: Identification of genes and epitopes in brown algae and their role in *Fucus serratus* embryo development. New Phytol. 2016;209:1428–41.

36. Linardić M, Cokus S, Pellegrini M, Braybrook S. Growth of the *Fucus* embryo: insights into wall-mediated cell expansion through mechanics and transcriptomics. bioRxiv.

2020;25107.

37. Farnham G, Strittmatter M, Coelho S, Cock JM, Brownlee C. Gene silencing in *Fucus* embryos: Developmental consequences of RNAi-mediated cytoskeletal disruption. J Phycol. 2013;49(5):819–29.

38. Hable WE. Rac1 signaling in the establishment of the fucoid algal body plan. Front Plant Sci. 2014;5(690).

39. Tarakhovskaya E, Lemesheva V, Bilova T, Birkemeyer C. Early embryogenesis of brown alga *Fucus vesiculosus* L. is characterized by significant changes in carbon and energy metabolism. Molecules. 2017;22(1509):doi:10.3390/molecules22091509.

40. Pearson GA, Hoarau G, Lago-Leston A, Coyer JA, Kube M, Reinhardt R, et al. An expressed sequence tag analysis of the intertidal brown seaweeds *Fucus serratus* (L.) and F. vesiculosus (L.) (Heterokontophyta, Phaeophyceae) in response to abiotic stressors. Mar Biotechnol. 2010;12(2):195–213.

41. Jueterbock A, Kollias S, Smolina I, Fernandes JMO, Coyer JA, Olsen JL, et al. Thermal stress resistance of the brown alga *Fucus serratus* along the North-Atlantic coast: Acclimatization potential to climate change. Mar Genomics. 2014;13:27–36.

42. Smolina I, Kollias S, Jueterbock A, Coyer JA, Hoarau G. Variation in thermal stress response in two populations of the brown seaweed, *Fucus distichus*, from the Arctic and subarctic intertidal. R Soc open Sci. 2016;3(1):150429.

43. Rugiu L, Panova M, Pereyra RT, Jormalainen V. Gene regulatory response to hyposalinity in the brown seaweed *Fucus vesiculosus*. BMC Genomics. 2020;21(1):42.

44. Mota CF, Engelen AH, Serrao EA, Coelho MAG, Marbà N, Krause-Jensen D, et al. Differentiation in fitness-related traits in response to elevated temperatures between

633 leading and trailing edge populations of marine macrophytes. PLoS One.
634 2018;13(9):e0203666.

635 45. Lago-Lestón A, Mota C, Kautsky L, Pearson GA. Functional divergence in heat shock
636 response following rapid speciation of *Fucus* spp. in the Baltic Sea. Mar Biol.
637 2010;157(3):683–8.

638 46. Pearson G, Serrão EA, Leonor Cancela M. Suppression subtractive hybridization for
639 studying gene expression during aerial exposure and desiccation in fucoid algae. Eur J
640 Phycol. 2001;36(4):359–66.

641 47. de Boer ME, de Boer TE, Mariën J, Timmermans MJ, Nota B, van Straalen NM, et al.
642 Reference genes for QRT-PCR tested under various stress conditions in *Folsomia candida*
643 and *Orchesella cincta* (Insecta, Collembola). BMC Mol Biol. 2009;10(1):54.

644 48. Dong M, Zhang X, Chi X, Mou S, Xu J, Xu D, et al. The validity of a reference gene is highly
645 dependent on the experimental conditions in green alga *Ulva linza*. Curr Genet.
646 2012;58(1):13–20.

647 49. Basu S, Sun H, Brian L, Quatrano RL, Muday GK. Early embryo development in *Fucus*
648 *distichus* is auxin sensitive. Plant Physiol. 2002;130:292–302.

649 50. Le Bail A, Billoud B, Kowalczyk N, Kowalczyk M, Gicquel M, Le Panse S, et al. Auxin
650 metabolism and function in the multicellular brown alga *Ectocarpus siliculosus*. Plant
651 Physiol. 2010;153:128–44.

652 51. Bogaert KA, Blommaert L, Ljung K, Beeckman T, De Clerck O. Auxin function in the brown
653 alga *Dictyota dichotoma*. Plant Physiol. 2018;pp.01041.2018.

654 52. Creis E, Delage L, Charton S, Goulitquer S, Leblanc C, Potin P, et al. Constitutive or
655 Inducible Protective Mechanisms against UV-B Radiation in the Brown Alga *Fucus*

vesiculosus? A Study of Gene Expression and Phlorotannin Content Responses. PLoS One. 2015;10(6):e0128003.

53. Gu C, Chen S, Liu Z, Shan H, Luo H, Guan Z, et al. Reference Gene Selection for Quantitative Real-Time PCR in *Chrysanthemum* Subjected to Biotic and Abiotic Stress. Mol Biotechnol. 2011;49(2):192–7.

54. Han X, Lu M, Chen Y, Zhan Z, Cui Q, Wang Y. Selection of Reliable Reference Genes for Gene Expression Studies Using Real-Time PCR in Tung Tree during Seed Development. PLoS One. 2012;7(8):e43084.

55. Cuevas JC, López-Cobollo R, Alcázar R, Zarza X, Koncz C, Altabella T, et al. Putrescine is involved in Arabidopsis freezing tolerance and cold acclimation by regulating abscisic acid levels in response to low temperature. Plant Physiol. 2008;148(2):1094–105.

56. Jain M, Nijhawan A, Tyagi AK, Khurana JP. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochem Biophys Res Commun. 2006 Jun 30;345(2):646–51.

57. Hoogewijs D, Houthoofd K, Matthijssens F, Vandesompele J, Vanfleteren JR. Selection and validation of a set of reliable reference genes for quantitative sod gene expression analysis in *C. elegans*. BMC Mol Biol. 2008;9:9.

58. Xiao L, Zhang L, Yang G, Zhu H, He Y. Transcriptome of protoplasts reprogrammed into stem cells in *Physcomitrella patens*. PLoS One. 2012;7(4).

59. Li M-Y, Wang F, Jiang Q, Wang G-L, Tian C, Xiong A-S. Validation and Comparison of Reference Genes for qPCR Normalization of Celery (*Apium graveolens*) at Different Development Stages. Front Plant Sci. 2016;7:313.

60. Pinto F, Pacheco CC, Ferreira D, Moradas-Ferreira P, Tamagnini P. Selection of Suitable

679 Reference Genes for RT-qPCR Analyses in Cyanobacteria. PLoS One. 2012;7(4):e34983.

680 61. Anderson RA. Algal culturing techniques. Vol. 53, Elsevier Academic Press. 2005. 596 p.

681 62. Apt KE, Clendennen SK, Powers DA, Grossman AR. The gene family encoding the
682 fucoxanthin chlorophyll proteins from the brown alga *Macrocystis pyrifera*. MGG Mol Gen
683 Genet. 1995;246(4):455–64.

684 63. Perkins J, Kohl M. NormqPCR: Functions for normalisation of RT-qPCR data. 2013;1–23.

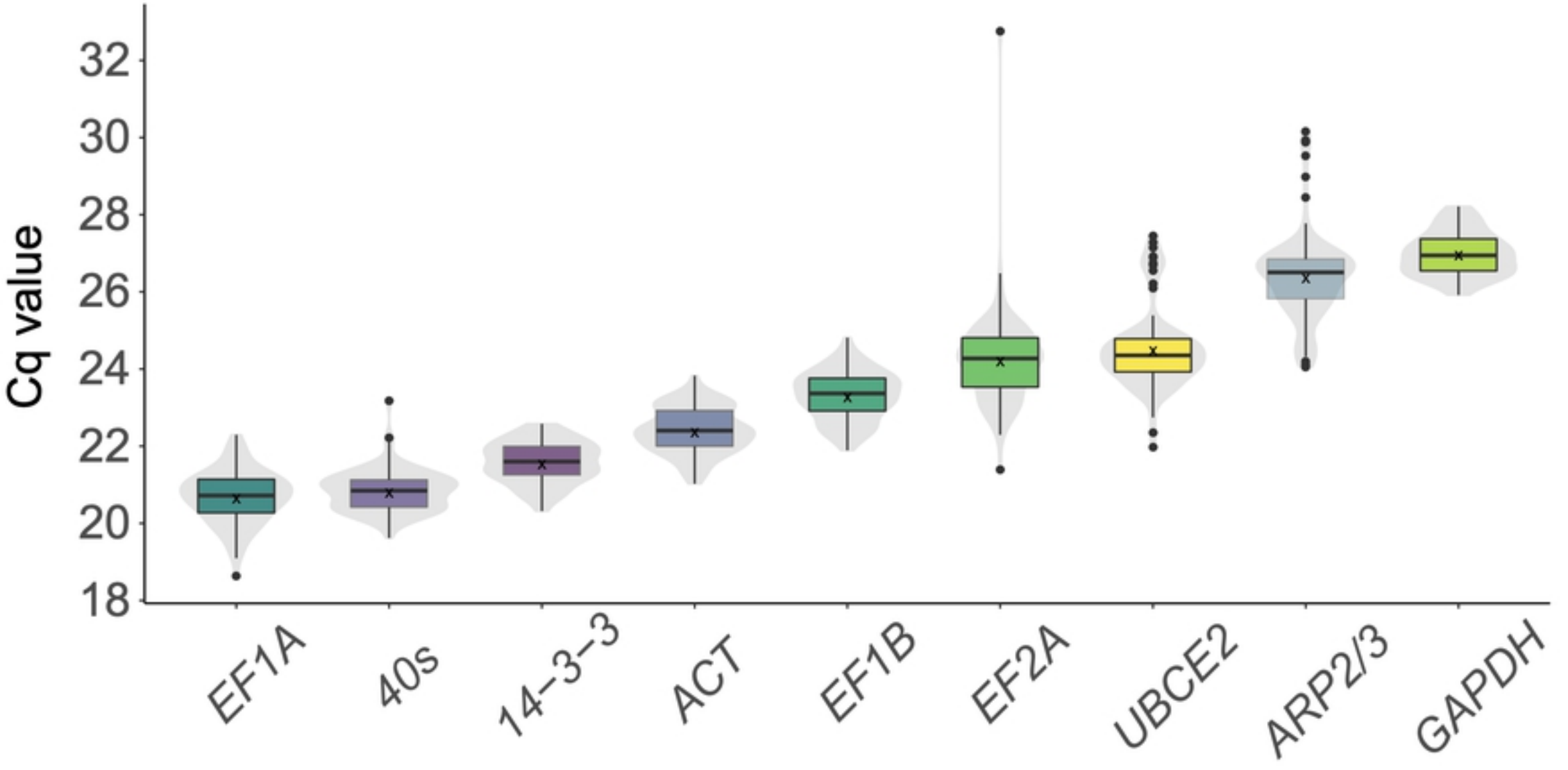
Cq value
32
30
28
26
24
22
20
18
EF1A
40s
14–3–3
ACT
EF1B
EF2A
UBCE2
ARP2/3
GAPDH

Figure 1

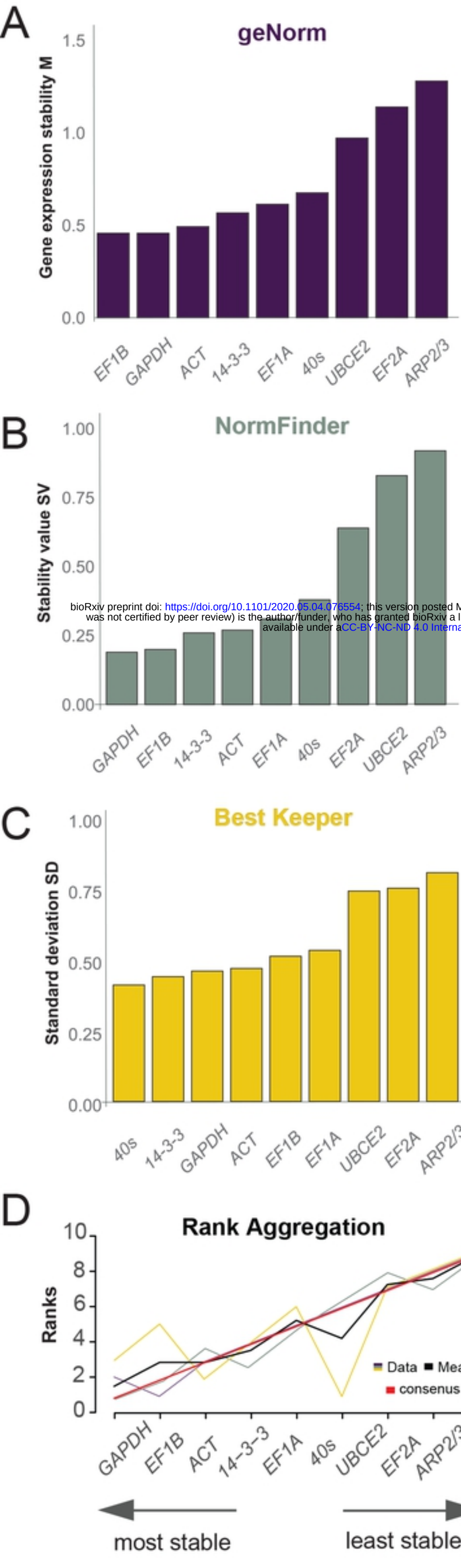

Figure 2

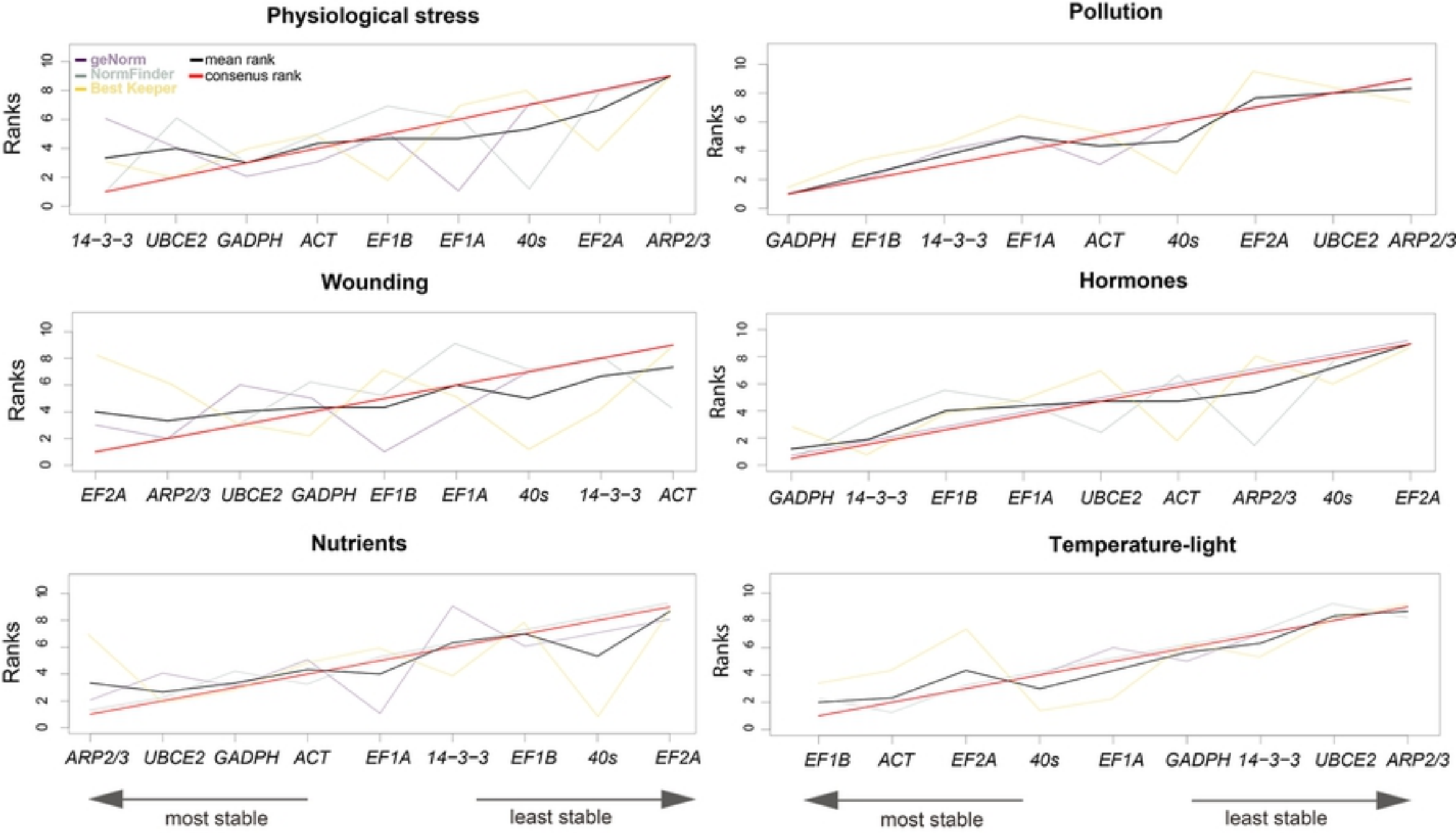
Physiological stress
geNorm
NormFinder
Best Keeper
mean rank
consenus rank
Ranks
14-3-3 UBCE2 GADPH ACT EF1B EF1A 40s EF2A ARP2/3
Pollution
GADPH EF1B 14-3-3 EF1A ACT 40s EF2A UBCE2 ARP2/3
Wounding
EF2A ARP2/3 UBCE2 GADPH EF1B EF1A 40s 14-3-3 ACT
Hormones
GADPH 14-3-3 EF1B EF1A UBCE2 ACT ARP2/3 40s EF2A
Nutrients
ARP2/3 UBCE2 GADPH ACT EF1A 14-3-3 EF1B 40s EF2A
most stable
least stable
Temperature-light
EF1B ACT EF2A 40s EF1A GADPH 14-3-3 UBCE2 ARP2/3
most stable
least stable

Figure 3

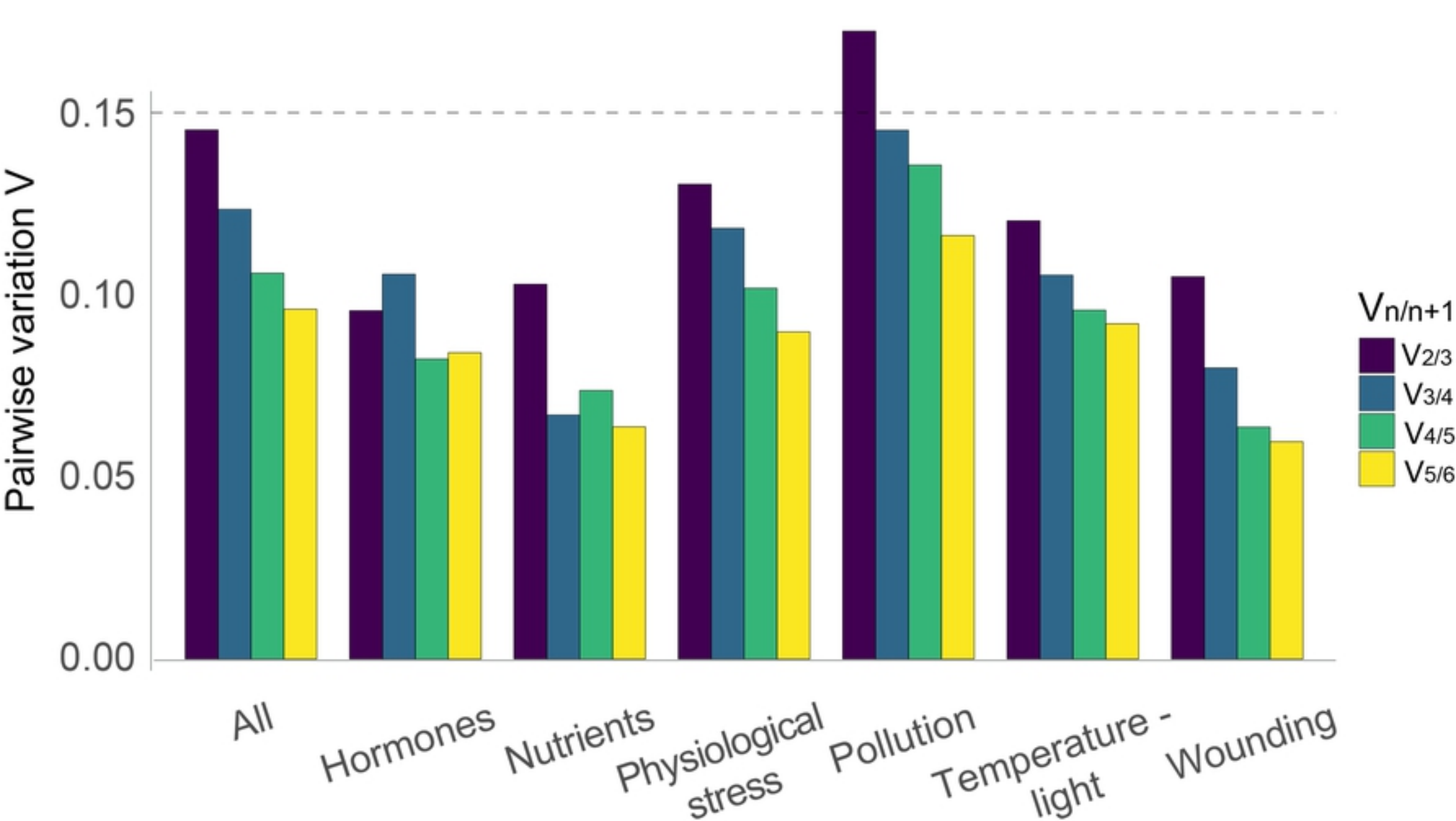
0.15
0.10
0.05
0.00
Pairwise variation V
All
Hormones
Nutrients
Physiological stress
Pollution
Temperature - light
Wounding
Vn/n+1
V2/3
V3/4
V4/5
V5/6

Figure 4

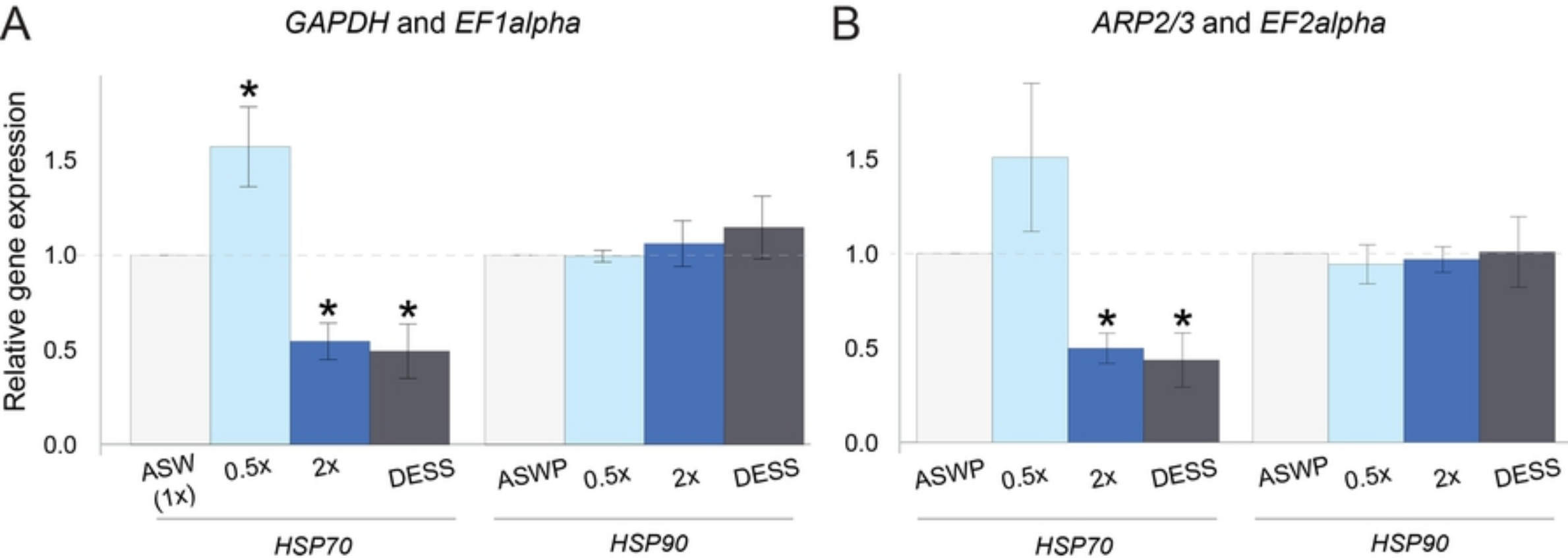
A
GAPDH and EF1alpha
Relative gene expression
1.5
1.0
0.5
0.0
*
*
*
ASW (1x)
0.5x
2x
DESS
ASWP
0.5x
2x
DESS
HSP70
HSP90
B
ARP2/3 and EF2alpha
1.5
1.0
0.5
0.0
*
*
ASWP
0.5x
2x
DESS
ASWP
0.5x
2x
DESS
HSP70
HSP90

Figure 5