Selection of reference genes for quantitative RT-PCR studies in striped dolphin (*Stenella coeruleoalba*) skin biopsies.

Giacomo Spinsanti[*], Cristina Panti[*], Silvia Casini[§], Letizia Marsili[§], Maria Cristina Fossi[§]

[*]Department of Evolutionary Biology §Department of Environmental Sciences - University of Siena - ITALY

The impact of POPs on marine mammals

Top predators, such as odontocete cetaceans, are known to accumulate high concentrations of persistent organic pollutants (POPs), including endocrine disrupting chemicals (EDCs), thereby incurring in high toxicological hazard. EDCs mime sex steroid hormones, both androgens and estrogens, binding to hormone receptors and influencing cellular pathways. Xenobiotic compounds exhibit lipophilic properties and tend to accumulate preferentially in the fat tissue of top predators. Previous ecotoxicological studies have assessed that cetacean skin biopsies constitute a suitable, non-lethal, biological material for the assessment of toxicological hazard in Mediterranean marine mammals. They enable the evaluation of levels of OCs with endocrine disrupting capacity and of Cytochrome P450 (CYP1A1) induction, as an early warning sign of exposure to organochlorines. Despite its sensitivity and reliability, qRT-PCR has never been used in cetacean skin biopsies to investigate gene induction. In this context, our work constitutes the first effort towards the routine application of this molecular technique for the evaluation of toxicological hazard in cetacean species. **Ten among the most common HKGs have been described and ranked, according to the stability of their expression, in skin biopsies of the striped dolphin.**

Stenella coeruleoalba

Cetacean skin biopsy with dermal (black) and fat (pink) tissues.

Materials and Methods

Partial nucleotide sequences of all selected HKGs have been determined in the striped dolphin. PCR primers were designed in conserved regions by comparing sequences of all genes available from GenBank for several mammal species. Primers were used in PCR reactions on cDNA retrotranscribed from *S.coeruleoalba* skin biopsies. Amplification products were sequenced on a automatic sequencer, checked for their specificity and deposited in GenBank (Tab 1). PCR primers for real-time assays were designed on the determined nucleotide sequences of each HKG, giving special attention to primer length, annealing temperature, base composition and 3'-end stability. To ensure optimal DNA polymerization efficiency, amplicons length ranged between 84 and 171 bp. For each different pair of primers, efficiency of RT-PCR (E), slope values, and correlation coefficients (R^2) were determined, using serial 1:5 dilutions of template cDNA.

Gene Symbol	Acc. Numb.	Gene Name
Act-B	DQ404535	β-actin
B2M	DQ533609	β-2-microglobin GAPDH
GAPDH	DQ404538	glyceraldehyde-3-phosphate dehydrogenase
HPRT1	DQ533610	hypoxantine phosphoribosyltransferase 1
PGK1	DQ533611	phosphoglycerate kinase 1 TFRC
RPL4	DQ404536	ribosomal protein L4
RPS18	DQ404537	ribosomal protein S18
SDHA	DQ533607	succinate dehydrogenase complex
TFRC	DQ533608	transferrin receptor
YWHAZ	DQ404539	tyrosine 3-monooxygenase

Tab 1: GenBank Accession Numbers of partially sequenced genes in *Stenella coeruleoalba*.

Skin biopsies, around 30 mg in weight, were homogenized with a TissueLyser; total RNA was subsequently extracted and checked with the Nano Drop® ND-1000 UV-Vis Spectrophotometer; absorbance ratio at 260/280 nm and 260/230 nm was used to assess purity of the RNA samples. Real-time amplifications were run in triplicate on 96-wells reaction plates with the iQ5 machine (Bio-Rad) using SYBR Green detection chemistry. Raw Ct values were transformed to quantities using an Excel spreadsheet based on the comparative Ct method. The data obtained have been converted into correct input files, according to the requirements of the software, and analysed using ***geNorm*** (version 3.4), ***NormFinder*** (*version* 0.953) and ***BestKeeper*** (*version 1*)*VBA applets.*

Results and Discussion

geNorm (Figs 1 and 2)

Selected HKGs were ranked according to the determined control gene-stability measure: GAPDH/YWHAZ, RPS18, RPL4, SDHA, Act-B, TFRC, PGK1, HPRT1, B2M. All studied genes reach a high expression stability with low M values, less than 1, below the default limit of M = 1.5. The *geNorm* software suggests that an accurate normalization factor of qRT-PCR data can be calculated by using the two most stably expressed genes. The addition of further HKGs will not significantly affect the reliability of the determined normalization factor, yielding a $V_{2/3}$ value of 0.141, lower than the default cut-off value of 0.15.

Figs 1 and 2: Candidate reference genes for normalization of qRT-PCR ranked according to their expression stability by the geNorm VBA applet and correspondent V values.

Ranking Order	Gene Name	Stability Value
1	GAPDH	0.155
2	YWHAZ	0.157
3	RPL4	0.278
4	RPS18	0.322
5	SDHA	0.343
6	Act-B	0.368
7	TFRC	0.385
8	PGK1	0.420
9	HPRT1	0.466
10	B2M	0.588

Tab 2: Rank of expression stability calculated by the *NormFinder* VBA applet.

NormFinder (Tab 2)

The ranking produced by *NormFinder* appears to be identical to the one previously determined using *geNorm*, except for the position of the two ribosomal protein genes S18 and L4, which are inverted. YWHAZ, GAPDH, RPL4 and RPS18 still occupy the highest positions, while TFRC, PGK1, HPRT1 and B2M are equally defined as the least reliable controls. As one would expect given their identical cellular function, the genes encoding for the ribosomal proteins S18 and L4 occupy the same position as in the *geNorm* rank.

BestKeeper (Tab 3 and 4)

All tested HKGs exhibit a SD value lower than 1 and have therefore been retained in the calculation of the *BestKeeper* (*BK*) index, which finally exhibit a moderate SD variation of 0.51. The 10 genes correlate well one with another, and also if compared with the *BK* index . The best correlation between the HKGs and the *BK* index is obtained for YWHAZ (r = 0.938), followed by Act-B and GAPDH. The statistically significant correlation shown by Act-B (r = 0.935) with the *BK* index appears inconsistent with the performance of this gene as assessed by *geNorm* and *NormFinder*. YWHAZ and GAPDH are still ranked as two of the most reliable control genes, showing respectively the best (0.938) and the third best (0.927) correlation values. It is also remarkable how TFRC, PGK1, HPRT1 and B2M are again classified as the least reliable HKGs, showing the worst correlations with the determined *BK* index. Calculation of the InVar parameter shows an overall good sample integrity.

	Act-B	B2M	YWHAZ	TFRC	SDHA	RPS18	RPL4	PGK1	HPRT1	GAPDH
N	30	30	30	30	30	30	30	30	30	30
GM [Ct]	18.66	21.23	18.95	24.10	24.82	16.83	16.13	21.36	23.04	17.82
AM [Ct]	18.69	21.26	18.97	24.11	24.85	16.84	16.14	21.37	23.05	17.83
Min [Ct]	16.60	18.79	17.29	22.26	22.49	15.49	15.05	19.78	21.05	16.59
Max [Ct]	20.72	23.17	20.56	26.04	26.69	18.63	17.25	23.83	24.12	19.12
SD [± Ct]	0.85	0.84	0.58	0.67	0.90	0.53	0.39	0.67	0.38	0.55
CV [% Ct]	4.57	3.97	3.07	2.77	3.60	3.13	2.43	3.13	1.67	3.10
BestKeeper vs.	Act-B	B2M	YWHAZ	TFRC	SDHA	RPS18	RPL4	PGK1	HPRT1	GAPDH
coeff. of corr. [r]	0.935**	0.668	0.938**	0.770	0.884	0.802	0.783	0.737	0.447	0.927**
p-value	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.013	0.001

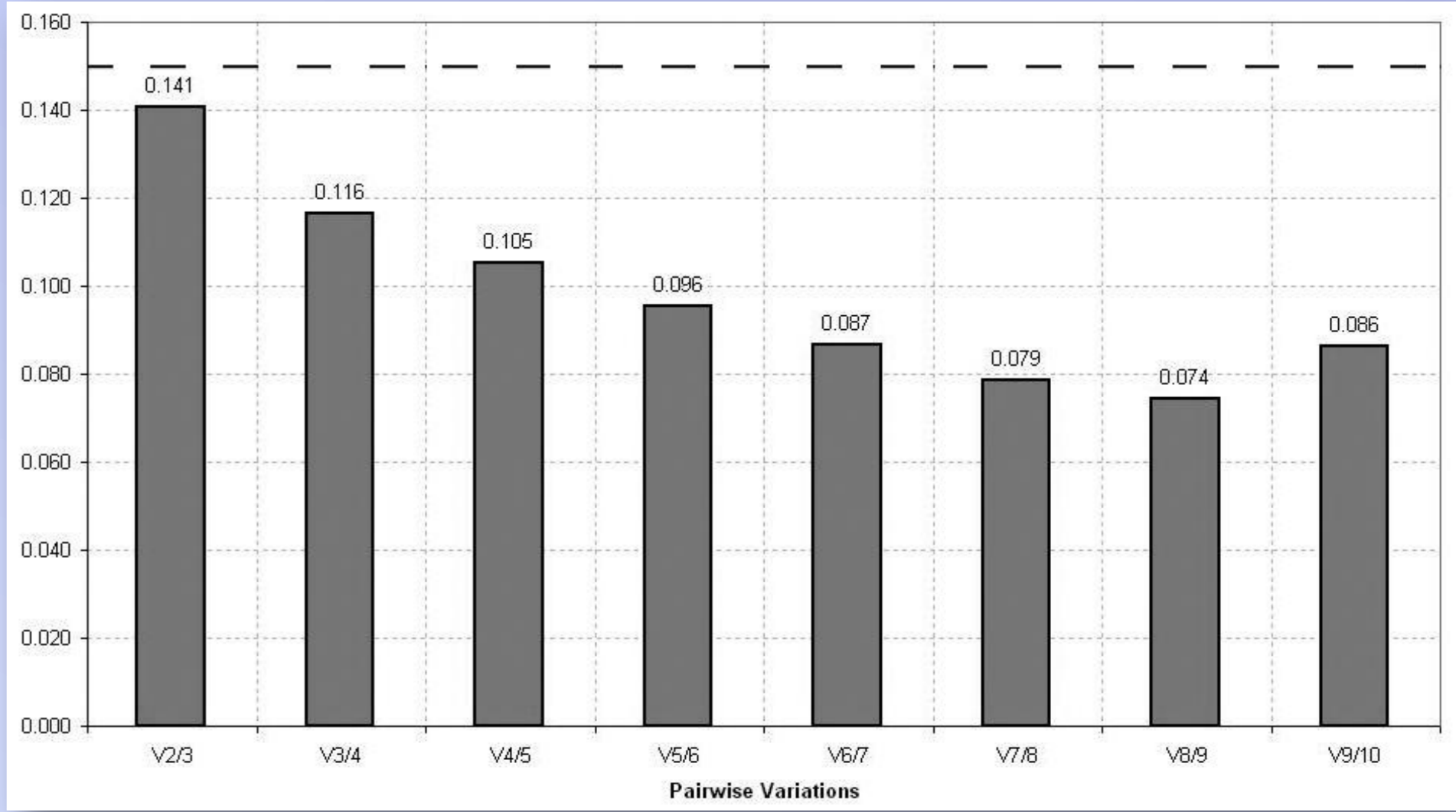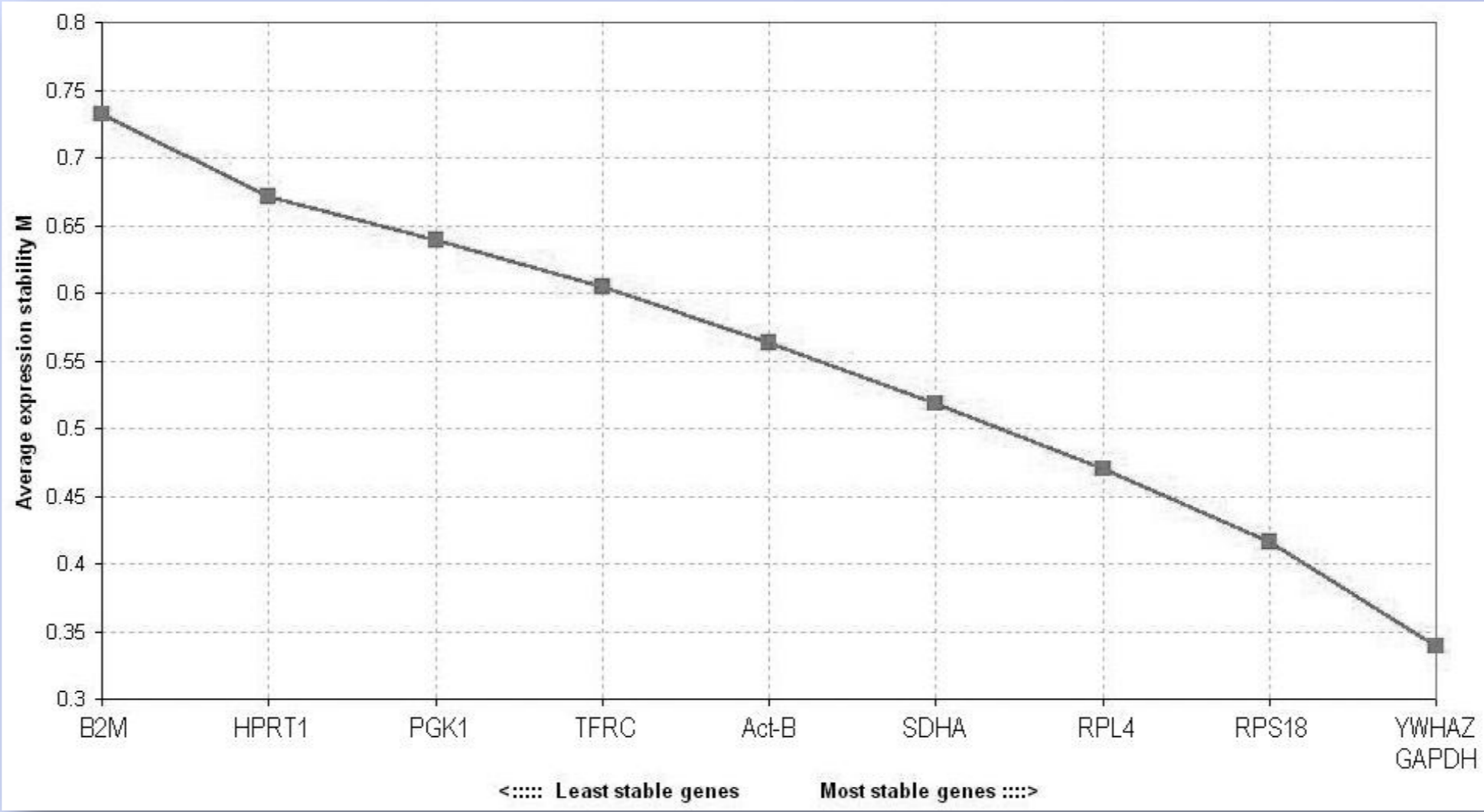Tab 3 and 4: Results from *BestKeeper* descriptive statistical and correlation analysis.

Conclusions

The three different specific VBA applets used to determine stability of selected control genes produce highly comparable results, even if based on different algorithms and analytical procedures. Our study let us conclude that GAPDH and YWHAZ are the most reliable HKGs of this set and we therefore strongly recommend their use in future qRT-PCR studies on the striped dolphin. Ribosomal protein genes RPL4 and RPS18 constitute additional useful HKGs. On the other hand, TFRC, PGK1, HPRT1 and B2M show unstable expression patterns and are always classified as the least reliable control genes of the group.