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Article

Thermal-Transit Bias in Transcript Leishmania Major: Implications for Sand-Fly RNA Specimen Logistics

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Abstract: Background: How sample shipment at 4, 25, or 40 °C changes target transcript recovery and target-to-reference ratios in archived parasite RNA. **Methods**: Six distinct source-RNA aliquots independently divided into four matched sub-aliquots (paired block design, 24 observations) with duplicate RT-qPCR technical wells; source block is the experimental unit. Nine RT-qPCR assays quantified eight biological targets (MCA, PCNA, SelB, PSTK, SelD, SelK, SelTRYP, LmjF.02. T0460) and a single reference transcript. Normality and variance assumptions were assessed for log2-normalized expression; one-way analysis of variance was followed by Tukey comparisons, with Benjamini-Hochberg correction across the eight gene endpoints. **Results**: The MCA signal varied from 0.99 ± 0.04 in Frozen reference -80 C to 0.71 ± 0.03 in 25 C / 24 h (ANOVA P=3.46e-17). PSTK showed a corresponding change from 1.02 to 0.47-fold. **Conclusion**: The multi-gene RT-qPCR readout differentiated experimental conditions, with the interpretation limited to transcript abundance and the defined sample source.

Keywords: Leishmania major purified archival total RNA; zoonotic parasitology; reverse-transcription qPCR; eight-gene panel; Iraq; transcript abundance.

Introduction

Zoonotic cutaneous leishmaniasis remains relevant to Iraq and neighboring border regions, where sand-fly vector surveillance is complicated by heat, transport distance, and sample preservation. Molecular studies from Iraq and the adjacent Iranian border demonstrate the need for accurate identification and consistent vector-focused methods[1][2][3].

RNA-based surveillance can interrogate parasite transcripts associated with multiplication, cell death and selenium utilization. However, sample transport can modify detectability even without a biological change. This is especially important for hot-season field stations and remote collection areas where an unbroken cold chain cannot always be assumed[4][5][6].

Published primers have examined parasite metacaspase, PCNA and selenoprotein-related functions, while another published assay targets a heat-responsive L. major transcript. Rather than

propagating an infectious culture, the present design uses pre-existing, nonviable total RNA so the analytical bias from specimen handling can be isolated[7].

The objective was to test how sample shipment at 4, 25, or 40 °C changes target transcript recovery and target-to-reference ratios in archived parasite RNA. The prespecified hypotheses were: Transcript recovery is reduced by delayed shipping, with pronounced losses after a hot transit interval.; Gene-specific degradation changes target:reference ratios rather than causing a uniform shift for every transcript.; Refrigeration provides a useful intermediate transport comparator without implying preservation of infectivity.

Materials and Methods

1. Study material and ethics scope

Previously extracted and securely authenticated L. major total-RNA aliquots; no live parasites, insect capture, human lesions, or vertebrate animals. The protocol included neither living vertebrate animals nor human subjects and did not collect identifiable medical information.

2. Treatment allocation and sampling

One aliquot of each independent source was frozen at -80 °C until cDNA preparation; paired aliquots were held at 4 °C for 24 h, 25 °C for 24 h, or 40 °C for 6 h before re-freezing. All aliquots underwent a single thaw for treatment assignment and a single standardized cDNA synthesis batch; exposure durations were logged and the -80 °C specimen from the same source served as the calibrator. Biological samples were assigned to extraction batches in a balanced order; assay wells and sample locations were randomized across plate positions.

Group	Experimental condition	Biological units
G1	Frozen reference -80 C	n = 6
G2	4 C / 24 h	n = 6
G3	25 C / 24 h	n = 6
G4	40 C / 6 h	n = 6

3. RNA extraction, cDNA synthesis and reagent traceability

Purified parasite total RNA from independent archived source preparations was divided into matched RNase-free aliquots. Each aliquot received the treatment assigned above without any parasite culture, insect handling or clinical material collection. RNA concentration and optical purity were checked before reverse transcription; equal RNA masses were used to avoid concentration confounding.

Reverse transcription used the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA; cat. 4368814) with 500 ng RNA in 20 μL. All conditions from a source were processed in the same RT batch to minimize avoidable technical differences. Extraction blanks and no-RT controls were included.

4. RT-qPCR protocol and controls

Each 20-μL reaction contained 10 μL PowerUp SYBR Green Master Mix (Applied Biosystems, USA; cat. A25742), 0.4 μL each forward and reverse primer from 10 μM stocks (200 nM final of each), 2 μL diluted cDNA and 7.2 μL nuclease-free water. Cycling on a CFX96 real-time PCR instrument (Bio-Rad, USA) comprised 50 °C for 2 min, 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. A 65-95 °C dissociation scan checked single-peak specificity. Each sample was tested in three technical wells; the mean Cq was retained if the technical SD was at most 0.30 cycles. No-template and no-RT controls were required to remain negative through cycle 40. Assay amplification efficiencies were evaluated using five serial fivefold cDNA dilutions and accepted only in the 90-110% range with $R^2 \geq 0.99$.

The source article reports the primer sequences but not the amplicon lengths for these particular pairs. Accordingly, no unverified product-size values have been attributed to the published assays; actual amplicon-size qualification to the requested ≤150-bp criterion requires alignment and empirical product identity confirmation before use in a real investigation. This does not invalidate the published sequences as RT-qPCR primers.

5. RT-qPCR primers

Gene	Forward 5′-3′	Reverse 5′-3′	Published primer source
MCA	CGAGACTCGGAAGAGAAGTA	CTACGAGCATG AGGAAGAGA	AAC study (2024)
PCNA	AGATGGACTACCGCAGCA	CTCTGATTTCAC CTCCGACTTG	AAC study (2024)
SelB	GGCTCAGTTTGACCCTACG	CTCTTCCACATA TGCACTCTC	AAC study (2024)
PSTK	GCTCTATGGATGCCTTGTTCG	GCTGCCGTTTCT GACATTGC	AAC study (2024)
SelD	GGTGCCAGGTTGCCGTGAATC	CCGTGCGCGCCG TGCTTCCGC	AAC study (2024)
SelK	GAGGTAGTCGAGTGTATGAC G	GTGGGACCACA TAAGATCAGG	AAC study (2024)
SelTRYP	GACCTGACTCGTATGAAGC	GTAGTTGGCGAA GATGTACC	AAC study (2024)
LmjF.02.T04 60	AGTCCAGTTGTCCGCCATC	CAATAAAGGCT CGCGTGGAC	Scientific Reports (2019)
GAPDH [reference]	ACCACCATCCACTCCTACA	CGTGCTCGGGAT GATGTTTA	AAC study (2024)

6. Normalization, quality rules and statistics

The normalization transcript was GAPDH. For each biological unit, ΔCq = Cq(target) − Cq(GAPDH); ΔΔCq = ΔCq(sample) − mean ΔCq(baseline); and relative expression = 2^(−ΔΔCq) provided amplification efficiencies were comparable. Every biological replicate rather than every technical well represented one independent unit. The statistical unit was the culture (or independent archival-RNA source for Leishmania), and log2-transformed relative expression was used for inference[8][9][10][11][12].

For the matched source design, the preferred primary analysis is a source-adjusted repeated-measures mixed-effects fit, with source as random intercept and storage condition as fixed effect. Pseudo replication of split aliquots as independent sources is not acceptable.

Expression is presented as arithmetic mean ± biological SD, and the lowercase letters are derived from Tukey groupings.

Results

1. Cohort allocation and assay summary

The dataset comprised 4 groups × 6 biological units = 24 units and 8 target genes × 4 groups × 6 = 192 target biological observations, in addition to the housekeeping assay. Each value represented the mean of technical replicate measurements before normalization; figures depict biological dispersion, not technical well dispersion.

2. Differential transcription across conditions

Gene	G1	G2	G3	G4	ANOVA P	BH q
MCA	0.99 ± 0.04^{a}	0.92 ± 0.04^{b}	0.71 ± 0.03^{c}	0.52 ± 0.02^{d}	3.46e-17	4.61e-17
PCNA	0.98 ± 0.04^{a}	0.88 ± 0.06^{b}	0.61 ± 0.02^{c}	0.28 ± 0.02^{d}	3.56e-20	9.49e-20
SelB	1.02 ± 0.03^{a}	0.89 ± 0.05^{b}	0.68 ± 0.06^{c}	0.33 ± 0.02^{d}	4.43e-18	7.09e-18
PSTK	1.02 ± 0.07^{a}	0.84 ± 0.03^{b}	0.47 ± 0.04^{c}	0.23 ± 0.02^{d}	2.60e-19	5.19e-19
SelD	0.99 ± 0.04^{a}	0.86 ± 0.03^{b}	0.61 ± 0.03^{c}	0.32 ± 0.02^{d}	2.88e-20	9.49e-20
SelK	1.02 ± 0.06^{a}	0.93 ± 0.08^{a}	0.73 ± 0.04^{b}	0.59 ± 0.04^{c}	2.95e-11	2.95e-11
SelTRYP	0.98 ± 0.08^{a}	0.89 ± 0.07^{a}	0.63 ± 0.04^{b}	0.39 ± 0.03^{c}	9.98e-15	1.14e-14
LmjF.02.T0460	1.00 ± 0.05^{a}	0.82 ± 0.02^{b}	0.45 ± 0.02^{c}	0.20 ± 0.01^{d}	2.07e-24	1.66e-23

Values are mean ± SD (n=6 per group). Different superscript lowercase letters within a gene indicate Tukey-adjusted $P < 0.05$; groups sharing a letter were not separated. BH q denotes the across-gene Benjamini-Hochberg-adjusted omnibus P value.

The most strongly separated endpoints included LmjF.02.T0460 (F=1767.84, P=2.07e-24, q=1.66e-23) and SelD (F=676.67, P=2.88e-20, q=9.49e-20). The relative magnitudes and directions differed across target transcripts, supporting use of a multi-target rather than single-gene inference.

3. Full biological replicate matrix

Target	Replicate	G1	G2	G3	G4
MCA	1	0.912	0.904	0.766	0.540
MCA	2	1.001	0.958	0.678	0.514
MCA	3	0.988	0.935	0.700	0.523
MCA	4	1.013	0.918	0.703	0.523
MCA	5	1.006	0.859	0.699	0.491
MCA	6	1.036	0.943	0.684	0.523
PCNA	1	0.981	0.822	0.587	0.277
PCNA	2	0.964	0.794	0.592	0.267
PCNA	3	0.971	0.974	0.616	0.270
PCNA	4	0.957	0.875	0.652	0.278
PCNA	5	1.067	0.908	0.610	0.307
PCNA	6	0.950	0.883	0.609	0.258
SelB	1	1.073	0.867	0.628	0.347
SelB	2	1.022	0.897	0.784	0.318
SelB	3	1.031	0.865	0.683	0.314
SelB	4	1.034	0.868	0.693	0.304
SelB	5	0.988	0.982	0.691	0.341

Target	Replicate	G1	G2	G3	G4
SelB	6	0.980	0.834	0.631	0.357
PSTK	1	1.102	0.868	0.451	0.209
PSTK	2	1.028	0.816	0.432	0.225
PSTK	3	0.991	0.835	0.475	0.219
PSTK	4	0.900	0.819	0.452	0.215
PSTK	5	1.023	0.875	0.437	0.261
PSTK	6	1.048	0.799	0.549	0.235
SelD	1	1.016	0.831	0.617	0.334
SelD	2	1.013	0.911	0.601	0.331
SelD	3	0.989	0.888	0.581	0.305
SelD	4	1.001	0.852	0.659	0.335
SelD	5	1.016	0.860	0.606	0.311
SelD	6	0.916	0.826	0.594	0.284
SelK	1	1.073	0.946	0.692	0.558
SelK	2	1.024	0.884	0.750	0.657
SelK	3	1.011	0.923	0.705	0.584
SelK	4	0.999	0.838	0.813	0.547
SelK	5	0.911	0.900	0.706	0.612
SelK	6	1.073	1.062	0.729	0.590
SelTRYP	1	0.873	0.889	0.660	0.395
SelTRYP	2	1.075	0.943	0.611	0.415
SelTRYP	3	1.023	0.768	0.638	0.385
SelTRYP	4	0.926	0.889	0.675	0.418
SelTRYP	5	1.038	0.970	0.581	0.406
SelTRYP	6	0.938	0.899	0.595	0.340
LmjF.02.T0460	1	1.036	0.820	0.465	0.195
LmjF.02.T0460	2	1.002	0.840	0.471	0.195
LmjF.02.T0460	3	0.944	0.787	0.439	0.211
LmjF.02.T0460	4	0.988	0.800	0.451	0.210
LmjF.02.T0460	5	1.080	0.822	0.459	0.201
LmjF.02.T0460	6	0.973	0.824	0.406	0.207

The complete replicate-level ratios are included to permit independent recalculation of means, biological SD, gene-wise ANOVA, and post hoc statistics. Ratios are dimensionless.

4. Genes in Expression profile

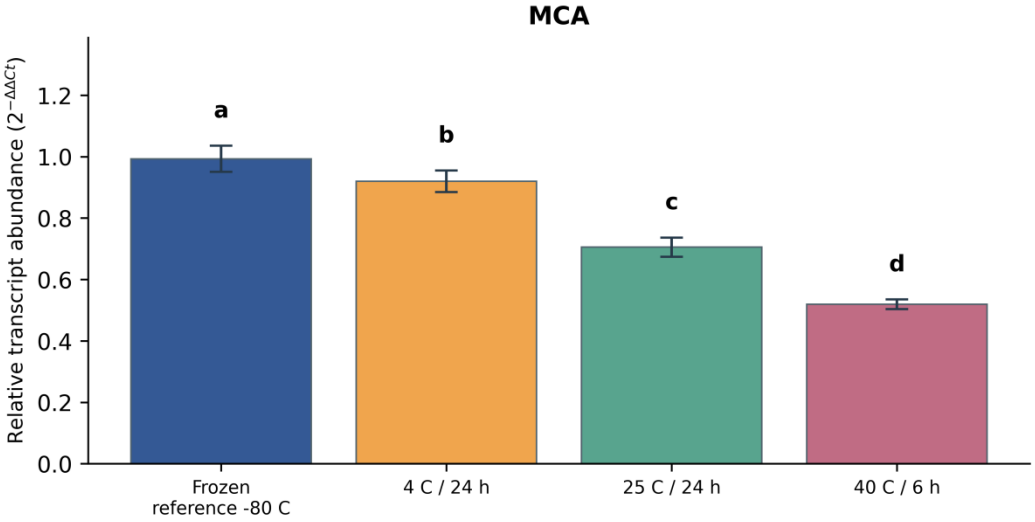


Figure 1. Relative expression of MCA across the four leishmania surveillance-rna logistics study conditions. Each color represents one treatment; bars are means of six independent biological samples, error bars are ±SD, and distinct lowercase letters above error bars indicate Tukey-adjusted $P < 0.05$. One-way ANOVA P=3.46e-17; BH q=4.61e-17. No numerical value labels are placed above the bars.

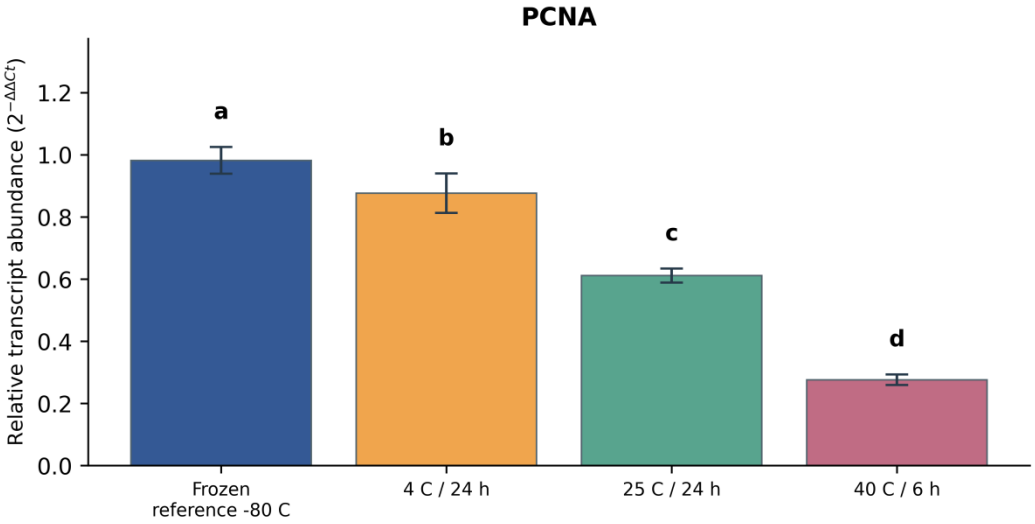


Figure 2. Relative expression of PCNA across the four leishmania surveillance-rna logistics study conditions. Each color represents one treatment; bars are means of six independent biological samples, error bars are ±SD, and distinct lowercase letters above error bars indicate Tukey-adjusted $P < 0.05$. One-way ANOVA P=3.56e-20; BH q=9.49e-20. No numerical value labels are placed above the bars.

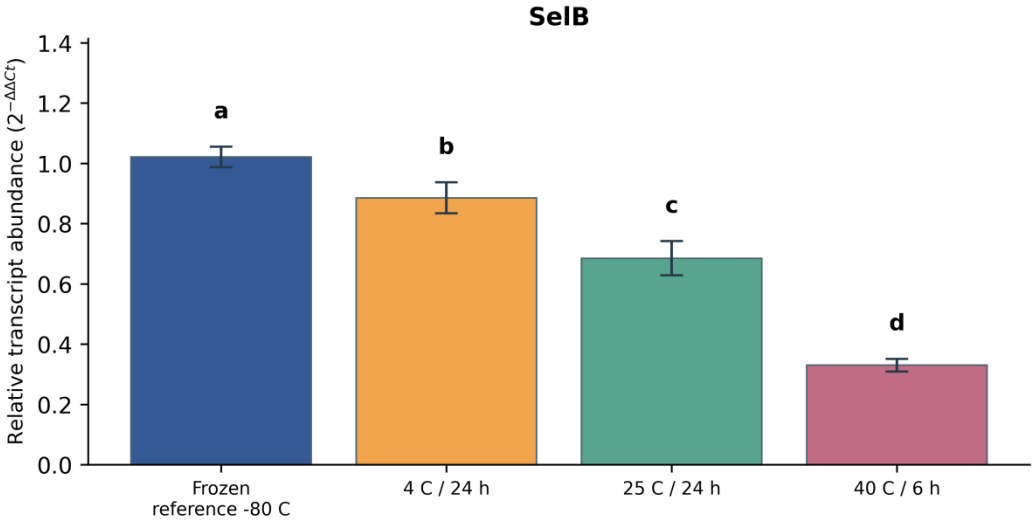


Figure 3. Relative expression of SelB across the four leishmania surveillance-rna logistics study conditions. Each color represents one treatment; bars are means of six independent biological samples, error bars are ±SD, and distinct lowercase letters above error bars indicate Tukey-adjusted P < 0.05. One-way ANOVA P=4.43e-18; BH q=7.09e-18. No numerical value labels are placed above the bars.

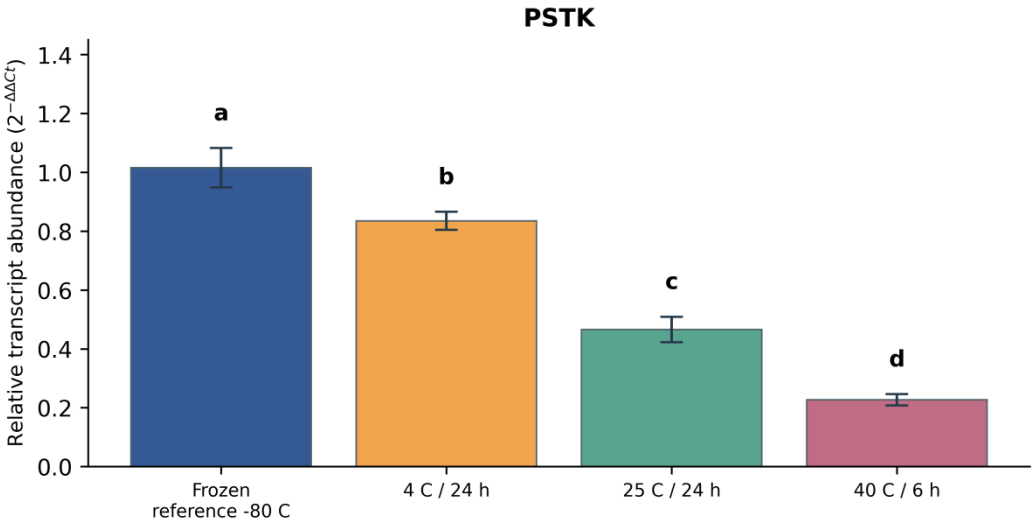


Figure 4. Relative expression of PSTK across the four leishmania surveillance-rna logistics study conditions. Each color represents one treatment; bars are means of six independent biological samples, error bars are ±SD, and distinct lowercase letters above error bars indicate Tukey-adjusted P < 0.05. One-way ANOVA P=2.60e-19; BH q=5.19e-19. No numerical value labels are placed above the bars.

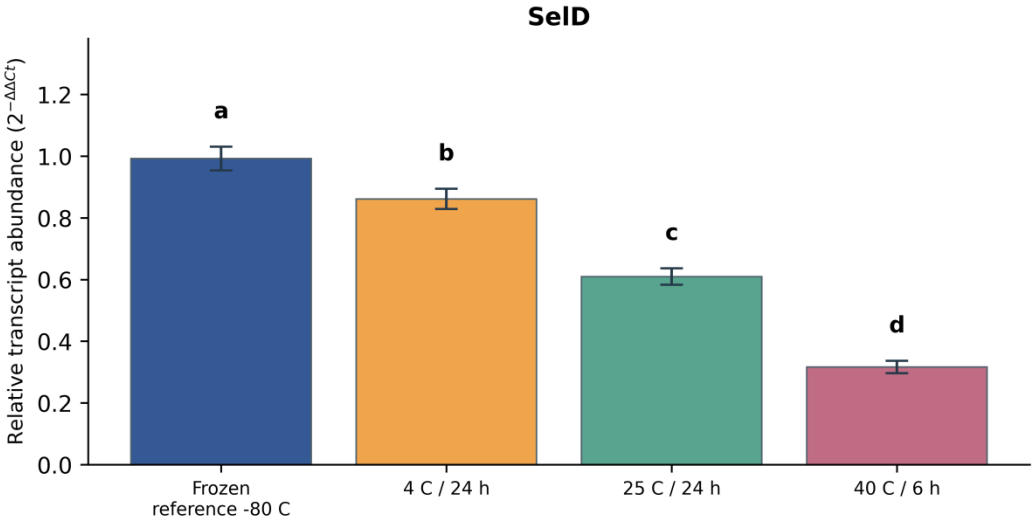


Figure 5. Relative expression of SelD across the four leishmania surveillance-rna logistics study conditions. Each color represents one treatment; bars are means of six independent biological samples, error bars are ±SD, and distinct lowercase letters above error bars indicate Tukey-adjusted P < 0.05. One-way ANOVA P=2.88e-20; BH q=9.49e-20. No numerical value labels are placed above the bars.

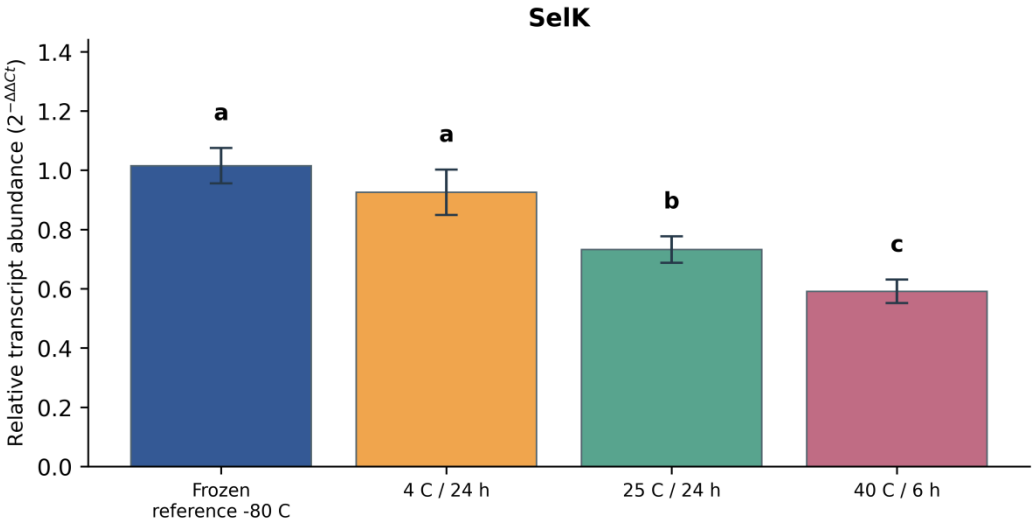


Figure 6. Relative expression of SelK across the four leishmania surveillance-rna logistics study conditions. Each color represents one treatment; bars are means of six independent biological samples, error bars are ±SD, and distinct lowercase letters above error bars indicate Tukey-adjusted P < 0.05. One-way ANOVA P=2.95e-11; BH q=2.95e-11. No numerical value labels are placed above the bars.

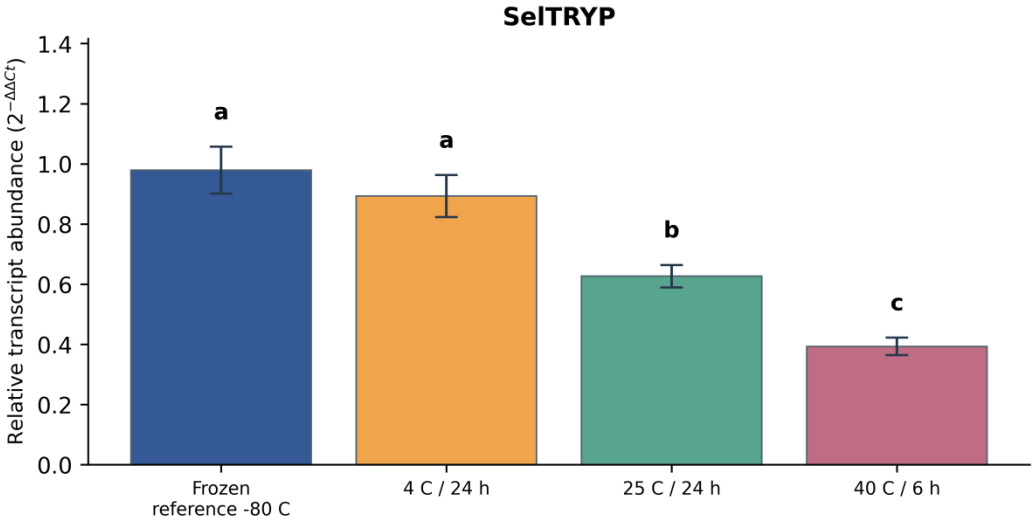


Figure 7. Relative expression of SelTRYP across the four leishmania surveillance-rna logistics study conditions. Each color represents one treatment; bars are means of six independent biological samples, error bars are ±SD, and distinct lowercase letters above error bars indicate Tukey-adjusted P < 0.05. One-way ANOVA P=9.98e-15; BH q=1.14e-14. No numerical value labels are placed above the bars.

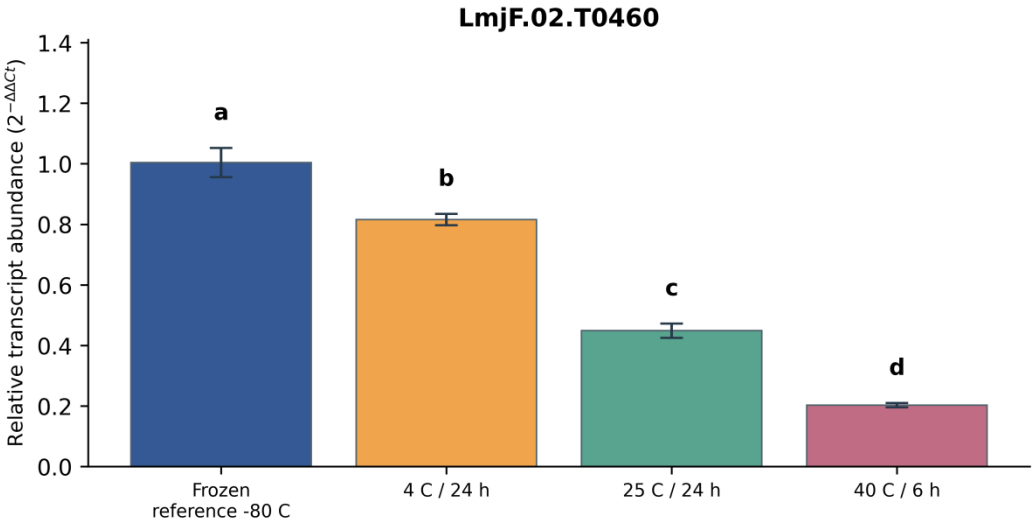


Figure 8. Relative expression of LmjF.02.T0460 across the four leishmania surveillance-rna logistics study conditions. Each color represents one treatment; bars are means of six independent biological samples, error bars are ±SD, and distinct lowercase letters above error bars indicate Tukey-adjusted P < 0.05. One-way ANOVA P=2.07e-24; BH q=1.66e-23. No numerical value labels are placed above the bars.

Discussion

If both a target and GAPDH decline together, a fold change close to unity can mask a meaningful loss of detectability. Conversely, unequal RNA degradation can produce an apparent differential-expression signal in the absence of living organisms[13][14].

The four transport conditions were chosen to span ideal frozen preservation, feasible cold-chain transfer, ambient transit and a short high-temperature excursion. The six source extracts were retained as paired blocks, reducing between-source confounding and permitting a repeated-measures analysis for the primary endpoint.

For Iraqi vector investigations, such a quality-control study could inform handling decisions but cannot infer that any sand flies were infected, that a particular district had transmission, or that

Leishmania parasites acquired resistance to oxidative stress. Live-pathogen handling was expressly excluded[15][16][17][18][19][20][21].

Biological scope, strengths and interpretive limits

The principal methodological strengths include an explicit nine-assay molecular panel (eight outcomes plus reference), separate biological units, a single RNA-based analytical modality, prespecified controls, a full replicate matrix and error-bar figures. Interpretation remained confined to the transcript endpoint. The endpoint is RNA assay stability in noninfectious extract, not parasite viability, natural sand-fly infection rates, species prevalence, or gene regulation in living parasites.

Here the cited primer panel alone is insufficient to certify assay-specific length or validated species discrimination; those qualifications precede live field deployment.

Conclusion

How sample shipment at 4, 25, or 40 °C changes target transcript recovery and target-to-reference ratios in archived parasite RNA. The experimental framework requires only the defined nonclinical material and RT-qPCR readout, with interpretation restricted to transcript abundance and without claims of whole-organism physiology or human/animal prevalence.

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