Supplementary Document to Mild temperatures differentiate while extreme temperatures unify gene expression profiles among populations of Dicosmoecus gilvipes in California. E.E. King and J.H. Stillman. DOI: 10.3389/fphys.2022.990390

Contents

Pg 1-9: Methods used to generate a de-novo transcriptome for the caddisfly Dicosmoecus gilvipes, and description of transcripts whose expression changed with laboratory exposure to warmer temperatures, including genes selected for assay of field-acclimatized populations using NanoString, as cited in the main text of the manuscript.

Pg 10-17: Supplementary figures and tables for the among-population analyses of gene expression profiles as cited in the main text of the manuscript.

Specimen sampling

Dicosmoecus gilvipes were collected from the surface of stones by hand on 19 and 20 July 2012 in the South Fork of the Eel River, 50-200m downstream of the Elder Creek confluence within the University of California Angelo Coast Range Reserve (http://angelo.berkeley.edu; 39°43'53.34"N; 123°38'41.92"W). Specimens were 5th stage instar larvae within their stone casing (staging was based on casing construction, as 5th instar larvae use minerals instead of plant material used in earlier stages (Limm and Power, 2011). Specimens were transported live to the laboratory within 24h of collection and held under common garden conditions at 11°C for two weeks before being transferred to tanks for different temperature conditions. Tank temperatures were thermally ramped over 4 days to temperatures ranging from 15.5°C to 28.6°C at which time they were held for 24 hours prior to sampling. Transcriptomic (RNA-seq) analyses were based on n=32 specimens, with n=8 per temperature treatment (see below table).
RNA-seq Methods: Library Construction, Sequencing and Bioinformatics

Animals were flash-frozen in liquid nitrogen and the thorax was dissected on dry ice and placed frozen into Tri Reagent (MRC). The thorax was used to maximize muscle tissue and minimize digestive tract symbiont contamination. Thorax tissue was homogenized under liquid N\textsubscript{2} using a TissueLyser II (Qiagen 85300) for 5-10 sec and RNA was extracted using the manufacturer’s polysaccharide/proteoglycan removal modified protocol, using BCP and High Salt Precipitation Solution (MRC). Purified RNA was assessed for quantity and quality using a Bioanalyzer (Agilent) and only samples with little to no degradation and adequate concentration were used in subsequent steps. For each exposure temperature, equal amounts of total RNA from each individual were used to make n=5 independent pooled RNA samples. Those n=5 pooled RNA samples were used to make RNAseq libraries following the Stillman laboratory’s modifications of the Illumina Tru-Seq RNA v2 kit (Stillman et al 2020). The pooled RNA allowed best sampling of mean levels of gene expression from a greater number of individuals, and to remove sources of technical variation from the ability to detect changes in gene expression.

Libraries size and concentration was determined on the Bioanalyzer using the high sensitivity DNA chip. Median library size was 400-450 bp across samples. Libraries were multiplexed and sequenced (100 bp paired-end) on the Illumina HighSeq 2000 platform at the Vincent J. Coates sequencing laboratory at UC Berkeley

| Temperature °C | # specimens in RNA-pool | # libraries made | Pool ID | # libraries passing filtration |
|----------------|-------------------------|-----------------|---------|-------------------------------|
| 15.5           | 8                       | 5               | Di_A   | 5                             |
| 20             | 8                       | 5               | Di_B   | 5                             |
| 25             | 8                       | 5               | Di_C   | 5                             |
| 28.6           | 8                       | 5               | Di_D   | 5                             |
Following qPCR to ensure equal concentrations of each sample, samples were multiplexed on a single sequencing lane.

In what follows we briefly summarize our bioinformatics pipeline. All scripts can be found on the GitHub page for Dr. Scott Fay (https://github.com/safay/RNA_seq/tree/master/blacklight_pipeline). RNAseq data were analyzed on the Pittsburg Supercomputer Center Blacklight. The mean number of processed reads in each library was $11.8 \pm 2.3 \times 10^6$ (mean $\pm$ SD). To prepare libraries for analysis, sequences were trimmed to remove Illumina adaptors (stringency = 1) and bases with a Phred quality score under 20 using Trim_Galore! (V 0.3.0). Trimmed reads were $32.0 \pm 1.2$ % of the total, $11.0 \pm 1.8$ % of which were quality trimmed, and $0.51 \pm 0.02$ % of the bases were trimmed. FLASH (V 1.2.8) was used to join overlapping paired end reads in order to make longer reads for de novo assembly and to eliminate artificial double counting of mapped read overlap regions. The number of reads processed by FLASH was $10.4 \pm 2.0 \times 10^6$, with $37.8 \pm 5.5$% of the reads combined, and $11.6 \pm 1.0$% of sequence pairs removed. De novo transcriptome assembly was performed using Trinity (V r2013-11-10) (http://trinityrnaseq.sourceforge.net/) (Grabherr et al., 2011). Trinity was used to conduct the assembly using a minimum kmer coverage = 2. The assembled transcriptome contained $n=135858$ contigs. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GJZL00000000. The version described in this paper is the first version, GJZL01000000. The transcriptome was annotated against the SwissProt database using Trinotate (V 1.1), with BLASTx of nucleotide sequences (resulting in $n=37464$ annotated contigs), and BLASTp ($n=31743$ annotated) and PFAM ($n=30225$ annotated) analyses of TransDecoder (V 2.0) produced protein sequences. Gene ontology annotation was found for $n=35024$ contigs.
Libraries were mapped to the *de novo* transcriptome for that species using Bowtie2 (V 2.0.6) and counted using eXpress (V 1.5.1) (http://bio.math.berkeley.edu/eXpress/), a read mapper that probabilistically assigns reads that ambiguously map to multiple loci, thus minimizing issues arising from redundant putative transcripts in *de novo* assembled transcriptomes (Roberts & Pachter, 2013). The unmodified eXpress output data are available at the NCBI Gene Expression Omnibus (NCBI GEO) under accession GSE206349 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE206349). Library size (# of mapped reads) was compared visually using boxplots to ensure that there was no library size variation. On average 93.8 ± 1.0 % of reads in each library mapped to the transcriptome.

Statistical analysis of differential gene expression was performed in R using the Bioconductor package EdgeR (V 1.2.4) (http://www.bioconductor.org/packages/release/bioc/html/edgeR.html) (Robinson et al, 2010) to identify genes with expression that varied across temperature. We used likelihood ratio tests with false-discovery rate correction to identify genes that had differential expression between any two acclimation treatments within a given species. Transcripts with expression of less than 2 reads/million mapped in less than 4 samples were removed from the analysis, which resulted in the retention of n=30092 transcripts in the analysis. Transcripts with a false-discovery rate corrected P-value of less than 0.05 and a fold-change ≥ 4 (for up-regulation) or ≤ 0.25 (for down-regulation) were identified as statistically differentially expressed genes (DEGs) in each LRT, which yielded a set of n=2586 DEGs. Differentially expressed transcripts were log2base transformed and median-centered based on the global within-species median. That set of transformed data is available at the NCBI GEO under accession GSE206349. Expression data were log2base transformed and median-centered based on the global within-species median.
Expression data were filtered again (MaxVal-MinVal ≥ 1.0), resulting in a final set of n=934 DEGs, and k-mean clustered (using default parameters) in Cluster (V 3.0; http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) and visualized using TreeView (http://jtreeview.sourceforge.net). Those clusters were visualized using TreeView (http://jtreeview.sourceforge.net) to determine the minimum number of k-means clusters that fully described the data. All subsequent data analysis of differentially expressed genes was performed in R.

**Gene Expression Clusters:**

Expression patterns for *D. gilvipes* are presented in the 8 k-means clusters named alphabetically “A” through “H” (Fig. S1; Table S1). Three of these clusters (A, B, and C) represented genes with a general pattern of down-regulation with increasing temperature, with cluster B showing the greatest downregulation at 30°C (approximately -2 log2-fold change on average; Fig. S1). All three of these clusters had a high representation of genes related to protein synthesis/degradation (Table S1). In addition, Cluster A had many genes related to transcription and RNA/DNA binding, Cluster B contained several genes related to immune/stress responses, oxidative metabolism, and storage, and Cluster C included many genes related to extracellular/cell-cell interaction/cuticular function and lipid modification (Table S1).

Three of the *D. gilvipes* clusters (D, E, and F) were associated with general increases in gene expression with rising temperature, particularly at 30°C. Clusters E demonstrated the strongest induction at 30°C, with a mean four-fold log2 increase in expression (Fig. S1). Cluster D was dominated by immune/stress response genes, including heat-shock proteins, as well as cellular chaperones typically unassociated with the stress response (Table S1). Cluster D also
contained a high proportion of genes related to cell cycle/development and transcription and DNA/RNA binding. Cluster E had a high representation of genes related to the cell cycle/development as well as the immune/stress response (Table S1). Cluster F had low and relatively even representation of genes across functional categories, but had the functions with the highest representation were related to amino acid metabolism, immune/stress response, and transcription and DNA/RNA binding (Table S1).

Two of the *D. gilvipes* clusters (G and H) had no strong pattern of change with temperature (Fig. S1). Cluster G contained only features in the “other” functional category, and cluster H had low and relatively even representation across functional categories, with no more than three features occurring for any given function (Table S1).

Table S1. Statistics on clustering of differentially expressed genes.

| Cluster                   | A   | B   | C   | D   | E   | F   | G   | H   | Total |
|---------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-------|
| Total No. DE features    | 139 | 163 | 108 | 264 | 25  | 136 | 22  | 77  | 934   |
| No. DE features annotated| 60  | 74  | 57  | 132 | 19  | 46  | 5   | 18  | 411   |
| No. unique annotations*  | 47  | 55  | 42  | 88  | 10  | 39  | 3   | 17  | 242   |
| biological rhythms        | 1   | 4   | 0   | 2   | 0   | 1   | 0   | 1   | 9     |
| carbohydrate metabolism  | 2   | 2   | 2   | 2   | 0   | 3   | 0   | 1   | 12    |
| cell cycle/development    | 5   | 0   | 2   | 24  | 9   | 4   | 0   | 1   | 45    |
| chaperones (not stress related) | 0  | 0  | 0   | 15  | 2   | 0   | 0   | 0   | 17    |
| digestion                 | 1   | 3   | 2   | 0   | 0   | 0   | 0   | 2   | 8     |
| extracellular/cell-cell interaction/cuticle | 7  | 13  | 0   | 1   | 0   | 0   | 0   | 1   | 22    |
| immune/stress response    | 3   | 5   | 14  | 39  | 7   | 5   | 0   | 3   | 76    |
| lipid modification        | 4   | 11  | 1   | 1   | 0   | 3   | 0   | 2   | 22    |
| membrane structure        | 0   | 0   | 0   | 0   | 0   | 1   | 0   | 0   | 1     |
| muscle function           | 0   | 3   | 2   | 0   | 0   | 2   | 0   | 0   | 7     |
| neuronal function         | 0   | 1   | 1   | 3   | 0   | 0   | 0   | 0   | 4     |
| osmotic/ionic regulation  | 0   | 0   | 0   | 2   | 0   | 0   | 0   | 1   | 3     |
| oxidative metabolism      | 2   | 8   | 6   | 6   | 0   | 2   | 0   | 1   | 25    |
| protein synthesis/degradation | 10 | 13  | 5   | 6   | 0   | 1   | 0   | 1   | 36    |
| storage                   | 0   | 4   | 8   | 0   | 0   | 0   | 0   | 0   | 12    |
| cytoskeleton              | 1   | 0   | 2   | 2   | 0   | 1   | 0   | 0   | 6     |
| transcription, DNA/RNA binding | 11 | 1   | 0   | 17  | 0   | 5   | 0   | 0   | 34    |
| transport                 | 7   | 0   | 1   | 1   | 0   | 1   | 0   | 0   | 10    |
| other                     | 5   | 4   | 9   | 10  | 1   | 9   | 5   | 1   | 44    |

* unique annotations in a cluster are specific to that cluster
Figure S1. Transcriptome profiles of *Dicosmoecus gilvipes* acclimated to different temperatures. Left, heat map of all data organized into k-Means clusters. Center, photo of *D. gilvipes* 5th instar larva, with stony case. Right, mean log$_2$-fold median-centered normalized FPKM expression difference ± 1 s.d. within k-Means clusters (left).
**Figure S2.** RNA-Seq data only for the genes used in the current study, organized by hierarchical clustering. The text colors represent log2 fold difference in expression, and indicate if we expected an increase (red) or decrease (blue) in transcript abundance with temperature in the current study. Two samples were removed from the 20°C group because of divergent expression patterns (see Fig. S1).
Bibliography

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, Chen Z, Mauceli E, Hacohen N, Gnirke A, Rhind N, di Palma F, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N and Regev A (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology 29, 644–652.

Limm MP and Power M (2011) The caddisfly Dicosmoecus gilvipes: Making a case for a functional role Journal of the North American Benthological Society 30(2):485-492 DOI:10.1899/10-028.1

Roberts A and Pachter L (2013) Streaming fragment assignment for real-time analysis of sequencing experiments. Nature Methods 10, 71–73.

Robinson MD, McCarthy DJ and Smyth GK (2010) Edger: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics (Oxford, England) 26, 139–140.

Stillman JH, Fay SA, Ahmad SM, Swiney KM, Foy RJ (2020). Transcriptomic response to decreased pH in adult, larval and juvenile red king crab, Paralithodes camtschaticus, and interactive effects of pH and temperature on juveniles. Journal of the Marine Biological Association of the United Kingdom 1–15. https://doi.org/10.1017/S002531541900119X
**Figure S3.** Heatmap of all genes and treatments, without any induction calculations. Genes are arranged in rows and grouped by similarity of expression value (dendrogram). Each column is an individual caddisfly, labeled by its warming treatment. White bars represent individuals in the cool control treatment. Black and grey bars correspond to the daily warming treatment and heat shock, respectively. The colors of the heat map cells represent the magnitude and direction of the change in expression, scaled and centered by row. Asterisk represents the individual left out of the PCA analysis.
Figure S4. Population and treatment differentiation along principal components 1 and 2. This figure and analysis are identical to Fig 2, except that it includes one outlier point from Sagehen control, represented by the asterisk. Data represent PC scores for all population and treatment combinations. Symbol shape represents population of origin, fill color represents the treatment.
**Figure S5.** Principal component loadings for A) PC 1 B) PC 2. Horizontal lines indicate significance level. Transcripts with significant loadings are in black. All others are in gray.
### Table S2. NanoString target and reference genes

| Target Transcripts                                      | Abbreviation             | Full Transcript Name                          | ContigID             | UniProtID  | Function                              | Expected Heat Δ |
|---------------------------------------------------------|--------------------------|-----------------------------------------------|----------------------|------------|---------------------------------------|-----------------|
| alpha-amylase                                           | Alpha-amylase A          | comp57363_c0_seq1                             | P08144              | carbohydrate metabolism             | down            |
| apoptosis inhibitor                                     | Apoptosis inhibitor      | comp58747_c0_seq1                             | P41436              | apoptosis inhibition               | down            |
| aquaporin                                               | Aquaporin AQPae.a        | comp64170_c1_seq1                             | Q9NHW7              | transporter activity               | down            |
| ATPase inhibitor                                        | ATPase inhibitor mai-2   | comp60498_c0_seq1                             | A8XZB0              | negative regulation of ATPase activity | up              |
| carbonic anhydrase                                      | Carbonic anhydrase 2     | comp61667_c0_seq1                             | Q8UWA5              | carbonate dehydratase activity      | up              |
| carbonyl reductase                                      | Carbonyl reductase [NADPH] | comp61657_c0_seq4                             | Q28960              | metabolic process                  | down            |
| chymotrypsin                                            | Chymotrypsin-1           | comp55087_c0_seq1                             | Q27289              | digestion                          | down            |
| circadian clock                                         | Circadian clock-controlled protein | comp57418_c0_seq3                             | O76879              | circadian rhythm                   | down            |
| copper chaperone                                        | Copper chaperone for superoxide dismutase | comp58560_c3_seq5                             | Q9JK72              | metal ion transport, superoxide radical removal | up              |
| GST                                                     | Glutathione S-transferase | comp58392_c0_seq1                             | P46437              | Transferase, antioxidant            | down            |
| hsp10                                                   | 10 kDa heat shock protein, mitochondrial | comp63721_c5_seq1                             | Q5DC69              | protein folding                    | up              |
| hsp23                                                   | Heat shock protein 23    | comp54992_c0_seq1                             | P02516              | protein folding                    | up              |
| hsp70                                                   | Heat shock protein 70 B2 | comp64140_c1_seq7                             | P41827              | stress response                    | up              |
| hsp70-3                                                 | Heat shock 70 kDa protein cognate 3 | comp58617_c2_seq1                             | P29844              | protein folding                    | up              |
| Protein Name          | Description                                                                 | Accession   | P200 number | Function               | Regulation |
|-----------------------|-----------------------------------------------------------------------------|-------------|-------------|------------------------|------------|
| hsp70-5               | Heat shock 70 kDa protein cognate 5                                           | comp63373_c1_seq4 | P29845      | protein folding        | up         |
| hsp90 activator       | Activator of 90 kDa heat shock protein ATPase homolog 1                      | comp63956_c0_seq2 | O95433      | co-chaperone of hsp90  | up         |
| MAPK-activator        | Arf-GAP with dual PH domain-containing protein 1                             | comp62480_c4_seq2 | O75689      | regulation of GTPase activity | up         |
| mito enolase          | Mitochondrial enolase superfamily member 1                                   | comp58198_c0_seq2 | Q7L5Y1      | amino acid and carbohydrate catabolism | down       |
| mobility group        | Mobility group protein 1A                                                    | comp38073_c0_seq1 | P40622      | DNA binding            | down       |
| PGM                   | Phosphoglucomutase                                                           | comp63948_c2_seq1 | Q7KHA1      | glucose metabolic process | down       |
| phenoloxidase 2       | Phenoloxidase subunit 2                                                      | comp61132_c0_seq1 | Q27452      | melanin biosynthesis, defense response | down       |
| phenoloxidase A3      | Phenoloxidase subunit A3                                                     | comp56149_c0_seq1 | Q9V521      | melanin biosynthesis, defense response | down       |
| procollagen           | Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1                           | comp61977_c0_seq1 | P24802      | collagen formation     | down       |
| protein henna         | Protein henna EC                                                             | comp64492_c0_seq6 | P17276      | amino acid metabolism  | up         |
| protein ubiquination  | E3 ubiquitin-protein ligase RNF139                                            | comp63534_c0_seq2 | Q8WU17      | protein ubiquination regulation | up         |
| RNA-binding           | RNA-binding protein Rsf1                                                      | comp55145_c0_seq1 | Q24491      | gene expression regulation | down       |
| TAM41                 | Mitochondrial translocator assembly and maintenance protein 41 homolog      | comp60449_c0_seq4 | Q8INF2      | cardiolipin biosynthesis | up         |
| TRAP-beta             | Translocon-associated protein subunit beta                                   | comp31633_c0_seq1 | P23438      | co-translational protein targeting to membrane | down       |
| trehalose transporter  | Facilitated trehalose transporter Tret1                                      | comp63446_c0_seq1 | A9ZSY3      | sugar transport        | down       |
|                |                                | ref         | acc   | process                   |   |
|----------------|--------------------------------|-------------|-------|---------------------------|---|
| xanthine dehydro | Xanthine dehydrogenase | comp64423_c0_seq2 | P08793 | xanthine catabolic process | up |
| zonadhesin     | Zonadhesin Flag | comp62844_c0_seq3 | O88799 | cell adhesion              | down |

**Reference Transcripts**

|                |                                | ref         | acc   | process                   |   |
|----------------|--------------------------------|-------------|-------|---------------------------|---|
| elongation 1B  | Elongation factor 1-beta      | comp51037_c0_seq1 | P29522 | Protein biosynthesis      | down |
| troponin       | Troponin I                     | comp62634_c1_seq14 | P36188 | Actin binding             | down |
| unknown ref    | NA                             | comp60946_c5_seq1 | NA    | NA                        | up  |
**Table S3.** Tukey multiple comparison tests. Significance codes for adjusted p-values: 0=***, 0.001=**, 0.01=*, 0.05=., 0.1=NS  
A) Population differentiation PC1  
B) Population differentiation PC2  
C) Angelo dates PC1  

| Population         | P adj |
|--------------------|-------|
| BigCreek-Angelo    | NS    |
| Sagehen-Angelo     | ***   |
| Sagehen-BigCreek   | ***   |

| Treatment          |       |
|--------------------|-------|
| HS-control         | *     |
| stream-control     | *     |
| stream-HS          | NS    |

| Population*Treatment |       |
|----------------------|-------|
| Sagehen:stream - Angelo:stream | ***   |
| Sagehen:stream - BigCreek:stream | ***   |
| Sagehen:stream - BigCreek:control | ***   |
| Sagehen:stream - Angelo:control  | ***   |
| Sagehen:stream - Sagehen:control | ***   |
| Sagehen:stream - Angelo:HS      | **    |
| Angelo:stream - BigCreek:HS     | **    |
| Sagehen:stream - Sagehen:HS     | **    |
| BigCreek:stream - BigCreek:HS   | **    |
| Angelo:stream - Sagehen:HS      | .     |
| BigCreek:HS - BigCreek:control  | .     |
| BigCreek:HS - Angelo:control    | .     |
| Sagehen:stream - BigCreek:HS    | NS    |
| BigCreek:stream - Sagehen:HS    | NS    |
| Sagehen:HS - Angelo:control     | NS    |

| Treatments Compared  |       |
|----------------------|-------|
| Sagehen:HS - BigCreek:control | NS    |
| Angelo:stream - Angelo:HS     | NS    |
| BigCreek:HS - Sagehen:control | NS    |
| BigCreek:stream - Angelo:HS   | NS    |
| Angelo:HS - Angelo:control    | NS    |
| Angelo:HS - BigCreek:control  | NS    |
| Angelo:stream - Sagehen:control | NS  |
| Sagehen:HS - Sagehen:control  | NS    |
| BigCreek:HS - Angelo:HS       | NS    |
| BigCreek:stream - Sagehen:control | NS  |
| Angelo:stream - BigCreek:control | NS  |
### Population

| Populations       | P adj |
|-------------------|-------|
| BigCreek - Angelo | ***   |
| Sagehen - Angelo  | NS    |
| Sagehen - BigCreek| ***   |

### Treatment

| Treatments       | P adj |
|------------------|-------|
| HS-control       | NS    |
| stream-control   | NS    |
| stream - HS      | NS    |

### Population*Treatment

| Interactions         | P adj |
|----------------------|-------|
| Angelo:HS - BigCreek:control | *     |
| Sagehen:stream - BigCreek:control | *     |
| BigCreek:control - Angelo:control | *     |
| Sagehen:control - BigCreek:control | ns    |
| BigCreek:stream - Angelo:HS      | NS    |
| BigCreek:stream - Angelo:control | NS    |
| BigCreek:stream - BigCreek:stream | NS    |
| BigCreek:stream - Angelo:stream | NS    |
| Sagehen:HS - BigCreek:control   | NS    |
| BigCreek:stream - Sagehen:control | NS    |
| BigCreek:stream - Sagehen:HS    | NS    |
| BigCreek:HS - BigCreek:control  | NS    |
| BigCreek:HS - Angelo:HS         | NS    |
| BigCreek:HS - Angelo:control    | NS    |
| Angelo:stream - BigCreek:HS     | NS    |
| Sagehen:stream - BigCreek:HS    | NS    |
| BigCreek:HS - Sagehen:control   | NS    |
| Angelo:stream - Angelo:HS       | NS    |
| Angelo:stream - Angelo:control  | NS    |
| Sagehen:stream - Sagehen:control | NS    |
| Angelo:stream - Angelo:stream   | NS    |
| Angelo:HS - Angelo:control      | NS    |
| Angelo:HS - Angelo:hs           | NS    |
| Angelo:stream - Angelo:stream   | NS    |
| Sagehen:stream - Sagehen:hs     | NS    |
| BigCreek:stream - BigCreek:hs   | NS    |
| Date          | P adj |
|---------------|-------|
| early-late    | ***   |

| Treatment     |       |
|---------------|-------|
| HS-control    | ***   |
| stream-control| NS    |
| stream-HS     | ***   |

| Date*Treatment |       |
|---------------|-------|
| late:stream - early:HS | ***   |
| early:HS - late:control | ***   |
| late:stream - late:HS | **    |
| late:HS - late:control | **    |
| early:stream - early:HS | *     |
| late:stream - early:control | .     |
| late:control - early:control | .     |
| early:HS - early:control | NS    |
| early:stream - late:HS | NS    |
| late:stream - early:stream | NS    |
| early:stream - late:control | NS    |
| late:HS - early:control | NS    |
| early:stream - early:control | NS    |
| late:HS - early:HS | NS    |
| late:stream - late:control | NS    |
| late:stream - early:control | NS    |